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**ALTERNATIVELY ACTIVATED MACROPHAGES IN *PSEUDOMONAS AERUGINOSA* PNEUMONIA: MODULATION OF THE NF- $\kappa$ B SIGNALING PATHWAY AND THE IMMUNOMODULATORY ROLE OF ARGINASE-1**

Dalia Haydar

*University of Kentucky*, [dalia.haydar@hotmail.com](mailto:dalia.haydar@hotmail.com)

Author ORCID Identifier:

 <https://orcid.org/0000-0002-7959-2699>

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Dalia Haydar, Student

Dr. David Feola, Major Professor

Dr. David Feola, Director of Graduate Studies

ALTERNATIVELY ACTIVATED MACROPHAGES IN *PSEUDOMONAS*  
*AERUGINOSA* PNEUMONIA:

MODULATION OF THE NF-KB SIGNALING PATHWAY  
AND THE IMMUNOMODULATORY ROLE OF ARGINASE-1

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DISSERTATION

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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

Dalia Haydar

Lexington, Kentucky

Director: Dr. David Feola, Associate Professor of Pharmacy Practice and  
Science

Lexington, Kentucky

2018

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## ABSTRACT OF DISSERTATION

### ALTERNATIVELY ACTIVATED MACROPHAGES IN *PSEUDOMONAS AERUGINOSA* PNEUMONIA: MODULATION OF THE NF-KB SIGNALING PATHWAY AND THE IMMUNOMODULATORY ROLE OF ARGINASE-1

**Background:** Azithromycin polarizes macrophages into an alternative phenotype and promotes a regulated immunity. Arginase is an important effector of these macrophages believed to play an essential role in decreasing injury and promoting repair.

**Hypothesis:** Decreases in inflammation in response to *Pseudomonas aeruginosa* (PA) pneumonia achieved by polarizing macrophages to an alternative phenotype is dependent upon the production of arginase.

**Methods:** Requirement of arginase was examined by pharmacological inhibition using S-(2-boronoethyl)- L-cysteine (BEC) or L-norvaline and by infecting arginase-1 conditional knock-out mice ( $Arg1^{flox/flox};Lyz2\text{-cre}$  ( $Arg1^{\Delta M}$ )) with PA intratracheally.  $Arg1^{\Delta M}$  and control  $Arg1^{flox/flox}$  mice were then dosed with azithromycin daily via oral gavage beginning four days prior to infection. Analysis of weight loss in addition to characterization of inflammatory cells and cytokine production via flow cytometry was performed. Macrophages were then stimulated with LPS and polarized with IL4/13, IFN $\gamma$ , or azithromycin plus IFN $\gamma$ . Western blot for signaling mediators, p65 translocation assay, and immunofluorescence were performed.

**Results:** Myeloid arginase-1 deletion resulted in greater morbidity along with more severe inflammatory response compared to the  $Arg1^{flox/flox}$  mice.  $Arg1^{\Delta M}$  mice had greater numbers of neutrophils, macrophages, and lymphocytes in their airways and lymph nodes compared to the  $Arg1^{flox/flox}$  mice. Conversely, global arginase inhibition resulted in greater weight loss along with greater neutrophil and macrophage infiltration compared to  $Arg1^{\Delta m}$  mice. BEC and L-norvaline treated mice had higher numbers of lymphocytes in their lymph nodes with variable effects on airway lymphocyte counts. Azithromycin treatment comparably reduced the acute inflammatory responses in both  $Arg1^{\Delta M}$  and

Arg1<sup>flox/flox</sup> mice. To evaluate this mechanism, we show in vitro that azithromycin decreases NF- $\kappa$ B activation by preventing p65 nuclear translocation and by decreasing STAT1 activation in a concentration-dependent manner. These effects were reversed with IKK $\beta$  inhibition.

Conclusions: Myeloid arginase is essential for control of inflammatory responses in PA pneumonia with potentially different effects of other cellular sources demonstrated with global arginase inhibition. Azithromycin reduces excessive inflammation even in the absence of arginase, potentially through a cross-inhibitory mechanism involving STAT1 and NF- $\kappa$ B pathways through IKK $\beta$ .

KEYWORDS: Azithromycin, Alternative Macrophages, Arginase-1, STAT1 and NF- $\kappa$ B pathways, Chronic *Pseudomonas aeruginosa* pneumonia

Dalia Haydar

June 12, 2018

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By

Dalia Haydar

Dr. David Feola

Director of Dissertation

Dr. David Feola

Director of Graduate Studies

07/25/2018

This thesis work is dedicated to my husband Nader and my sister Katia who have been a constant source of support and encouragement during the challenges of graduate school and life. I am truly thankful for having you in my life. This work is also dedicated to my parents, Abir and Chawki, and my brothers, Mohammed and Haydar, who have always loved me unconditionally and whose good examples have taught me to work hard for the things that I aspire to achieve.

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## Chapter 1: Introduction

### I. Cystic fibrosis and infection

Cystic fibrosis is a hereditary progressive disease of the secretory glands acquired by inheriting the mutated genes of a chloride ion transport channel called the cystic fibrosis transmembrane conductance regulator (CFTR) [1]. The autosomal recessive mutation of the CFTR gene on chromosome 7 affects about 1 in 3300 white newborns with lower incidence in other races [2]. People with defective CFTR suffer from abnormal thickening of their mucoid secretions. Thickened mucus constitutes a major problem in the lungs although other organs can be affected including the kidneys, intestines, pancreas, and liver [2-6]. In the lungs, a vicious cycle of thickened mucus, repetitive infections, and extensive inflammation result in mucoid plugging of the airways, lung injury, and progressive pulmonary function decline [1-5, 7].

According to the Cystic Fibrosis Foundation, more than 70,000 people in the world suffer from cystic fibrosis [2, 6]. In the United States, more than 30,000 people live with cystic fibrosis with an expected increase of 1000 new patients annually. Today, patients suffering from this life-shortening disease have a median survival of 37 years with an anticipated increase in survival beyond 50 years of age [1, 7]. This is considered a major advancement since 1962 when patients used to die by 10 years of age due to extensive mucus accumulation in their lungs and inability to breathe [2-6]. At least 50% of the cystic fibrosis patients in the United States are above 18 years of age according to the 2017 Cystic Fibrosis Foundation Patient Registry [2, 6].

Newborns with cystic fibrosis develop structural abnormalities, mucus accumulation and obstruction of the airways, along with hypertrophy of the mucosal glands [8-11]. Radiological evidence of lung destruction is strongly correlated with chronic lung infections. The increased risk for chronic bacterial

colonization along with the exaggerated inflammation result in progressive airway wall thickening and lumen dilatation along with permanent scarring in the lungs [12-16]. Additionally, early signs of inflammation occur shortly after birth preceding structural changes and are predictive of the future disease progression [14]. The mucosal glands in cystic fibrosis are hypertrophied and ducts of these glands are obstructed due to mucus cell hyperplasia. Early structural changes in cystic fibrosis also include increased smooth muscle content and smooth muscle cell hyperplasia in the airway walls in early disease stages [12]. These structural abnormalities are also responsible for changes in the velocity and resistance of the airflow in cystic fibrosis lungs compared to healthy lungs [13]. Additionally, elevated pro-inflammatory mediators in the patients' lungs constitute a positive indicator for the early development of bronchiectasis, a condition where the lung structure is lost due to irreversible damage with thickened and dilated airways [15]. Bronchiectasis occurs in end-stage cystic fibrosis and results in respiratory failure and death.

Additionally, the lungs of cystic fibrosis patients are plugged with dense mucus. The mucus becomes abnormally heavy and thick due to loss of water content resulting from the abnormal ion transport across the mutated CFTR channels. Besides the ion and water abnormalities, some reports suggest increased mucus secretion in cystic fibrosis due to increased expression of mucin genes (e.g. Muc1, Muc6, Muc5AC, and Muc5B) [13]. Mucins are large glycoproteins that normally constitute about 1-2% of mucus along with water which constitutes about 98%. When mucin concentration in mucus increases, it results in mucus hyperconcentration and dehydration [13]. Collectively, the increased mucin concentration and the disrupted chloride channels increase the mucus viscosity in cystic fibrosis to  $10^4$ - $10^5$  times higher than that of water. Increased mucus accumulation in cystic fibrosis complicates the disease via mechanically obstructing the airways, trapping bacteria and pathogens in the lungs, therefore driving inflammation.

Infection and inflammation are the other two main pathological aspects of cystic fibrosis to be extensively discussed in this dissertation. Chronic infections are the leading cause of mortality in these patients with the most predominant pathogen being *Pseudomonas aeruginosa* (PA) [17-19]. In fact, about 60% of patients with cystic fibrosis are infected with PA, which chronically colonizes the lungs of 80% of the patients older than 18 years of age [17-19]. According to Davies et al., for a given bacterial load, a person with cystic fibrosis will have up to 10 times more inflammation than a person with a lower respiratory tract infection but without the disease [15]. Many scientists initially approached the inflammatory aspect of cystic fibrosis as a consequence of the chronic and repetitive infections. However, it has become clear that inflammation is an independent factor of cystic fibrosis pathophysiology. Yet, it is not very well understood why the immune system responds in an exaggerated manner to the infections.

Several factors increase the infection risk in cystic fibrosis lungs including environmental, structural, and immune abnormalities. The resultant dense and viscous mucus along with the dampened mucociliary clearance are believed to create a favorable environment for the growth and replication of bacteria and microorganisms in the lower respiratory tract [20]. It is hypothesized that the excessively high salt concentrations in the airways due to the malfunctional CFTR channels are responsible for pulmonary infections. The high salt concentrations inactivate host antimicrobial defensin proteins [21, 22]. Additionally, the malfunctional channels disrupt the homeostatic balance of the water volume in the airways. The inability to efficiently transport the sodium and chloride across the airway epithelium disrupts the isotonic state and results in volume and water depletion. This eventually results in increased viscosity and malfunctional mucociliary clearance; thus, invading microbes are trapped in the lower respiratory tract [23, 24].

Patients with cystic fibrosis are also more susceptible to bacterial infections due to altered macrophage function [20]. The specific macrophage alterations in

cystic fibrosis are extensively discussed in later sections. However, reduced macrophage autophagosome function and the reduced ability to engulf and phagocytize pathogens contribute to increased susceptibility for pulmonary infections. The loss of CFTR channels on the surface of these immune cells is in part responsible for the loss of macrophage functions [25-35].

Moreover, the structural abnormalities discussed above also contribute to the increased risk of infection [20]. Structural changes blunt the effectiveness of physical defense mechanisms in clearing invading pathogens. Additionally, these changes make the sites of infection in the lower respiratory tract inaccessible for macrophages and other immune cells [11, 25-28, 30-36]. Specific changes in the trachea, airway size, and wall thickness can also contribute to the increased infection risk. Long et al. and Meyerholz et al. found dilated and thickened airways and bronchioles in children with cystic fibrosis before any infectious and pulmonary symptoms occur [11, 36]. These changes promote colonization of the initially sterile lower respiratory airways and thus comprise challenges to the normal host-defense mechanisms. This “otherwise harsh lung environment” with limited oxygen and nutrient supply for microbiota favors the development of evolving survival tools for bacteria. This drives the bacteria to mutate in order to adapt and colonize the lower airways for prolonged durations [37-42].

Acidification of the lung microenvironment also increases the risk of pulmonary infections in patients with cystic fibrosis [20]. Mucus in cystic fibrosis lungs is of gel-rubber consistency which results in ciliary dyskinesia and increased acidity.

Acidification of the lung environment impairs the host-defense mechanisms which are sensitive to pH and creates an aerobic condition favorable for microbes and harmful for the patients’ lung function [11, 20-24, 29-36].

Collectively, these factors contribute to an excessive inflammatory response in an anatomical region of the lung which is normally free of any immune reactions. Thus, the inflammatory response itself constitutes an additional factor for prolonged bacterial colonization of the lower respiratory tract in cystic fibrosis.

Similarly, infections with PA are predominant in cystic fibrosis due to microbial, environmental, and immune factors [43-48]. PA is a rod-like bacterium which benefits from the tremendous pulmonary changes. This microorganism undergoes genotypic and phenotypic changes which make it able to survive in the cystic fibrosis lungs for prolonged periods. PA is able to combat the oxidative and nutrient stresses and it develops efficient and specialized antibiotic resistance mechanisms. Additionally, PA is able to change its lifecycle by reducing its metabolic rate, decreasing its motility and dampening its growth and replication cycles. Moreover, PA chronically colonizing the lungs are characterized by reduced quorum sensing and increased alginate production [20]. PA commonly found the lungs of patients with cystic fibrosis have highly evolved virulence factors along with increased ability to line the airways with biofilm formation. Thus, these facultative anaerobic Gram-negative bacteria can alter the cystic fibrosis lung environment to favor its survival and replication [45-47].

Chronic infections with PA are also related to the unique ability of this microorganism to down- and up-regulate the expression of different genes related to virulence and infectivity [48-54]. PA colonizing cystic fibrosis airways can compete with other pathogens by regulating the nutrient levels and by releasing antimicrobial substances capable of killing other bacteria. For instance, PA competes with the second most common pathogen in cystic fibrosis, *Staphylococcus aureus* [49] by inhibiting its growth and replication through tight regulation of free iron concentrations and by lysing *S. aureus* through the release of bactericidal substances [49]. Conversely, infections with other pathogens including bacteria (*Burkholderia cenocepacia*, *Mycobacterium smegmatis*, *Haemophilus influenzae*, and *Staphylococcus aureus*), viruses (Respiratory syncytial virus), fungi and certain yeast strains (*Aspergillus*, *Candida*, *Exophiala dermatitidis* and *Lomentospora prolificans*) have been shown to promote

infections and colonization with PA by directly altering the micro-environment and disturbing the immune response against PA [50-54].

Altered CFTR channels also favor PA acquisition in patients with cystic fibrosis [55-59]. CFTR channels act as receptors for the uptake of PA by the airway epithelium; therefore, the inability to internalize PA can evade its clearance and the antigen presentation for incoming immune cells [60-62]. This strong adherence to mutated CFTR channels contributes to chronic and prolonged infections with PA [55-57]. Epithelial cells with mutated or deleted CFTR are also prone to increased activation of the pro-inflammatory transcription factor, NF- $\kappa$ B when stimulated with PA in-vitro compared to normal epithelial cells [57, 58]. Increased NF- $\kappa$ B activation is associated with significantly lower levels of IFN $\gamma$  release by the cystic fibrosis epithelial cells challenged with PA. IFN $\gamma$  is an essential pro-inflammatory cytokine required for an efficient activation of the inflammatory response. Thus, dampening the IFN $\gamma$  response is a unique tool by which PA can evade its immune recognition and clearance [59].

Additionally, PA forms biofilms to evade immune response and to cause prolonged infections. PA is characterized with biofilm formation and strong adherence to the thick mucus which prevents its recognition and phagocytosis by neutrophils [63, 64]. Moreover, PA is able to utilize the neutrophil extracellular traps (NETs) to favor its own survival. As the name implies, NETs are network-shaped traps made of extracellular fibers, primarily composed of DNA from neutrophils and used to bind pathogens. PA utilizes these neutrophil traps to secrete a highly virulent and pro-inflammatory toxin called Pyocyanin [65, 66]. Pyocyanin released by PA can kill other competing bacteria and alter the activation and function of immune cells.

Finally, PA benefits from the skewed T cells responses to evade clearance and to chronically colonize the lungs. The adaptive immune response in cystic fibrosis promotes PA infections as it is skewed towards a Th2/Th17 response which is

believed to favor and even prime infections with PA [67-70]. Despite all the studies about the different variants of PA and the different characteristics related to the infection and host-defense mechanisms in cystic fibrosis; the exact machinery and environmental characteristics that favor the competition with other pathogens and survival of PA are very poorly understood.

## II. Inflammation and immune response in cystic fibrosis

The inflammatory response in cystic fibrosis is dysregulated and significantly contributes to disease pathology [63]. Epithelial cells and resident macrophages lining the airways release pro-inflammatory mediators and chemokines when an invading pathogen is sensed. A normal immune system responds to the released inflammatory mediators by recruiting innate immune cells (like neutrophils and macrophages) which are non-specific but show up early to contain and clear the infection. The innate immune cells then activate adaptive immunity in a very well-coordinated and controlled manner [63]. Adaptive immunity is antigen-specific and is highly efficient in clearing infections. However, the immune system in patients with cystic fibrosis responds in an abnormally excessive and inefficient manner against the invading pathogens. Consequently, altered inflammation fails to clear the infection and results in lung injury. Additionally, the non-resolving pulmonary inflammation creates niches which favor further chronic infections and colonization with highly virulent and opportunistic pathogens. The following section discusses the specific alterations in the innate immune response of cystic fibrosis (also summarized in Figure 1.1 a and b). The main elements of the innate immune response which are altered in cystic fibrosis include the epithelium, the NF- $\kappa$ B pro-inflammatory signaling pathway, the neutrophil response, as well as the macrophage response.

## i. Epithelium

The bronchial epithelium is the first line of defense against invading pathogens and its physical, chemical, and immunologic functions are disrupted in cystic fibrosis. The epithelium functions to physically remove and prevent the spread of inhaled microbes by cough, mucociliary clearance, as well as by forming tight physical and chemical barriers [63, 71-73]. Appropriate composition, hydration, and viscosity of the mucus are essential for the release and transport of mucus in the airways. The malfunctional CFTR channels, as mentioned previously, prevent chloride and bicarbonate transport. This increases the viscosity and acidity of the mucus thus hindering the mucociliary clearance process. Eventually, the thick mucus along with the trapped pathogens remain adherent to the epithelium and fail to be expelled by cough and mucociliary clearance [13, 74, 75]. Another way by which the epithelium prevents invading pathogens from spreading and establishing an infection is by the release of antimicrobial products and peptides. However, these are inactivated at the acidified airway surface liquid (ASL). The ASL pH drops drastically due to ion flux imbalance by the mutated CFTR channels [63, 76]. The inactivation of epithelial microbicidal products prevents the killing of invading bacteria, viruses, and yeasts. Additionally, the tight epithelial barrier is cleaved and digested in the presence of elevated neutrophil elastases and proteases [63, 77, 78], thereby increasing paracellular permeability of pathogens into the lung interstitium [79, 80]. Collectively, CFTR channel dysfunction and the altered inflammatory environment impair the physical and chemical functions of the epithelial barrier.

The immunologic function of the epithelium also includes the activation of innate immunity via antigen presentation and pro-inflammatory cytokine release. The cellular innate immune response is activated when the pathogen-associated molecular patterns (PAMPs) are recognized by the Toll-like receptors (TLR) expressed on the epithelial cells [81-84]. This primary response occurs when the non-self antigens of invading pathogens are presented by the pattern recognition

receptors. Several TLRs recognize microbial components of PA, which expresses numerous PAMPs, including lipopolysaccharides (LPS) and flagellin. TLR2 and TLR4 detect LPS while TLR5 detects flagellin. Along with the cytokine stimulation of their corresponding receptors, binding of PAMPs to TLRs activates efficient downstream signaling cascades [81]. Activation of the nuclear transcription factor NF- $\kappa$ B is one of the main pro-inflammatory signaling cascades triggered in the stimulated epithelial cells. However, functional CFTR mutations are associated with increased NF- $\kappa$ B activation, reduced TLR4 expression and stimulation, as well as reduced antigen processing and presentation [85-88]. The increased NF- $\kappa$ B activation in epithelial cells is therefore partly responsible for the dysregulated immunologic function of the epithelium as it results in excessive release of pro-inflammatory cytokines and mediators. Pro-inflammatory mediators then act to recruit and activate exaggerated numbers of innate immune cells which can lead to inflammatory lung injury [85-88].

#### ii. The NF- $\kappa$ B pro-inflammatory signaling pathway

Increased NF- $\kappa$ B activation is a major immunologic alteration in cystic fibrosis. As mentioned previously, NF- $\kappa$ B is a nuclear transcription factor in epithelial cells and in many other immune cells including macrophages, neutrophils, as well as B and T lymphocytes. Disruption of NF- $\kappa$ B activation in the epithelial cells and in the macrophages is a very well described pathological factor in cystic fibrosis [89-91]. The NF- $\kappa$ B family of proteins includes different transcription factors which control genes involved in inflammation, immunity, cell proliferation, differentiation, and survival.

The NF- $\kappa$ B family of transcription factors is divided into the canonical vs the non-canonical pathways. The canonical pathway is mainly involved in inflammation while the non-canonical pathway is involved in immune cell differentiation and maturation. Each NF- $\kappa$ B transcription factor consists of a heterodimer or a homodimer of the five NF- $\kappa$ B subunits: RelA (also named p65), RelB, cRel, p50,

and p52 (p50 and p52 subunits are synthesized as precursor proteins, p105 and p100) [91]. Activation of the canonical NF- $\kappa$ B pathway mainly involves I $\kappa$ B- $\alpha$  degradation and p50/p65 subunits (p50/p65 heterodimers and p65 homodimers). Alternatively, activation of the non-canonical NF- $\kappa$ B mainly involves RelB/p52 subunits and is independent of I $\kappa$ B- $\alpha$  degradation (instead it is dependent on the processing of the precursor proteins p100 and p105). Moreover, the activation of the canonical pathway frees pre-existing subunits while the non-canonical pathway allows the synthesis of new subunits. Finally, the two pathways are induced and regulated through independent mechanisms. The canonical pathway involves the activation of a NEMO-dependent IKK complex (a trimeric complex composed of two catalytic subunits, IKK $\alpha$  and IKK $\beta$ , and a regulatory subunit, IKK $\gamma$ ) [91]. Conversely, the non-canonical pathway is mediated through the activation of a NEMO-independent kinase complex including IKK1 and the NF- $\kappa$ B -inducing kinase (NIK) [89]. Inappropriate activation of the canonical pathway results in inflammatory and auto-immune diseases while the disruption of non-canonical NF- $\kappa$ B signaling results in lymphoid malignancies. Since the canonical pathway is mainly involved in inflammation and in cystic fibrosis, whenever NF- $\kappa$ B is mentioned in this dissertation it refers to the activation of p65 subunits through the canonical pathway unless otherwise specified [91].

In the absence of TLR and cytokine receptor stimulants, NF- $\kappa$ B activation is suppressed by an inhibitory subunit (I $\kappa$ B- $\alpha$ , I $\kappa$ B- $\beta$ , or I $\kappa$ B- $\epsilon$ ) which binds the NF- $\kappa$ B subunits (p50 or p65) and prevents them from translocating to their site of action in the nucleus. Stimulation through TLR allows the phosphorylation and activation of the I $\kappa$ B kinase (IKK $\beta$ ) which phosphorylates the inhibitory I $\kappa$ B- $\alpha$  subunits. Once phosphorylated, the I $\kappa$ B- $\alpha$  subunits undergo rapid ubiquitination and proteasomal degradation, thus releasing p50/p65 from the inhibited state. Free p50/p65 subunits undergo further phosphorylation in the cytoplasm, dimerize, and translocate to the nucleus where they bind to the NF- $\kappa$ B DNA promoter region. This promoter region controls several genes for pro-inflammatory cytokines and mediators including TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IL-8, IL-12,

iNOS, IFN $\gamma$ , adhesion molecules (VCAM1, ICAM1), as well as many other immune and non-immune related genes [81, 92-95]. The release of those inflammatory cytokines and mediators results in the recruitment and chemoattraction of neutrophils and macrophages to the site of infection.

NF- $\kappa$ B activation in epithelial cells plays an essential role in initiating and modulating the immune response. In addition to recruiting innate immune cells, the inflammatory cytokines also polarize different inflammatory signaling cascades in the recruited immune cells including further activation of the NF- $\kappa$ B pathway in the recruited neutrophils and macrophages. Thus, any deviation from the normal NF- $\kappa$ B activation levels can result in serious pathology [96]. For instance, regulation of NF- $\kappa$ B activation mainly involves tight control of the activity and synthesis of the I $\kappa$ B- $\alpha$  and IKK $\beta$  proteins as well as controlling subunit nuclear translocation and DNA binding [91]. Synthesis of I $\kappa$ B- $\alpha$  subunits and IKK $\beta$  are sensitive to NF- $\kappa$ B activation through a negative feedback regulation. Binding of p50/p65 subunits to the DNA shuts down the IKK $\beta$  synthesis and stimulates the synthesis of new I $\kappa$ B- $\alpha$  subunits thus critically controlling the duration of the NF- $\kappa$ B response. I $\kappa$ B- $\alpha$  controls the nuclear translocation of p50/p65 subunits by masking the nuclear localization sequence (NLS) of p65. Therefore, terminating the NF- $\kappa$ B response is disrupted in the case of I $\kappa$ B- $\alpha$  deficiency [91]. Alternatively, IKK $\beta$  activation requires phosphorylation of two serines; thus, regulation of IKK $\beta$  activation involves tight control of its trans-autophosphorylation as well as its phosphorylation by the upstream kinases [91-94]. IKK $\beta$  deficiency abolishes the activation of an NF- $\kappa$ B response while IKK $\beta$  accumulation shuts down other signaling pathways which release pro-inflammatory cytokines including the STAT-1 pathway [91]. Additionally, post-translational modifications of NF- $\kappa$ B subunits affect their nuclear translocation, dimerization, and DNA binding. For instance, several modifications have been described like Ser276 phosphorylation of p65 which facilitates its DNA binding, while Ser536 phosphorylation affects its nuclear translocation [91]. Other modifications like methylation, acetylation, and phosphorylation of additional sites

affect p65 interaction with other enzymes and NF- $\kappa$ B subunits as well as inhibiting the transcriptional activity of p65. In fact, abnormal NF- $\kappa$ B activation has been described in many diseases like cancer, Alzheimer's disease, multiple sclerosis, and cystic fibrosis [96].

Increased NF- $\kappa$ B activation in epithelial cells is well described in cystic fibrosis and is caused by mutated CFTR channels as well as changes in the inflammatory environment. Continual NF- $\kappa$ B activation in cystic fibrosis contributes to chronic infection, neutrophil inflammation, and lung injury [97]. Unopposed NF- $\kappa$ B activation is attributed to the significantly high levels of IL-8 and the suppressed levels of IL-10. Carrabino et al. found that in the absence of any stimulation, isolated cystic fibrosis nasal epithelial cells release high levels of IL-8 [98]. Under basal conditions, cells from cystic fibrosis nasal epithelium express significantly higher activity of NF- $\kappa$ B compared to normal epithelial cells. Co-stimulation of epithelial cells from cystic fibrosis patients with PA and IL-1 $\beta$  produced a two-fold increase in NF- $\kappa$ B activation and at least six-folds greater increase in IL-8 levels compared to normal epithelial cells [98]. Additionally, CFTR mutations in epithelial cells and macrophages are related to increased NF- $\kappa$ B activation. Specifically, the most common CFTR mutation is the deletion of phenylalanine 508 ( $\Delta$ F508), which results in its endoplasmic reticulum associated degradation (ERAD) by the ubiquitin-proteasome system. The inability of  $\Delta$ F508-CFTR to translocate to the cell surface is associated with exaggerated NF- $\kappa$ B activation [97]. According to Chanson et al., the  $\Delta$ F508 mutation enhances NF- $\kappa$ B activation due to lack of gap junctional communication (GJIC) of CFTR with inflammatory receptors [97]. Additionally, CFTR binds to and colocalizes with TRADD (the key adaptor molecule in TNF- $\alpha$  signaling) while  $\Delta$ F508 CFTR cannot bind TRADD thus preventing the degradation of TRADD, an event that is necessary for turning off TNF- $\alpha$ -induced NF- $\kappa$ B activation [99].

Additionally, appropriate CFTR localization within the lipid-rafts formed in response to infection is essential for raft clustering and regulated signaling

through the NF- $\kappa$ B pathway [100]. Mutated CFTR disrupts lipid-raft formation and clustering thereby inducing uncontrolled activation of the NF- $\kappa$ B signaling pathway. Tabary et al. also show that disrupted calcium signaling is associated with increased NF- $\kappa$ B activation in cystic fibrosis epithelial cells [100]. They show that cells with mutated CFTR channels respond to IL-1 $\beta$  stimulation with a significant increase in calcium concentrations along with increased NF- $\kappa$ B activation. This effect was reversed with thapsigargin treatment which prevents calcium release from the endoplasmic reticulum. Tabary et al. suggest that epithelial cells with mutated CFTR are endogenously primed for excessive release of calcium which results in abnormal lipid-raft formation and disrupts NF- $\kappa$ B signaling [100]. Finally, Saadane and colleagues infected normal and cystic fibrosis mice with PA intratracheally [101]. Although infection is cleared in both groups with similar kinetics; cystic fibrosis mice have greater neutrophil influx along with higher levels of pro-inflammatory cytokines compared to wild-type mice. Saadane found that cystic fibrosis mice lack the ability to re-synthesize new I $\kappa$ B- $\alpha$  once it was degraded resulting in prolonged and excessive activation of NF- $\kappa$ B which lasted for 6 days compared to normal mice which had significantly lower levels of NF- $\kappa$ B which rapidly decreased by day 4 post-infection [101]. Collectively, aberrant activation of the NF- $\kappa$ B pathway is a hallmark of cystic fibrosis pathology.

### iii. Neutrophilic inflammation

The third element of the disrupted innate immunity in cystic fibrosis is the excessive recruitment and influx of neutrophils. Neutrophils are thought to greatly contribute to morbidity and mortality in cystic fibrosis. This is due to the overwhelming numbers of neutrophils which migrate to the lungs in large numbers due to persistent infection and continuous stimulation. Inflammation in cystic fibrosis is characterized by elevated concentrations of neutrophil chemokines including IL-17, IL-8, and IL-6 [8]. Neutrophils exert their pro-inflammatory function by releasing powerful antimicrobial substances, free

radicals, and antioxidants which the cystic fibrosis lungs are unable to clear. This is aggravated by the death of neutrophils which further releases its granular contents resulting in inflammatory lung injury and damage. The resultant lung injury and oxidative stress eventually recruit more neutrophils thus causing an excessive inflammation that is characteristic of cystic fibrosis [102-105].

CFTR channels are also expressed on the neutrophil cell surface and CFTR mutations impair the degranulation of important neutrophil microbicidal products from the secondary and tertiary granules (lysozyme, lactoferrin, and cathepsins) [102]. Additionally, there is an excessive amount of neutrophil elastases and proteases that overwhelm the antiproteases that protect the lungs. In fact, even milder forms of cystic fibrosis are associated with greater than 1000 times the concentration of  $\alpha$ 1-protease inhibitors compared to healthy individuals [103-105]. Thus, neutrophils contribute to lung injury by releasing excessive amounts of neutrophil elastases which break down the connective tissues and matrix proteins leading to a structural loss in the small airways [106-110]. Additionally, unopposed neutrophil elastases are also capable of cleaving non-structural proteins like essential immune cell surface receptors (CFTR, CD4, CD8, complement receptors, and antigen presentation receptors) [8, 111, 112]. Also, elastases can cleave the macrophage phosphatidylserine receptors which prevents compensatory anti-inflammatory mechanisms due to impaired efferocytosis, the process by which macrophages clear dead and apoptotic neutrophils [113-115].

The changes in the lung microenvironment and the ion imbalance result in decreased phagocytic and degranulation capacities of neutrophils. New studies suggest that neutrophils express CFTR in their phagolysosomes. Thus, mutations in CFTR impair neutrophils ability to kill engulfed pathogens. This occurs due to impaired chlorination of the internalized pathogen [111, 112, 116]. So, there is an excessive number of neutrophils in the cystic fibrosis lungs that

are unable to clear the invading pathogens and simultaneously contribute to excessive inflammatory injury.

Additionally, unregulated death of neutrophils contributes to severe inflammatory lung injury. The excessive numbers of neutrophils in cystic fibrosis is associated with failure of some neutrophils to undergo regulated cell death or apoptosis. Large numbers of recruited neutrophils undergo necrosis which releases massive intracellular contents like inflammatory mediators and cytokines, oxidants and proteases, in addition to large DNA fragments and actin [117]. Neutrophils also form chromatin traps called NETs. The purpose of these traps is to facilitate the catching and killing of bacteria. However, these NETs contain high amounts of DNA which are released upon death of the neutrophils and the released DNA further increases the mucus viscosity and reactivity [118]. Thus, the death of neutrophils releases tremendous amounts of intracellular contents that are extremely harmful to the lungs.

Neutrophils in cystic fibrosis can also undergo metabolic changes which affect their pro-inflammatory functions and half-life. According to Ingersoll and his colleagues, neutrophils in cystic fibrosis can develop metabolic adaptations which contribute to their altered function [119]. Changes in metabolic cycles affect the ability of neutrophils to engulf and phagocytize pathogens. It also results in additional production of toxic metabolites and prolongs the otherwise short half-life of neutrophils. In fact, neutrophils are short-lived immune cells with a lifespan ranging from a few hours to 5 days. In cystic fibrosis, several factors prolong the neutrophil lifespan including the prolonged pro-survival signals and the delayed apoptosis signal (due to mutated CFTR) [120]. These changes impair the neutrophil phagocytic capacities and extend their half-life while enhancing the neutrophil chemotaxis and migratory abilities.

Neutrophils also control T cell responses in cystic fibrosis. One mechanism through which neutrophils can regulate T cell proliferation is by controlling

arginine concentration. Arginine is a conditionally essential amino acid required for T cell function and proliferation. In cystic fibrosis, neutrophils release excessive amounts of arginase-1 which depletes arginine and renders the airway lumen a highly inhibitory milieu for T cells. Additionally, neutrophil arginase-1 cleaves T cell receptors and prevents their activation [119-125]. Conversely, neutrophils in cystic fibrosis can exert positive regulation of T cell responses by increasing the neutrophil expression of T cell activation receptors, major histocompatibility complex II, co-activator CD80, and prostaglandin D2 receptor (CD294). Neutrophils have been shown to particularly promote the expansion of Th2 and Th17 lymphocytes [119, 123]. Th17 lymphocytes will then recruit more neutrophils by releasing IL-17, the major cytokine involved in neutrophil chemotaxis and cystic fibrosis pathology (to be discussed in later sections). Thus, excessive influx of neutrophils in cystic fibrosis also impact the development and function of other immune cells, mainly T lymphocytes [119].

In summary, neutrophils constitute a major threat in the cystic fibrosis inflammatory process as they release toxic substances, reactive oxygen and nitrogen species, as well as DNA [82, 119]. Along with proteinases, these toxic neutrophil substances result in progressive inflammatory lung injury.

#### iv. Macrophage alterations

Macrophage function is an important element of innate immunity and is disrupted in cystic fibrosis. Changes in lung micro-environment affect macrophage polarization, phagocytic function, and antigen presentation. In normal circumstances, macrophage subpopulations are recruited to the lungs at different timepoints during the infection. These macrophage populations are essential for initiation, control, coordination, and resolution of inflammation [126]. Therefore, any disruption of a specific macrophage population can drastically affect the initiation and control of the immune response against infections. In this section,

changes in macrophage polarization and functions in patients with cystic fibrosis will be discussed.

#### a. M1 and M2 macrophage activation

Macrophage populations are separated into two distinct subpopulations based on their role in inflammation. Classically activated macrophages (designated M1 macrophages) are inflammatory while alternatively activated macrophages (designated M2 macrophages) are regulatory. M1 macrophages produce high levels of pro-inflammatory cytokines and mediate resistance to pathogens by releasing reactive nitrogen and oxygen intermediates, phagocytizing invading organisms, and promoting Th1 responses. In contrast, M2 macrophages control parasitic and helminth infections, express scavenger mannose receptor and anti-inflammatory cytokines, promote tissue remodeling and immune regulation, clear apoptotic cells and debris, and promote Th2 responses [127, 128]. Activation of M2 macrophages inhibits M1 macrophage proliferation and function and suppresses Th1-type cell-mediated immune responses. The latter is achieved through a characteristic pattern of gene up-regulation that includes the production of the type II cytokine TGF $\beta$  and the effector molecule arginase-1. By functioning to orchestrate remodeling and repair mechanisms, arginase-1 and TGF $\beta$  are also important in controlling lung homeostasis, inflammation, and subsequent damage [128-132]. It is important to note that M2 macrophages have recently been further characterized into M2a, M2b, M2c and M2d subsets based on their distinct gene expression profiles [127, 128]. However, in this dissertation we refer to the M2a subtype elicited by IL-4 and IL-13 as M2 macrophages. In cystic fibrosis, both macrophage populations are required. M1 macrophages are essential for protecting against infections and intracellular pathogens, while M2 macrophages are essential for inflammation resolution and for tissue regeneration and airway remodeling.

The activation of M1 and M2 macrophages are governed by distinct signaling mediators. M1 macrophages are activated by TNF- $\alpha$  or IFN- $\gamma$  when stimulated by non-self foreign antigens (such as LPS in the case of Gram-negative bacteria) [127, 128]. Conversely, M2 macrophages are activated by IL-4, IL-13, or IL-10. Signaling through the interferon regulatory transcription factor and the signal transducer and activator of transcription proteins (IRF/STAT) is the central governing mechanism of macrophage M1–M2 polarization [118, 127-129]. LPS signaling through TLR4 activates several signaling cascades which involve two adaptors, MyD88 and TRIF. MyD88 signaling activates several kinases including IRAK4, TRAF6, and IKK $\beta$  which ultimately activate NF- $\kappa$ B, the main M1 macrophage transcription factor [127, 128]. Alternatively, TRIF signaling promotes the secretion of type I interferons through IRF3 activation. Consequently, secreted interferons bind receptors on macrophages and stimulate the phosphorylation and activation of the second M1 transcription factor, STAT-1. STAT proteins form dimers and translocate to the nucleus [128]. While NF- $\kappa$ B activation depends on I $\kappa$ B- $\alpha$  and IKK $\beta$  regulation, STAT signaling is mainly controlled by the suppressor of cytokine signaling (SOCS) family. SOCS modulates the sensitivity of cytokine receptors and alters the signaling pathways induced in response to TLR stimulation [127-129].

In contrast to M1 macrophages, M2 macrophages are activated upon IL-4, IL-13, or IL-10 binding to their receptors, IL-4R, IL-13R, or IL-10R [108, 128, 129]. Binding to the receptors activates JAK1 and JAK3. Activation of JAK signaling through IL-4R $\alpha$  activates STAT-6 while IL-10 stimulation through IL-10R activates STAT-3 transcription factor. The activation of STAT-6 and STAT-3 induce their phosphorylation, dimerization, and nuclear translocation which results in an anti-inflammatory M2 macrophage phenotype [108, 127]. STAT-6 and STAT-3 activation is regulated via SOCS proteins and protein inhibitors of activated STAT (PIAS). SOCS proteins form a negative feedback loop whereby SOCS genes are induced following cytokine stimulation and inhibit cytokine signaling. Conversely,

PIAS regulate STAT signaling by binding the activated STATs and preventing them from binding to the DNA [108, 128].

M1 and M2 macrophages have distinct effectors and secrete different cytokines and mediators upon stimulation. Activation of STAT-1 and NF- $\kappa$ B signaling in M1 macrophages promotes the release of TNF- $\alpha$ , IL-1, IL-6, IL-12, Type I IFN, CXCL1-3, CXCL-5, and CXCL8-10. TNF- $\alpha$  and IL-1 $\beta$  are pro-inflammatory cytokines responsible for inducing fever, tissue destruction, hematopoiesis, leukocyte chemotaxis, and interferon secretion [108, 127, 128]. IL-6 is a pleiotropic cytokine with a wide range of pro-inflammatory effects and some regenerative and anti-inflammatory activities. IL-12 is a potent pro-inflammatory cytokine and it promotes the activation of cytotoxic T lymphocytes and natural killer cells. CXCL-8 is also called IL-8 and it is the major neutrophil chemotactic factor. Last but not least, IFN $\gamma$  promotes type 1 T helper cell responses (Th1 immunity). The other cytokines mediate inflammation, recruitment and migration of immune cells, and release of pro-inflammatory mediators [127, 128, 133].

Alternatively, activation of STAT-6 and STAT-3 promotes the release of anti-inflammatory mediators. This includes the expression of IL-10, YM1, and FIZZ1 by M2 macrophages. Additionally, activation of STAT-6 and STAT-3 upregulates the expression of Dectin-1, DC-SIGN, mannose receptor, scavenger receptor A, scavenger receptor B-1, and CD163 [128]. IL-10 is an anti-inflammatory cytokine which suppresses the expression of pro-inflammatory cytokines, inhibits NF- $\kappa$ B signaling, suppresses Th1 immunity and promotes a regulatory T cell phenotype. Ym1 and FIZZ1 promote Th2 cytokine expression, wound healing, and allergic airway inflammation. Moreover, mannose receptor functions as a pattern recognition receptor, plays a role in antigen uptake and presentation, mediates phagocytosis, scavenges unwanted mannoglycoproteins, and clears pro-inflammatory mediators during inflammation resolution. Additionally, scavenger receptors remove debris and waste materials [108, 127-130, 133, 134].

M1 and M2 macrophages also express distinct enzymes which are believed to play important immunomodulatory functions [127, 131, 132, 135, 136]. M1 macrophages release nitric oxide and reactive oxygen byproducts due to the upregulation of iNOS enzyme which metabolizes arginine into nitric oxide and citrulline. M2 macrophages express arginase instead which leads to the production of prolines and polyamines through arginase conversion of arginine into ornithine and urea [128]. The iNOS/arginase paradigm has been studied for its role in modulating the immune response. This hinges upon the competition between iNOS and arginase for the same substrate, arginine, as well as the distinct immunomodulatory effects of arginase [127-132, 135, 136].

#### b. Arginase-1 immunomodulatory properties

Arginase is a unique effector of alternative macrophages believed to have an immunomodulatory function, particularly through its competition with iNOS for their common substrate, arginine. iNOS induced by LPS and Th1 cytokines generates tremendous amounts of nitric oxide (NO). This results in an aggravated inflammatory response, contributes to bacterial clearance, and causes tissue injury [129-132]. Thus, the protective role of arginase-1 was initially expressed in terms of its competition with iNOS for arginine to limit the NO-derived inflammatory damage. This hypothesis has recently been challenged because of the important role of NO in macrophage cytotoxic function and bacterial clearance [132, 135]. Hopkins and colleagues addressed this issue by distinguishing the role of NO in chronic and acute PA lung infection [136]. Acutely, the exaggerated generation of NO, the reactive nitrogen species, peroxynitrite, and superoxide radicals does not only contribute to the clearance of pathogens but also results in serious lung injury and fibrosis. NO is essential for angiogenesis, smooth muscle relaxation, and inhibition of neutrophil and leukocyte chemotaxis and migration to the lung [136].

The anti-inflammatory role of arginase-1 is believed to be far beyond limiting NO generation. In fact, increased arginase-1 expression is associated with down-regulation of the immune response in several conditions including solid and hematological tumors, leishmaniasis, schistosomiasis, autoimmune encephalomyelitis, sepsis, pregnancy, trauma and experimental glomerulonephritis [137-150]. The immunomodulatory effects of arginase have been described in these disease models and they include: (1) suppressing Th1 mediated pathology; (2) modulating the interplay of Th1/Th2 cytokines; (3) shifting the macrophage/neutrophil balance; (4) suppressing immune recognition and rejection of tumor cells; (5) reducing CD8+ and CD4+ T cell proliferation; and (6) decreasing NO-mediated injury [137-150].

The most commonly described immunomodulatory property of arginase-1 includes the induction of T cell hyporesponsiveness through arginine depletion [151-161]. Arginine depletion results in decreased T cell proliferation, decreased T cell effector function, and significantly decreased cytokine release (IFN $\gamma$ , IL4, IL5, and IL10) [151, 152, 154-156, 157 2004, 158-161]. Activated T cells under arginine depleted conditions fail to express CD3 $\zeta$  and CD3 $\epsilon$  which results in decreased receptor-mediated tyrosine phosphorylation [162]. CD3 $\zeta$ , CD3 $\epsilon$ , and CD3 $\delta$  form the CD3 co-receptor essential for T cell activation and transduction of intracellular signaling pathways upon antigen stimulation. Decreased expression of CD3 impairs the immune response and impairs T cell development, lineage determination, and activation [153 2004, 154, 155, 156 2004, 158-161]. Decreased CD3 $\zeta$  expression in arginine-depleted conditions is associated with decreased cyclin D3 mRNA stability and expression which in turn prevents progression in the cell cycle and arrests T cells in the G0-G1 phase [157]. Impairment of T cell activation under arginine depleted conditions is T cell receptor (TCR) independent and is not mediated via inhibitory effects of CTLA4, IL10, PDL1, or TGF $\beta$  [144]. Moreover, the decreased T cell proliferation is not associated with an increase in apoptosis or T cell death [151]. Additionally, arginase-1 promotes a regulatory T cell phenotype independent of IL2, TGF $\beta$ ,

IL10, CTLA4, or PDL1 [144]. Although arginine depletion results in decreased expression of T cell activation markers (CD28, CD62L, and CD25), it does not affect macrophage activation [160]. Arginine depletion by arginase-producing M2 macrophages does not affect the expression of other M2 macrophage effectors (IL-10, Ym1, and Fizz1) [144, 160]. In most cases, arginase-mediated T cell suppression is localized to the site of infection and does not affect naïve T cells in lymph nodes and other secondary lymphoid organs [138-144]. Importantly, arginine depletion does not affect the expression of iNOS or arginase. Finally, this T cell suppression has been demonstrated to be reversible with arginase inhibition or with L-arginine supplementation in a variety of disease models [137-150].

Arginase-1 also plays an essential role in wound healing and protection against inflammatory injury [135, 163]. Arginase is thought to promote its effects on tissue remodeling by metabolizing arginine into prolines and polyamines thereby promoting collagen synthesis, cell proliferation, and tissue regeneration [163]. Inhibiting or deleting arginase is associated with delayed wound healing along with defects in matrix deposition in several disease models. Campbell et al. show a macroscopic increase in cutaneous wound area along with decreased reepithelization in arginase conditional knock-out mice (Tie2-cre-mediated deletion from endothelial cells) [163]. In cystic fibrosis, the increased risk for chronic bacterial colonization along with the exaggerated inflammation result in progressive airway wall thickening and lumen dilatation along with permanent scarring of the lungs. Arginase is thought to protect against the resultant scarring and to mediate airway remodeling [135, 163].

Collectively, arginase-1 is an effector of M2 macrophages which exerts immunomodulatory and anti-inflammatory properties. Arginase-1 was evaluated in different diseases where it is shown to suppress T cell immunity and to promote a regulatory phenotype. Thus, it is critical to define the role of arginase-1 in the setting of dysregulated immunity in cystic fibrosis. It is reported that

patients with cystic fibrosis express significantly high levels of arginase in their sputum along with decreased levels of NO. The increased arginase expression in patients with cystic fibrosis is believed to be mainly of neutrophil origin. Moreover, increased sputum arginase activity is reported in patients with end-stage disease, patients with disease exacerbations, and in patients with severe lung disease and bronchiectasis [109, 119, 123-126, 135, 143, 164]. According to Grasemann et al., patients with stable disease have significantly lower levels of arginase expression and arginase activity is significantly increased upon hospital admission for exacerbation. Thus, arginase activity negatively correlates with lung function [109]. Current clinical studies link arginase expression with cystic fibrosis pathology due to significantly reduced levels of NO required for bacterial clearance [109, 119, 123-126, 135, 143, 165]. However, the immunomodulatory properties of arginase in cystic fibrosis and its ability to regulate the immune response in chronic infections have not been investigated.

### c. Altered macrophage polarization

Proportional and balanced macrophage polarization is essential for effective control of inflammation. In response to any infection, different populations of macrophages are activated to play specialized roles in clearing bacteria and dead cells by phagocytosis, recruiting neutrophils and antigen presenting cells to the site of infection, as well as establishing and coordinating an efficient adaptive immune response.

In patients infected with PA, there are increased levels of M2 macrophages in their bronchoalveolar lavage fluids while M1 macrophages predominate in the nasal cavities of these patients [166, 167]. Similarly, macrophages in sputum samples of cystic fibrosis patients show reduced expression of M2 markers and tend to be predominantly M1 polarized [168, 169]. Additionally, Tarique et al. show that macrophages from cystic fibrosis patients as well as macrophages with inhibited CFTR fail to respond to IL-4 and IL-13; therefore, they fail to polarize to

an M2 phenotype [27]. However, polarization to an M1 phenotype is unaffected [27]. Thus, CFTR mutations contribute to the altered macrophage phenotype and promote an M1 predominant polarization. According to Levi, stimulation of cystic fibrosis macrophages with IFN- $\gamma$  and LPS induces a significantly greater increase in STAT-1 phosphorylation and activation compared to normal macrophages. This was also associated with significantly greater levels of pro-inflammatory cytokine release compared to normal macrophages [27, 167, 169]. Importantly, several studies report excessive and dysregulated stimulation of NF- $\kappa$ B transduction pathway in macrophages and epithelial cells [170].

Additionally, the lung environment in cystic fibrosis skews macrophage polarization depending on the stability of the disease and the nature of infections at specific timepoints. The regulatory environment in stable disease and the Th2 mediated immune responses drive macrophage polarization towards an M2 phenotype. Infections which favor a Th1 response are associated with an aberrant polarization of M1 macrophages [171]. These macrophages are recruited in excessive numbers and are more sensitive to external stimuli, resulting in robust signal transduction and excessive release of their characteristic cytokines. Gao and colleagues show that alveolar macrophages with mutated or inhibited CFTR have exaggerated activation of NF- $\kappa$ B and increased release of TNF- $\alpha$  [25]. Gao also shows increased chemotaxis and migratory abilities of macrophages with mutated CFTR [25]. Therefore, skewing macrophage polarization in cystic fibrosis is uncontrolled and results in an exaggerated immune response [134, 170-172].

#### d. Altered macrophage functions

Macrophage phagocytic function is affected by the absence or mutation of CFTR receptors normally expressed on the cell surface. Malfunctional CFTR receptors impair the ability of cystic fibrosis macrophages to clear engulfed pathogens [134, 172, 173]. CFTR is essential to control phagosomal pH which is critical for

macrophage bactericidal functions. This was demonstrated in CFTR knock-out mice as alveolar macrophages from these animals fail to acidify their phagolysosomes and therefore lack the ability to efficiently kill internalized microbes [174]. Additionally, cystic fibrosis causes a disruption in macrophage lysosome function and the ability to recognize PAMPs. Bruscia and colleagues show that cystic fibrosis macrophages are characterized by defective endosomes with delayed endosome/lysosome maturation during LPS challenge [106, 170]. This prevents trafficking of TLR4 from the plasma membrane to the lysosome resulting in profound stimulation of these macrophages and increased release of inflammatory mediators compared to wild-type macrophages [170]. Additionally, this defect contributes to long-term colonization of the cystic fibrosis lungs by PA which is able to mutate their recognition sequences in order to escape the recognition by macrophage TLRs [25, 175, 176].

Macrophages in cystic fibrosis also have decreased ability to clear dead neutrophils. Dead neutrophils are normally cleared from the airways by being expelled in the coughed mucus or through phagocytosis by alveolar macrophages. However, the impaired macrophage signaling pathways, the increased intraphagosomal pH, as well as the impaired trafficking of recognition receptors to the surface of macrophages render these cells inefficient in clearing the excessive number of dead neutrophils [134, 170-172]. Neutrophils also form extracellular traps which interact with macrophages and further impair the macrophage functions. Therefore, clearance of dead neutrophils is impaired in cystic fibrosis leading to accumulation of highly toxic substances [20, 25, 63, 133, 134, 170-175].

#### e. Altered alveolar and interstitial macrophages

Two major macrophage populations are normally present in the lungs, the alveolar macrophages and the interstitial macrophages. Alveolar macrophages are found in the airway lumen while interstitial macrophages reside in the lung

parenchyma [63, 133, 134, 172-174]. When alveolar and interstitial macrophages are activated they release mediators to recruit other macrophages from the circulation to assist in pathogen clearance. Alveolar macrophages are inherently suppressive and maintain homeostasis by clearing debris, recycling surfactant molecules, and preventing inflammation. However, they are the first line of defense which is initiated by the release of pro-inflammatory cytokines upon encountering a pathogen [63, 133]. Conversely, interstitial macrophages are very heterogeneous with versatile regulatory functions including the activation of IL-10 mediated adaptive responses. Alveolar and interstitial macrophages in cystic fibrosis are intrinsically modified with metabolic hyperactivity, excessive cytokine release, as well as the production of ciliary dyskinesia and tissue-damaging substances [63, 133, 177-182]. Additionally, several reports describe a remarkable increase in the numbers of tissue macrophages in cystic fibrosis and strongly correlate their altered functions with increased mucus accumulation and pulmonary exacerbations [63, 133, 134, 172-174, 183-185]. Moreover, tissue macrophages are very sensitive to CFTR mutations. A single allelic CFTR mutation is sufficient to exacerbate macrophage pro-inflammatory functions. Alveolar macrophages in cystic fibrosis have reduced ability to secrete IL-10 while they release tremendous amounts of IL-6, IL-8, IL-1, and TNF- $\alpha$ . Therefore, alveolar macrophages in cystic fibrosis are unable to exert their regulatory functions and they fail to establish homeostasis in the lungs. Additionally, alveolar and interstitial macrophages in cystic fibrosis express high levels of TLR-4 which is associated with significantly elevated and sustained signal transduction and activation of the NF- $\kappa$ B and MAPK pathways [63, 133, 177, 186, 187]. Tissue macrophages in the cystic fibrosis lungs also have impaired phagocytosis, impaired autophagy, impaired lipid raft composition, impaired vesicle trafficking, as well as impaired pH in the trans-Golgi, endosome, and lysosome [63, 133, 184, 185]. Collectively, these impairments render the tissue macrophages unable to engulf and clear pathogens [63, 133, 178-182].

In summary, macrophages present in the cystic fibrosis lungs fail to exert the essential functions required for pathogen clearance while coordinating an inflammatory response that is excessive and dysregulated. These changes are evident even before any signs of infection and continue to accumulate with non-resolving infections. The phagocytic and regulatory functions of these macrophages are impaired and thus are drastically deleterious to the host.

### III. Adaptive immunity in cystic fibrosis

The activation of an efficient and specialized adaptive immune response is crucial to completely clear and eradicate infections. There are two main categories of cells which carry out adaptive immunity: B cells and T cells. Both are antigen-specific and selectively expand in lymphoid organs after recognition of foreign antigens presented to them by antigen presenting cells (APC). Similar to the innate system, adaptive immune cells exert regulatory functions which terminate the inflammatory response. Additionally, adaptive immunity provides long-lasting immunological memory. Disruption of adaptive immune responses in patients with cystic fibrosis is associated with disease pathology and is a result of the disturbed communication with the innate immune cells, environmental factors which skew T and B lymphocyte responses, as well as intrinsic factors like CFTR mutations and altered signaling [63, 133, 182]. The following section discusses the main elements of the adaptive immune response and how they are altered in patients with cystic fibrosis.

#### i. Impaired communication between innate and adaptive immunity

In cystic fibrosis, innate immune cells display a decreased efficiency in antigen presentation and to provide necessary costimulation for B and T cell selection. The communication, whether direct or indirect, between the innate and adaptive immune cells is essential to selectively induce the expansion of antigen-specific B and T lymphocytes. Positive selection requires antigen presentation through

the MHC receptors on APC. Macrophages and dendritic cells process antigens from the site of infection and present them for lymphocyte selection in the lymphoid organs. When T or B cells get activated, they require costimulation by a second signal in order to expand and differentiate. Costimulation involves cytokine signaling for lymphocyte differentiation and proliferation (like IL-2, IL-4, IFN) as well as costimulatory receptors (like B7 and CD28) that govern cognate interactions between cells [182]. In cystic fibrosis, non-CFTR genetic mutations disrupt the communication between innate and adaptive immunity. In fact, Wright and colleagues show that disease severity is directly correlated with the MHC gene polymorphisms in cystic fibrosis [182]. These polymorphisms are related to the failure of appropriate antigen processing and presentation. For instance, dendritic cells from cystic fibrosis patients express lower levels of MHC-II on their surface compared to dendritic cells from healthy volunteers [63, 133, 183]. Specific mutations were also associated with increased infections with PA including the DR7/DQA\*0201 mutation while HLA-DQB1\*0201 increases the risk for diabetes in cystic fibrosis patients. Additionally, genetic variations of MHC alleles predict susceptibility to PA infections. Expression of HLA-DR7 in cystic fibrosis patients is associated with increased risk for PA colonization while HLA-DR4 expression is associated with reduced frequency of PA colonization [188]. In addition, neutrophil elastases are capable of cleaving MHC receptors from antigen-presenting cells. Additionally, B lymphocytes are essential for antigen presentation required for effector and memory T cell priming and induction. B cells from cystic fibrosis patients are characterized with an inefficient uptake, processing, and antigen presentation on their B-cell receptors [189-191]. Additionally, T and B cells with mutated CFTR lose the appropriate membrane polarization potential due to abnormal ion flux across the CFTR channels thus losing the essential ion clustering along the membrane for stabilizing the interaction between these lymphocytes and the antigen presenting cells.

While immune cells fail to present antigens effectively, costimulatory signals are also altered in cystic fibrosis lungs [133, 182, 183, 188-192]. Evidence suggests

that PD-L1/PD-1 interaction between airway epithelial cells and T cells is suppressed with reduced PD-L1 expression on cystic fibrosis epithelial cells. Additionally, epithelial cells control costimulation by intrinsically modulating their cytokine release. They skew T lymphocytes into a Th2 response by releasing IL-4 and they control T cell proliferation by releasing IL-2. Finally, the local release of different pro- and anti-inflammatory cytokines, as well as growth factors, by different immune cells directly influence the clonal selection and the differentiation of T and B lymphocytes [189-192]. Collectively, genetic and inflammatory factors contribute to the failure of coordinated activation of the adaptive immune response in cystic fibrosis.

## ii. Skewed T cell responses

Differentiation of T lymphocytes into different effector cells is altered in cystic fibrosis due to CFTR mutation, intrinsic alterations, and environmental factors (summarized in Figure 1.1b). T cell responses involve the activation of T helper (Th) lymphocytes which are divided into different subpopulations. Type-1 or Th1 lymphocytes are pro-inflammatory and are polarized by IFN- $\gamma$  predominantly produced by natural killer (NK) and natural killer T (NKT) cells and by Th1 and cytotoxic T lymphocytes [192-194]. Th1 cells are involved in clearance of intracellular pathogens and they polarize macrophages into an M1 phenotype thus promoting their microbicidal function [192-194]. Th1 lymphocytes are stimulated through IFN- $\gamma$  mediated activation of the STAT-1 signaling pathway which activates T-bet, the main Th1 transcription factor. Th1 lymphocytes release IL-2, IFN- $\gamma$ , TNF- $\alpha$ , macrophage inflammatory protein-1 alpha (MIP-1 $\alpha$ ), and MIP-1 $\beta$  [192-194]. Additionally, IL-12 from M1 macrophages can activate STAT-4 which binds and activates T-bet. Conversely, Type-2 or Th2 lymphocytes are polarized by IL-13 or IL-4 and release anti-inflammatory cytokines, mediate humoral immunity, and clear extracellular bacteria and parasites. IL-4 activates the STAT-6 signaling pathway which stimulates GATA3 activation, the Th2 transcription factor. Additionally, IL-4 is a negative regulator of STAT-4 while IFN-

$\gamma$  is a negative regulator of STAT-6; thus, the polarization into a specific Th cell subset negatively regulates the other [192-194].

A third population of T helper cells includes the Th17 lymphocytes which are strongly associated with morbidity in cystic fibrosis. Th17 lymphocytes are polarized in the presence of IL-6, IL-23, and TGF- $\beta$  and are responsible for the production of neutrophil chemokines, IL-17 and IL-8 [193]. TGF- $\beta$  along with IL-6 activate STAT-3 which stimulates the Th17 transcription factor, ROR $\gamma$ t. IL-23 also activates STAT-3 by stimulation of IL-23R. Th17 lymphocytes are considered pro-inflammatory due to the potent ability of IL-17 to induce the release of TNF- $\alpha$ , IL-1 $\beta$ , and MCP-1 [194]. Th17 lymphocytes and their contribution to inflammatory lung injury and pathogenesis in cystic fibrosis are discussed in later sections. However, Th17 lymphocytes are the main source of neutrophil chemoattractant factors. IL-17A and IL-17F are elevated in the sputum of patients with cystic fibrosis and correlate with the excessive neutrophil recruitment [164, 194-207]. Chronic infections with PA and other Gram-negative bacteria skew the T cell responses towards a Th17 predominant response [194].

The fourth subset of T helper lymphocytes is referred to as regulatory T cells (Tregs). Tregs naturally exist in the thymus and can be polarized peripherally in the presence of TGF- $\beta$  [195]. Tregs express CD25 (IL-2 $\alpha$ ) and FoxP3, both of which are essential for the suppressive activity of these lymphocytes. Tregs release anti-inflammatory mediators upon activation including IL-10, IL-35, and TGF- $\beta$ . Tregs are significantly decreased in the peripheral blood and airways of patients with cystic fibrosis compared to healthy individuals. Moreover, patients with chronic infections like PA have reduced numbers of Tregs [195, 205] and the numbers of Treg lymphocytes in cystic fibrosis lungs positively correlate with lung function [195, 205-207]. Additionally, Treg lymphocytes with mutated CFTR fail to suppress T cell responses and promote regulated immunity. According to Hector et al., infections with PA promote suppressed Treg numbers, impaired Treg regulatory functions, and impaired Treg memory development [207].

T lymphocytes exhibit unique plasticity and are able to convert from one phenotype to another [164, 194-207]. For instance, IL-6 can downregulate FoxP3 expression in Tregs and increase their secretion of IL-17 thus converting them into Th17 phenotype [164, 194-207]. Additionally, an intermediate phenotype of Th17 and Th1 lymphocytes has been described where cells co-express IFN- $\gamma$  and IL-17 [194]. Th2 lymphocytes stimulated with IFN- $\gamma$  also co-express GATA3 and T-bet. It is important to note that two new subsets of Th lymphocytes were discovered, and they include Th9s which release IL-9 and Th22s which release IL-22 [194]. Th9 lymphocytes mediate immunopathology and mitigate allergic inflammation and fibrosis in patients with cystic fibrosis. Th22 lymphocytes observed to play a role are in bacterial infections and help in microbe clearance [164, 194-208].

In cystic fibrosis, T helper lymphocyte populations contribute to pulmonary pathology and excessive inflammation. The specific role of each population and the different environmental factors that alter these responses are discussed in the following sections.

#### a. CFTR in lymphocytes

CFTR is expressed on B and T lymphocytes and its mutation contributes to the alteration of B and T cell responses. Lack of functional CFTR expression on B cells is associated with suppressed ability to produce  $\lambda$  light chain upon stimulation [196]. Lack of  $\lambda$  light chain significantly affects B cell response against PA and the clearance of this pathogen [164, 196-206]. Additionally, lack of functional CFTR expression on T cells is associated with exaggerated IgE levels and a skewed proallergic Th2 response [164, 197-199]. Increased IL-4, IL-13 and IgE levels in CFTR deficient T cells results in a Th2 dominated response which is effective against parasites but cannot clear PA infections [164, 196-199]. Several studies in CFTR knock-out mice and in CFTR-deficient lymphocytes show the

direct correlation between CFTR mutations and allergic inflammation in cystic fibrosis [164, 197-199]. CFTR knock-out mice infected with PA produce defective Th1 lymphocytes which can release IL-12 but do not exhibit other Th1 properties. This was explained by the altered calcium flux in T cells which lack CFTR [196, 200-202]. Increased calcium signaling alters the expression of calcium-sensitive genes including those involved in Th1/Th2 differentiation. According to Mueller, increased calcium signaling in T cells activates NFAT transcription factor which increases Th2 cytokines (IL-4, IL13, and IL-6) along with increased IgE synthesis in B cells leading to suppression of Th1 cytokines and therefore creating an allergic inflammatory environment in the cystic fibrosis lungs [200, 201]. Moreover, the altered ion flux across the CFTR deficient T cells results in membrane depolarization due to retention of intracellular chloride concentrations thus altering the surface signaling pathways and resulting in abnormal gene expression [202]. Collectively, CFTR mutation in cystic fibrosis skews lymphocytic responses by altering the different signaling pathways and machineries in B and T cells.

#### b. Intrinsic and environmental lymphocyte alterations

Lymphocytes in cystic fibrosis are skewed into a Th2 and T17 predominant immune response due to inherent predisposition as well as abundance of inflammatory signals and cytokines which polarize these cells. Inherent predisposition is described by the ability of naïve T cells isolated from cystic fibrosis patients to be polarized into Th2 and Th17 phenotypes [203-206]. Moreover, there is a significant imbalance between driving and opposing signals and cytokines involved in Th2 and Th17 polarization. In fact, Th2 and Th17 lymphocytes are present in excessive amounts and are believed to mediate pathology in cystic fibrosis [203, 204]. Kushwah et al. provide evidence on the intrinsic tendency of naïve T cells from cystic fibrosis patients to polarize towards the Th17 phenotype [205]. Naïve T cells were isolated from mice and humans and examined in response to different polarizing cytokines and stimulants [205].

They found that T cells, with or without mutated CFTR channels, can equally differentiate under the influence of appropriate cytokines into Th1 and Treg phenotypes. However, when T cells with CFTR mutation were polarized with Th17 stimulants, there was at least a 2 times higher and faster shift into the Th17 phenotype compared to wild-type lymphocytes. Similarly, at least double the number of naïve lymphocytes from cystic fibrosis patients polarized into Th17 phenotype compared to lymphocytes from healthy volunteers. Simultaneously, there was equal differentiation of naïve T lymphocytes into Th1 and Treg phenotypes from healthy and diseased populations [205]. Interestingly, a case report from a 16 year old female diagnosed with cystic fibrosis shows T cell unresponsiveness despite the fact that she was not receiving steroids or any other immunomodulatory therapy. T cells isolated from her blood failed to produce IFN- $\gamma$  and ex vivo stimulation of naïve T cells failed to differentiate into either Th1, Treg, or Th17 phenotypes [206]. This patient had greater severity and faster progression of her disease. While intrinsic impairment in T cell differentiation might be in part related to CFTR mutations, the exact intrinsic mechanisms by which naïve T cells from cystic fibrosis patients polarize to a specific phenotype are yet to be elucidated [206].

Several other factors drive lymphocyte polarization in patients with cystic fibrosis including the suppressed Treg numbers and the exaggerated release of cytokines released by epithelial cells and macrophages. The numerous Th2 and Th17 lymphocytes are attributed to the fact that there are minimal numbers of Tregs [207]. The deficiency in Tregs creates an imbalance in the pro- and anti-inflammatory cytokines. Thus, the lack of IL-10 from Tregs and the pro-inflammatory lung environment in cystic fibrosis promote polarization of T lymphocytes into the Th17 subtype [205-207, 209]. Additionally, lower numbers of Tregs in cystic fibrosis patients are associated with increased Th2 lymphocytes and reduced lung function. M2 macrophages and epithelial cells release TGF- $\beta$  and IL-6 which further polarize T cells towards a Th17 phenotype. Moreover, excessively elevated levels of IL-1 $\beta$ , TNF- $\alpha$ , and IL-21 in the cystic

fibrosis lungs, along with TGF- $\beta$ , strongly drive Th17 polarization [67, 207, 209-219]. Th17s also secrete IL-21 which results in a vicious cycle of continual Th17 stimulation. IL-6 by itself upregulates IL-23R expression, the latter is stimulated with IL-23 binding which further promotes the expansion and maintenance of Th17 lymphocytes. Other environmental factors which contribute to increased Th17 polarization include the change in lung microbiota and prolonged infections [209]. In fact, high concentration of antigen and microbial stimuli synergistically stimulate Th17 differentiation by upregulating CD40 expression on T cells and increasing IL-6 release by dendritic cells. Strong CD40/CD40 ligand interaction in this way preferentially drives Th17 polarization [209-214]. Collectively, intrinsic and environmental alterations skew the T lymphocytes in cystic fibrosis towards Th2/Th17 phenotype.

Th17 lymphocytes and IL-17 production are associated with poor prognosis and play an early role in disease pathology. In fact, IL-17 has been found to be involved in several inflammatory diseases including multiple sclerosis, rheumatic diseases, inflammatory bowel disease, asthma, and atopic dermatitis [210]. In cystic fibrosis, human lung lavage and sputum samples show elevated concentrations of IL-17 and IL-23, especially in patients infected with PA infections [211-213]. IL-17 is strongly associated with disease pathology via upregulation of mucin-producing genes [214, 215]. Th17 cells provide a continuous signal for sustained neutrophil inflammation which further aggravates lung injury [216-220]. Th2 and Th17 lymphocyte populations also predict disease progression. In a study of T cell phenotypes in 57 cystic fibrosis patients, stable patients had augmented numbers of Th1, Th2, and Th17 lymphocytes compared to normal individuals. Cystic fibrosis patients with PA colonization had significantly higher numbers of Th17 lymphocytes compared to non-cystic fibrosis patients [67]. Moreover, symptomatic patients with PA or fungal infections had significantly higher concentrations of IL-17, IL-13, and IL-5 compared to asymptomatic patients. Immune responses in symptomatic patients were Th2/Th17 skewed and correlated with increased neutrophils and high-resolution

computed tomography changes (fibrosis) [67]. These changes, interestingly, were independent of IFN- $\gamma$  levels. There were higher levels of Th2 and Th17 cytokines in the bronchoalveolar lavage fluids of patients prior to infections with PA and the levels of these cytokines positively predicted the development of PA infections [67]. Moreover, decreased IFN- $\gamma$  levels and defective Th1 responses were also predictive of increased PA infection frequency [67]. Another study shows increased Th17 cytokines in the lungs of PA infected cystic fibrosis patients, and the authors report a strong negative correlation between Th17 levels and lung function. This relationship remained significant even after antimicrobial treatment [221].

It is important to note that Th17s and IL-17 are key elements of cystic fibrosis pathology as they are present very early in the disease and are maintained and remain elevated throughout the course of the disease. As the disease reaches the end stages, high IL-17 concentrations persist. This is partially explained by the fact that Th17 cells exhibit immunological memory and that other immune cells secrete IL-17 [222]. Non-Th17 cells which secrete IL-17 are thought to play a role in later more progressive stages of the disease and include the NKT cells and the  $\gamma\delta$  T-cells [211-213]. IL-17 producing  $\gamma\delta$  T-cells result in hyperinflammatory granulomatous disease and are associated with severe and fatal lung damage [209]. Cystic fibrosis patients infected with PA have increased numbers of  $\gamma\delta$  T-cells which release large amounts of IL-17, TNF- $\alpha$  and IFN- $\gamma$  thereby aggravating inflammation and lung injury [67, 223, 224]. Similarly, NKTs are pro-inflammatory cells which function to maintain a profound activation of the immune response by attracting other immune cells. NKT cells express ROR $\gamma$ t and recruit large numbers of neutrophils by releasing IL-17. In cystic fibrosis, a significant decrease in the Treg/NKT ratio is observed. This further relates to the constantly activated inflammatory response with persistent neutrophilia and airway hyperresponsiveness [209, 225, 226].

In summary, adaptive immunity is altered in cystic fibrosis and is directly related to CFTR mutations as well as to intrinsic T and B cell alterations. Changes in ion flux and signaling transduction along with changes in the lung microenvironment and the predominance of specific cytokines drive lymphocyte polarization and dysfunction. Th2 and Th17 lymphocytes predict lung function and acquisition of PA infections while they contribute to cystic fibrosis pathology and lung damage.

#### IV. Dysregulated inflammation

There is evidence that inflammation exists in cystic fibrosis even before any signs of infection or lung disease [186]. Scientists originally thought of excessive inflammation in cystic fibrosis as a consequence of chronic and repetitive infections. However, in a review of primary literature and clinical studies by Rao et al., the authors propose that the cause of inflammation in cystic fibrosis extends far beyond infection [186]. Rao presents inflammation as an important pathological component of cystic fibrosis. Inflammation in cystic fibrosis is exaggerated, results in lung injury, persists after pathogen clearance, and is present even before the first pulmonary infection [186]. Human sputum and bronchoalveolar lavage samples reflect an established pro-inflammatory state in cystic fibrosis lungs independent of the presence of an active infection. Several clinical studies show at least 10 fold more neutrophils in uninfected cystic fibrosis lungs compared to normal humans with or without lower respiratory tract infections. Moreover, there is an elevated presence of pro-inflammatory cytokines including TNF- $\alpha$ , IL-6, IL-8, and LTB-4 in un-infected cystic fibrosis lungs as well [173, 227, 228]. There is an increased number of macrophages in un-infected cystic fibrosis lungs. These macrophages are believed to have a more profound ability to release mediators and to induce an inflammatory state compared to macrophages from non-cystic fibrosis lungs [186]. Studies show that in the absence of any infection, macrophages in cystic fibrosis lungs are polarized towards a pro-inflammatory M1 phenotype [173, 184]. Naïve human

macrophages from cystic fibrosis patients stimulated with LPS show significantly higher stimulation of the NF- $\kappa$ B and MAPK signaling pathways, higher levels of pro-inflammatory cytokine release, and a decreased ability to produce the anti-inflammatory cytokine IL-10 [134, 172-174, 181, 229-232]. Similarly, T lymphocytes from cystic fibrosis lungs have an increased pro-inflammatory potential in culture when compared to T lymphocytes from healthy volunteers [231].

In addition, cells that make up tracheal and epithelial tissues are also capable of producing dysregulated inflammation. In a study by Tirouvanziam et al., naïve tissues from newborns with cystic fibrosis were grafted into immunocompromised mice to investigate the inflammatory potential of cystic fibrosis lung tissues [233]. When fetal tracheal tissues were transplanted into severe combined immunodeficient mice, there was an eight-fold greater increase in IL-8 secretion as compared to mice which received non-cystic fibrosis tracheal grafts. When these mice were infected with PA, there was a rapid increase in leukocyte migration and spread of PA into the lamina propria within 3 hours in mice which received grafts from diseased neonates [233]. In mice with grafts from normal neonates, the inflammatory response occurred later and at a slower rate after 6 hours of infection. The inflammation in mice grafted with tissues from cystic fibrosis neonates was associated with severe exfoliation of the lung epithelium along with damage in the mucosa which was not otherwise observed in mice with normal grafts. These pulmonary changes are believed to create a niche for PA growth and replication which is thought to resemble the chronic infection in humans [233]. This study supports the idea that CFTR mutations predispose to an inflammatory state in cystic fibrosis patients independent of infection. And that in the presence of an infection, the already primed and inflamed lungs of cystic fibrosis patients respond in an exaggerated manner to invading pathogens.

Additionally, Khan et al. analyzed lavage fluids from the lungs of 16 human newborns with cystic fibrosis whose cultures were negative for any possible

infection (bacterial, viral, or fungal) [234]. In comparison with normal infants, the lavage fluids of the diseased population had significantly higher levels of neutrophils, neutrophil elastases, anti-protease inhibitors, and IL-8. Collectively, this study by Khan, in addition to many similar studies, confirm the inverse relation between neutrophil counts and lung function in cystic fibrosis which is again independent of the infection state [185, 234-240].

Cystic fibrosis patients also have a reduction in the typical anti-inflammatory set point of the pulmonary environment, represented by a decrease in the concentrations of IL-10, nitric oxide, and lipoxin-A4. IL-10 acts to stop the inflammatory response by suppressing the pro-inflammatory transcription factors, shutting down the synthesis of inflammatory mediators, and inducing the regulated-cell death of neutrophils. The reduction in nitric oxide prevents bacterial killing and relaxation of the airways. And finally, lipoxin-A4 is essential to limit the neutrophil-mediated inflammatory response [228, 241-250]. These changes exist along with the pre-described pro-inflammatory state. Thus, there is a disrupted balance of the inflammatory state in patients with cystic fibrosis.

Another factor that clearly relates to the inflammatory picture of cystic fibrosis which is independent of infection is the presence of reactive airway (asthma-like) signs and symptoms. In fact, smooth muscle cells express CFTR receptors. When the latter are mutated they are believed to impair the smooth muscle contractility due to impairment of ion flux [248]. Additionally, smooth muscle cells in children and adults with cystic fibrosis were observed to be affected with hyperplasia and hypertrophy which impairs their contraction [12, 249]. The airway hyper-responsiveness and the asthma-like phenotype are also attributed to inflammatory mediators which induce smooth muscle contractions. In response to these inflammatory cytokines, smooth muscle cells can release IL-8 which further contributes to the inflammatory process. Additionally, the excessive release of IL-4 and IL-13 by T lymphocytes with mutated CFTR (Th2s) drives airway hyper-responsiveness directly and through the increased production of

IgE [68]. This presents an inflammatory pulmonary disease overlapping with cystic fibrosis pathology and independent of infection state [248-250].

Collectively, the evidence presented distinguishes the inflammatory process as a unique pathological element of cystic fibrosis. This is supported by the facts that inflammation is (1) present prior to any infection; (2) is excessive compared to the number of infective microbes; (3) contributes to lung injury and damage; (4) and is innately skewed towards a pro-inflammatory response. This further emphasizes that dysregulated inflammation is a key factor in cystic fibrosis pathology to be closely considered for therapeutic targeting and treatment.

## V. Current treatments for cystic fibrosis

Current treatment options for patients with cystic fibrosis include supportive treatment to improve breathing and lung function, as well as symptomatic treatment targeting the infections and pulmonary inflammation. Most of the currently available anti-inflammatory and immunomodulatory therapies fail to effectively resolve the dysregulated inflammation without drastically impairing the immune response [84 2001, chmiel, 1999, 97, 250-279]. Several elements of the disrupted inflammation and the dysregulated innate and adaptive immune responses have been considered for development of new therapeutic targets.

### i. CFTR based therapies

Novel therapeutic approaches aim at targeting CFTR function. As discussed earlier, CFTR mutations contribute to altered inflammation in several aspects [250-256]. Patients with cystic fibrosis have 2 mutated CFTR alleles which result in decreased number and malfunction of the CFTR channels. Genotype-directed therapies use genetic approaches to restore expression and function of CFTR channels. The personalized approaches have been successful in restoring epithelial and phagosomal functions; however, they are of limited usefulness in

advanced disease stages [250-253]. Additionally, small molecules have been developed as CFTR modulators. These modulators target specific CFTR variants and mutations that are associated with a minimum level of CFTR protein expression. Therefore, these CFTR modulators potentiate and improve the function of CFTR expressed on cell surface level [250-253]. Ivacaftor, lumacaftor/ivacaftor, and tezacaftor/ivacaftor are FDA approved CFTR potentiators. They improve ion flux across the channels, improve lung function, and reduce morbidity and mortality. However, the use of these CFTR modulators remains limited for patients with a particular mutation [254-256].

## ii. Anti-inflammatory therapies

### a. Corticosteroids

Corticosteroids are the main anti-inflammatory agents used in cystic fibrosis patients [81, 257]. They are widely available and efficiently dampen airway inflammation in a global and non-specific way. There are several oral, inhaled, and intravenous corticosteroid formulations that are extensively used in patients with cystic fibrosis [81, 256]. Corticosteroids are effective in slowing disease progression and suppressing excessive inflammation. However, long-term steroid use is associated with many adverse events including growth retardation and even decline in lung function [256-259]. Nonetheless, steroids are broadly used in cystic fibrosis as targeted anti-inflammatory drugs are not available.

### b. Therapies targeting neutrophil recruitment

Multiple approaches aim to correct specific alterations of the immune response [8]. Clinicians and immunologists are attempting to target the signaling pathways involved in neutrophil recruitment. For instance, a human monoclonal antibody against IL-8 is proving to be promising in clinical trials [260]. Besides targeting IL-8, studies in mice show that neutralizing IL-17 is also promising and it effectively

reduces neutrophil influx in response to PA [260-262]. However, targeting IL-17 with neutralizing antibodies is challenging due to the importance of this cytokine in the clearance of pathogens [8, 260-262].

Other therapies directed against neutrophils include the attempt to deliver or stimulate the endogenous release of anti-proteinases ( $\alpha$ 1-proteinase inhibitor) [105, 263-267]. Several studies are evaluating the potential to formulate appropriate dosage forms or genetic approaches to achieve therapeutic levels of these proteinase inhibitors [105, 263-266]. A recent study just passed phase-II clinical trials where they show limited toxicity with an inhaled form of  $\alpha$ -1 antitrypsin (a human anti-protease) [267].

Moreover, new therapies are directed to clear the pathogenic DNA released from dead neutrophils and bacteria [268, 269]. The release of DNA fragments results in increased mucus thickness and activation of several inflammatory cascades. Recombinant DNase was evaluated in several human trials where it proved to reduce mucus thickness, decrease neutrophil influx and recruitment, improve lung function, and decrease exacerbations [268, 269]. In fact, some DNases are FDA approved for use in cystic fibrosis.

### c. Therapies against NF- $\kappa$ B signaling pathway

Several approaches target specific pathways involved in exaggerated inflammation including that governed by NF- $\kappa$ B [84 2001, chmiel, 1999, 97, 257-259, 270, 271, 279]. Recombinant IL-10 succeeds in regulating inflammation by inhibiting NF- $\kappa$ B activation in murine models of PA pneumonia. However, it has not yet been assessed in humans [280]. A phase-II clinical trial is currently evaluating the efficacy of Genestein which is a tyrosine kinase inhibitor that stimulates CFTR and inhibits NF- $\kappa$ B activation [270, 271]. Other therapies in development which target NF- $\kappa$ B activation in cystic fibrosis include (1) HE3286, which is currently in phase I/II clinical trials; (2) azithromycin, which passed a

phase III clinical trial and is suggested to inhibit NF- $\kappa$ B and AP1 activation; (3) curcumin, which is proven to block NF- $\kappa$ B activation and rescue mutated CFTR from degradation; (4) and ibuprofen and fenretinide/docosahexaenoic acid (in phase II clinical trial), which are shown to regulate NF- $\kappa$ B activation and lipid-raft formation and clustering [97].

A major concern for developing an NF- $\kappa$ B inhibitor is the threat to suppress immunity and pathogen clearance. Attempts are being made to target NF- $\kappa$ B activation locally in the lungs by developing dosage forms that deliver the inhibitors to the lungs directly. Alternative approaches include screening for specific molecular targets that would provide controlled inhibition of NF- $\kappa$ B without global inhibition. Therefore, optimizing currently available therapies by identifying specific pathways by which they exert their beneficial effects is essential to overcome the failure and adverse drug effects of the current therapies.

#### d. Cell-based therapies

Cellular therapy is also an interesting and promising venue for cystic fibrosis treatment [272-274]. Clinical studies show a significant reduction of bacterial replication and infectivity along with increased soluble bactericidal substances in the airways of cystic fibrosis patients treated with human mesenchymal stem cells. Other cellular therapies include the restoration and transfer of engineered epithelial cells, monocytes, or macrophages [272-274]. Some of these approaches made it into clinical trials while others are still at the level of experimental animal models [272-274].

#### iii. Antimicrobial therapies

Scientists are developing new antimicrobial molecules and evolving better ways to deliver the currently available antibiotics [275-277]. New dosage forms deliver

high concentrations of antibiotics locally into the lungs which can allow the compounds to reach into the inaccessible areas of infection in the cystic fibrosis airways. Rapamycin and azithromycin can be successfully delivered in high concentrations packed in engineered nano-particles [275, 276]. Other therapies directed against the infecting microorganisms include the development of viral phages to kill the colonizing bacteria [277]. However, resolution of chronic infection is promising, no evidence is available on how helpful it would be because inflammation is dysregulated independent of the infection status.

In summary, cystic fibrosis pathology is based on the chronic infections and the non-resolving pulmonary inflammation. Our increased understanding of the different components of cystic fibrosis pathology and the specific alterations in the immune system and the lung microenvironment identifies novel therapeutic targets. In fact, many therapies in development are targeting these specific alterations including (1) therapies which restore CFTR function; (2) therapies which control neutrophil inflammation; (3) therapies which control NF- $\kappa$ B activation; (4) therapies which restore functional immune cells; (5) and therapies which clear non-resolving infections. Additionally, the advancement in therapeutic options necessitates the consideration of combination therapy that would target infection, inflammation, genetic, and symptomatic components of this complex disease [84 2001, chmiel, 1999, 97, 250-278].

## VI. Azithromycin use in cystic fibrosis

Macrolide antibiotics constitute a unique therapeutic option proven to be tremendously effective in cystic fibrosis patients colonized with PA [281-285]. Macrolides exhibit anti-inflammatory properties and are used to control dysregulated inflammation. Macrolide antibiotics include azithromycin, erythromycin, clarithromycin, and roxithromycin. Macrolide antibiotics inhibit bacterial growth by binding and inhibiting the 50s ribosomal subunit thereby inhibiting protein synthesis by blocking the translation of mRNA into proteins in

bacteria [281]. However, the beneficial effects of macrolide antibiotics in cystic fibrosis are independent of their antibacterial activity. In fact, several studies evaluated the anti-inflammatory properties of the four macrolides mentioned. In a study by Ianaro et al., treatment with either macrolide reduced leukocyte infiltration, exudate volume, as well as reducing the pro-inflammatory cytokine burst in an acute pleuritic model in rats. The four macrolides reduce TNF- $\alpha$ , IL-1 $\beta$ , IL-6, and nitric oxide levels in normal and in inflamed lungs [282]. The four macrolides were also evaluated in several inflammatory diseases in humans where they proved to control neutrophil migration, oxidative burst in phagocytes, as well as pro-inflammatory cytokine release [281-285]. Additionally, the four macrolides share the ability to accumulate intracellularly in macrophages and neutrophils suggesting a possible interaction with leukocytes to control inflammation [282-285]. However, the specific mechanism underlying the anti-inflammatory properties remains unclear. In cystic fibrosis, azithromycin received special attention and it proved to exert clinically beneficial effects without considerable adverse reactions. This section summarizes the clinical studies which evaluate azithromycin anti-inflammatory properties in cystic fibrosis and presents the cellular and molecular mechanisms involved.

#### i. Azithromycin antimicrobial spectrum and pharmacokinetic properties

Azithromycin is effective against some Gram-positive, some Gram-negative, and some atypical bacteria [281-295]. The drug's spectrum of activity covers some strains of *Staphylococcus*, *Streptococcus*, *Haemophilus*, *Chlamydia*, *Mycoplasma*, *Neisseria*, *Moraxella*, *Legionella*, and *Prevotella*. Additionally, azithromycin is orally bioavailable and peaks within 2-3 hours in adult humans [281, 282]. Azithromycin accumulates in tissues and intracellularly- particularly in macrophages. Thus, tissue levels are at least 50 fold greater than plasma concentrations. Additionally, azithromycin is characterized with a very long half-life estimated to be about 35-40 hours and can be as high as 68 hours following a single 500 mg dose [286-289]. Despite its large therapeutic window,

azithromycin can cause gastrointestinal toxicity and cardiotoxicity (including QT-prolongation and arrhythmias). The FDA has issued a warning concerning the cardiotoxicity with azithromycin which indicates that the risk is very low in patients with no coexisting risk factors [289-293]. However, the use of azithromycin should be closely monitored in patients with pre-existing cardiac problems, arrhythmias, baseline QT prolongation, electrolyte disturbances, and kidney problems [290-295].

## ii. Anti-inflammatory effectiveness of azithromycin

Several clinical studies have evaluated the effectiveness of azithromycin in lung infection and inflammation [296]. Azithromycin is particularly beneficial in inflammatory lung diseases with non-resolving infections. In fact, azithromycin was used in 1982 against panbronchiolitis where patients suffer from chronic pulmonary inflammation and PA infections. Azithromycin has proven in randomized clinical trials in patients with panbronchiolitis to improve lung function and outcomes in patients infected with PA. PA virulence was shown to be reduced with long-term azithromycin treatment, primarily its ability to form biofilms [297, 298]. However, it is important to note that azithromycin does not inhibit or kill PA. Thus, these studies provided early evidence of an alternative mechanism involved with azithromycin beneficial effects which is independent of azithromycin antibacterial activity [296].

In patients with cystic fibrosis, five large clinical trials have evaluated the safety and anti-inflammatory efficacy of chronic azithromycin administration. Southern et al. performed a meta-analysis of these clinical trials comparing azithromycin therapy against placebo and including at least 959 patients from different ages [296]. The following section presents the main outcomes of the five clinical trials.

#### a. Improved lung function

Chronic azithromycin administration in patients with cystic fibrosis improves lung function. Three of the clinical trials show a significant improvement in lung function in patients on azithromycin (measured by forced expiratory volume, FEV1) [299-301], while the other two trials reported that azithromycin treatment did not alter the FEV1 in patients with cystic fibrosis [302-305]. Nevertheless, the grouped meta-analysis of all the data from the above-mentioned trials supports the fact that azithromycin improves lung function in adults and children with cystic fibrosis at a 6-month treatment timepoint [296]. Additionally, there was a significant improvement in the forced vital capacity (FVC) in patients who received azithromycin. However, these studies did not distinguish the effects of azithromycin on FEV1 or FVC in the context of chronic PA infections. The five trials either excluded the patients with PA infections or included them randomly along with other patients not infected with PA [296]. Therefore, there are no studies that directly compare the effects of azithromycin vs placebo in cystic fibrosis patients infected with PA.

#### b. Reduced exacerbations and improved quality of life

Another endpoint evaluated in these trials include the effects of azithromycin on the exacerbation frequency [296]. Exacerbations recorded in these trials were either protocol defined or physician determined and they were reported in terms of frequency and time to exacerbation. In the five clinical trials mentioned above, there was a significant reduction in the number of exacerbations and hospital visits in patients who were on azithromycin [299-305].

Additionally, chronic azithromycin use in patients with cystic fibrosis improved quality of life [296]. There were slight differences among the five clinical trials as the quality of life (QOL) endpoint was analyzed using different QOL assessment questionnaires. However, each trial showed improvement in certain measurable

components including the physical performance, body image, mental health, and social involvement [299-305].

#### c. Reduced need for antibiotics

Although long-term antibiotic use could lead to antimicrobial resistance and other issues of collateral damage, the chronic use of azithromycin reduced the need for antibiotics and infection risk [296]. According to the five clinical trials, there was a significant reduction in the need for initiation and duration of oral and IV antibiotics in the azithromycin group. Patients on azithromycin acquired PA infections at a minimal rate similar to the placebo arm. Moreover, patients in the azithromycin group had a significantly lower rate of *Staphylococcus aureus* infections [299-305]. Conversely, the azithromycin group was at higher risk for infections with macrolide-resistant *Staphylococcus aureus* while the infection rate with MRSA was not different. Additionally, the risk for acquiring other infections was similar between the two groups in all the trials [299-305].

#### d. Reduced inflammatory parameters

The five clinical trials evaluated the anti-inflammatory effects of chronic azithromycin use in patients with cystic fibrosis [296]. There was a significant reduction in pulmonary inflammation with azithromycin use. For instance, there was a significant drop in C-reactive protein (CRP) levels at 3, 6, and 12 months intervals. Additionally, the trials reported reduced neutrophilia and pro-inflammatory cytokine concentrations with azithromycin treatment [299-305].

#### e. Adverse events

Chronic azithromycin administration in patients with cystic fibrosis was safe and well tolerated [296]. Although patients on azithromycin had reduced fever, cough, and other respiratory symptoms, they had a higher incidence of gastrointestinal

side effects compared to the placebo group [299-305]. None of the patients on azithromycin developed any severe side effects while a few patients had moderate side effects that required treatment discontinuation (in the 5 trials 0,1,1,5, & 12 dropouts were recorded for an overall percentage of 0.5–11% of the total number of subjects enrolled). Finally, there were no deaths reported in any of the studies [296, 299-305].

In summary and despite the heterogeneity of the study populations, the meta-analysis supported the use of azithromycin treatment in cystic fibrosis patients. The improved lung function, reduced exacerbations, reduced need for antibiotics, and the decreased inflammatory parameters (CRP) were all clinically significant. However, these studies had some limitations including: (1) none of the studies evaluated the effects of azithromycin in patients with PA infections; (2) only one study evaluated azithromycin use at a 12-months timepoint and the remaining were for 6-months treatment durations; (3) different daily and weekly doses of azithromycin were evaluated in different age groups; (4) and none of the studies stratified the effects of azithromycin based on the CFTR mutation category [296, 299-305]. Therefore, more studies are required to distinguish the beneficial effects of azithromycin among patients with PA infections and patients within specific age groups.

### iii. Current treatment guidelines for azithromycin use in cystic fibrosis

In Europe, and based on this meta-analysis by Southern et al., the off-label use of azithromycin in cystic fibrosis patients was added to the National Institute for Health and Care Excellence (NICE) antibiotic treatment guidelines [306]. A 6-month treatment in patients with deteriorating disease or as determined by physician judgment is suggested. The regimen for the off-label use as per the NICE clinical guidelines suggests a 3 times weekly dose of azithromycin [306].

In the United States, and according to the Cystic Fibrosis Pulmonary Guidelines, azithromycin long-term use in cystic fibrosis is divided into two recommendations [307, 308]. The guidelines suggest “high” benefit in patients with PA infections who are 6 years of age and above. The Cystic Fibrosis Foundation recommends the “chronic use of azithromycin to improve lung function and reduce exacerbations” in these patients chronically colonized with PA. Alternatively, the guidelines suggest “moderate” benefit in patients not infected with PA. The Cystic Fibrosis Foundation recommends for these patients, 6 years of age and above, without evidence of PA in their lungs to consider long-term azithromycin treatment to reduce exacerbations [308].

#### iv. Anti-inflammatory cellular and molecular mechanisms of azithromycin

The mechanisms underlying the clinical benefits of azithromycin and the substantial improvement in lung function and inflammatory parameters are yet to be elucidated. As discussed in sections (i) and (ii), macrolides and specifically azithromycin exert a clinically beneficial role in cystic fibrosis patients by regulating the pro-inflammatory cytokine release and the oxidative burst and migration of phagocytes [309]. Additionally, azithromycin has unique pharmacokinetic properties allowing it to accumulate intracellularly in these phagocytes. This drove many researchers to believe that the macrolide anti-inflammatory mechanisms involve specific targets in neutrophils and macrophages. Yet, the specific cellular and molecular targets of azithromycin had not been described.

In 2008, Murphy et al. successfully advanced our knowledge by demonstrating the effects of azithromycin on macrophage polarization [310]. This report by our group examined the effects of azithromycin on macrophage phenotype by stimulating J774 murine macrophages invitro. Macrophages were polarized into an M1 phenotype with IFN $\gamma$  or into an M2 phenotype with IL-4 and IL-13. After

stimulation with LPS, polarized J774 macrophages expressed different surface markers that distinguish either macrophage phenotype. Interestingly, azithromycin in the presence of IFN $\gamma$  and LPS was able to shift the macrophage polarization away from an M1 towards an M2-like phenotype [310]. This was based upon reduced CCR7 expression (M1 marker) and increased MR and CD23 expression (M2 markers) compared to IFN $\gamma$  alone and similar to IL-4 and IL-13 treated macrophages. Additionally, azithromycin treated macrophages produced significantly less IL-12 and IL-6 pro-inflammatory cytokines and more of the anti-inflammatory cytokine IL-10 compared to IFN $\gamma$  treated cells [310]. Moreover, Murphy and his colleagues showed that azithromycin also modulates other important effectors of macrophage polarization, iNOS and arginase-1 [166, 310]. Specifically, azithromycin blunts the increase in iNOS expression observed with IFN $\gamma$  and overcomes the suppressed arginase expression in these otherwise M1-polarized macrophages. Azithromycin, in the presence of IFN $\gamma$  and LPS signals, was shown to significantly increase arginase-1 (M2 effector) expression and activity similar to IL-4 and IL-13 treated macrophages [310]. In summary, this report by our group was the first to show that azithromycin directly affects macrophage phenotype and drives macrophages into an alternative M2 phenotype in-vitro.

To examine the physiological relevance of these findings, azithromycin's effect on macrophage phenotype was then evaluated in a mouse model of PA pneumonia [311]. In a 2010 report by Feola et al., C57BL/6 mice were infected with a clinical mucoid strain of PA (M57-15) [311]. The PA was incorporated in agarose beads prior to intratracheal instillation in anesthetized mice. The PA-impregnated agarose beads result in a prolonged infection similar to that in cystic fibrosis patients. In order to examine the effects of azithromycin, infected mice received the drug via oral gavage four days prior to infection and daily thereafter. Azithromycin treatment of infected mice did not significantly alter bacterial burden while protecting against excessive weight loss and improving survival. Importantly, the azithromycin-treated group had significantly higher numbers of

CD11b+ immune cells infiltrating into their alveolar spaces, lower number of neutrophils in their airways, and higher number of CD4+ lymphocytes [311].

The phenotype of these CD11b+ monocytes migrating into the lungs and alveolar spaces was then evaluated. Similar to the in-vitro observations, macrophages in the azithromycin-treated mice exhibited higher levels of M2 markers like the increased MR surface expression and the increased arginase activity.

Additionally, CD11b+ monocytes in the azithromycin-treated mice exhibited greater production of IL-10 and significantly lower production of TNF- $\alpha$ , CCL2 and IL-6. Subsequently, the effects of azithromycin on lung fibrosis and inflammatory changes were evaluated in H&E stained lung sections.

Histologically, azithromycin treatment was associated with reduced inflammatory infiltrates around the bronchioles of PA infected mice compared to untreated mice at day seven post-infection. Additionally, the inflammatory infiltrates in infected mice were predominantly monocytes in the azithromycin-treated group versus neutrophils in the untreated group [311].

In summary, azithromycin treatment blunts the neutrophilic inflammation and shifts the compartmentalization of monocytes infiltrating the lungs and alveolar spaces in response to PA intratracheal infection. Collectively, these data support the hypothesis that azithromycin shifts macrophage polarization into an alternative anti-inflammatory phenotype in response to PA pneumonia.

The next step was to evaluate the clinical applicability of the developing central hypothesis [166]. A pilot clinical study was conducted to evaluate the immunomodulatory properties of azithromycin and the role of alternatively activated macrophages in the pathophysiology of cystic fibrosis. The study objective was to characterize the expression of classical and alternatively activated macrophage markers in the context of PA infection, immunomodulatory drug therapy and pulmonary function [166]. Sputum and lung lavage samples were collected from forty-eight cystic fibrosis patients. Flow cytometry was used

to evaluate the expression of surface macrophage proteins. Additionally, analysis of arginase activity and cytokine levels were evaluated in the sputum and lavage samples [166].

Patients with chronic PA infections had a significant increase in M2 macrophage markers. Cystic fibrosis patients infected with PA had higher levels of MR expressing monocytes compared to patients with no history of PA. Additionally, patients infected with PA had increased arginase activity compared to uninfected patients [166]. These observations emphasize the role of alternative macrophages in the pathophysiology of PA infections in patients with cystic fibrosis and suggest that the cytokine environment in patients with chronic PA infections skew the macrophage polarization towards an alternative phenotype. However, whether alternative macrophage effectors contribute to improved lung function or to pathology is yet to be elucidated [166].

In this pilot study, there were no significant differences in the pro- and anti-inflammatory cytokines between the azithromycin and the PA infected subgroups when analyzing gene expression [166]. While patients infected with PA had significantly higher levels of IL-8, IL-1, and IL-12, there were no significant differences in TNF- $\alpha$ , IL-6, and IL-10 compared to the uninfected group. Moreover, the increased expression of alternative macrophage effectors or the increased arginase expression were inversely correlated with lung function [166]. However, it is hard to correlate these observations with any direct effects of azithromycin due to the observational nature of this study and the fact that empiric azithromycin therapy is very likely to be prescribed to patients with declining lung function. In fact, patients infected with PA had a greater chance of being on long-term azithromycin therapy compared to uninfected patients. Therefore, it was difficult to separate the effects of azithromycin versus the effects of PA infections due to the overlap of these two parameters [166].

Importantly, this pilot study examined the effects of azithromycin on alternative macrophages in patients with cystic fibrosis [166]. Azithromycin treatment was not associated with significant differences in terms of arginase activity, cytokine levels, or expression of alternative macrophage proteins. However, data generated in the azithromycin group were analyzed based on the PA infection status, a significant relationship between arginase activity and lung function and between lung function and the expression of alternative macrophage effectors was observed in the azithromycin-treated patients with PA infection and not in the other groups [166]. These observations emphasize the potential immunomodulatory mechanism of azithromycin which involves suppression of exaggerated inflammation rather than promoting anti-inflammatory mediators. Additionally, these data validate that patients with chronic PA infections might benefit the most from azithromycin therapy [166].

Finally, a combination of parameters was used to predict lung function. Lung function (FEV1) was best interpreted from a linear combination of the patient's age, PA infection status, azithromycin treatment status, macrophage MR surface expression, and arginase activity [166]. In fact, MR expression and arginase activity were significant predictors of lung function in cystic fibrosis patients along with azithromycin treatment and PA infection status. These data suggest that azithromycin alters macrophage polarization in patients infected with PA and emphasize the ability of azithromycin to drive macrophage polarization independently of the cytokine environment in the lungs. However, the specific mechanisms involved with alternative macrophage polarization with azithromycin are yet to be elucidated [166].

This was a novel study by our group which was the first to show evidence of alternative macrophage function and its relation to azithromycin use in cystic fibrosis. It is important to note that due to the limitations of this study, it was difficult to have definitive answers for the specific anti-inflammatory effects and mechanisms of azithromycin in the context PA pneumonia in cystic fibrosis [166].

In summary, the Feola lab group provided evidence that azithromycin polarizes macrophages into an alternative phenotype in vitro, and that alternative macrophages predominated in the lungs of azithromycin treated mice infected with PA and in cystic fibrosis patients with PA pneumonia [166, 310, 311]. Azithromycin increases alternative macrophage effectors including MR expression and arginase activity while suppressing pro-inflammatory cytokine release. However, the specific mechanisms by which azithromycin promotes an alternative macrophage phenotype remain unknown.

## VII. Summary and specific aims

The previous sections summarize our current understanding of cystic fibrosis pathology and the available therapeutic options. Patients suffer from genetic abnormalities to the CFTR gene which predispose them for chronic and repetitive infections. Infections with PA are the most common in patients 18 years and older and they are characterized by a chronic, dysregulated, inflammatory response with aberrant T cell immunity and a predominant recruitment of neutrophils and pro-inflammatory macrophages [312]. This continual immune response lowers patients' life expectancy by 30 years as a result of lung injury and damage, deteriorated lung function, and decreased quality of life [313].

Azithromycin, a macrolide antibiotic, exerts anti-inflammatory and immunomodulatory effects in cystic fibrosis patients. We have previously shown that azithromycin alters macrophage polarization and shifts the macrophages into an alternatively activated phenotype, both in vitro and in a mouse model of PA pneumonia [166, 310, 311]. Murine experiments with an early polarization of macrophages into an alternative phenotype show decreased neutrophil influx and pulmonary injury in mice infected with PA. Conversely, abolishing alternative macrophage polarization was associated with a profound acute immune response along with exaggerated neutrophil influx and an altered T cell

phenotype. The absence of alternative macrophages was also associated with increased morbidity and mortality in our model of murine PA pneumonia [166, 310, 311].

These findings from in vitro, murine, and human experiments were the basis for the central hypothesis that the alternatively activated macrophages decrease pulmonary inflammation in PA infection, an effect that is dependent upon production of TGF $\beta$  and arginase-1. The objective is to determine whether alternative macrophages induced with azithromycin are essential to regulate the exaggerated inflammatory response against PA pneumonia. Additionally, our objective is to determine which specific macrophage effectors are essential for immunomodulation and whether this involves control of other immune cells like neutrophils and T cells. The second objective of our research is to define the specific mechanisms by which azithromycin modulates these macrophages. The long-term goal is to utilize these mechanisms to optimize the current therapeutic options and to identify new therapeutic targets for patients with non-resolving pulmonary inflammation.

Work presented in this dissertation is based on our previous findings and aims to identify the specific effectors of alternative macrophages that are crucial for regulating inflammation and modulating T cell disposition. Closely examining the different effectors of alternative macrophages revealed an important role of arginase-1. Arginase-1 expression and activity are increased in alternative macrophages polarized with azithromycin. Preliminary experiments using arginase-1 conditional knock-out mice verified that arginase deficiency is associated with greater morbidity in terms of more significant weight loss. Additionally, arginase-1 has unique immunomodulatory properties (discussed earlier) and include controlling NO-mediated injury, suppression of T cell function and proliferation, as well as promoting a Treg phenotype [151, 152, 154, 157, 312-316]. In cancer, myeloid suppressor cells producing arginase inhibit T cell responses against tumor cells [317, 318]. In pregnancy, arginase is essential to

prevent maternal immune reactions against the fetus [319]. Arginase also plays an important role in sepsis, trauma, surgery, certain infections, and some inflammatory and autoimmune diseases [151, 152, 154, 157, 314-316]. However, arginase-1 mediated modulation of the inflammatory response against PA pneumonia has not been investigated.

The work presented here also investigates the specific pathways involved with azithromycin macrophage polarization. It is unknown how azithromycin modulates the expression of different pro- and anti- inflammatory macrophage effectors. The expression of these inflammatory cytokines and mediators is controlled via different transcription factors induced in response to distinct stimuli. Th1 cytokines can induce M1 macrophage activation by stimulation of the STAT-1 and NF- $\kappa$ B transcription factors. Alternatively, Th2 cytokines activate alternative M2 macrophages through STAT-6 activation. While some reports suggest that azithromycin can inhibit NF- $\kappa$ B activation and polarize macrophages into an M2 phenotype, a link between these effects has not been established. Theodore Cory, a previous graduate student in the Feola lab, conducted a preliminary study of azithromycin using an in-vitro model of J774 murine macrophages. He showed that azithromycin affects non-canonical NF- $\kappa$ B activation. He showed that azithromycin increases the level of inactive NF- $\kappa$ B p105 subunit while the active p50 subunit was down-regulated. The work in this dissertation ties the preliminary data that Dr. Cory generated with specific regard to the effects of azithromycin on the canonical NF- $\kappa$ B subunit, p65. Additionally, the research in this dissertation evaluates cross-talk between the canonical NF- $\kappa$ B pathway and the STAT-1 pathway.

The purpose of the work presented in this dissertation is to specifically investigate the role of arginase-1 in the immunomodulatory functions of alternative macrophages and to define the molecular anti-inflammatory mechanism of azithromycin. In addition to evaluating my hypotheses in murine models of PA pneumonia, I also address the translatability of my observations by

evaluating the immunomodulatory properties of azithromycin in cystic fibrosis patients.

The long-term goal is to define key regulators of the exaggerated immune response that can be of therapeutic value for immunotherapy in cystic fibrosis. The objective of this project is to investigate the regulatory role of arginase-1 in the immune response to PA pneumonia and its modulation of T cell immunity. Additionally, this project examines the effects of azithromycin on the transcription factors involved with macrophage polarization, specifically NF- $\kappa$ B and STAT-1.

The central hypotheses to be tested in the following chapters are:

- I- Decreases in inflammation in response to PA pneumonia achieved by polarizing macrophages to an alternatively-activated phenotype is dependent upon the production of arginase-1.
- II- Azithromycin polarizes macrophages to an M2 phenotype via inhibition of STAT1 through cross-talk from NF- $\kappa$ B signaling mediators.
- III- The ability of azithromycin-induced M2 macrophages to control inflammation in response to PA pneumonia is dependent on arginase-1 expression.

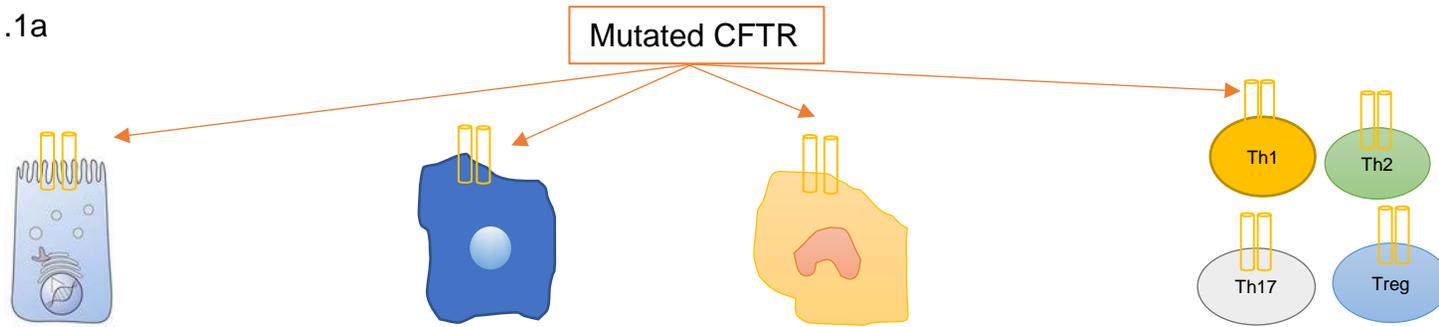
I found that decreases in inflammation in response to PA pneumonia are dependent upon the production of arginase-1. Arginase-1 deletion resulted in exaggerated neutrophil influx and in skewing of the T cell responses towards a Th1 and Th17 responses.

Additionally, azithromycin polarized macrophages to an M2 phenotype via inhibition of STAT1 through cross-talk from the NF- $\kappa$ B signaling mediators.

Azithromycin inhibited p65 nuclear translocation which resulted in IKK $\beta$  accumulation due to suppressed negative feedback. Thus, inhibiting the NF- $\kappa$ B signaling pathway cross-inhibited the STAT-1 pathway.

Finally, azithromycin regulated the immune responses in PA pneumonia via mechanisms independent of arginase. Azithromycin protected against excessive morbidity and exaggerated inflammation by controlling the influx of neutrophils and inflammatory macrophages in mice with macrophages that could not express arginase-1. Additionally, azithromycin balanced the Th17/Treg responses in mice infected with PA, an effect that was also independent of arginase production.

Figure 1.1a



Defective epithelium

- Impaired mucociliary clearance
- Increased NF-κB signaling
- Decreased antigen presentation
- Inactivation of antimicrobial substances
- Loosened junctions and increased paracellular permeability

Defective macrophages

- Impaired phagocytic function
- Impaired antigen presentation
- Impaired clearance of dead neutrophils
- Altered M1/M2 macrophage polarization
- Exaggerated NF-κB activation
- Exaggerated pro-inflammatory cytokine release

Defective neutrophils

- Impaired phagocytic function
- Excessive release of granule products and superoxide anions
- Increased neutrophil elastases and proteases
- Protease >>> anti-protease
- Destruction of connective tissue and matrix proteins
- Unregulated cell death and release of intracellular DNA

Defective adaptive immunity

- Impaired antigen presentation and necessary costimulation
- Impaired membrane depolarization and signaling transduction
- Intrinsic skewing towards Th17 predominant phenotype
- Th17 >>> Treg

Figure 1.1b

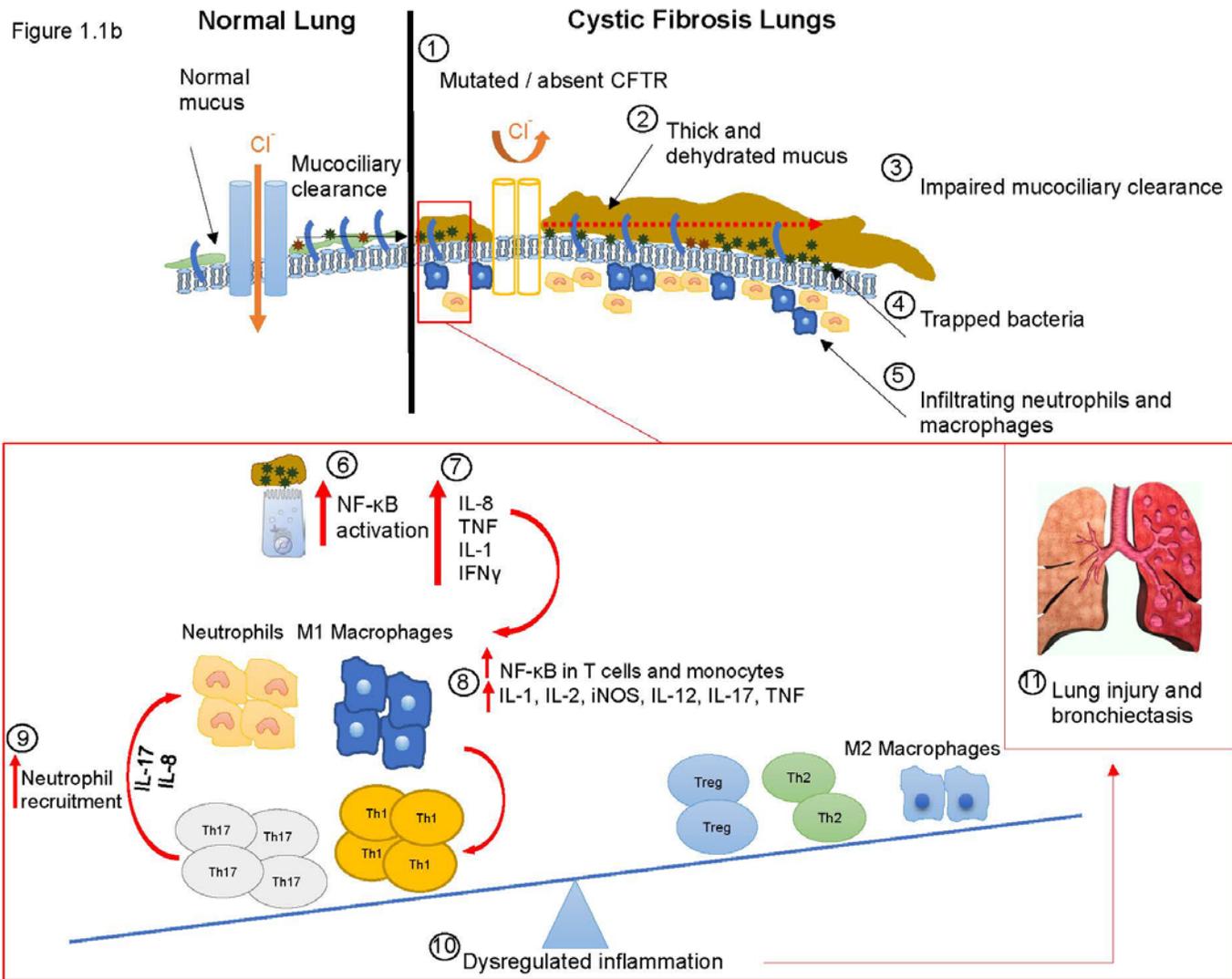


Figure 1.1. Cystic fibrosis pathology.

Cystic fibrosis patients suffer from a defective and dysregulated immune response. (a) The main alterations of the cystic fibrosis epithelium, macrophages, neutrophils, and adaptive immune cells. (b) Figure depicts the progression of cystic fibrosis pathogenesis from a functional CFTR mutation (1) to the development of end-stage bronchiectasis (11).

## Chapter 2: Methods

### I. Mice

#### i. Arginase-1 conditional knock-out mice

The function of macrophage arginase was studied by genetically modifying C57BL/6 mice to delete arginase-1 expression in myeloid cells. To generate arginase-1 conditional knock-out mice, C57BL/6-*Arg1<sup>tm1Pmu</sup>/J* (or *Arg1<sup>flox</sup>*) mice were utilized along with B6.129P2-*Lyz2<sup>tm1(cre)lfo</sup>/J* (or *LysMcre*) mice (The Jackson Laboratory, Bar Harbor, ME). *Arg1<sup>flox</sup>* are floxed mutants with *LoxP* sites flanking exons 7 and 8 of the arginase-1 gene. When these mice are bred with the *LysMcre* mice expressing Cre recombinase under the control of *Lyz2* promoter, floxed arginase-1 gene in myeloid cell lineages is deleted. All animals were bred in-house starting with the homozygous *loxP* mice (*Arg1<sup>flox</sup>*) and the transgenic Cre mice. These generate heterozygous *loxP*, hemizygous Cre mice; the latter were backcrossed with the homozygous *loxP* mice to generate homozygous *loxP*, hemizygous Cre mice (arginase-1 conditional knock-out mice or *Arg1<sup>Δm</sup>*), and homozygous *loxP*, non-carrier mice (littermate controls that do not have Cre recombinase to excise the flanked allele, *Arg1<sup>flox/flox</sup>*). All studies were approved by the University of Kentucky Institutional Animal Care and Use Committee. The mice were housed under conditions of pathogen-free isolation and were transferred to a biosafety level 2 housing unit after infection. All mice utilized in the infection experiments were about 6-8 weeks of age and were randomized into different treatment groups with comparable weight and sex. Arginase deletion from different immune cells is shown in Figure 2.1

#### ii. BALB/cJ mice

BALB/cJ mice were utilized in experiments with pharmacological arginase inhibition. The rationale behind using BALB/cJ mice is that they are

immunologically modified and demonstrate a Th-2 biased immune response with a significant expression of arginase-1 post infection. BALB/cJ females 4-6 weeks of age were purchased from Jackson Laboratory (Bar Harbor, ME). Mice were allowed to acclimate after shipment for 1 week prior to use. All mice utilized in the infection experiments were 6-8 weeks of age and were randomized into different treatment groups.

## II. Murine infection and drug dosing

### i. PA-laden agarose beads

Mice were infected with PA incorporated into agarose beads to cause a prolonged infection similar to that in cystic fibrosis patients. PA M57-15 is a clinical mucoid strain of PA isolated from a patient with cystic fibrosis and obtained as a generous gift from Anna Van Heeckeren from Case Western University.

PA M57-15 were frozen in glycerol stocks at -80 °C. Prior to each infection, a fresh batch of beads was prepared. A sterile inoculating loop was used to scrape bacteria off the frozen bacterial stock. The scraped bacteria (1, 2, and 3 loops) were allowed to grow overnight in three 125 mL flasks with 30 mL of sterile Trypticase soy broth (TSB). The bacterial cultures were allowed to grow for 18 hours (to reach the late log phase or early stationary phase) in a shaking incubator at 37 °C and 220 RPM. According to the growth curves and the optical density (OD) analysis of the PA M57-15 cultures, the starting OD of the bacterial culture to be incorporated into the beads for optimal infective beads is 1.0 – 2.0. The OD of each bacterial culture was measured using the spectrophotometer at 620 nm wavelength and by normalizing the OD to that of TSB. After determining which bacterial culture to use, two stirring hot plates were placed in a hood. Agarose powder (0.5 g) was dissolved in 25 mL of sterile phosphate buffered saline solution (PBS) by microwaving for 1 minute. The agarose solution was

placed on a hot plate with a slow stirring magnet bar and a thermometer. Simultaneously, 40 mL of mineral oil was heated on the second hot plate with a slow stirring magnet bar and a thermometer. The temperatures were monitored closely to assure that the agarose solution cooled down to 50 °C and the mineral oil heated up to the same temperature of 50 °C. Once the target temperature was reached, 15 mL of the bacterial culture was slowly added to the agarose solution and allowed to mix for 3-5 minutes. Subsequently, the stirring speed of the mineral oil was increased, and 10 mL of the bacteria-glycerol mixture was transferred to the mineral oil- 1 mL at a time. The mineral oil flask was then cooled immediately by adding ice to the secondary beaker and allowing the mixture to stir at maximal speed for 10 minutes. The rapid speed allows the agarose to break into beads trapping the bacteria inside. Different sizes of agarose beads form, each containing different amounts of PA. After cooling the oil-agarose-bacteria mixture and mixing briskly, a milky and frothy mixture forms. This mixture was collected into 50 mL canonical tubes and several washes were done by centrifuging with different buffers at 600 RCF for 4 minutes. Two initial washes with 0.5% and 0.25% deoxycholic acid (DCA) were performed to remove the mineral oil. Two more washes with PBS were then performed to remove the DCA. Four more washes with PBS were used to settle the large beads and collect the floating smaller beads. The latter constitutes the bead stock. A sample of the bead stock was then homogenized on ice to break the beads. The homogenized beads were serially diluted and plated on dry TSA (Trypticase soy agar) plates using a spiral plater. Inverted plates were incubated overnight at 37 °C. The concentration of PA in the bead stock was then determined by counting the colonies growing on the incubated plates using a QCount Colony Counter. The bead stock was then diluted to make the infecting solution with the desired inoculum of  $2 \times 10^5$  CFU in 100  $\mu$ L for C57BL/6 mice and  $2 \times 10^6$  CFU in 100  $\mu$ L for BALB/c mice. This inoculum was determined to result in significant murine pneumonia without causing severe morbidity or mortality in each mouse strain. The target inoculum was calculated as the 10% of the lethal CFU or LD10.

## ii. Infection

Mice were lightly anesthetized in a chamber connected to an isoflurane vaporizer and an oxygen source. Mice were then placed on the infection stand and the tongue was pulled out gently with forceps to visualize the trachea. A curved and blunted 24-gauge needle was used to infect the mouse by introducing 100  $\mu$ L of the infecting bead solution intratracheally. Importantly, the infecting bead solution was allowed to mix on a stirring plate during the infection procedure and each 100  $\mu$ L was pulled individually to make sure an even bead sample was used to infect each mouse. The infecting bead solution was then homogenized and plated on Pseudomonas isolation agar (PSA) plates and the colony count was determined after 24 hours of incubation at 37 °C to confirm the concentration of PA that was instilled into the mice. Experimental designs for each infection experiment are depicted in Figures 2.2 a, b, and c.

## iii. Animal dosing

### a. Azithromycin

To evaluate azithromycin effects in arginase-1 conditional knock-out mice, the drug was dosed via oral gavage starting four days prior to infection and daily thereafter. Azithromycin was dosed based on murine body weight at 40 mg/Kg. The murine dose of azithromycin was prepared by suspending crushed azithromycin tablets (Teva, Petah Tikva, Israel) in 2% methylcellulose. Alternatively, the control group was dosed with the vehicle (2% methylcellulose). To administer the drug, the head of each mouse was gently restrained and kept in an upright position. A stainless-steel bulb-tipped gavage needle was then gently rolled along the back of the tongue into the esophagus and towards the stomach. The drug was then delivered orally by instilling 150  $\mu$ L of the azithromycin suspension into the stomach of each mouse.

## b. L-norvaline and BEC

In select experiments, the arginase inhibitors L-norvaline or S-(2-Boronoethyl)- L-cysteine hydrochloride (BEC) were given to mice to evaluate the immunomodulatory effects of pharmacological arginase inhibition in PA pneumonia. L-norvaline and BEC were dosed via oral gavage starting 1 day prior to infection and daily thereafter.

BEC was synthesized in the lab of our collaborator Dr. Sylvie Garneau-Tsodikova lab. The thiol-ene reaction strategy was used to synthesize S-(2-boronethyl)- L-cysteine. The single step reaction between di-(*n*-butyl)-vinylboronate and L-cysteine in the presence of a radical initiator 2,2'-azobis(2-methylpropionitrile) resulted in the formation of BEC at a 53% yield [320]. Detailed synthesis procedure is described in Figures 2.3 and 2.4.

The BEC dose was calculated based on the daily water consumption of BALB/cJ mice and the suggested dosing of 0.2% BEC in water. A dose of 0.2% represents 0.2 g of BEC per 100 mL of water intake. The reported volume of water consumption in BALB/cJ mice is about 6 mL per day [321]. Therefore, the daily volume of water consumed by each mouse contains about 0.012 g of BEC. To ensure each mouse receives an equivalent dose of the arginase inhibitor, a daily dose of 0.012 g of BEC was administered in 100  $\mu$ L of water via oral gavage (same as described in *II.iii.a*).

L-norvaline was purchased from Sigma-Aldrich (St. Louis, MO). The murine dose of L-norvaline was calculated from the rat dose of 50 mg/kg [322]. Animal equivalent dose calculation based on body surface area resulted in an estimated dose of 100 mg/kg in mice [323]. Appropriate L-norvaline dose was administered daily in 100  $\mu$ L of water via oral gavage (same as described in *II.iii.a*).

### III. Tissue harvest and processing

Murine body weight was monitored daily to assess morbidity post infection. Mice were euthanized and excluded from the experiment if they lose 20% or more of their body weight prior to infection along with 1 sign of morbidity (immobility, hunched posture, or lack of response to handling). If mice suffered from cyanosis, dyspnea, or loss of righting reflex, they were euthanized immediately.

Mice which survived and lost at least 5% of their body weight were included in the analysis. A representative sample of the infected mice was humanely killed at different timepoints post-infection (select experiments had timepoints at different days post-infection. Death was ensured using two methods (intraperitoneal injection of pentobarbital and aortic exsanguination).

#### i. Murine lung lavage

Lung lavage was performed on each mouse to collect cells from the airway and alveolar spaces. Lavage was performed by connecting two 5 mL syringes to a three-way stopcock with a lavage catheter inserted into a small puncture in the exposed trachea. Each lung was then lavaged with 5 mL of PBS (with 30  $\mu$ M EDTA) in 1 mL aliquots. The lavage samples were then centrifuged at 1200 RPM for 7 minutes at 4 °C. The supernatants were saved while the cells were incubated for 2 minutes with a lysis buffer to lyse the red blood cells. The lysis buffer was then washed away by centrifuging the samples with fresh PBS (for 2 times). The final pellet was suspended in 1 mL of PBS and cells were counted prior to being aliquoted into 5 mL flow cytometry tubes for processing for flow cytometry analysis.

## ii. Tracheobronchial lymph nodes

Tracheobronchial lymph nodes draining the site of infection were excised and placed in 1 mL of PBS. Lymph nodes were then pushed through a 70  $\mu\text{m}$  cell strainer with a syringe plunger to create a single cell suspension. Similar to the lavage samples, the lymph nodes were centrifuged and the pellet was incubated with red blood cell lysis buffer for 2 minutes. After washing away the lysis buffer with PBS, the final pellet was suspended in 1 mL of PBS and the cells were counted prior to being aliquoted into 5 mL flow cytometry tubes for processing for flow cytometry analysis.

## iii. Interstitial lung tissues

To collect cells from the interstitial spaces, the lung tissues were collected after being lavaged and the lymph nodes harvested. Each lung was collected in 2 mL of RPMI with 5% Fetal Bovine Serum (FBS). Lungs were then minced with scissors and incubated with 1 mg/mL of collagenase and 50 U/ml of DNase for 1 hour at 37 °C. After digestion, the lungs were then pushed through a 70  $\mu\text{m}$  cell strainer with a syringe plunger to create a single cell suspension. The lung samples were centrifuged, and the pellet was incubated with red blood cell lysis buffer for 2 minutes. The lysis buffer was then washed away by centrifugation with PBS (twice). The final pellet was suspended in 1 mL of PBS and the cells were counted prior to being aliquoted into 5 mL flow cytometry tubes for processing for flow cytometry analysis.

## IV. Histology

Lungs from infected mice were collected for histologic analysis. Importantly, the lung lavage procedure was not performed when the lungs were to be sectioned. Lungs were sectioned using two different methods, cryosectioning and paraffin-embedding.

#### i. Tissue cryosectioning

Lungs for cryosectioning were insufflated with 2-3 mL of formalin- optimal cutting temperature (OCT) solution (50% OCT and 50% formalin (10%)). The lungs were inflated to the appropriate degree so as to not cause bursting of the alveoli. The formalin-OCT solution was slowly and gently infused into the lungs after the trachea was cannulated with an 18G catheter needle. When the lungs were fully inflated, the trachea was tied with a suture and the lungs were carefully removed and placed in 25 mL of 5% formalin overnight. The next day, lungs were transferred into a 15% sucrose solution and incubated overnight. Finally, the lungs were transferred to a 30% sucrose solution and incubated overnight. Subsequently, lungs were placed in a tissue mold and submerged OCT. Tissue molds were then snap frozen at -80 °C. Frozen tissues were cryosectioned at the Biospecimen Procurement & Translational Pathology Shared Resource Facility of the University of Kentucky Markey Cancer Center (P30CA177558). Specimens were sectioned on a cryostat at 8 µm. Hematoxylin and eosin stain (H&E) was performed according to the standard Harris' hematoxylin protocol on a Leica XL Autostainer and mounted with permanent mounting media [324].

#### ii. Sectioning of paraffin-embedded tissues

Lungs for paraffin embedding were collected after the mice were euthanized. Harvested lungs were gently inflated with 2-3 mL of 10% formalin. Excised lungs were then kept overnight in 25 mL of 10% formalin. Lungs were then transferred into 70% ethanol. Tissues were processed and paraffin embedded according to standard procedures described by Morton et al. [325]. Lungs were trimmed in from the ventral face until all 5 lobes were visible. H&E staining was performed according to the standard Harris' hematoxylin protocol. Paraffin embedding and staining were performed at the COBRE Pathology Core of the University of Kentucky which is supported by an Institutional Development Award (IDeA) from

the National Institute of General Medical Sciences of the National Institutes of Health under grant number P20GM103527.

### iii. Lung injury scoring

Histologic analysis of sectioned lungs was performed to evaluate acute inflammatory lung injury after infection. Lung injury scoring was calculated according to the American Thoracic Society guidelines [326]. Briefly, 20 random high-power fields were independently and blindly scored. The scoring system takes into consideration the number of neutrophils in the alveolar and interstitial spaces, the presence of hyaline membranes and proteinaceous debris, as well as septal wall thickening. The scoring system is summarized in Table 2.3. To generate a lung injury score, the sum of each of the five independent variables shown in Table 2.3 were weighted according to the significance factor of each variable and then normalized to the number of fields evaluated. The following formula was used to calculate the lung injury score:  $\text{Score} = [(20 \times A) + (14 \times B) + (7 \times C) + (7 \times D) + (2 \times E)] / (\text{number of fields} \times 100)$ . Scoring was performed by an independent pathologist, Dr. Therese Bocklage, MD.

## V. Flow cytometry

### i. Surface staining

Samples of the processed lung lavage, lung digest, and lymph nodes were counted using the TC10 Automated Bio-Rad Cell Counter with Tryptan Blue to determine viability. Aliquots of  $1 \times 10^5$  -  $1 \times 10^6$  cells were transferred into each flow cytometry tube (5 mL polystyrene round-bottom tubes). Cells were then washed with PBA (PBS with bovine serum albumin and sodium azide) by centrifuging at 1200 RPM for 7 minutes at 4 °C. Tubes were then decanted and the pellets were incubated with 20  $\mu$ L of the appropriately diluted fluorochrome-conjugated antibodies. The latter were diluted according to the manufacturer

instructions or as determined by the antibody titer experiments. Cell-antibody mixtures were incubated on ice and in the dark for 20 minutes. Subsequently, unbound antibodies were washed away with PBA. When fixation was required for some experiments, the stained cells were fixed by incubating with 200  $\mu$ L of 5% formalin for 20 minutes in the dark. Excess formalin was then removed by washing with PBS and fixed cells were suspended in PBS for flow cytometry.

## ii. Intracellular staining

After tissue processing and cell counting, samples for intracellular staining were aliquoted into flow cytometry tubes (5 mL polypropylene round-bottom tubes). Cells were then incubated in 2 mL of RPMI (with 5% FBS and 1% Penicillin/Streptomycin). Ionomycin (1  $\mu$ g/mL final concentration) and PMA (Phorbol 12-myristate 13-acetate, 50 ng/mL final concentration) were added to the media for stimulation of intracellular cytokine expression. Cells were incubated for 2 hours at 37 °C. After 2 hours, Brefeldin A was added into each tube (2  $\mu$ L of 1000x) in order to enhance intracellular cytokine staining by blocking protein transport. Cells were incubated with Brefeldin A for additional 2 hours at 37 °C after which they were centrifuged and washed with PBA. Staining for surface markers was performed similarly to the procedure described in (a) followed by fixation with 5 % formalin when required. After washing away the excess formalin with PBA, cells were incubated with permeabilization buffer (PBA + 0.5% saponin) for 10 minutes at room temperature. Cells were then centrifuged and the pellet was incubated with Fc block (10  $\mu$ L of 1:10 diluted stock) for 10 minutes at room temperature. This was followed by adding 20  $\mu$ L of the appropriately diluted fluorochrome-conjugated antibodies specific for intracellular proteins. Unbound antibodies were washed away by centrifuging twice with permeabilization buffer followed by a third wash with PBA. Cells were then suspended in PBA for flow cytometry analysis.

### iii. Flow cytometry analysis

Surface and intracellularly stained cells were analyzed using either the Attune Flow Cytometer (Applied Biosystems, Foster City, CA) at the Feola lab or the BD LSR II Flow Cytometer (BD Biosciences, Franklin Lakes, NJ) at the UKY flow core facility. Attune Performance Tracking Beads were used to run a performance test prior to each experiment in order to define the baseline performance of each laser and to set the intensity level of each detection channel. Unstained and single-stained control tubes were used to adjust the instrument settings by running a compensation for each set of antibodies used in a multicolor panel. During compensation setup, the threshold and voltages of the fluorescence channels were adjusted to visualize the desired populations. Proper compensation is necessary to correct for spillover due to the overlap of the emission spectra of the used fluorochromes. Subsequently, each sample was run according to the collection criteria set during the compensation setup in order to record a minimum of 50,000 events per sample. Further analysis of the recorded events was performed using FlowJo software (FlowJo, LLC, Ashland, Oregon). FlowJo allows analysis of single live cells and performs statistics on the desired populations. Additionally, FlowJo allows analysis for multiple surface and intracellular markers in single cells. Therefore, FlowJo was used to generate percentages and counts of specific cells that were gated by selecting on the positively stained versus the negatively stained events for specific markers within the identified parent populations and sub-populations. For graphs with cell counts, the FlowJo percentages were multiplied by the number of cells added into each tube. Table 2.4 illustrates the specific markers of different immune cell populations analyzed.

#### iv. Flow panels

##### a. Surface staining panels

Immune cells recruited into the lungs and lymph nodes were identified by staining for surface markers. The antibodies listed below were used to identify these specific cell markers. Importantly, in certain experiments the antibody panels or fluorochromes varied from the list below as will be indicated in the Results section.

Table 2.1. Surface staining panels

Tissue Sample	Antibody- Fluorochrome
Lung lavage and lung digest	F4/80- APC CD11c- PE/Cy7 CD11b- PerCP/Cy5.5 Ly6G- APC/Cy7 CD68- FITC MR- PE
Lung lavage, lymph nodes, and lung digest	CD4 PE/Cy7 CD44- FITC CD62L- PE CD69- APC CD25- PerCP-Cy5.5
Lung lavage, lymph nodes, and lung digest	LIN- FITC CD90.2- APC IL33R- PE CD127- PE/Cy7 CD25- PerCP-Cy5.5

b. Intracellular staining panels

Cytokine production and expression of lineage transcription factors by immune cells in the lungs and lymph nodes were identified by staining for intracellular proteins. The antibodies listed below were used to identify surface and intracellular proteins. Importantly, in certain experiments the antibody panels or fluorochromes varied from the list below as will be indicated in the Results section.

Table 2.2. Intracellular staining panels

Tissue Sample	Antibody-Fluorochrome
Lung lavage and lung digest	F4/80- PerCP-Cy5.5 CD11c- PE/Cy7 TNF- $\alpha$ - FITC CCR7- APC iNOS- PE
Lung lavage and lung digest	F4/80- PerCP-Cy5.5 CD11c- PE/Cy7 IL10- PE ARG1- APC Ly6G- APC/Cy7
Lung lavage, lymph nodes, and lung digest	CD4- PE/Cy7 IFN $\gamma$ - APC IL17- PE ROR $\gamma$ t- PerCP-Cy5.5 CXCR3- FITC
Lung lavage, lymph nodes, and lung digest	CD4- PE/Cy7 CD25- FITC FOXP3- PE TGF $\beta$ - PerCP-Cy5.5 ARG1-APC

## VI. Macrophage polarization

In vitro assays used to study azithromycin's mechanism of action were performed using the murine macrophage cell line J774A.1 (ATCC, Manassas, VA). J774 macrophages were allowed to grow and reach confluency in appropriate media (Dulbecco's Modified Eagle's Medium (DMEM) + 10% FBS + 1% Sodium Pyruvate + 1% penicillin/streptomycin).

Confluent cells were scrapped, counted, and plated in 24-well plates at a concentration of  $2.5 \times 10^5$  cells per 1ml of media. Cells were allowed to adhere for 4-6 hours and then polarized to an M1 phenotype with IFN $\gamma$  (final concentration 20 ng/mL) or to an M2 phenotype with both IL-4 and IL-13 (final concentration 10 ng/mL of each). Additionally, azithromycin was added to select wells along with IFN $\gamma$  at concentrations ranging from 5 to 100  $\mu$ M. Additionally, IKK-16, an IKK $\beta$  inhibitor, was added to select wells with azithromycin and IFN $\gamma$  (final concentrations 50 or 100 nM). Cells were then incubated overnight at 37 °C with 5% CO $_2$ .

Polarized cells were then stimulated with LPS (final concentration 100 ng/mL). The duration of LPS stimulation ranged from 0, 2, 5, 10, 15, 30, and 60 minutes up to 24 hours depending on the experimental goals. LPS and other cytokines were then washed away with PBS. Cells were scraped, enumerated, and lysed in 0.1% (v/v) Triton X-100 (protease and phosphatase inhibitors were added to the lysis buffer prior to use). Protein concentrations were quantified utilizing the Pierce BCA reaction kit. Alternatively, in some experiments, stimulated cells were fractionated into nuclear and cytoplasmic contents; or, homogenized with trizol for RNA extraction (procedures described in the following sections).

## VII. RNA isolation and quantitative RT-PCR.

RNA isolation was performed using trizol reagent (Invitrogen, Carlsbad, CA) and RNeasy Mini Kits (QIAGEN, Valencia, CA). Polarized cells were homogenized with 1 mL of trizol reagent by pipetting up and down several times to lyse the cells. Lysates were allowed to incubate at room temperature in trizol, then 0.2 mL of chloroform was added. The lysate-trizol-chloroform mixture was vortexed and incubated for 2-3 minutes. The mixture was then centrifuged at 12,000 g for 15 minutes at 4 °C. The last step separated the mixture into 2 fractions: an aqueous transparent layer and a pink phenol-chloroform layer. The aqueous (top) layer containing the RNA was transferred to a new tube. Subsequently, RNA was purified using Spin technology. Briefly, the isolated RNA was mixed with 245  $\mu$ L of 100% ethanol. The mixture was then transferred to the spin columns provided by the RNeasy Mini Kit. Columns were centrifuged at 8,000g for 30 seconds which allows the isolated RNA to be trapped in the columns. The columns were then washed twice by centrifuging for 30 seconds with 700  $\mu$ L of RW1 and 500  $\mu$ L of RPE buffers respectively. A third wash with 500  $\mu$ L of RPE buffer was performed by centrifuging for 2 minutes. After the 3 washes with RW1 and RPE buffers, the flow-through eluents were discarded and the columns were transferred into new tubes. RNA was eluted from the columns by adding 40  $\mu$ L of RNase-free water and by centrifuging the columns at 8,000g for 1 minute. Isolated RNA was quantified using Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE).

Equal amounts of RNA were then reverse transcribed into cDNA using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA) according to manufacturer's protocols. cDNA samples were then used for quantitative real-time PCR using the TaqMan gene expression arrays for murine Arg1 (arginase-1), Ikbkb (IKK $\beta$ ) and GAPDH. An epMotion 5070 robot was used to accurately pipette the PCR reaction components (cDNA template, forwards and reverse primers, TaqMan Gene Expression Master Mix, and RNase-free water) into 384-well plates. Plates

were centrifuged briefly and transferred into the ABI Prism 7900HT Fast Real Time-PCR System (Applied Biosystems, Foster City, CA) set for 40 standard thermal PCR cycles. The generated cycle threshold (CT) values were used to quantify gene expression.  $\Delta$ Ct values were calculated by normalizing the target gene expression (Arg1 and Ikbkb) to the housekeeping gene expression (GAPDH).  $\Delta\Delta$ Ct was then calculated by comparing the expression of the experimental condition to the control condition. Power analysis of the generated  $\Delta\Delta$ Ct values was then used to interpret the fold change in gene expression.

#### VIII. RelA translocation assay.

Analysis for cellular localization of p65 subunit reflects on the activation of the NF- $\kappa$ B signaling pathway. Therefore, a nuclear translocation assay was used to interpret NF- $\kappa$ B activation by quantifying the amount of translocated subunit in the nucleus versus the cytoplasmic levels. Polarized and stimulated J774 macrophages (as described in VI) were washed with PBS to remove polarizing cytokines and then scraped manually using sterile well scrapers. Cells were then counted and fractionated into nuclear and cytoplasmic fractions using the NF- $\kappa$ B Assay Kit (FivePhoton Biochemicals, San Diego, CA) and according to the manufacturer protocol. Briefly, collected cells were treated with ice-cold Cytoplasmic Fractionation Reagent (CFR) containing protease inhibitors and centrifuged at 2500 RPM for 4 minutes at 4 °C. The supernatant was then collected as the cytoplasmic fraction. The pellet was then washed a second time with the CFR by centrifugation. The washed pellet was resuspended in ice-cold Nuclear Fractionation Reagent (NFR) containing protease inhibitors. The pellet was allowed to incubate in the NFR for 10 minutes on ice and then centrifuged at maximum speed for 10 minutes. The final supernatant containing the nuclear fraction was then collected. RelA (or p65 subunit of NF- $\kappa$ B) was then quantified in the nuclear and cytoplasmic fractions by Western blot.

## IX. Immunofluorescence staining and analysis

Immunostaining was used to visualize the NF- $\kappa$ B subunit localization in stimulated cells. Macrophages were polarized as described in VI except that round glass coverslips (12 mm) were added to each well of the 24-well plates. Cells were allowed to attach to the glass coverslips overnight. After polarization and stimulation, the coverslips were washed three times with PBS++ (PBS with 0.5 mM CaCl<sub>2</sub> and MgCl<sub>2</sub>). Cells were then fixed and permeabilized by adding 1 mL of ice-cold methanol and incubating for 5 minutes. Methanol was then aspirated, and the coverslips were washed three times with PBS++. Primary and secondary antibodies were diluted at appropriate concentrations in 3% BSA as determined by the titration experiments. A humidified chamber was used for the antibody incubations. Coverslips were removed using thin tip forceps and incubated over a 50  $\mu$ L drop of the primary antibody for 45 minutes at room temperature. Unbound primary antibodies were washed away by repeating the 3 washes with PBS++. Subsequently, coverslips were incubated over a 50  $\mu$ L drop of the fluorochrome-conjugated secondary antibody for 45 minutes at room temperature and in the dark. Excess secondary antibodies were washed away with PBS++ and the coverslips were finally incubated in DAPI nucleic acid stain to visualize the nuclei. About 300  $\mu$ L of the 300 nM DAPI stock solution (Invitrogen, Carlsbad, CA) were added to completely cover each coverslip. After 5 minutes incubation with the DAPI stain, the coverslips were washed with PBS++ several times and mounted using an antifade reagent.

Stained cells were visualized using a Zeiss fluorescent microscope (Oberkochen, Germany) at the 100X objective. The scoring system described in Table 2.5 was used to interpret the activation of the NF- $\kappa$ B signaling pathway based on the localization of the subunits. The scoring system gives each cell a number based on the location of the subunit relative to the DAPI stained nuclei. At least 100 cells were scored per each replicate coverslip. Scores were then averaged and compared to the control condition.

## X. Arginase assay.

Arginase enzymatic activity was assessed using the urea assay. Arginase is an enzyme which metabolizes arginine into ornithine and urea; therefore, urea concentrations directly correlate with the activity and expression level of arginase. J774 murine macrophages were polarized and lysed with 0.1% Triton X-100 (containing protease and phosphatase inhibitors) as described in VI. The enzyme was activated by incubating 50  $\mu$ L of the cell lysate with 50  $\mu$ L of the arginase activation solution (10 mM  $\text{MnCl}_2$  in 50 mM Tris HCl, pH 7.5) for 10 minutes at 55  $^\circ\text{C}$ . Subsequently, 25  $\mu$ L of the previous reaction mixture was added to 25  $\mu$ L of the arginase substrate solution (0.5 M L-arginine in water, pH 9.7). This mixture was allowed to incubate at 37  $^\circ\text{C}$  for 6 hours. The reaction was then terminated by adding 400  $\mu$ L of the acid mixture ( $\text{H}_2\text{SO}_4$ ,  $\text{H}_3\text{PO}_4$ , water at a ratio of 1:3:7) followed by the addition of 25  $\mu$ L of alpha-isonitrosopropiophenone (9% w/v). The reaction mixture was heated at 100  $^\circ\text{C}$  for 45 minutes. Optical density was then read at 540 nm wavelength using a spectrophotometer (the intensity of color change of the urea-chromogen complex was measured). A standard curve was used to interpret the results by repeating the assay described above using standard stock solutions with known urea concentrations.

Readings were normalized to the optical density of blank sample and water. Arginase activity was then calculated in units where 1 unit of arginase activity is equal to the conversion of 1  $\mu$ mole of L-arginine to ornithine and urea per 1 minute; arginase activity was then normalized to the protein concentrations of each sample.

## XI. Western blot analysis.

Western blot analysis was performed to determine the effect of macrophage polarization on the protein mediators of NF- $\kappa$ B and Stat1 signaling pathways. Cell lysates obtained from the polarization assay described in VI were quantified.

Samples of 20-30 µg of protein were denatured by heating for 5 minutes at 95 °C in loading buffer (Bio-Rad, Hercules, CA) containing β-mercaptoethanol. Denatured samples were loaded onto 4-15% precast polyacrylamide gels (Bio-Rad, Hercules, CA). Proteins were then separated by electrophoresis at 100 V for 1-2 hours and transferred onto a methanol-activated and wetted Immobilon®-FL PVDF membrane at 100-200 V for 90 minutes (LI-COR Biosciences, Lincoln, NE). The membranes were rinsed with PBS and then blocked for 1 hour at room temperature with Tris-buffered saline (TBS) based Odyssey® Blocking Buffer (LI-COR Biosciences, Lincoln, NE). Membranes were then incubated overnight at 4°C with primary antibodies specific for p65, IκB-α, IKKβ, phospho-IKKβ (Abcam, Cambridge, UK), phospho-Stat1 (Santa Cruz, Dallas, TX), Stat1 (ThermoFisher, Wilmington, DE), or actin (LI-COR Biosciences, Lincoln, NE) at recommended dilutions. Unbound primary antibodies were washed away with PBS + 0.1% Tween 20 (repeated 3 times with gentle shaking). Subsequently, membranes were incubated with the appropriate IRDye Subclass Specific secondary antibody for 1 hour at room temperature (IRDye 680RD Goat anti-rabbit or IRDye 800CW Goat anti-mouse, LI-COR Biosciences, Lincoln, NE). Excess secondary antibodies were washed away with PBS + 0.1% Tween 20. Membranes were finally rinsed with PBS to remove residual detergents and then imaged and analyzed using the Odyssey® CLx Imaging System (LI-COR Biosciences, Lincoln, NE).

## XII. Human study protocol

### i. Study design

To evaluate azithromycin anti-inflammatory mechanisms in cystic fibrosis patients, a prospective, unblinded, non-randomized study was designed in which each patient served as his or her own control. The study was approved by the Institutional Review Board of the University of Kentucky. Patients with cystic fibrosis who were on chronic azithromycin therapy and meet the

inclusion/exclusion criteria were consented (alternatively children were assented) and instructed to stop azithromycin use for 60-90 days. After informed consent was obtained, sputum was induced voluntarily or via inhalation of hypertonic saline. Samples were collected at 3 regular clinician visits which were scheduled every 1-3 months. At the 1<sup>st</sup> visit, samples were collected, and patients started their 60-90 day azithromycin holiday. At the 2<sup>nd</sup> visit and by the end of the 60-90 day drug holiday period, sputum was collected again and patients re-initiated azithromycin therapy. Finally and at the 3<sup>rd</sup> visit, samples were collected again 60-90 days after azithromycin re-initiation. During the drug holiday and re-initiation periods, patients were encouraged to report any illness and potential adverse reactions to the pulmonologist on call. In addition, check-up calls were done every 2 weeks during the azithromycin holiday to make sure there were no adverse effects that require medical attention.

Demographic information, CFTR mutation type, pulmonary function status, current medication regimen, blood glucose measurements, culture data, and any comorbidities were recorded via chart review for each subject (Data collection form in appendix 1).

## ii. Study population

The University of Kentucky Children's' Hospital pulmonology group provides care for a cystic fibrosis population of approximately 300 patients in our targeted age range between 12 to 50 years. The target of this study was to recruit up to 25 subjects during the period starting from January 2018 until December 2019 (if necessary). Subjects were included without regard to gender, race, or ethnic background according to the following criteria:

#### a. Inclusion criteria

- Age from 12 to 50 years
- Confirmed diagnosis of CF
- Stable disease with FEV1 > 40 % (no CF pulmonary exacerbation, no intravenous antibiotics, no hospitalization, and stable clinical symptoms for past 1 month)
- Chronically colonized with Gram-negative bacterial pathogens based on 1 previous infection and 2 positive cultures on regular visits in the absence of an exacerbation.
- Able and willing to provide the induced sputum clinical samples
- Clinically stable and is on azithromycin for at least 1 year

#### b. Exclusion criteria

- History of cancer
- HIV infection
- Pregnancy
- Breastfeeding
- Any other major illness/disease like heart failure, liver failure
- Systemic corticosteroid treatment
- Participation in other clinical studies
- Undergoing change of therapy during the 4-month study period (including immunomodulatory therapy or steroids)

The above criteria were selected to minimize variability in immune function due to parameters including age, medication exposure, and other disease states.

Aspects of pulmonary immune function have been demonstrated to decline at older ages. Additionally, the above immunomodulatory disease states affect the parameters that were measured; therefore, exclusion of these patients was necessary.

### iii. Human sample processing

Fresh samples were transported on ice to the laboratory for analysis of inflammatory cell populations and their function. Sputum samples were liquefied and homogenized with an equal volume of Sputasol (Thermo Fisher Scientific, Waltham, MA) containing dithiothreitol. Samples were then filtered using a 48- $\mu$ m nylon mesh and centrifuged to separate cells from the fluid phase. Cells were then enumerated and divided for different experimental purposes.

#### a. TaqMan microfluidic cards for gene expression assays

RNA was isolated as described in section VII and used for microarray analysis to compare expression of inflammatory genes at different treatment timepoints. Microarrays were performed using TaqMan® Array Micro Fluidic Cards (Life Technologies, Grand Island, New York). Life Technologies Corporation manufactured gene microarrays that are specific to a given cellular function, pathway, or disease state. These arrays each consisted of 47 genes associated with expression of the inflammatory immune reactions.

RT-qPCR reactions were performed using cDNA reverse transcribed from the purified RNA to amplify the specific genes of interest (as described in VII). When the 3 samples are obtained from each patient, genetic information will be compared between different timepoints to determine if alterations exist in relative expression levels as a result of azithromycin treatment. Scatter plots will be generated to analyze fold differences in relative expression levels for each gene when patients are on and off azithromycin. A list of genes included in the assessment can be found in Appendix 2.

### b. Flow cytometry analysis of immune cells in the sputum

Single-cell suspensions from processed sputum were stained and analyzed with flow cytometry as described in V. Cells were stained for multiple surface and intracellular markers listed below:

- TLR
- CD25
- CD19
- CD11c
- CD4
- CD8
- Foxp3
- ROR $\gamma$ t
- CD11b
- ARG1
- ARG2

### c. Cytometric Bead Array (CBA)

Cytokine concentrations in cell lysates from sputum samples were evaluated using CBA kits. After sputum processing, cells were lysed in a hypotonic buffered solution, and the lysates were frozen at -80°C for simultaneous analysis of all samples within 6 months. Concentrations of the cytokines listed below were quantified using BD CBA Kits (BD Biosciences, San Jose, CA) according to the manufacturer protocol. Briefly, the CBA Kit contains bead populations with distinct fluorescence intensities that are coated with capture antibodies specific for each cytokine to be measured. These beads were incubated with fluorochrome-conjugated detection antibodies and then incubated with 50  $\mu$ L of each sample for 3 hours at room temperature. Sandwich complexes formed, after which the beads were washed, and the fluorescence intensity was assayed by flow cytometry. These intensities were then compared to a standard curve generated for each cytokine to determine the concentration in each sample.

- TNF- $\alpha$
- IL-1 $\beta$
- IFN $\gamma$
- IL-2
- IL-4
- IL-6
- IL-10
- IL-12p40
- IL-5
- IL-8

### XIII. Statistical analysis.

Statistical analysis was performed utilizing GraphPad Prism (GraphPad Software, La Jolla, CA). Comparison between groups was made via one-way ANOVA with Tukey's test multiple comparisons, paired sample T-test with McNemar's test, or via two-way ANOVA with Sidak's multiple comparisons test. Repeated-measures ANOVA with Tukey's multiple comparison tests were utilized for time course experiments. Kaplan-Meier survival curves were compared using Log-rank (Mantel-Cox) and Gehan-Breslow-Wilcoxon tests. The trend of survival curves was also compared using Log-rank test.

Gene and protein expression levels in sputum samples were analyzed through principal component analysis. The expression levels were compared among paired samples from the 3-collection time-points when patients are on and off azithromycin. Results were then correlated to clinical outcomes (demographic information, pulmonary function tests, requirement for hospitalization, requirement for antibiotics, etc.) acquired through retrospective review of the chart. Paired sample T-test with McNemar's test and two-way ANOVA were used to perform statistical analysis for paired human samples and among groups.

Table 2.3. Lung injury scoring guide

Parameter	Score per field		
	0	1	2
A. Neutrophils in the alveolar space	None	1-5	>5
B. Neutrophils in the interstitial space	None	1-5	>5
C. Hyaline membranes	None	1	>1
D. Proteinaceous debris filling the airspaces	None	1	>1
E. Alveolar septal thickening	<2x	2x-4x	>4x

Table 2.4. Key markers for different immune cell populations.

Immune Cell Populations	Key Markers
Infiltrating Monocytes	F4/80 <sup>+</sup> , CD11c <sup>-</sup> , CD11b <sup>+</sup>
Alveolar Macrophages	F4/80 <sup>+</sup> , CD11c <sup>+</sup> , CD11b <sup>-</sup>
Interstitial Macrophages	F4/80 <sup>+</sup> , CD11c <sup>+</sup> , CD11b <sup>+</sup>
Neutrophils	F4/80 <sup>-</sup> , Ly6G <sup>+</sup>
Type 2 innate lymphoid cells (ILC2)	LIN <sup>-</sup> , CD90.2 <sup>+</sup> , CD127 <sup>+</sup> , IL33R <sup>+</sup>
Activated CD4 <sup>+</sup> T cells	CD4 <sup>+</sup> , CD44 <sup>Hi</sup> , CD62L <sup>lo</sup>
Th1 lymphocytes	CD4 <sup>+</sup> , CXCR3 <sup>+</sup> , IFN $\gamma$ <sup>+</sup>
Th17 Lymphocytes	CD4 <sup>+</sup> , ROR $\gamma$ t <sup>+</sup> , IL-17 <sup>+</sup>
Treg Lymphocytes	CD4 <sup>+</sup> , FOXP3 <sup>+</sup> , TGF $\beta$ <sup>+</sup>

Table 2.5. P65 nuclear translocation scoring scale.

Score	Location of p65 in a single cell
0	Cytoplasmic signal only
1	Evenly distributed signal (nuclear and cytoplasmic)
2	Mainly nuclear with faint cytoplasmic signal
3	Nuclear signal only

Table 2.5. P65 nuclear translocation scoring scale.

Polarized J774 murine macrophages were immunostained on glass coverslips and visualized using Zeiss fluorescent microscope. Different fields were evaluated to score at least 150 cells per each replicate coverslip by 2 blinded investigators. Each cell was evaluated to determine the localization of the p65 signal with respect to the nucleus.

Figure 2.1

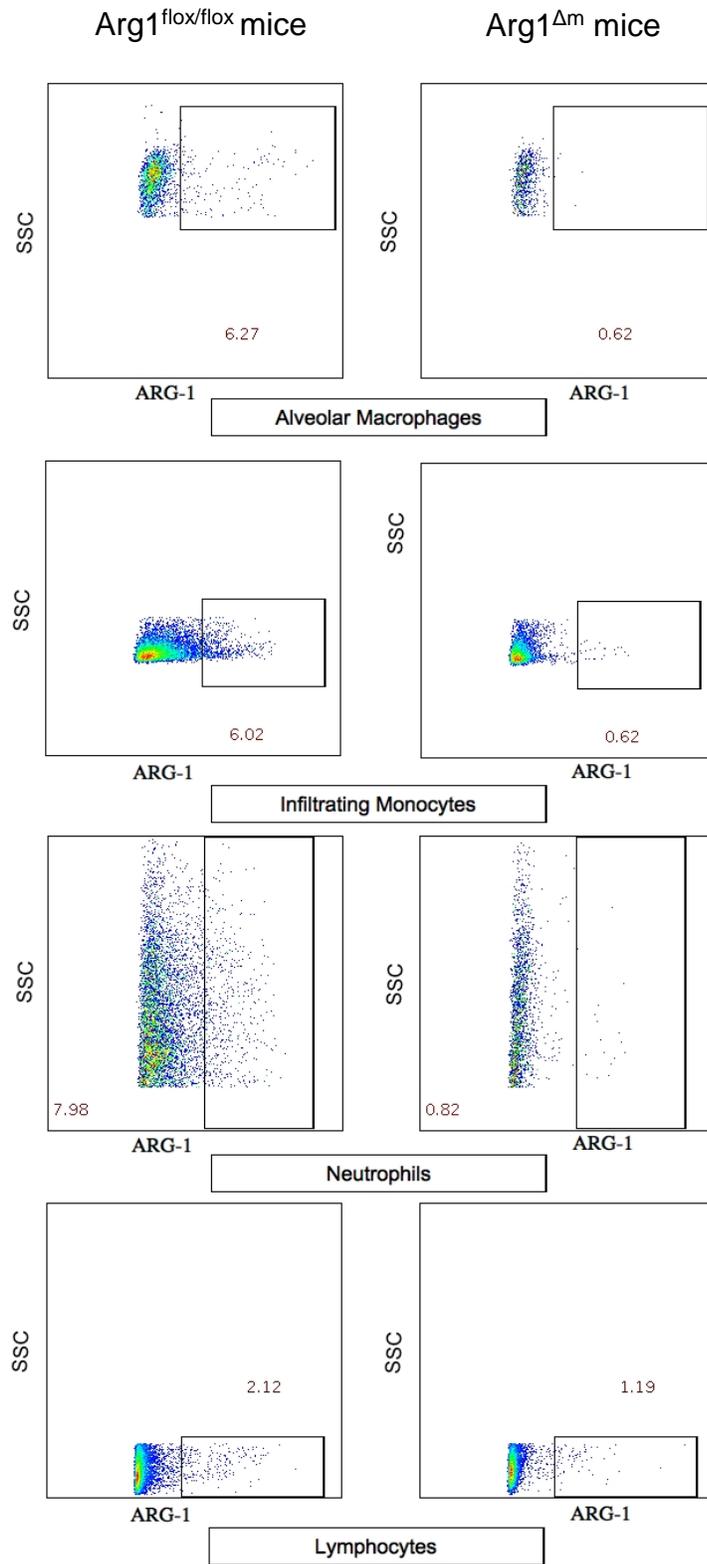


Figure 2.1. Arginase deletion from Arg1<sup>Δm</sup> mice.

Lungs from Arg1<sup>flox/flox</sup> and Arg1<sup>Δm</sup> mice were lavaged with PBS as described and the lung tissues were collected. Cells were then processed into single cell suspensions for flow cytometry analysis. Figure shows representative images of arginase-1 expression in alveolar macrophages, infiltrating monocytes, neutrophils, and lymphocytes from Arg1<sup>flox/flox</sup> and Arg1<sup>Δm</sup> mice.

Figure 2.2a

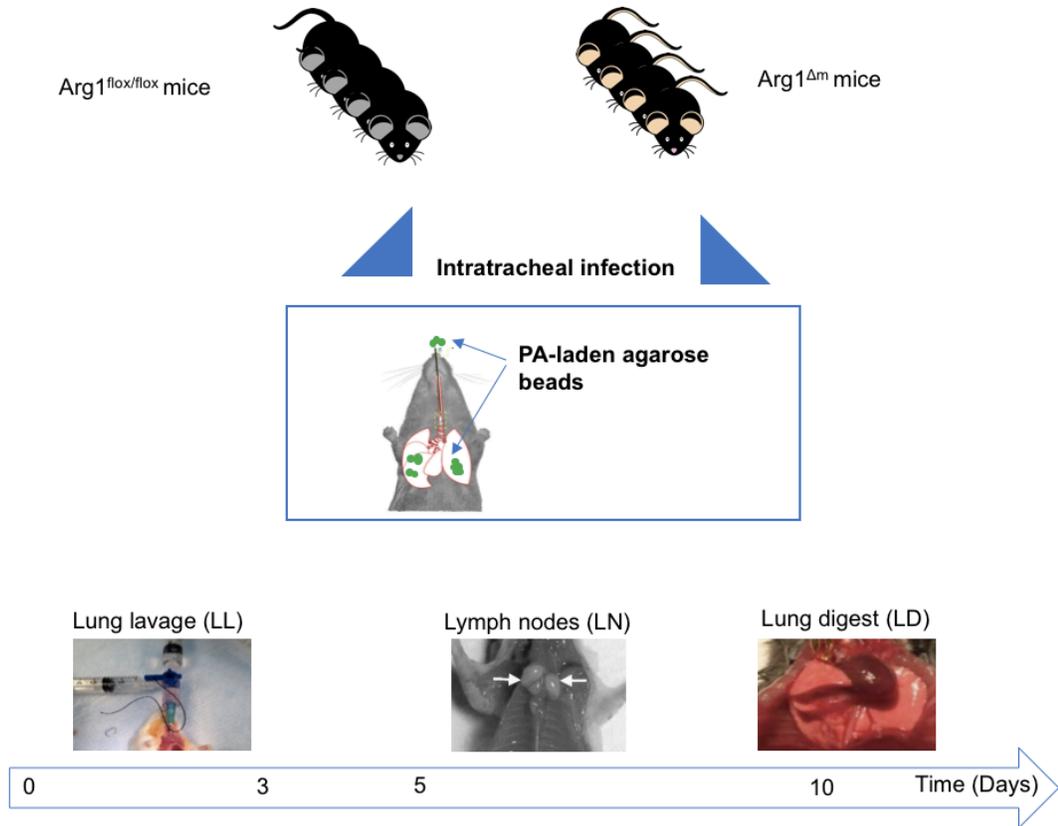


Figure 2.2b

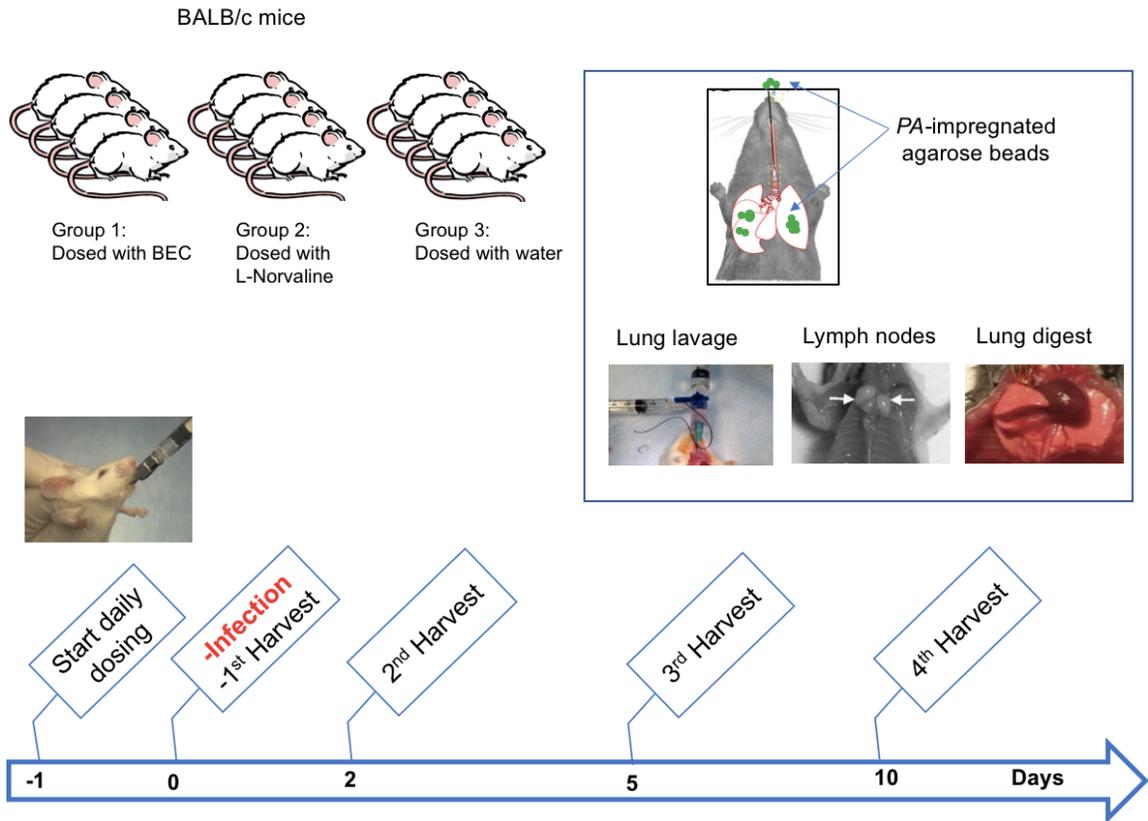


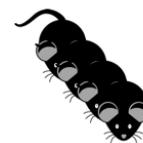
Figure 2.2c



Group 1:  
AZM-treated Arg1<sup>Δm</sup> mice



Group 2:  
AZM-treated Arg1<sup>flox/flox</sup> mice



Group 3:  
Control Arg1<sup>Δm</sup> mice

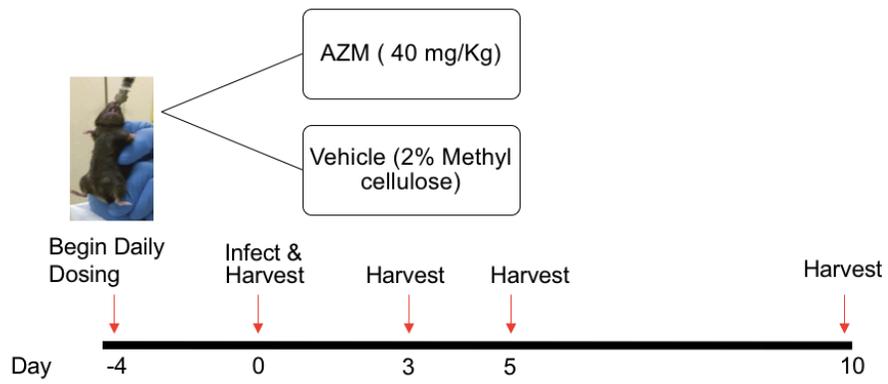
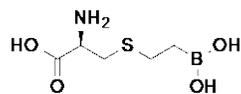


Figure 2.2. Murine experimental design.

(a) and (b) figures depict the experimental design for experiments in chapter 3.

(c) Experimental design for experiments in chapter 5.

Figure 2.3. Synthesis of BEC



Synthesis of BEC. Di-(*n*-butyl)-vinylboronate (303 mg, 1.65 mmol) and L-cysteine (200 mg, 1.65 mmol) were mixed in MeOH (5 mL) and H<sub>2</sub>O (3 mL) under N<sub>2</sub>. 2,2'-Azobis(2-methylpropionitrile) (AIBN) (30 mg) was added and the mixture was heated at 80 °C for 1 h. An additional portion of AIBN (20 mg) was further added and the mixture was heated for an additional 6 h at 80 °C. The solvents were evaporated and the crude product was purified by flash column chromatography (SiO<sub>2</sub> gel, 1:4:0.5/CH<sub>2</sub>Cl<sub>2</sub>:MeOH:NH<sub>4</sub>OH) to afford BEC as white solid (170 mg, 53%): <sup>1</sup>H NMR (400 MHz, D<sub>2</sub>O, Figure 2.3b) δ 3.74 (dd, *J*<sub>1</sub> = 7.6 Hz, *J*<sub>2</sub> = 4.2 Hz, 1H), 2.96 (dd, *J*<sub>1</sub> = 14.8 Hz, *J*<sub>2</sub> = 4.2 Hz, 1H), 2.84 (dd, *J*<sub>1</sub> = 14.8 Hz, *J*<sub>2</sub> = 7.6 Hz, 1H), 2.54 (t, *J* = 7.9 Hz, 2H), 2.84 (td, *J*<sub>1</sub> = 7.9 Hz, *J*<sub>2</sub> = 2.4 Hz, 2H); <sup>13</sup>C NMR (100 MHz, D<sub>2</sub>O, Figure 2.4c) δ 172.9, 53.4, 31.7, 26.8, 14.8.

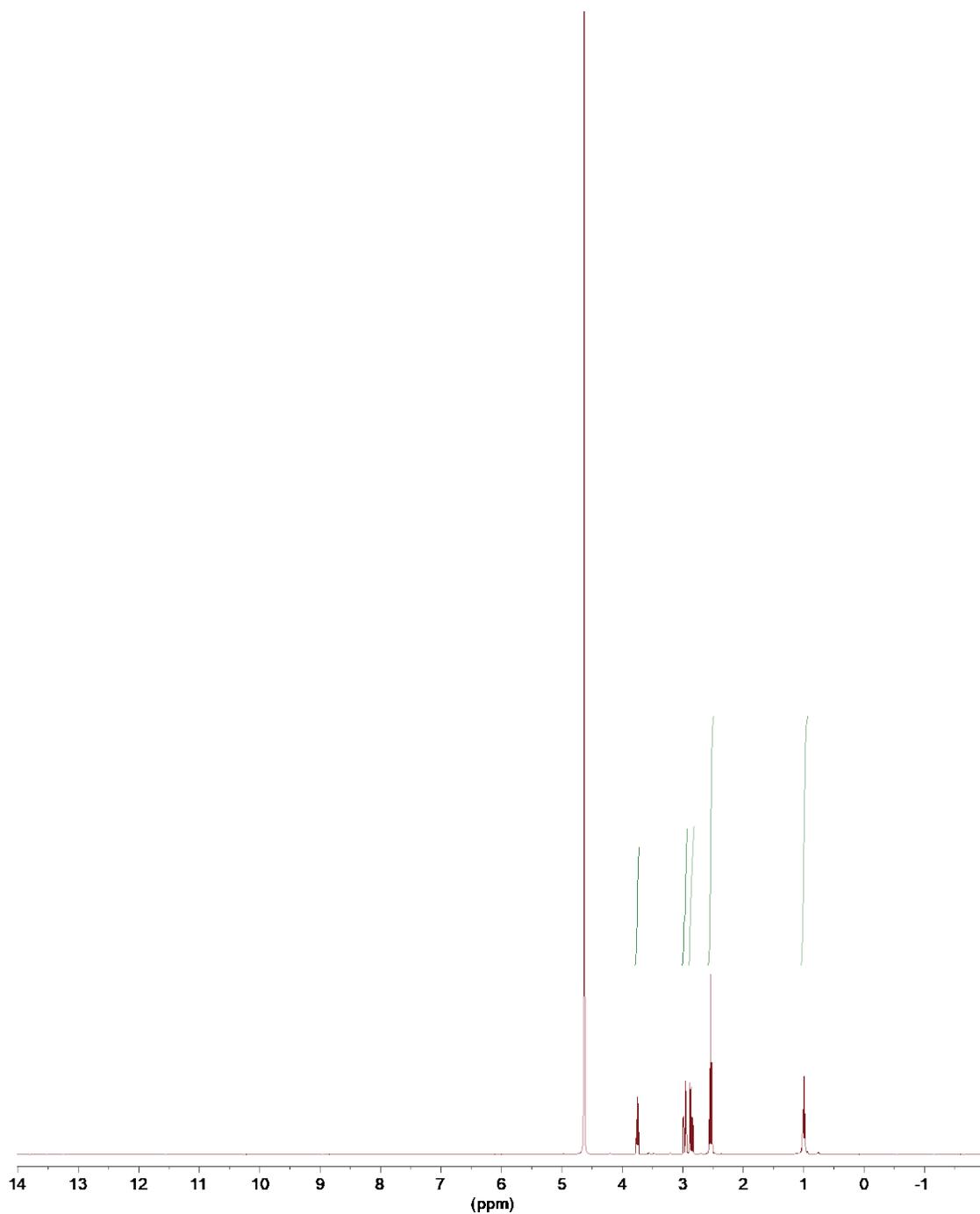


Figure 2.3b  $^1\text{H}$  NMR of BEC in  $\text{D}_2\text{O}$  (400 MHz).

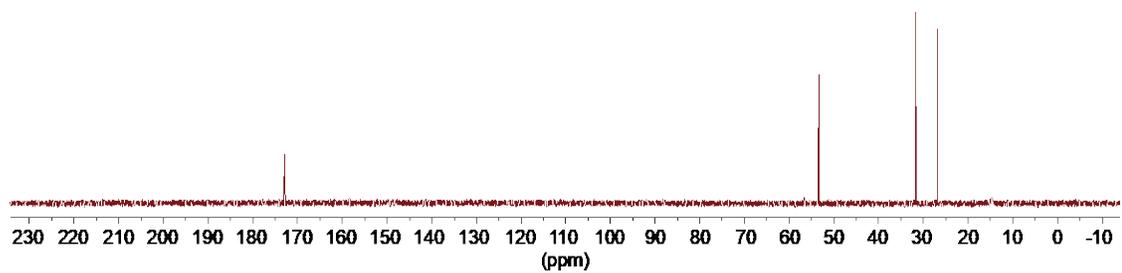


Figure 2.3c  $^{13}\text{C}$  NMR of BEC in  $\text{D}_2\text{O}$  (100 MHz).

## Chapter 3: Requirement of arginase in host protection against excessive inflammation

### I. Introduction

Macrophages are important sentinel cells of the immune system that play highly complex and coordinated functions during inflammatory responses [131]. In addition to macrophages that reside in tissues, activated monocytes are recruited from the bone marrow and infiltrate tissues in response to injury, infections, or other inflammatory stimuli. However, both tissue and infiltrating macrophages are not terminally differentiated; they undergo phenotypic changes which can direct their functions into killer (M1) macrophages or into repair (M2) macrophages [327]. The balance between these two macrophage phenotypes is essential for proper activation and termination of the immune response and for maintaining tissue homeostasis. In fact, a shift towards M1 macrophage polarization is associated with exaggerated inflammation and autoimmune disorders; while a shift towards M2 macrophage polarization is associated in some instances with suppressed immunity and inability of the immune system to combat infections [327]. Therefore, macrophage phenotypic changes are viewed as a spectrum of changes which are driven by the two major classes of lymphocytes [327]. Th1 cytokines (IFN $\gamma$ ) polarized macrophages towards an M1 or classical phenotype while M2 or alternative macrophages are polarized in the presence of Th2 cytokines (IL-4 and IL-13). M1 macrophages are pro-inflammatory, they are characterized with bactericidal and phagocytic properties and they release pro-inflammatory cytokines and mediators. Alternatively, M2 macrophages are viewed as anti-inflammatory cells with important functions for limiting the immune response and driving wound healing and tissue repair. Additionally, M1 and M2 macrophages have distinct metabolic properties which drive their different immune functions. The two macrophage populations metabolize arginine, a unique conditionally essential amino acid, differently through the expression of different enzymes. Nitric oxide (NO) is the characteristic inflammatory and

microbicidal mediator of M1 macrophages which is generated with the induced expression of nitric oxide synthase enzyme (iNOS). Conversely, M2 macrophages express arginase-1 enzyme which metabolizes arginine into proline and polyamines essential for cell proliferation and wound healing [328].

Thus, arginine metabolomics in macrophages had been evaluated as a key regulator of the immune response. While 80% of circulating arginine comes from diet and proteolysis, the remaining 15-20% are from citrulline-derived synthesis in the kidneys [130]. Under physiological conditions, arginine primarily participates in the urea cycle for the elimination of toxic ammonia compounds. However, under certain inflammatory conditions, arginine becomes semi-essential where it is metabolized by arginase-1, arginase-2, or iNOS [130]. Both arginase isoforms convert arginine into ornithine and urea. Cytosolic arginase-1 is predominantly expressed in hepatocytes and certain immune cells, whereas mitochondrial arginase-2 is found in renal cells, enterocytes, neurons, and mammary gland cells. Constitutive expression of arginase-2 is mainly involved in the generation of ornithine precursor for the synthesis of proline (for collagen synthesis and repair) and polyamines (for cell proliferation and regeneration). Conversely, arginase-1 expression is induced in macrophages, neutrophils, and other immune cells where it competes with iNOS thereby regulating NO generation and limiting NO-mediated inflammatory injury [129-132, 135, 136, 317-319]. However, recent studies evaluating the immunomodulatory role of arginase-1 suggest that its beneficial properties reach beyond its effects on limiting NO generation. In contrast to iNOS which does not deplete the arginine pool due to the citrulline recycling pathway, arginase depletion of arginine was found to have important effects on T cell function and proliferation [152, 154, 157, 314, 315, 316 2008, 317]. Arginine depletion by arginase arrests T cell proliferation in the G0-G1 phase of the cell cycle, decreases its CD3 $\zeta$  expression, affects the development of memory T cells, and suppresses the T cell effector pro-inflammatory functions [151-161]. By this mechanism, arginase suppresses the secretion of Th1 cytokines (IFN $\gamma$ ), as well as Th2 cytokines (IL4, IL5, and

IL10) [151-161]. Arginine deprived T cells also decrease their expression of activation markers (CD25, CD28, and CD62L) [160]. Those changes promote a regulatory T cell phenotype and suppress T cell immunity [129-132, 135, 136, 317-319]. Additionally, arginase expression by M2 macrophages stimulates autophagy by increasing the conversion of microtubule-associated protein light chain 3 (LC3) from LC3-I to LC3-II [329]. Thus, arginase expression mediates the formation of autophagosomes and induces caspase-independent apoptosis. Autophagy induction by arginase is cytoprotective and it plays a critical regulatory role that downregulates inflammation [329-334]. Collectively, arginase expression by macrophages and other immune cells depletes arginine, controls T cell responses, prevents inflammatory injury, induces autophagy, and modulates the immune response.

The immunomodulatory role of arginase had been evaluated in several disease models. In cancer, myeloid suppressor cells producing arginase inhibit T cell responses against tumor cells [151-162]. In pregnancy, arginase is essential to prevent maternal immune reactions against the fetus [319]. Arginase also plays an important immunomodulatory role in sepsis, trauma, surgery, certain infections, and some inflammatory and autoimmune diseases (e.g. leishmaniasis, streptococcus pneumonia infection, schistosomiasis, autoimmune encephalomyelitis, asthma, and experimental glomerulonephritis) [151, 152, 316]. However, arginase mediated modulation of the inflammatory response against *Pseudomonas aeruginosa* (PA) pneumonia has not been investigated.

About 60% of patients with cystic fibrosis (CF) are infected with PA which chronically colonizes the lungs of 80% of the patients older than 18 years [17-19]. Infections with PA are characterized by a chronic dysregulated inflammatory response with aberrant T cell immunity and a predominant recruitment of neutrophils and pro-inflammatory macrophages [312]. Epithelial cells with mutated CFTR channels release excessive amounts of cytokines and chemokines (IL8, IL1, and TNF- $\alpha$ ) recruiting tremendous amounts of neutrophils

and pro-inflammatory macrophages which further release more inflammatory mediators. Neutrophil death results in the release of proteases and neutrophil elastases which digest the structural airway proteins and result in bronchiectasis. Death also causes the release of neutrophil intracellular DNA resulting in increased mucus viscosity, oxidative stress, and free radical formation. In addition to the exaggerated responses of pro-inflammatory neutrophils and macrophages, T cell responses against PA pneumonia in CF are also dysregulated, with a Th1 predominant response believed to be disproportionate to that required to respond to infection [312, 335]. This exaggerated inflammation had previously been attributed to abnormal NF- $\kappa$ B activation, decreased negative feedback and regulatory responses, disproportional cytokine release, and irregular immune cell activation [312].

Our group has previously demonstrated that azithromycin-driven polarization of macrophages into an alternative phenotype controls several aspects of the immune response in mice infected with PA-impregnated agarose beads [166, 310, 311]. Previously published data suggest that azithromycin treatment polarizes macrophages towards an M2 phenotype, shifts the immune response in infected mice into a monocyte predominant response, blunts neutrophil influx, and shifts the T cell response away from the Th1 phenotype [166, 310, 311].

Closely examining the different effectors of those M2 macrophages revealed an important role of arginase-1. Azithromycin treatment significantly increases arginase expression and activity in vitro and in vivo. In mice treated with azithromycin, peaks in arginase expression in the lungs at days 3 and 14 post-infection were consistent with reduced morbidity and decreased airway damage. In this chapter, we evaluate the hypothesis that the decreases in inflammation in response to PA pneumonia achieved by polarizing macrophages to an M2 phenotype is dependent upon the production of arginase-1. The possible role and mechanism of arginase modulation of inflammation and T cell immunity in PA pneumonia have not been previously investigated. Many studies focus on

changes in arginase expression and its competition with iNOS in pulmonary inflammatory conditions, but to our knowledge, none of the studies previously examined the possible protective role of arginase in PA pneumonia. This study presents a novel approach for examining the immune-protective role of arginase-1 in PA pneumonia by utilizing arginase-1 conditional knock-out mice and two pharmacological inhibitors of arginase (L-norvaline and S-(2-boronoethyl)-L-cysteine (BEC)). We show that macrophage arginase-1 is essential for control of inflammatory responses in PA pneumonia with potentially different effects of other cellular sources as shown with global arginase inhibition.

## II. Results

Increased morbidity and acute inflammation in arginase-1 conditional knock-out mice. We evaluated the impact of arginase deletion from myeloid cells upon the acute inflammatory response against PA pneumonia. We conditionally deleted arginase-1 in macrophages and neutrophils of C57BL/6 mice. Arg1<sup>Δm</sup> and control Arg1<sup>flox/flox</sup> mice were infected intratracheally with PA-laden agarose beads to cause a prolonged infection similar to the chronic infections in patients with cystic fibrosis. Morbidity in infected mice was evaluated by measuring their body weight daily (Figure 3.1a). Mice were then humanely killed for assessment of inflammation and lung injury at different timepoints post-infection (Figure 3.2). Lungs were lavaged to collect cells from the alveolar spaces and the lung tissues were harvested to collect cells from the lung interstitium. Lavage and lung tissue samples were processed for flow cytometry analysis to assess neutrophil recruitment, macrophage disposition, as well as macrophage polarization over time post-infection. Arg1<sup>Δm</sup> mice lost more weight compared to their littermate controls. There was a significantly faster and greater decline in murine body weight at days 1 and 2 post-infection in the arginase conditional knock-out mice (Figure 3.1a). Increased morbidity in the Arg1<sup>Δm</sup> mice was associated with an average weight loss of 13.33% of their baseline body weight versus an average weight loss of 8.09% in the control mice (Figure 3.1, day 2, p-value < 0.0001).

Conversely, control mice recovered faster than the Arg1<sup>Δm</sup> mice which slowly regained their weight over time (Figure 3.1a, day 3, littermate control mice at 96.02% of their baseline weight vs 90.38% in the conditional knock-out mice, p-value = 0.0002).

Interestingly, there were no significant differences in terms of bacterial clearance and mortality between the two groups (Figure 3.1 b and c). Lung bacterial clearance was evaluated at different timepoints post-infection by counting the number of viable PA colonies in homogenized lung tissues. Arg1<sup>Δm</sup> mice cleared PA at a comparable rate similar to the Arg1<sup>flox/flox</sup> mice. Both groups failed to completely clear the infection and PA was still present in the lungs by day 10 post-infection. Similarly, arginase deletion from myeloid cells was not associated with increased mortality (Figure 3.1c). Importantly, the infective inoculum used in these experiments was selected to cause murine pneumonia without causing severe morbidity or mortality in each mouse strain. Mice were euthanized and excluded from the analysis if they lose 20% or more of their body weight prior to infection along with 1 sign of morbidity (e.g. immobility, hunched posture, or lack of response to handling).

Additionally, the impact of arginase production by myeloid cells upon the acute inflammatory response to the infection was evaluated (Figure 3.2). Figure 3.2a explains the gating scheme used in FlowJo to identify different immune cell populations activated in response to the infection. Arg1<sup>Δm</sup> mice had significantly greater recruitment and infiltration of neutrophils into their lung interstitial and alveolar spaces. Arg1<sup>Δm</sup> mice had a greater peak in neutrophil counts at day 3 post-infection compared to their littermate control mice (Figure 3.2b, p-value = 0.0033 in the alveolar spaces and lung interstitium). Similarly, there was a significantly larger number of macrophages in the lungs of arginase conditional knock-out mice (Figure 3.2c). Littermate control mice had significantly lower numbers of tissue macrophages at day 3 post-infection in their alveolar spaces compared to the Arg1<sup>Δm</sup> mice (p-value = 0.0042). The latter recruited and

maintained a larger number of macrophages into their lung interstitium at days 3 through 5 post-infection (Figure 3.2c, day 3, p-value = 0.0003; day 5 p-value < 0.0001).

Macrophage polarization was also evaluated in the arginase conditional knock-out mice. The expression of different M1 and M2 macrophage effectors was assessed to determine the phenotype of macrophages recruited in response to the infection (Figure 3.2 d, e, f). Arg1<sup>Δm</sup> mice recruited larger numbers of all macrophage phenotypes. There were significantly higher numbers of TNF-α producing macrophages in the lungs of arginase conditional knock-out mice (Figure 3.2d, day 5, p-value = 0.0063 (alveolar and infiltrating)). Additionally, there was a significantly greater number of IL-10 producing alveolar macrophages in the lungs of arginase conditional knock-out mice compared to their littermate controls (Figure 3.2e, day 3, p-value = 0.0225). However, the number of infiltrating monocytes producing IL-10 were comparable in both groups with no significant difference. Similarly, there was no significant difference in the number of mannose receptor expressing alveolar macrophages between the 2 groups (Figure 3.2f). However, the arginase conditional knock-out mice recruited significantly larger numbers of mannose receptor expressing macrophages into their lung interstitium later at 10 post-infection compared to the control mice (Figure 3.2f, day 10, p-value = 0.0493). Interestingly, the ratios of TNF-α and IL-10 producing cells were comparable in both groups (Figure 3.2g, A). However, Arg1<sup>Δm</sup> had at least 2 times more neutrophils than macrophages in their lungs by day 3 post-infection compared to Arg1<sup>flox/flox</sup> mice (Figure 3.2g, B, day 3, p-value = 0.0387).

Arginase conditional knock-out mice respond to PA pneumonia with greater T cell recruitment and activation. The impact of arginase production by neutrophils and macrophages upon the T cell responses was evaluated in Arg1<sup>Δm</sup> mice infected with PA-laden agarose beads intratracheally. T cell responses and activation profiles at different timepoints post-infection were evaluated in the

tracheobronchial lymph nodes that drain the site of infection, in the lung lavage, and in the lung tissue samples (Figure 3.3). Figure 3.3a and 3.4a explain the gating scheme used in FlowJo to identify different T cell populations. Although the arginase conditional knock-out mice responded to the infection with a greater increase in T cell numbers in their tracheobronchial lymph nodes by day 5 post-infection, the differences were not statistically significant (Figure 3.3b). Similarly, there was a greater increase in T cell activation in the lymph nodes of arginase conditional knock-out mice at day 5 post-infection which was not statistically significant. Conversely, there were significantly greater numbers of CD4+ T cells and activated CD4+ T cells in the alveolar spaces of arginase conditional knock-out mice compared to their littermate controls (Figure 3.3c, day 5, p-value = 0.0369 and 0.0092, respectively). However, the arginase conditional knock-out mice responded with comparable T cell recruitment and activation in their interstitial spaces compared to their littermate controls with no significant differences (Figure 3.3d).

Additionally, we analyzed the phenotypes of activated T cells in the lymph nodes and lungs overtime post-infection (Figure 3.4). Th1 lymphocytes were defined as the CD4+ T cells which express CXCR3 and IFN $\gamma$ . In the lymph nodes of arginase conditional knock-out mice, there was a greater increase in the number of Th1 lymphocytes compared to the littermate control mice, but the differences were not statistically significant (Figure 3.4b). However, there were significantly higher numbers of Th1 lymphocytes in the lungs of arginase conditional knock-out mice at days 3 and 5 post-infection (Figure 3.4c, p-value = 0.0345 and 0.0371, respectively). Finally, Th17 lymphocytes were defined as the CD4+ T cells which express ROR $\gamma$ t and IL-17. There were significantly larger numbers of Th17 lymphocytes in the lymph nodes and in the lungs of arginase conditional knock-out mice compared to the littermate control mice (Figure 3.4 b and c, day 3, p-value = 0.0191 and 0.0488, respectively).

Additionally, we estimated the ratios of pro- and anti-inflammatory cytokines expressed by all lymphocytes. The lymphocyte population contains CD4<sup>+</sup> T cells as well as other lymphoid cells including the innate lymphoid cells, CD8<sup>+</sup> T cells, and NK cells. Figure 3.2g shows the ratios of IL-17, IFN $\gamma$ , and IL-10 cytokines from lymphoid origin. The ratio of IL-17 to IL-10 producing lymphocytes was significantly higher in Arg1 $\Delta^m$  mice at days 3, 5, and 10 post-infection (Figure 3.2g, C, p-value < 0.0001). However, the ratios and trend of IFN $\gamma$  to IL-10 expressing lymphocytes were comparable among the 2 groups with similar trends (Figure 3.2g, D).

Pharmacological arginase inhibition is associated with comparable morbidity and acute inflammatory response. We evaluated the impact of pharmacologic arginase inhibition upon the inflammatory response to PA pneumonia. BALBc/J mice were dosed with one of 2 pharmacological arginase inhibitors (BEC or L-norvaline) via oral gavage starting 1 day prior to infection and daily thereafter. Control BALBc/J mice were dosed with water daily. Mice were infected with PA-laden agarose beads as described in the methods section. Figure 3.5a shows morbidity at different tested infective inoculums. No significant morbidity was attained at the lowest inoculum of  $5 \times 10^6$  CFU/mL (Figure 3.5a, B) while the highest inoculum of  $5 \times 10^7$  CFU/mL resulted in severe morbidity (Figure 3.5a, D). Most of the mice infected with the inoculum of  $1 \times 10^7$  CFU/mL lost at least 10% of the baseline body weight (Figure 3.5a, C). Therefore, we selected an inoculum between  $1 \times 10^7$  CFU/mL and  $5 \times 10^7$  CFU/mL for our experiments. Statistical significance was determined using two-way ANOVA ((x) BEC treated group significantly different than water treated group; (#) L-norvaline treated group significantly different than water treated group; (+) BEC treated group significantly different than L-norvaline treated group; p-value < 0.05).

Morbidity post-infection was evaluated by measuring daily weight and evaluating weight loss as compared to the baseline prior to infection. There were no significant differences in terms of acute weight loss in mice treated with water

versus mice treated with the arginase inhibitors. However, control mice recovered slowly and incompletely compared to the mice treated with the arginase inhibitors (Figure 3.5b). While the BEC and L-norvaline treated mice regained their lost weight at day 6 post-infection up to an average of 89.94% and 91.5% of their baseline body weight respectively, the water treated mice failed to recover with an average body weight at day 6 of 83.2% of their original weight prior to infection (Figure 3.5b, day 6, (x) p-value = 0.0391, (#) p-value = 0.0187). Similarly, BEC and L-norvaline treated mice recovered with an increase in their weight up to 98.21% and 99.4% of their baseline body weight at day 10 post-infection while the water treated mice remained at 88.5% of their original weight (Figure 3.5b, day 10, (x) p-value = 0.025, (#) p-value = 0.0161). Interestingly, there were no significant differences in terms of mortality or bacterial clearance among the 3 groups (Figure 3.5 c and d).

Additionally, we evaluated the impact of pharmacologic arginase inhibition on the inflammatory response and recruitment of innate immune cells (Figure 3.6). Surface and intracellular staining of lung lavage and lung tissue samples collected at different timepoints post-infection were performed and analyzed using flow cytometry. While the water treated mice had significantly higher numbers of neutrophils recruited into their alveolar spaces compared to the mice treated with BEC and L-norvaline (Figure 3.6a, day 2, (x) p-value = 0.0264, (#) p-value = 0.0221); neutrophil recruitment into the lung interstitium was comparable among the 3 groups of mice. Similarly, water treated mice had significantly higher numbers of macrophages in their alveolar spaces at day 2 post-infection (Figure 3.6b, day 2, (x) p-value = 0.0263, (#) p-value = 0.0053). Macrophage infiltration into the lung interstitium was comparable with no significant differences among the 3 groups (Figure 3.6b).

To evaluate the phenotype of macrophages recruited into the lungs of mice treated with pharmacologic arginase inhibitors we stained for different M1 and M2 macrophage effectors and cytokines. There were no statistically significant

differences in either alveolar or infiltrating macrophages expressing TNF- $\alpha$ , IL-10, or iNOS (Figure 3.6c, d, f) among the 3 groups of mice. However, mice treated with BEC or L-norvaline had significantly lower numbers of MR expressing alveolar macrophages compared to water treated mice (Figure 3.6e, day 2, (x) p-value = 0.038, (#) p-value = 0.0165). Similarly, mice treated with pharmacologic arginase inhibitors presented with significantly lower numbers of infiltrating M1 macrophages at day 2 post-infection compared to water treated mice (Figure 3.6g, day 2, (x) p-value = 0.0253, (#) p-value = 0.0349). Conversely, alveolar M1 macrophages and infiltrating macrophages expressing MR were comparable among the 3 groups. Interestingly, the ratio of CCR7 and MR expressing macrophages and of TNF- $\alpha$  and IL-10 producing cells were comparable among the 3 groups (Figure 3.6h, A and B).

Finally, we stained for type 2 innate lymphoid cells (ILC2) which are a major source of arginase in addition to neutrophils and macrophages (Figure 3.6i). ILC2s are derived from a lymphoid progenitor, but they lack B and T cell receptors. ILC2s are found in the skin, respiratory and gastrointestinal systems and they play a role in asthma, allergy, and parasitic infections. They release Th2 cytokines and express arginase-1 constitutively [336]. Control mice had a greater increase in ILC2 recruitment to the alveolar spaces at day 2 post-infection which was not significantly different than the ILC2 recruitment in mice treated with arginase inhibitors. Similarly, the numbers of ILC2 infiltrating into the lung interstitium were comparable among the 3 groups of mice.

Pharmacologic arginase inhibition is associated with increased T cell recruitment and activation in the lymph nodes of infected mice with potentially reduced chemotaxis to the lungs. We evaluated the impact of arginase inhibition in mice infected with PA pneumonia upon the activation of different T cell responses. The total number of CD4<sup>+</sup> T cells and the number of CD4<sup>+</sup> T cells expressing activation markers were quantified at different time points post-infection. In the tracheobronchial lymph nodes of water treated mice, there was a slightly higher

acute recruitment and activation of CD4<sup>+</sup> T cells at day 2 post-infection compared to mice treated with arginase inhibitors (Figure 3.7a, day 2, p-value > 0.05). The latter had a significantly increased recruitment and activation of CD4<sup>+</sup> lymphocytes in their tracheobronchial lymph nodes at day 10 post-infection (Figure 3.7a, day 10, Total T cells: (x) p-value = 0.0017, (#) p-value = 0.0066; Activated T cells: (x) p-value = 0.0292, (#) p-value = 0.0044). Conversely, water treated mice had significantly higher numbers of CD4<sup>+</sup> lymphocytes in their alveolar spaces at day 2 post-infection compared to the 2 groups treated with arginase inhibitors (Figure 3.7b, day 2, (x) p-value = 0.0349, (#) p-value = 0.0285). However, there were no significant differences in T cell recruitment to the alveolar spaces at later timepoints post-infection. Similarly, the number of activated T cells in the alveolar spaces were comparable at all timepoints post-infection with no significant differences among the 3 groups of mice (Figure 3.7b). As for the T cell responses in the lung interstitium, the 2 groups of mice treated with arginase inhibitors have slightly higher numbers of CD4<sup>+</sup> lymphocytes compared to the water-treated mice (Figure 3.7c, Total T cells, p-value > 0.05). However, mice treated with L-norvaline had significantly higher numbers of activated T cells in their interstitial spaces compared to both BEC and water treated mice (Figure 3.7c, activated T cells, day 2 (+) p-value = 0.0081, (#) p-value = 0.0016; day 5 (+) p-value = 0.0256, (#) p-value = 0.0179). Although no statistical significance was observed, the trend of increased numbers of CD4<sup>+</sup> T cells observed on day 10 post-infection in the lymph nodes holds true for both the airways and the lung interstitium.

Next, we sought to determine the phenotype of T cells recruited and activated in the lymph nodes and lungs of mice treated with pharmacologic arginase inhibitors. We stained for different surface receptors, lineage-specifying transcription factors, and intracellular cytokines. Mice treated with BEC or L-norvaline had higher recruitment of Th17 lymphocytes in their lymph nodes with significantly higher numbers at day 10 post-infection (Figure 3.8a, day 10, (x) p-value = 0.0079, (#) p-value < 0.0001). However, there were no significant

differences in the number of Th17 lymphocytes in the interstitial spaces among the 3 groups of mice; whereas, the water treated mice had significantly higher numbers of Th17 lymphocytes in their alveolar spaces compared to the L-norvaline treated mice only (Figure 3.8a, day 2, (#) p-value = 0.0127).

As for the type 1 T helper lymphocytes, the numbers were comparable in the lymph nodes with no significant differences among the 3 groups of mice.

However, water treated mice had significantly higher numbers of Th1 lymphocytes in the alveolar and interstitial spaces at day 2 post-infection compared to mice treated with BEC and L-norvaline (Figure 3.8b, day 2, lung interstitium (x) p-value = 0.0002, (#) p-value < 0.0001; alveolar spaces (x) p-value = 0.0066, (#) p-value = 0.0165).

Lastly, water-treated mice had higher numbers of regulatory T cells in their lymph nodes, alveolar spaces, and lung interstitium (Figure 3.8c). While the number of Treg lymphocytes were not significantly different among the groups treated with BEC versus the groups treated with L-norvaline, the latter had significantly lower numbers of Treg lymphocytes compared to the water treated mice (Figure 3.8c, day 2, lymph nodes (#) p-value = 0.0317; lung interstitium (#) p-value = 0.0128).

Additionally, we estimated the ratios of pro- and anti-inflammatory cytokines expressed by CD4<sup>+</sup> T cells as well as other lymphocytes. Figure 3.6h shows the ratios of total IL-17, IFN $\gamma$ , TGF $\beta$ , and IL-10 expressing lymphoid cells in the alveolar spaces. The ratio of IL-17 to IL-10 producing lymphocytes was significantly higher in water treated mice at day 3 post-infection compared to the L-norvaline treated group (Figure 3.6h, C, (#) p-value = 0.0014). However, the ratios and trend of IFN $\gamma$  to TGF $\beta$  expressing lymphocytes were comparable among the 3 groups (Figure 3.6h, D).

### III. Discussion

Patients with cystic fibrosis are genetically predisposed for chronic and repeated infections against which the immune system responds in an exaggerated but inefficient manner. The excessive immune response fails to adequately clear the infection while damaging the lungs and aggravating the pathology. The resultant lung injury further activates the immune response resulting in a vicious cycle of continuous inflammation and decline in lung function. Because of this, pulmonary infections are the leading cause of mortality in cystic fibrosis [1]. Despite our increased understanding of the altered immune response, life expectancy in patients with cystic fibrosis is still limited to 40 years in male patients and 37 years in female patients.

Several drugs against individual elements of the altered immune response are in clinical trials; yet, there is no defined target that can globally modulate the multiple mechanisms involved. Arginase-1 is an immunomodulatory enzyme with a versatile power in regulating multiple elements of the immune system. Additionally, we consistently see increased arginase expression and activity with improved inflammatory response and decreased lung pathology. Our group has previously shown increased arginase expression in mice treated with azithromycin along with improved morbidity and mortality [166, 310, 311]. Additionally, we have previously shown increased arginase expression in patients with cystic fibrosis and in J774 murine macrophages polarized with azithromycin in vitro [166, 310, 311]. Yet, whether arginase plays a role in modulating immunity in cystic fibrosis has not been investigated. And if so, the specific mechanisms involved in arginase modulation of the immune response are yet to be elucidated. The current study investigates the hypothesis that regulating excessive inflammation in PA pneumonia is dependent upon arginase production. We provide novel evidence concerning the different aspects of the immune response modulated with arginase expression, broadening our

knowledge of the specific mechanisms involved in regulating the immune response against infections in cystic fibrosis patients.

We utilized an arginase-1 conditional knock-out mouse model as well as two global pharmacological arginase inhibitors to investigate the impact of arginase deletion or inhibition upon the inflammatory response to PA pneumonia. Our group has previously developed an effective method for establishing a prolonged infection with PA in mice. We incorporate bacteria isolated from cystic fibrosis patients into agarose beads which are instilled intratracheally in mice to cause a chronic PA lung infection.

We show that arginase deletion from myeloid cells is associated with increased morbidity in mice infected with PA-laden agarose beads. Arginase conditional knock-out mice got sicker and lost more weight after the infection compared to mice whose macrophages and neutrophils can produce arginase. Additionally, arginase-1 conditional knock-out mice had a more severe response to the infection compared to their littermate controls. Arginase deletion from myeloid cells resulted in an excessive recruitment of neutrophils and macrophages into the lungs of infected mice. The increased macrophage infiltration into the lungs of arginase conditional knock-out mice was predominantly skewed towards an M1 pro-inflammatory phenotype with excessive numbers of TNF- $\alpha$  producing macrophages. These results suggest that arginase production is essential to control the acute inflammatory response and that absence of arginase precludes regulatory mechanisms essential for limiting the immune response and preventing exaggerated activation and recruitment of immune cells. One of the possible mechanisms by which arginase could lead to reduced recruitment of pro-inflammatory cells into the lungs is by protecting against permeability edema [337]. It is suggested that arginase reduces NO concentrations, through competition with iNOS, thereby reducing NO-mediated capillary endothelial hyperpermeability which is associated with excessive infiltration of neutrophils and macrophages from the blood into the alveoli. Therefore, arginase protects

against dysfunction of the alveolar-capillary barriers which limit the influx of inflammatory cells into the lungs [337]. Moreover, it is very likely that the arginase-mediated regulation of T cell responses that we observed in our model is responsible for limiting the excessive recruitment and chemotaxis of inflammatory cells into the lungs.

The most described immunomodulatory properties of arginase include its modulation of T cell responses. Consistently, we demonstrate abruptly increased proliferation and activation of CD4<sup>+</sup> T lymphocytes in the Arg1<sup>Δm</sup> mice. The deletion of arginase from myeloid cells resulted in increased T cell numbers in the lymph nodes and alveolar spaces of infected mice. This can be attributed to the increased arginine concentrations in the absence of arginase-mediated arginine depletion. These findings are consistent with research showing that arginase-1 expression induces T cell hypo-responsiveness through arginine depletion by the enzyme [130-132, 135, 136, 338-340]. In fact, arginase-mediated arginine depletion was shown to be associated with down-regulation of the T cell mediated immune responses in several conditions including (1) solid and hematological tumors, (2) virus-induced diseases, (3) leishmaniasis, (4) dextran sodium sulfate induced intestinal inflammation, (5) Behçet disease, (6) streptococcus pneumonia infections, (7) schistosomiasis, (8) autoimmune encephalomyelitis, (9) filarial infections, (10) sepsis, (11) asthma, (12) trauma, and (13) experimental glomerulonephritis [137-150, 341, 342]. Arginase-mediated effects on T cell responses were variable among the disease models listed above ranging from suppression of Th1 mediated pathology, to modulating the interplay of Th1/Th2 cytokines, shifting the macrophage/neutrophil balance, suppressing immune recognition and rejection of tumor cells, reducing CD8<sup>+</sup> and CD4<sup>+</sup> T cell proliferation, and modulating the Treg/Th17 balance [137-150, 341, 343]. According to Denning et al., arginase expression by lamina propria macrophages controls T cell differentiation into Treg and T17 phenotypes [344]. Overall our findings are in accordance with findings reported by Denning and several other studies which show that arginine depletion by arginase decreases

T cell proliferation and T cell effector functions [151-161]. Previous studies show that T cells activated under arginine depleted conditions fail to express CD3 $\zeta$  and CD3 $\epsilon$ . This results in decreased receptor-mediated tyrosine phosphorylation; however, it does not affect Ca<sup>2+</sup> flux [162]. The decreased CD3 $\zeta$  expression is not due to protein degradation or decreased mRNA but due to decreased global protein synthesis [157]. It is attributed to decreased cyclin D3 mRNA stability and expression which in turn prevents forward progression in the cell cycle and arrests T cells in the G0-G1 phase [157]. Impairment of T cell activation under arginine depleted conditions is T cell receptor (TCR) independent and is not mediated via inhibitory effect of CTLA4, IL10, PDL1, or TGF $\beta$  [144]. Moreover, the decreased T cell proliferation is not associated with an increase in apoptosis or T cell death [151]. Previous literature suggests that in most cases the arginase-mediated suppression of T cells is localized to the site of infection and does not affect naïve T cells in lymph nodes and other secondary lymphoid organs ensuring that this suppression is not due to T cell intrinsic defects [138-143]. Additionally, T cell suppression due to arginine depletion is reversed with arginase inhibition or with the L-arginine supplementation [137-150]. Thus, the increased arginine availability in Arg1 $\Delta^m$  mice is most likely driving increased T cell proliferation and contributing to the robust and excessive numbers of T cells in infected mice. Additionally, we show that deletion of arginase is associated with excessive numbers of activated T cells emphasizing the important role of this immunomodulatory enzyme in controlling the expression of T cell activation markers and limiting T cell responses in PA pneumonia.

Additionally, our results show that arginase deletion from myeloid cells skews the T helper cell responses towards a Th1/Th17 predominant response consistent with previous reports of arginase-mediated shift towards a regulatory T cell phenotype along with suppression of pro-inflammatory cytokine production [129]. We show that in a murine model of PA pneumonia, Arg1 $\Delta^m$  mice respond with excessive activation of Th1 and Th17 pro-inflammatory lymphocytes in their lymph nodes and in their lungs. In addition to the previously discussed evidence

of arginase effects on T cell phenotype mediated via arginine depletion, several other mechanisms could be involved. According to Obermajer and colleagues, iNOS mediates Th17 induction and stabilization via NO mediated pathways [345]. NO secretion by myeloid-derived suppressor cells expressing iNOS promoted the differentiation of ROR $\gamma$ t<sup>+</sup> IL-17<sup>+</sup> CD4<sup>+</sup> T cells from naïve and memory human T cells. These effects of NO on T cell polarization were mediated via signaling through the canonical cyclic guanosine monophosphate (cGMP)-dependent protein kinase (cGK) pathway within CD4<sup>+</sup> T cells. Inhibition of iNOS or cGMP–cGK signaling prevents the polarization towards a Th17 phenotype [345]. Obermajer reports that suppression of NO levels significantly reduces IL-17 concentrations in patients with ovarian cancer [345]. Therefore, comparing our results to the findings by Obermajer et al., it is likely that arginase deletion shifts the arginine metabolism towards the iNOS pathway thereby increasing NO levels which drive T cell polarization towards the Th17 phenotype. Additionally, it is likely that an arginase-mediated shift of T cell response towards a regulatory phenotype suppresses Th17 and Th1 responses. Geiger and colleagues suggest that arginase controls several metabolic pathways in T cells which promotes their regulatory functions [346]. Arginase modulates glycolysis and oxidative phosphorylation in activated T cells which shifts their function towards regulatory and memory cells by direct effects on several transcription regulators including BAZ1B, PSIP1, and TSN [346]. Tregs suppress Th1 and Th17 responses and directly inhibiting the secretion of polarizing cytokines [346, 347]. Therefore, it is likely arginase deletion shifts the Treg/Th17 balance in Arg1 $\Delta^m$  mice by promoting Th17 polarization and suppressing Treg numbers.

Skewing of the T cell responses towards a Th17 and Th1 predominant response partially explains the increased recruitment of neutrophils and M1 macrophages. It is very likely that the excessive amounts of IFN $\gamma$  and IL-17 released by the Th1 and Th17 lymphocytes are responsible for polarizing macrophages towards an M1 pro-inflammatory phenotype and for recruiting excessive numbers of neutrophils. According to Disteldorf et al., Th17 responses attract destructive

neutrophils which cause renal tissue injury [348]. In a murine model of crescentic glomerulonephritis, authors report that CXCL5 drives the excessive influx of pathogenic neutrophils into sites of inflammation via a Th17-dependent pathway in later chronic stages of the disease [348]. Similarly, Th17 responses recruit neutrophils both of which mediate immunopathogenesis in Leishmaniasis. This is mediated via cooperation of IL-17 with other cytokines which recruits neutrophils in excessive numbers during Leishmania infection [349]. Therefore, our results emphasize the importance of arginase-mediated regulation of Th17 responses thereby limiting the pathogenic neutrophil influx into the lungs of patients with cystic fibrosis. Our data support that arginase is a mediator which controls Th17-associated inflammation providing new targets to manipulate Th17 responses in cystic fibrosis, cancer, autoimmunity, and other inflammatory diseases.

Additionally, results show significantly increased ratio of IL-17/IL-10 producing lymphoid cells at days 3 through 10 post-infection in Arg1<sup>Δm</sup> mice. We observed marked expression of IL-17 cytokine in CD4<sup>+</sup> T cells expressing non Th17 cytokines or lineage markers as well as in non CD4<sup>+</sup> lymphoid cells. This reflects upon the potential of arginase-1 to control IL-17 production through mechanisms additional to Th17 regulation. According to Hayes et al., IL-17 is required to control chronic infection with PA and is released by Th17 cells, innate lymphoid cells,  $\gamma\delta$ -T cells, and NK cells [289]. Hayes and colleagues infected IL-17RA knock-out mice with PA-laden agarose beads to cause a chronic infection with a mucoid PA strain similar to our procedure. Authors found that IL-17RA knock-out mice had significantly lower morbidity and faster recovery while the bacterial loads in their lungs were higher at 2 weeks post-infection compared to wild-type mice. Additionally, there were no significant differences in the number of neutrophils and the histologic lung injury scores between wild-type and IL-17RA knock-out mice [289]. The decreased morbidity in IL-17RA knock-out mice is attributed to the lack of IL-17 direct effects on pro-inflammatory cytokines like IL-6 and TNF- $\alpha$ . Additionally, they show a CD3<sup>+</sup> population expressing both IFN $\gamma$  and IL-17 in the lymph nodes of infected mice. About 90% of the cells expressing

IL-17 in the lymph nodes at 2 weeks post-infection had a phenotype consistent with ILC3 cells while the remaining 5-10% of the cells had B cell markers [289]. However, in the lungs of wild-type mice, there was a significant increase in IL-17 expressing cells. About 50% of these cells were consistent with Th17 and  $\gamma\delta$ -T cells while the majority of the remaining cells were consistent with ILC3 cells. A small fraction of about 3.83% of all IL-17 expressing cells in the lungs had B cell markers and was consistent with B1 cell population [289]. Accordingly, our results from Arg1<sup>Δm</sup> mice show significantly increased IL-17 expression along with increased morbidity. We observed a significant IL-17 expression by lymphoid and non-lymphoid cells. Although we did not specifically stain for ILC3 cells, it is very likely that ILC3 cells contribute to the increased IL-17 expression by non-CD4+ T cells. Group 3 ILCs produce IL-22 and IL-17A and express ROR $\gamma$ t, they present antigens and control CD4+ T cell responses [350, 351]. According to von Burg et al., ILC3 cells can regulate lymphoid tissue development and can alter the adaptive immune response by directly stimulating CD4+ lymphocytes [350]. ILC3 cells prime CD4+ T cells and induce their proliferation and activation by directly presenting antigen and secreting cytokines. Therefore, regulating IL-17 production by ILC3 cells constitutes a novel approach to control T cell immune responses [350, 351]. Accordingly, it is essential to closely examine the effects of arginase mediated regulation of IL-17 secretion and whether it involves regulation of ILC3 development and subsequent regulation of T cell mediated immunity.

Importantly, the absence of arginase does not inhibit anti-inflammatory responses, as we see recruitment of IL-10 and MR expressing M2 macrophages. The latter are likely to be elevated in the absence of arginase as a regulatory feedback that is failing to downregulate the excessive inflammation. Despite the increased levels of the anti-inflammatory cytokine IL-10, the anti-inflammatory response is still unable to protect against the excessive inflammation caused by the deletion of arginase. We have consistently observed this in past experiments in mice that lack alternative macrophage activation. Also, in-vitro when TNF- $\alpha$

production increases as a result of increased M1 polarization, IL-10 is also increased in a compensatory manner [166, 310, 311]. According to Stenvinkel et al., systemic inflammation responsible for pathogenesis in cardiovascular disease and end-stage liver disease is associated with elevated concentrations of IL-10, IL-6, and TNF- $\alpha$ . Authors report that IL-10 secretion is delayed and always follows the release of TNF- $\alpha$  with a latency of a few hours. The coupling of excessive inflammatory mediators with the delayed secretion of IL-10 ensures that inflammation will securely be down-regulated [352]. Additionally, several reports describe complex signaling mechanisms involved with TNF- $\alpha$  induced IL-10 secretion which involves activation of protein kinase C [353-355]. Exclusive secretion of IL-10 without preceding secretion of pro-inflammatory cytokines seems to be a rare event. Our results were broadly in agreement with the previous findings which demonstrate that excessive TNF- $\alpha$  release induces increased IL-10 concentrations as a regulatory mechanism to control excessive inflammation. However, in our model the increased IL-10 was not enough to protect against the increased morbidity in the Arg1 $\Delta^m$  mice.

Our model of Arg1 $\Delta^m$  mice allowed us to address our hypothesis concerning macrophage and neutrophil specific arginase expression. Arginase is expressed by many other immune and non-immune cells; therefore, we next sought to confirm the results by globally inhibiting arginase by administering pharmacologic arginase inhibitors. BALBc/J mice are known to be Th2-dominant compared to C57BL/6 mice which are on a Th1-dominant. BALBc/J mice respond to the infection with significant expression and activation of arginase. Hence, we decided to globally inhibit arginase in these mice to investigate the impact of inhibiting arginase from all cellular sources on the immune response to PA pneumonia. Mice treated with global arginase inhibitors did not lose more weight compared to mice which were dosed with water. However, it is important to note that mice with global arginase inhibition lost more weight generally compared to mice with conditional arginase deletion from myeloid cells. The maximum weight loss in arginase conditional knock-out mice was about 13.33% achieved by day 2

post-infection, versus a maximum average weight loss of about 20% in BALBc/J mice treated with arginase inhibitors and achieved by day 3 post-infection. This is a trend that we observed in our experiments, but we did not directly compare the 2 strains with simultaneous infection.

Global arginase inhibition with both pharmacologic inhibitors suppressed the acute inflammatory response in infected mice. There were significantly lower numbers of neutrophils in the alveolar spaces of mice treated with BEC or L-norvaline compared to mice which were dosed with water only. However, in the lung interstitial spaces there was a delayed and slightly elevated influx of neutrophils into the lungs of mice treated with arginase inhibitors at day 5 post-infection. Results suggest that while arginase inhibitors can acutely attenuate neutrophil influx in the alveolar spaces, the global arginase inhibition is associated with a delay in neutrophil infiltration into the lung interstitium.

Importantly, the total number of neutrophils recruited in the 3 groups of BALBc/J mice were much higher than in the arginase conditional knock-out mice ( $1.5-9 \times 10^6$  versus  $0.5-1.5 \times 10^6$  in the alveolar spaces;  $8-9 \times 10^6$  versus  $1-3 \times 10^6$  in the lung interstitium). Whether this difference is significant, and if so, whether this is due to global inhibition of arginase versus deleting it from myeloid cells is a question to be answered. Additionally, this difference might be due to a more infective batch of PA-laden agarose beads as we prepare these prior to each infection and there is always a chance of the bacteria changing its virulence or becoming more or less infective. Moreover, the difference in the weight loss and in the inflammatory response to the infection might be solely due to the different genetic background of the two strains of mice. Therefore, a clear and definitive assumption about the effect of global arginase inhibition on morbidity and neutrophil influx cannot be concluded from these results.

Similarly, global arginase inhibition was associated with different effects on alveolar and infiltrating macrophages. While mice treated with BEC or L-norvaline had reduced numbers of alveolar macrophages activated in response to the

infection, they had higher numbers of infiltrating macrophages in their lung interstitium. Again, the total number of macrophages recruited in the 3 groups of BALBc/J mice were much higher than in the arginase conditional knock-out mice (1 - 4 x 10<sup>6</sup> versus 0.25 - 0.5 x 10<sup>6</sup> in the alveolar spaces; 0.5 - 2 x 10<sup>7</sup> versus 1 - 5 x 10<sup>6</sup> in the lung interstitium) and this is likely due to the use of BALB/c mice, a strain in which we consistently observe higher macrophage numbers as compared to neutrophils. Moreover, the numbers of TNF producing macrophages were comparable in mice treated with water versus mice treated with global arginase inhibitors. As for IL-10 producing macrophages, they were slightly lower in the BEC and L-norvaline groups without being statistically significant. Similarly, there were lower levels of MR expressing alveolar macrophages in the groups treated with arginase inhibitors while the infiltrating monocytes were not significantly different among the 3 groups. Moreover, there were no significant differences in iNOS expressing alveolar and infiltrating macrophages. While the numbers of alveolar M1 pro-inflammatory macrophages were comparable among the 3 groups of mice, the numbers of infiltrating M1 macrophages were significantly lower in mice treated with arginase inhibitors. Our results suggest that arginase inhibitors have potentially different effects on resident versus infiltrating macrophages. This might be explained by potentially different local, anatomical, or cellular effects of the pharmacologic inhibitors. Additionally, these observations may be a mere effect of the murine strain used in these experiments. According to Loke and colleagues, C57BL/6 mice and BALB/c mice have inherent differences in PDL1 and PDL2 expression on the surface of macrophages [356]. The expression of these markers, as well as other surface receptors essential for migration of leukocytes, is governed by Th1 and Th2 cytokines. These differences account for a significant variation in leukocyte recruitment and transmigration as well as to differences in T cell activation among the 2 strains of mice [356, 357]. Therefore, it is important to consider the differences among the 2 strains of mice used in our experiments besides the effects of the arginase inhibitors. This urges us to repeat the arginase inhibitor experiments in C57BL/6 mice to better draw informative conclusions about the

specific effects of these inhibitors on leukocyte recruitment and activation in response to PA pneumonia.

Collectively, global arginase inhibition had different effects on alveolar versus interstitial macrophages. Our results do not support a definite conclusion as to whether arginase inhibition modulates compartmentalization of immune cells recruited in response to the infection. Additionally, BALBc/J mice are on a Th2 background which drives a completely different immune response compared to Arg1<sup>Δm</sup> mice which are on a C57BL/6 background. Stevenson and colleagues suggest resistance of BALBc/J to infections with PA whereas the C57BL/6 mice were more susceptible to the infection. This group shows increased cellularity and a shift towards a neutrophilic response in C57BL/6 while BALBc/J had a monocytic predominant influx with less cellularity [358]. However, both groups in this report were infected at the same CFU while in our experiments there was a 10-fold difference in the CFU required to cause a comparable infection in both strains. Additionally, a mucoid PA strain was used which is not the same that was used in our experiments which might result in differences in the immune response activated. Moreover, our BALBc/J were dosed with arginase inhibitors which might have contributed to the difference in the inflammatory response observed. Furthermore, the difference in susceptibility to the infection is also related to the differences in clearance due to altered macrophage activation and chemotaxis between C57BL/6 mice and BALBc/J mice. According to Watanabe et al., in vitro infection of naïve macrophages from both strains of mice with live bacteria shows a significant impairment of bactericidal functions of BALB/c macrophages compared to macrophages from C57BL/6 mice [359]. This effect was explained by the impaired ability of BALBc/J macrophages to upregulate lysosomal enzyme and NO essential for bacterial killing. Authors show that in a murine model of septic peritonitis, BALBc/J mice fail to clear the infection despite the excessive recruitment of leukocytes. Similar to our observations, BALBc/J mice recruited tremendous numbers of macrophages into the peritoneal cavity with significantly higher levels of pro-inflammatory cytokines and mediators

compared to C57BL/6 mice thereby resulting in exaggerated systemic inflammation. Watanbe and colleagues suggest significant differences in innate immune responses between C57BL/6 mice and BALB/c mice depicted by increased cellularity but decreased functionality of immune cells recruited in response to infections in BALB/c mice compared to the C57BL/6 mice [359]. Therefore, it is important to distinguish the effects of the pharmacological arginase inhibitors on leukocyte activation and migration versus the inherent immune differences in the BALBc/J mice.

Additionally, we evaluated the effects of the pharmacologic arginase inhibition upon the T cell responses. As expected, mice treated with BEC or L-norvaline had a significantly higher number of T cells in their lymph nodes similar to the effects with myeloid arginase deletion. This can be explained by the increased arginine pool and the lack of arginase-dependent suppression of T cell activation as we explained in the Arg1<sup>Δm</sup> murine experiments. However, in contrast to the observations with Arg1<sup>Δm</sup> mice, there were lower numbers of T cells and activated T cells in the alveolar spaces of mice treated with global arginase inhibitors (differences not statistically significant). Yet, there was a greater infiltration and activation of T cells in the interstitial spaces of mice treated with BEC or L-norvaline. Therefore, global arginase inhibition increases T cell numbers in the lymph nodes and in the lung interstitium but not in the alveolar spaces. This may be explained by the potentially different spatial effects of the arginase inhibitors and the potential effects of arginase inhibitors on chemotaxis and migration of T cells between the lymph nodes and the different lung compartments. It is likely that BEC and L-norvaline modulate effectors involved in T cell migration into the alveolar spaces. Future studies will evaluate the effects of arginase inhibitors on T cell migration and chemotaxis. This includes looking for transmigration effectors and proteins in addition to performing T cell migration assays using transwells and Boyden chambers. Additionally, in vivo T cell migration assays to evaluate the effects of arginase inhibition of T cell recruitment to the lungs involve adoptively transferring T cells into mice as well

as tracking radiolabeled T cells according to the methods proposed by Campanella and colleagues [360-362].

Lastly, we evaluated the specific phenotypes of T cells affected by pharmacologic arginase inhibition. Similar to the results from Arg1<sup>Δm</sup> mice, there was a significantly higher number of Th17 lymphocytes in the lymph nodes of mice treated with BEC or L-norvaline. Conversely, arginase inhibitors did not significantly increase the numbers of Th17 lymphocytes in the lungs like in the Arg1<sup>Δm</sup> mice. There were comparable numbers of Th17 lymphocytes in the alveolar spaces and lower numbers in the interstitial spaces compared to the water treated mice. Similarly, there were higher numbers of Th1 lymphocytes in the lymph nodes of mice treated with arginase inhibitors while they were considerably lower in the interstitial and alveolar spaces compared to the water treated mice. These observations are also different than the effects of arginase deletion from myeloid cells in the Arg1<sup>Δm</sup> mice which promotes Th1 responses both in the lungs and the lymph nodes. Additionally, global arginase inhibition suppressed the development of regulatory T cells. There were significantly lower numbers of Treg lymphocytes in the lymph nodes and interstitial spaces of mice treated with arginase inhibitors versus mice treated with water. In the alveolar spaces, arginase inhibitors reduced the numbers of Treg lymphocytes; however, the differences were not statistically significant. Therefore, results from experiments with global arginase inhibition validate our previous hypotheses that global arginase inhibition has different effects on the lung compartments versus the lymph nodes and that T cell migration and chemotaxis is potentially altered in mice treated with BEC or L-norvaline. It is likely that arginase inhibitors prevent migration of activated T helper cells (including Th1, Th17, and Tregs) from the lymph nodes to the lungs of infected mice. This will be confirmed using the chemotaxis assay proposed earlier. However, it is also possible that the arginase inhibitors affect local T cell activation in the lungs via effects on leukocyte activation and expression of co-stimulatory molecules. It has been reported that PDL1 and PDL2 promote peripheral tolerance in viral infections and in cancer. In

a murine model of *Trypanosoma cruzi* infection, peritoneal macrophages suppressed proliferation of T cells at the site of infection through a PD-1/PDL1 dependent mechanism [363]. This effect was mediated by modulation of the iNOS/arginase metabolic pathways and it results in suppression of peripheral T cell proliferation and cytokine release. Therefore, future experiments will evaluate the effects of arginase inhibitors on macrophage expression of T cell inhibitory receptors and whether effects of arginase inhibitor are mediated through effects on transmigration versus monocyte-mediated peripheral tolerance.

In summary, results from Arg1<sup>Δm</sup> mice experiments confirm the importance of myeloid arginase in the regulation of the inflammatory response, while experiments with global arginase inhibition suggest different and broad effects of other cellular sources of arginase. Besides neutrophils and macrophages, many other immune cells express arginase which can potentially influence our observations. Monticelli et al. show that arginase deletion from type 2 innate lymphoid cells can suppress Th2 mediated inflammation and reduce cytokine release. They suggest that arginase deletion from these cells changed multiple metabolic pathways with potential effects on several inflammatory and immune responses [364]. Additionally, Bando et al. show that arginase expression in ILC2s is governed by distinct signaling pathways as compared to the M2 macrophages and that ILC2s express arginase at rest and during inflammation. Bando's group show that altered arginase expression affects the development and expansion of the ILC2 population besides affecting arginase expression in M2 macrophages [365]. Moreover, CD4<sup>+</sup> and CD8<sup>+</sup> T cells as well as B cells can express arginase. In our experiments with BEC and L-norvaline we inhibited arginase from all of the above-listed sources and therefore it is difficult to tease apart the specific effects of different arginase sources on the immune response to PA pneumonia.

Our results confirm that global arginase inhibition has a different effect on different elements of the immune response. Our results suggest that arginase

inhibition affects compartmentalization of both myeloid and lymphoid cells recruited in response to the infection. While arginase inhibition did not result in increased weight loss, mice which received BEC and L-norvaline did have multiple signs of increased morbidity, reduced motility, and increased inflammation. However, comparing these results to the experiments with Arg1<sup>Δm</sup> mice confirms our hypothesis that myeloid arginase is essential in controlling the inflammatory response. Therefore, we provide strong evidence that myeloid arginase specifically, and not other arginase sources, is important for controlling inflammation in PA pneumonia.

Importantly, results were comparable among the two arginase inhibitors used, BEC and L-norvaline. Both inhibitors are arginine analogs and they compete with the substrate and irreversibly inhibit arginase enzymatic activity. We used 2 inhibitors to confirm that our observations are due to arginase inhibition rather than other pleiotropic effects of the inhibitor. However, some reports suggest that L-norvaline and not BEC might have some anti-inflammatory effects independent of arginase inhibition [366]. Ming et al. suggest that L-norvaline can have anti-inflammatory effects by suppressing the S6K1 pathway thereby reducing TNF- $\alpha$  secretion. However, their results are from in vivo experiments with endothelial cells which might be valid but insignificant in our murine model of PA pneumonia. Yet, it is possible that some of our results with BEC and L-norvaline might be driven by different alternative mechanisms that either of the inhibitors can exert. Moreover, it is possible that the inhibitors act differently on different immune cells and in different anatomical compartments and that this difference is responsible for our observations in mice treated with either BEC or L-norvaline. Additionally, although the doses of both arginase inhibitors used in our study were adapted from literature and established to result in comparable enzymatic inhibition, it is possible that the potency of these drugs in our mouse model is different and that this drives some of our results.

The effects of arginase inhibitors on the migration and chemotaxis of myeloid cells and T lymphocytes to the lungs might be attributed to the effects on NO production. Both BEC and L-norvaline are shown to affect NO production by NOS and to modulate the endothelium junctions and affect dilation and relaxation of the blood vessels [367]. While arginase inhibition can affect the expression of surface markers on immune cells, the changes in the endothelium and blood vessels with our inhibitors can potentially be responsible for reduced transmigration and trafficking of immune cells across the vessel walls.

Some of the other limitations in our study include the “leaky” LysM cre system and the difference in murine strains used in both experiments. The deletion of arginase in our murine model is controlled by lysozyme expression. Lysozyme M is expressed in neutrophils and macrophages. So, it is hard to delineate the difference between neutrophil versus macrophage arginase function as both are deleted. Additionally, new reports show different levels of lysozyme expression between tissue and infiltrating macrophages thus accounting for different levels of arginase deletion from these two populations system [368]. Suggested models which may be associated with even deletion of arginase from different macrophage population would be the *Arg1 flox/floxTie2cre* system [358]. *Arg1 flox/floxTie2cre* mice delete Arg1 in all macrophage populations and they are available on both the BALB/c and C57BL/6 genetic backgrounds making it possible to examine the influence of Arg1 activity on two distinct backgrounds. Tie2cre system is associated with >99% deletion of Arg1 from all macrophage populations as the Tie2 endothelial-specific receptor tyrosine kinase promoter expression allows the deletion of floxed sequences in endothelial and myeloid cells during embryogenesis and adulthood [143, 369].

Future experiments will define the contribution of arginine to the modulation of T cell phenotype. This will be approached by evaluating whether L-citrulline, the arginine precursor, can rescue the T cell responses in PA pneumonia. The hypothesis is that L-citrulline metabolism in T cells increases CD4+ cell

recruitment, proliferation, and Th17 effector function following PA. This is based on preliminary data suggesting that L-citrulline rescues T cell proliferation and differentiation in arginine-depleted environments. Furthermore, we will test if T cells require L-citrulline to L-arginine synthesis to regulate neutrophil recruitment and defense to PA. This will be achieved using  $Asl^{\Delta T_{cell}}$  mice, a novel mouse model in which T cells cannot synthesize L-arginine from L-citrulline.

Moreover, the arginase conditional knock-out mice are on a C57BL/6 background which is known to express a lower level of arginase as compared to BALBc/J mice as discussed earlier. Therefore, we will examine the effects of conditional arginase deletion from myeloid cells in BALBc/J mice. Especially that it is hard to make any comparison between the two experiments with obvious differences among the two strains of mice. Moreover, global arginase inhibition with pharmacologic inhibitors does not provide a clear explanation of the role of arginase from non-myeloid cells. Therefore, utilizing different mouse models with conditional arginase deletion from other immune cells is essential to answer the question of whether myeloid arginase functions differently as compared to other cellular sources. Additionally, our models did not specifically distinguish the role of different arginase isoforms. Future work aims at examining the immunomodulatory effects of different arginase isoforms. Studies to differentiate the functions of arginase-1 versus arginase-2 are needed.

Moreover, correlating our results with their applicability in humans is essential. In fact, the role of arginase in humans is complex as there is a large discrepancy between the cellular sources of arginase in humans. Studies in cystic fibrosis suggest that neutrophils may be the predominant source of arginase in these patients. Because arginase is consistently elevated during disease exacerbation and in end-stage deteriorated and severe disease states, arginase has been correlated with pathology [109, 135, 164, 370]. Additionally, many reports suggest distinct effects of arginase-1 versus arginase-2 in humans. For instance, several studies show that arginase-1 induces autophagy while some reports

show that arginase-2 suppresses autophagy [248, 371]. Moreover, some data in systemic lupus erythematosus and in sickle cell anemia suggest that arginase promotes Th17 responses and neutrophil influx contributing to immunopathogenesis [372-375]. However, it is likely that arginase upregulation follows the activation of exaggerated inflammation and that arginase induction is a regulatory mechanism to downregulate the activated inflammation. Therefore, extending our studies to validate the immunomodulatory mechanisms of arginase in humans is crucial.

Our results show for the first time that myeloid arginase has a role in regulation of the immune response to chronic PA pneumonia. We show that arginase production by macrophages and neutrophils is essential to protect against excessive morbidity and inflammation. To our knowledge, we are the first to show that deletion of arginase from myeloid cells is associated with an acute influx of neutrophils and pro-inflammatory macrophages which are likely to be driven by the altered T cell response. Our results emphasize the effects of myeloid arginase on T cell polarization and skewing the T helper response towards a Th1 and Th17 predominant response in mice infected with PA pneumonia. Additionally, we show that global arginase inhibition has different effects on immune cell recruitment, activation, and chemotaxis. We show that pharmacologic arginase inhibition might not be the best way to control inflammation in chronic infections with PA pneumonia. Results from experiments with global arginase inhibition suggest close consideration of the effects of different cellular sources on the immune response and how this could potentially influence treatment of excessive inflammation and regulation of altered immune responses.

We conclude that myeloid arginase is essential for control of inflammatory responses in PA pneumonia.

Figure 3.1a

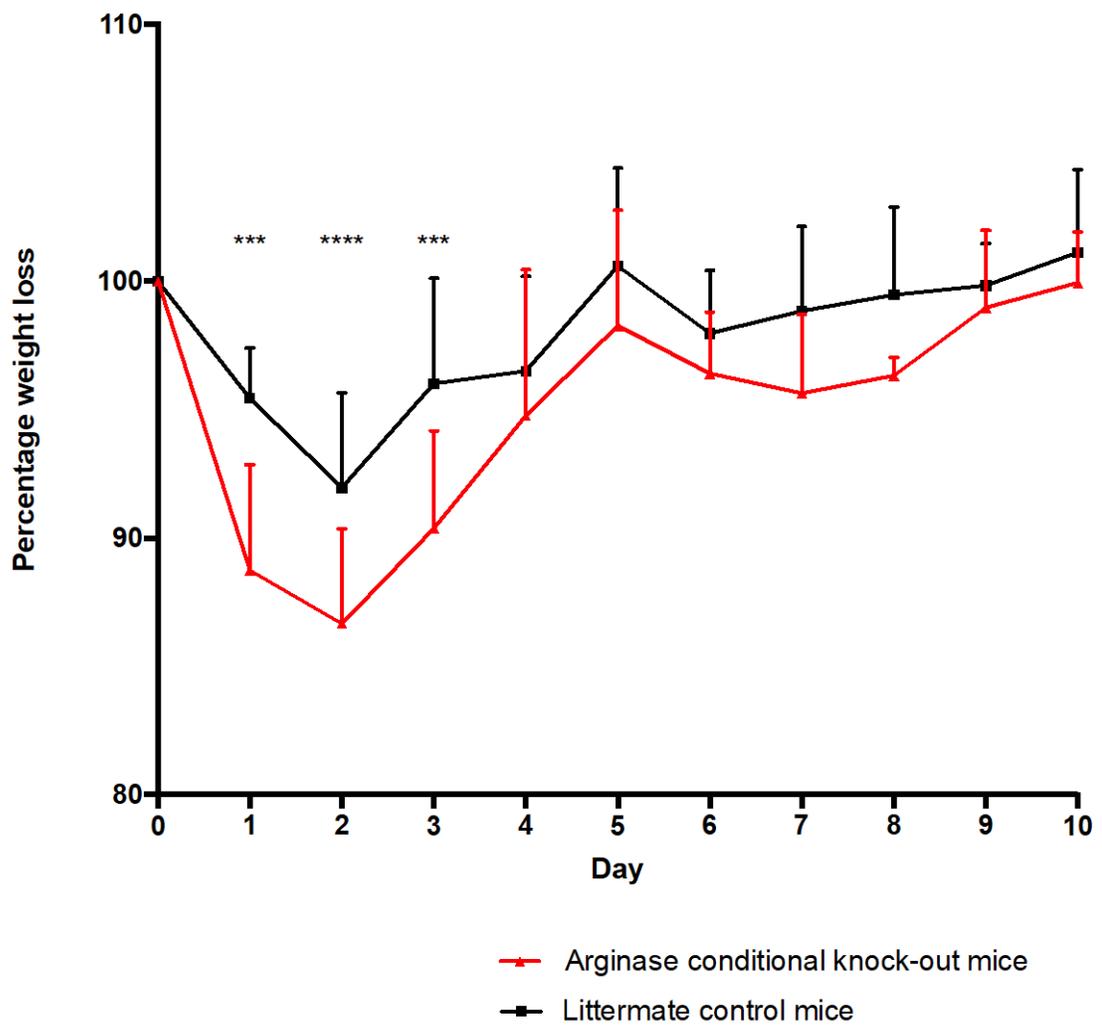


Figure 3.1b

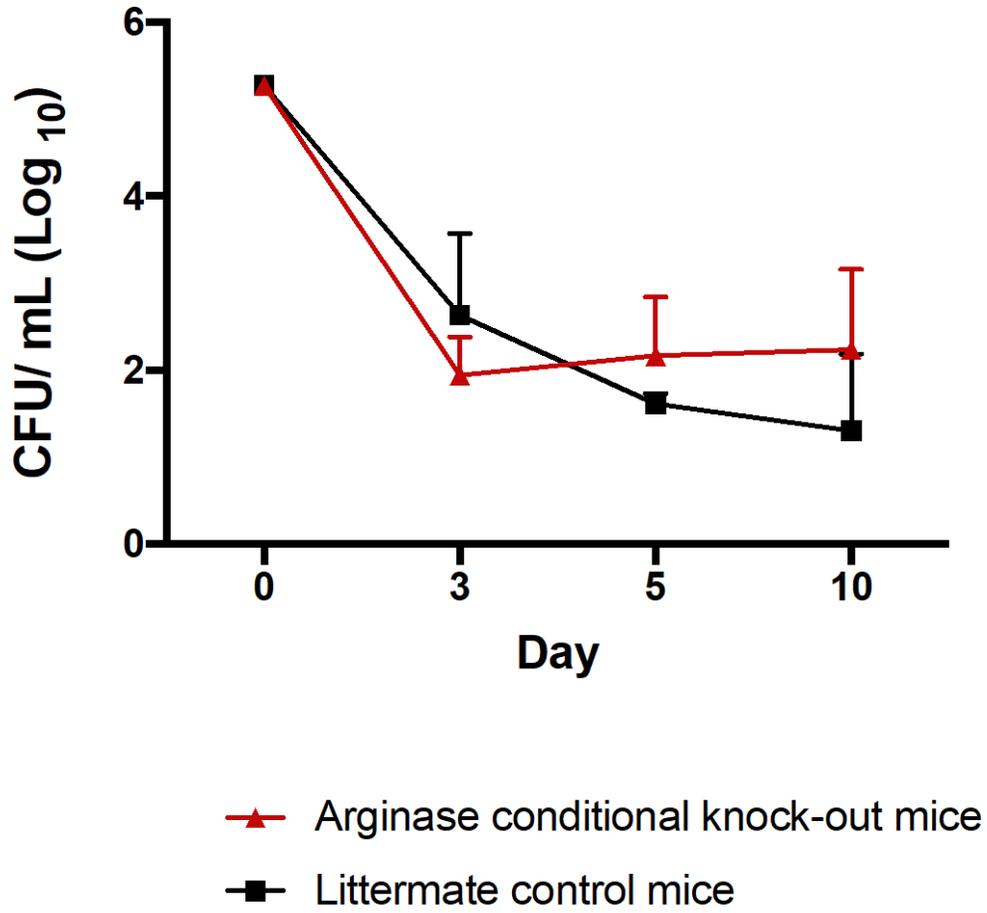


Figure 3.1c

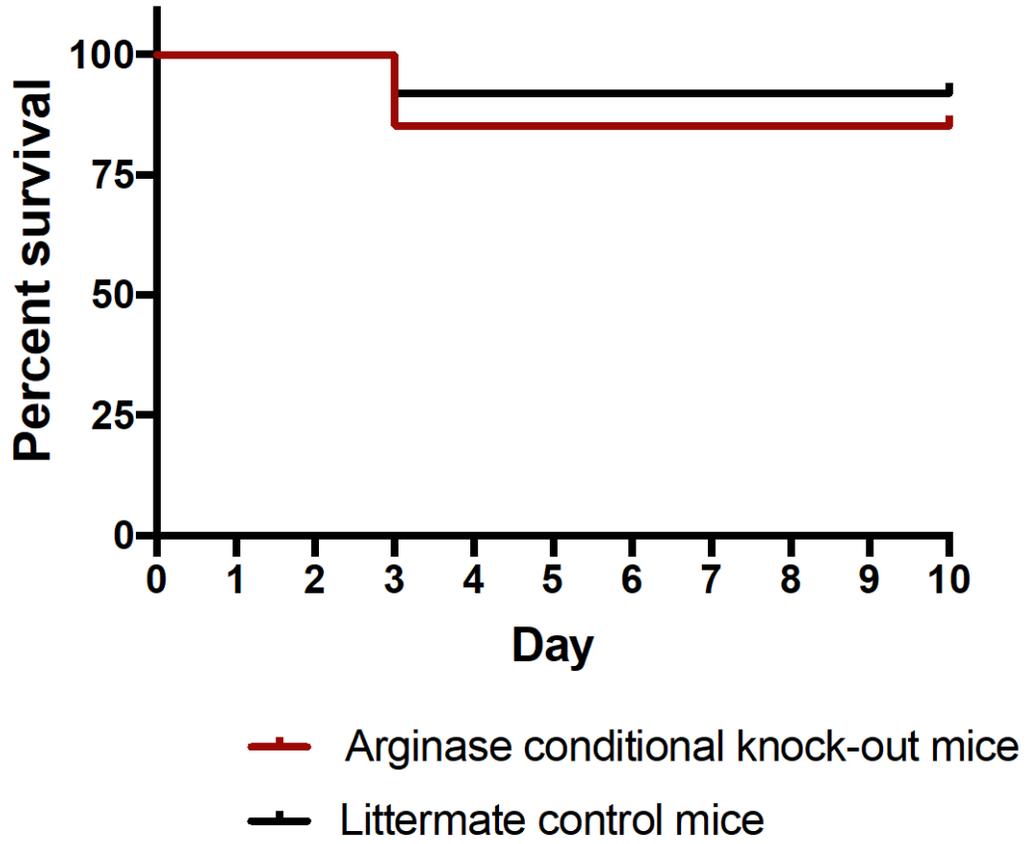


Figure 3.1. Arginase conditional knock-out mice lose more weight post intratracheal infection with PA compared to their littermate controls. Mice were infected with PA-laden agarose beads through intratracheal instillation of  $2 \times 10^6$  CFU/ mL as described. Murine morbidity post infection was evaluated in terms of weight loss measured at least once daily before and after infection. (a) The graph represents percentage weight loss normalized to the baseline body weight prior to infection. (b) The graph represents bacterial clearance from the lungs. (c) Kaplan-Meier curve represents the survival of mice post-infection. Data represents mean  $\pm$  SD. Data are representative of 3 independent experiments. Statistical significance determined by two-way ANOVA (p-value < 0.05 (\*); p-value < 0.0005 (\*\*); p-value < 0.0001 (\*\*\*\*)). Graphs plotted using GraphPad Prism 7.

Figure 3.2a

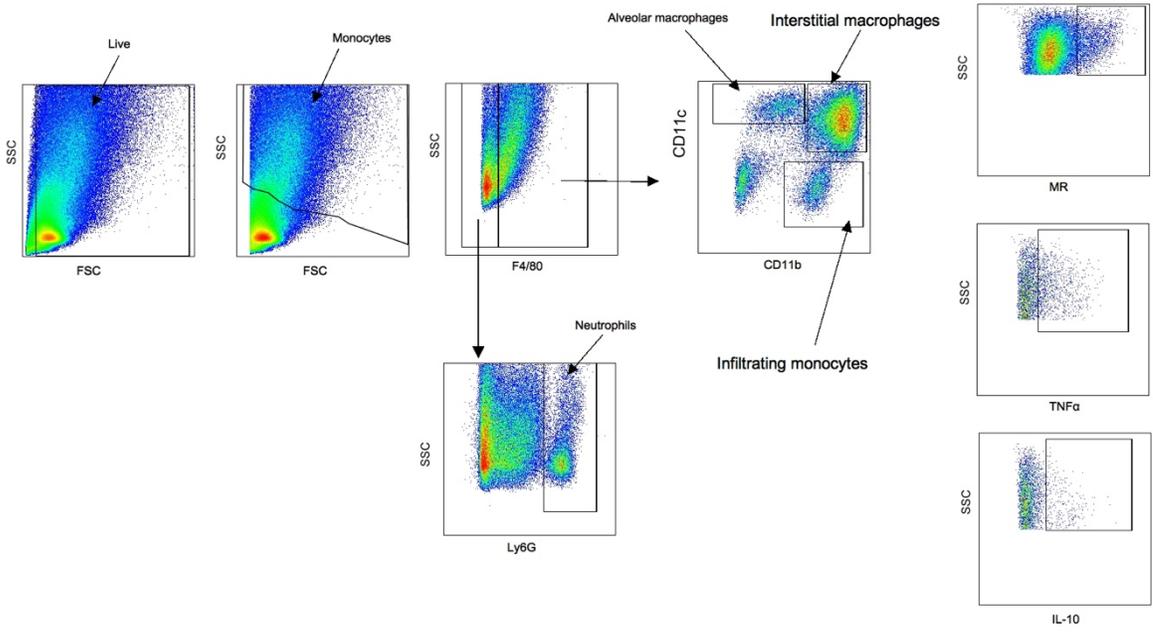
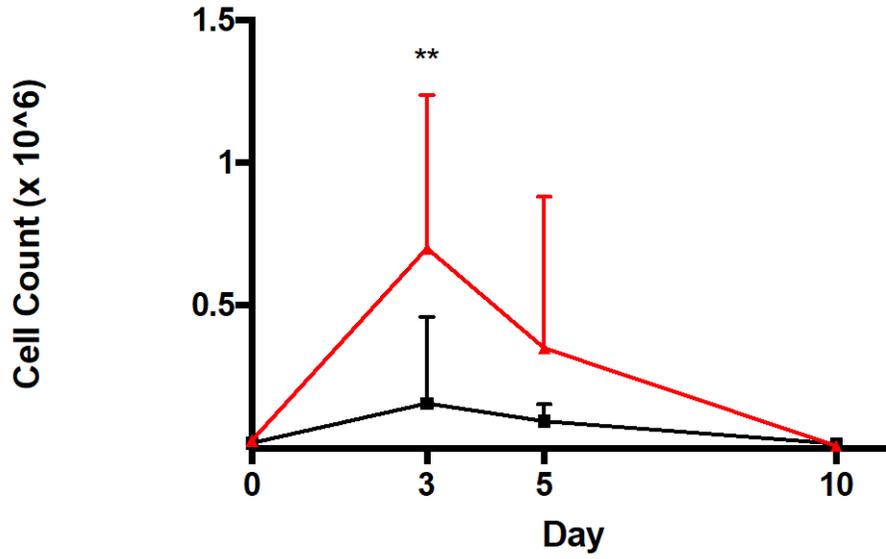


Figure 3.2b

### Neutrophils \_ Alveolar Spaces



### Neutrophils\_Lung Interstitium

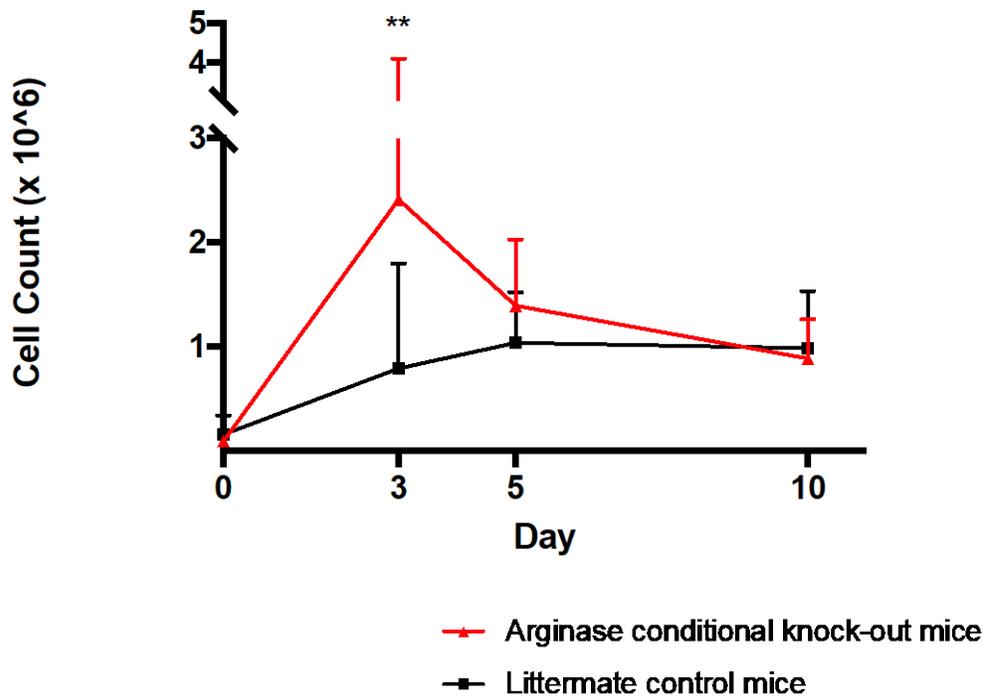


Figure 3.2c

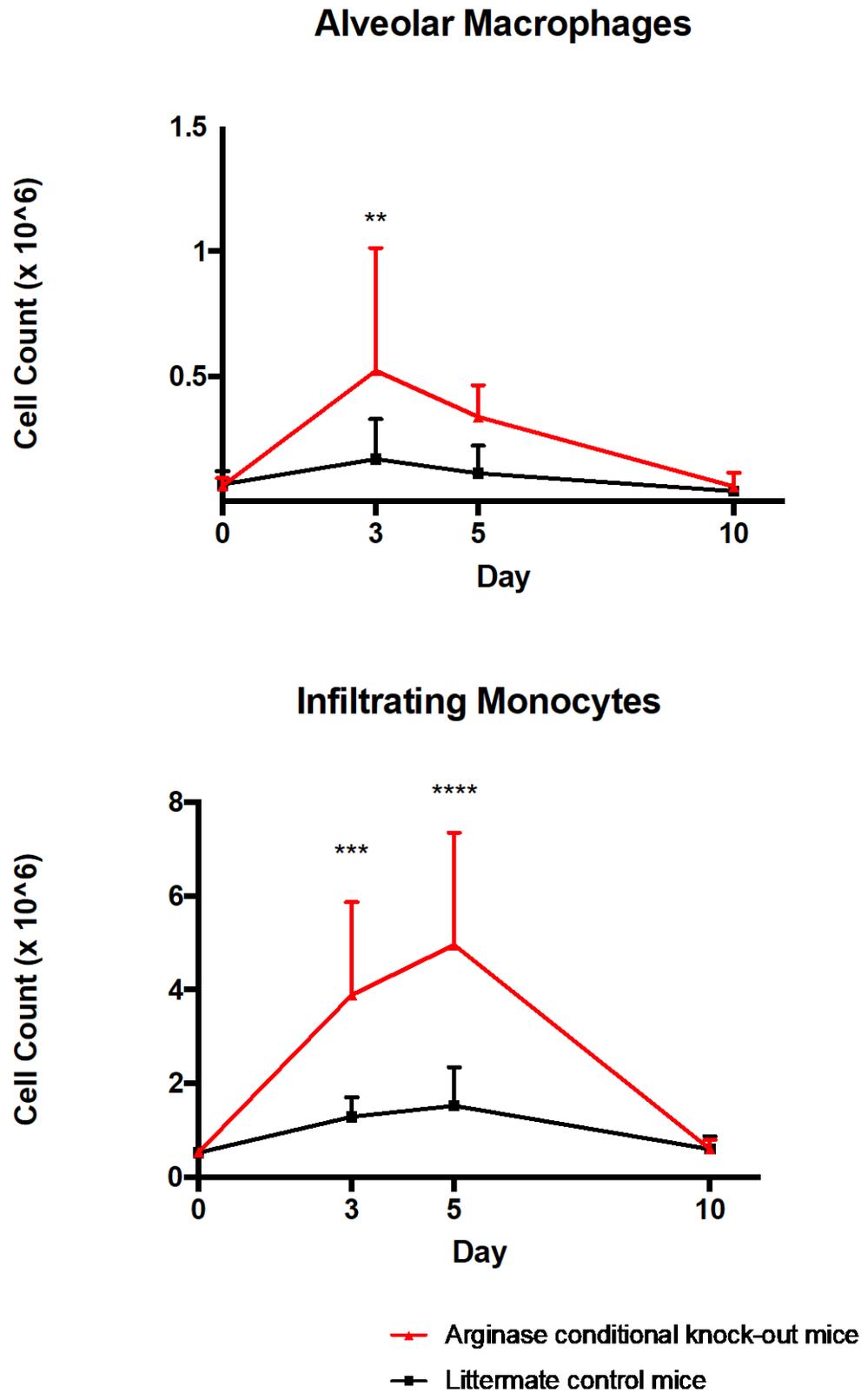
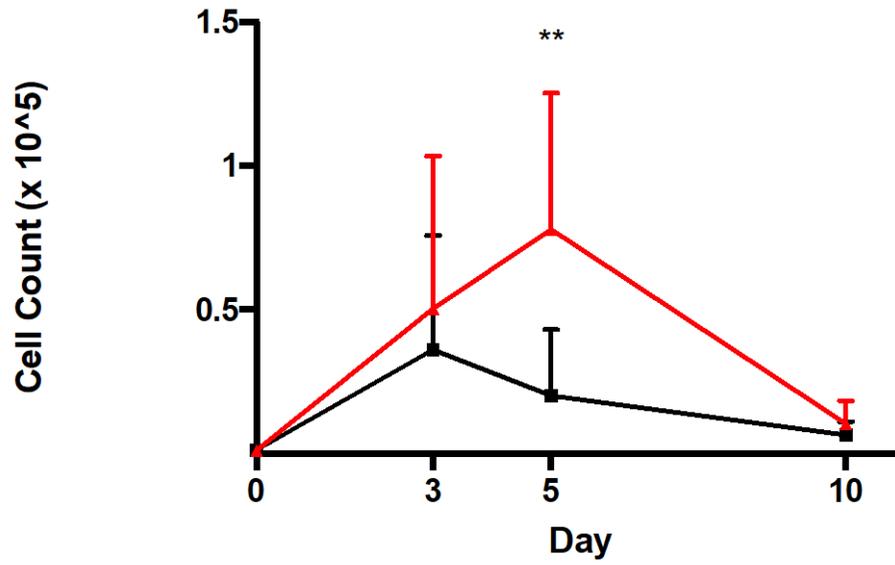


Figure 3.2d

### TNF $\alpha$ + Alveolar Macrophages



### TNF $\alpha$ + Infiltrating Monocytes

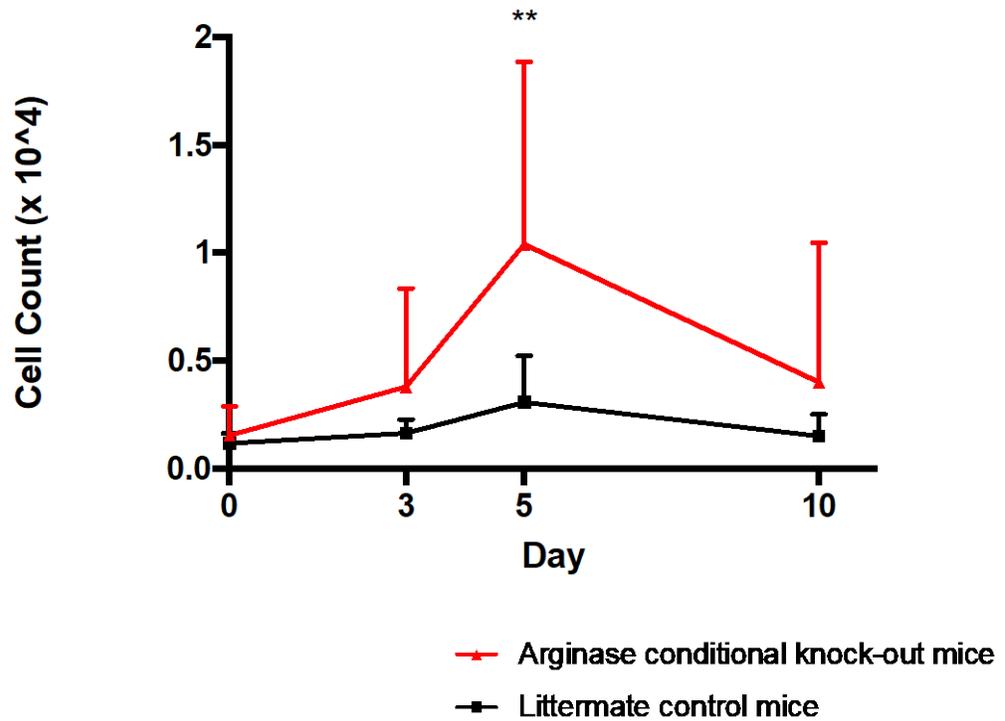


Figure 3.2e

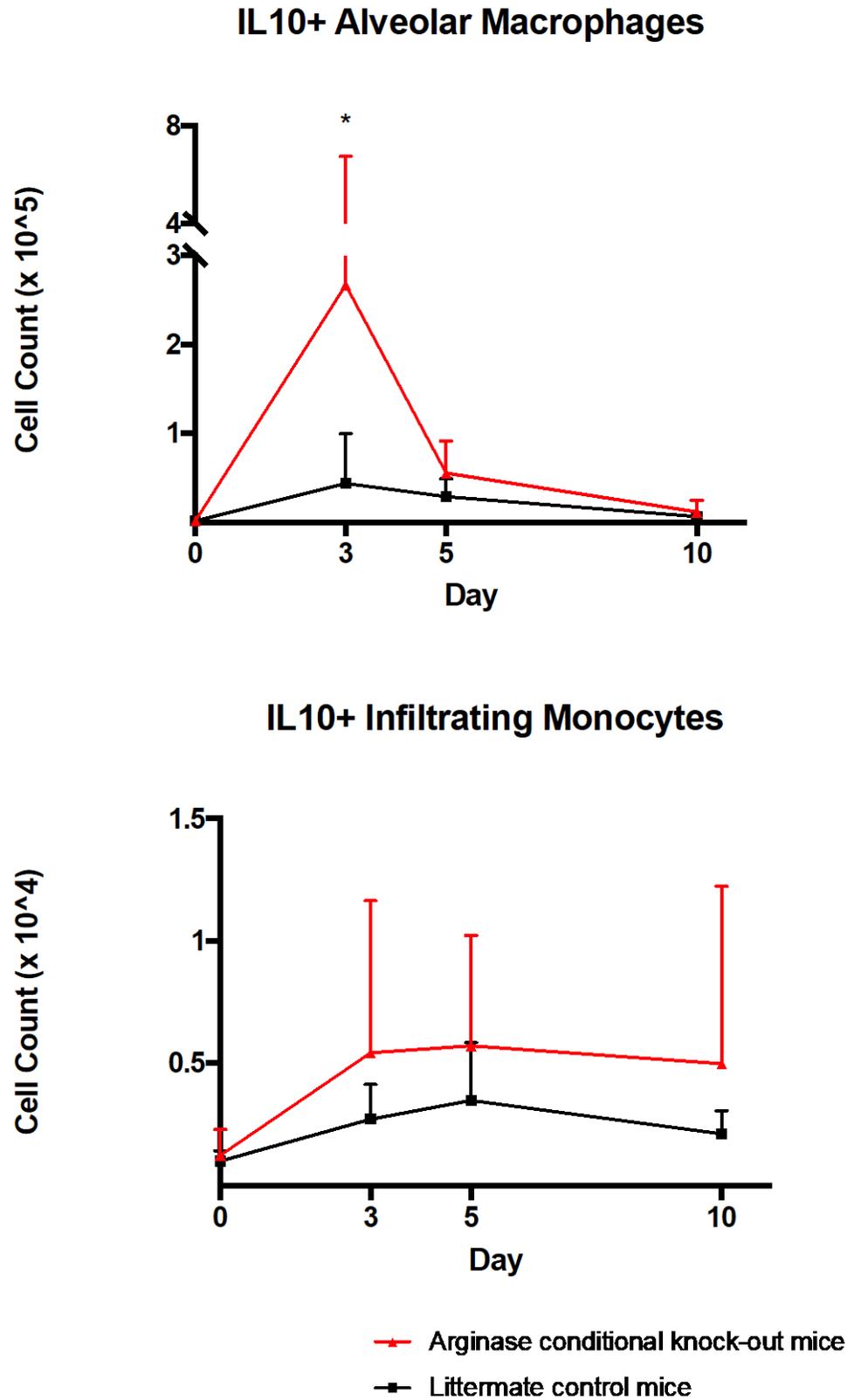


Figure 3.2f

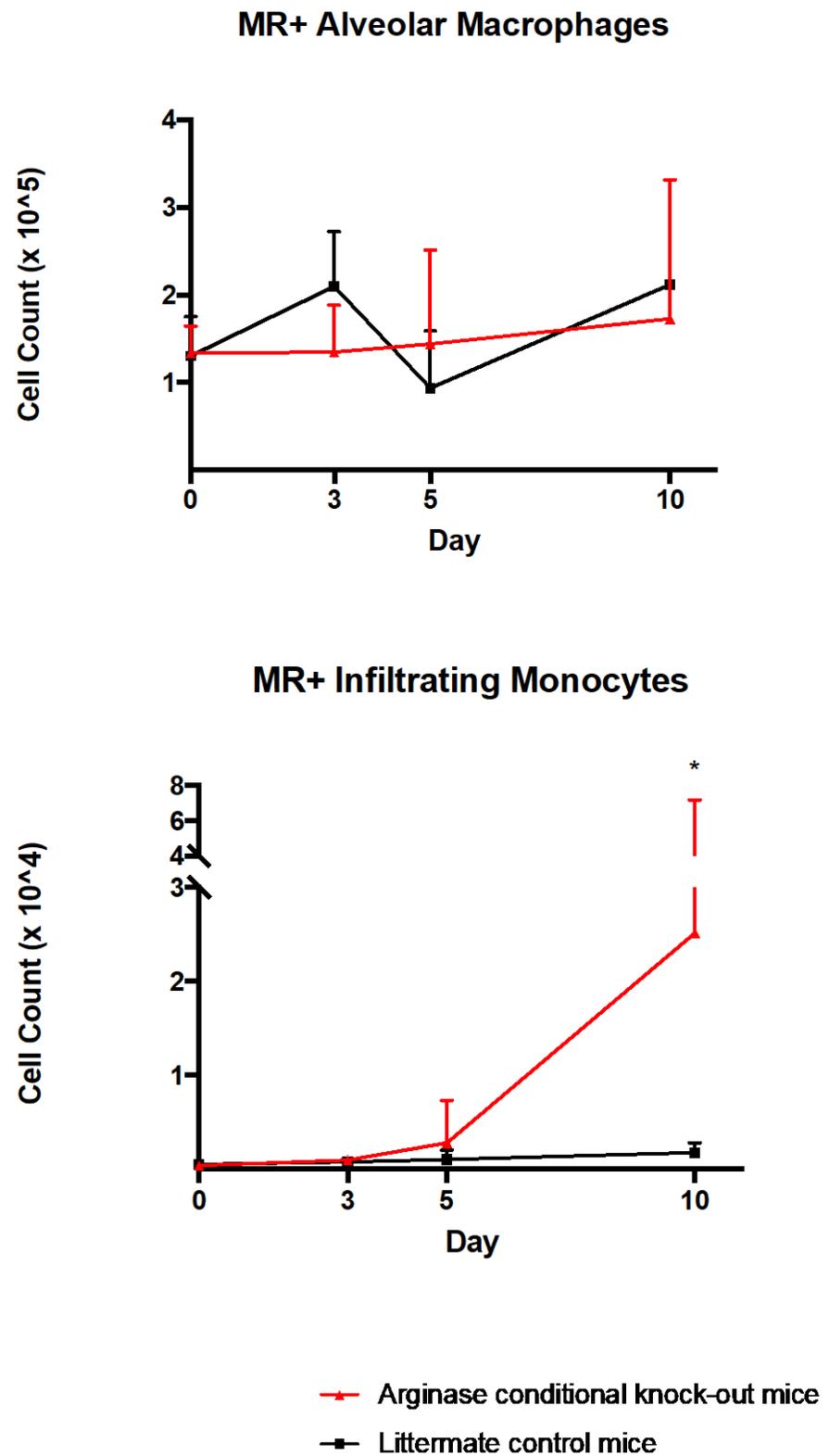


Figure 3.2g

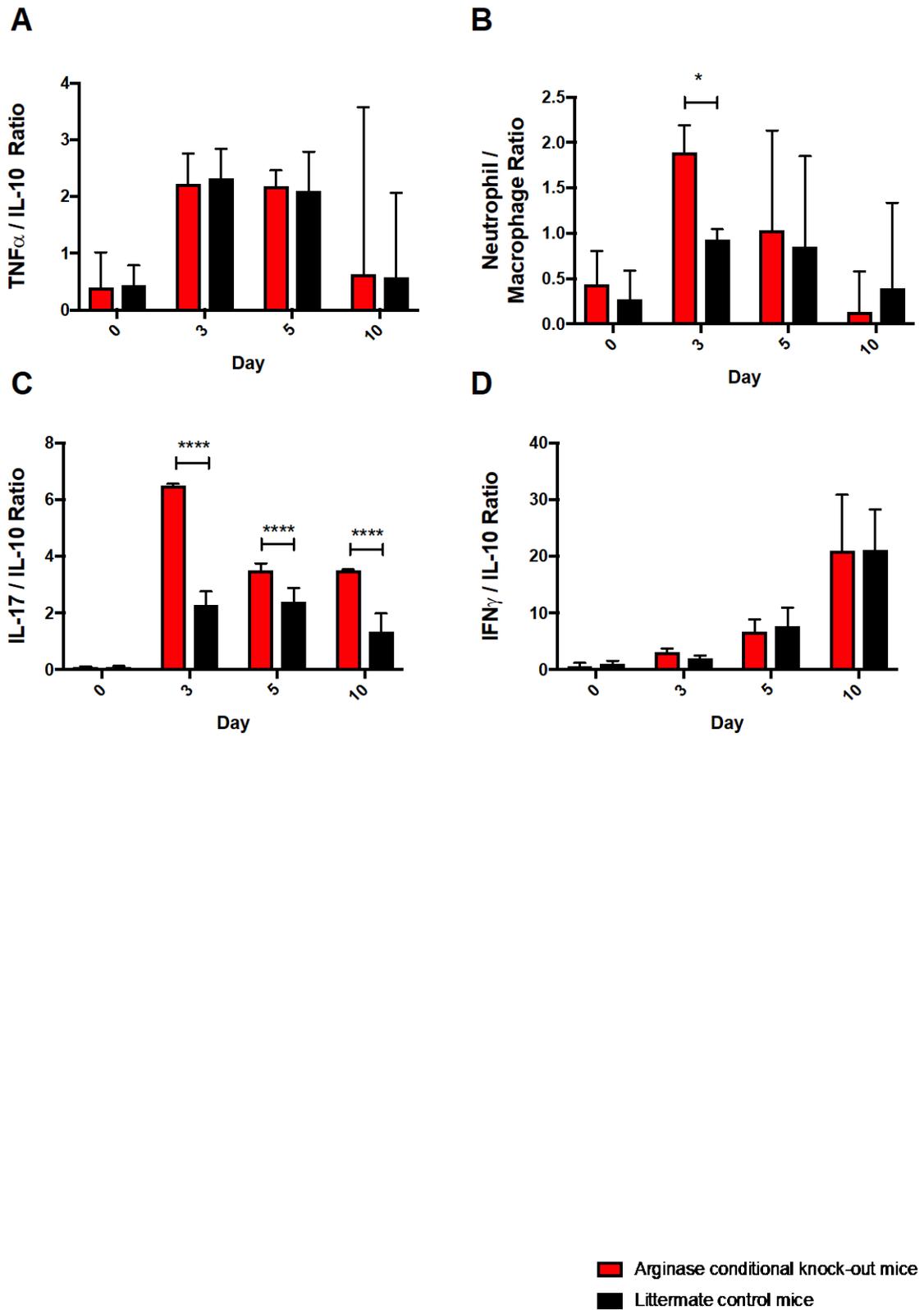


Figure 3.2. Arginase conditional knock-out mice respond with a profound recruitment of innate immune cells.

Lungs from infected mice were lavaged with PBS to collect cells from the alveolar spaces and lung tissues were then harvested to collect cells from the lung interstitium. Lavage and lung tissue samples were collected from at least 4 mice per timepoint per group. Harvested samples were processed into single cell suspensions and stained for flow cytometry analysis as described. At least 50,000 events per sample were analyzed using the FlowJo software to quantify different immune cell populations. Data represent total count of each cell population as a fraction of the total number of live cells analyzed. (a) Gating scheme representing the FlowJo analysis performed to identify different immune cell populations activated in response to the infection. (b) Neutrophil recruitment into the alveolar and lung interstitial spaces (c) Total number of tissue and infiltrating macrophages. (d, e, f) Total counts of alveolar and infiltrating macrophages producing TNF- $\alpha$ , IL-10, or expressing Mannose receptor (MR). (g) Bar graphs represent ratios of TNF- $\alpha$  to IL-10 expressing monocytes in the alveolar spaces (A); ratios of neutrophils to macrophages in the alveolar spaces (B); ratios of IL-17 to IL-10 expressing lymphocytes in the alveolar spaces (C); and ratios of IFN $\gamma$  to IL-10 expressing lymphocytes in the alveolar spaces (D). Data represents mean  $\pm$  SD. Data are representative of 3 independent experiments. Statistical significance determined by two-way ANOVA (p-value < 0.05 (\*); p-value < 0.0005 (\*\*); p-value < 0.0001 (\*\*\*)). Graphs plotted using GraphPad Prism 7.

Figure 3.3a

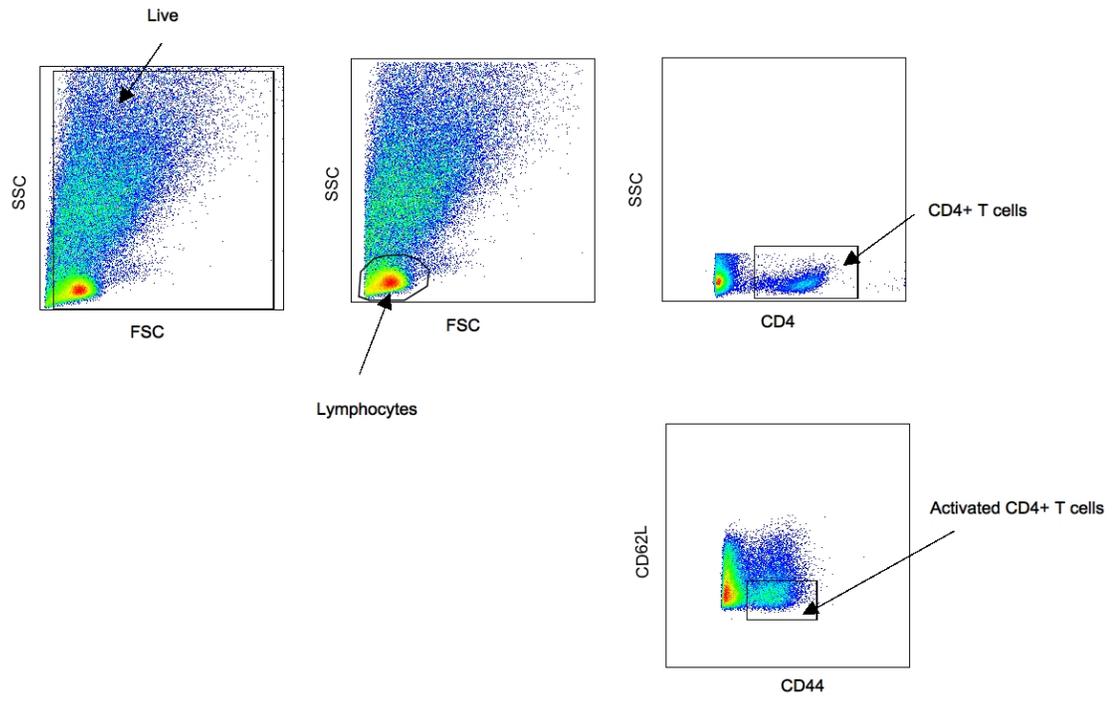


Figure 3.3b

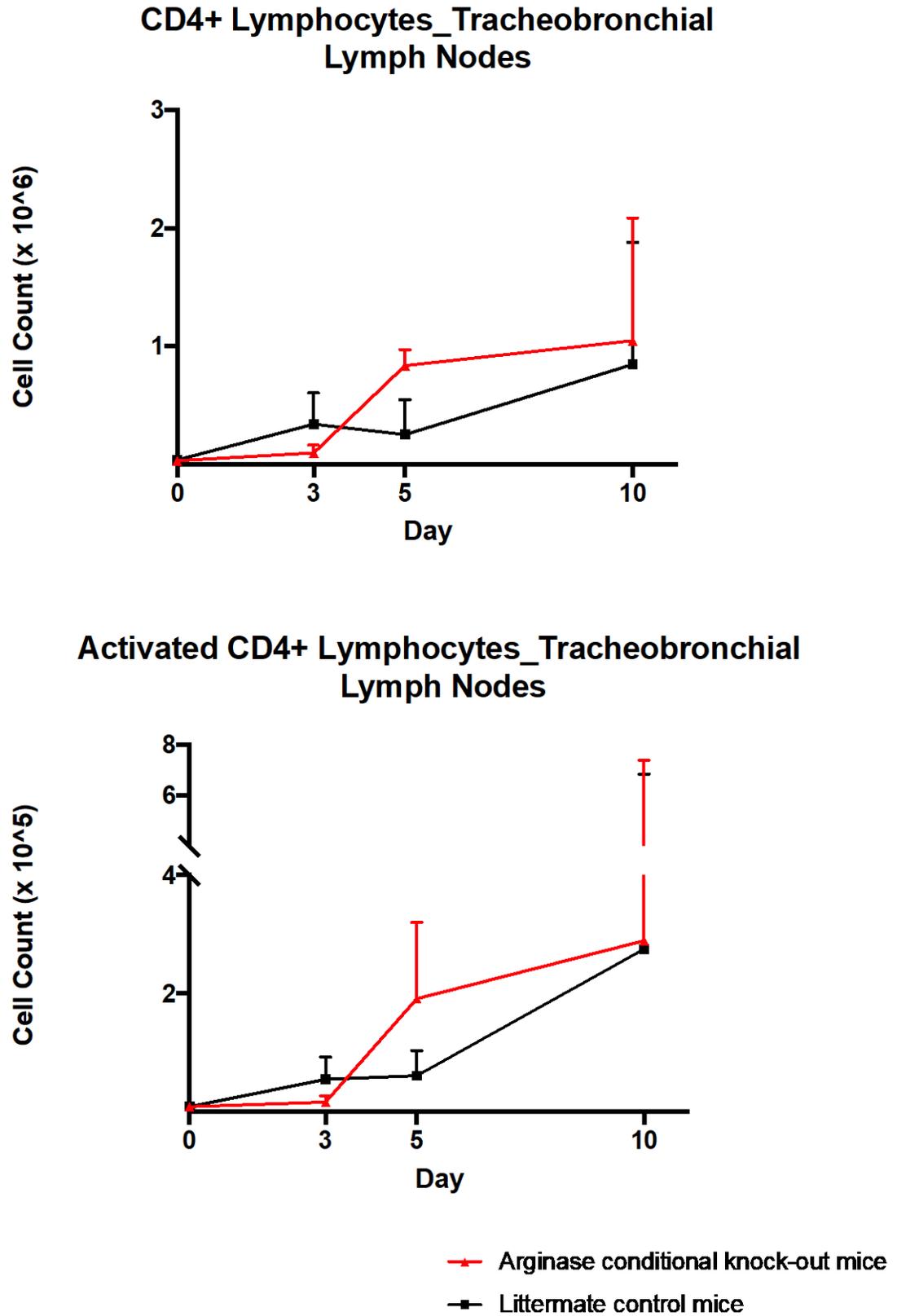
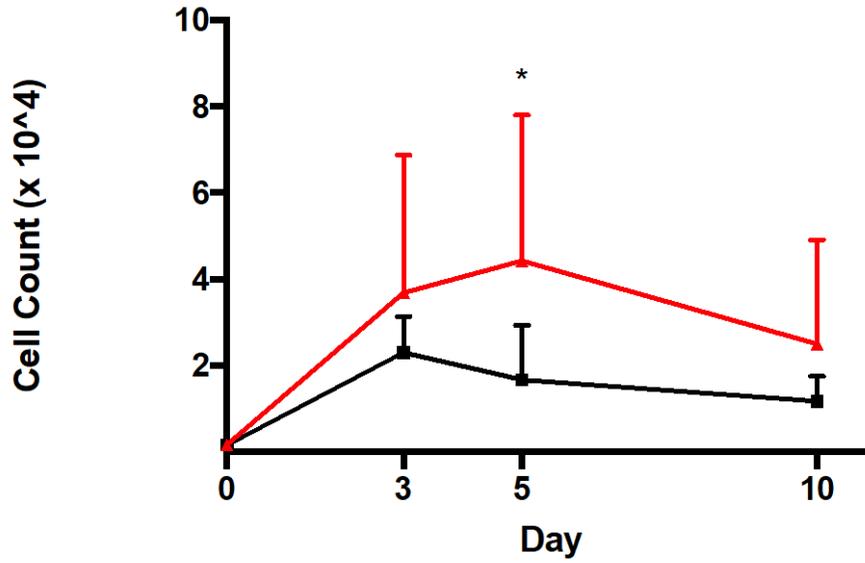
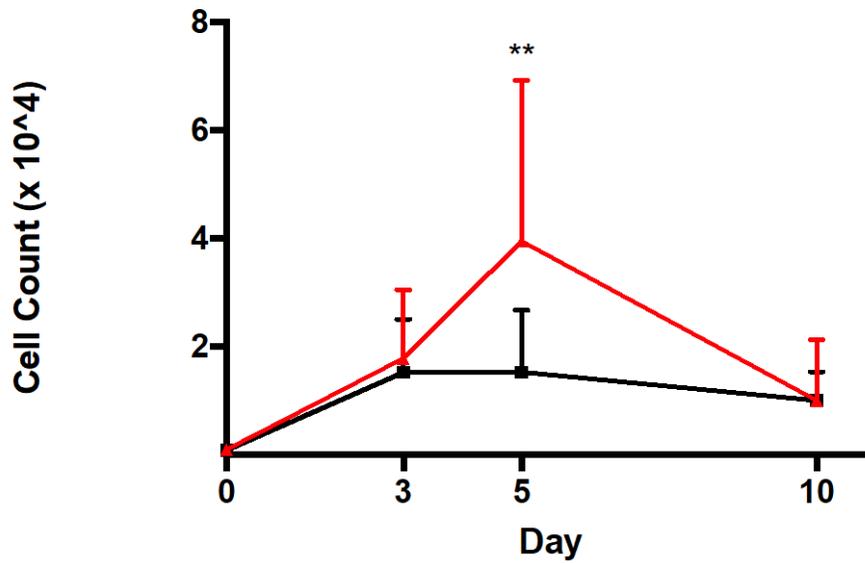


Figure 3.3c

### CD4+ Lymphocytes\_Alveolar Spaces



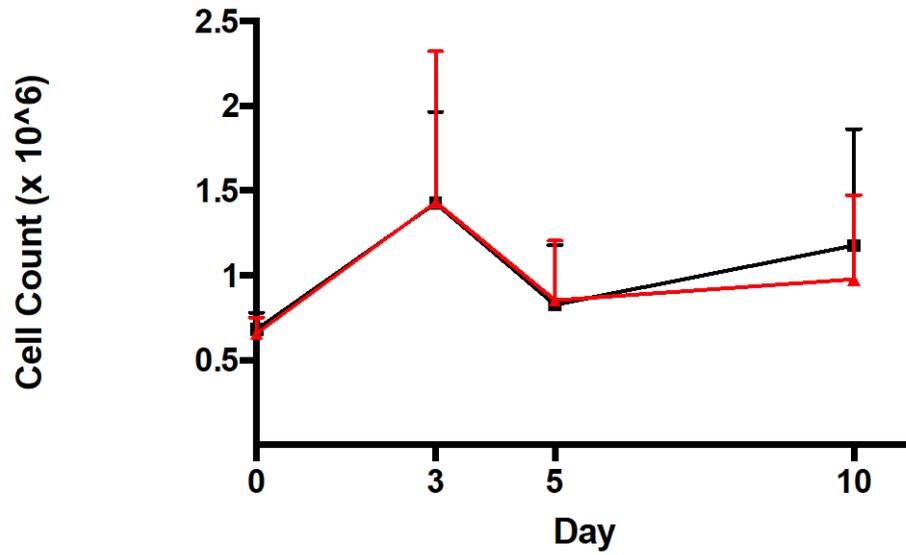
### Activated CD4+ Lymphocytes\_Alveolar Spaces



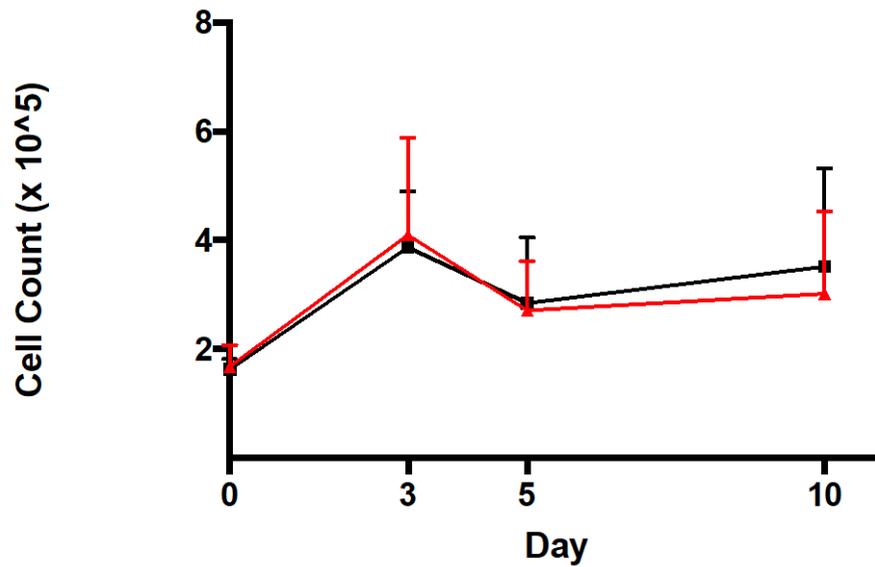
—\*— Arginase conditional knock-out mice  
—■— Littermate control mice

Figure 3.3d

### CD4+ Lymphocytes\_Lung Interstitium



### Activated CD4+ Lymphocytes\_Lung Interstitium



- ▲— Arginase conditional knock-out mice
- Littermate control mice

Figure 3.3. Greater T cell recruitment and activation in response to PA pneumonia in arginase conditional knock-out mice.

Tracheobronchial lymph nodes draining the site of infection were harvested and processed into single cell suspensions by passing through mesh strainers. Lung lavage and lung tissue samples were collected and processed as described.

Single cell suspensions were stained for flow cytometry analysis as described. At least 50,000 events per sample were analyzed using the FlowJo software to quantify different immune cell populations. Data represent total count of each cell population as a fraction of the total number of live cells analyzed. (a) Gating scheme representing the FlowJo analysis performed to identify different immune cell populations activated in response to the infection (b, c, d) Total number of CD4<sup>+</sup> T cells recruited and activated in the lymph nodes, alveolar spaces, and lung interstitium of infected mice. Data represents mean  $\pm$  SD. Data are representative of 3 independent experiments. Statistical significance determined by two-way ANOVA (p-value < 0.05 (\*); p-value < 0.0005 (\*\*); p-value < 0.0001 (\*\*\*\*)). Graphs plotted using GraphPad Prism 7.

Figure 3.4a

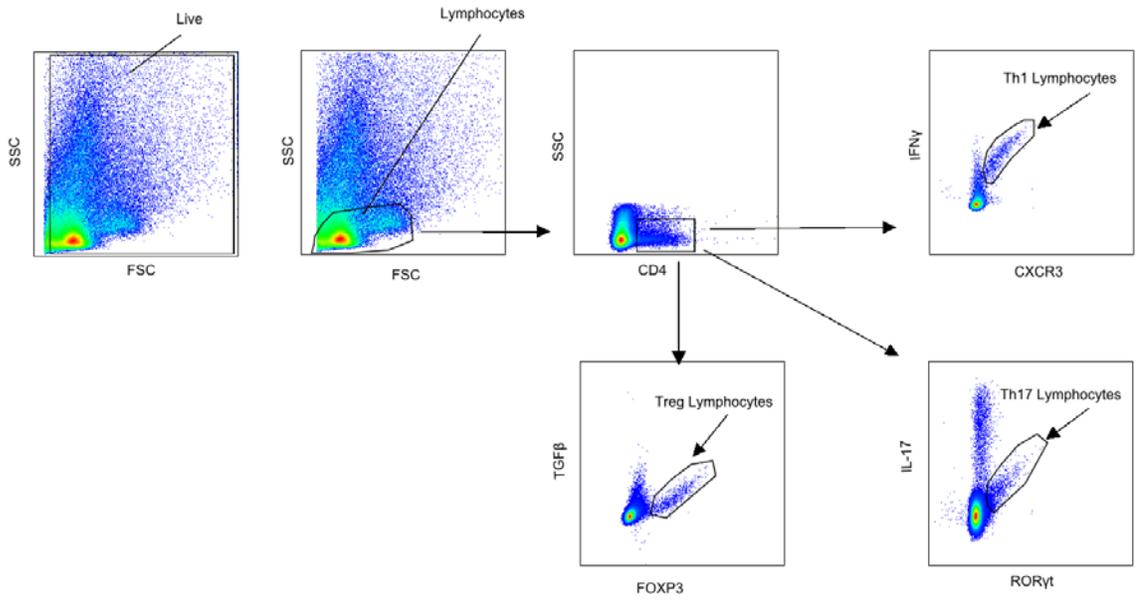
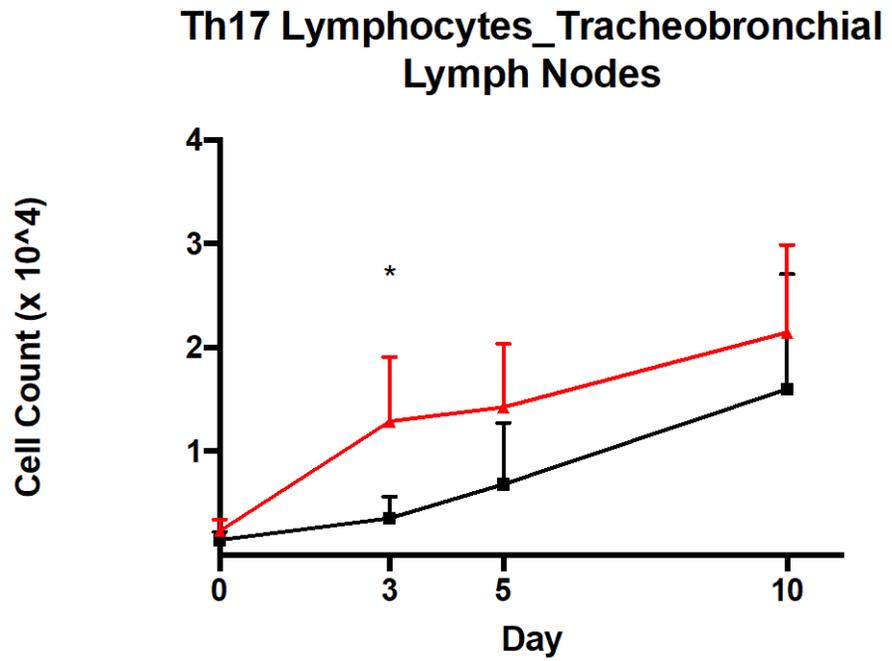
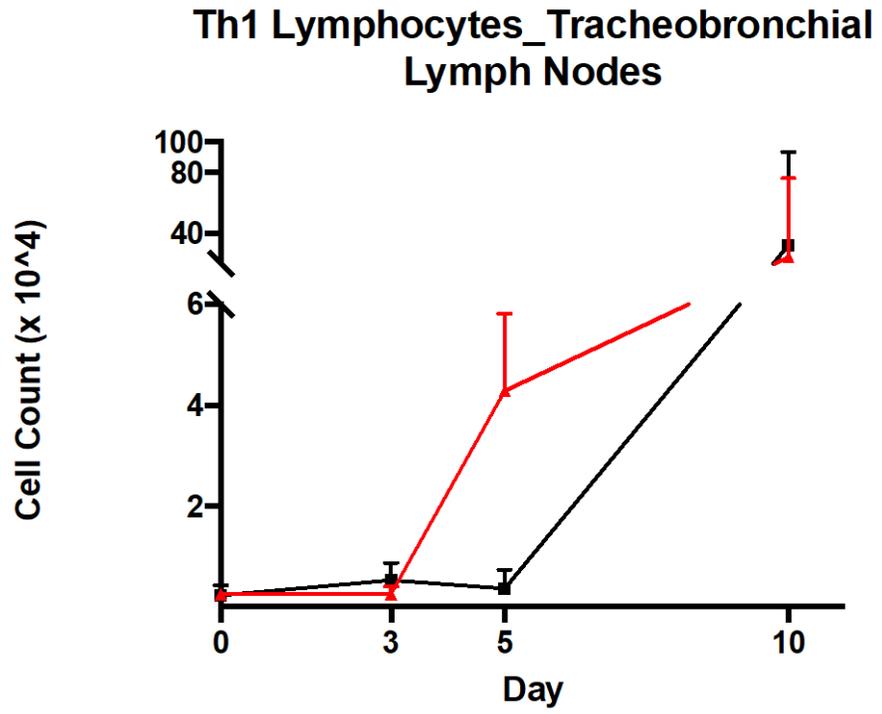


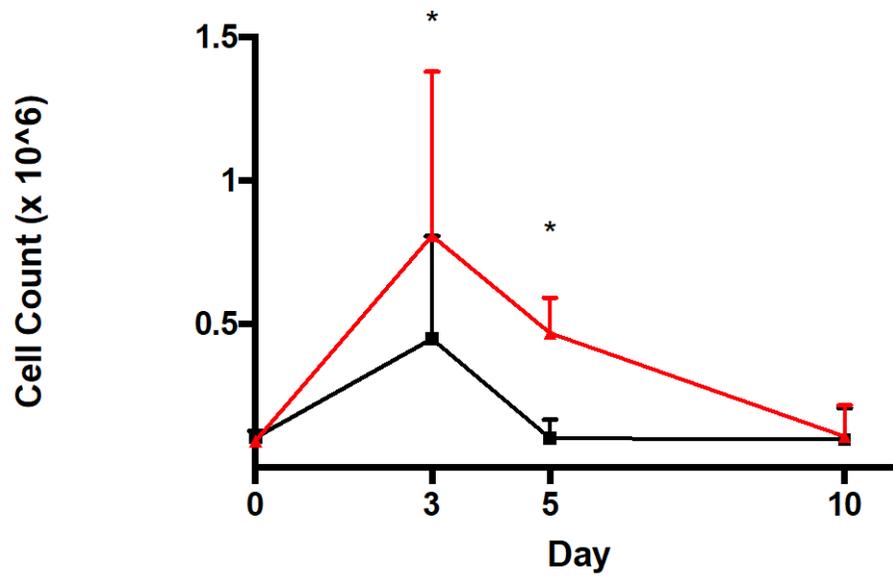
Figure 3.4b



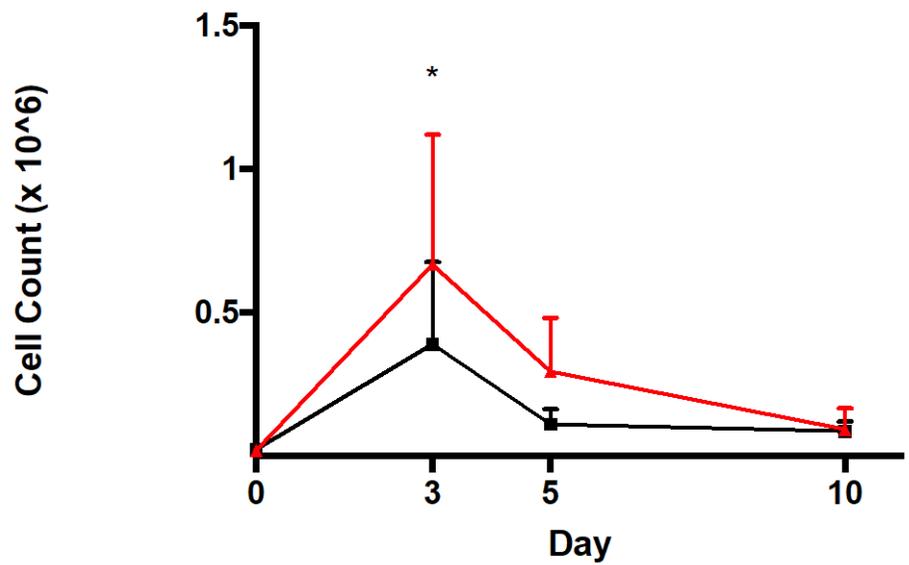
—\*— Arginase conditional knock-out mice  
—■— Littermate control mice

Figure 3.4c

### Th1 Lymphocytes\_Lung Interstitium



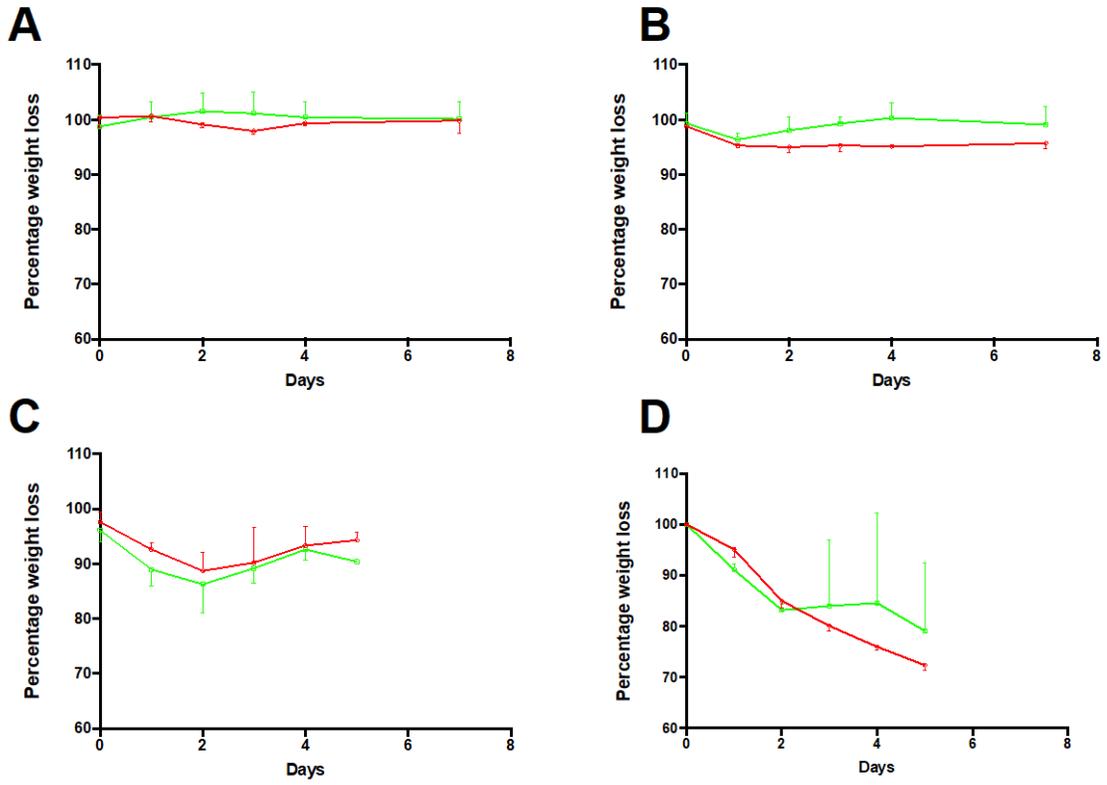
### Th17 Lymphocytes\_Lung Interstitium



- \*— Arginase conditional knock-out mice
- Littermate control mice

Figure 3.4. Arginase conditional knock-out mice respond to PA pneumonia with an excessive recruitment of Th1 and Th17 pro-inflammatory lymphocytes. Tracheobronchial lymph nodes draining the site of infection and lung tissue samples were collected and processed as described. Single cell suspensions were stained for flow cytometry analysis as described. At least 50,000 events per sample were analyzed using the FlowJo software to quantify different immune cell populations. Data represent total count of each cell population as a fraction of the total number of live cells analyzed. (a) Gating scheme representing the FlowJo analysis performed to identify different lymphocyte populations activated in response to the infection (b, c) Total number of Th1 and Th17 lymphocytes in the lymph nodes and lung interstitium of infected mice. Data represents mean  $\pm$  SD. Data are representative of 3 independent experiments. Statistical significance determined by two-way ANOVA (p-value < 0.05 (\*); p-value < 0.0005 (\*\*); p-value < 0.0001 (\*\*\*\*)). Graphs plotted using GraphPad Prism 7.

Figure 3.5a



— BEC treated  
— Water treated

Figure 3.5b

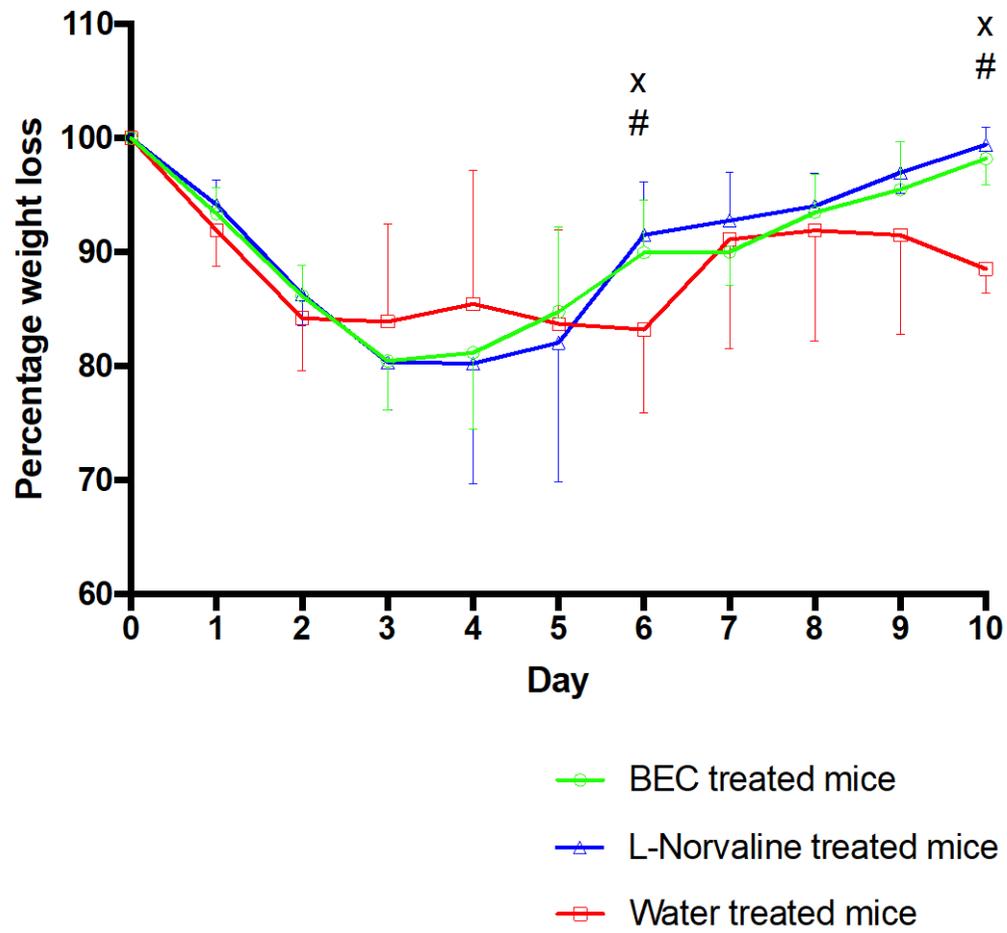


Figure 3.5c

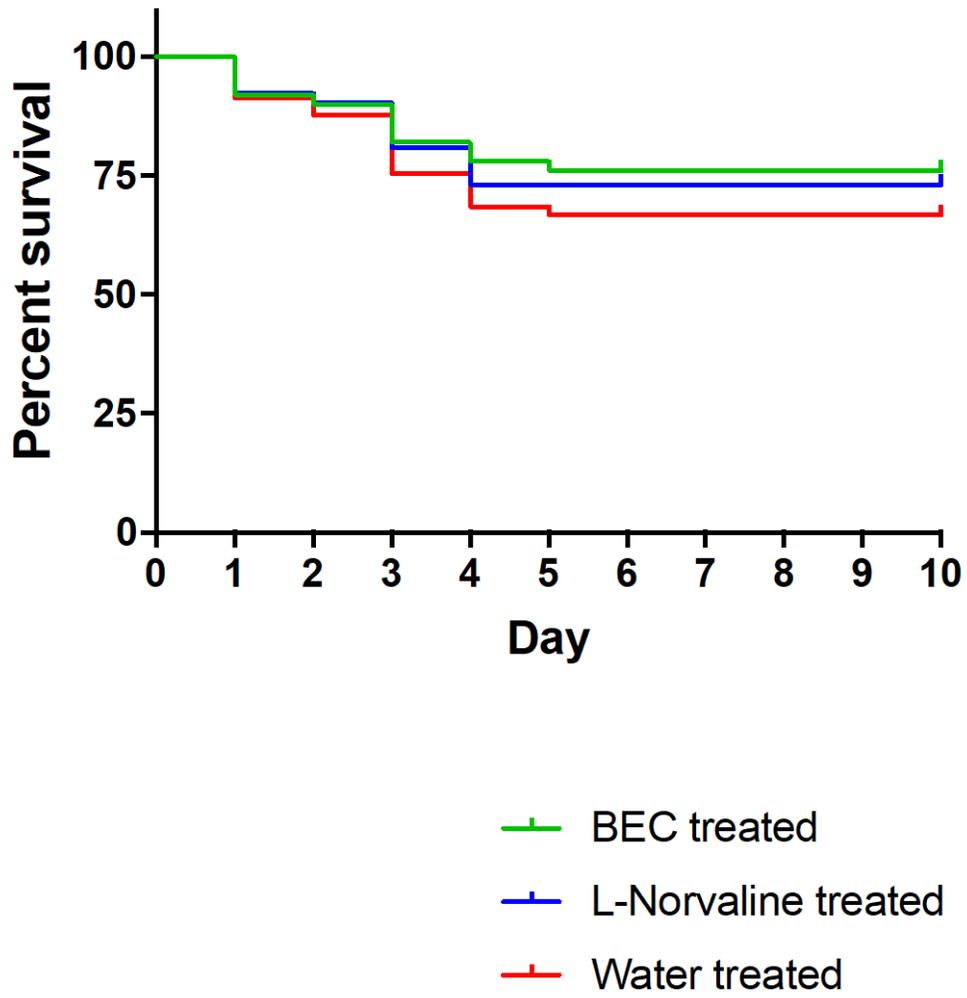


Figure 3.5d

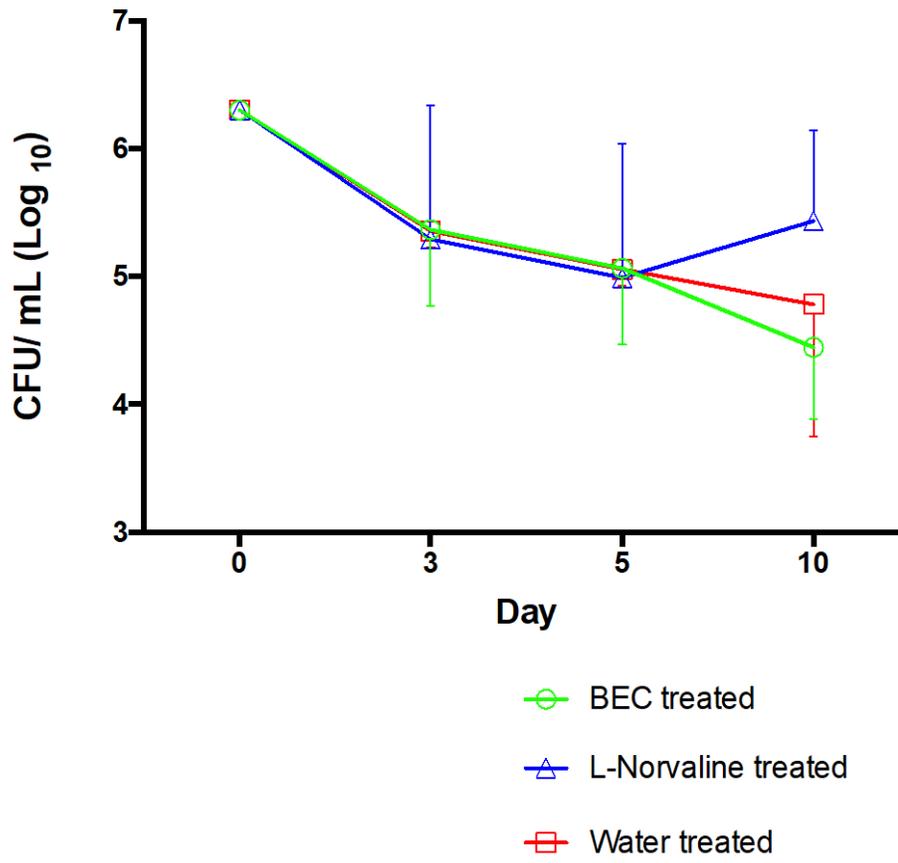


Figure 3.5. Pharmacological arginase inhibition results in comparable acute morbidity with faster recovery.

BALB/cJ mice were infected intratracheally with PA-laden agarose beads as described. Murine morbidity post infection was evaluated in terms of weight loss measured at least once daily before and after infection. (a) Graph represents percentage weight loss normalized to the baseline body weight prior to infection at different infective inoculums (A: 0 CFU/mL; B:  $5 \times 10^6$  CFU/mL; C:  $1 \times 10^7$  CFU/mL; D: of  $5 \times 10^7$  CFU/mL). (b) The graph represents percentage weight loss normalized to the baseline body weight prior to infection at the chosen optimal infective inoculum of  $2 \times 10^7$  CFU/mL. (c) Kaplan-Meier curve representing mortality post-infection in mice infected with  $2 \times 10^7$  CFU/mL. (d) The graph represents bacterial clearance after infection with  $2 \times 10^7$  CFU/mL. Data represents mean  $\pm$  SD. Data are representative of 3 independent experiments. Statistical significance determined by two-way ANOVA ((x) BEC treated group significantly different than water treated group; (#) L-norvaline treated group significantly different than water treated group; p-value < 0.05). Graphs plotted using GraphPad Prism 7 (Note: for some points, the error bars are shorter than the height of the symbol. In these cases, Prism simply does not draw the error bars).

Figure 3.6a

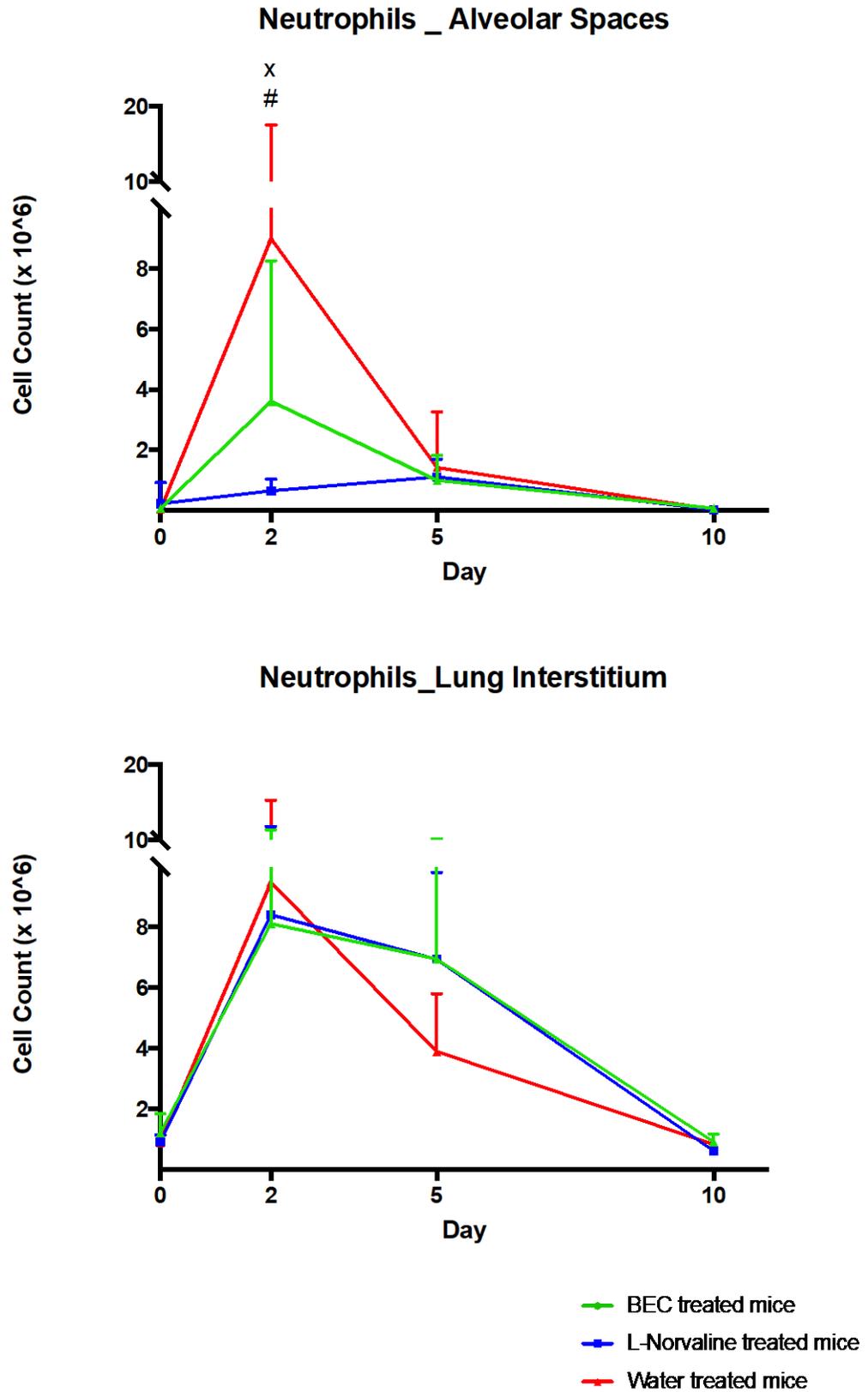


Figure 3.6b

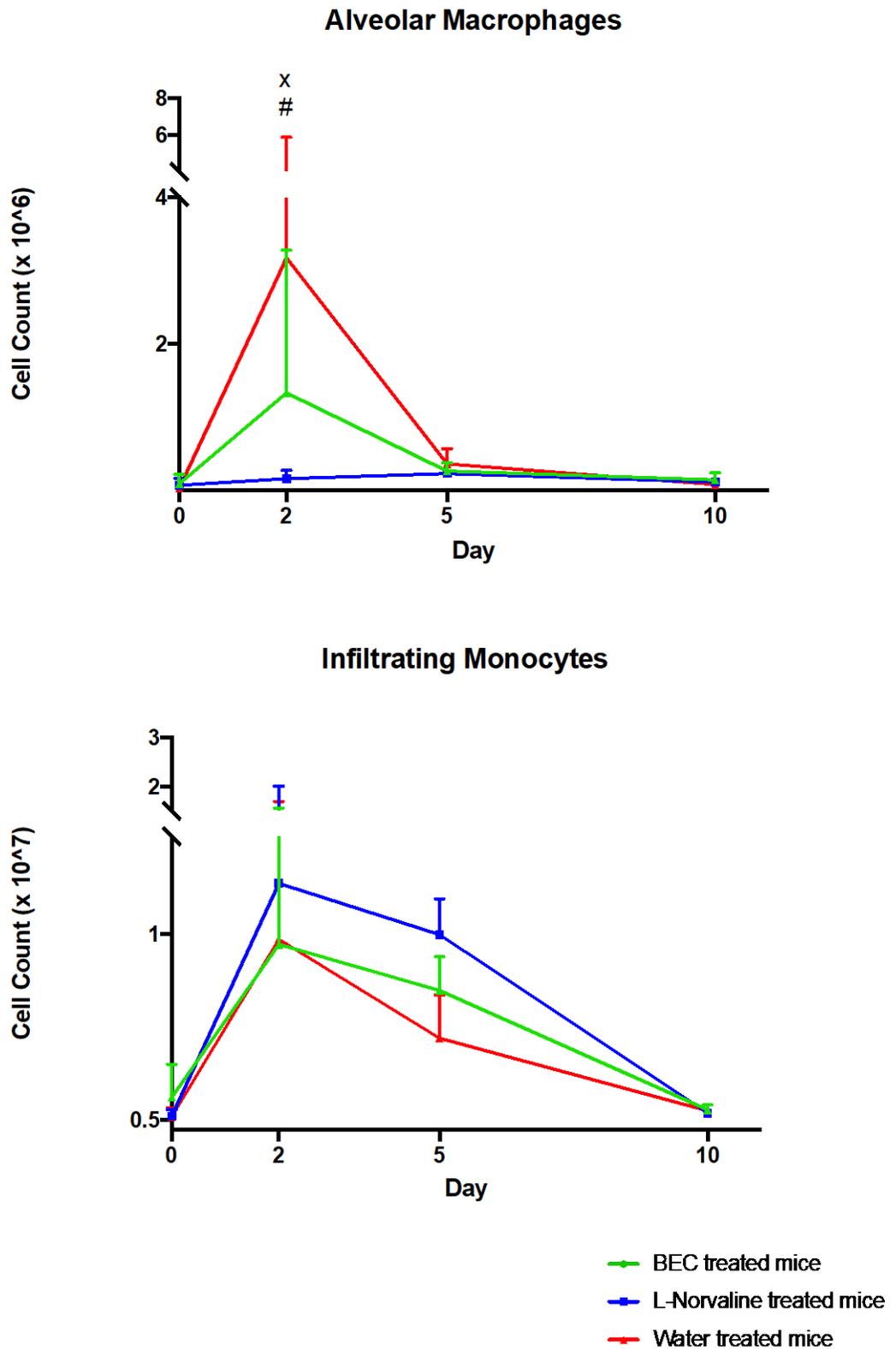


Figure 3.6c

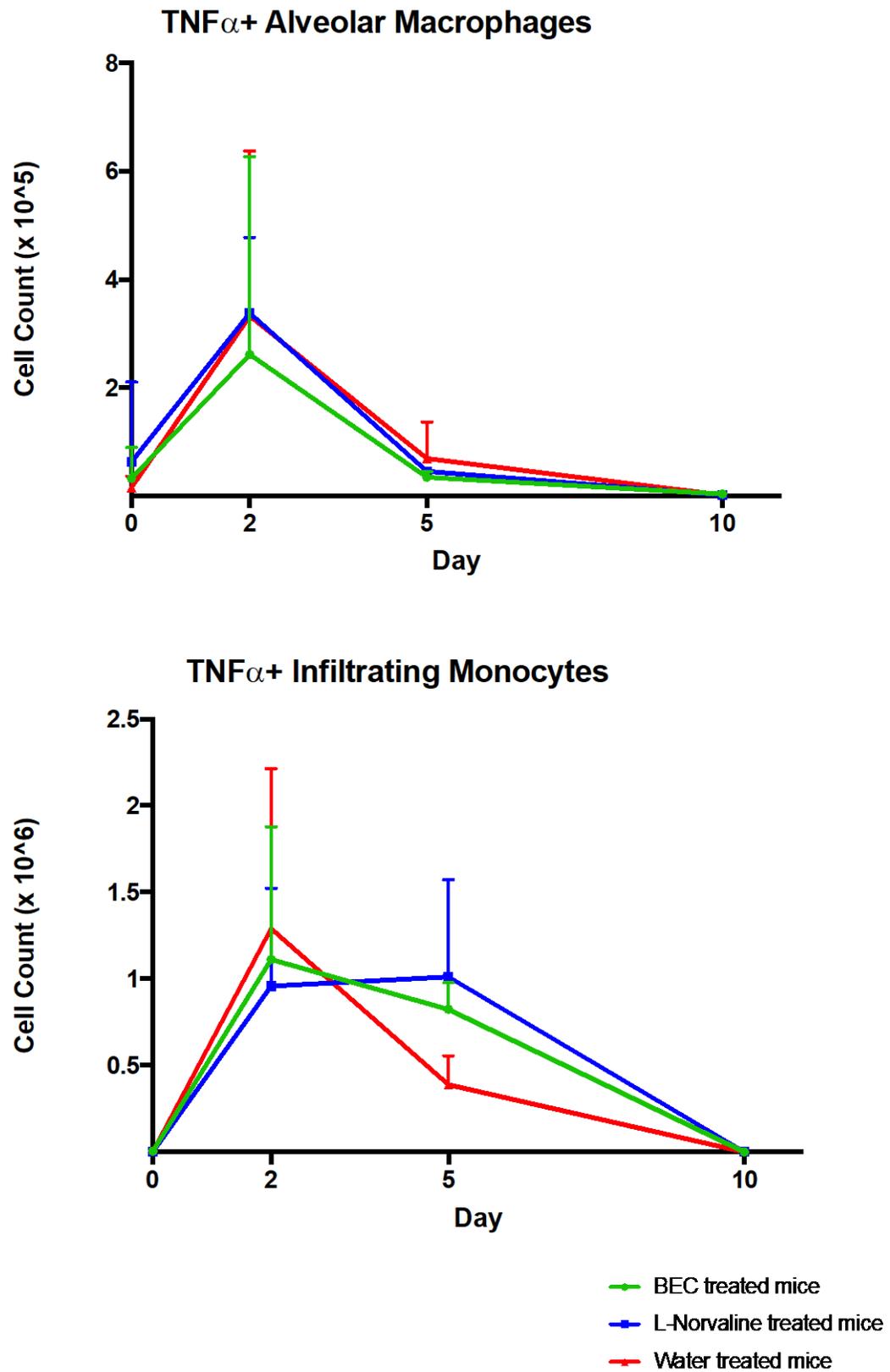


Figure 3.6d

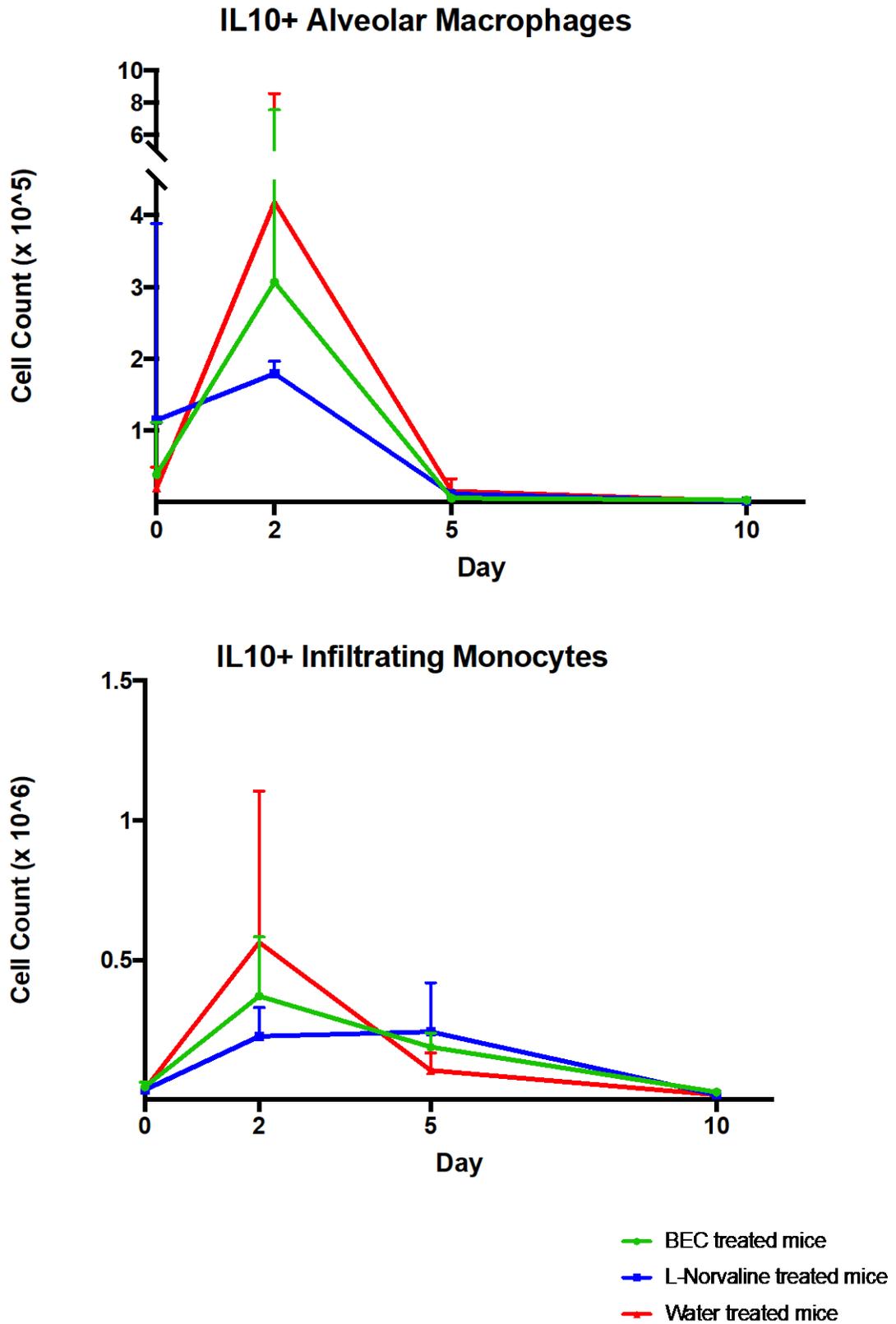


Figure 3.6e

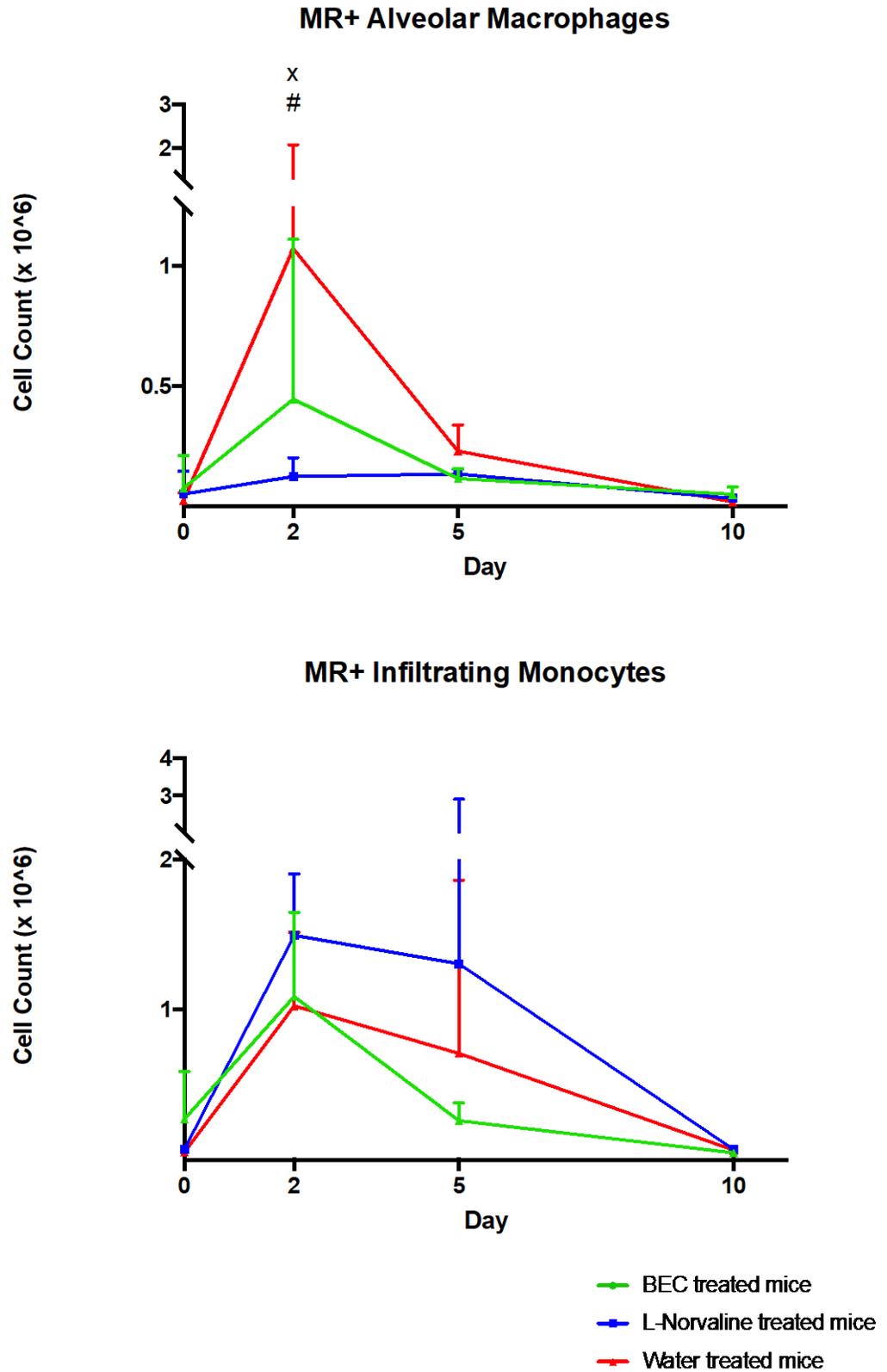


Figure 3.6f

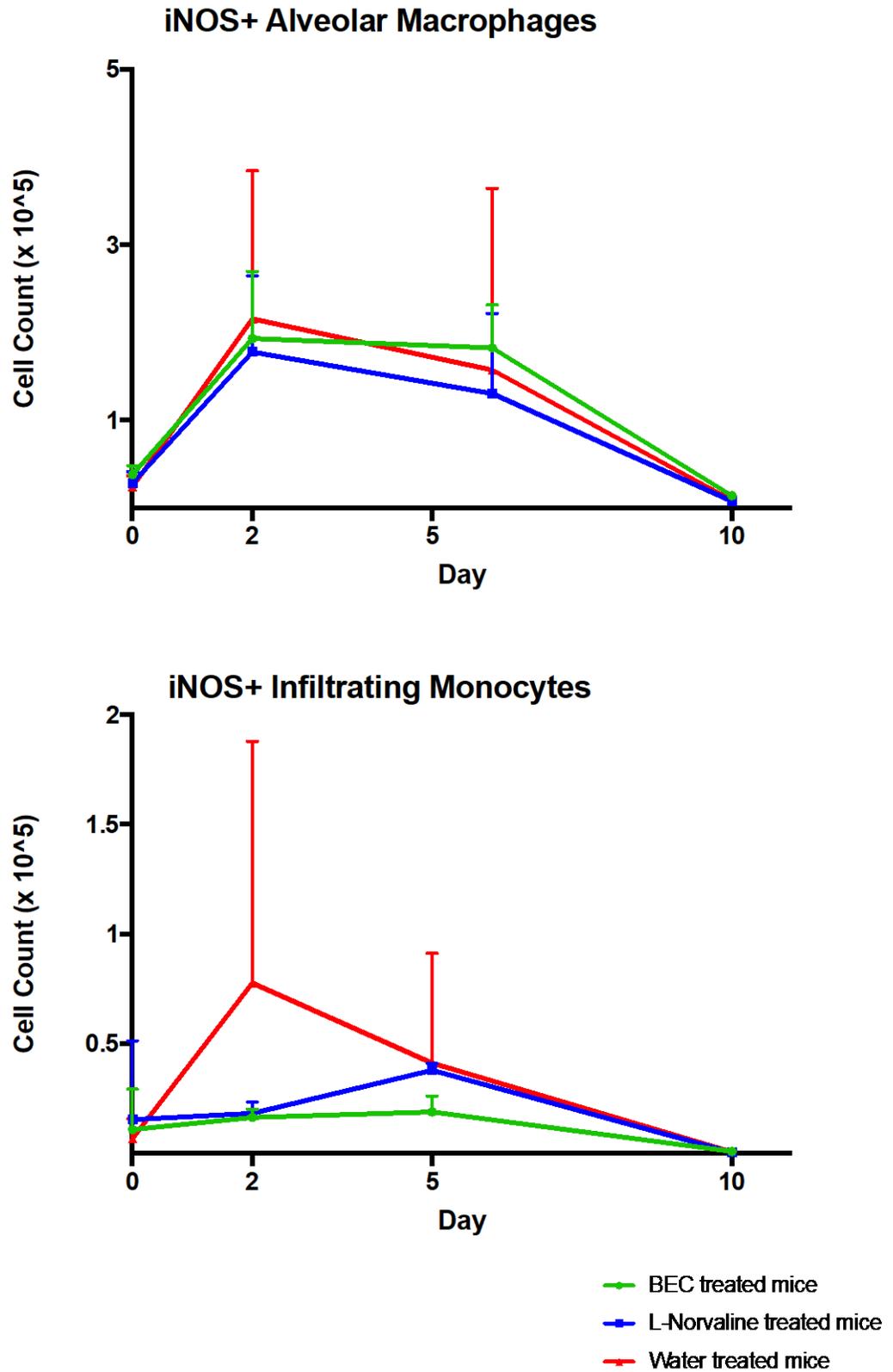


Figure 3.6g

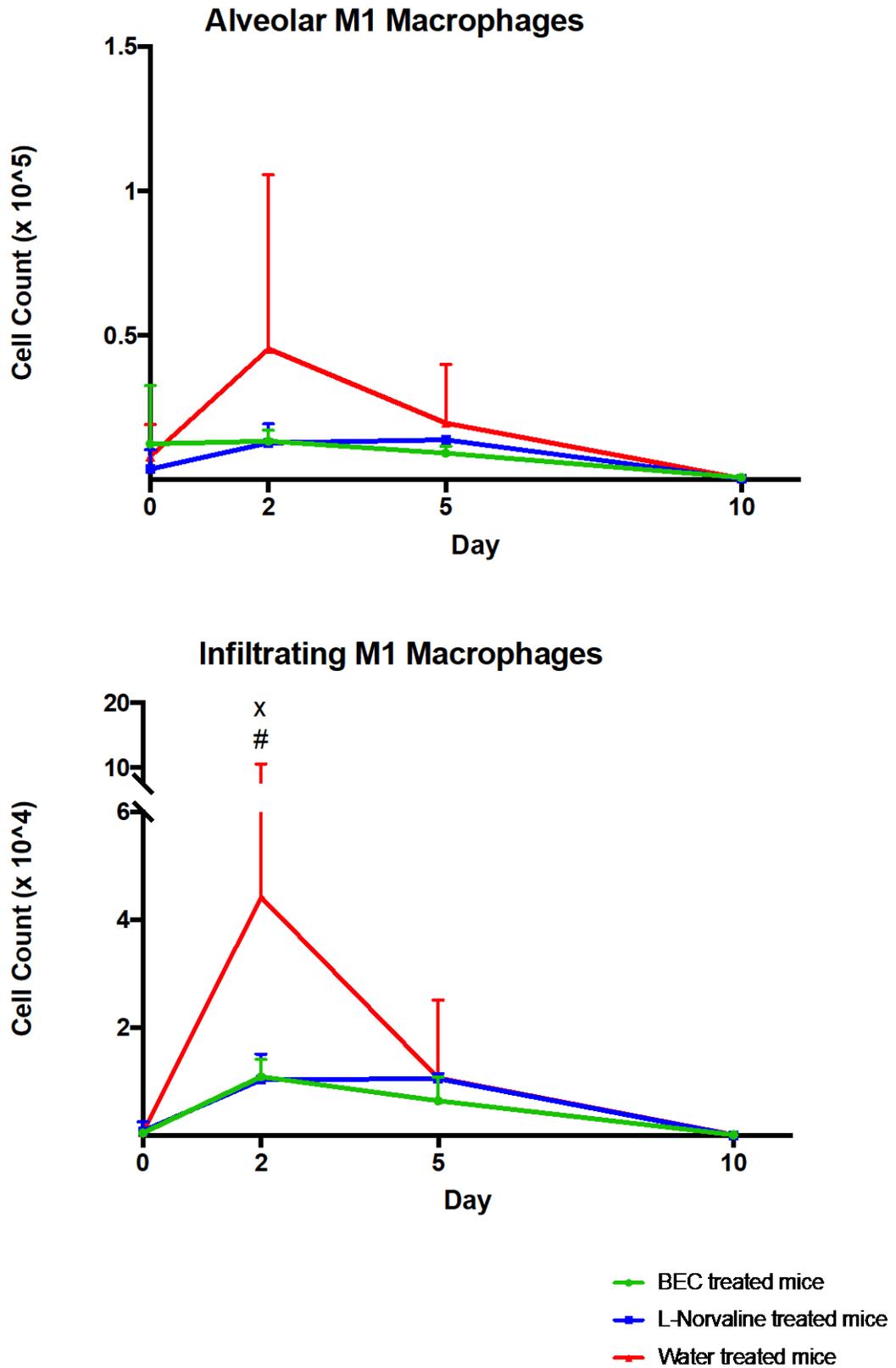


Figure 3.6h

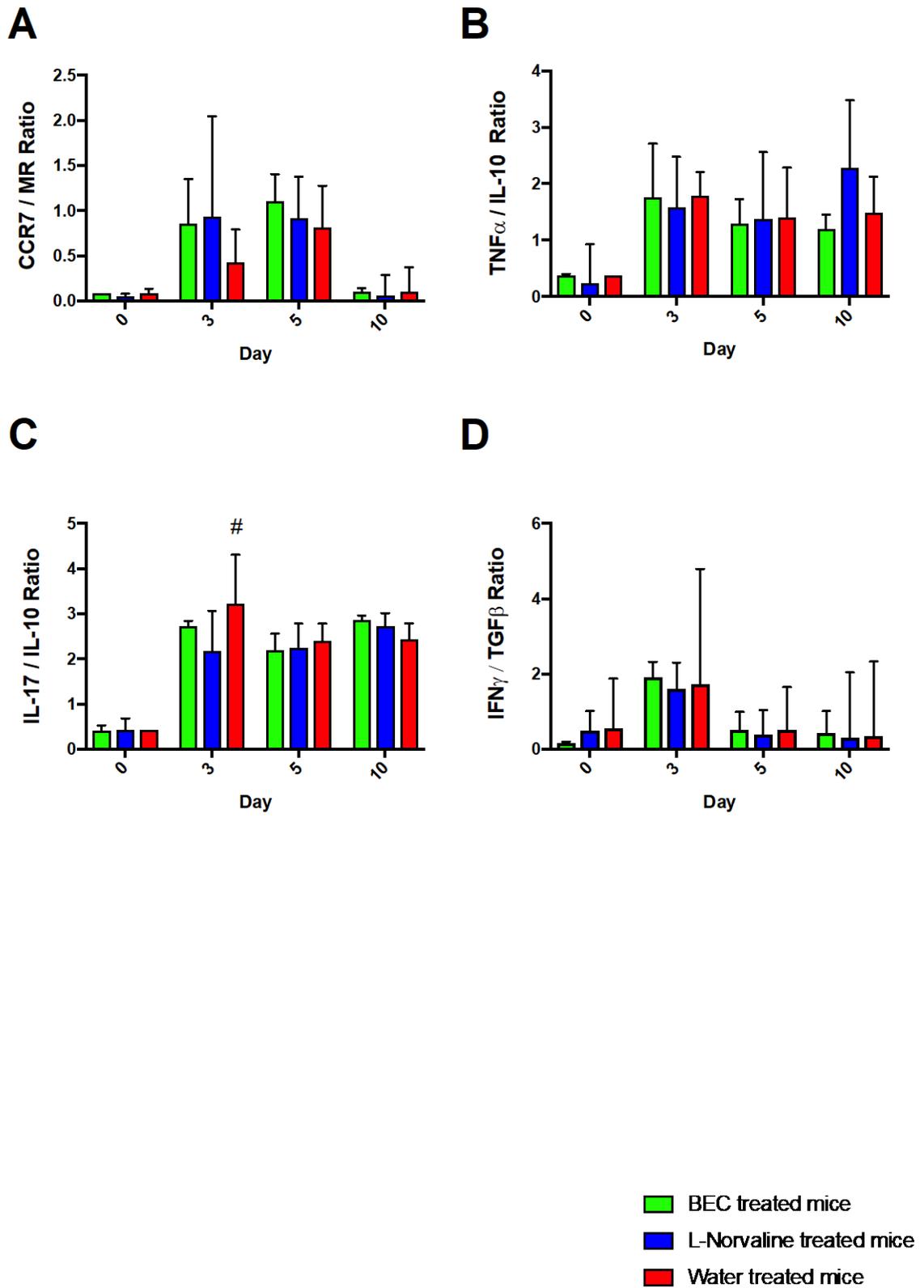


Figure 3.6i

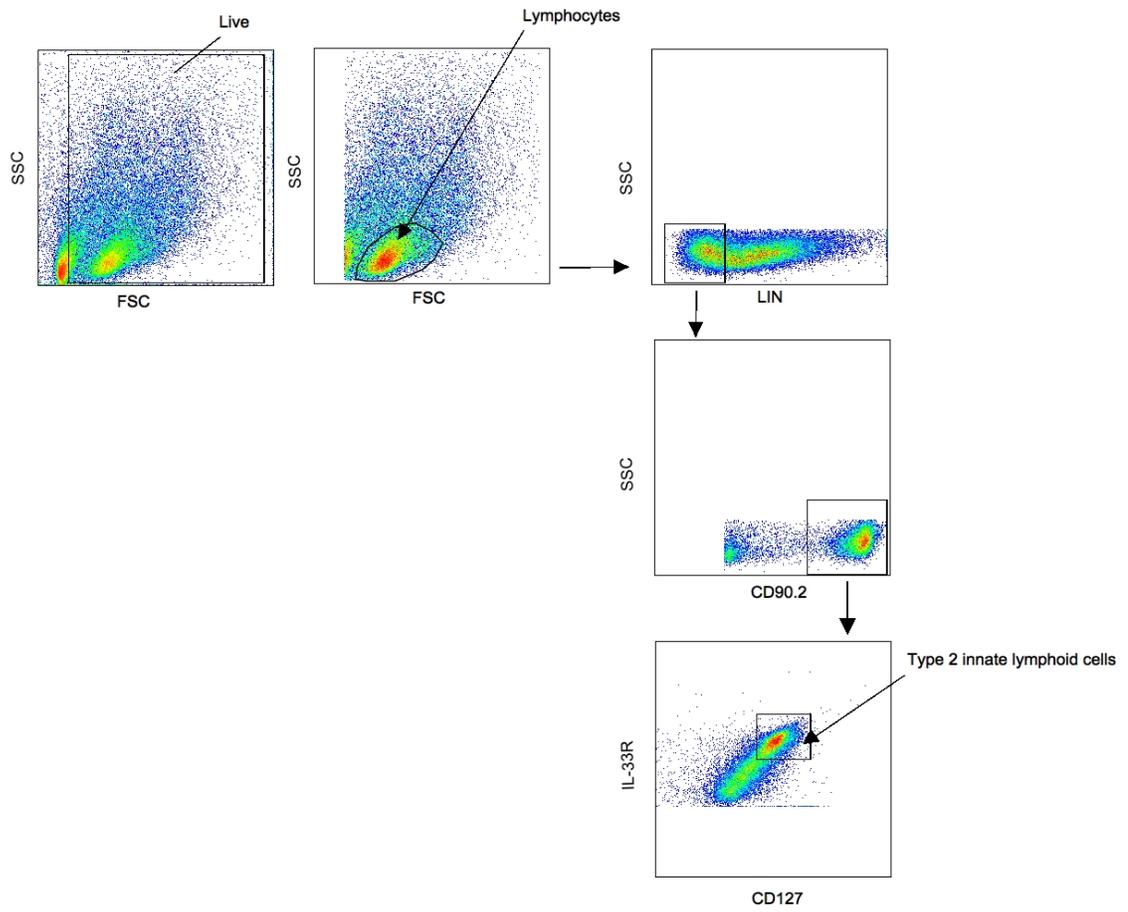
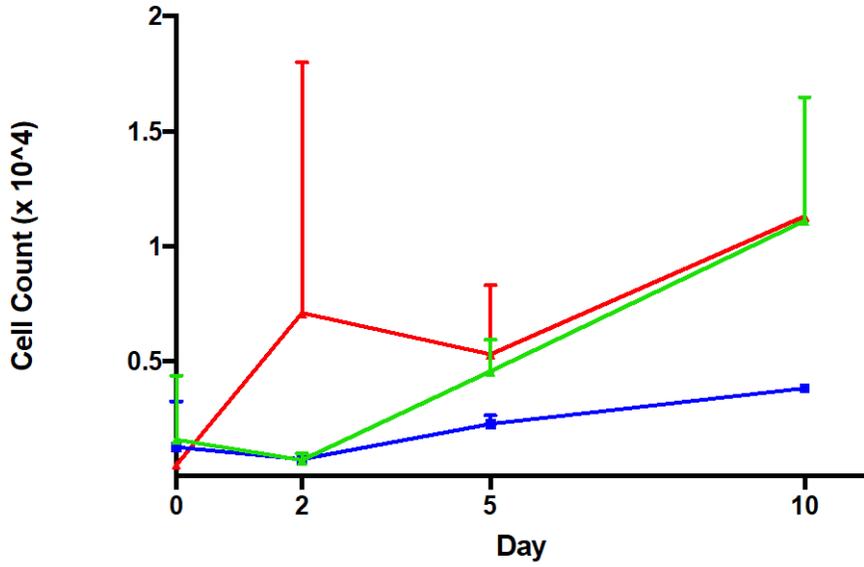
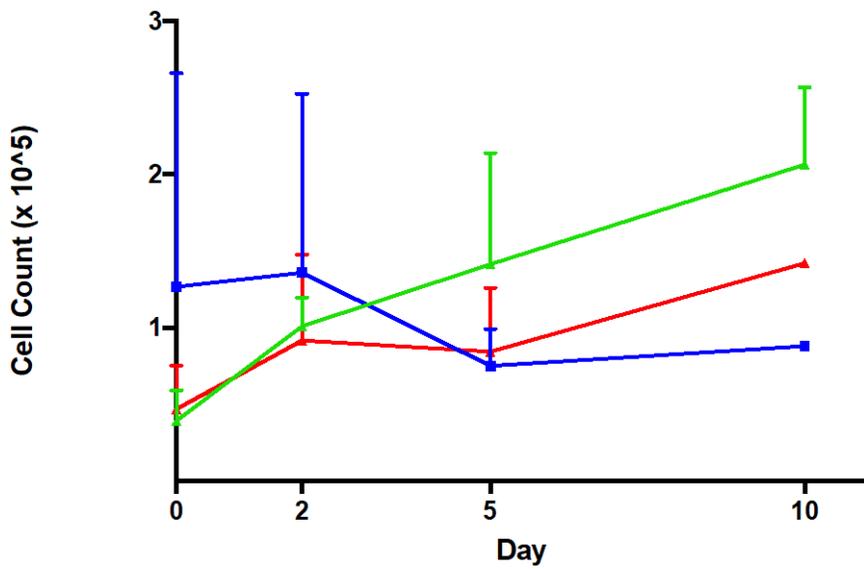


Figure 3.6i (continued)

### Innate Lymphoid Cells Type 2 \_Alveolar Spaces



### Innate Lymphoid Cells Type 2 \_Lung Interstitium



- BEC treated mice
- L-Norvaline treated mice
- ▲— Water treated mice

Figure 3.6. BALB/cJ mice with global arginase inhibition respond to PA pneumonia with comparable but slightly attenuated recruitment of innate immune cells.

Lungs from infected mice were lavaged with PBS to collect cells from the alveolar spaces and lung tissues were then harvested to collect cells from the lung interstitium. Lavage and lung tissue samples were collected from at least 4 mice per timepoint per group. Harvested samples were processed into single cell suspensions and stained for flow cytometry analysis as described. At least 50,000 events per sample were analyzed using the FlowJo software to quantify different immune cell populations. Data represent total count of each cell population as a fraction of the total number of live cells analyzed. (a) Neutrophil recruitment into the alveolar and lung interstitial spaces (b) Total number of tissue and infiltrating macrophages in the lungs of infected mice. (c, d, e, f) Total number of alveolar and infiltrating macrophages producing TNF- $\alpha$ , IL-10, expressing MR, or expressing iNOS enzyme respectively. (g) The total number of M1 macrophages expressing CCR7 receptor and positive for iNOS and TNF- $\alpha$ . (h) Bar graphs represent ratios of CCR7 to MR expressing monocytes in A; ratio of TNF- $\alpha$  to IL-10 expressing monocytes in B; ratio of IL-17 to IL-10 expressing lymphocytes in C; and the ratio of IFN $\gamma$  to TGF $\beta$  expressing lymphocytes in D. (i) The total number of type 2 innate immune cells recruited into the alveolar and lung interstitial spaces. Data represents mean  $\pm$  SD. Data are representative of 3 independent experiments. Statistical significance determined by two-way ANOVA ((x) BEC treated group significantly different than water treated group; (#) L-norvaline treated group significantly different than water treated group; (+) BEC treated group significantly different than L-norvaline treated group; p-value < 0.05). Graphs plotted using GraphPad Prism 7 (Note: for some points, the error bars are shorter than the height of the symbol. In these cases, Prism simply does not draw the error bars).

Figure 3.7a

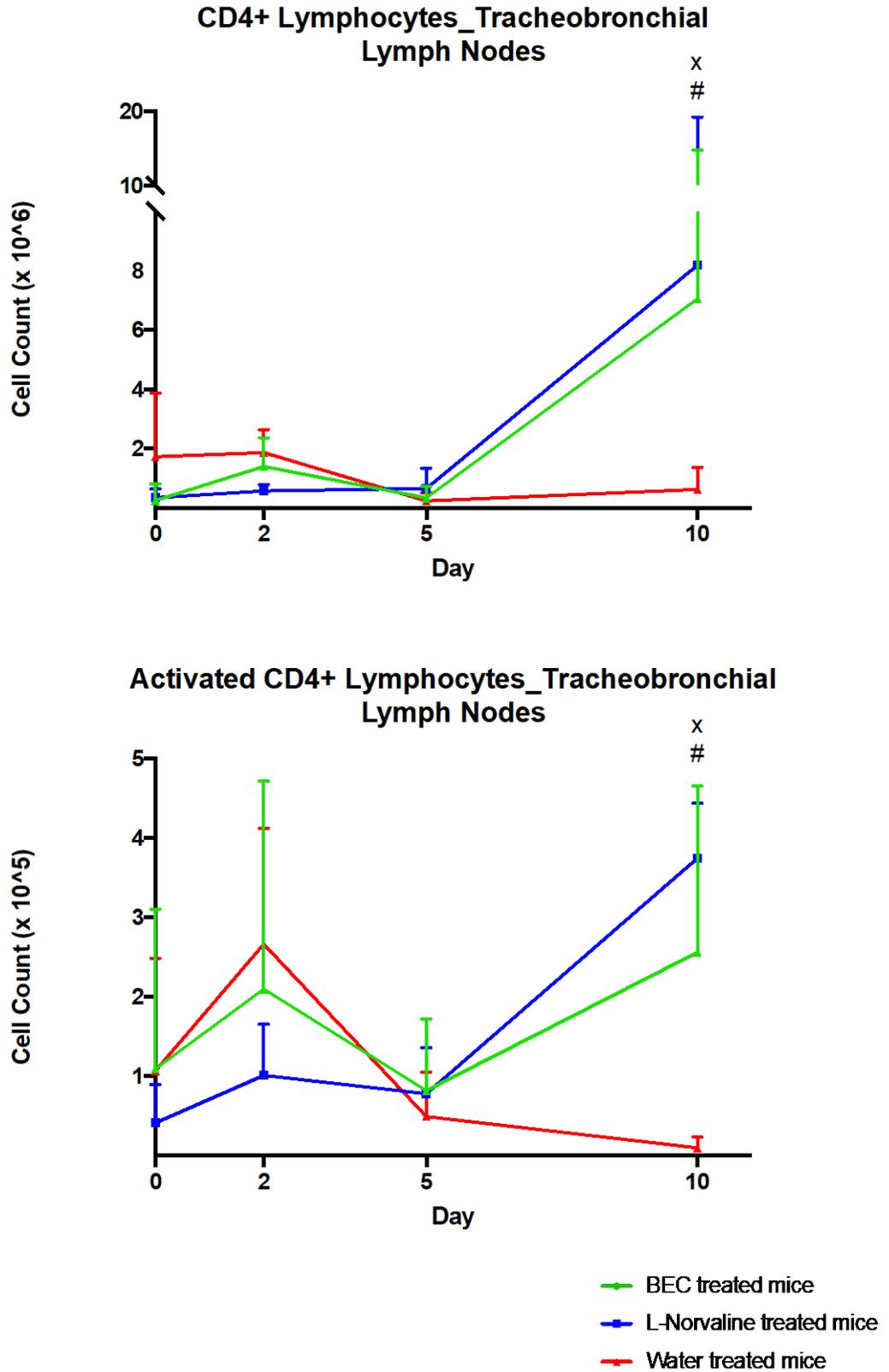
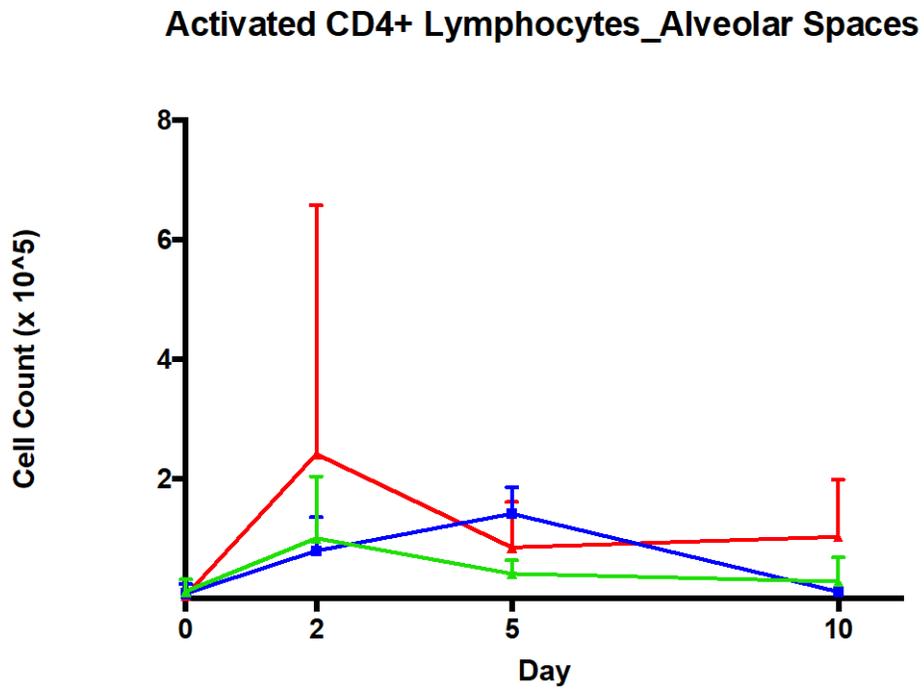
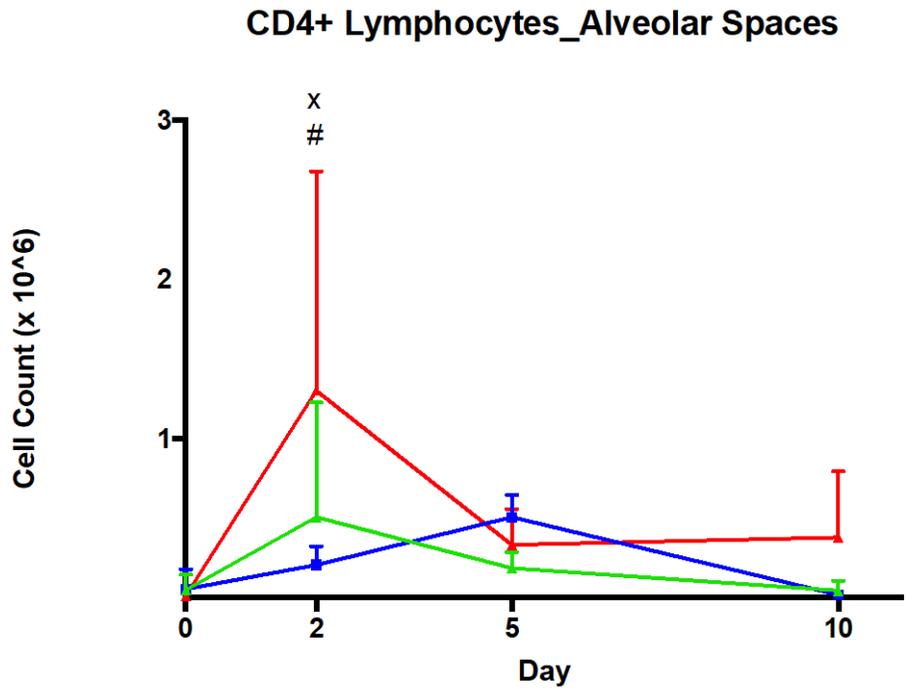


Figure 3.7b



- BEC treated mice
- L-Norvaline treated mice
- Water treated mice

Figure 3.7c

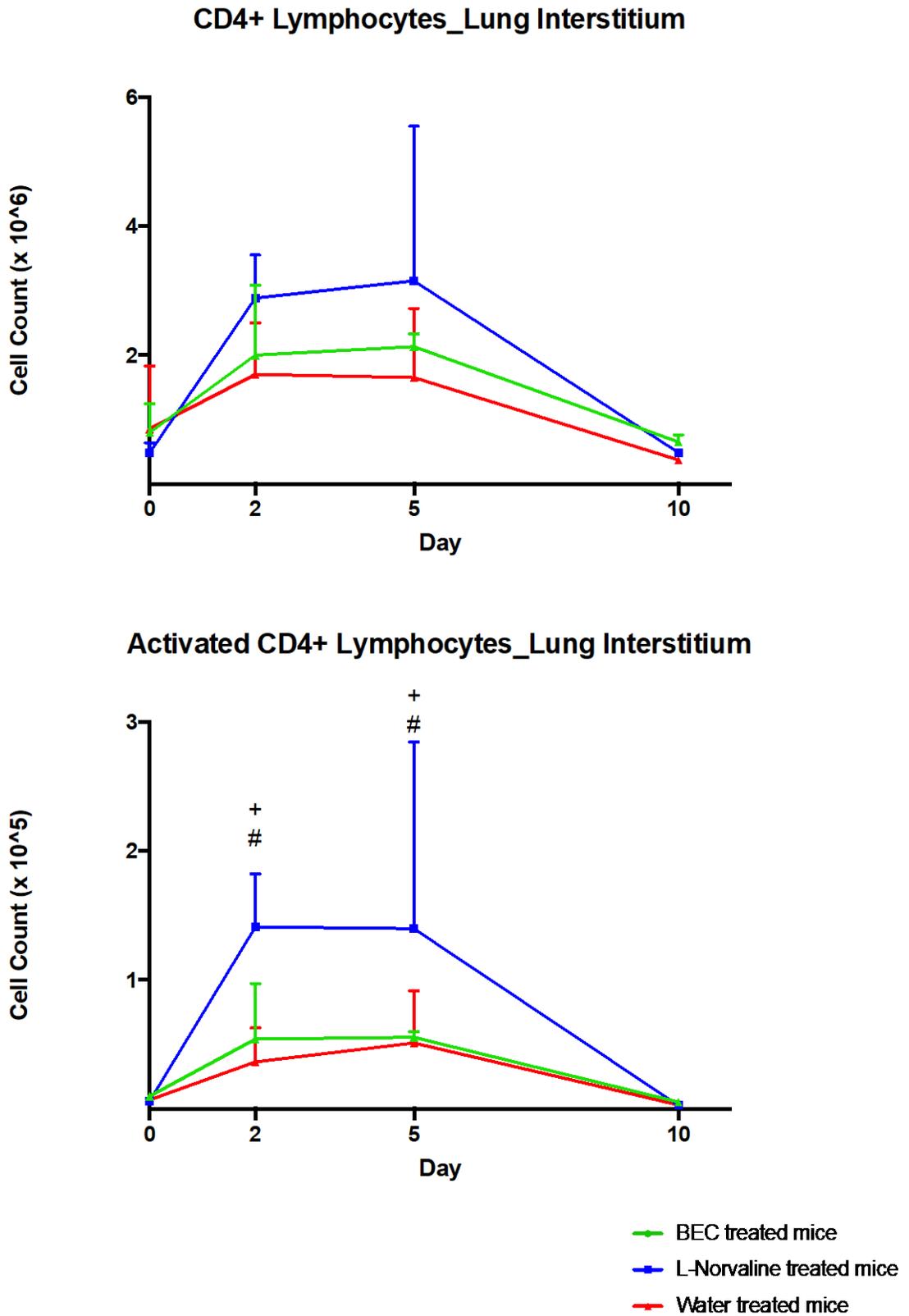


Figure 3.7. Global arginase inhibition results in greater T cell recruitment and activation in the lymph nodes but not the lungs of infected mice.

Tracheobronchial lymph nodes draining the site of infection were harvested and processed into single cell suspensions by passing through mesh strainers. Lung lavage and lung tissue samples were collected and processed as described.

Single cell suspensions were stained for flow cytometry analysis as described. At least 50,000 events per sample were analyzed using the FlowJo software to quantify different immune cell populations. Data represent total count of each cell population as a fraction of the total number of live cells analyzed. (a, b, c) The total number of CD4<sup>+</sup> T cells recruited and activated in the lymph nodes, alveolar spaces, and lung interstitium of infected mice. Data represents mean  $\pm$  SD. Data are representative of 3 independent experiments. Statistical significance determined by two-way ANOVA ((x) BEC treated group significantly different than water treated group; (#) L-norvaline treated group significantly different than water treated group; (+) BEC treated group significantly different than L-norvaline treated group; p-value < 0.05). Graphs plotted using GraphPad Prism 7 (Note: for some points, the error bars are shorter than the height of the symbol. In these cases, Prism simply does not draw the error bars).

Figure 3.8a

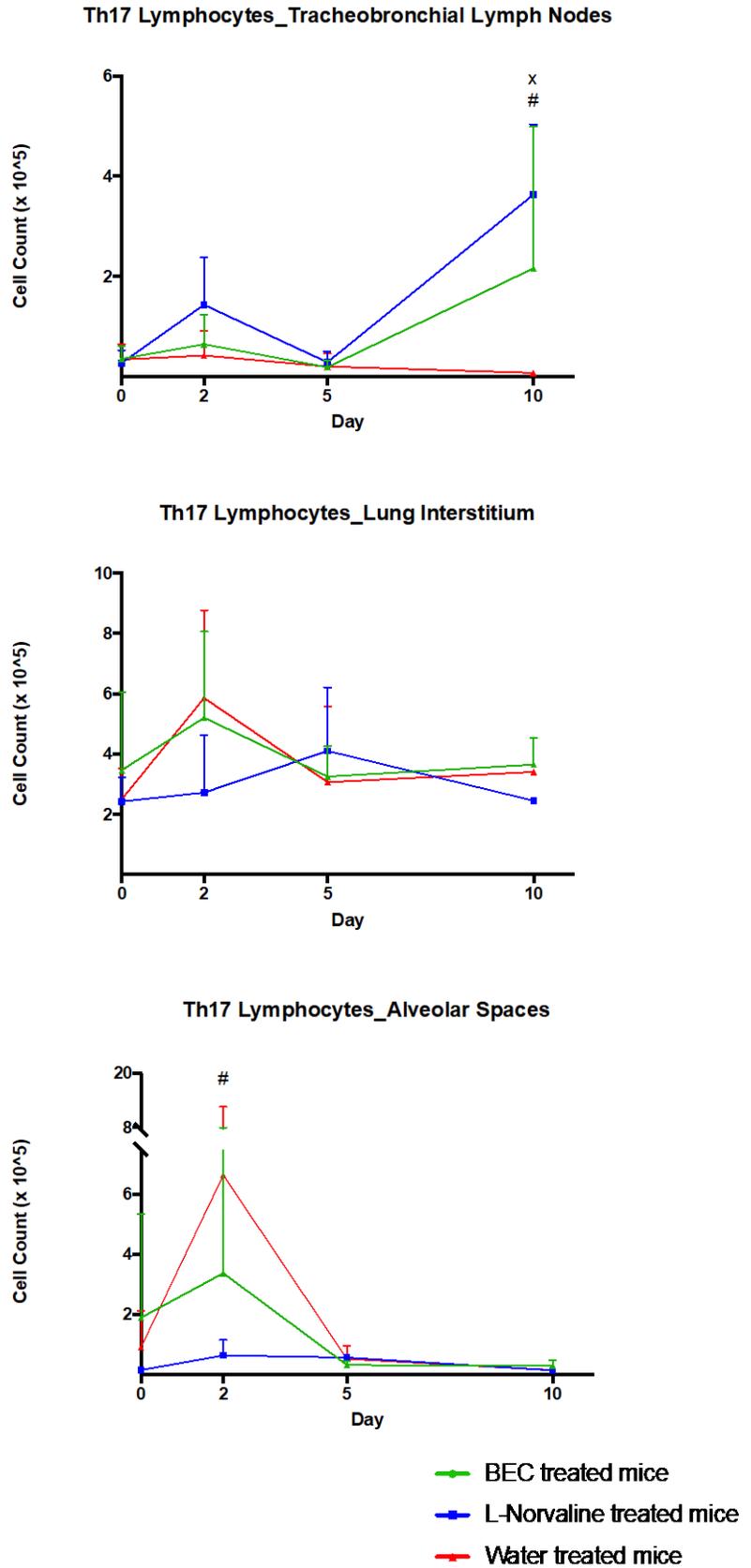


Figure 3.8b

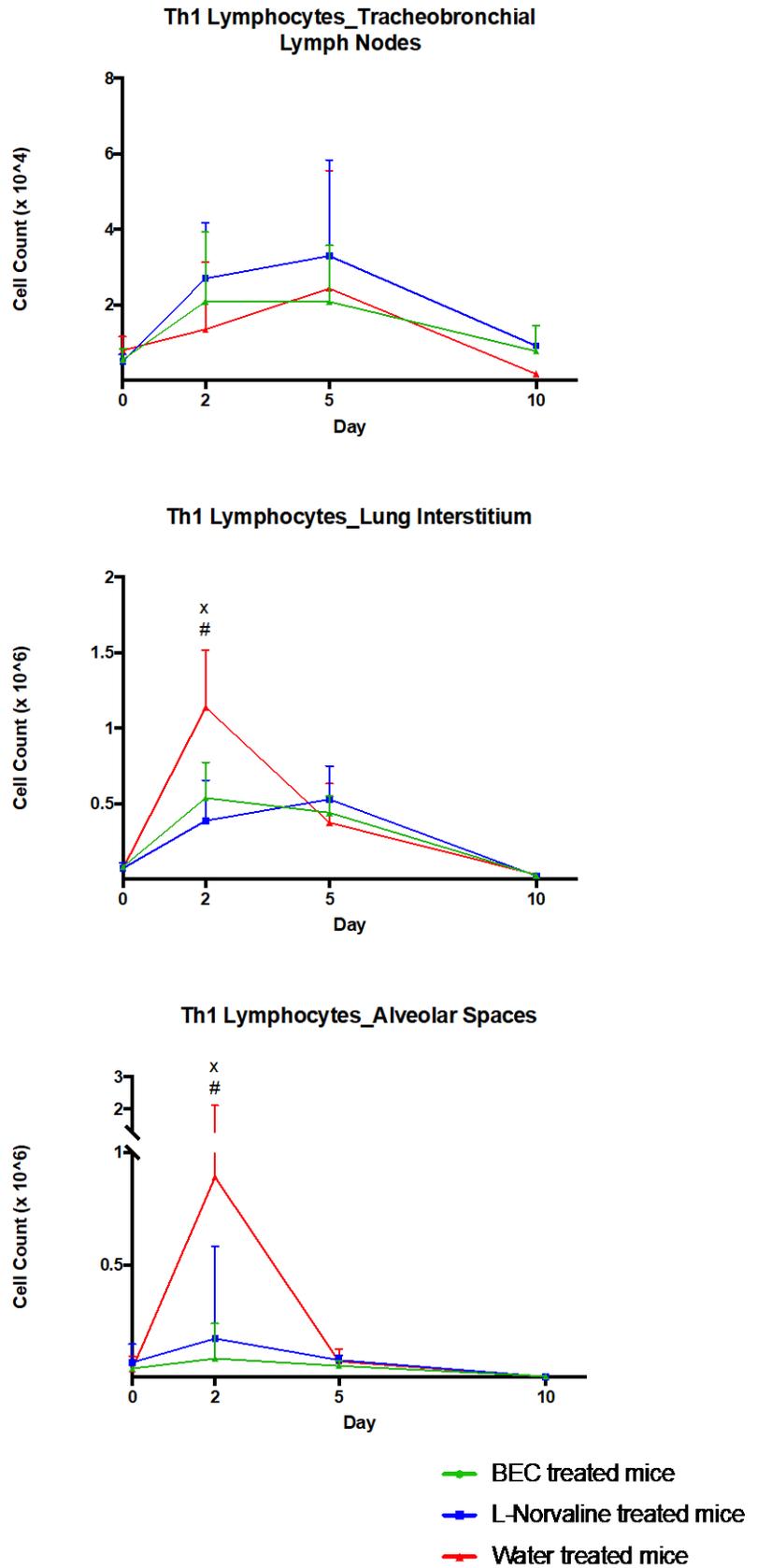


Figure 3.8c

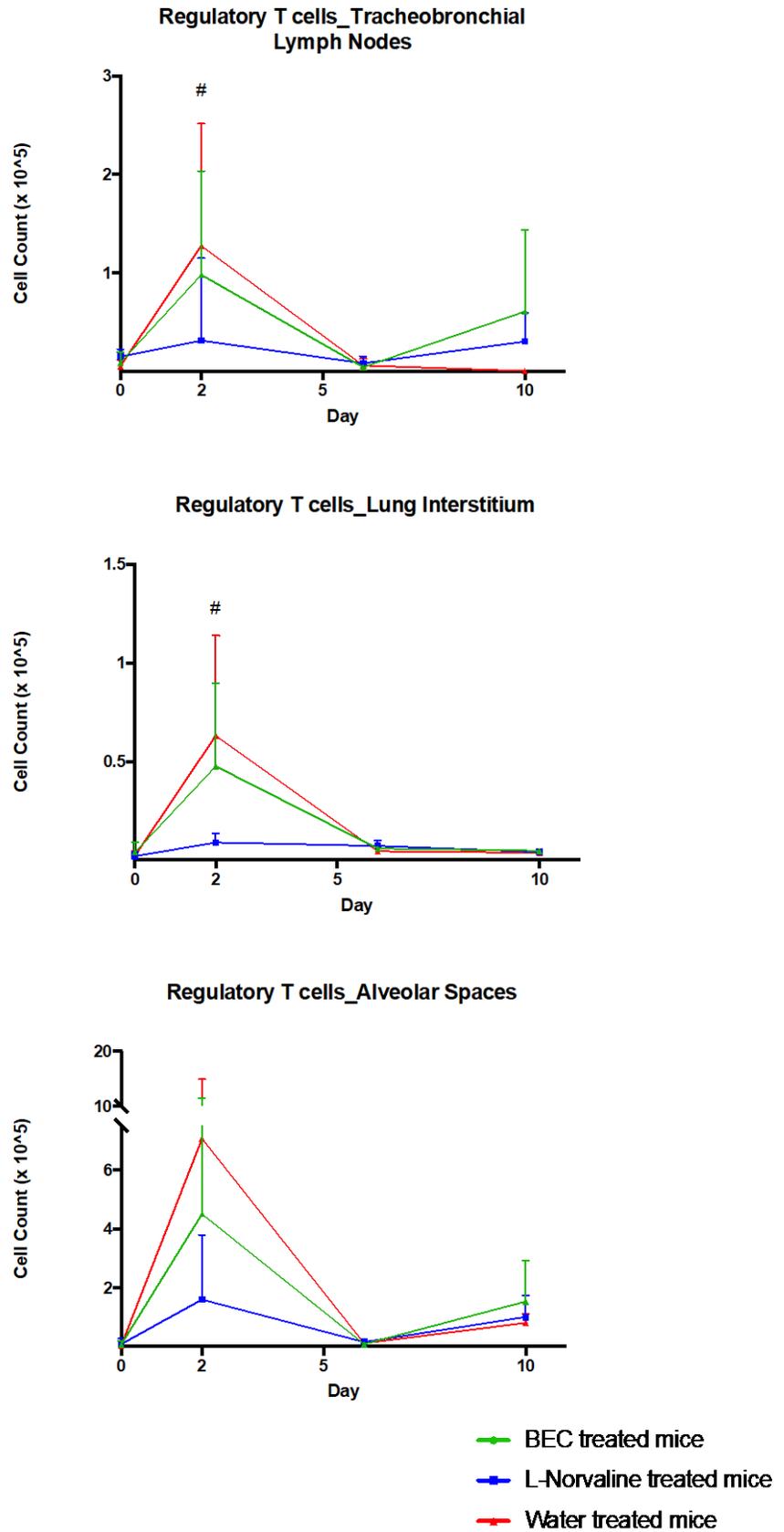


Figure 3.8. Global arginase inhibition is associated with increased activation of Th1, Th17 and regulatory T lymphocytes in the lymph nodes of infected mice with potentially reduced migration into the lungs.

Tracheobronchial lymph nodes draining the site of infection, lung lavage, and lung tissue samples were collected and processed as described. Single cell suspensions were stained for flow cytometry analysis as described. At least 50,000 events per sample were analyzed using the FlowJo software to quantify different immune cell populations. Data represent total count of each cell population as a fraction of the total number of live cells analyzed. (a, b, c) Total number of Th1, Th17, and Treg lymphocytes in the lymph nodes, alveolar spaces, and lung interstitium of infected mice. Data represents mean  $\pm$  SD. Data are representative of 3 independent experiments. Statistical significance determined by two-way ANOVA ((x) BEC treated group significantly different than water treated group; (#) L-norvaline treated group significantly different than water treated group; (+) BEC treated group significantly different than L-norvaline treated group; p-value < 0.05). Graphs plotted using GraphPad Prism 7 (Note: for some points, the error bars are shorter than the height of the symbol. In these cases, Prism simply does not draw the error bars).

## Chapter 4: Azithromycin polarizes macrophages to an M2 phenotype via inhibition of STAT1 through cross-talk from NF- $\kappa$ B signaling mediators

### I. Introduction

While the function of alternatively activated macrophages (M2-polarized) has been primarily evaluated in host responses to disease processes that elicit Th2-type immune mechanisms [143, 376-378], little is known of their function in regulating inflammation in response to extracellular Gram-negative bacterial infections. While primarily functioning to orchestrate remodeling and repair, M2-polarized macrophages produce effector molecules such as arginase-1 and TGF $\beta$  that control lung homeostasis, inflammation, and subsequent pulmonary damage associated with pneumonia [143, 379]. We have demonstrated that the immunomodulatory antimicrobial azalide azithromycin can induce macrophage characteristics that are consistent with an M2 polarization, both in vitro and in a mouse model of *Pseudomonas aeruginosa* infection of the lungs [135, 310, 311]. The subsequent improvement observed in the mortality and severity of lung destruction in this infection model has direct bearing on chronic inflammatory lung conditions such as cystic fibrosis, where *P. aeruginosa* relapsing pneumonias contribute to the decline of pulmonary function over time [380, 381].

Macrophages are polarized to distinct functional phenotypes via signaling from two separate pathways. Classical, or M1 activation requires signaling through both the cytokine IFN $\gamma$  and a pattern recognition receptor, which, in the case of responses to Gram-negative bacterial pathogens, is mainly toll-like receptor 4 (TLR4). IFN $\gamma$ -dependent signaling results in phosphorylation and dimerization of STAT-1, allowing for translocation to the nucleus of the cell. Similarly, IFN $\gamma$  and LPS signal through interferon receptors and TLR4 respectively, inducing a series of phosphorylation steps which result in NF- $\kappa$ B activation and nuclear translocation. NF- $\kappa$ B is the prototypical pro-inflammatory transcription factor activated through TLR and inflammatory cytokine signaling. Stimulation through TLR, IL1R, or other TNF receptor family members results in a series of

phosphorylation reactions activating the canonical pathway kinase I $\kappa$ B kinase (IKK) $\beta$  [382]. Activated IKK $\beta$  phosphorylates and releases the NF- $\kappa$ B inhibitory subunit, I $\kappa$ B- $\alpha$ , which is subsequently ubiquitinated and degraded. This frees the nuclear translocation signal on the NF- $\kappa$ B p65 subunits. After translocating to the nucleus, together with the activated subunit of STAT-1, phospho-p65 (RelA) induces the transcription of many inflammatory mediators and cytokines [383, 384]. Nuclear fractions of RelA are representative of NF- $\kappa$ B activation.

Alternative, or M2 polarization, occurs through the binding of either interleukin (IL)-4 or IL-13 to their respective receptors leading to the phosphorylation and dimerization of STAT-6. Upon activation, STAT-6 translocates to the nucleus of the cell and upregulates the expression of genes associated with anti-inflammatory processes [158, 385-388]. Through our work characterizing the effects of azithromycin, we found that the drug is only able to polarize macrophages to an M2-like phenotype when they are stimulated with LPS [310]. This characteristic provides the basis for our hypothesis that the mechanism of the drug's ability to polarize cells lies in its interference with these signaling cascades. Other groups have shown that azithromycin decreases the activation of NF- $\kappa$ B signaling and subsequent production of pro-inflammatory cytokines and other inflammatory effectors [389, 390]. While these effects may help to explain the beneficial effects of azithromycin in patients with CF, the mechanism by which azithromycin is able to polarize macrophages towards an M2-like phenotype is unknown. Because of work by others that demonstrates a cross-talk between the NF- $\kappa$ B and STAT-1 signaling cascades through IKK $\beta$  [391], we hypothesized that azithromycin polarizes macrophages to an M2 phenotype via inhibition of STAT-1 through cross-talk from NF- $\kappa$ B signaling mediators.

In the present study, we demonstrate that azithromycin inhibits the nuclear translocation of p65. Concurrently, total IKK $\beta$  levels were increased, and through this mediator STAT-1 phosphorylation was directly inhibited. In cultured macrophages, inhibition of IKK $\beta$  resulted in a reversal of M2-macrophage

polarization. These results provide insights into the immunomodulatory mechanism of azithromycin and support studies by other groups that demonstrate a cross-talk between the 2 pathways.

## II. Results

Azithromycin prevents p65 nuclear translocation while increasing IKK $\beta$  levels. We first assessed the effects of azithromycin on the activation of transduction proteins involved in the NF- $\kappa$ B signaling cascade. Cells were incubated overnight with IFN $\gamma$  alone or with azithromycin at concentrations ranging from 5 to 100  $\mu$ M. Cells were then stimulated with LPS for durations ranging from 0 to 60 minutes. Polarized macrophages were fractionated into nuclear and cytoplasmic fractions to assess the effects of azithromycin on the translocation of p65 subunits to the nucleus. (Figure 4.1). Importantly, overnight polarization with IFN $\gamma$  alone prior to LPS stimulation (Figure 4.1a, time 0) induced p65 nuclear translocation to a lower extent with a peak in nuclear p65 fractions at 30 minutes post LPS stimulation (Figure 4.1a, time 30). However, azithromycin treatment in the presence of IFN $\gamma$  completely and significantly prevented p65 translocation to the nucleus at all timepoints. Treatment with azithromycin at all concentrations tested was associated with accumulation of p65 in the cytoplasm, shown for azithromycin concentrations of 5  $\mu$ M and 30  $\mu$ M (Figure 4.1a). This was coupled with decreased p65 fractions in the nucleus of cells treated with azithromycin compared to cells treated with IFN $\gamma$  only, with the ratios of p65 nuclear to cytosolic fractions shown over time in Figure 4.1b.

To confirm the observations above, immunostaining was used to visualize the localization of p65 subunits relative to the nucleus at 30 minutes post LPS stimulation (Figure 4.2). NF- $\kappa$ B p65 subunits were stained with a FITC-conjugated antibody (green). The p65 signal was overlaid with the DAPI stained nuclei (blue) to determine the localization of the subunits in the polarized macrophages (Figure 4.2a). Similar to the observations in Figure 4.1, a strong

nuclear signal was observed in IFN $\gamma$  polarized macrophages while azithromycin treatment was associated with a strong cytoplasmic signal. The nuclear translocation scoring (Figure 4.2b) shows a significant decrease in p65 nuclear translocation with azithromycin treatment at all the concentrations tested compared to IFN $\gamma$  polarized macrophages.

The impact of azithromycin upon the expression of NF- $\kappa$ B associated proteins was then assessed. The amounts of I $\kappa$ B- $\alpha$ , IKK $\beta$ , and pIKK $\beta$  were measured over time after stimulation with LPS as described. For cells incubated with IFN $\gamma$ , LPS caused the expected decrease in I $\kappa$ B- $\alpha$  within 2 minutes, with a steady reemergence over time (Figure 4.3). This is due to activation of this pathway, because when I $\kappa$ B- $\alpha$  is phosphorylated it is rapidly ubiquitinated and degraded. The presence of azithromycin at concentrations as low as 5  $\mu$ M prevented this decrease in I $\kappa$ B- $\alpha$ , as graphically represented in Figure 4.3b. The amount of IKK $\beta$  present in cell lysates was increased at all timepoints even before the addition of LPS in cells polarized with azithromycin and IFN $\gamma$  (Figure 4.3a). The phosphorylated form of IKK $\beta$  was also increased by azithromycin (Figure 4.3a).

This increase in IKK $\beta$  is likely a result of the inhibition of p65 translocation to the nucleus, as transcription of the IKK $\beta$  gene (*Ikkb*) is normally inhibited when p65 is in the nucleus as a feedback mechanism [89, 91, 382, 392-394]. To test this, we compared messenger RNA expression for *Ikkb* via RT-PCR and found that azithromycin treatment caused an increase as compared to the control condition (Figure 4.3c). Significant differences were observed only at higher azithromycin concentrations at 0 and 5 minutes post LPS stimulation while all azithromycin concentrations were associated with significantly higher levels of *Ikkb* expression at 30 minutes after LPS stimulation.

Inhibition of IKK $\beta$  activation prevents azithromycin from polarizing macrophages to the M2 phenotype. Because azithromycin increased the amount of IKK $\beta$  present, we sought to determine whether azithromycin exerts its effect on

macrophage polarization via this mechanism. Previous reports have shown that excessive IKK $\beta$  activation prevents the activation of pro-inflammatory characteristics of macrophages [391]. We treated macrophages with cytokines and azithromycin to polarize them into either M1 or M2 cells, and in addition added IKK-16, an inhibitor of IKK $\beta$  (Figure 4.4). This inhibitor displays a high affinity for IKK $\beta$ , with an IC<sub>50</sub> of 40 nM. At higher concentrations, it can also inhibit the activation of the entire IKK complex [395]. Arginase-1 (Arg1) is an important effector of M2 macrophages and it is a marker of M2 macrophage polarization induced in response to Th2 cytokines [396]. Azithromycin increased Arg1 gene expression in cells incubated with IFN $\gamma$  and exposed to LPS (Figure 4.4a). The addition of the IKK $\beta$  inhibitor significantly negated the effect of azithromycin on Arg1 gene expression. Interestingly, the decrease in Arg1 gene expression was not statistically significant when IKK-16 was added to wells treated with IL4 and IL13 (the M2 control condition) ( $p=0.115$ ). These data suggest that azithromycin's ability to induce expression of Arg1, an important M2 effector, is dependent on its ability to activate IKK $\beta$ .

We next assessed the effect of IKK-16 on arginase protein activity (Figure 4.4b). In this series of experiments cells were treated with 100 nM of IKK-16, a concentration chosen due to its maximal inhibition. Once again azithromycin increased the amount of arginase activity as previously published, but not to the extent of the M2 control condition of IL4 and IL13 treatment. The inhibitor had no effect on arginase activity in cells treated with IL4 and IL13 plus LPS, but for the cells incubated with azithromycin, treatment with IKK-16 decreased arginase activity to similar levels as cells that were not exposed to the drug. When cells were treated with increasing concentrations of IKK-16, the inhibition of azithromycin-induced arginase activity was consistent over a wide range of concentrations, with statistically significant decreases from 12.5 to 200 nM (Figure 4.4c). The inhibition of IKK $\beta$  had a little effect however on IL4 and IL13-dependent arginase production over the broad range of concentrations.

Inhibition of STAT-1 phosphorylation by azithromycin is dependent upon IKK $\beta$ . We then assessed the effect of azithromycin on the phosphorylation of STAT-1. Phosphorylated STAT-1 represents the activated form. It dimerizes and translocates to the nucleus of the cell where it initiates the transcription of several cytokines and inflammatory genes, most of which are associated with M1 macrophage activation [383, 384]. J774 macrophages were polarized under conditions to drive them to an M1 (IFN $\gamma$ ) or an M2 (IL4/13) phenotype. Cells were also polarized using different azithromycin concentrations (5, 30, 60, and 100  $\mu$ M) in the presence of IFN $\gamma$ . After stimulation with LPS we assessed the levels of phospho-STAT-1 and STAT-1 (Figure 4.5a). As shown in Figure 4.5b, IFN $\gamma$  activated STAT-1 leading to an increase in the phosphorylated form whereas IL4 and IL13 blunted this increase in phosphorylation and resulted in suppressed levels of pSTAT-1 as expected. Azithromycin treatment also blunted IFN $\gamma$ -dependent STAT-1 phosphorylation decreasing pSTAT-1 levels in a concentration-dependent manner. These results support our previous observation that azithromycin blunts NF- $\kappa$ B activation and subsequently shifts macrophage polarization away from the M1 phenotype. Importantly, inhibiting IKK $\beta$  via IKK-16 was associated with a reversal of azithromycin's effect on pSTAT-1 levels. The addition of IKK-16 to the azithromycin polarized macrophages prevented the suppression of pSTAT-1 levels and resulted in a restoration of STAT-1 phosphorylation.

### III. Discussion

Macrophages constitute the first line of defense for pathogen infiltration into the lungs through their ability to initiate inflammation. This is accomplished in the case of Gram-negative pathogens primarily through the binding of TLR4 to bacterial LPS [397]. Together with IFN $\gamma$ , this initiates NF- $\kappa$ B signal activation that leads to the transcription of inflammatory genes including cytokines and chemokines. The NF- $\kappa$ B signaling cascade works along with STAT-1 activation to polarize macrophages to a classically activated phenotype to govern this process

[383, 384]. Here we demonstrate that the immunomodulatory mechanism of azithromycin involves elements of both of these pathways, stemming from the inhibition of the translocation of p65 to the nucleus. This leads to an increase in IKK $\beta$  which then in turn blocks the phosphorylation of STAT-1, increasing the expression of M2 effectors.

In the case of PA, influx of neutrophils into infected tissues is the primary mechanism to prevent the pathogen's replication and spread. In chronic inflammatory lung conditions, responses to bacteria like PA induce exaggerated or prolonged neutrophilia leading to pulmonary damage and fibrosis caused by excessive neutrophil elastase concentrations, an imbalance in the protease-antiprotease ratio, and a vicious cycle of excessive inflammation that leads to scarring [186, 312, 398-400]. While many groups have demonstrated that azithromycin reduces NF- $\kappa$ B activation [390, 401-404], we showed that azithromycin also polarizes macrophages to an M2-like phenotype, both in vitro and in vivo during PA infection [310, 311]. Subsequently, we demonstrated in a mouse model of PA infection that polarizing the macrophage response to one in which the M2 phenotype predominates early in the inflammatory process reduces neutrophil influx, decreases inflammation, and reduces fibrotic changes that correlate to improved morbidity and survival [311]. This includes decreased production of iNOS and an increased production of the M2 effectors, mannose receptor and arginase-1. Utilizing the anti-inflammatory effects of azithromycin, it appears that by polarizing the macrophage response early in the infection, lung damage is controlled, and importantly the clearance kinetics of the pathogen are not altered [311]. The efficacy of azithromycin in this setting is also reflected in clinical practice, as this agent is used as a chronic therapy for patients with cystic fibrosis. Quality of life is improved according to multiple clinical trials that utilized azithromycin chronically. Additionally, extended treatment with azithromycin slowed the pulmonary function decline in patients with CF who are colonized with PA [299-301]. We have observed in our mouse model that the clearance of PA is not altered, and likewise no changes in the incidence of bacterial infection have

been observed in these studies that place subjects on azithromycin long-term [299-301].

Future studies will investigate whether the modulation of macrophage phenotype with azithromycin via cross-inhibition of the NF- $\kappa$ B and STAT-1 signaling pathways is beneficial in patients with cystic fibrosis. Several groups have studied the impact of macrolides including azithromycin, clarithromycin, and erythromycin on ERK1/2 and p35 MAPK signaling cascades which result in decreased downstream NF- $\kappa$ B and AP-1 signaling. Azithromycin was shown in vivo and in vitro, both in human and murine cell culture models, to decrease NF- $\kappa$ B activation, decrease its nuclear translocation, and decrease NF- $\kappa$ B and Sp1 transcription factor binding to DNA [390, 402-405]. These effects are associated with a significant reduction in inflammatory cell infiltration into infected lungs, and a profound decrease in pro-inflammatory cytokine concentrations in the alveolar space. Groups studying the anti-inflammatory mechanisms of azithromycin show decreases in NF- $\kappa$ B binding to DNA along with suppressed induction of pro-inflammatory genes and cytokine production in different murine and in vitro models of various inflammatory and infectious diseases [389, 390, 401-406]. We expanded these studies to address the specific effects on the upstream mediators of the NF- $\kappa$ B signaling cascade and their cross-talk with the other inflammatory signaling pathways.

Previous studies demonstrate that the nuclear translocation of phospho-p65 is inhibited by azithromycin [403, 404]. Additionally, Vrancic et al. showed no overall impact of azithromycin on the phosphorylation of p65—this is also consistent with our findings when factoring in the increase in phospho-p65 in the cytoplasmic fraction, as this group did not fractionate the nuclear compartment [402]. They also demonstrated that azithromycin inhibits the phosphorylation of STAT-1. We extend these studies here in our model to prove a direct relationship between increased IKK $\beta$  protein concentrations and this effect on STAT-1 through experimentally blocking IKK $\beta$  through competitive inhibition.

Evidence from the work by Fong and colleagues alludes to a potential link between the NF- $\kappa$ B and STAT-1 signaling pathways. This group demonstrated that the NF- $\kappa$ B signaling molecule IKK $\beta$  can inhibit STAT-1 signaling in macrophages in a model of group B streptococcus (GBS) infection [391]. While deletion of IKK $\beta$  in airway epithelial cells led to a decrease in inflammation, macrophage-specific deletion of IKK $\beta$  caused a resistance to GBS infection that was associated with increased expression of M1-associated inflammatory molecules including IL-12, iNOS, and MHCII [391]. Additionally, in macrophages infected with GBS and in macrophages incubated with IFN $\gamma$ , the absence of IKK $\beta$  led to an increase in phosphorylation of STAT-1 [391]. These findings, along with our data, suggest that the increase in IKK $\beta$  expression in macrophages by azithromycin may be the mechanism through which polarization to the M2 phenotype occurs.

There are other examples of small molecules that inhibit the translocation of NF- $\kappa$ B including aspirin, non-steroidal anti-inflammatory drugs, and 1,8-Cineol [407-410]. The nuclear binding of p65 normally provides a feedback signal to shut down the production of IKK $\beta$  [91, 411-417]. Therefore, it is likely that the inhibition of p65 translocation is causing the increase in IKK $\beta$  production. Additionally, IKK $\beta$  degradation occurs through a mechanism of autophosphorylation. Because p65 concentrations are high in the cytoplasm, this autophosphorylation process is likely decreased, which turns the degradation pathway off [411, 416-423]. We have shown by PCR that message expression for *Ikbkb* is increased, therefore a reversal of the feedback loop is at least partly responsible. However, message expression is not increased until 30 minutes after LPS stimulation at lower azithromycin concentrations, whereas the IKK $\beta$  protein expression is increased earlier, even at time zero, suggesting other potential mechanisms.

Despite the evidence concerning macrophage polarization, the primary immunomodulatory mechanism of azithromycin remains to be discovered. Because increases in IKK $\beta$  expression and phosphorylation should lead to increased NF- $\kappa$ B activation, it is highly likely that the effect of azithromycin is below this step in the pathway, involving either I $\kappa$ B itself or other mechanisms that regulate nuclear translocation. Clearly this process is highly dependent upon the degradation of I $\kappa$ B [411, 413, 416, 424]. Our data shows that with azithromycin treatment, degradation of I $\kappa$ B- $\alpha$  is inhibited, leading to decreases in p65 nuclear translocation. I $\kappa$ B- $\alpha$  concentration is decreased upon stimulation with LPS for 30 minutes, and then rebounds to baseline concentrations (Figure 4.3), with p65 translocation peaking at the corresponding 30-minute timepoint (Figure 4.1)—all of which is blocked by azithromycin. However, nuclear translocation of p65 also depends upon a number of other factors. Alteration of the nuclear location sequence of p65 can occur through a number of mechanisms, including changes in the dimerization or improper folding, which are both required. The function of importins and other nuclear shuttling machinery [425-427], and permeability characteristics of the nuclear membrane can be disrupted [428-433]. Additionally, acetylation of the activated subunit in the nucleus at multiple lysine residues is required for translocation and is governed by histone regulation and coactivators such as CREB-binding protein [434, 435, 436 1994, 437]. We are continuing to evaluate the impact of azithromycin on these mechanisms.

In conclusion, the immunomodulation of macrophage function by azithromycin is a complex effect associated with the alteration of STAT1 signaling through the inhibition of NF- $\kappa$ B mediators, linked through cross-talk via IKK $\beta$ . Macrolides have been demonstrated to have an effect through the polarization of macrophages to a regulatory phenotype in several models of inflammation. Studies have been extended to investigations of spinal cord injury, stroke, and other cerebrovascular events. An improved understanding of the mechanisms associated with these agents could lead to promising therapeutic target discovery in these and other devastating disease processes. And, defining interactions

between signaling pathways will lead to a better understanding of cellular biology and provide the impetus for future drug discovery.

Figure 4.1a

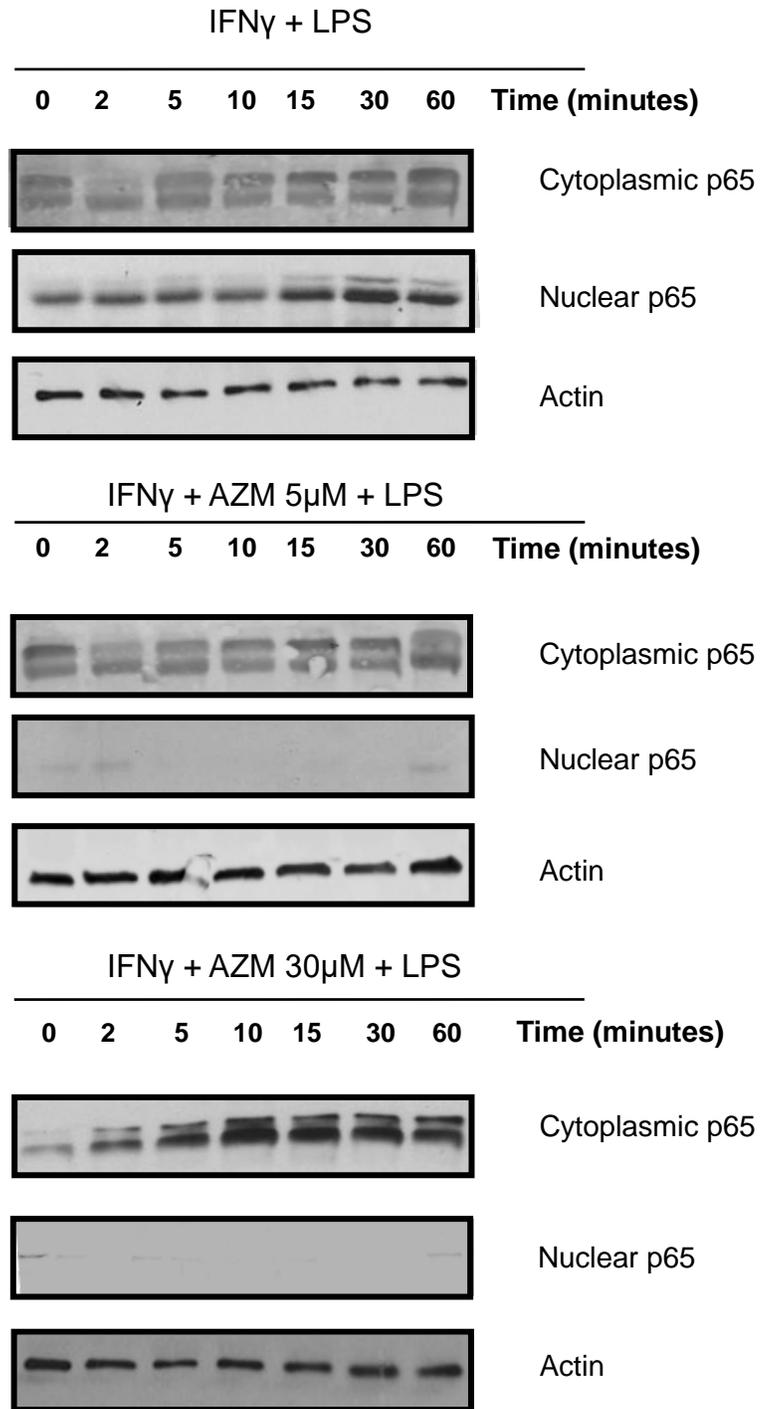


Figure 4.1b

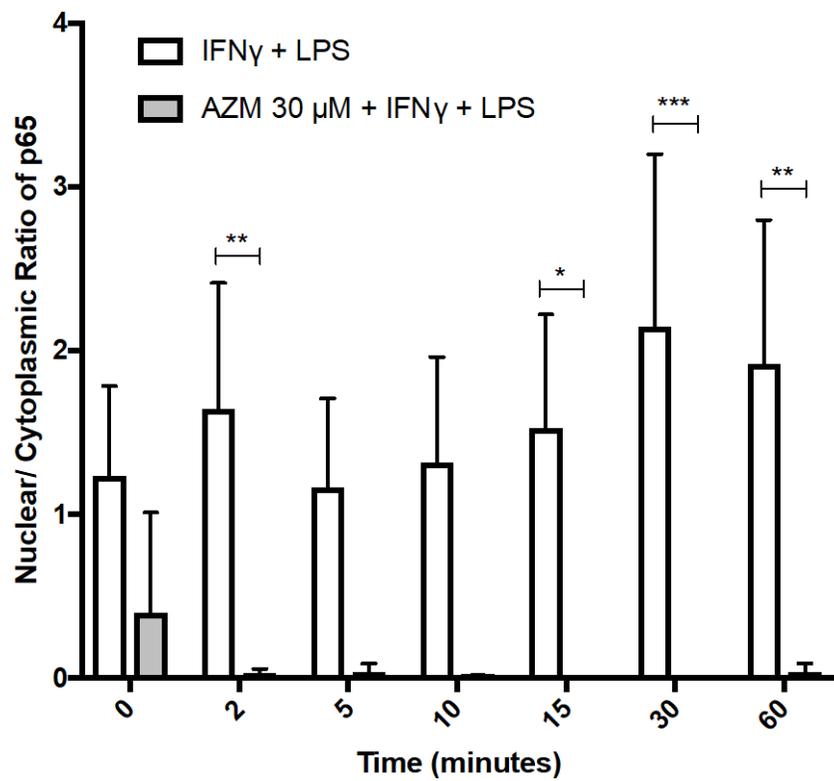
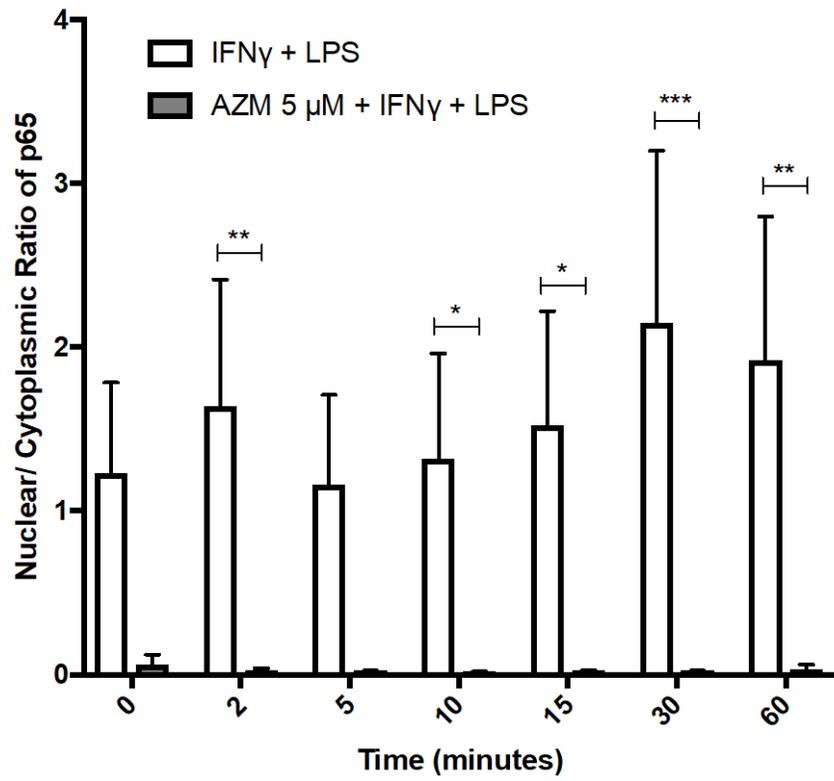


Figure 4.1. Azithromycin decreases NF- $\kappa$ B activation and prevents p65 nuclear translocation.

J774 cells were plated at  $2.5 \times 10^5$  cells per 1 ml of DMEM in 24 well plates. Cells were allowed to attach for 8 hours and then polarized overnight with INF $\gamma$  (50U/ml) alone or with azithromycin (5, 10, 15, 30, 60, and 100  $\mu$ M). Cells were then stimulated with LPS (10 nM) for 0, 2, 5, 10, 15, 30, and 60 minutes. (a) Cells were harvested by scrapping and NF- $\kappa$ B Assay Kit (FivePhoton Biochemicals, San Diego, CA) was used to collect nuclear and cytoplasmic fractions according to the manufacturer protocol. Western blots for p65 in nuclear and cytoplasmic fractions were performed. (b) Bar graph represents the ratio of nuclear to cytoplasmic fractions of p65 from (a). Data represents mean  $\pm$  SD. Data is representative of 3 independent experiments. Statistical significance determined by two-way ANOVA with Sidak's multiple comparisons test (p-value < 0.05 (\*); p-value < 0.005 (\*\*); p-value < 0.0005 (\*\*\*)). Bar graphs plotted using GraphPad Prism 7. Western Blots quantified using ImageJ.

Figure 4.2a

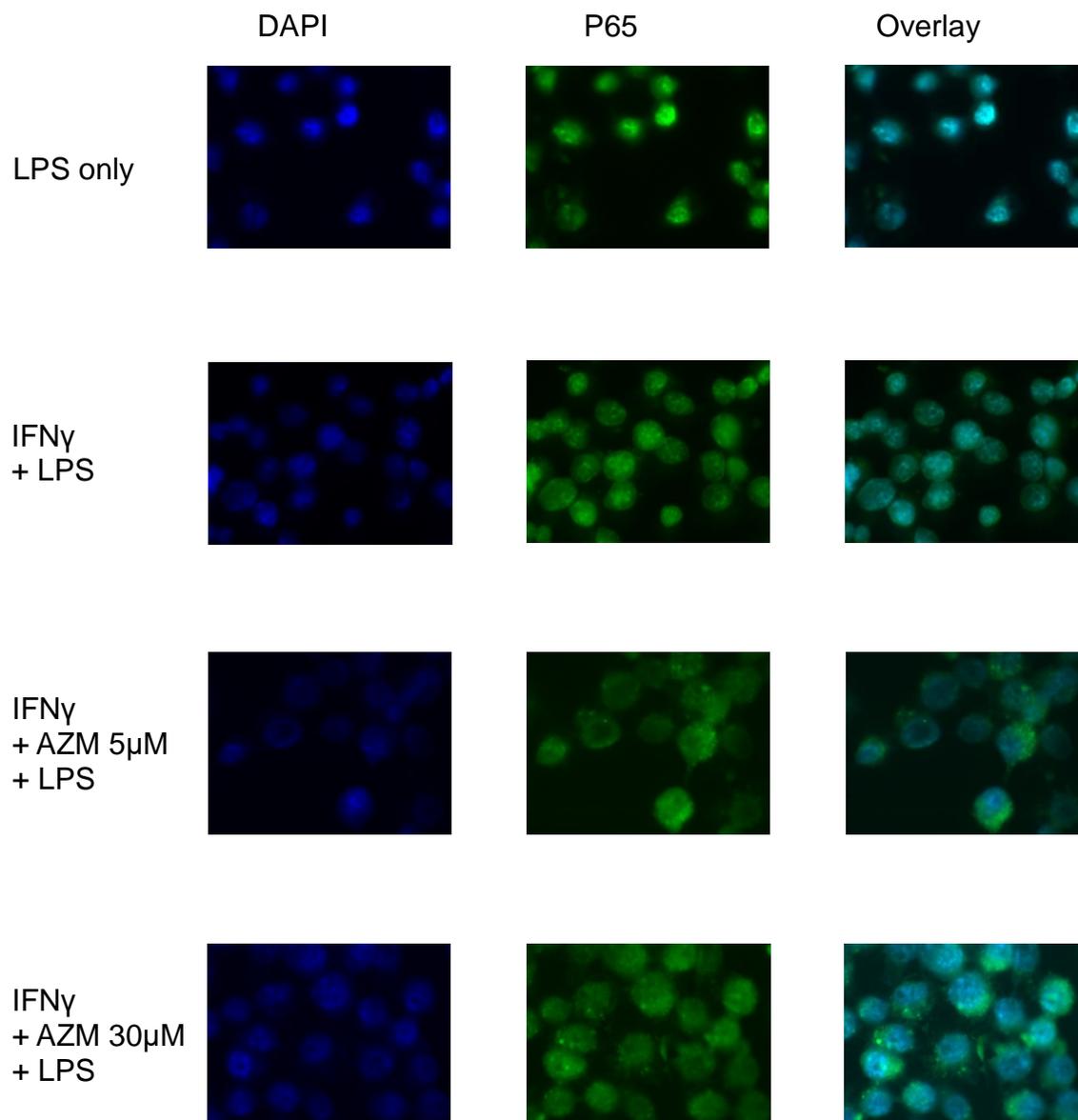


Figure 4.2b

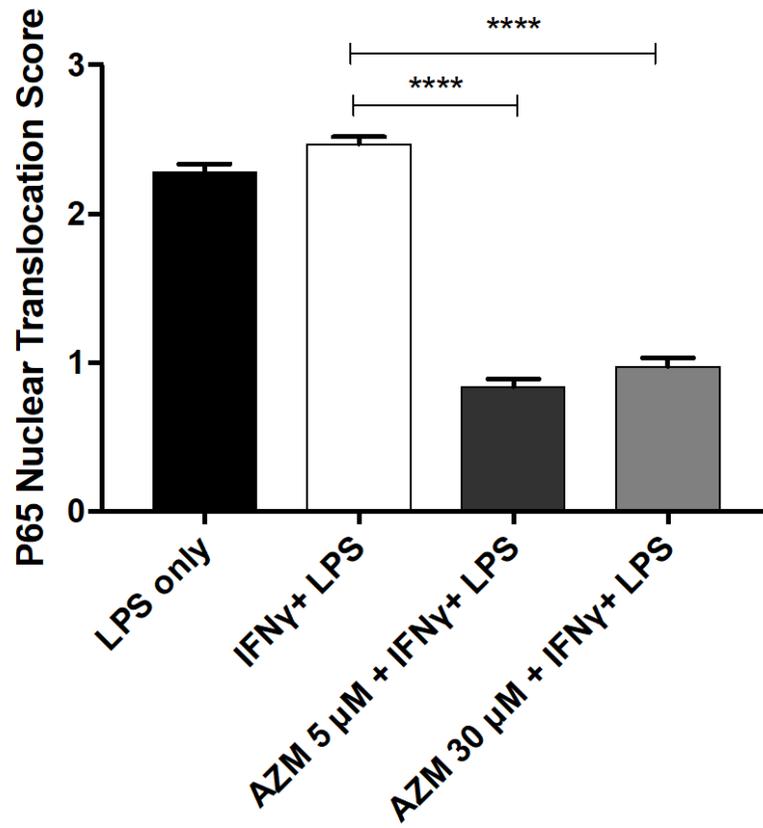


Figure 4.2. NF- $\kappa$ B p65 subunit accumulates in the cytoplasm around the nuclear membrane in azithromycin treated macrophages.

J774 murine macrophages were plated at  $2.5 \times 10^5$  cells on round glass coverslips. Cells were allowed to attach to the glass and then polarized overnight with INF $\gamma$  (50 U/mL) alone or with azithromycin (5, 10, 15, 30, 60, and 100  $\mu$ M). Additionally, some azithromycin polarized macrophages were treated with IKK-16. Cells were then stimulated with LPS (10 nM) for 0, 2, 5, 10, 15, 30, and 60 minutes. (a) Immunostaining for p65 subunit was performed on the glass coverslips in a wet chamber. Images were taken using Zeiss fluorescent microscope at 100X oil objective. Images show NF- $\kappa$ B p65 subunits stained in green (FITC) overlaid with DAPI nuclear staining. (b) Bar graphs represent the nuclear vs cytoplasmic fractions of p65 quantified using the scoring system in Table 2.5. Data represents mean  $\pm$  SD. Data is representative of 3 independent experiments. Statistical significance determined by two-way ANOVA (p-value < 0.05 (\*); p-value < 0.0005 (\*\*); p-value < 0.0001 (\*\*\*\*)). Bar graphs plotted using GraphPad Prism 7.

Figure 4.3a

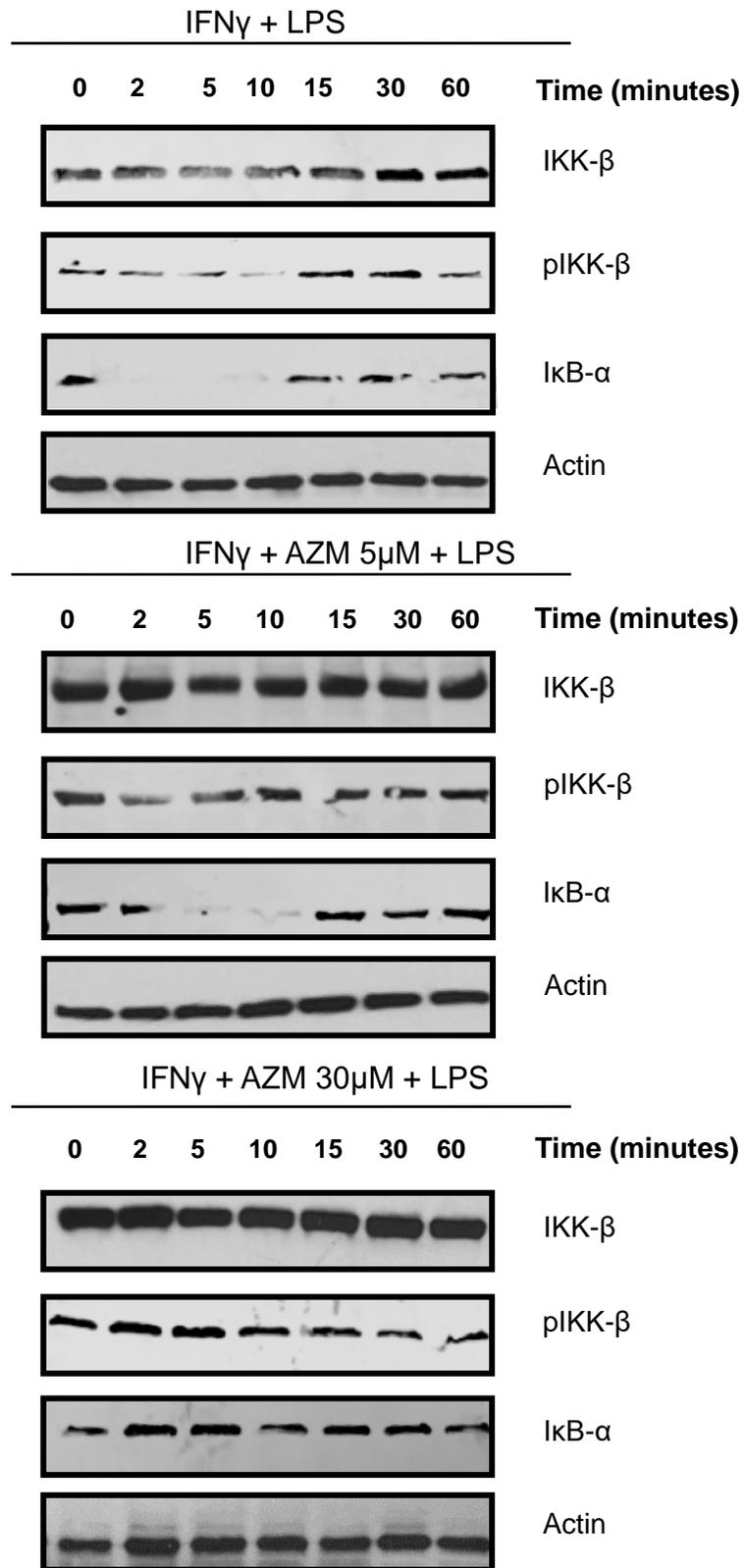


Figure 4.3b

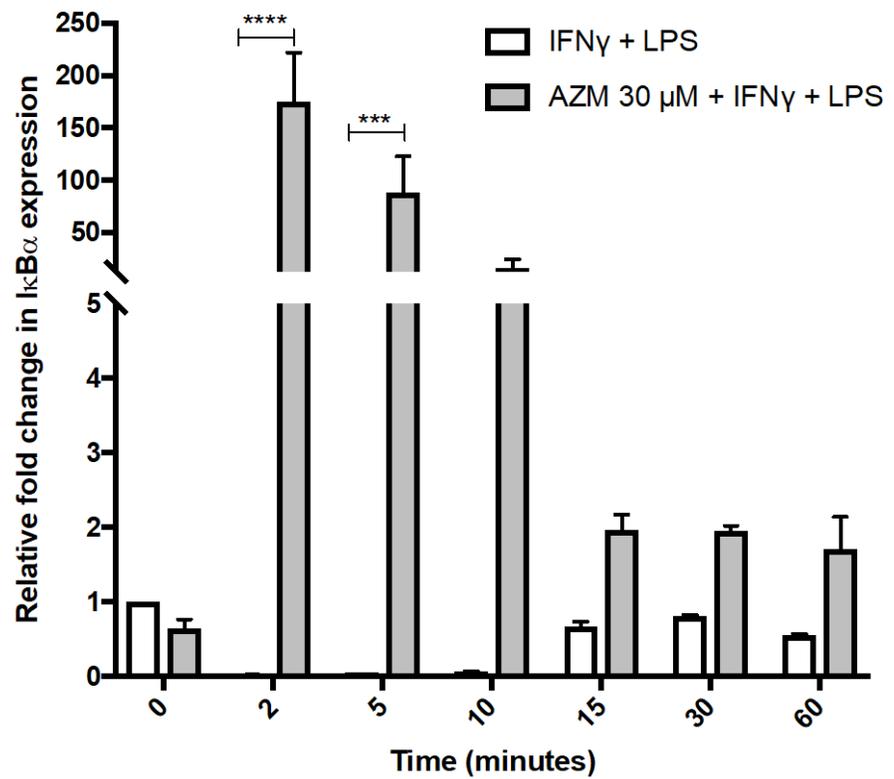
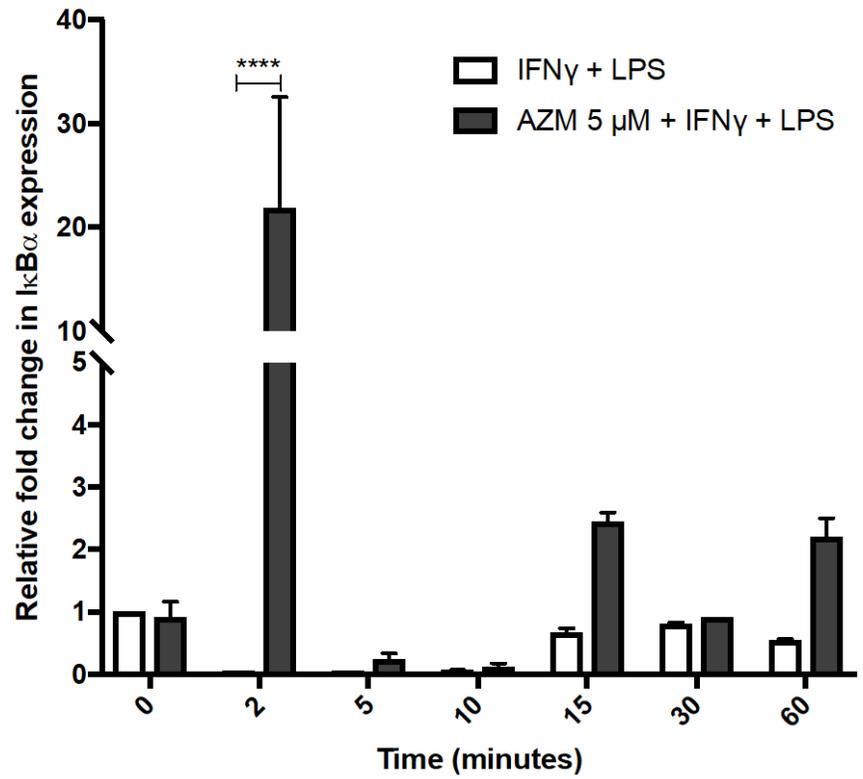


Figure 4.3c

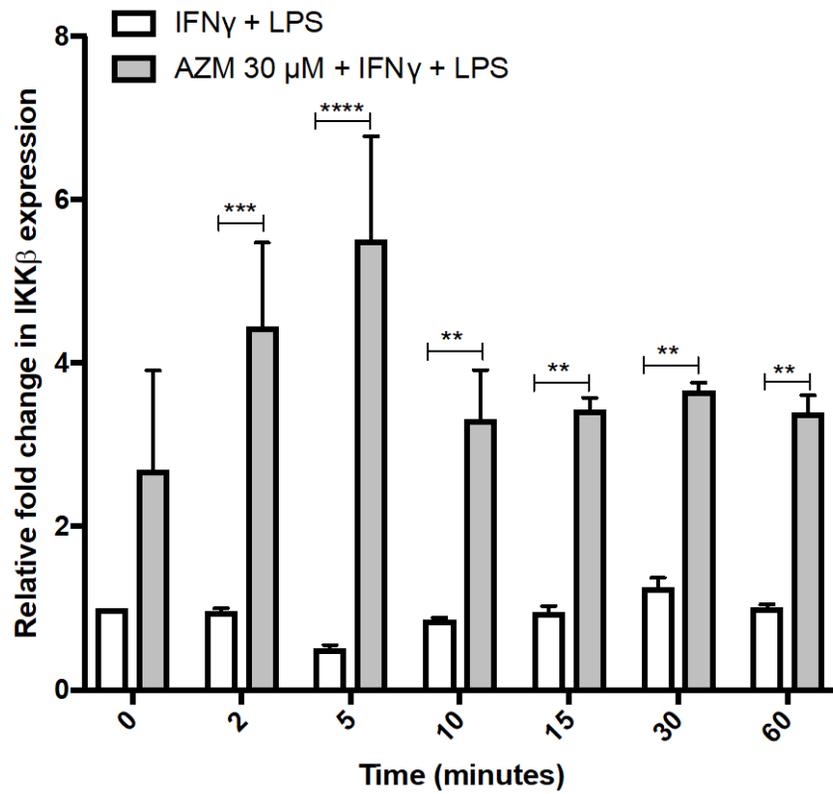
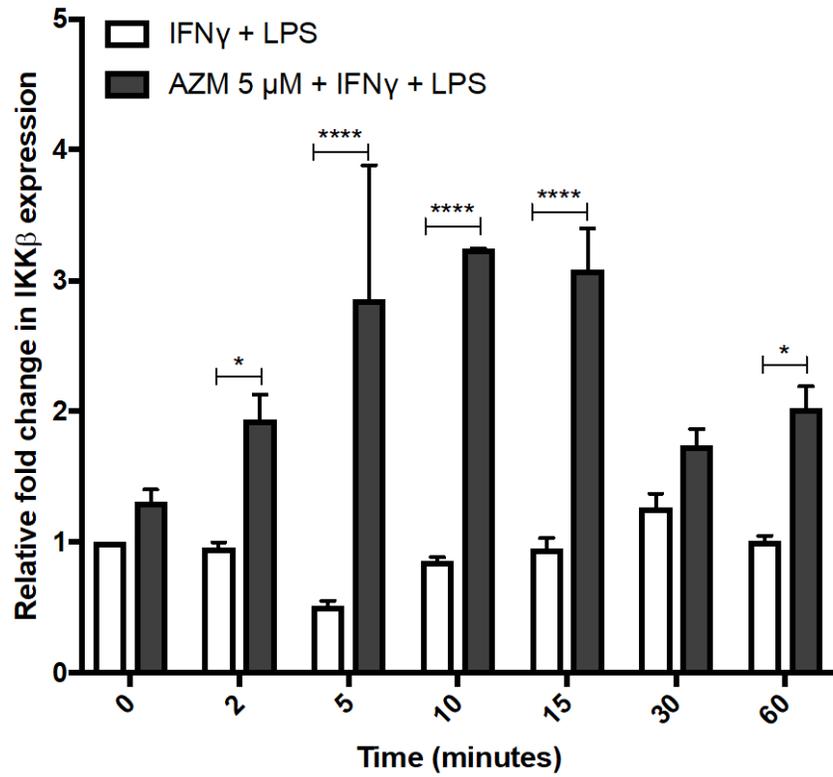


Figure 4.3d

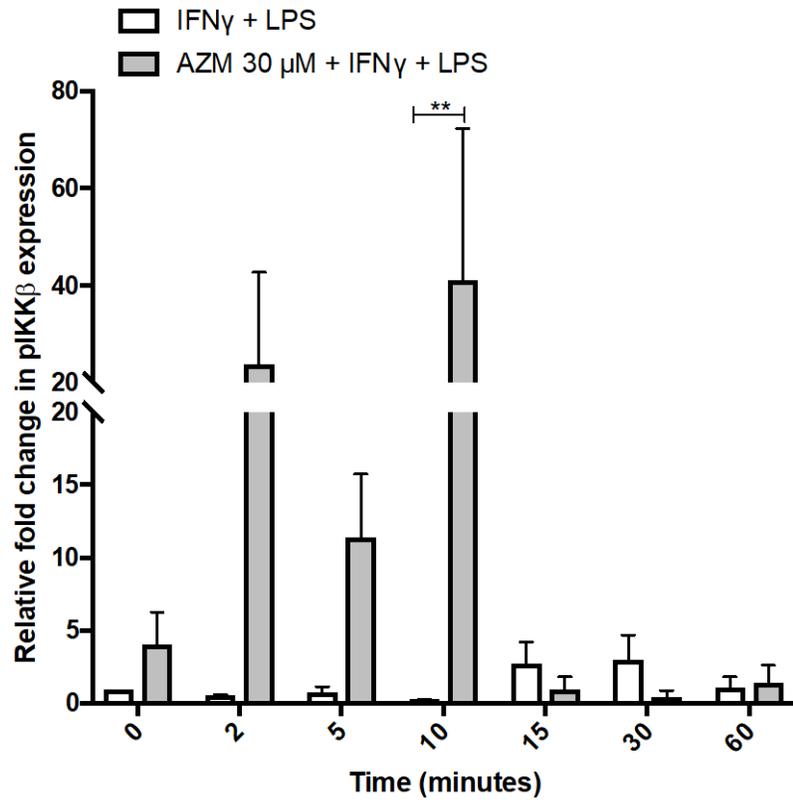
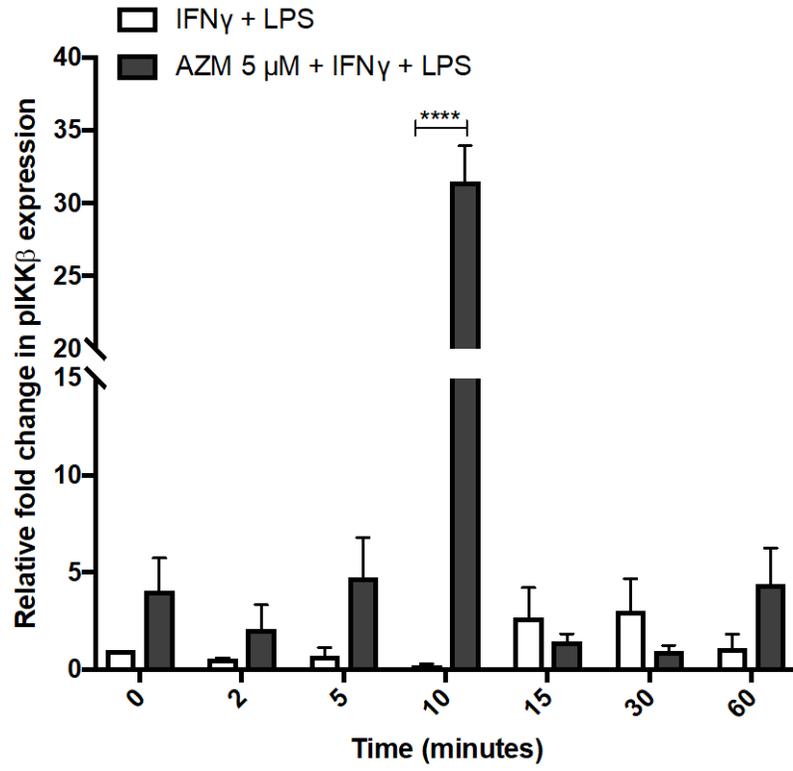


Figure 4.3e

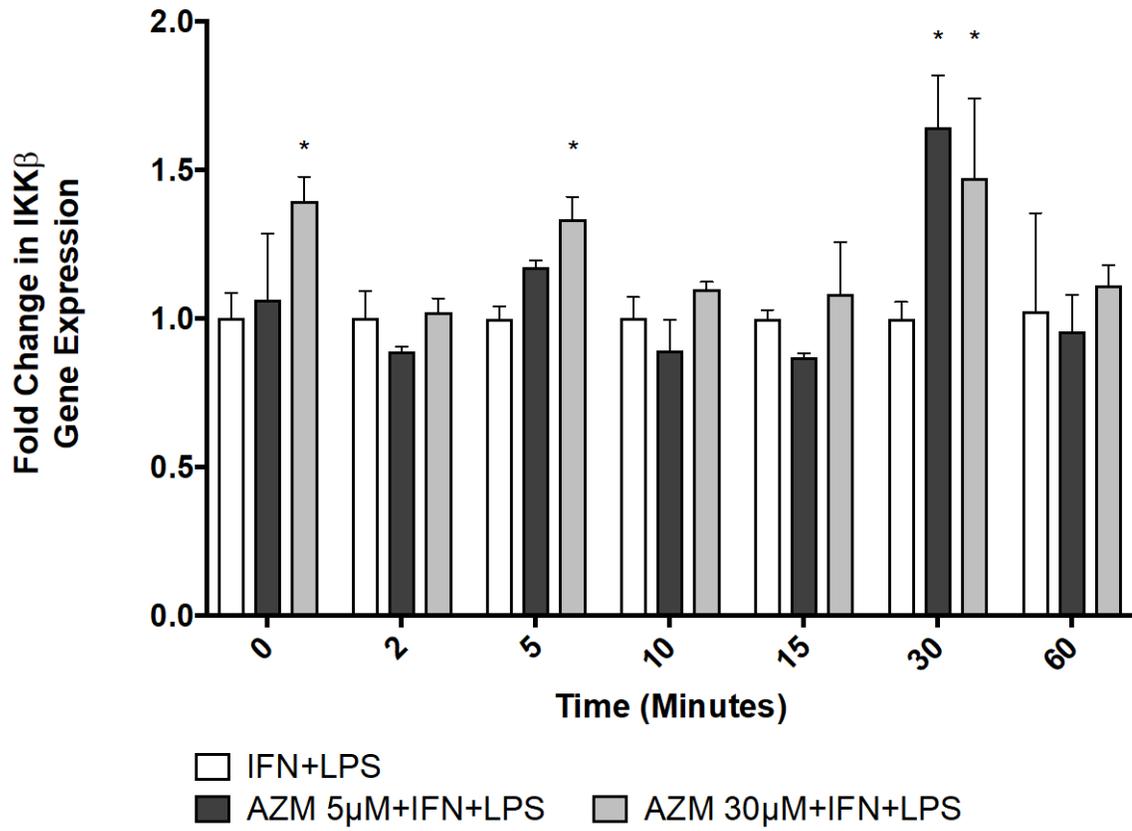


Figure 4.3. Azithromycin prevents I $\kappa$ B- $\alpha$  degradation while accumulating IKK $\beta$ . J774 cells were plated at  $2.5 \times 10^5$  cells per 1 ml of DMEM in 24 well plates. Cells were allowed to attach for 8 hours and then polarized overnight with INF $\gamma$  (50 U/mL) alone or with azithromycin (5, 10, 15, 30, 60, and 100  $\mu$ M). Cells were then stimulated with LPS (10 nM) for 0, 2, 5, 10, 15, 30, and 60 minutes. Proteins and RNA were then collected and probed for mediators in the NF- $\kappa$ B signaling cascade using western blot and RT-PCR. (a) Western blots for I $\kappa$ B- $\alpha$ , IKK $\beta$ , and p-IKK $\beta$ . (b) Bar graphs represent the relative fold change in protein concentration over time in INF $\gamma$  treated macrophages versus azithromycin and INF $\gamma$  treated macrophages. (c) Bar graphs represent fold change in IKK $\beta$  gene expression calculated from the  $\Delta\Delta$ Ct values and normalized to GAPDH and compared to INF $\gamma$  treated macrophages over time. Data represents mean  $\pm$  SD. Data is representative of 3 independent experiments. Statistical significance determined by two-way ANOVA with Sidak's multiple comparisons test (p-value < 0.05 (\*); p-value < 0.0005 (\*\*\*) ; p-value < 0.0001 (\*\*\*\*)). Bar graphs plotted using GraphPad Prism 7. Western Blots quantified using ImageJ.

Figure 4.4a

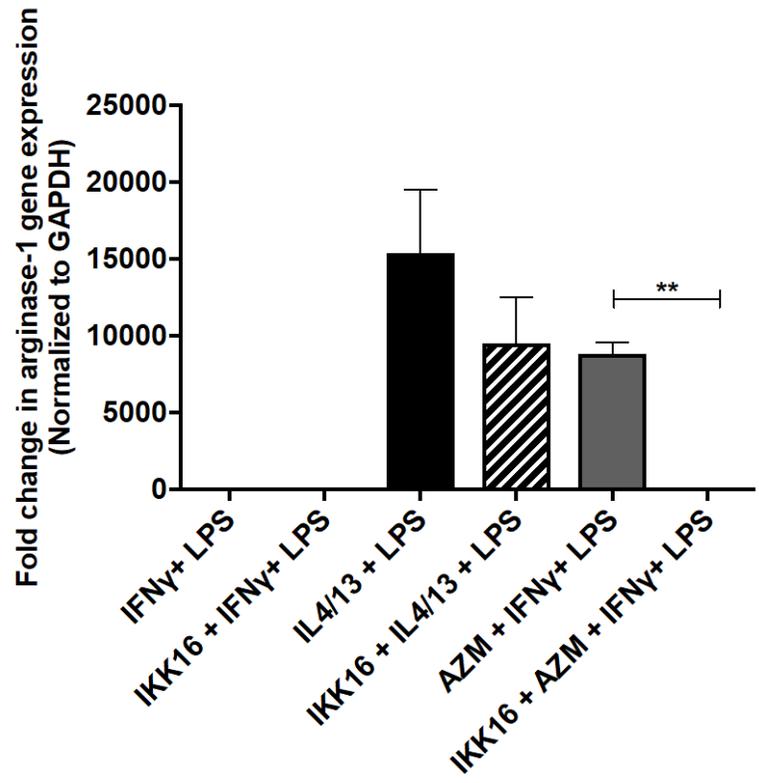


Figure 4.4b

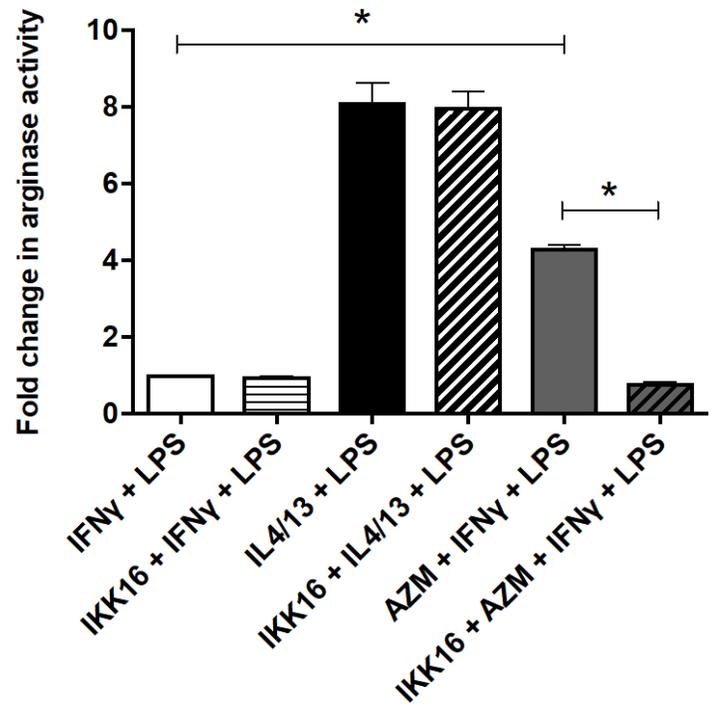


Figure 4.4c

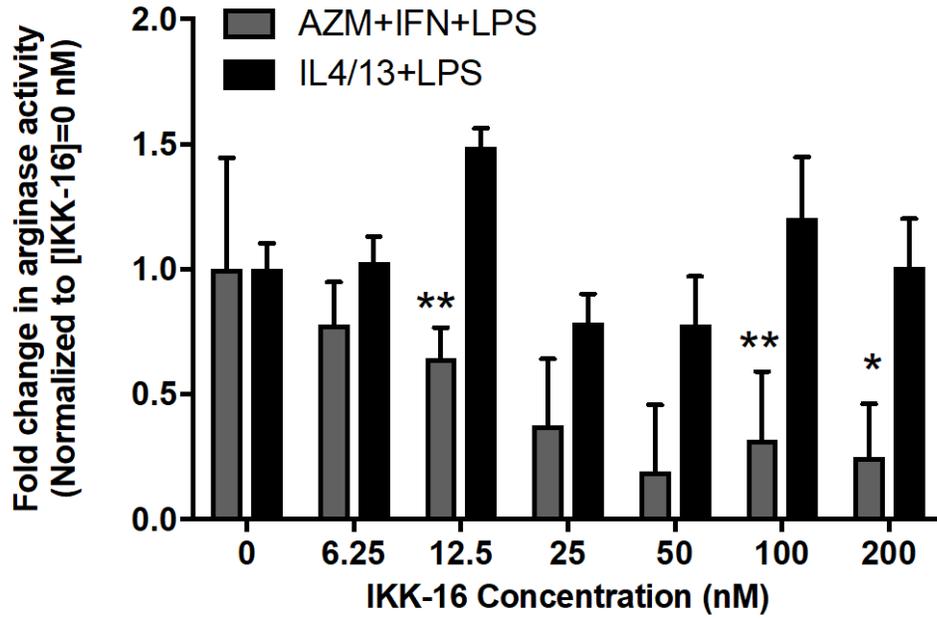


Figure 4.4. Azithromycin induced arginase gene expression and activity are reversed with IKK $\beta$  inhibition.

J774 cells were stimulated overnight with IFN $\gamma$ , IL4 and IL13, or with azithromycin (concentration 10  $\mu$ M shown here) and IFN $\gamma$  in the presence or absence of the IKK $\beta$  inhibitor, IKK-16. Cells were then stimulated with LPS for 24 hours. (a) Arginase-1 gene expression was analyzed by qRT-PCR. Bar graphs represent fold change in arginase-1 gene expression calculated from the  $\Delta\Delta$ Ct values normalized to GAPDH and compared to IFN $\gamma$  treated macrophages. (b) Arginase activity was determined using an enzymatic assay in lysates from polarized macrophages. Bar graph represents fold change in arginase activity calculated from the standard curve under different polarization conditions compared to IFN $\gamma$  treated macrophages. (c) Bar graph represents percentage change in arginase activity with increasing IKK-16 concentrations compared to no inhibitor treatment. Data represents mean  $\pm$  SD. Data is representative of 3 independent experiments. Statistical significance determined by two-way ANOVA (p-value of < 0.05 (\*); p-value of < 0.0001 (\*\*\*\*)). Bar graphs plotted using GraphPad Prism 7.

Figure 4.5a

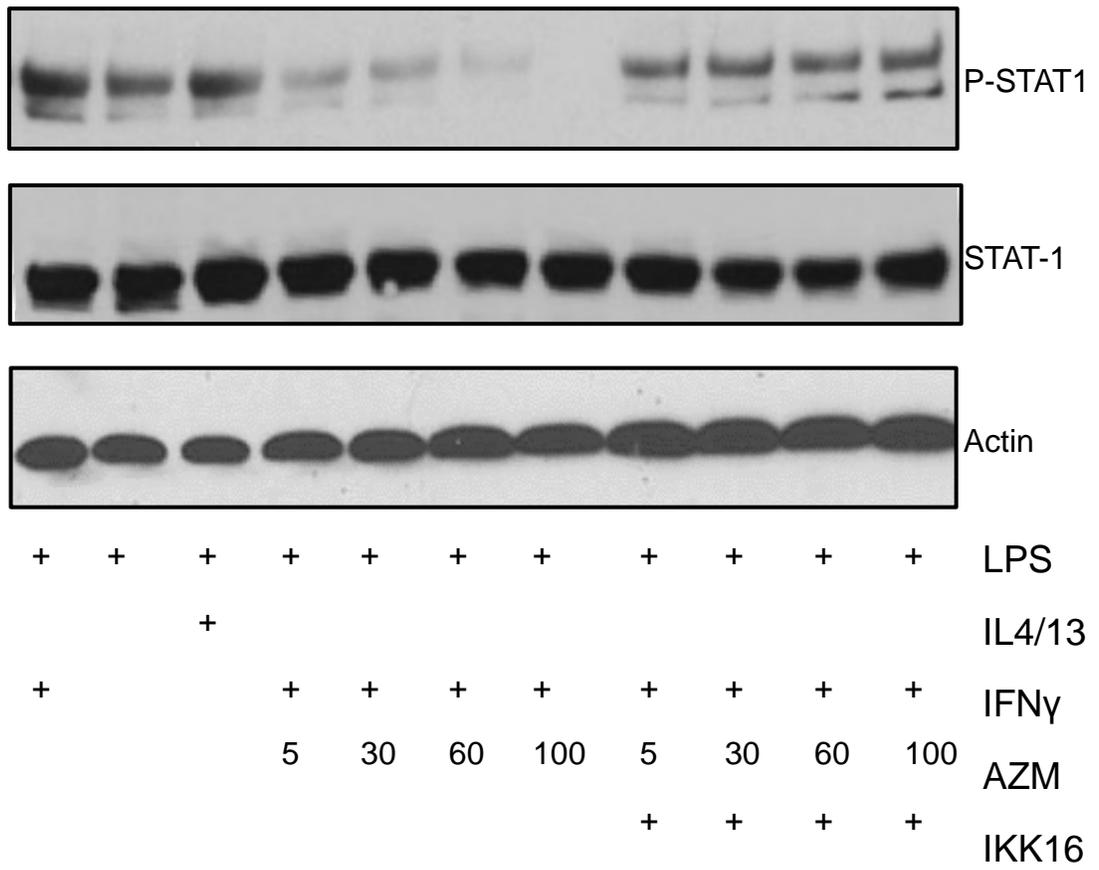


Figure 4.5b

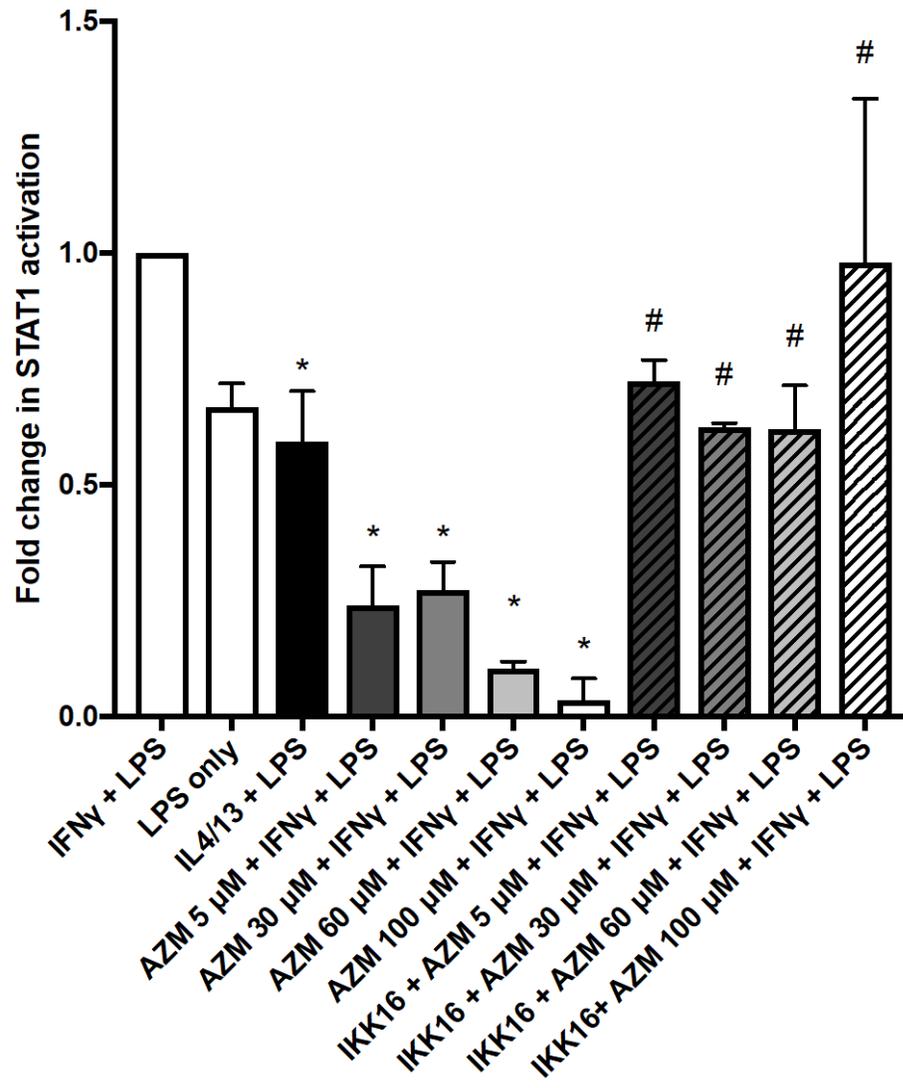


Figure 4.5. Azithromycin prevents STAT-1 activation in an IKK $\beta$  dependent mechanism.

J774 cells were plated at  $2.5 \times 10^5$  cells per 1 ml of DMEM in 24 well plates. Cells were then polarized with IL4 and IL13 (10 nM each), or with INF $\gamma$  (50 U/mL) alone, or with INF $\gamma$  and azithromycin (5, 30, 60, and 100  $\mu$ M) with/without IKK-16 (100 nM). After overnight polarization cells were stimulated with LPS (10 nM) for 15 minutes and proteins were harvested by cell lysis. (a) Western blots for the active form pSTAT-1 and the inactive STAT-1 subunits were performed. (b) Bar graph represents fold change in STAT-1 phosphorylation under different polarization conditions compared to INF $\gamma$  and LPS stimulated macrophages (normalized to actin and STAT-1 levels). Data represents mean  $\pm$  SD. Data is representative of 3 independent experiments. Statistical significance determined by two-way ANOVA with Sidak's multiple comparisons test ((\*) denotes significant difference compared to INF $\gamma$ +LPS; (#) denotes significant difference compared to the corresponding AZM concentration with no IKK16 treatment; p-value < 0.05). Bar graphs plotted using GraphPad Prism 7. Western Blots quantified using ImageJ.

Figure 4.6

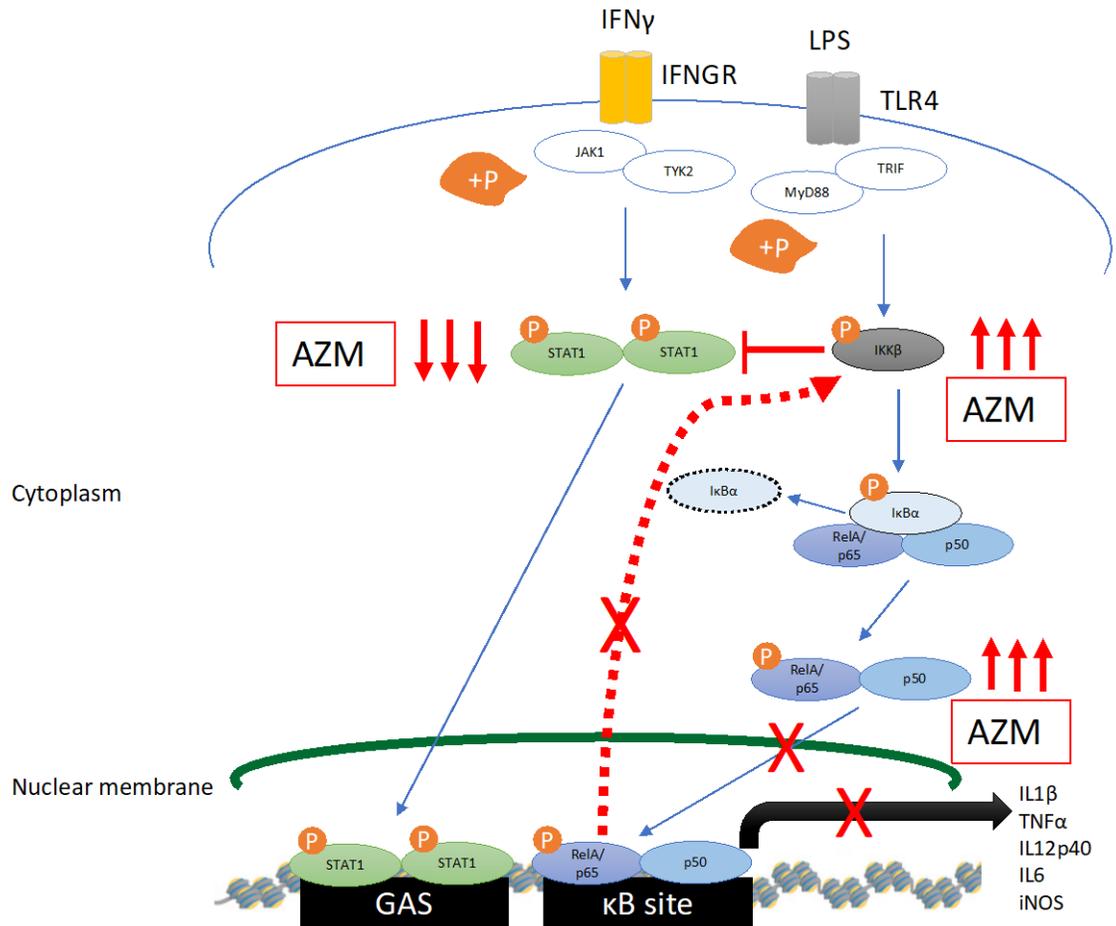


Figure 4.6. The proposed interaction induced by azithromycin (AZM) is depicted. Azithromycin inhibits IFN $\gamma$ -induced STAT-1 activation through cross-inhibition of the LPS induced NF- $\kappa$ B signaling mechanism. The nuclear translocation of p65 otherwise required for induction of pro-inflammatory gene transcription is inhibited by azithromycin. This prevents the negative feedback which otherwise shuts down the inflammatory signal by decreasing IKK $\beta$  production through decreased Ikbkb gene expression. Simultaneously, the lack of p65 nuclear translocation results in sustained I $\kappa$ B- $\alpha$  levels. Accumulated IKK $\beta$  protein cross-

inhibits the STAT-1 signaling pathway by decreasing STAT-1 phosphorylation thereby decreasing the associated pro-inflammatory gene transcription and increasing the expression of the M2-associated protein arginase-1.

## Chapter 5: Dependence of azithromycin-induced M2-like macrophage phenotype on arginase-1 to alter inflammation

### I. Introduction

Cystic fibrosis (CF) is a chronic inflammatory lung disease caused by genetic mutations which make the lung a favorable environment for chronic bacterial colonization. The dysfunctional mucociliary clearance, persistent lung injury, and accumulation of mucoid and endobronchial secretions promote repeated infections [312]. In fact, 90% of these patients die due to chronic lung infections with the most predominant pathogen being *Pseudomonas aeruginosa* (PA) [2, 6, 18, 19]. Additionally, the immune system in these patients responds in an exaggerated manner to infections with a characteristic chronic dysregulated inflammatory response along with aberrant T cell immunity and a predominant recruitment of neutrophils and pro-inflammatory macrophages [312]. While it is known that a Th1 response is important for the clearance of PA and other extracellular Gram-negative pathogens, patients with CF have a dysregulated T cell response that emphasizes pro-inflammatory T cell phenotypes [438]. It is possible that this dysregulation could lead to the increased susceptibility to Gram-negative colonization and infection observed in this patient population. Thus, the continual exaggerated immune response contributes to the shortening of patient life expectancy by 30 years as a result of lung injury and damage, deteriorates lung function, and decreases the quality of life [2, 6, 18].

Recently, there has been much interest in the use of azithromycin in patients with CF who are chronically infected with PA. Three randomized, placebo-controlled trials have shown an improvement in lung function with 6-12 months of treatment [299-301]. To date, the mechanisms of action of azithromycin in patients with CF remain unknown and long-term studies to demonstrate clinical benefit are ongoing.

*In vitro* studies have demonstrated that macrolides reduce several virulence factors of PA infection including the disruption of biofilm formation and altering expression of structural proteins such as flagellin [2, 6, 17, 18, 312, 439-441]. From other *in vitro* data, the anti-inflammatory effects of azithromycin have been postulated to occur by inhibiting pro-inflammatory cytokine production; decreasing chemotaxis, oxidative burst and adhesion of neutrophils; and accelerating pro-apoptotic state of monocytes. Our group studied azithromycin immunomodulatory mechanism *in vitro* where azithromycin polarized macrophages into an alternative phenotype via a mechanism that is dependent on cross-inhibition of the NF- $\kappa$ B and STAT1 signaling pathways. This modulation was dependent on IKK $\beta$ , a signaling molecule in the NF- $\kappa$ B pathway, and resulted in subsequent suppression of inflammatory gene activation while promoting an anti-inflammatory phenotype (Chapter 4).

Moreover, we have demonstrated that azithromycin-driven polarization of macrophages into an alternative phenotype controls several aspects of the immune response in mice infected with PA. Results demonstrate that alternatively activated macrophages shift the immune response into a monocyte predominant influx, decreasing the number of neutrophils that enter the lungs post-infection, and shifting the T cell response away from the Th1 phenotype [166, 310, 311]. Thus, our results are consistent with previous literature supporting the immunoregulatory role of azithromycin and support a macrophage reprogramming that could be responsible for a large portion of the beneficial effects observed when using the drug.

Our published *in vitro* and *in vivo* data show that azithromycin significantly upregulates arginase expression and activity [166, 310, 311]. Arginase is an important effector of M2 macrophages normally induced in response to Th2 cytokines (IL4, 10, and 13) [128-132, 135-138, 198, 331]. Its competing enzyme, iNOS, is induced in M1 macrophages in response to Th1 cytokines (e.g. IFN $\gamma$ ). Arginase metabolizes arginine into ornithine and urea while iNOS metabolizes

arginine into nitric oxide (NO) and citrulline [151, 152, 315, 316]. In addition to competing with iNOS for the same substrate and controlling its generation of NO, arginase is believed to be an immunomodulatory effector with important effects on T cell function and proliferation [151, 152, 154, 157, 315-318, 442]. Our group has shown a 10-fold increase in arginase activity in J774 murine macrophages polarized with IFN $\gamma$  and azithromycin [166, 310, 311]. Azithromycin-induced arginase expression *in vitro* was associated with reduced M1 pro-inflammatory macrophage effectors (e.g. IL-6, IL-12, TNF- $\alpha$ , iNOS, and CCR7) along with increased M2 anti-inflammatory macrophage effectors (e.g. IL10, MR, and CD23) [166, 310, 311]. Additionally, we have shown that azithromycin treatment in mice infected with PA increases arginase expression which is associated with reduced morbidity and decreased airway damage. Therefore, we consistently observe increased arginase expression with azithromycin along with reduced inflammatory parameters and a shift in macrophage polarization into an alternative phenotype.

Moreover, results show that arginase deletion from macrophages and neutrophils results in exaggerated inflammation (Chapter 3). Experiments using Arg1 $\Delta^m$  mice verified that arginase deficiency is associated with greater morbidity in terms of more significant weight loss. Arg1 $\Delta^m$  mice had greater neutrophil, macrophage, and lymphocyte infiltration with higher numbers of IL-10 and TNF- $\alpha$  producing macrophages compared to their littermate control mice. Moreover, Arg1 $\Delta^m$  mice had higher numbers of CD4+ T-cells, activated T-cells, IFN $\gamma$  and IL-17 producing CD4+ T-cells. These results validate that the production of arginase-1 by M2 macrophages is essential to regulate the inflammatory response against PA pneumonia. However, whether the production of arginase by macrophages is essential for the immunomodulatory mechanism of azithromycin has not been investigated.

Our group has previously studied the transcriptional profiles of inflammatory genes in patients with cystic fibrosis treated with azithromycin. Although

azithromycin treatment was not associated with an increase in M2 anti-inflammatory genes, the group of patients treated with azithromycin did have significantly reduced pro-inflammatory macrophage gene expression [442]. Yet, these observations could not be correlated to clinical outcomes or to definitive mechanisms by which azithromycin is thought to exert its beneficial immunomodulatory role. This was because of the small sample size and the fact that most of the subjects colonized with PA were on chronic azithromycin therapy making it difficult to distinguish the specific effects of azithromycin therapy on macrophages and inflammatory gene expression [442].

The study described herein evaluates the dependence of azithromycin anti-inflammatory mechanisms on arginase production by alternative macrophages to alter inflammation. We investigated whether the beneficial response to azithromycin is dependent upon arginase-1.  $Arg1^{\Delta m}$  and control mice were dosed with azithromycin and infected with PA-laden agarose beads. Moreover, we developed a clinical study which improves upon the limitations and design of the previous studies by generating matched data from subjects while on and off of azithromycin. We tested the hypothesis that azithromycin balances the immune response by controlling the inflammatory gene expression and the dependence of these mechanisms on arginase-1 production. While the human study is still in its early phases, murine experiments suggest that azithromycin is protective regardless of arginase production.

## II. Results

Azithromycin treatment in mice infected with PA protects against excessive morbidity and inflammation regardless of arginase production. Three groups of mice were infected with PA-laden agarose beads as described. Four days prior to infection, one group of  $Arg1^{\Delta m}$  mice and one group of littermate control mice received a dose of azithromycin via oral gavage. Azithromycin dosing continued daily thereafter. As a control, a group of  $Arg1^{\Delta m}$  mice received daily doses of the

vehicle only (2% methylcellulose). Each group of mice consisted of at least four mice per timepoint. Infected mice were humanely killed, and tissues were harvested and processed for flow cytometry analysis. Additionally, we monitored morbidity post-infection by comparing the daily weight to the baseline murine body weight prior to infection (Figure 5.1a). The control Arg1<sup>Δm</sup> mice lost weight faster and to a greater degree compared to the other two groups treated with azithromycin. While the control Arg1<sup>Δm</sup> mice lost the maximum amount of weight by day 2 post-infection with an average of 82.52% of their initial weight; maximum weight loss in the azithromycin treatment group was attained at day 3 post-infection with an average of 85.95% of their baseline body weight (Figure 5.1a, day 3 (x) p-value = 0.0059; day 3 (x) p-value < 0.0001, (#) p-value = 0.0008).

We also evaluated the effects of azithromycin on bacterial clearance and mortality in Arg1<sup>Δm</sup> mice (Figure 5.1 b and c). Lung bacterial clearance was evaluated at different timepoints post-infection by counting the number of viable PA colonies in homogenized lung tissues. Azithromycin treated groups cleared PA at a rate comparable to the control Arg1<sup>Δm</sup> mice. Additionally, the three groups failed to completely clear the infection and PA was still present in the lungs by day 10 post-infection (Figure 5.1b). Importantly, azithromycin treatment protected against mortality in both Arg1<sup>Δm</sup> and Arg1<sup>flox/flox</sup> groups (Figure 5.1c). Importantly, the infective inoculum used in these experiments was selected to cause murine pneumonia without causing severe morbidity or mortality in each mouse strain. Mice were euthanized and excluded from the analysis if they lose 20% or more of their body weight prior to infection along with one sign of morbidity (e.g. immobility, hunched posture, or lack of response to handling).

Azithromycin has been previously shown to shift the cellular influx towards a monocytic response and to reduce neutrophil infiltration in response to the infection. To examine the dependence of these mechanisms on arginase-1 expression, we used flow cytometry to characterize populations of neutrophils

and macrophages (Figure 5.2). Azithromycin treatment protected against excessive inflammation in both Arg1<sup>Δm</sup> and Arg1<sup>flox/flox</sup> groups. There was a significant attenuation of neutrophil recruitment into the alveolar spaces of azithromycin treated groups compared to the control Arg1<sup>Δm</sup> group at day 3 post-infection (Figure 5.2a, day 3, alveolar spaces (x) p-value = 0.0038, (#) p-value = 0.0017). While neutrophil numbers in the lung interstitium were lower in the azithromycin group, the differences were not statistically significant. However, azithromycin treatment, regardless of arginase deletion in macrophages, decreased the total number of tissue and infiltrating macrophages in the lungs of infected mice at day 3 post-infection (Figure 5.2, day 3, alveolar macrophages (x) p-value = 0.0469, (#) p-value = 0.0068; infiltrating macrophages (x) p-value = 0.0055, (#) p-value = 0.0457). Similarly, azithromycin treatment in both knock-out and control groups significantly reduced recruitment of M1 pro-inflammatory macrophages (tissue and infiltrating) into the alveolar spaces at day 3 post-infection (Figure 5.2c, day 3, (x) p-value = 0.0107, (#) p-value = 0.0026 and Figure 5.2d, day 3, (x) p-value = 0.046, (#) p-value = 0.0419). However, in the lung interstitium, both Arg1<sup>Δm</sup> groups had significantly lower numbers of M1 macrophages (tissue and infiltrating) compared to the azithromycin-treated Arg1<sup>flox/flox</sup> mice (Figure 5.2c, day 3, (+) p-value = 0.0091, (#) p-value = 0.0189 and Figure 5.2d, day 3, (x) p-value = 0.0118, (+) p-value < 0.0001, (#) p-value = 0.0288).

Additionally, we used flow cytometry analysis to define the expression of specific M1 and M2 macrophage effectors. Consistently we observed lower levels of resident and infiltrating TNF-α producing macrophages in the azithromycin-treated groups compared to the control Arg1<sup>Δm</sup> mice (Figure 5.2e, day 3, (x) p-values = 0.0408 and 0.0166, (#) p-values = 0.0243 and 0.0466, respectively). While azithromycin reduced the number of alveolar macrophages expressing iNOS in both Arg1<sup>Δm</sup> and Arg1<sup>flox/flox</sup> groups (Figure 5.2f, (x) p-value = 0.0439, (#) p-value = 0.0416), only the Arg1<sup>Δm</sup> mice treated with azithromycin had lower

number of infiltrating macrophages expressing iNOS (Figure 5.2f, (x) p-value = 0.0003, (+) p-value = 0.0003).

To determine the effects of azithromycin treatment on M2 macrophage polarization, we used two M2 macrophage markers, mannose receptor (MR) and IL-10. There were no statistically significant differences in MR expressing macrophages among the three groups of mice (Figure 5.2g). However, both azithromycin treated groups had significantly lower numbers of IL-10 producing alveolar and infiltrating macrophages compared to the control Arg1<sup>Δm</sup> mice (Figure 5.2h, day 5, (x) p-values = 0.0461 and 0.0399, (#) p-values = 0.0325 and < 0.0001 respectively). Interestingly, the ratio of pro- and anti-inflammatory monocytes were comparable among the three groups with similar trends including the ratio of CCR7/MR expressing macrophages (Figure 5.4d, A), IL-17/IL-10 expressing monocytes (Figure 5.4d, B), and TNF- $\alpha$ /IL-10 producing monocytes (Figure 5.4d, C).

We then evaluated the role that arginase expression plays in the ability of azithromycin to protect against inflammatory lung injury. Lung sections from mice treated with azithromycin and infected with PA-laden agarose beads were stained with H&E. An independent pathologist evaluated the lung sections blindly using a scoring system adapted from the American Thoracic Society guidelines [326]. The scoring system takes into consideration (1) the number of neutrophils in the alveolar spaces; (2) the number of neutrophils in the interstitial spaces; (3) the formation of hyaline membranes; (4) accumulation of proteinaceous debris in the airspaces; and (5) septal wall thickening. The inflammatory lung injury at day 2 post-infection was comparable among the 3 groups of mice. However, both azithromycin treated groups had significantly lower mean lung injury score compared to the control Arg1<sup>Δm</sup> mice. The lung injury score decreased in the azithromycin-treated Arg1<sup>Δm</sup> mice to an average of  $0.1065 \pm 0.031$  at day 5 post-infection and in the azithromycin-treated Arg1<sup>flox/flox</sup> mice to an average of 0.2016

$\pm 0.146$  versus a persistent lung injury score of  $0.446 \pm 0.113$  in the control Arg1 $\Delta^m$  mice (Figure 5.2i, day 5, (x) p-value = 0.0012, (#) p-value = 0.0078).

Azithromycin treatment in arginase conditional knock-out mice reduces T cell recruitment and activation in response to PA pneumonia. Our results in chapter 3 show that arginase is required for modulation of T cell responses to PA pneumonia and that deletion of arginase in Arg1 $\Delta^m$  mice skews the T cell responses towards Th1/Th17 predominance. However, because azithromycin is protective in part by increasing arginase in normal infected mice, we next evaluated whether azithromycin required arginase expression for its effects on T cells. Arg1 $\Delta^m$  mice were dosed with azithromycin and infected with PA-laden agarose beads intratracheally. Tracheobronchial lymph nodes draining the site of infection, in addition to the lung lavage and lung tissue samples were stained for surface and intracellular markers to evaluate T cell responses and activation profiles at different timepoints post-infection (Figures 5.3 and 5.4). Azithromycin treated Arg1 $\Delta^m$  mice responded to PA pneumonia with lower numbers of CD4+ and activated CD4+ T cells in their tracheobronchial lymph nodes (Figure 5.3a, day 14, Total CD4+ T cells (+) p-value = 0.0059; activated T cells (x) p-value = 0.0079, (+) p-value = 0.0017). Similarly, both azithromycin treated groups had significantly lower numbers of CD4+ lymphocytes and activated CD4+ lymphocytes in their interstitial spaces (Figure 5.3b, day 10 Total CD4+ T cells (x) p-value = 0.0228, (#) p-value = 0.0426; activated T cells day 14 (x) p-value < 0.0001, (#) p-value < 0.0001). However, there were no statistically significant differences in the numbers of CD4+ T cells and activated CD4+ T cells in the alveolar spaces in the 3 groups (Figure 5.3c).

Next, we used flow cytometry to analyze the different T helper cell lineages modulated with azithromycin treatment in Arg1 $\Delta^m$  mice (Figure 5.4). We observed a consistent decrease in Th17 recruitment with azithromycin treatment in both Arg1 $\Delta^m$  and Arg1<sup>flx/flx</sup> groups compared to untreated control Arg1 $\Delta^m$  mice (Figure 5.4a). In the lymph nodes, only the azithromycin-treated Arg1<sup>flx/flx</sup> mice had

significantly lower numbers compared to the control Arg1<sup>Δm</sup> mice (Figure 5.4a, day 3, (#) p-value = 0.0489). While in the lung tissues, both azithromycin treated groups had significantly reduced numbers of Th17 lymphocytes at day 3 post-infection (Figure 5.4a, day 3, (x) p-value = 0.0047, (#) p-value = 0.0079; day 10 (#) p-value = 0.0463). Similarly, both azithromycin treated groups had significantly lower numbers of Th17 lymphocytes recruited into their alveolar spaces at day 3 post-infection (Figure 5.4a, day 3, (x) p-value = 0.0047, (#) p-value = 0.0079; day 10 (#) p-value = 0.0463).

Additionally, azithromycin treatment was associated with attenuation of the type 1 T helper lymphocyte counts in both Arg1<sup>Δm</sup> and Arg1<sup>flox/flox</sup> groups. In the lymph nodes, azithromycin treatment significantly lowered the number of Th1 lymphocytes activated (Figure 5.4b, day 3, (x) p-value = 0.0002, (#) p-value = 0.0004). Similarly, both azithromycin treated groups had significantly lower numbers of Th1 lymphocytes recruited into their interstitial spaces at day 10 post-infection (Figure 5.4b, day 10, (x) p-value = 0.0314, (#) p-value = 0.0121). While in the alveolar spaces, both azithromycin treated groups had significantly reduced numbers of Th1 lymphocytes at day 3 post-infection (Figure 5.4b, day 3, (x) p-value < 0.0001, (+) p-value 0.007, (#) p-value < 0.0001; day 10 (#) p-value = 0.0208).

Finally, azithromycin treatment was associated with increased regulatory T cell numbers in both Arg1<sup>Δm</sup> and Arg1<sup>flox/flox</sup> groups. In the lymph nodes, untreated control Arg1<sup>Δm</sup> mice had significantly higher numbers of Treg lymphocytes compared to both azithromycin treated groups at day 5 post-infection (Figure 5.4c, day 5, (x) p-value = 0.0139, (#) p-value = 0.034). However, in the interstitial and alveolar spaces of both azithromycin treated groups, we observed increased numbers of Treg lymphocytes at days 3 through 10 compared to the untreated control Arg1<sup>Δm</sup> mice (Figure 5.4c, lung interstitium and alveolar spaces, p-values > 0.05).

Additionally, we estimated the ratios of pro- and anti-inflammatory cytokines expressed by all lymphoid cells. The lymphocyte population contains CD4+ T cells as well as other lymphoid cells including the innate lymphoid cells, CD8+ T cells, and NK cells. Figure 5.4d shows the ratios of total IL-17, IFN $\gamma$ , TGF $\beta$ , and IL-10 expressing lymphoid cells in the alveolar spaces. The ratio of IL-17 to IL-10 producing lymphocytes was significantly higher in the control Arg1 $\Delta^m$  mice at day 3 post-infection compared to the azithromycin groups (Figure 5.4d, D, (x) and (#) p-values < 0.0001). Similarly, azithromycin treated mice had decreased IFN $\gamma$  to TGF $\beta$  ratios at days 0 and 3 post-infection compared to the control Arg1 $\Delta^m$  group (Figure 5.4d, E, day 0, (x) p-value = 0.0151; day 3 (x) p-value = 0.004 and (#) p-value = 0.0024). Additionally, azithromycin treated mice had decreased Th17 to Treg lymphocyte ratios at days 10 post-infection compared to the control Arg1 $\Delta^m$  group (Figure 5.4d, F, day 10, (x) p-value = 0.0002, (#) p-value = 0.001).

### III. Discussion

Azithromycin is a well-known and widely used macrolide antibiotic with significantly important anti-inflammatory properties. The use of azithromycin as an immunomodulatory drug in cystic fibrosis patients is a common practice today in patients chronically infected with PA. The clinical benefits of this drug are well established and the clinical trials showing its anti-inflammatory efficacy has led to a recommendation for its use in the European NICE guidelines and the American Cystic Fibrosis Pulmonary guidelines [306, 307]. Yet, very little is known about azithromycin anti-inflammatory mechanisms. Despite the extended use of this drug in CF patients, it is still unclear how azithromycin exerts its immunomodulatory functions. Therefore, there are no clear-cut guidelines on when to use this drug and if there is a specific population that would benefit from this drug more than another. Our limited understanding of azithromycin anti-inflammatory mechanisms and the specific targets involved limits our judgment on the duration of use and whether we can adjust the dosing or modify the structure for increased benefit.

Our group has shown that azithromycin shifts the macrophage polarization away from the M1 pro-inflammatory phenotype towards an M2 anti-inflammatory phenotype. We consistently observe suppression of pro-inflammatory effectors and cytokines with azithromycin (including iNOS, TNF- $\alpha$ , IL-6, and IFN $\gamma$ ), and an increase in anti-inflammatory effectors (including IL-10 and arginase-1) in both in-vitro and in-vivo PA infection models [166, 310, 311]. We have demonstrated that arginase-1 production by alternative macrophages is essential for regulating excessive inflammation in PA pneumonia and by limiting Th1 and Th17 cell responses (chapter 3). Here, we have focused on the question of whether azithromycin's anti-inflammatory mechanisms require arginase-1 for regulation of inflammation.

Results from experiments with arginase conditional knock-out mice treated with azithromycin negate this hypothesis. Our results show that azithromycin protects against excessive morbidity and inflammation in mice infected with PA pneumonia regardless of arginase production. We have previously shown that arginase conditional knock-out mice do worse compared to their littermate controls with excessive neutrophil and pro-inflammatory macrophage influx in response to infections with PA pneumonia. The current study shows that azithromycin is equally protective in both arginase conditional knock-out mice and in their littermate controls. Azithromycin equally blunted neutrophil influx in infected mice regardless of arginase production. Moreover, azithromycin reduced macrophage infiltration and polarization towards an M1 phenotype regardless of arginase deletion from myeloid cells. Therefore, azithromycin suppresses excessive neutrophil influx and pro-inflammatory macrophage polarization through mechanisms that are independent of arginase. Although we see increased arginase expression and activation with azithromycin treatment, it is most likely a consequence rather than a mechanism by which azithromycin exerts its clinical benefits.

Our in vitro data suggest that azithromycin cross-inhibits NF- $\kappa$ B and STAT-1 pro-inflammatory transcription factors rather than promoting M2 anti-inflammatory signaling mediators. Consistently, we see suppression of pro-inflammatory macrophage effectors with azithromycin treatment rather than an increase in anti-inflammatory effectors. There were no significant differences in MR expressing M2 macrophages, and azithromycin equally downregulated IL-10 expressing macrophages in both arginase conditional knock-out mice and their littermate controls. It is very likely that azithromycin suppresses pro-inflammatory mediators and cytokines rather than increasing anti-inflammatory mediators; thus, it adjusts the balance of the pro- and anti-inflammatory effectors and protects against excessive inflammation.

Similar to arginase-mediated modulation of the T cell phenotype, azithromycin modulates the T cell responses, but independently of arginase. Our previous data suggest that arginase production by neutrophils and macrophages is essential for limiting Th1 and Th17 pro-inflammatory responses. However, the current study shows that azithromycin can suppress excessive Th1 and Th17 responses regardless of arginase deletion from myeloid cells. Additionally, we saw reduced T cell numbers in the interstitial spaces with azithromycin treatment while the effects were variable in the lymph nodes and alveolar spaces. Results indicate that azithromycin affects T cell polarization and disposition independent of arginase production. It is likely that azithromycin affects compartmentalization of T cell responses and that effects of azithromycin on T cell polarization are cytokine mediated. Future studies evaluating effects of azithromycin in the presence of neutralizing cytokine antibodies are essential to determine the major cytokines driving the effects of the drug on T cells and other immune responses to PA pneumonia.

Lastly, azithromycin treatment promoted a regulatory T cell phenotype in the lungs of mice infected with PA regardless of arginase. While azithromycin reduced the numbers of Treg lymphocytes in the lymph nodes of infected mice,

there were more Treg lymphocytes in the lungs and alveolar spaces. It is possible that the attenuation of the Th17/Th1 responses tilts the balance towards a Treg predominant phenotype. The specific mechanisms by which azithromycin affects the Th17/Treg balance is independent of arginase-mediated modulation of T cell phenotype. It is possible that azithromycin might have direct effects on T cell proliferation and expression of activation markers. Yet, the specific mechanisms involved are yet to be elucidated.

Collectively, our results show that azithromycin protects against excessive inflammation in PA pneumonia by attenuating the release of pro-inflammatory cytokines and thus shifting the T cell responses away from the Th1/Th17-mediated inflammation. This modulation is associated with reduced neutrophil influx and is independent of arginase production. Additionally, we have extended our studies to show protective effects of azithromycin in myocardial infarction, spinal cord injury, stroke, and other diseases with potential inflammatory pathology [443-446]. It is clear that arginase production in these models can modulate the immune response; however, we show here that azithromycin exerts its anti-inflammatory mechanism independent of its ability to increase arginase. It is possible that the increased arginase expression and activity with azithromycin assists in better modulation of the immune response and in speeding the recovery and inducing wound healing and repair. However, azithromycin mediates faster recovery via non-arginase pathways. We hypothesize that azithromycin regulates inflammation via increased TGF $\beta$  secretion thereby modulating the T cell subset distribution and function in response to PA infection. In support of this notion, we observed increased TGF- $\beta$  expression in azithromycin-treated mice. TGF $\beta$  is an important type II cytokine which inhibits T cell activation and alters the differentiation of naïve T cells into effector phenotypes [447]. According to Cottrez et al., TGF- $\beta$  expression suppresses T cell proliferation and cytokine secretion. These effects are potentiated with increased IL-10 concentrations which increase the surface expression of TGF- $\beta$  receptors [448]. According to Cottrez and colleagues, IL-10 restores TGF- $\beta$

inhibitory effects on activated and memory T cells [448]. Therefore, it is very likely that the increased IL-10 concentrations with azithromycin treatment along with the induced TGF- $\beta$  levels, act synergistically to modulate T cell responses and to induce a regulatory phenotype. In a murine model of helminth infection, mice colonized with the nematode, *Heligmosomoides polygyrus*, have increased IL-10 and TGF- $\beta$  concentrations which inhibits the release of Th1 cytokines [449]. Ince and colleagues show that increase TGF- $\beta$  mediates a Treg predominant immune response via an IL-10 dependent mechanism and suppresses Th1 and Th2 responses thereby protecting against chronic colitis in mice infected with *H. polygyrus* [449]. Similarly, IL-10/TGF- $\beta$  synergism promotes an increase in Treg numbers in active *Mycobacterium tuberculosis* infections [450]. Feruglio and colleagues suggest that early inhibition of IL-10/TGF- $\beta$  pathway facilitates *M. tuberculosis* clearance by rescuing the Th1 responses [450]. Additionally, deletion of TGF- $\beta$  drastically affects Treg development and promotes Th1 responses. This effect is dependent on the TGF- $\beta$  mediated regulation of T-bet, the Th1 transcription factor, expression [447, 451]. Therefore, it is possible the azithromycin induces a Treg phenotype through TGF- $\beta$  dependent mechanisms thereby limiting the Th1 and Th17 responses. Moreover, it is likely that TGF- $\beta$  is driving fibrogenesis and wound healing which we otherwise see with arginase. In fact, TGF- $\beta$  is a master regulator of fibrosis through signaling pathways independent of arginase. It is possible that azithromycin-mediated modulation of TGF- $\beta$  affects the latter signaling through Smad and non-Smad pathways which regulates myofibroblast activation and extracellular matrix deposition [452]. Future studies utilizing TGF- $\beta$  neutralizing antibodies in azithromycin-treated Arg1 $\Delta^m$  mice will help validate this hypothesis. Additionally, future studies will examine the effects of azithromycin on non-arginase mediated signaling pathways involved with wound healing including the Smad pathway [452]. This will be achieved by utilizing Lck-creSmad4 mice. These mice lack the Smad4 intracellular signaling molecule in lymphoid cells. This protein is responsible for the signaling cascade through the receptor that binds to members of the TGF $\beta$  superfamily [453].

Some of the limitations of our study include the “leakiness” of the LysMcre system and the deletion of arginase from both neutrophils and macrophages as discussed in Chapter 3. Briefly, the deletion of arginase in our murine model is controlled by lysozyme expression. Lysozyme M is expressed in neutrophils and macrophages. So, it is difficult to delineate the difference between neutrophil versus macrophage arginase functions as both are deleted. Additionally, new reports show different levels of lysozyme expression between tissue and infiltrating macrophages thus accounting for different levels of arginase deletion from these two populations [368].

Importantly, it is possible that azithromycin induces arginase expression in non-myeloid cells. Hence, non-myeloid sources of arginase may be rescuing the effects of its deletion from neutrophils and macrophages in Arg1<sup>Δm</sup> mice. Therefore, it is essential to study the effects of azithromycin in other models of conditional arginase deletion or global inhibition. For instance, ILC2s are an important source of arginase which may be induced with azithromycin thereby counteracting the effects of deleted arginase from myeloid cells. In fact, Amantea et al. show that pharmacologic arginase inhibition in peritoneal macrophages reversed the neuroprotective effects of azithromycin in ischemic stroke [445]. The authors suggest that polarization of migratory macrophages towards an M2 phenotype with azithromycin is essential for the reduced ischemic injury which requires arginase production by these macrophages. This group shows that azithromycin does not alter microglia or tissue macrophages but affects infiltrating macrophages. These results are similar to our observation that differential effects of azithromycin on alveolar versus interstitial macrophages in the lungs. However, this group shows a 10-fold increase in M2 anti-inflammatory macrophage numbers rather than a suppression of the pro-inflammatory M1 macrophage numbers which we see in our model. Therefore, it might be possible that azithromycin acts differently in different disease models. While a shift in macrophage polarization towards the M2 phenotype underlies the beneficial effects of the drug in ischemic stroke thus requiring arginase function for

neuroprotection, a shift in macrophage polarization away from the M1 phenotype underlies the beneficial effects of the drug in PA pneumonia thus functioning independent of arginase.

Finally, we designed a clinical study to evaluate the applicability of our observations in patients with cystic fibrosis. As discussed in chapter 1, the immunomodulatory role of arginase-1 in humans is controversial. It is well established that increased arginase expression promotes suppressed immunity and increased inhibitory Treg responses in cancer, type I diabetes, rheumatoid arthritis, and many other auto-immune diseases [454-456]. However, increased arginase expression is also associated with exaggerated inflammation and exacerbations in patients with cystic fibrosis as well as in patients with systemic lupus erythematosus, myasthenia gravis, autoimmune encephalomyelitis, and obesity [109, 135, 164, 165, 248, 370-375, 457]. Additionally, several studies suggest that the two arginase isoforms function differently in humans [248, 371]. However, a direct link between arginase expression and pathogenesis of the above-listed diseases have not been elucidated yet. It is likely that arginase upregulation follows the activation of exaggerated inflammation and that arginase induction is a regulatory mechanism. Therefore, future studies are needed to validate the immunomodulatory mechanisms of arginase in humans. Additionally, the specific mechanisms by which azithromycin exerts its clinically beneficial effects and whether arginase is required for azithromycin anti-inflammatory mechanisms are yet to be elucidated. Our proposed clinical study will help answer these questions and will improve our understanding of azithromycin's clinical benefits. This will provide the opportunity to individualize therapy for maximal benefits in different patient populations. Additionally, knowing the effectors involved in modulating inflammation identifies future drug targets for new and improved immunomodulatory agents for cystic fibrosis and many other inflammatory and autoimmune diseases.

The clinical study was approved by the Institutional Review Board at the University of Kentucky. We are currently pre-screening cystic fibrosis patients between 12 and 50 years of age, who are clinically stable and have been on azithromycin for at least a year. We hope to recruit about 20 subjects within the next 1 year. Patients who meet our inclusion/exclusion criteria will take a drug holiday discontinuing azithromycin treatment for 2-3 months. We will utilize the methods proposed in Chapter 2 including flow cytometry, gene expression assays, and cytometric bead arrays to compare immune signatures and inflammatory responses in patients when on and off azithromycin. Additionally, we will use principal component analysis and paired sample T-tests to compare the matched samples over time after re-initiating azithromycin therapy in these patients. We will also utilize statistical analysis to correlate our results with clinical outcomes (including demographic information, pulmonary function tests, requirement for hospitalization, and requirement for antibiotics).

Along with the *in vitro* and *in vivo* data, our research leads the way towards fully describing the role and mechanism of azithromycin in modulating the immune response to PA infections. Completion of this study will have a positive impact by defining the immune-regulatory mechanisms of azithromycin in the context of chronic inflammatory lung disease. Additionally, it will provide evidence on the mechanisms of arginase-mediated regulation of the immune response. Results from this pilot clinical study will broaden our knowledge of the specific role of arginase in the anti-inflammatory mechanisms of azithromycin. With this, we hope to identify essential regulatory pathways and specific downstream signaling cascades which modulation can be of therapeutic value for future drug targets in patients with non-resolving pulmonary infections and in other chronic and acute inflammatory pathologies.

Figure 5.1a

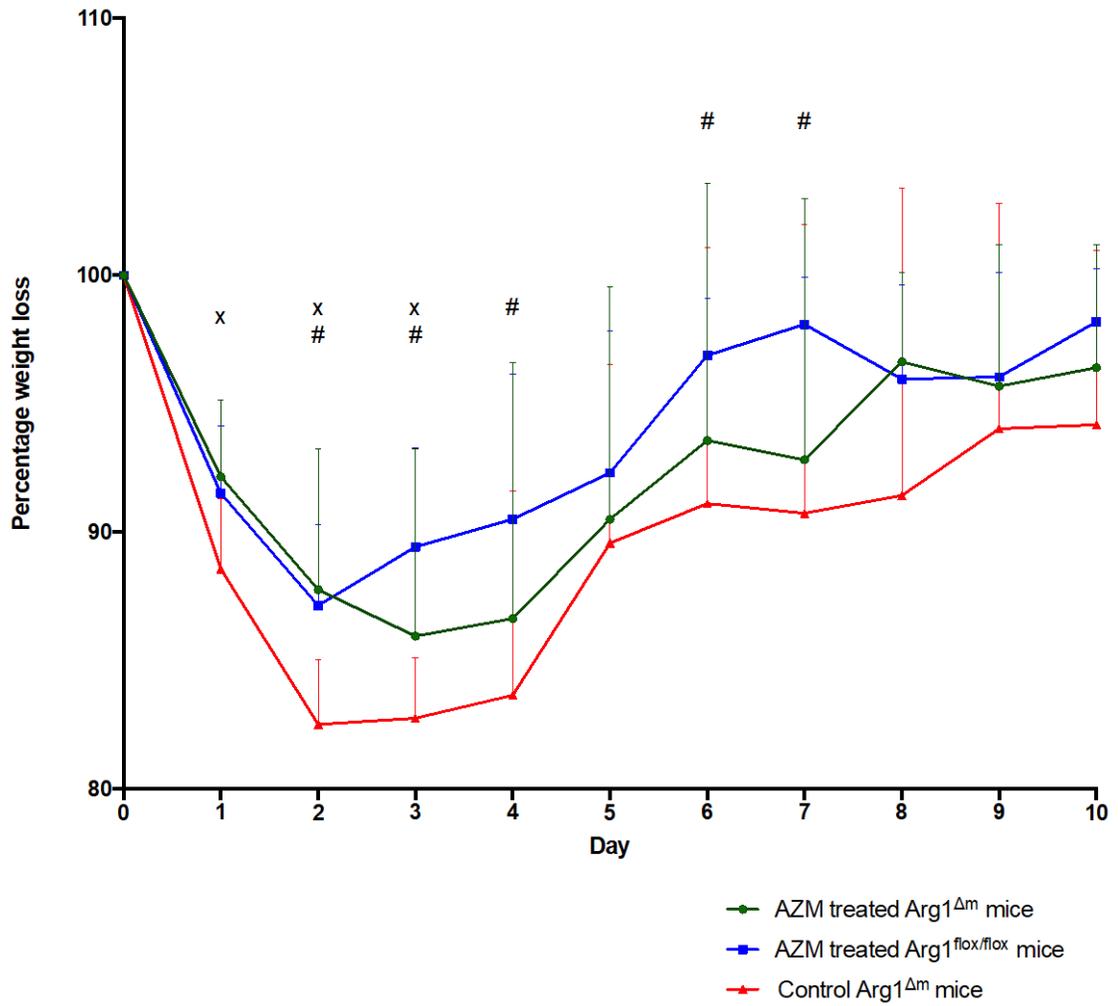


Figure 5.1b

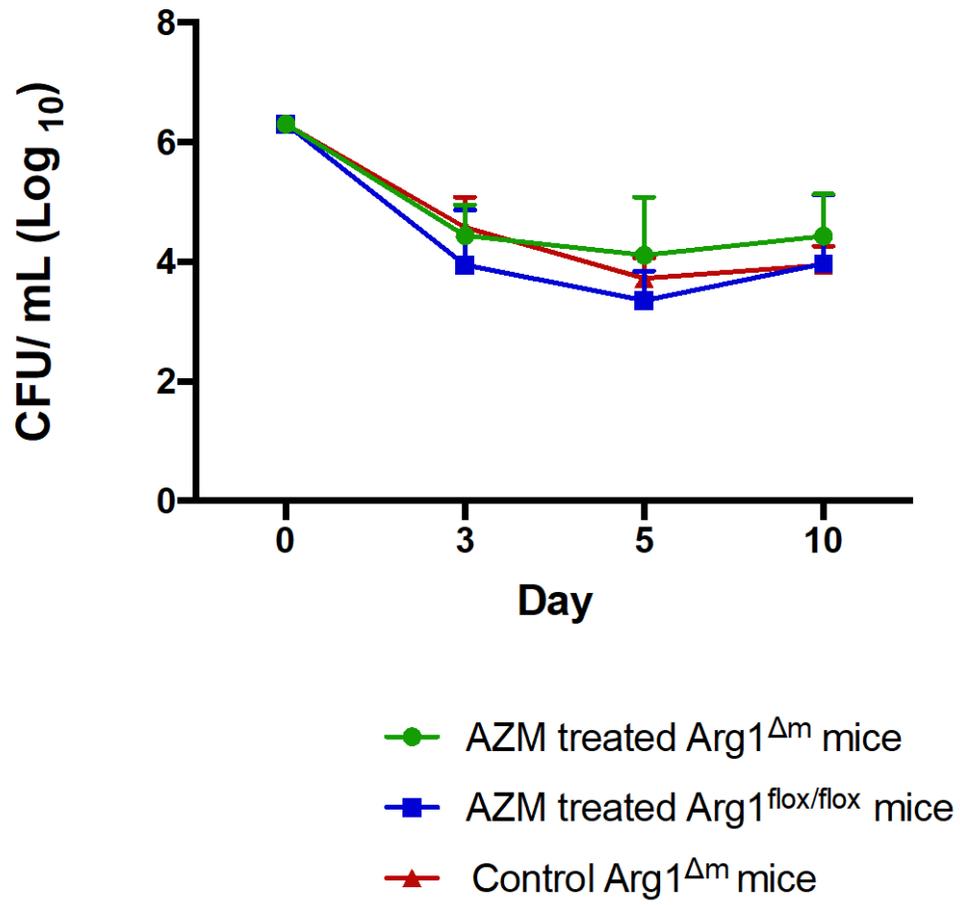


Figure 5.1c

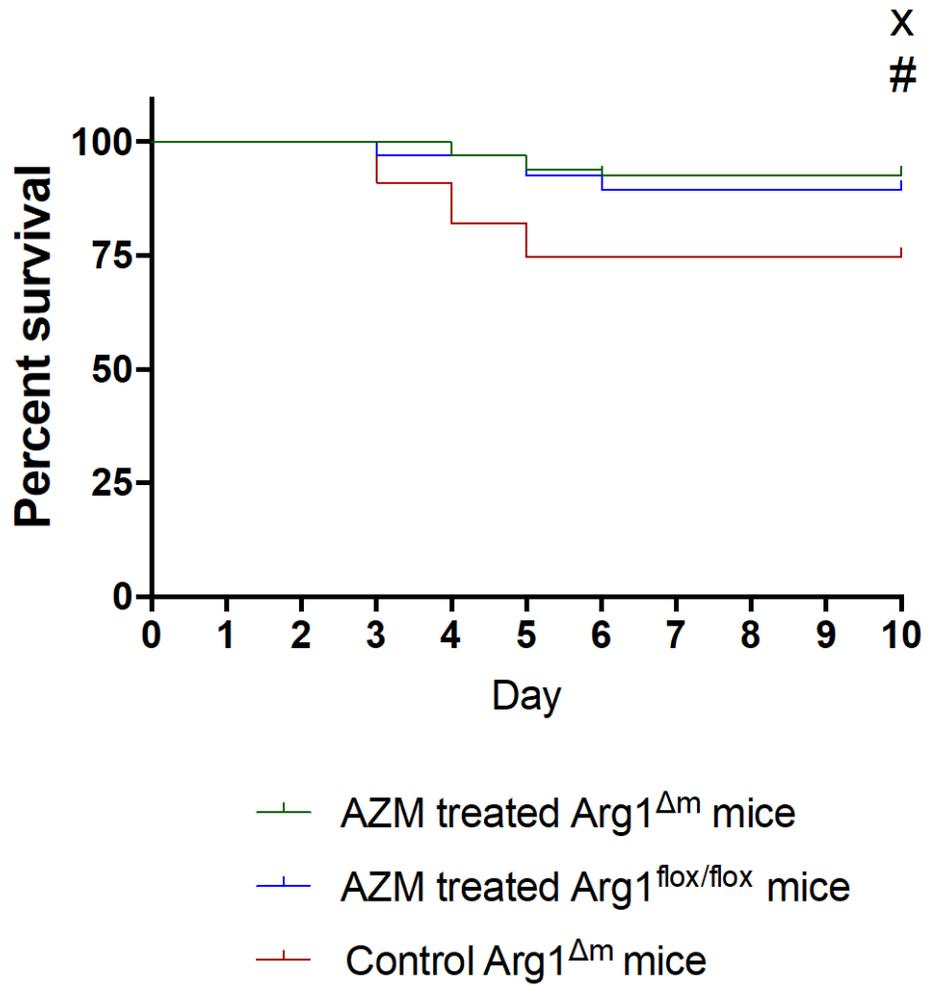


Figure 5.1. Azithromycin treatment protects against excessive morbidity and weight loss in arginase-1 conditional knock-out mice.

$Arg1^{\Delta m}$  and their littermate  $Arg1^{flox/flox}$  mice were treated with azithromycin starting 4 days prior to infection and daily thereafter. A third control group of  $Arg1^{\Delta m}$  mice was dosed with the vehicle (2% methylcellulose). The 3 groups of mice were infected with PA-laden agarose beads through intratracheal instillation of  $2 \times 10^6$  CFU/ mL as described. Murine morbidity post infection was evaluated in terms of weight loss measured at least once daily before and after infection. (a) The graph represents the percentage weight loss normalized to the baseline body weight prior to infection. (b) The graph represents bacterial clearance from the lungs post-infection. (c) Kaplan-Meier graph represents murine survival post-infection. Data represent mean  $\pm$  SD and is representative of 3 independent experiments. Statistical significance was determined by two-way ANOVA ((x) azithromycin treated arginase conditional knock-out mice significantly different than control arginase conditional knock-out mice; (#) azithromycin treated littermate control mice group significantly different than control arginase conditional knock-out mice; (+) azithromycin treated arginase conditional knock-out mice significantly different than the azithromycin-treated littermate control mice; p-value < 0.05). Graphs plotted using GraphPad Prism 7 (Note: for some points, the error bars are shorter than the height of the symbol. In these cases, Prism simply does not draw the error bars).

Figure 5.2a

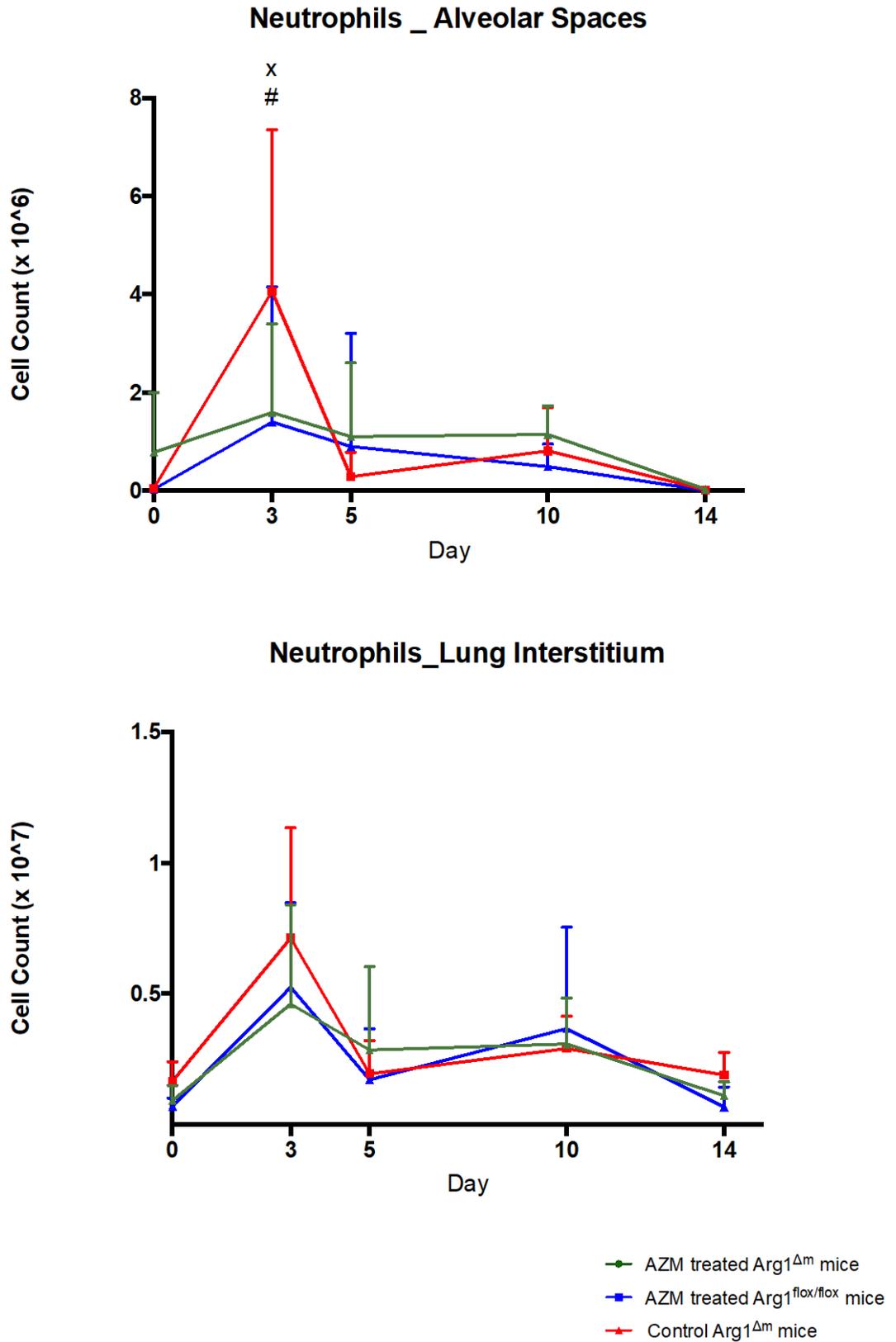


Figure 5.2b

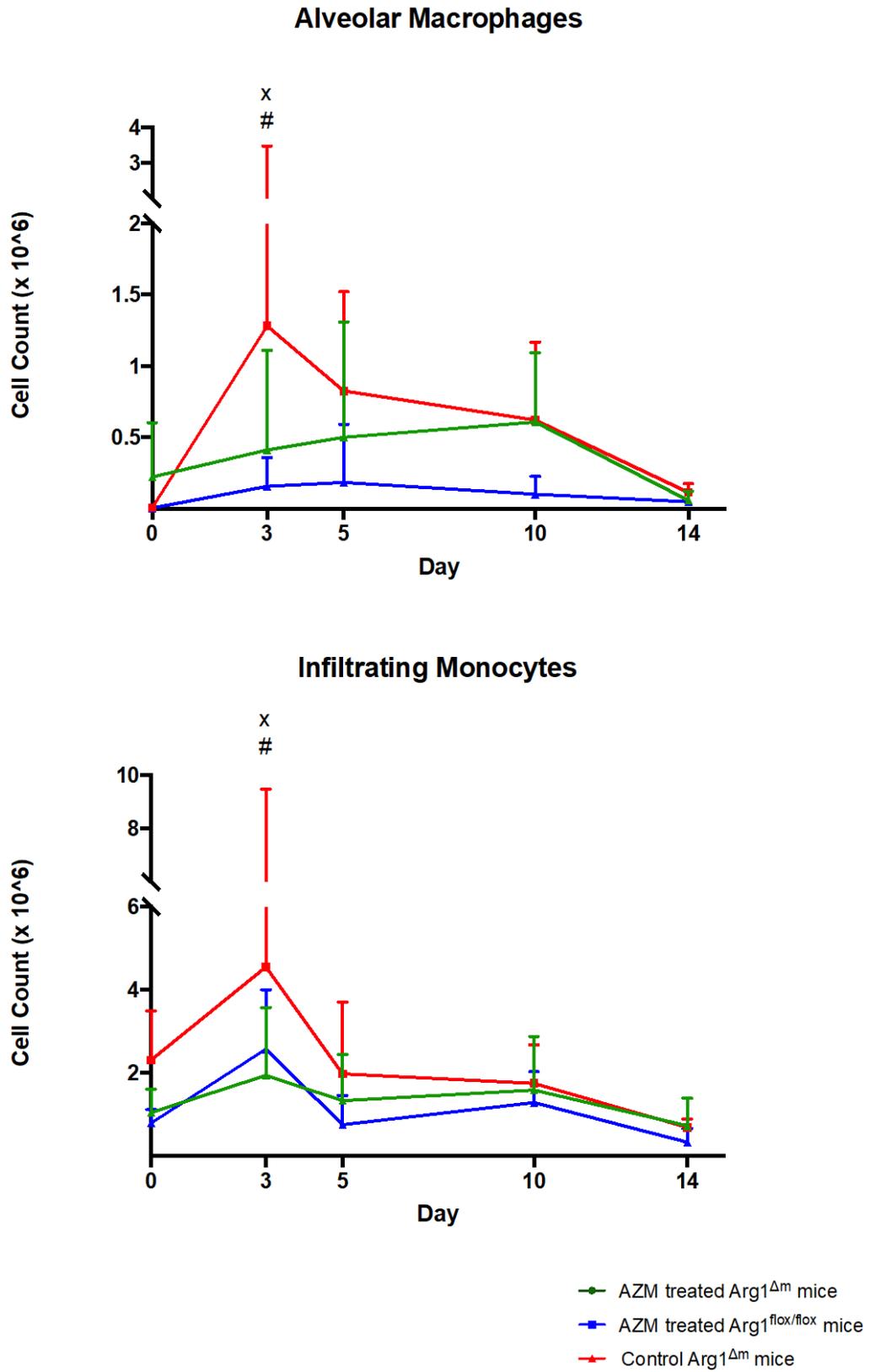
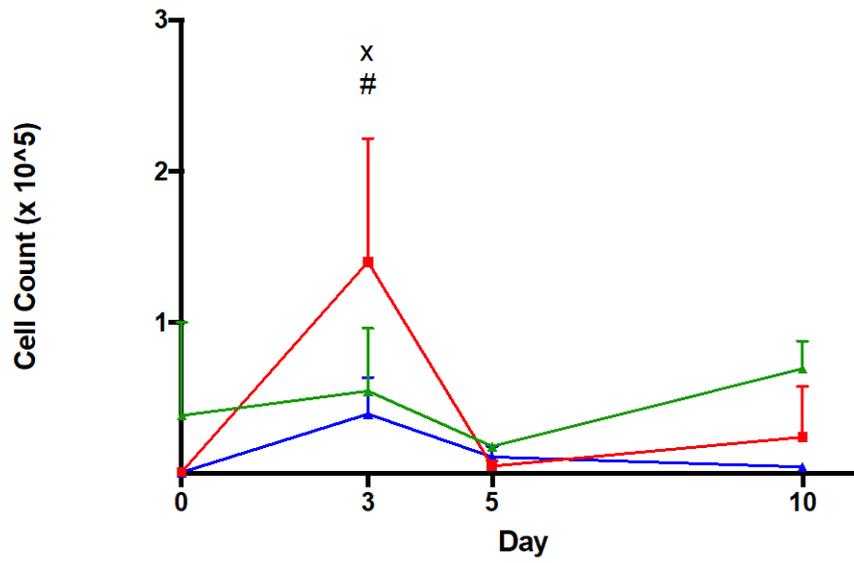
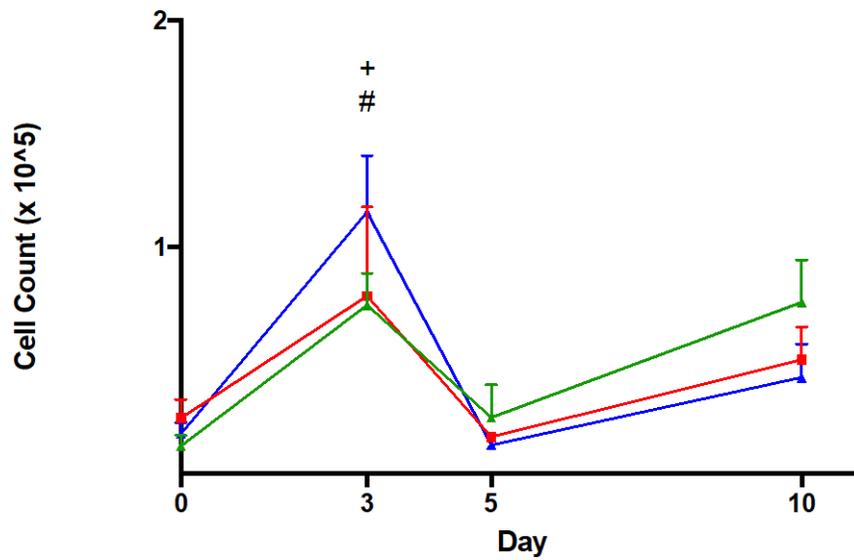


Figure 5.2c

### M1 Tissue Macrophages \_Alveolar Spaces



### M1 Tissue Macrophages \_Lung Interstitium



- AZM treated  $Arg1^{\Delta m}$  mice
- AZM treated  $Arg1^{flx/flx}$  mice
- Control  $Arg1^{\Delta m}$  mice

Figure 5.2d

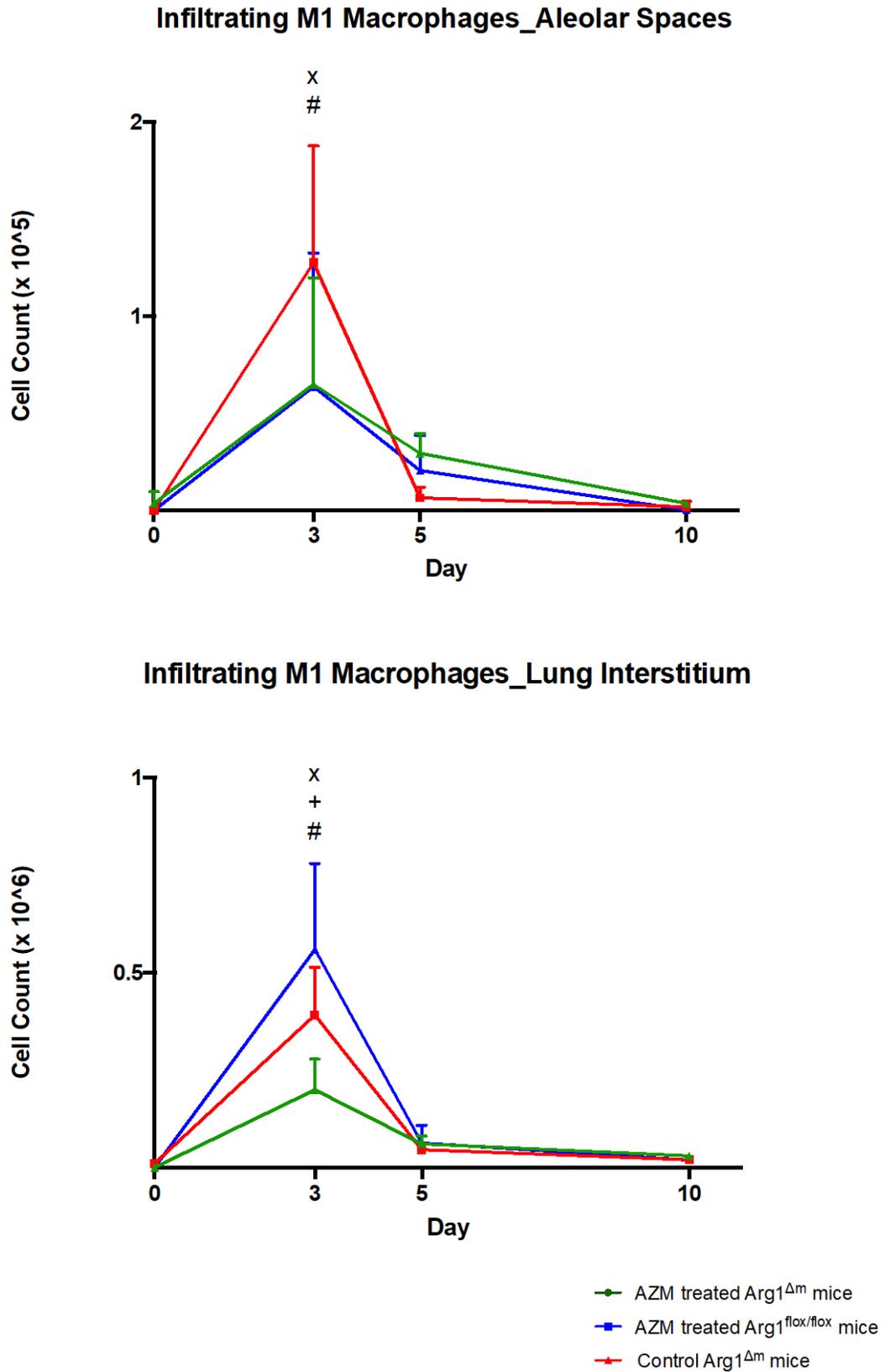


Figure 5.2e

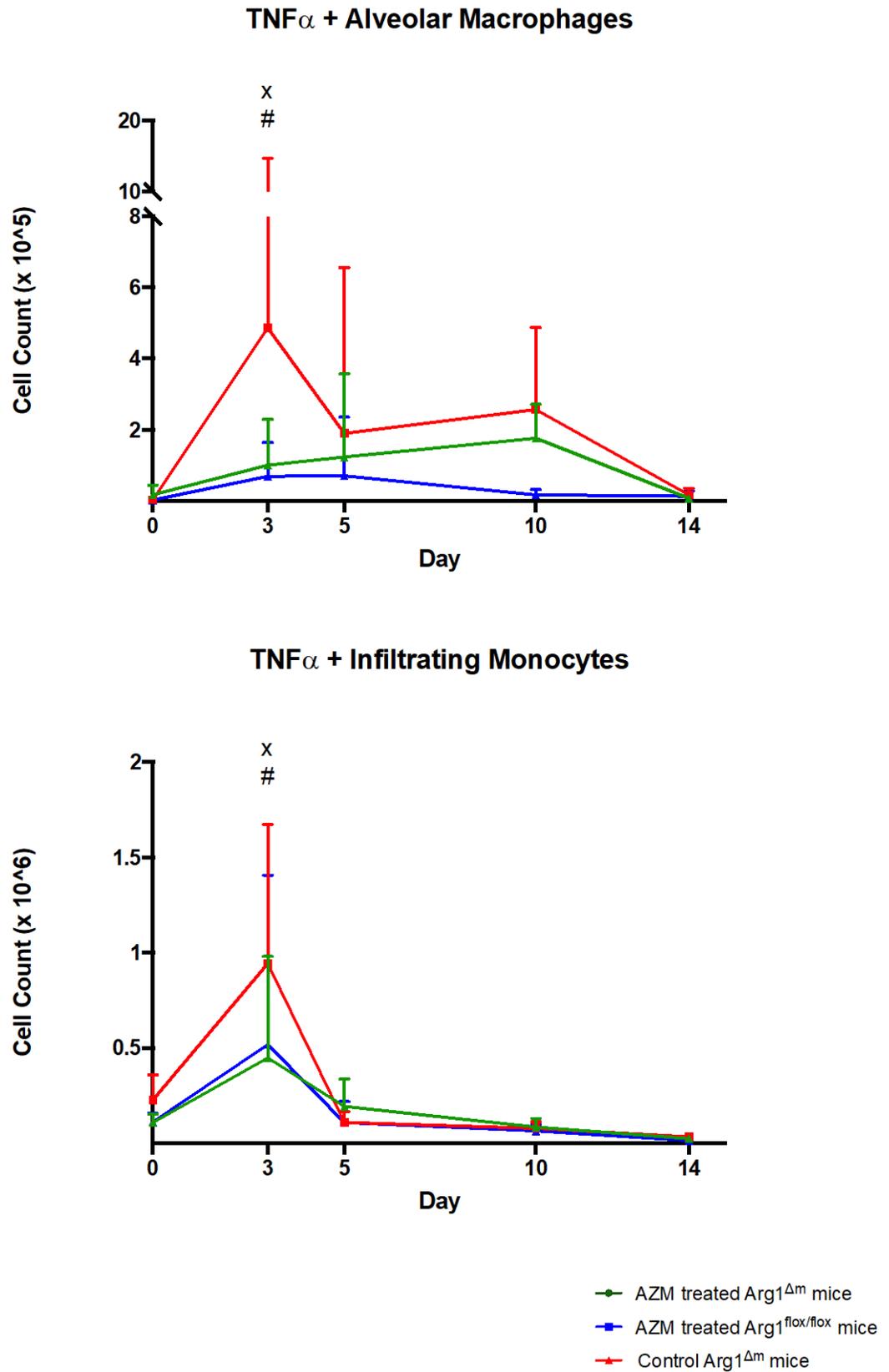


Figure 5.2f

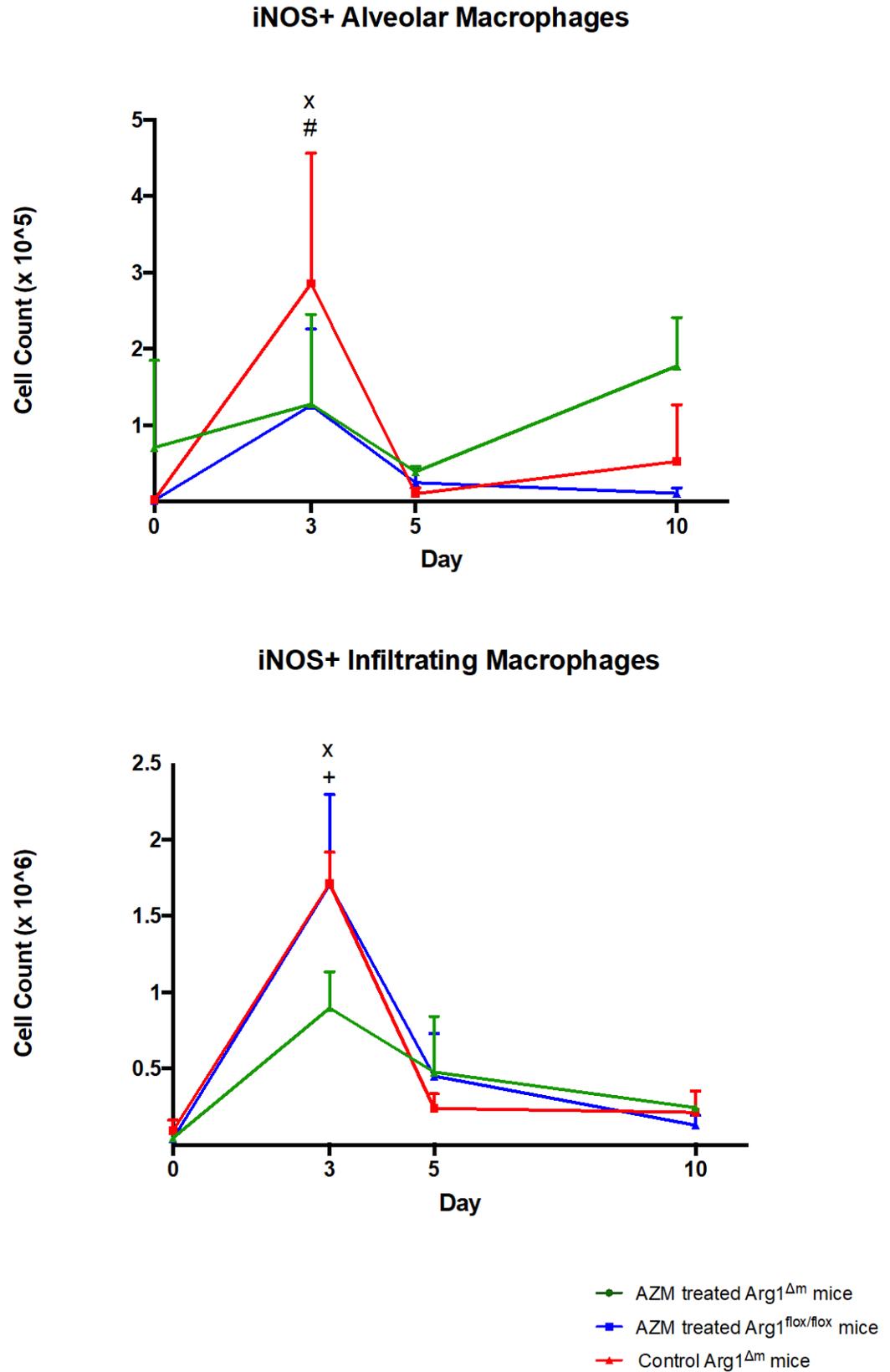


Figure 5.2g

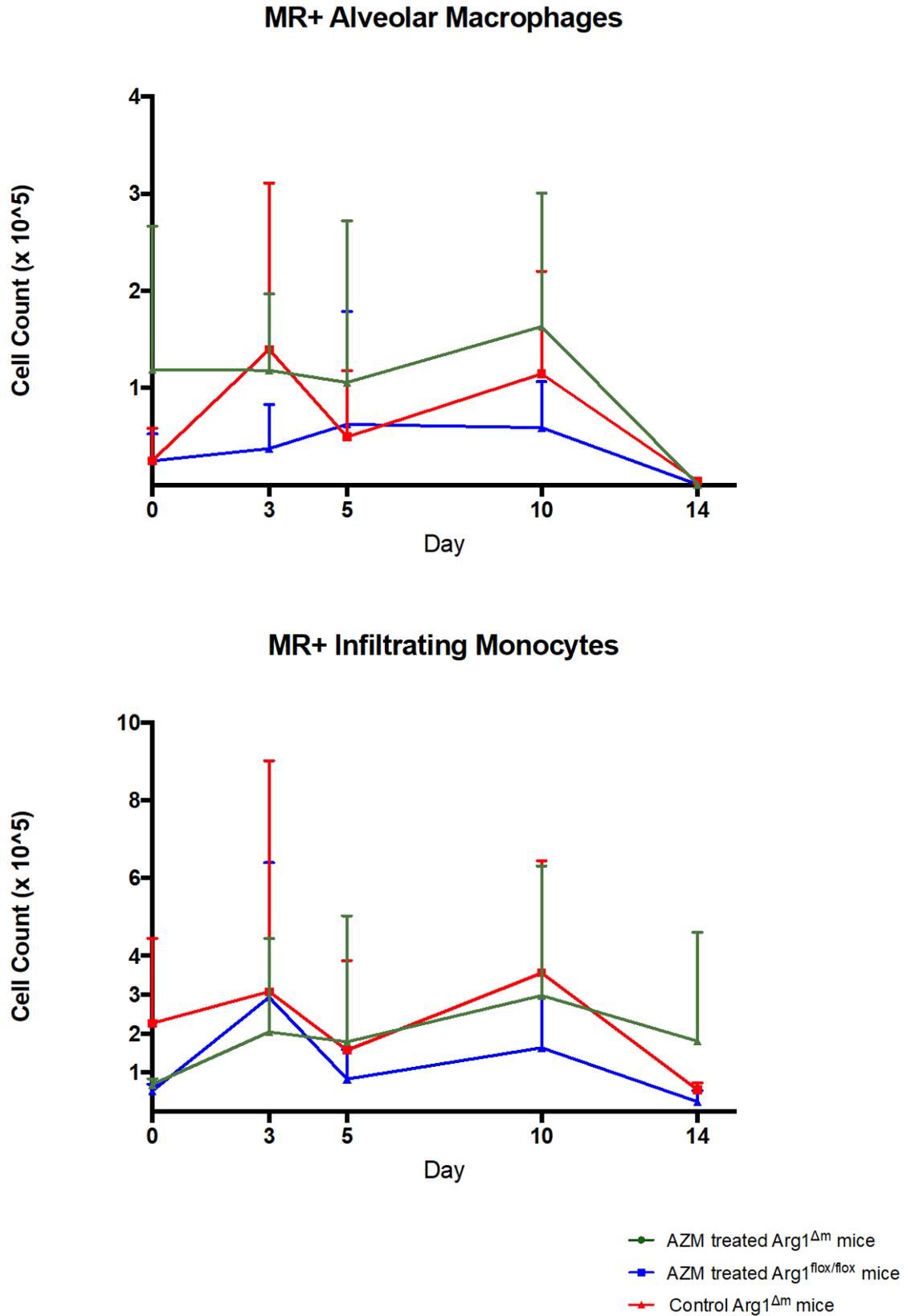


Figure 5.2h

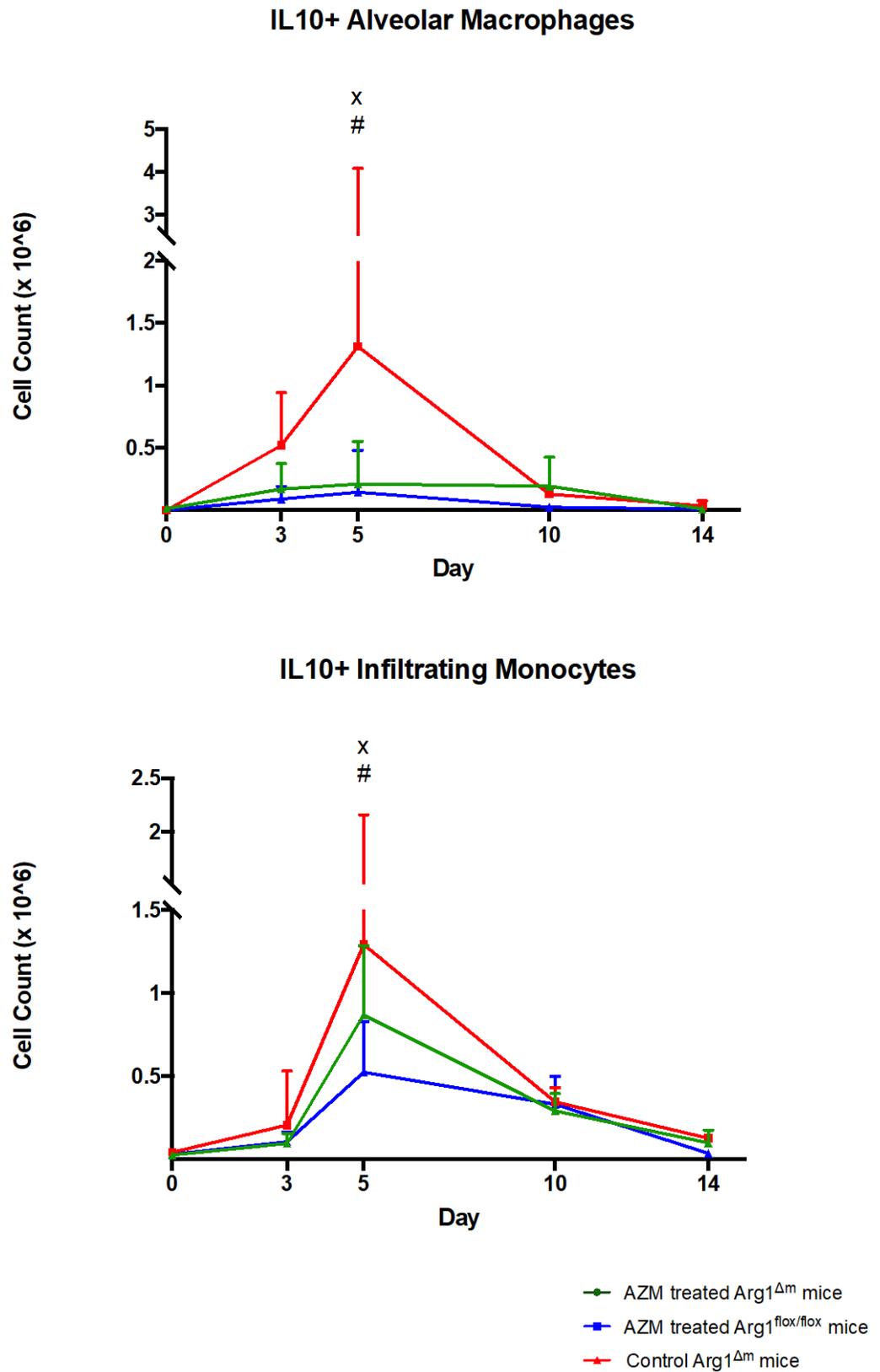


Figure 5.2i

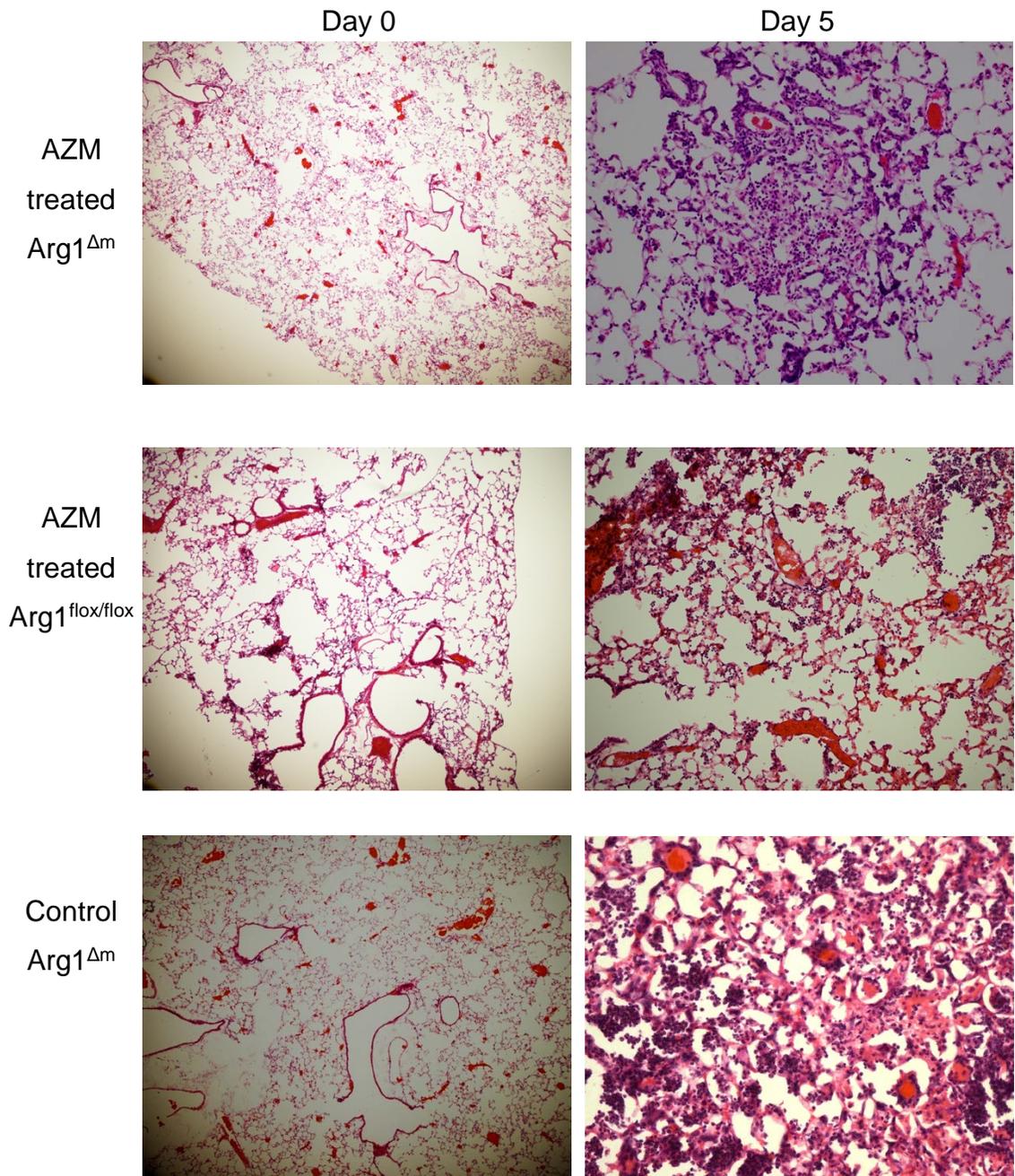


Figure 5.2i (continued)

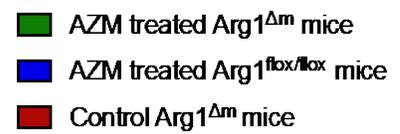
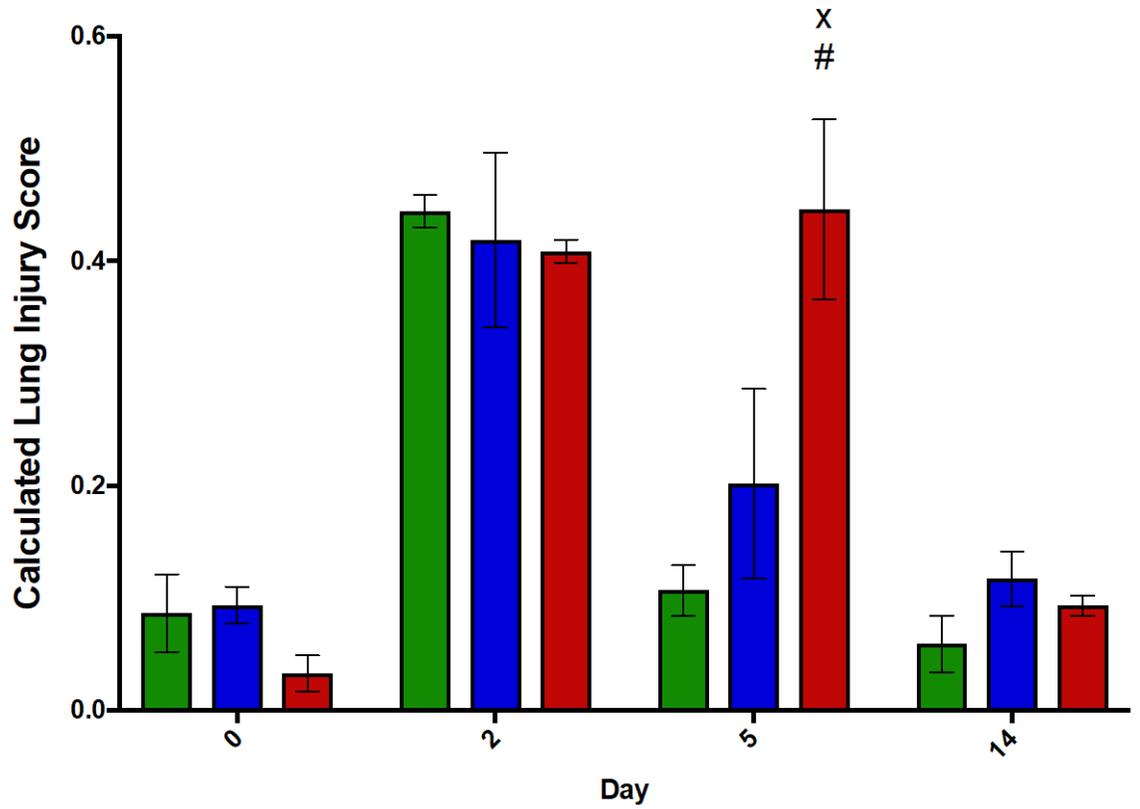


Figure 5.2. Azithromycin treatment attenuates acute inflammatory infiltration of innate immune cells in arginase conditional knock-out mice.

Lungs from infected mice were lavaged with PBS to collect cells from the alveolar spaces and lung tissues were then harvested to collect cells from the lung interstitium. Lavage and lung tissue samples were collected from at least four mice per timepoint per group. Harvested samples were processed into single cell suspensions and stained for flow cytometry analysis as described. At least 50,000 events per sample were analyzed using the FlowJo software to quantify different immune cell populations. Data represent total count of each cell population as a fraction of the total number of live cells analyzed. (a) Neutrophil recruitment into the alveolar and lung interstitial spaces (b) Total number of tissue and infiltrating macrophages. (c, d) The total number of tissue and infiltrating M1 macrophages expressing CCR7 receptor and positive for iNOS and TNF- $\alpha$ . (e, f, g, h) Total counts of alveolar and infiltrating macrophages producing TNF- $\alpha$ , iNOS, IL-10, or expressing Mannose receptor (MR). (i) Lungs were insufflated and fixed in paraformaldehyde for sectioning. Lung sections were stained with H&E and lung injury was assessed by an independent blinded investigator. Lung injury was scored as described in methods. The score ranges from 0-1 (inclusive). Data represent mean  $\pm$  SD and is representative of 3 independent experiments. Statistical significance determined by two-way ANOVA ((x) azithromycin treated arginase conditional knock-out mice significantly different than control arginase conditional knock-out mice; (#) azithromycin treated littermate control mice group significantly different than control arginase conditional knock-out mice; (+) azithromycin treated arginase conditional knock-out mice significantly different than the azithromycin-treated littermate control mice; p-value < 0.05). Graphs plotted using GraphPad Prism 7 (Note: for some points, the error bars are shorter than the height of the symbol. In these cases, Prism simply does not draw the error bars).

Figure 5.3a

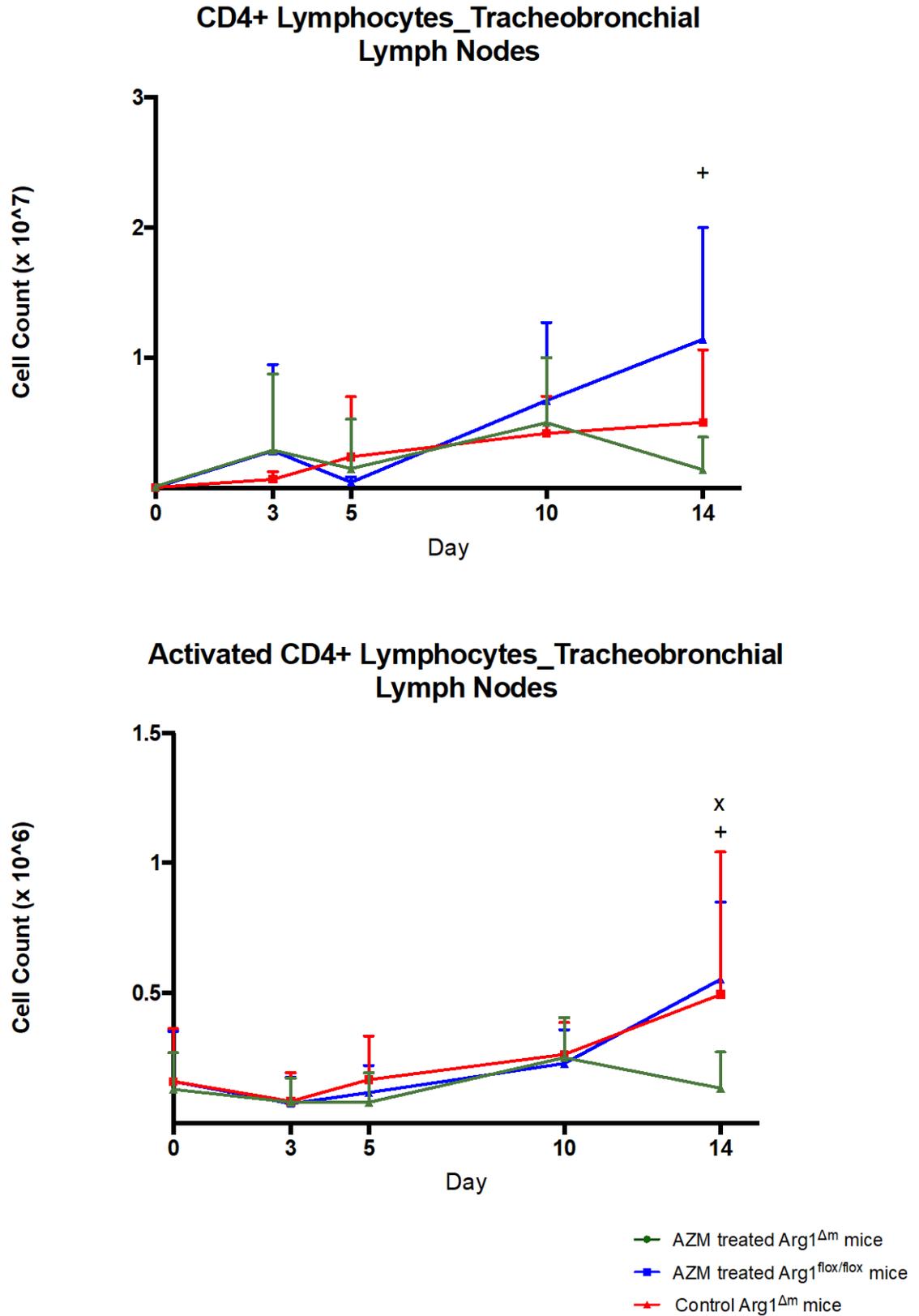


Figure 5.3b

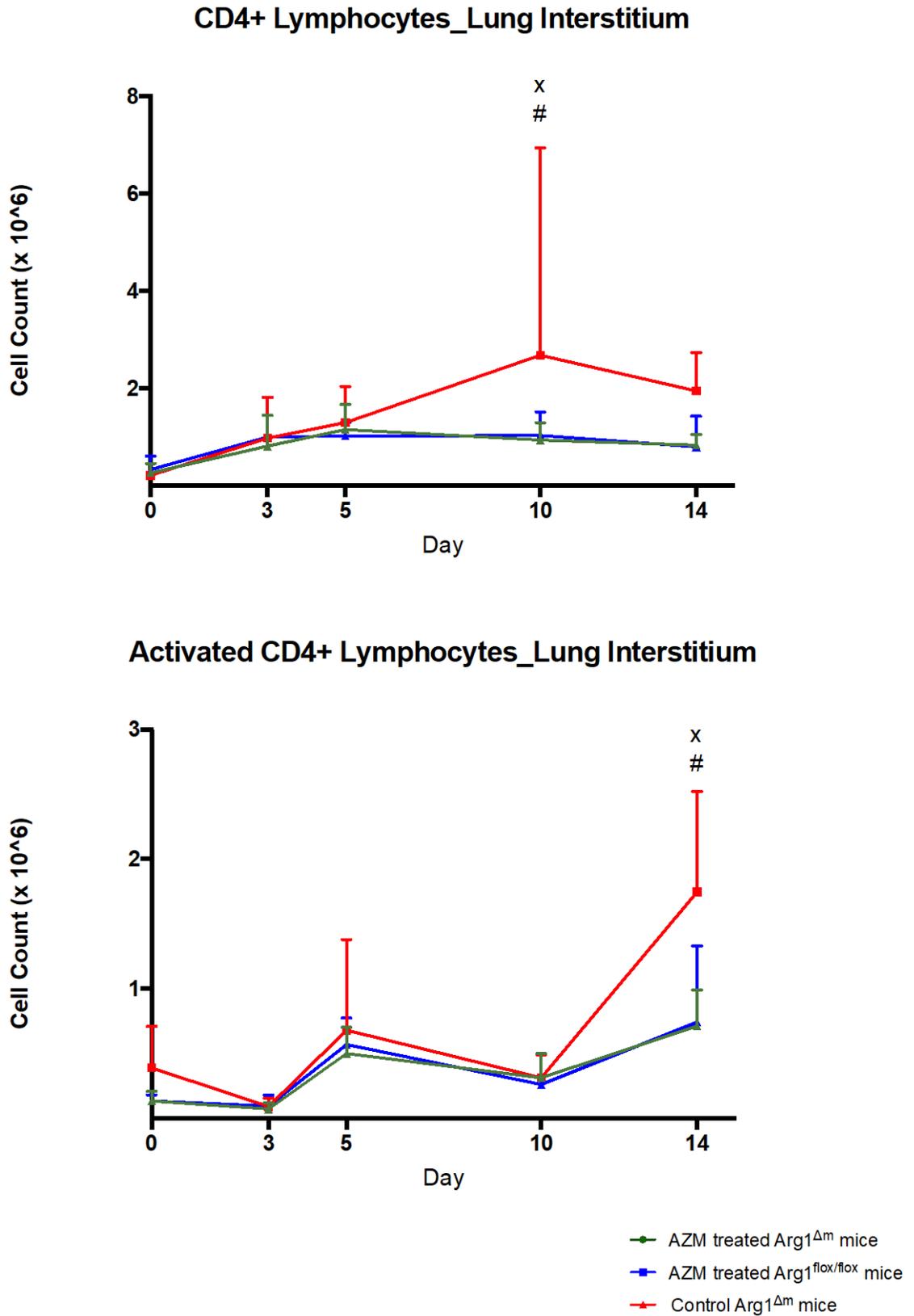
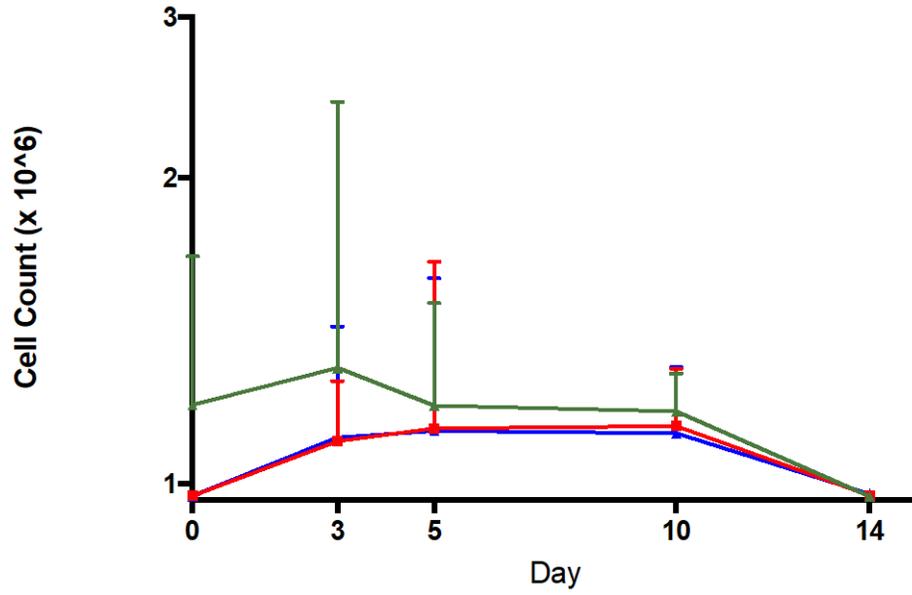
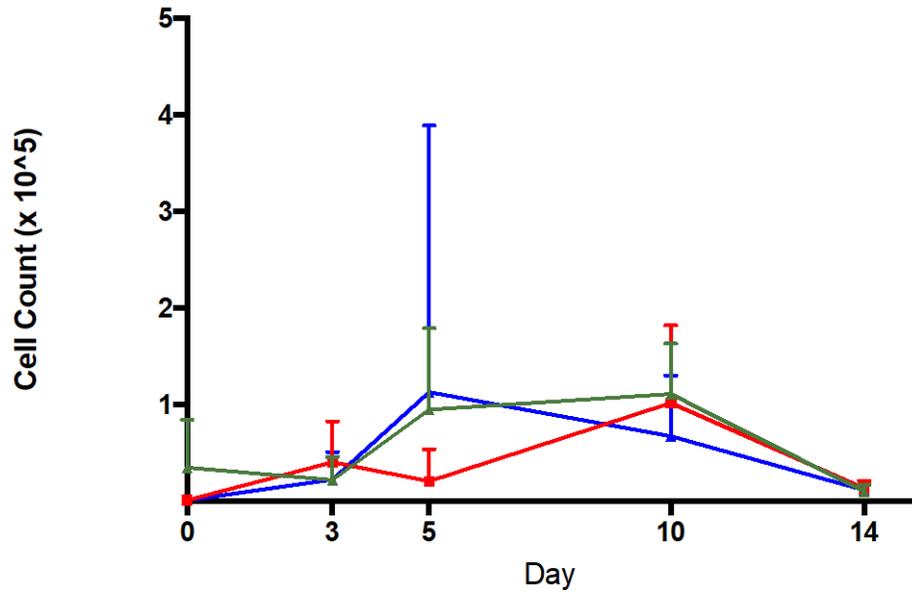


Figure 5.3c

### CD4+ Lymphocytes\_Alveolar Spaces



### Activated CD4+ Lymphocytes\_Alveolar Spaces



- AZM treated  $Arg1^{\Delta m}$  mice
- AZM treated  $Arg1^{flox/flox}$  mice
- Control  $Arg1^{\Delta m}$  mice

Figure 5.3. Azithromycin treatment in arginase conditional knock-out mice is associated with decreased CD4<sup>+</sup> lymphocyte recruitment and activation. Tracheobronchial lymph nodes draining the site of infection were harvested and processed into single cell suspensions by passing through mesh strainers. Lung lavage and lung tissue samples were collected and processed as described. Single cell suspensions were stained for flow cytometry analysis as described. At least 50,000 events per sample were analyzed using the FlowJo software to quantify different immune cell populations. Data represent total count of each cell population as a fraction of the total number of live cells analyzed. (a, b, c) Total number of CD4<sup>+</sup> T cells recruited and activated in the lymph nodes, alveolar spaces, and lung interstitium of infected mice. Data represent mean  $\pm$  SD. Data are representative of 3 independent experiments. Statistical significance determined by two-way ANOVA ((x) azithromycin treated arginase conditional knock-out mice significantly different than control arginase conditional knock-out mice; (#) azithromycin treated littermate control mice group significantly different than control arginase conditional knock-out mice; (+) azithromycin treated arginase conditional knock-out mice significantly different than the azithromycin-treated littermate control mice; p-value < 0.05). Graphs plotted using GraphPad Prism 7 (Note: for some points, the error bars are shorter than the height of the symbol. In these cases, Prism simply does not draw the error bars).

Figure 5.4a

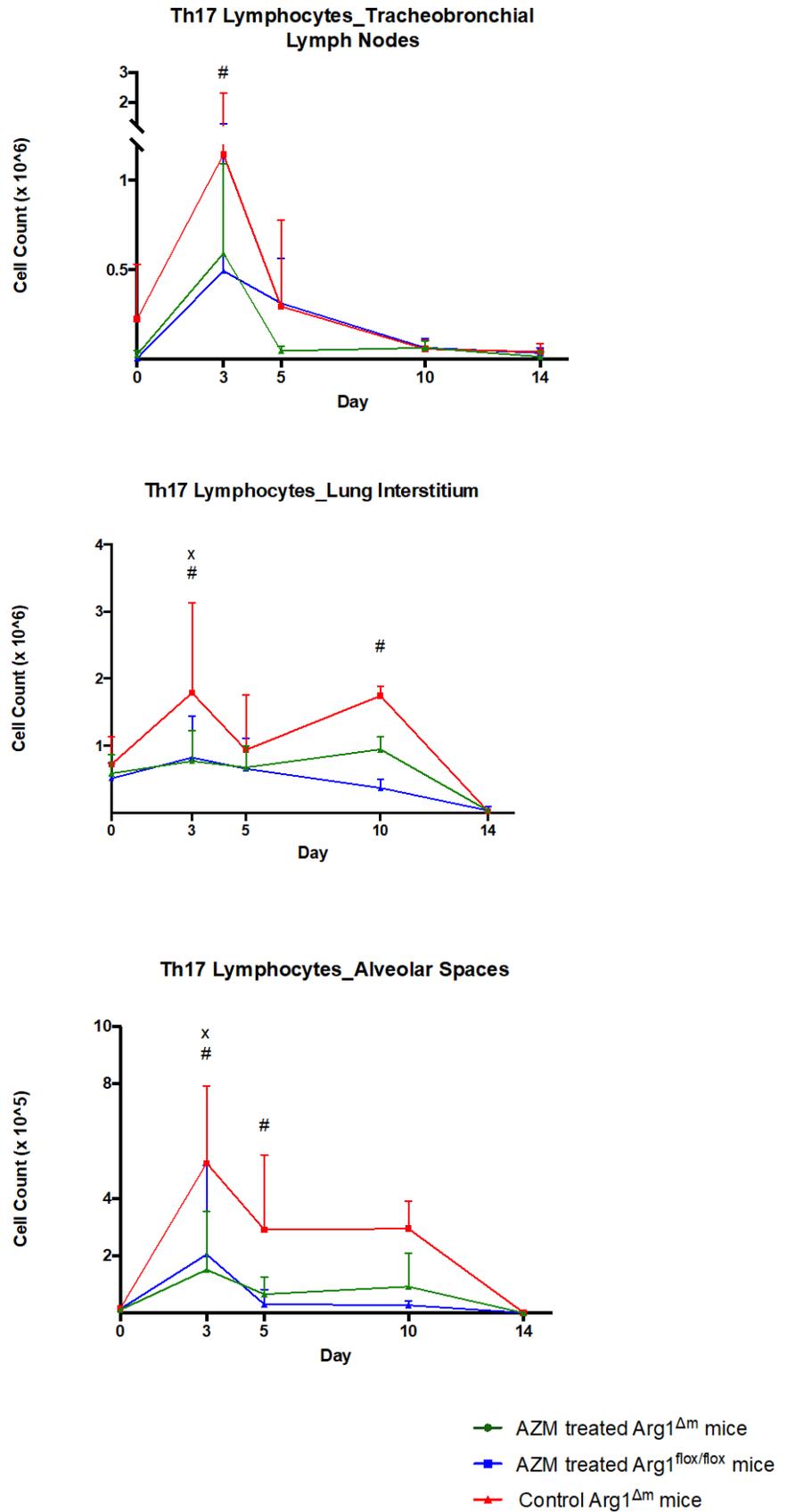
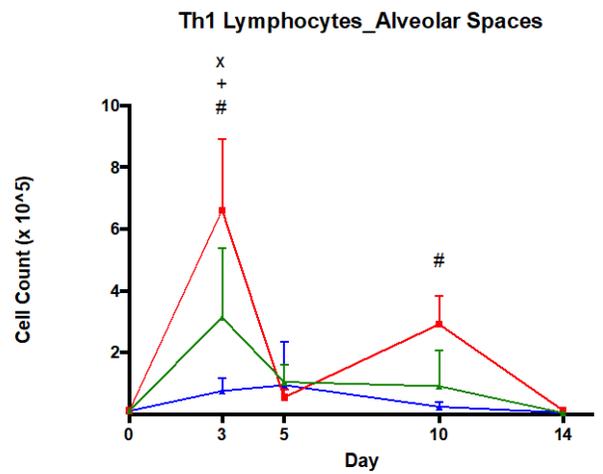
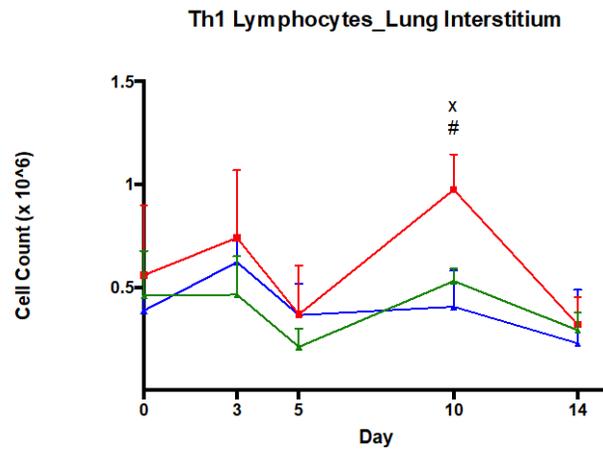
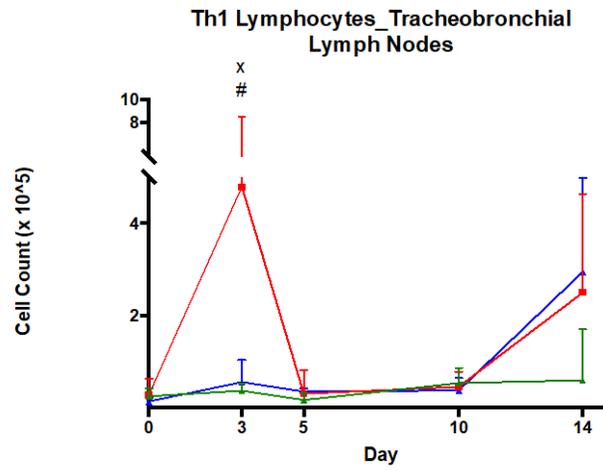
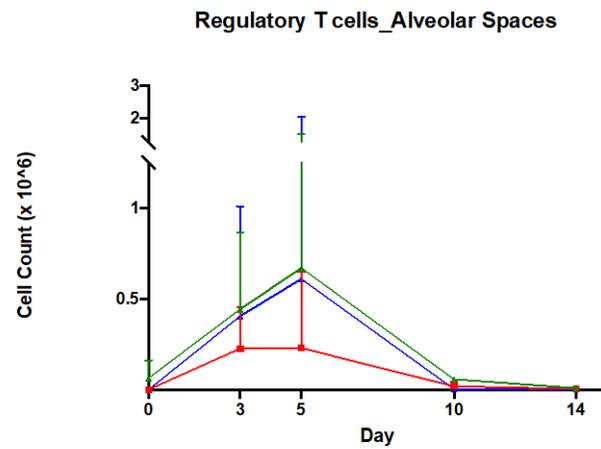
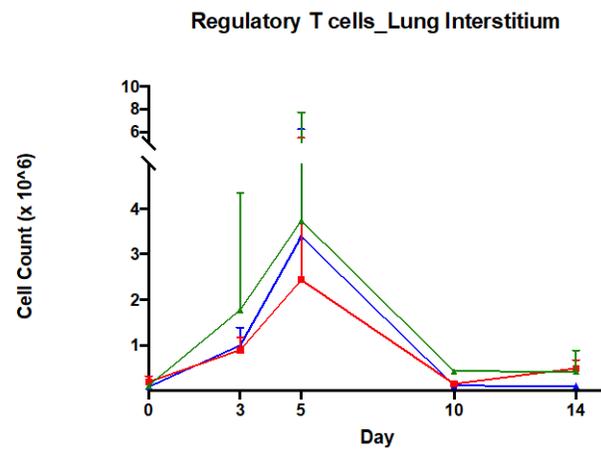
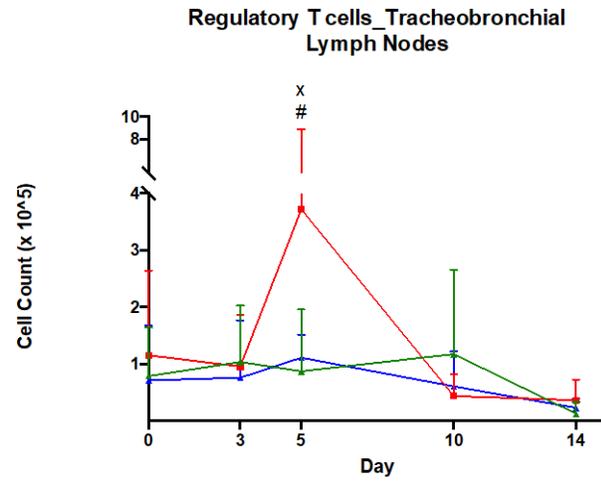


Figure 5.4b



- AZM treated Arg1<sup>Δm</sup> mice
- AZM treated Arg1<sup>flox/flox</sup> mice
- ▲— Control Arg1<sup>Δm</sup> mice

Figure 5.4c



- AZM treated Arg1 $\Delta^m$  mice
- AZM treated Arg1<sup>flox/flox</sup> mice
- Control Arg1 $\Delta^m$  mice

Figure 5.4d

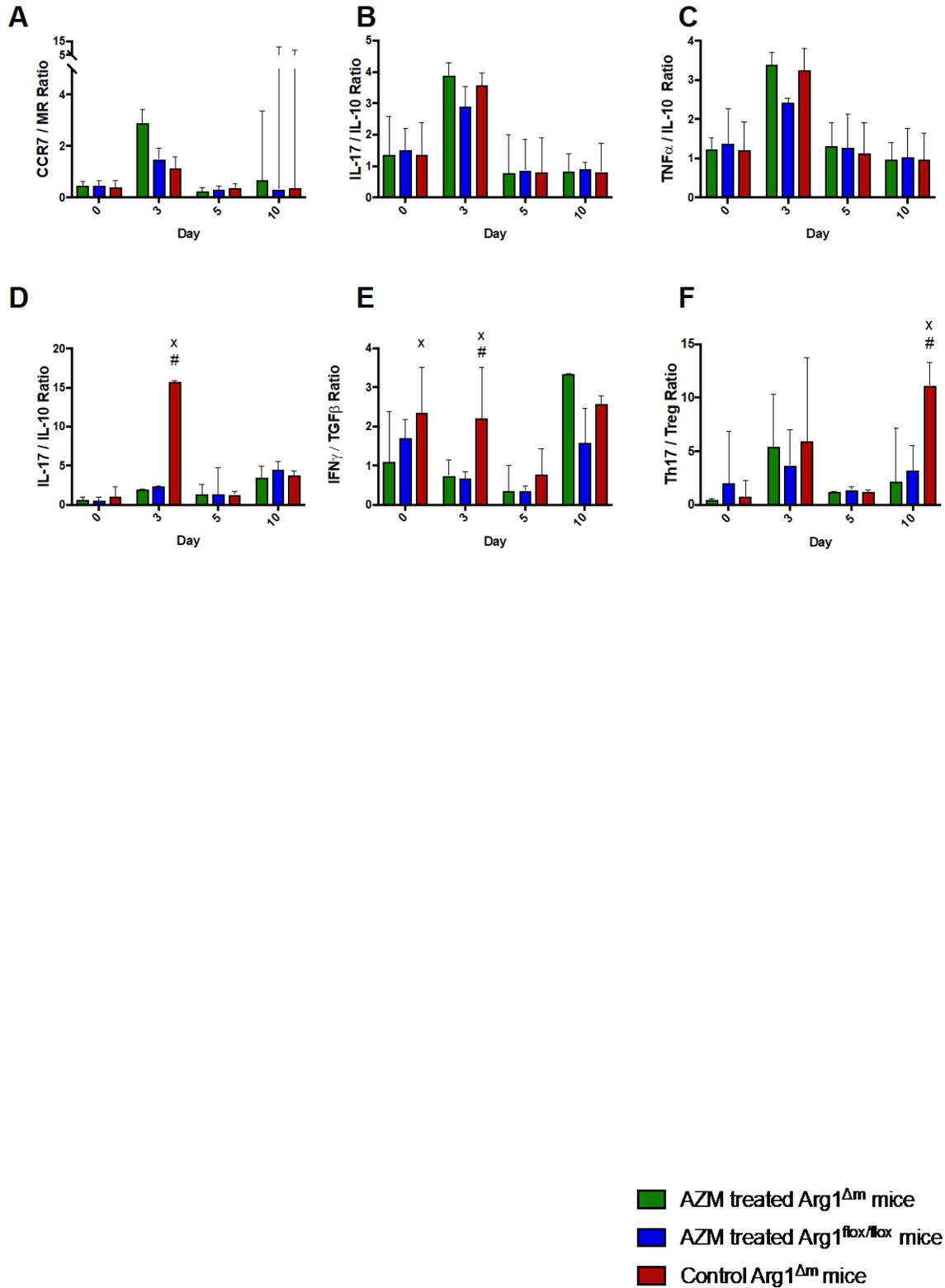


Figure 5.4. Azithromycin treatment in the arginase conditional knock-out mice suppresses the exaggerated recruitment of inflammatory lymphocytes. Tracheobronchial lymph nodes draining the site of infection and lung tissue samples were collected and processed as described. Single cell suspensions were stained for flow cytometry analysis as described. At least 50,000 events per sample were analyzed using the FlowJo software to quantify different immune cell populations. Data represent total count of each cell population as a fraction of the total number of live cells analyzed. (a, b, c) Total number of Th17, Th1, and regulatory T lymphocytes in the lymph nodes, alveolar spaces, and lung interstitium of infected mice. (d) Bar graphs represent ratios of CCR7 to MR expressing cells in A; ratio of IL-17 to IL-10 expressing monocytes in B; ratio of TNF- $\alpha$  to IL-10 expressing macrophages in C; ratio IL-17 to IL-10 expressing lymphocytes in D; ratio of IFN $\gamma$  to TGF $\beta$  expressing lymphocytes in E, and ratio of Th17 to Treg cells in F. Data represent mean  $\pm$  SD. Data are representative of 3 independent experiments. Statistical significance determined by two-way ANOVA ((x) azithromycin treated arginase conditional knock-out mice significantly different than control arginase conditional knock-out mice; (#) azithromycin treated littermate control mice group significantly different than control arginase conditional knock-out mice; (+) azithromycin treated arginase conditional knock-out mice significantly different than the azithromycin-treated littermate control mice; p-value < 0.05). Graphs plotted using GraphPad Prism 7 (Note: for some points, the error bars are shorter than the height of the symbol. In these cases, Prism simply does not draw the error bars).

## Chapter 6: Summary and conclusions

### I. Project Overview

Research work presented in this dissertation aimed at defining the immunomodulatory functions of the alternative macrophage effector, arginase-1, as well as defining the immunomodulatory mechanisms of azithromycin. We utilized genetically modified mouse models, pharmacological inhibitors, in-vitro assays, and clinical approaches to better understand the immune response to infections with PA pneumonia.

Infections with PA pneumonia are the leading cause of morbidity and mortality in patients with cystic fibrosis [1-6]. As discussed in the introduction, cystic fibrosis is an autosomal recessive disorder caused by mutations of the CFTR gene. This mutation is associated with disrupted ion flux across the epithelium resulting in the production of abnormally thick and dense mucus which obstructs the airways, pancreatic ducts, intestines, and other organs [1-7]. Additionally, patients suffering from this mutation develop structural airway abnormalities including increased smooth muscle content and smooth muscle cell hyperplasia [12]. These changes predispose the patients to chronic and repetitive lung infections against which the immune system responds in an exaggerated manner. The increased risk for infections is attributed to the failure of the epithelium to perform its role as the first line of defense against inhaled microbes. This happens due to the abnormally viscous mucus which causes ciliary dyskinesia thereby hindering the mucociliary clearance essential for expelling trapped pathogens [63, 133]. Additionally, the altered airway surface liquid and the increased pH inactivate the epithelial microbicidal substances essential for killing the invading bacteria, viruses, and yeasts [63-91, 102-105, 133, 458]. Additionally, CFTR mutations potentiate the signaling through the NF- $\kappa$ B pathway in epithelial cells which results in a robust and vigorous release of pro-inflammatory mediators upon activation by non-self antigens [63-75, 77-95, 102-105, 133, 458, 459]. These

pro-inflammatory cytokines function to recruit innate and adaptive immune cells which are essential for containing the infection and resolving the immune response. However, several alterations of the innate and adaptive immune responses occur in cystic fibrosis rendering the inflammatory response disproportionate to the severity of the infection.

First, neutrophils are recruited in excessive numbers due to the persistent infections and continuous stimulation. These neutrophils are affected by the CFTR mutations which impair the degranulation of important neutrophil microbicidal products from the secondary and tertiary granules [102]. Additionally, neutrophils release excessive amounts of neutrophil elastases and proteases in the cystic fibrosis lungs which overwhelm the antiproteases that protect the lungs. The excessive amounts of neutrophil elastases break down the connective tissues and matrix proteins leading to a structural loss in the small airways [106-110]. Additionally, neutrophils in cystic fibrosis patients fail to undergo programmed cell death and the resultant necrosis releases the massive intracellular contents like inflammatory mediators and cytokines, oxidants and proteases, in addition to large DNA fragments and actin [117]. Thus, the death of neutrophils releases tremendous amounts of intracellular contents that are extremely harmful to the lungs.

Second, macrophages are recruited into the lungs of cystic fibrosis patients in response to the infection. However, changes in lung microenvironment and the altered inflammatory signals result in altered macrophage polarization, phagocytic function, and antigen presentation. Several reports observed intrinsic macrophage alterations which result in an exaggerated release of pro-inflammatory mediators from cystic fibrosis macrophages compared to normal human macrophages [27, 166-170]. This is attributed to the potentiated NF- $\kappa$ B and STAT-1 signaling pathways in these cells. Additionally, the lung environment in cystic fibrosis skews macrophage polarization depending on the stability of the disease and the nature of infections at specific timepoints. This results in a

disproportional balance of M1/M2 macrophages in patients with cystic fibrosis. Moreover, malfunctional CFTR on the surface of macrophages impairs their microbicidal functions by dysregulating the formation of phagosomes with appropriate pH and by impairing the late endosome/lysosome maturation essential for trafficking of surface pattern recognition receptors [25, 134, 170-176]. Hence, mutated CFTR on the surface of macrophages drastically affects their ability to present antigens for incoming immune cells.

Third, altered adaptive immune responses in patients with cystic fibrosis are attributed to the impaired communication between innate and adaptive immune cells and to the skewing of the T cell responses towards a Th2 and Th17 predominant response. Innate immune cells fail to present antigens and provide necessary costimulation for B and T cell selection. This results from non-CFTR genetic mutations of the MHC complex which affects efficient antigen presentation. Moreover, suppressed PD-L1/PD-1 interaction between airway epithelial cells and T cells results in uncontrolled activation of the adaptive immune response [63, 133, 182, 183, 188-192]. Additionally, naïve T cells isolated from cystic fibrosis patients are inherently predisposed to be polarized towards Th2 and Th17 phenotypes [203, 204]. Moreover, there is a significant imbalance of signals and cytokines which drive T cell differentiation towards a Th17 predominant phenotype versus signals essential for limiting this response. In fact, T cells with mutated CFTR can equally differentiate under the influence of appropriate cytokines into Th1 and Treg phenotypes. However, when T cells with CFTR mutations are polarized with Th17 stimulants, there is a higher and faster shift into the Th17 phenotype compared to wild-type lymphocytes. Importantly, Th17 lymphocytes and IL-17 production are associated with poor prognosis as they play an early role in disease pathology. They are associated with sustained neutrophil recruitment and a decline in lung function [67, 210-219, 221].

Collectively, intrinsic and environmental alterations result in an exaggerated immune response. These alterations impair the function of immune cells thereby

failing to contain and clear the infections. This results in a vicious cycle of exaggerated inflammation, inflammatory lung injury, and persistent infections [185, 233, 235-247, 460]. Our long-term goal is to define key regulators of this exaggerated immune response that can be of therapeutic value for immunotherapy in cystic fibrosis. Our group has previously demonstrated that azithromycin, a macrolide antibiotic, drives macrophages into an alternative M2 phenotype in-vitro [166, 310, 311]. Additionally, murine experiments with early polarization of macrophages into an alternative phenotype show decreased neutrophil influx and pulmonary injury in mice infected with PA. Alternatively, abolishing alternative macrophage polarization was associated with a profound acute immune response along with exaggerated neutrophil influx and an altered T cell response. The absence of alternative macrophages was also associated with increased morbidity and mortality in our murine model of PA pneumonia [166, 310, 311]. Closely examining the different effectors of alternative macrophages revealed an important role of arginase-1. Arginase-1 expression and activity are increased in alternative macrophages polarized with azithromycin. Moreover, preliminary experiments using arginase-1 conditional knock-out mice verified that arginase deficiency is associated with greater morbidity in terms of more significant weight loss. Additionally, arginase-1 has unique immunomodulatory properties where it is shown in other disease models to control of NO-mediated injury, to suppress T cell function and proliferation, and to promote a Treg phenotype [17, 151, 152, 154, 157, 312, 314-318].

Hence, the objective of this project was to investigate the regulatory role of arginase in the immune response to PA pneumonia and its modulation of T cell immunity. Additionally, our second objective was to evaluate the anti-inflammatory mechanisms of azithromycin and the dependence of these mechanisms on arginase. Our central hypothesis was that regulation of exaggerated inflammation achieved by polarizing macrophages into an alternative anti-inflammatory phenotype is dependent on arginase production.

Our second hypothesis was that azithromycin controls immunity in PA pneumonia via cross-inhibition of the NF- $\kappa$ B and STAT-1 signaling pathways.

## II. Results overview

### i. Myeloid arginase production is essential for regulation of excessive inflammation in PA pneumonia

Our results show that arginase production by myeloid cells is essential to regulate immunity against PA pneumonia. We utilized Arg1 $\Delta^m$  mice with conditional arginase deletion from macrophages and neutrophils along with two global pharmacological arginase inhibitors to examine the effects of arginase on the immune response. Mice were infected with PA-laden agarose beads to cause a prolonged infection similar to the chronic PA pneumonia in patients with cystic fibrosis. We used flow cytometry analysis to identify the immune cell populations activated and recruited in response to the infection. Our results show that arginase deletion from myeloid cells resulted in increased morbidity and weight loss post infection, while global arginase inhibition resulted in exaggerated weight loss (Chapter 3). Additionally, Arg1 $\Delta^m$  mice responded to the infection with an amplified influx of neutrophils and pro-inflammatory macrophages into their lungs and alveolar spaces. BALBc/J mice dosed with arginase inhibitors responded with significantly higher numbers of neutrophils and macrophages compared to the Arg1 $\Delta^m$  mice. Additionally, Arg1 $\Delta^m$  mice responded with significantly increased numbers of CD4<sup>+</sup> T cells and activated T cells along with a predominant polarization towards a Th1 and Th17 response. Conversely, global arginase inhibition was associated with increased numbers of CD4<sup>+</sup> T cells and activated T cells in the lymph nodes and in the lung interstitial spaces of infected mice while these effects were reversed in the alveolar spaces. This trend also applied to the Th1, Th17, and Treg polarized lymphocytes in the lymph nodes with potentially reduced transmigration of these lymphocytes to the lung interstitial and alveolar spaces of mice treated with BEC or L-norvaline.

Therefore, our results show that myeloid arginase is essential to limit T cell proliferation and activation and to protect against disproportionate polarization towards a Th1 and Th17 predominant response which contributes to excessive neutrophil and pro-inflammatory macrophage recruitment. However, global arginase inhibition results in exaggerated morbidity and inflammation along with blunted lymphocyte recruitment and chemotaxis to the lungs.

ii. Azithromycin balances the M1/M2 macrophage polarization by cross-inhibiting the M1-associated transcription factors, NF- $\kappa$ B and STAT-1

Our results show that azithromycin acts directly on macrophages by cross-inhibiting the NF- $\kappa$ B and STAT-1 signaling pathways. We utilized an in vitro model of J774 murine macrophages which were polarized with azithromycin and cytokines to drive macrophage polarization towards an M1 or an M2 phenotype. Using a p65 translocation assay coupled with western blot analysis of p65 protein in the nuclear versus the cytoplasmic compartments and using immunofluorescence assay to visualize p65 localization in the cell upon stimulation, we demonstrated that azithromycin significantly suppresses p65 translocation to the nucleus of stimulated macrophages. We also showed that azithromycin results in increased IKK $\beta$  protein concentrations along with blunted I $\kappa$ B- $\alpha$  degradation. Our results also show increased IKK $\beta$  gene expression with azithromycin treatment suggesting that increased IKK $\beta$  protein concentration results from the inhibited p65 nuclear translocation which blunts the negative feedback response essential to downregulate IKK $\beta$  and to turn off the NF- $\kappa$ B pathway. Additionally, we showed that accumulated IKK $\beta$  was associated with cross-inhibition of the STAT-1 pathway with azithromycin. Moreover, our results show that IKK $\beta$  played a critical role in azithromycin anti-inflammatory mechanisms. Results from experiments with IKK-16, an IKK $\beta$  inhibitor, showed a reversal of azithromycin effects on arginase expression and activity as well as on the suppressed STAT-1 activation. Therefore, azithromycin polarizes

macrophages towards an alternative M2 phenotype by cross-inhibiting the NF- $\kappa$ B and STAT-1 signaling pathways through a mechanism dependent on IKK $\beta$ .

iii. Azithromycin protects against excessive morbidity and inflammation through mechanisms independent of arginase-1 production

Our results show that azithromycin regulates neutrophil influx and T cell responses in mice infected with PA independent of myeloid arginase production. Arg1 $\Delta^m$  mice were pre-treated with azithromycin starting four days prior to the infection and daily thereafter. Azithromycin protected against excessive morbidity and weight loss in Arg1 $\Delta^m$  and Arg1<sup>flox/flox</sup> mice. Additionally, azithromycin suppressed the excessive recruitment of neutrophils and pro-inflammatory macrophages independent of myeloid arginase production. Importantly, azithromycin suppressed Th1 and Th17 responses and skewed towards a Treg phenotype equally in Arg1 $\Delta^m$  and Arg1<sup>flox/flox</sup> mice. Additionally, azithromycin treatment in Arg1 $\Delta^m$  and Arg1<sup>flox/flox</sup> mice was associated with faster recovery in terms of earlier resolution of inflammatory lung injury compared to untreated mice. Therefore, azithromycin protects against excessive morbidity and inflammation in chronic PA pneumonia through mechanisms independent of arginase production by macrophages and neutrophils.

### III. Significance

i. Myeloid arginase, a therapeutic target to control inflammation

Arginase production by myeloid cells was essential to limit inflammation and to regulate the influx of pro-inflammatory neutrophils and lymphocytes into the lungs in response to PA infections. We provided evidence that exaggerated inflammation can be regulated by directly modulating the production of arginase by myeloid cells while other cellular sources of arginase exert potentially different functions. The significance of this evidence lies in the potential of myeloid

arginase production to regulate the influx of neutrophils and Th17 lymphocytes, both of which are highly pathogenic in patients with cystic fibrosis. The increased IL17 release by Th17 lymphocytes is associated with increased disease severity, decline in lung function, and end-stage bronchiectasis [461]. According to Mulcahy et al., cystic fibrosis is a Th17-mediated disease [221]. They evaluated T cell responses in 42 cystic fibrosis patients ranging from 6 months to 53 years of age. They observed a very strong association between Th17 numbers and poor lung function. Malcahy et al. suggest that peripheral blood Th17 levels may be a surrogate marker of lung function in cystic fibrosis [221]. Additionally, Th17 lymphocytes contribute to pathology by recruiting neutrophils in cystic fibrosis as well as in other disease models like Leishmaniasis, rheumatoid arthritis, inflammatory bowel disease, and crescentic glomerulonephritis [348, 349, 462]. Neutrophils are immunopathogenic in these disease models where they contribute to inflammatory tissue injury as a result of the extensive release of proteinases, neutrophil elastases, DNA, and oxidative free radicals [348, 349, 462]. In the case of PA infections in cystic fibrosis, Dubin and colleagues conclude from an extensive literature review that the inability to clear PA results in chronic IL-17 production and the development of a Th17 phenotype [163, 215-218, 222, 463]. The persisting neutrophilia fails to clear the pathogen [222, 464, 465], which triggers the recruitment of more neutrophils that result in inflammatory lung injury and a decline in lung function [466, 467]. Therefore, identifying a pathway that can limit Th17 lymphocyte activation thereby controlling the influx of neutrophils represents a very promising target to manipulate excessive inflammation in cystic fibrosis, cancer, autoimmunity, and other inflammatory diseases. Our data suggest that arginase can serve as a novel target to control immunity and to prevent inflammatory lung injury in cystic fibrosis.

In fact, there are recombinant human arginase formulations in clinical trials being evaluated in multiple disease models. A pegylated recombinant human arginase (BCT-100) is currently in phase I/II trial where its safety and efficacy in children

and young adults with relapsed/refractory leukemia, neuroblastoma, sarcoma and high-grade gliomas are being evaluated [468]. Moreover, there are several small molecules that are being evaluated for inducing endogenous expression of arginase by signaling through pathways involved with arginase expression [208, 469-472]. However, whether those approaches can control immunity in cystic fibrosis without dampening the essential functions of the immune system is yet to be elucidated. It is important to develop new approaches that would balance the immune response rather than suppressing it and preventing bacterial clearance. Therefore, it is pertinent to closely evaluate the effects of arginase administration in humans on the immune system and to consider targeted approaches to deliver arginase to the sites of infection or to stimulate arginase expression in particular cells without globally promoting suppressed immunity.

ii. NF- $\kappa$ B signaling pathway, a targeted approach to limit pro-inflammatory macrophage polarization

We show that azithromycin controls pro-inflammatory macrophage activation by cross-inhibiting the NF- $\kappa$ B and STAT-1 signaling pathways. The beneficial clinical effects of azithromycin are well established in patients with cystic fibrosis [296-304]. Azithromycin is known to regulate pro-inflammatory cytokines and to improve lung function in these patients [296, 297, 299-304, 306, 307]. Our results are significant in that they identify the specific molecular mechanisms that can be targeted to modulate the M1/M2 macrophage balance thereby regulating inflammation. Targeting macrophage-specific NF- $\kappa$ B and STAT-1 signaling pathways provide a novel approach that would protect against the unwanted side effects of azithromycin and against the potential antimicrobial resistance and other issues of collateral damage. Additionally, it protects against global NF- $\kappa$ B inhibition that would result in suppressed immunity. According to Hoesel et al., inhibition of the NF- $\kappa$ B pathway is a very effective approach to limit chronic inflammation and to promote anti-tumorigenic effects of many chemotherapeutic agents [394]. However, NF- $\kappa$ B is activated in several cell lineages and is

essential to induce sufficient inflammation needed to clear infections and to promote regulated cell death and other physiological functions activated through the NF- $\kappa$ B signaling pathway [394]. For example, dexamethasone, prednisone, methylprednisolone, aspirin, cyclosporin A, indomethacin, and ibuprofen are all reported to inhibit NF- $\kappa$ B [416, 417]. However, their effects are generalized and non-macrophage specific thereby resulting in suppressed immunity and inability of the immune system to clear the invading pathogen. Additionally, azithromycin inhibits the NF- $\kappa$ B pathway through a unique mechanism which cross-inhibits the pro-inflammatory STAT-1 transcription factor. According to Breuer a STAT1 gain of function mutation promotes severe bronchiectasis even in non-cystic fibrosis patients [473]. Additionally, Kretzmer and colleagues report that increased STAT-1 phosphorylation was associated with recurrent pneumonia, exaggerated inflammation, and severe bronchiectasis in a 5-year-old child [474]. Whereas Kreiselmeier et al., report that cystic fibrosis pathology is associated with altered STAT-1 activation which mediates pathogenesis by altered iNOS induction while statin-mediated correction of STAT-1 signaling may represent a potential avenue for therapeutic intervention in cystic fibrosis [475]. Therefore, the ability of azithromycin to target two transcription factors involved in immunopathogenesis and exaggerated inflammation in cystic fibrosis constitutes a favorable approach to limit pathology using a single agent. Identifying the cross-inhibitory mechanism of azithromycin mediated via IKK $\beta$  presents a promising approach to be considered for the development of immunomodulatory agents that target several pathological mediators.

Additionally, identifying the molecular mechanisms of azithromycin is essential to improve the efficacy and functionality of the drug in other disease models. Our group has shown promising cardioprotective effects with azithromycin pretreatment in myocardial infarction (MI). Unpublished preliminary data show a significant reduction in mortality in a murine model of MI. Additionally, azithromycin pretreatment resulted in attenuated inflammation and enhanced recovery post-MI in mice. Our collaborators have shown in a murine model of

spinal cord injury that azithromycin promotes an anti-inflammatory phenotype and improves tissue sparing and recovery of gross and coordinated locomotor functions [443]. However, myocardial infarctions and traumas resulting in spinal cord injury in humans happen suddenly; therefore, it is essential to identify azithromycin analogs that exert comparable beneficial anti-inflammatory effects post-injury or to develop formulations that are associated with fast attainment of steady-state concentrations essential for azithromycin's immunomodulatory effects. Moreover, our group is working with medicinal chemists to develop azithromycin analogs that lack the antimicrobial activity and improve upon the anti-inflammatory properties. Thus, identifying azithromycin molecular targets and developing new analogs or small molecules that would exert immediate beneficial effects are crucial to extend the usefulness of this drug.

iii. Arginase production by myeloid cells is not required for azithromycin anti-inflammatory effects

Azithromycin blunts the Th1 and Th17 immune responses thereby protecting against excessive neutrophil recruitment and inflammatory lung injury in response to PA infections. Importantly, azithromycin does not require myeloid arginase production to exert its anti-inflammatory effects. These effects were associated with increased secretion of TGF- $\beta$  and IL-10 which are most likely responsible for the induction of the regulatory T cell phenotype thereby suppressing Th1 and Th17 responses [447-453]. Therefore, the significance of these results includes the identification of alternative pathways for targeted immunotherapy against aberrant T cell responses and exaggerated inflammation in cystic fibrosis. According to Kramer and colleagues, TGF- $\beta$  targeted therapeutics can be complicated in cystic fibrosis due to differential effects on this cytokine on different cellular mechanisms [476]. For instance, Sun et al., show that TGF- $\beta$  disrupts the function and the numbers of CFTR channels along the cystic fibrosis epithelium [477]. Similarly, Arkwright suggests that increased TGF- $\beta$  concentrations are associated with a decline in lung function in patients

with cystic fibrosis [478]. These effects were mediated via direct effects of TGF- $\beta$  on the lung parenchyma and ion flux across the CFTR channels. Therefore, developing targeted TGF $\beta$ -dependent therapeutics is required to prevent the unwanted detrimental effects [477, 478]. Accordingly, we have identified azithromycin as a potentially novel and targeted TGF $\beta$ -modulator which regulates T cell responses without broadly impacting other necessary TGF $\beta$  signaling effects.

Additionally, the ability of azithromycin to exert protective effects independent of arginase suggests possible synergistic mechanisms between these two powerful immunomodulatory effectors. New therapeutic approaches focus on combination therapies to target the different elements of the altered immune response. According to Randhawa et al., combination immunotherapy including azathioprine and cyclosporine which act through independent mechanisms resulted in significantly improved lung function and reduced hospitalization along with reduced need for IV antibiotics [479]. Similarly, Obaid and colleagues report that combination immunotherapy including IL-21 and anti-IL10 antibodies act synergistically by correcting the different aspects of the altered immune response in patients with Hepatitis C Virus (HCV) infections [480]. Additionally, the NICE clinical guidelines recommended a combination of nivolumab and ipilimumab both of which boost the activity of the immune system against cancer cells [481]. Therefore, combination immunomodulatory therapies are widely considered in several disease models where different aspects of the immune response are altered. Here we identify two distinct immunomodulators which act through independent mechanisms to be considered for new potential combinations.

#### IV. Future directions

##### i. Evaluating the contribution of L-arginine synthesis in modulating T cell responses to PA infections

Our results show that arginase controls T cell proliferation and polarization towards Th1 and Th17 phenotypes. As discussed in Chapter 3, these effects are very likely to be mediated with L-arginine depletion. Future studies will evaluate the hypothesis that eliminating T cell L-arginine synthesis will impair Th17 proliferation, recruitment, and cytokine production in response to PA infection. The studies will focus on whether L-citrulline metabolism regulates the accumulation and effector profile of T cells during PA infection, and whether PA-associated pathophysiology or burden is regulated by T cell L-citrulline metabolism. L-citrulline is an essential precursor for L-arginine synthesis. Recent evidence supports the contribution of L-citrulline during immune cell activity, especially during host defense to infectious disease [482]. We will test whether T cells require L-citrulline to L-arginine synthesis to regulate neutrophil recruitment and defense to PA. This will be achieved using  $Asl^{\Delta Tcell}$  mice, a novel mouse model in which T cells cannot synthesize L-arginine from L-citrulline. We anticipate decreased CD4<sup>+</sup> T cell accumulation and Th17 responses in  $Asl^{\Delta Tcell}$  mice as compared to littermate controls. We also expect decreased neutrophil accumulation and increased PA burden in the lungs of  $Asl^{\Delta Tcell}$  mice as compared to littermate controls. If our hypothesis is correct, this will validate the effects of arginase on T cell activation and neutrophil recruitment via arginine depletion.

##### ii. Evaluating the requirements of non-myeloid arginase in the anti-inflammatory mechanisms of azithromycin

It is important to note that we evaluated the effects of azithromycin in a murine model with conditional arginase deletion from macrophages and neutrophils. It is possible that other cellular sources of arginase can rescue the depleted myeloid

arginase. It is also possible that azithromycin promotes arginase expression in other immune cells. Therefore, it is essential to evaluate the effects of azithromycin with global arginase inhibition and in other models of conditional deletion from non-myeloid cells. We will evaluate the effects of azithromycin on the immune response in BALB/cJ mice and C57BL/6 mice dosed with BEC and L-norvaline. Also, will evaluate the immunomodulatory effects of azithromycin in  $Asl^{\Delta T_{cell}}$  mice. Therefore, we will test if azithromycin can control inflammation and modulate T cell disposition in these models.

Additionally, we will evaluate the requirement for TGF- $\beta$  for azithromycin protective effects. This will be achieved by utilizing TGF- $\beta$  neutralizing antibodies in azithromycin-treated  $Arg1^{\Delta m}$  mice. Additionally, future studies will examine the effects of azithromycin on non-arginase mediated signaling pathways involved with wound healing including the Smad pathway [452]. This will be achieved by utilizing Lck-creSmad4 mice. These mice lack the Smad4 intracellular signaling molecule in lymphoid cells. This protein is responsible for the signaling cascade through the receptor that binds to members of the TGF $\beta$  superfamily [453].

### iii. Evaluating the effects of arginase deletion in cystic fibrosis mouse models

Work presented in this dissertation evaluated the immune response against chronic PA pneumonia in non-cystic fibrosis murine models. We used a mucoid PA strain isolated from a cystic fibrosis patient and we incorporated the bacteria in agarose beads to imitate the infection in humans. Heeckeren and colleagues show that this method causes a chronic infection in mice that mimics the colonization in humans. Intra-tracheal instillation of PA immobilized in agarose beads has proven to be very successful in revealing cystic fibrosis defects in bacterial clearance and it ensues an exaggerated inflammatory response [483]. However, it is important to take into consideration the various alterations of the immune response caused by CFTR mutations in humans (discussed in chapter

1). Therefore, future studies will utilize CFTR knock-out mice to validate that arginase controls inflammation and that azithromycin is effective in mice with mutated CFTR. At least 11 models of CFTR knock-out mice are available targeting the most common mutations  $\Delta F508$  and G551D [484]. Earlier models of CFTR knock-out mice were limited by the very low survival rate, lack of cystic fibrosis pulmonary manifestations, resistance to certain pathogens, and development of severe intestinal obstruction [484-489]. We will use CFTR<sup>tm1G551D</sup> and CFTR<sup>tm1EU</sup> to examine the effects of arginase and azithromycin in mice with the most common mutations, G551D and  $\Delta F508$ . If our clinical studies reveal any preferential effects with specific mutations in humans, we will consider mouse models representative of these mutations.

iv. Evaluating the clinical applicability of azithromycin anti-inflammatory mechanisms

Investigating the clinical applicability of our results is still ongoing. In order to study the immune changes induced with azithromycin in patients with cystic fibrosis we proposed a prospective, unblinded, non-randomized study in which each patient will serve as his or her own control. Patients with cystic fibrosis currently taking azithromycin who meet the inclusion/exclusion criteria will be consented/assented. Enrolled subjects will be taken off of azithromycin for 60-90 days. Three sputum samples induced via inhalation of hypertonic saline will be collected from each patient at 3 different timepoints. The first sputum sample will be collected after the patient had been on azithromycin for at least a year and prior to the initiation of the 60-90 day azithromycin holiday. The second sputum sample will be collected by the end of the 60-90 day drug holiday period and patients will re-initiate azithromycin therapy. Finally, the third sample will be collected again 2-3 months after azithromycin re-initiation. This pilot clinical study is unique in that it generates matched data from subjects while on and off of azithromycin. Data generated from this study will be very useful in identifying pathways affected by azithromycin in humans. Additionally, this study will allow

us to identify specific genes and immune cells induced with azithromycin and the possible correlation with clinical effectiveness by measuring the patient's performance when on and off of azithromycin. The study protocol has been fully established and the study was approved by the Institutional Review Board at the University of Kentucky. Three leading pulmonologists at the KY clinic are involved in the study and are pre-screening potential study patients. Our inclusion/exclusion criteria have been very carefully selected to minimize variability in immune function due to parameters including age, medication exposure, and other disease states. Therefore, recruiting patients into our study has been challenging due to the strict inclusion/exclusion criteria. Future work aims to screen more potential subjects and to reassess the inclusion/exclusion criteria if no subjects were eligible.

#### v. Additional future studies

Future work includes extending our human studies and closely evaluating the effects of arginase on T cell responses and transmigration. We hope to broaden our human experiments to evaluate changes in arginase expression and its effects on T cell immunity in cystic fibrosis as well as other disease models. Additionally, future studies aim to delineate the NOS/arginase paradigm and how disruptions in arginine/NO balance can affect the beneficial effects of arginase expression in non-resolving inflammatory conditions. Moreover, future studies will evaluate the mechanisms involved in skewing T cell responses with azithromycin and with arginase and whether it is cytokine-mediated versus a specific direct effect on T cell activation. It is also essential to define the mechanisms by which arginase and azithromycin affect T cell disposition and compartmentalization in the alveolar versus the interstitial spaces. Further studies are also needed to define the effects on T cell chemotaxis and transmigration from the lymph nodes to the sites of infection. Last but not least, it is crucial to define the specific effects of arginase-1 versus arginase-2 isoforms and whether one and not the other modulates T-cell immunity in humans.

## V. Conclusions

### I- Arginase-1 immunomodulatory role in PA pneumonia

We conclude that decreases in inflammation in response to PA pneumonia achieved by polarizing macrophages to an alternatively-activated phenotype is dependent upon the production of arginase-1. Arginase-1 is an important immunomodulatory enzyme which suppresses exaggerated inflammation in PA pneumonia by controlling the Th1 and Th17 responses and therefore protecting against the excessive influx of neutrophils and pro-inflammatory macrophages.

### II- Azithromycin anti-inflammatory mechanism of action

We conclude that azithromycin polarizes macrophages to an M2 phenotype via inhibition of STAT1 through cross-talk from the NF- $\kappa$ B signaling mediators. Azithromycin inhibits p65 nuclear translocation which results in IKK $\beta$  accumulation due to suppressed negative feedback. Thus, inhibiting the NF- $\kappa$ B signaling pathway cross-inhibits the STAT-1 pathway. This modulation is dependent on IKK $\beta$  and results in subsequent suppression of inflammatory gene activation while promoting an anti-inflammatory phenotype.

### III- Dependence of azithromycin-induced M2 macrophage phenotype on Arginase-1 to alter inflammation

Azithromycin balances the immune responses in PA pneumonia via mechanisms independent of arginase. Azithromycin protects against excessive morbidity and exaggerated inflammation by regulating the Th17/Treg balance and thus controlling the influx of neutrophils and inflammatory macrophages to the lungs regardless of arginase production.

Collectively, the work presented in this dissertation significantly improves our knowledge of the immune system in cystic fibrosis. Defining the immunomodulatory mechanisms of arginase and of azithromycin identifies new molecular targets for immunotherapy in patients with non-resolving pulmonary inflammation. Therefore, our research provides hope for the development of new targeted therapies that control inflammation and prevent lung injury without the need for long-term antibiotic use. Moreover, the ability of azithromycin to balance macrophage polarization provides a unique approach for controlling the function of the immune system without blunting the immune response or promoting severe systemic immune suppression like with steroids and other non-specific immunosuppressants.

Additionally, the significance of this work extends to other immune-mediated diseases. In fact, immune dysregulation has been shown to be involved in many disorders where the role of the immune system in disease pathogenesis has not been previously considered. Immune dysregulation has been found to contribute to pathology in solid and hematological tumors, Alzheimer's disease, Parkinson's disease, major depressive disorder, autism, obsessive-compulsive disorders, as well as epilepsy, stroke, and brain injury [490, 491]. Therefore, defining key regulators of the immune response and the primary regulatory mechanisms involved identifies new drug targets for other diseases.

Research from this dissertation provides insight into specific downstream signaling cascades and mechanisms involved with arginase and azithromycin's immunomodulatory functions. We identify specific pathways which modulation can be of therapeutic value for targeted immunotherapy that could be generalizable to multiple immune-mediated disorders.

APPENDIX A: DATA COLLECTION FORM

Subject Study Code Number \_\_\_\_\_

Visit 1: Patient on Azithromycin

Specimen Date \_\_\_\_\_

Specimen collection: \_\_\_\_\_ Induced  
 \_\_\_\_\_ Spontaneous

Inclusion/Exclusion

Age \_\_\_\_\_ CF diagnosis age \_\_\_\_\_ CFTR  
 mutation \_\_\_\_\_

Receiving immunomodulatory agent (steroids—date last dose received \_\_\_\_\_) Yes No

Pregnant, lactating Yes No

Immunomodulatory disease (cancer, DM, obesity, malnutrition, SLE, HIV-infection, transplantation) Yes No

Azithromycin Treatment

Receiving azithromycin Yes No

Dose \_\_\_\_\_

Start \_\_\_\_\_

Duration \_\_\_\_\_

Demographics

Wt \_\_\_\_\_ Ht \_\_\_\_\_ Gender \_\_\_\_\_

Ethnicity \_\_\_ white \_\_\_ black \_\_\_ Asian \_\_\_ other Hispanic \_\_\_yes \_\_\_no

Current medications:

Drug	Start	Stop
_____	_____	_____
_____	_____	_____
_____	_____	_____
_____	_____	_____
_____	_____	_____
_____	_____	_____

Subject Study Code Number \_\_\_\_\_

Other drugs:

Non-prescription \_\_\_\_\_

Other vitamins, supplements \_\_\_\_\_

Labs: Date \_\_\_\_\_

Metabolic panel: Glucose\_\_\_\_ BUN\_\_\_\_ SrCr\_\_\_\_

WBC\_\_\_\_ Hgb\_\_\_\_ Hct\_\_\_\_ Diff: N\_\_\_\_ L\_\_\_\_ E\_\_\_\_ B\_\_\_\_

Pulmonary Function Tests:

Date \_\_\_\_\_ FEV<sub>1</sub> \_\_\_\_\_ FVC \_\_\_\_\_

Date \_\_\_\_\_ FEV<sub>1</sub> \_\_\_\_\_ FVC \_\_\_\_\_

Date \_\_\_\_\_ FEV<sub>1</sub> \_\_\_\_\_ FVC \_\_\_\_\_

Pulmonary Cultures

Date	Result/organism	Sensitivities
------	-----------------	---------------

_____	_____	
_____	_____	
_____	_____	
_____	_____	
_____	_____	

Subject Study Code Number \_\_\_\_\_

Visit 2: After Azithromycin Holiday

Specimen Date \_\_\_\_\_

Specimen collection: \_\_\_\_\_ Induced  
\_\_\_\_\_ Spontaneous

Demographics

Wt \_\_\_\_\_ Ht \_\_\_\_\_

Current medications:

Drug	Start	Stop
_____	_____	_____
_____	_____	_____
_____	_____	_____
_____	_____	_____
_____	_____	_____
_____	_____	_____

Other drugs:

Non-prescription \_\_\_\_\_  
\_\_\_\_\_

Other vitamins, supplements \_\_\_\_\_  
\_\_\_\_\_

Labs: Date \_\_\_\_\_

Metabolic panel: Glucose \_\_\_\_\_ BUN \_\_\_\_\_ SrCr \_\_\_\_\_

WBC \_\_\_\_\_ Hgb \_\_\_\_\_ Hct \_\_\_\_\_ Diff: N \_\_\_\_\_ L \_\_\_\_\_ E \_\_\_\_\_ B \_\_\_\_\_

Pulmonary Function Tests:

Date \_\_\_\_\_ FEV<sub>1</sub> \_\_\_\_\_ FVC \_\_\_\_\_

Date \_\_\_\_\_ FEV<sub>1</sub> \_\_\_\_\_ FVC \_\_\_\_\_

Date \_\_\_\_\_ FEV<sub>1</sub> \_\_\_\_\_ FVC \_\_\_\_\_

Subject Study Code Number \_\_\_\_\_

Pulmonary Cultures

Date	Result/organism	Sensitivities
_____	_____	
_____	_____	
_____	_____	
_____	_____	

Visit 3: After Azithromycin Restart

Specimen Date \_\_\_\_\_

Specimen collection: \_\_\_\_\_ Induced  
\_\_\_\_\_ Spontaneous

Demographics

Wt \_\_\_\_\_ Ht \_\_\_\_\_

Current medications:

Drug	Start	Stop
_____	_____	_____
_____	_____	_____
_____	_____	_____
_____	_____	_____
_____	_____	_____

Other drugs:

Non-prescription \_\_\_\_\_  
\_\_\_\_\_

Other vitamins, supplements \_\_\_\_\_

Subject Study Code Number \_\_\_\_\_

Labs: Date \_\_\_\_\_

Metabolic panel: Glucose\_\_\_\_ BUN\_\_\_\_ SrCr\_\_\_\_

WBC\_\_\_\_ Hgb\_\_\_\_ Hct\_\_\_\_ Diff: N\_\_\_\_ L\_\_\_\_ E\_\_\_\_ B\_\_\_\_

**Pulmonary Function Tests:**

Date \_\_\_\_\_ FEV<sub>1</sub> \_\_\_\_\_ FVC \_\_\_\_\_

Date \_\_\_\_\_ FEV<sub>1</sub> \_\_\_\_\_ FVC \_\_\_\_\_

Date \_\_\_\_\_ FEV<sub>1</sub> \_\_\_\_\_ FVC \_\_\_\_\_

**Pulmonary Cultures**

Date	Result/organism	Sensitivities
------	-----------------	---------------

_____	_____	
_____	_____	
_____	_____	
_____	_____	
_____	_____	

## APPENDIX B: HUMAN GENES FOR ARRAY PLATES

384 well Micro Fluidic Card; 47 genes + 1 mandatory control;  
Format 48, Part # 4342253

Well	Gene	Fisher #
A1	CD86	Hs01567026_m1
A2	CD64/CD64	Hs00417598_m1
A3	ITGAM	Hs00167304_m1
A4	CD14	Hs02621496_s1
A5	CD68	Hs02836816_g1
A6	CD23/FCER2	Hs00233627_m1
A7	CD40	Hs01002915_g1
A8	STAT1	Hs01013996_m1
A9	SOCS3	Hs02330328_s1
A10	SLAMF1	Hs00234149_m1
A11	RNA18S5	manufacturing control (housekeeping gene)
A12	TNF	Hs00174128_m1
A13	IL6	Hs00174131_m1
A14	IL12B	Hs01011518_m1
A15	IL1B	Hs01555410_m1
A16	IL10	Hs00961622_m1
A17	TGFB1	Hs00998133_m1
A18	IL12A	Hs01073447_m1
A19	IL8/CXCL8	Hs00174103_m1
A20	IL23A	Hs00372324_m1
A21	IL4RA	Hs00965056_m1
A22	IL27RA	Hs00945029_m1
A23	HLA-DRA	Hs00219575_m1
A24	MARCO	Hs00198937_m1
B1	CCL17	Hs00171074_m1
B2	CCR2	Hs00704702_s1
B3	TREM2	Hs00219132_m1
B4	CCL22	Hs01574247_m1
B5	CCL18	Hs00268113_m1
B6	NOS2	Hs01075529_m1
B7	RETNLB	Hs00395669_m1
B8	ARG1	Hs00163660_m1

B9	PPARG	Hs01115513_m1
B10	IKBKB	Hs01559460_m1
B11	ARG2	Hs00982833_m1
B12	IDO1	Hs00984148_m1
B13	IRF1	Hs00971965_m1
B14	MRC1/CD206	Hs00267207_m1
B15	NLRP1	Hs00248187_m1
B16	CASP1	Hs00354836_m1
B17	MAP1LC3B	Hs00797944_s1
B18	AKT1	Hs00178289_m1
B19	PCNA	Hs00427214_g1
B20	ATG5	Hs00169468_m1
B21	MTOR	Hs00234508_m1
B22	GCN2/EIF2AK 4	Hs01010957_m1
B23	GAPDH	Hs02786624_g1
B24	CCND1/cyclin D1	Hs00765553_m1

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## VITA

Place of birth:

Tyre, Lebanon

Education:

Lebanese American University

Pharm.D.

University of Kentucky

Ph.D in Pharmaceutical Sciences (expected), Clinical and Experimental Therapeutics

University of Kentucky

Graduate Degree+ in Leadership, Team Building, and Communication for careers

Professional positions:

University of Kentucky

August 2013- December 2017

Teaching Assistant

University of Kentucky

July 2013 - current

Research Assistant

Honors and Awards:

October 2017: Peter G. Glavinos Jr. Ph.D. Memorial Travel Award

August 2017: Pharmaceutical Sciences Excellence in Graduate Achievement Fellowship

November 2016: AAI Young Investigator Award

June 2016: First Place Excellence Award for the Podium Presentation at the *48th Annual Pharmaceutics Graduate Student Research Meeting*

November 2015: Third Place Outstanding Elevator Speech Presentation at the *Symposium on Drug Discovery and Development 2015*

July 2015: AAPS Student Chapter of the Year Award

Publications:

August 2017:

Azithromycin Therapy Reduces Cardiac Inflammation and Mitigates Adverse Cardiac Remodeling after Myocardial Infarction: Potential Therapeutic Targets in Ischemic Heart Disease

Ahmed Al-Darraj, Dalia Haydar, Lakshman Chelvarajan, Himi Tripathi, Bryana Levitan, Shaojing Ye, Vincent J. Venditto, John C. Gensel, David J. Feola and Ahmed Abdel-Latif

*PLOS ONE*

July 2018 (submitted):

Azithromycin Decreases Stat-1 Phosphorylation via Cross-talk with NF- $\kappa$ B Signaling Pathway.

Dalia Haydar, Theodore J. Cory, Susan E. Birket, Brian S. Murphy, Keith R. Pennypacker, and David J. Feola

*Scientific Reports*

December 2017 (under construction):

Impact of azithromycin treatment on pulmonary gene expression in a murine

model of chronic *Pseudomonas aeruginosa* infection

Rene Gonzalez, Dalia Haydar, John Gensel, Cynthia Mattingly, David J. Feola

June 2018 (under construction):

Macrophage Arginase-1 is essential for regulation of excessive inflammation in  
*Pseudomonas aeruginosa* pneumonia

Dalia Haydar, Nishad Thamban Chandrika, Rene Gonzalez, Beth Garvy,  
Sylvie Garneau-Tsodikova, and Dave J. Feola

June 2018 (under construction):

Azithromycin balances the immune responses in PA pneumonia via  
mechanisms independent of arginase.

Dalia Haydar, Rene Gonzalez, Beth Garvy, David J. Feola

Typed name:

Dalia Haydar