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Imatinib mesylate as a host directed therapeutic for mycobacterial infection

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Imatinib mesylate as a host directed therapeutic for mycobacterial infection

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An abstract of
A dissertation submitted to the Faculty of the
James T. Laney School of Graduate Studies of Emory University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy
in Immunology and Molecular Pathology
2023

Abstract

Imatinib mesylate as a host directed therapeutic for mycobacterial infection

By Tesia L. Cleverley

Mycobacterial infections remain an important cause of morbidity and mortality in humans; for example, *Mycobacterium tuberculosis*, the cause of tuberculosis, kills ~1.5 million and newly infects ~10 million each year. Most people effectively combat mycobacterial infections with only 10% of people exposed tuberculosis developing active disease and infections with non-tuberculosis mycobacteria being predominate in immunocompromised individuals. Treatment is compromised in at-risk individuals by an inadequate immune response and chronic inflammation, which results in chronic infection and tissue damage. Granulomas form to encase bacteria thus limiting spread of the infection. However, these structures impair tissue function and limit access of antibiotics to bacteria, which engenders antibiotic resistance. Antibiotic resistance is an ongoing concern in mycobacterial infections as the bacteria are intrinsically resistant to many antibiotics and readily acquired resistance. Current antibiotic therapies used to treat mycobacterial infections are required to be taken for months leading to side effects and poor compliance. Therefore, there is a need to develop new therapies to combat the rise of antibiotic resistance or shorten treatment regimes. Imatinib mesylate, a host directed therapeutic, has shown efficacy against mycobacteria in cell culture and mouse model systems. Imatinib was originally conceived as a cancer therapeutic that inhibits Abl and related tyrosine kinases, however inhibition of Abl has been shown to alter intracellular transit of bacteria during infection. Low doses of imatinib have also been shown to induce myelopoiesis in mice. Using systems biology approaches in conjunction with murine infections with *Mycobacterium marinum*, a close genetic relative of *M. tuberculosis* that forms tail granulomas, we report that imatinib does not fundamentally alter the anti-mycobacteria immune response at the site of infection, but rather accelerates development of the immune response. In addition, imatinib limits granuloma formation and growth resulting in less inflamed tissue. In the absence of caspase 8, imatinib is unable to limit granuloma growth. These data highlight imatinib as a possible host directed therapeutic for mycobacterial infections with the capacity to augment the immune response in at-risk individuals, and limit granuloma growth, thereby limiting tissue damage.

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Part 1: Antibiotic Resistance in Mycobacterial infection

Mycobacterial diseases

Various species of mycobacteria have been well known human pathogens. The mycobacterium genus includes historically well know and problematic pathogens *Mycobacterium tuberculosis*, the causative agent of tuberculosis (Tb), and *Mycobacterium leprae*, the pathogen that causes leprosy. However, with the advent of antibiotics and increased public health measures in the 20th century, rates of these illnesses have dropped significantly in the United States. Only 150-250 new cases of leprosy are identified a year in the United States, and only ~100,000- 150,000 cases are identified worldwide (WHO, 2022, January 11). In the United States, ~9,000 cases of Tb are identified each year, however worldwide it is estimated that ~10million people fall ill with Tb each year, and 1.6 million people died of Tb in 2021, making Tb the second leading infectious disease killer after COVID-19 the peak of the COVID pandemic (WHO, 2022, October 27).

Other human pathogens within the mycobacterium genus are referred to as nontuberculosis mycobacteria (NTM). This includes many human pathogens such as *Mycobacterium avium complex, Mycobacterium kansasii, Mycobacterium abscessus, Mycobacterium chelonae, Mycobacterium fortuitum, Mycobacterium terrae, Mycobacterium xenopi, Mycobacterium simiae, Mycobacterium ulcerans* and *Mycobacterium marinum*. Incidence of disease caused by NTM is less common with incidence rates at 1-10 per 100,00 people (Wu et al., 2018), and usually occurs in individuals that are immune compromised or have underlying chronic lung disease such as cystic fibrosis. While most NTM infections occur in the

lungs, many of these pathogens can infect various tissues such as skin, soft tissues, lymph nodes, and surgical sites. While incidence of these diseases remains low, the incidence is increasing (Kilinç et al., 2021). Treatment success rates for NTM infections is low, at 30-60% treatment success based on the species of mycobacteria and the age of the patient(Kim et al., 2022; Kwak et al., 2017; Mirsaeidi et al., 2014). Treatment for all mycobacterial diseases often require a minimum of three antibiotics to be given over months. Antibiotic resistance is also common in mycobacterium, leading to a need to develop new therapies. Most research related to mycobacterium to date has been on *M. tuberculosis* as it poses the largest threat to human populations, however many of the lessons we learned for *M. tuberculosis* can cautiously be applied to other pathogenic mycobacteria.

It is estimated that *M. tuberculosis* first became a human pathogen around 70,000 years ago, with some modeling systems placing the emergence of tuberculosis even earlier evolutionarily spurred when humans began to utilize fire (Chisholm et al., 2016). However, the origin, evolution, and spread of *M. tuberculosis* is still greatly debated. Genetic evidence of *M. tuberculosis* has been found in human remains up to 6,000 years ago, with evidence of *M. tuberculosis* in bison 17,000 years ago (Rothschild et al., 2001). Despite the long history of tuberculosis, it was not until 1689 that the tubercles formed in the lung was established as a pathology, and not until the 1820's that tuberculosis was identified as a single disease. This could be due to the rise of the disease in Europe in the 1600s peaking in the 1800's when it is estimated that tuberculosis was the cause of 25% of deaths in Europe (CDC, 2016 December 12). The first vaccine against *M. tuberculosis* was developed in 1906 from an attenuated stain of bovine tuberculosis, *Mycobacterium bovis*. The Bacillus Calmette–Guérin (BCG) vaccination is still used

today with minor changes. The rates of protection this vaccination provides is variable, vaccination of infants is able to provide protection against disseminated and pulmonary tuberculosis infection in young children but confers variable efficacy when given to adults to protect against pulmonary tuberculosis. Protection conferred by the BCG vaccination can lasts between 15 and 60 years (Dockrell & Smith, 2017).

It was not until the advent of antibiotics in the 1950's that the prevalence of tuberculosis truly started to diminish. Today it is estimated that one third of the world's population is latently infected with *M. tuberculosis*, with about 10 million people falling ill with tuberculosis every year and between 1.5 and 2 million deaths every year. Most cases and deaths occur in the developing world. About one third of the deaths caused by tuberculosis occur in patients co-infected with HIV (WHO, 2022, October 27). Though the prevalence of tuberculosis disease has been decreasing steadily since the 1800's, the addition of the HIV epidemic and antibiotic resistance has threatened the decline in cases so that more must be done to combat the global health risks.

Characteristics of mycobacterium

The genus mycobacterium is composed of a variety bacterium, some pathogenic some not. All the mycobacteria are gram positive, aerobic, and acid fast. The most distinguishable characteristic of mycobacterium is the thick, waxy cell wall. The cell wall consists of an inner layer of peptidoglycan followed by a layers of arabinogalactan polysaccharides, then mycolic acids and an outermost layer of lipids (Jarlier & Nikaido, 1994). This thick and waxy cell wall is highly hydrophobic and limits absorption of hydrophilic compounds into the bacteria. Hydrophilic antibiotics need to cross in the cell via porins which are limited in number in the mycobacterial

cell wall leading to innate resistance to many antibiotics (Mailaender et al., 2004; Stephan et al., 2004). One emerging theory about the differences in virulence between members of the mycobacterium genus, relates to the composition of the cell wall. The genus is roughly divided into two main groups, fast growing mycobacterium such as *M. abscessus* and slow growing mycobacterium such as *M. tuberculosis, M. leprae, and M. marinum*. Fast growing mycobacteria in general have lower virulence and human infections with these bacteria are usually only seem patients with compromised immune systems or other underlying health problems (De Groote & Huitt, 2006). Pathogenic mycobacteria tend to be slow growing, with the exception of opportunistic pathogens such as *M. abscesses*. One of the main differences between the fast growing and slow growing mycobacteria is the composition of the cell wall, and specifically, the capping of LAM (Shaler et al., 2012). Fast growing mycobacterium will have LAM capped with phosphoinositol or uncapped. Slow growing mycobacterium have mannosylated LAM which has been shown to have anti-inflammatory properties creating a more tolerant T cell response (Shaler et al., 2012). Though this is not the only factor that influences mycobacterium virulence, it might be worth further examination.

Immune Evasion with Mycobacteria

Immune responses to mycobacterium have been primarily studied during *M. tuberculosis* infection in either human or mouse model systems. Studying host responses to NTM has been more challenging as infection rates are low, only 30-60% of NTM infections are successfully treated in humans, and mice have low susceptibility to these bacteria often requiring mice to have known immune defects, such as knocking out the IFN-γ gene, to get productive infection.

To explore the role of the host immune response against mycobacterial infection, we will focus on what is known about the host response to *M. tuberculosis*.

Early infection

M. tuberculosis is transmitted from a human host with active Tb disease via aerosol to another host. An aerosol droplet containing as few as 10 bacilli can spur a productive infection. Once in the airways, the bacilli will be taken up by an alveolar macrophage. Early in the infection the bacteria will reside inside professional phagocytes: macrophages, monocytes, neutrophils and dendritic cells (Kang et al., 2011). Recognition and immune response to the bacteria is dependent on TLR2 (Banaiee et al., 2006) and TLR9 (Bafica et al., 2005) in a redundant way so that knocking out of either or both receptors is not detrimental, however knocking out the MyD88 adaptor protein, involved in propagating signal from either of these TLRs, results in a lethal infection in mice (Holscher et al., 2008). Several other receptors have been identified to be important in the recognition and immune response to *M. tuberculosis*. These receptors include members of the C-type lectin receptor family which utilize the CARD9 adaptor molecule, DC-SIGN, Dectin1, the mannose receptor, mincle, cytosolic-NOD2, and NLRP3 (Ernst, 2012). Overall *M. tuberculosis* is redundantly recognized in a way such that any single receptor KO is unlikely to result in lethal infection however knocking out a set of receptors may lead to loss of immune control.

Once inside the professional phagocyte, *M. tuberculosis* can make the usually hostile environment suitable to allow bacterial replication. *M. tuberculosis* can prevent phagosome maturation, allowing to bacteria to reside in the phagosome instead of pushing the bacteria into the lysosome (Bruns et al., 2012; Chackerian et al., 2002; Napier et al., 2011). The mechanism

that *M. tuberculosis* employs to prevent phagosome maturation is not fully understood, however a key component of the phagosome arrest is lipoarabinomannan (LAM). LAM is a glycolipid component of cell wall of bacteria in the mycobacterium genus. It has been demonstrated that LAM is involved in inhibiting phagosome maturation by preventing phosphorylation of PI3P (Briken et al., 2004; Deretic et al., 2006). *M. tuberculosis* also produces two superoxide dismutase, sodA and sodC, in order to deal with reactive oxygen species (ROS) in the phagosome (Ehrt & Schnappinger, 2009). Once in the secured phagosome the bacteria replicate freely and activate other virulence programs.

Some virulent mycobacteria also able to induced phagosome rupture allowing the mycobacteria into the cytosol where they can activate other cell host defense systems. Phagosome escape was first witnessed with pathogenic *M. marinum* (Smith et al., 2008), however it was confirmed that *M. tuberculosis* utilizes the type VII secretion system ESX-1 to escape the phagosome in a similar manner (Houben et al., 2012; Simeone et al., 2012). Once the phagosome is ruptured IFN-β is indued through the cGAS-STING pathway, and NLRP3 inflammasome activation enhances secretion of IL-1β (Wassermann et al., 2015). Triggering these inflammatory pathways via phagosome rupture results in an increase in cell death of the infected host cells and contributes to the virulence of the pathogen (Aguilo et al., 2013; Augenstreich et al., 2017; Majlessi & Brosch, 2015).

During the early phase of infection, the macrophages are not sequestered at one site of infection and are able to further spread the infection within the lung. The ESX-1/RD1 virulence locus helps with the early dissemination of the bacteria by inducing production of proteins that are essential in resulting in a long term chronic infection (Davis & Ramakrishnan, 2009) as well as

inducing the necrotic death of neutrophils (Pym et al., 2002). Three of the major proteins encoded on the ESX1 virulence locus are ESAT-6, culture filtrate protein 10 (CFP-10)(Gao et al., 2004; Tan et al., 2006), and EspA (Fortune et al., 2005). ESAT-6 is a secreted protein and major T cell antigen that has been associated with many virulent functions of *M. tuberculosis* including preventing phagosome maturation in conjunction with CFP-10 (Tan et al., 2006). ESAT-6 has also been shown to induce host epithelial cells to secretion of matrix metalloproteinase-9 (MMP9) an enzyme involved in degrading the extracellular matrix (Volkman et al., 2010). The induction of MMP9 could have multiple implication, however the early effects result in an influx of macrophages into the site of infection. Taking all this into account, *M. tuberculosis* have developed effective ways to initiate infection. Being an intracellular pathogen, *M. tuberculosis* ensures that host phagocytes will ingest the bacterium by appealing to a wide range of receptors. Once inside the phagocyte *M. tuberculosis* is able to arrest the host defenses to increase the number of bacteria and call more phagocytes to house the bacteria released from the necrotic phagocytic cells. In this way the bacteria are establishing a foothold in the host.

Onset of Adaptive Immunity

The adaptive immune response to *M. tuberculosis* is delayed. In humans, a positive tuberculosis skin test is not detectible until 42 days after exposure to *M. tuberculosis* (Poulsen, 1950; Wallgren, 1948). In mice, the induction of *M. tuberculosis* specific T cells occurs between day14 and 21 after infection (Clay et al., 2008; Flynn et al., 1995). The onset of the adaptive immune response relies on presentation of antigen and recognition/response to the antigen. In *M. tuberculosis*, there are defects in both the presentation of the antigen and the T cells response to the antigen that results in a delay in the activation of the adaptive immune system. In order

to get an adaptive response to *M. tuberculosis*, live, antigen producing, bacteria must be brought to the draining lymph node (Khader et al., 2006; Wolf et al., 2008). However, arrival of the *M. tuberculosis* exposed dendritic cells in the draining lymph node is delayed. In mice *M. tuberculosis* infected dendritic cells take 8-10 days to arrive at the draining lymph node, while, comparatively, during an influenza infection dendritic cells carrying antigen will arrive at the draining lymph node in 20 hours (Ernst, 2012).

The migration of these dendritic cells does not appear to be delayed, however activation preceding migration appears to be delayed. Migration of dendritic cells in an *M. tuberculosis* infection is highly reliant on the expression of IL12p40 in dendritic cells (Khader et al., 2006). *M. tuberculosis* also induced highly responsive IL12 receptors in the dendritic cells (Robinson et al., 2010). The migratory capacity of the dendritic cells is not impaired if anything it is increased during an *M. tuberculosis* infection. The problem is with dendritic cell activation. TNF has been shown to be a critical factor during the early *M. tuberculosis* infection by inducing reactive nitrogen species (Flynn et al., 1995) and restricting *M. tuberculosis* growth (Clay et al., 2008). Host TNF-α has been implicated in necrotic death of macrophages in early and late stages of the disease through RIP1 and RIP3 via production of ROS from the mitochondria (Roca & Ramakrishnan, 2013). Necrotic death of infected macrophages can lead to bacteria dissemination. Lipomannan, a membrane ganglioside on mycobacterium, is able to block TNF production (Rajaram et al., 2011). Indicating that the bacteria may use TNF as a means of self-controlling the spread of infection. The mycobacterium infected dendritic cells are also deficient in MHCII (Pecora et al., 2009), HLA-DM, and CIITA (Fulton et al., 2004), all important molecules in CD4+ T cell activation. *M. tuberculosis* also impairs the expression of CD1b and DC-SIGN on

dendritic cells (Balboa et al., 2010) indicating an impaired ability of these dendritic cells to respond and communicate with CD4+ T cells. When stimulated externally via or aerosol LPS into the lungs of mice, the *M. tuberculosis* infected dendritic cell population did not decrease the time it took for the adaptive immune response to develop (Wolf et al., 2008). The delay in dendritic cell migration to the draining lymph node, and the impaired antigen presentation is able to hinder the development of the adaptive immune system which is required to arrest bacterial growth.

A controversial area of research on tuberculosis has to do with the role of neutrophils. Neutrophils have long been associated with lung damage in mice (Ong et al., 2014) as well as in humans (Ong et al., 2015). Early enhanced recruitment of neutrophils has been associated with higher disease severity in different strains of mice (Eruslanov et al., 2005; Keller et al., 2006). However neutrophils also confer protection for the host and promote granuloma formation. One study found that apoptotic neutrophils may play an important role in activating dendritic cells and inducing migration to the lymph nodes to activate the adaptive immune response (Blomgran & Ernst, 2011). Though it is still uncertain the exact role that neutrophils may play in tuberculosis infection, neutrophils are likely required in a balanced way. Too many highly active neutrophils my result in increased tissue damage while too few or underactive neutrophils may result in lack of immune control.

T cell priming is also delayed during an *M. tuberculosis* infection. Another virulence factor produced by *M. tuberculosis* is the cells wall cord factor, trehalose dimycolate. The cord factor is thought to be important in holding the arrangement of the bacteria in long slender formations. It has also been shown to be able to impair CD40, CD80, and CD86 expression (Kan-Sutton et al., 2009). Impaired expression of these molecules would cause an impaired co-stimulatory signal

when attempting to activate adaptive immune cells. *M. tuberculosis* also induced IL10 in order to skew the T cell response away from a Th1 or Th17 response and towards a Th2 type response that is more moderate and focuses more on healing damage than eliminating bacteria. The *M. tuberculosis* PPE18 protein is able to induce IL10 utilizing TLR2 (Nair et al., 2009), but only when the *M. tuberculosis* is alive (O'Leary et al., 2011). As the T cells are being activated in the lymph node, the IL10 is able to further delay T cell response by inducing antigen specific T regulatory cells (Shafiani et al., 2010). This induces a state where the host is being trained to tolerate the bacteria more similar to a commensal bacterium than an invading pathogen in the lung.

Once the adaptive immune system is activated, the host is able to arrest bacterial growth but not eliminate the bacteria. The arrival of T and B cells into the lung directs the development of the solid granuloma by forming a lymphocytic cuff at the periphery of the infection site and giving the granuloma a solid structure (Ulrichs et al., 2004). Though the CD4+ T cells are not required for early infection, mice with knocked out CD4+ T cells have reduced survival, poorly formed granulomas, and low recruitment of immune cells into the lung (Saunders et al., 2002). The role of CD4+ T cells is widely recognized as a critical component to control a tuberculosis infection, however the amount to which other T cell types contribute to controlling the infection is still under debate. In mice with CD1 or CD8 knocked out, the infection resembles that of an infection in a wild type mouse, however if an essential component of MHC I, the beta-2 microglobulin gene, is knocked out the mice obtain higher colonization of the bacteria and have no infiltrating protective lymphocytes in the lesion (D'Souza et al., 2000). While αβ T cells have been shown to be essential in tuberculosis control, γδ T cells are not essential for control of infection but may play a role in mediating infection induced inflammation (Mogues et al., 2001).

The type of CD4+ T cell has also shown to be important in control of tuberculosis infection. Th2 type responses have not been shown to be beneficial for long term control of tuberculosis infection, but Th1 and Th17 immune responses have shown to be critical for long term control of infection (Desvignes & Ernst, 2009; Nandi & Behar, 2011). The T cell response to *M. tuberculosis* is an area of great interest to researchers due to the implications that a successful T cell vaccine may have on the disease. Being a disease where the most important protective factor of the adaptive immune system is the T cell response, vaccination to induce "better" T cells is a highly researched area.

Latent infection

After the granuloma has formed, the host and bacteria enter a silent battle to maintain equilibrium. *M. tuberculosis* will downregulate antigen expression and switch over to producing proteins that will allow the bacteria to survive inside the granuloma (Bold et al., 2011). Meanwhile the macrophages that surround the infected center of the granuloma undergo an epithelia reprograming that switches the junctions between the macrophages from loose mesenchymal adhesions, to strong epithelial junctions characterize by the presence of e-cadherin (Cronan et al., 2016). The epithilialization of the macrphages may serve two purposes 1) to prevent bacterial disemination, and 2) to reduce traffic of immune cells in and out of the infected region. As a result, granulomas limit antigen presentation and T effector function (Egen et al., 2011). The caseous and cavitary centers of the granuloma are composed primarily of pro-inflamatory eicosanoids while the regions near the periphery show anti-inflamatory pathways that lead to tolerance, such as the upregulation of indolamine 2,3-dioxygenase (IDO) which catabolizes tryptophan and has been shown to encourage the development of CD4+ T regulatory

cells and limit CD4+ T effector cell function (Gautam et al., 2018; Marakalala et al., 2016). At any one point the host will have many granulomas in varying states (Lin et al., 2014; Matty et al., 2015). Each granuloma is its own system with the forces for the bacteria and host working independently on it to build up and take apart the granuloma. During this latent phase the balance between bacteria and host has been likened to a delicate equilibrium. To make things more complicated, it has been observed that there is no correlation between the state of granuloma destruction and the disease state in primates (Lin et al., 2009). Taking the limited antigen presentation, immune- inhibitory atmosphere of the outer granuloma, and the limited access to the inner granuloma, fluctuations in the granuloma state seems to be one way that the host is able to re-encounter antigens and revamp the immune response. This continuously perilous state of tug-of-war leaves the host in a dynamic equilibrium of granuloma weakening and strengthening. While the host is most benefited when the bacteria are locked away, fluctuations in immune response can lead to weakening in the granuloma leading to antigen escape and allowing the immune system to be re-primed.

Reactivation: The Balance between host and bacteria

With a latent infection being the result of an equilibrium between host defenses and bacterial virulence, all it would take for reactivation of disease would be a loss of balance. The exact cause of reactivation is unknown, but the field is leaning away from a single cause and towards a series of causes that lead to an imbalance in the immune response. Only ~10% of people infected with Tb develop disease. This usually happens within a few months to 2 years after exposure after a lengthy and variable incubation period (Behr et al., 2018). There are currently three defined triggers that have been shown to lead to tuberculosis disease at a high

rate. The acquisition of the human immunodeficiency virus (HIV) is one of the most well characterized causes of reactivation. HIV will lead to a drop in CD4+ T cell counts which greatly increases the chances of developing tuberculosis (Glynn et al., 2010). The second trigger is taking TNF inhibitors for auto immune disorders while latently infected with *M. tuberculosis*. Neutralizing TNF with rituximab can cause a loss of immune control of latent *M. tuberculosis* infection (Alkadi et al., 2017; Harris & Keane, 2010). The third potential trigger is type II diabetes mellitus. With the increasing number type II diabetes mellitus patients in the developing world, *M. tuberculosis* reactivation has been shown to happen at a higher frequency in patients suffering from type II diabetes. The mechanism behind this is still under investigation, but it is though that this association is due to the immune dysfunction caused by type II diabetes (Sola et al., 2016). All three of these known triggers either knock out a major component of immune control of *M. tuberculosis* (CD4+ T cells) or alter the immune system in a way to throw-off the carefully balanced equilibrium between host and pathogen. In order to understand reactivation, it is important to understand the contributions of both the host and bacteria.

The host immune system must coordinate many moving parts in the proper proportions in order to keep the bacteria under control. The granuloma has been thought to be one of the major host defenses and to be solely host protective against the bacteria (Chao & Rubin, 2010; Saunders & Cooper, 2000). Mouse and zebra fish model systems have shown that granuloma caseation or granuloma dysregulation is linked to an increase in bacterial proliferation (Swaim et al., 2006). However *M. tuberculosis* also participates in directing granuloma formation via ESX-1 (Davis & Ramakrishnan, 2009). Once inside the granuloma *M. tuberculosis* also has many ways to help its survival. The DosR regulon in the bacteria induced a programing that allows *M.*

tuberculosis to survive in the hypoxic environment of the granuloma (Leistikow et al., 2010). *M. tuberculosis* is also able to induce angiogenesis in order to increase the supply of nutrients into the area of the granuloma (Oehlers et al., 2015). *M. tuberculosis* is also able to make up for essential nutrients in order to prolong the standoff. While the host T cells try to starve the bacteria of tryptophan, *M. tuberculosis* can produce its own using TrpE enzyme (Zhang et al., 2013). Though the granuloma may protect the host, it is unlikely that it is solely host protective. The bacteria come equipped and ready for a long period of time inside the granuloma. The granuloma could provide a protective niche for the bacteria to proliferate and survive in. Much is still left to learn about the interactions and functions of *M. tuberculosis* during the latent phase of infection. This is in part due to the limited research that has been performed looking at the bacteria during a latent infection. Few model systems can accurately replicate this stage and those that can are costly and not often used to examine the basic pathogenesis of the bacteria at this stage (Ernst, 2012).

Host factors that are critical in the maintenance of a latent *M. tuberculosis* infection are somewhat easier to study in part because factors important early in the infection tend to remain important in retaining latency. TNF is a major factor in *M. tuberculosis* control (Chakravarty et al., 2008; Flynn et al., 1995), with the introduction of rituximab to treat autoimmune disorders, such as arthritis gravis, *M. tuberculosis* reactivation is more likely (Alkadi et al., 2017). IFNγ is another important factor for immune control, when the IFNγ gene is disrupted, mice were unable to control or contain the bacteria (Cooper et al., 1993). Mice with knocked out IL12p40 were unable to control bacterial infection and the onset of the adaptive immune system was delayed (Cooper et al., 1997). The transcription factor important in the development of Th1 cells, STAT4, was also

shown to be an important factor, with knock out mice having a huge influx of neutrophils into the lungs and eventually succumbing to infection (Sugawara et al., 2003). The MyD88 adaptor protein has been shown to be critical in recognizing and initiating an immune response to *M. tuberculosis* at initial infection and during latency (Fremond et al., 2007; Fremond et al., 2004; Scanga et al., 2004). This list of host factors critical to infection control is not complete. The host has a complex immune system that is redundant is many ways to help protect against pathogens, however at various points of signal integration, critical factors appear. Understanding what these factors are and what minor factors may contribute to the maintenance of these factors could shed light on treatment and lifestyle choices that may help reduce the risk of reactivation.

Model systems

Most of what we know about mycobacterial infection has been determined in various model system. Studies in humans often deal with clinical trials, or endpoint analysis of patients that have succumb to tuberculosis infection. In order to study different phases of tuberculosis infection, different model systems are used. *M. tuberculosis* can be grown *in vitro* in macrophages or dendritic cells isolated from humans or mice as well as mouse macrophage cells lines. These types of experiments can only give information on innate immune responses and are most likely only representative of early responses to infection.

Non-human primates are the best *in vivo* model system because they can replicate all stages of the disease, however they are used sparingly because of the cost. *Cynomolgus macaques* and *Rhesus macaques* are the most commonly used models for *M. tuberculosis* infections (Kaushal et al., 2012). *Cynomolgus macaques* can be infected with low dose aerosol

infection, with about 1/3 of primates developing latent infections. Adaptive immune responses to *M. tuberculosis* can be measured after 4 weeks of infection. Non-human primates can also be used in studies involving SIV to model HIV infection and utilizing vaccines such as the BCG vaccine. Lung lesions in the non-human primates resemble the variety of lesion types seen in humans with *M. tuberculosis* infection such as caseous necrotic lesions, and fibrotic lesions (Kaushal et al., 2012). Guinea pigs, rabbits, mice, and zebra fish are most often used to study early infection. Guinea pigs develop symptoms and pathology similar to humans, however they are also more susceptible to disease (Padilla-Carlin et al., 2008). Rabbits also human like pathology, including cavitary lesions (Nedeltchev et al., 2009). However, both these model systems can be difficult to work with as few molecular and immunological tools have been developed to work within these model systems.

Mice infected with *M. tuberculosis* are often used as a model of tuberculosis infection due largely to the ease of mouse models, the numerous molecular and immunological tools available, and ease of genetic manipulations, however this model is not always the most accurate. Mice infected *M. tuberculosis* with do not develop a latent infection and eventually succumb to disease (Guirado & Schlesinger, 2013). However mice infected with the fish mycobacterium, *M. marinum*, do develop latent disease as indicated by a reduction in inflammation, and healing of diseased tissue while still maintaining a low level of bacteria in the granulomas (Lienard & Carlsson, 2017). However, *M. marinum* does not replicate the lung pathology in mice, instead granulomas form on the tails of the mice possibly due to the lower temperature which is ideal for *M. marinum* growth. While *M. tuberculosis* grows optimally at 37 degrees Celsius, the optimal growth temperature for *M. marinum* is 30-32 degrees Celsius. Granulomas resulting for *M. marinum*

infection are also similar to human Tb granulomas as they develop a macrophage cuff around central necrosis that limits access of CD3+ T cells into the central regions of the lesions where the bacteria are found (Carlsson et al., 2010). Because *M. marinum* does not cause tuberculosis in humans, it is much safer to utilize this pathogen and as a result this pathogen can be used to study antibiotic resistance in mycobacterium while *M. tuberculosis* cannot.

Though neither mouse model is ideal, both can be used to investigate various aspects of the disease. Zebra fish have proven to be a very important model system to study granuloma formation (Sullivan et al., 2017). Zebra fish embryos can be infected with *M. marinum* and develop granulomas. The benefit of using zebra fish would be that they are the natural host of *M. marinum*, and they have translucent skin to allow the granuloma to be imaged as it is forming. A newer model system involving ferrets is currently being designed and utilized to study tuberculosis disease. In this model system ferrets developed lung pathology based on inoculum dose over 7 weeks. This model also shows signs of ferret-to-ferret transmission of bacteria (Gupta et al., 2022).

Many models have been used for antibiotic resistance tests and drug development with NTM, including amoebas, drosophila, *Galleria mellonella* larvae, zebrafish, and mice (Rampacci et al., 2020). Amoebas can be natural hosts to many NTM and intracellular mycobacteria can show higher resistance to antibiotics (Miltner & Bermudez, 2000). Drosophila, and *Galleria mellonella* larvae can be used to study antibiotic efficacy in biological systems, however disease in these insect models does not resemble disease seen in humans histopathologically. Zebrafish provide the best non-mammalian model for NTM infection developing granuloma-like structures and chronic infections. One benefit of this system is the ability to image host-pathogen

interactions at a cellular level in a living system(Bernut et al., 2014; Fenaroli et al., 2014). Mouse models have been the most valuable mammalian model system for NTM infections as transgenic and knock-out mouse lines are widely available, as most immune competent mice develop short infections with rapid clearance of fast-growing NTM (Obregón-Henao et al., 2015; Ordway et al., 2008). Research has focused on mice with significant immune deficiencies such as severe combined immunodeficiency (SCID) mice, granulocyte monocyte-colony stimulating factor (GM-CSF-/-) deficient mice, and mice with significant deficiencies in B cells, T cells and NK cells, NOD.CB17-Prkdc^{scid}/NCrCrl mice. With significant immune deficiencies present, *M. abscessus* infection results in lung infection more closely resembling lung disease in humans. Immune competent mice are more susceptible to slow-growing NTM such as *M. avium* complex (Andréjak et al., 2015) and *M. marinum* (Lienard & Carlsson, 2017). Each model system can give insight into host pathogen interactions with mycobacteria; however, every discovery must be taken with caution as no model system is ideal to study disease in humans. Discoveries will need to be confirmed with in human populations to the best of their abilities with the limitations posed by studying diseases in humans.

Antibiotic resistance in mycobacterium

The signs of bacterial resistance were present from the advent of the very first antibiotics. Utilizing antibiotics applies selective pressure to the bacteria forcing the bacteria to either change of die out. Only the bacteria with the most favorable genes can survive the pressure. However, in order to reduce the incidence of antibiotic resistance, antibiotics are normally given at “high” doses over extended periods of time that leaves the low potential for the bacteria to develop

resistance. Antibiotic resistance does occur though. Two major factors can contribute to the development of antibiotic resistance in the community: 1) over prescription of antibiotics for illnesses that are either not caused by bacteria or easily cleared with no antibiotics, 2) non-compliance of patients taking antibiotics to finish their regiment. Granuloma induced by the bacteria have also been shown to aid in the development of antibiotic resistance, as the structure of the granuloma limits the concentration of antibiotics that are able to be reach the bacteria with in the granuloma (Sarathy & Dartois, 2020; Sarathy et al., 2018), thus necessitating the long treatment periods with high doses of antibiotic to ensure that bacteria is cleared from the granuloma.

Mycobacteria utilize three main mechanisms that contribute to antibiotic resistance— an impermeable cell wall, ability to modify antibiotics, and efflux systems to remove antibiotics. The Mycobacterial cell wall is a thick lipid rich cellular envelope with 60% by weight made up by lipids, particularly long-chain fatty acids known as mycolic acids (Jarlier & Nikaido, 1994). This makes the cell wall highly hydrophobic limiting water soluble, hydrophilic antibiotics from entering the cell. The cell wall contains porins to allow hydrophilic molecules to pass through the membrane, however these porins are low in number (Mailaender et al., 2004), thus limiting the concentrations of antibiotics that are able to penetrate the cell wall. Mycobacteria can also utilize enzymes that are able to modify the structure of antibiotics or alter the antibiotics to render them ineffective (Chambers et al., 1995; Quinting et al., 1997; Wang et al., 2006; Warrier et al., 2016). For example, some mycobacteria contain an enzyme β-lactamase which can hydrolyze the β-lactam ring of antibiotics such as penicillin. This enzymatic degradation of β-lactam antibiotics renders this class of antibiotics useless against *M. tuberculosis* (Chambers et al., 1995; Quinting

et al., 1997). *M. tuberculosis* also contains a protein, the enhanced intracellular survival proteins (Eis), that, when over expressed, can acetylate various aminoglycosides and can lower the concentrations of second line antibiotics such as kanamycin A (Kambli et al., 2016; Zaunbrecher et al., 2009). Finally, mycobacteria have elaborate efflux systems that are able to pump antibiotics out of the cell and potentially lead to antibiotic resistance (Adams et al., 2011; Gupta et al., 2010; Li et al., 2015). Various studies have shown that with pressure from antibiotics, such as rifampicin, efflux pumps will be up regulated at the mRNA level in *M. tuberculosis*. These various innate methods of mycobacteria, particularly *M. tuberculosis*, are concerning and require antibiotics to be applied carefully to treat disease.

The rise of antibiotic resistance is concerning in all bacteria, but it is of particular concern in mycobacteria. *M. tuberculosis* and NTMs possess natural resistance to many antibiotics and are developing resistances to current antibiotics. In most cases tuberculosis is curable with a cocktail of antibiotic taken over 6 months. The first line drugs against *M. tuberculosis* are ethambutol, isoniazid, pyrazinamide, and rifampicin. These four drugs have limited side effects and are well tolerated. Often patients will feel better after a couple weeks on treatment however the treatment must continue for 6 to 9 months to ensure the bacteria are eliminated and reduce the risk of developing antibiotic resistant strains. Tb that is determined to be resistant to rifampicin (RR-tb) or multiple first line antibiotics (MDR-Tb) effects around 0.5 million people. If it is determined that the patient has Tb unresponsive to the first line antibiotics, then second line antibiotics are used where treatment success rates are around 60%. The second line antibiotics are fluoroquinolones, injectable antibiotic (such as amikacin, kanamycin, and capreomycin) and newer antibiotics linezolid and bedaquiline (Guglielmetti et al., 2017). Treatment for MDR

tuberculosis can last up to 20 months and second line antibiotics are associated with somewhat more severe side effects. About one third of patients on MDR tuberculosis treatment reported some side effect requiring intervention with the most common side effect being gastrointestinal issues (Yang et al., 2017).

For NTM infections, treatment starts with macrolide- based antibiotics clarithromycin or azithromycin with the addition of ethambutol and rifampin for slow-growing mycobacteria(Sim et al., 2010) or aminoglycosides and cefoxitin, imipenem or tigecycline for fast growing NTMS (Wallace et al., 2014). Treatment can last as long at 18 months and cure rates range from at 30-60% treatment success based on the species of mycobacteria and the age of the patient(Kim et al., 2022; Kwak et al., 2017; Mirsaeidi et al., 2014). Treatment failure rates are often attributed to the high level of antibiotic resistance found in the NTM. Of particular concern, patients with cystic fibrosis are at high risk of pulmonary infection leading to extensive antibiotic usage. Treatment of NTM infections from these patients is often complicated by antibiotic resistance (Laudone et al., 2021), leading to a need for new therapies.

A new regiment has recently been approved and put into trial to treat MRD tuberculosis. The Bangladeshi regiment consists of and initial 4 to 6 months of kanamycin, moxifloxacin, prothionamide, clofazimine, pyrazinamide, high-dose isoniazid, and ethambutol, followed by 5 months of moxifloxacin, clofazimine, pyrazinamide, and ethambutol (Guglielmetti et al., 2017). In the three major trials of this regiment so far, approximately 90% of the patients were successfully treated (Aung et al., 2014; Piubello et al., 2014; Van Deun et al., 2010). The main side effects of this treatment were vomiting and impaired hearing. The prospects for treatment of MDR tuberculosis are improving, but that does not mean it is time to stop innovation. The

bacteria will gain resistance to these antibiotics as well as has already been seen with extensively drug resistant (XDR) tuberculosis. Innovation should focus not only on developing new regiments, antibiotics, and vaccinations, but also host directed therapeutics to attack the bacteria on all fronts and eliminate this disease once and for all.

The rise in antibiotic resistance in *M. tuberculosis* can be related to many factors. *M. tuberculosis* is predominately in poorer countries where treatment is harder to come by and often patients are restricted from taking their full course of medicine by lack of supply, or cost. The antibiotics used against *M. tuberculosis* also have debilitating side effects making patients more likely to stop taking their antibiotics when they feel better. The rise of antibiotic resistance *M. tuberculosis* will continue until something changes. Treatments need to be developed to treat existing cases of antibiotic resistance as well as prevent new antibiotic resistant bacterium from developing. New drugs are urgently needed to target resistant strains, shorten the duration of treatment, and target different stages of the disease to control the disease.

Part 2: Imatinib as a host directed therapeutic

Host Directed Therapeutics for Tb infection

While antibiotics have been the main focus of drug development against bacterial infection since the discovery of penicillin in 1928, host directed therapeutics [HDTs] are now being explored to cover the deficiencies of antibiotics treatments. Antibiotics target bacterial specific factors leading to direct selective pressure from which, over time, the bacteria will evolve to escape. Utilizing drugs to target the host has the potential to enhance the effectiveness of the host response against the pathogen and induce proven defenses to give the host an advantage against invading bacteria. Likewise, HDTs could synergize with antibiotics to induce better clearance and shorten antibiotic regimens and even be used against drug resistant bacterial strains. While most antibiotics have limited effect on dormant or latent bacteria, HDTs may retain activity against these persisters. Two general strategies for HDT development against tuberculosis deal with 1) disrupting bacterial pathogenesis in macrophages and 2) immunomodulatory HDTs to induce a protective immune response (Napier et al., 2012). Pathogenic mycobacteria are experts at manipulating the host immune system by limiting phagolysosome fusion, escaping into the cytosol, redirecting host metabolism, and preventing antigen presentation, thus delaying an adaptive immune response (Kilinç et al., 2021). These mechanisms allow the bacteria to establish long term infections. Studied host directed therapeutics against tuberculosis infection have targeted a variety of pathways involved in inflammation, intracellular killing, autophagy, phagosome maturation, macrophage polarization, antigen processing and presentation, T cell polarization and immune exhaustion (Kilinç et al.,

2021; Mahon & Hafner, 2015). These host directed therapeutics have been chosen and tested based on known deficiencies in the host response to pathogenic mycobacteria.

Other host directed therapeutics have been used in animal models of tuberculosis to mediate infection. Eicosanoids have been targeted though prostaglandin E2 levels in order to moderate the effects of IL-1 and IFNs on tuberculosis infected mice (Mayer-Barber et al., 2014). Ibuprofen has also been shown to reduce bacterial burden in tuberculosis infected mice by limiting inflammation (Vilaplana et al., 2013). Other therapeutics include inhibitors for VEGFA, inhibiting VEGFA inhibits angiogenesis in order to act with synergistically with antibiotics to kill bacteria by starving them inside the granuloma (Oehlers et al., 2015). 6-FABA can be used to block *M. tuberculosis*'s tryptophan producing enzyme, TrpE, making *M. tuberculosis* more susceptible to immunological stress (Zhang et al., 2013). Many host directed therapeutics have been considered and are at various stages of being tested for use against *M. tuberculosis* in human patients.

Though TNF is a critical host defense factor against tuberculosis, high levels of TNF have been associated with increased pathology in zebrafish and humans (Roca & Ramakrishnan, 2013; Tobin et al., 2012; Tobin et al., 2010). One potential therapy for dysregulated TNF levels is the introduction of TNFα inhibitors. These therapies have shown some effectiveness but also indicates that TNF inhibitors may contribute to reactivation of active disease in mice (Skerry et al., 2012). Likewise, studies conducted looking at historical data of patients taking infliximab, found that the TNF inhibitor may have increased the risk of reactivation (Keane et al., 2001; Wallis et al., 2004). This implies that careful regulation of TNFα would be needed to successfully utilize TNF-inhibitors to treat Tb effectively without risking detrimental effects.

The goal of a HDTs is to supplement a host immune response to be better prepared to eliminate a Tb infection. However, the problem drug developers encounter when attempting to devise HDTs towards Tb infection is that the ideal response to Tb are still poorly understood with many factors being implicated as potentially important. For example, granulomas formation has been associated with control of the infection (Chao & Rubin, 2010; Saunders & Cooper, 2000; Swaim et al., 2006), but chronic inflammation from lasting bacterial antigens and an exuberant inflammatory response can cause excessive tissue damage that can lead to scaring and lung dysfunction (Hnizdo et al., 2000; Meghji et al., 2020; Plit et al., 1998). Research in zebrafish has shown that deconstructing the granuloma is required in order to allow immune cells to reach the bacteria and increases host survival (Cronan et al., 2016). Likewise, the granuloma has shown to limit antibiotic penetrance into the infected tissues leading to lower concentrations of antibiotics in caseous regions of the granuloma where bacteria reside (Sarathy & Dartois, 2020; Sarathy et al., 2018).

This leads to the question of what sort of immune reaction should we be striving to induce utilizing HDTs. In other diseases, vaccines and supportive treatments are often designed based on what a successful immune response to the disease looks like. However, with Tb it is more difficult to classify what a successful immune response looks like. Only about 10% of patients with tuberculosis will reactivate the disease, while 90% of patients with live with latent disease that may indicated successful clearance of the pathogen or an immunological stalemate between the host and pathogen. Reactivation of disease is often associated with immunocompromising conditions such as HIV infection or immunosuppressive drugs for other conditions (Kilinç et al., 2021). However it is only during reactivation that the bacteria can spread and become a public

health concern (Ernst, 2012), and thus research has focused on individuals with reactivated disease. A beneficial immune response to Tb could be exemplified in the 90% of patients that can prevent reactivation of the bacteria, however even patients that maintain control of Tb or are treated with antibiotics to eliminate the bacteria, can become re-infected (Hnizdo et al., 2000; Hunter et al., 2007). While a preventative immune response to prevent Tb via vaccination is highly desirable, a more easily achievable goal may instead be to utilize what is known about the protective host immune response to Tb seen in most patients to instead develop HDTs to support host immune responses in patients that struggle to maintain control of infection. Utilizing what we know about naturally beneficial immune responses to Tb, we can evaluate the potential of new HDTs.

Host tyrosine kinases and pathogens

To survive and proliferate in a host, viruses and intercellular bacteria take advantage of host factors to work in their favor. One way that both bacteria and viruses do this is by conscripting host factors to modify actin polymerization. Poxviruses, vaccinia, variola, monkeypox, and likely smallpox, utilize Abl- and Src-family kinases to manipulate actin polymerization and move across the surface of the cells on a pedestal (Reeves et al., 2005; Reeves et al., 2011). Release of the viruses from the cell surface requires Abl- family kinases. By utilizing Abl kinase inhibitors, poxvirus dissemination can be reduced in culture, and survival can be increased in infected mice (Reeves et al., 2005). Likewise budding and release of the Ebola virus has been shown to be dependent on c-Abl1, another member of the Abl tyrosine kinase family (Garcia et al., 2012). Polyomaviruses also rely on Abl-family kinases but in a slightly different way,

the kinase will regulate ganglioside sialyation which in turn regulates the cells susceptibility to infection (Swimm et al., 2010). It is possible that many other viruses take advantage of host tyrosine kinase pathways to increase pathogenicity and modulate host response.

Bacteria also take advantage of host tyrosine kinases in order to survive and disseminate. The obligate intercellular parasite *Chlamydia trachomatis* utilizes Abl-family kinases and PDGFR-β redundantly in order to gain entry into the cellular host (Elwell et al., 2008). *Pseudomonas aeruginosa* and *Shigella flexneri* utilize host Abl- family kinases in order to regulate actin motility to be internalized by non-phagocytic cells (Burton et al., 2003; Pielage et al., 2008). In the context of *S. flexneri*, Abl phosphorylates N-WASP in order to induce comet tail formation and allow for the bacteria to move around the cell (Burton et al., 2005). Enteropathogenic *Escherichia coli* (EPEC) have bacterial virulence factors Tir and intimin which are able to interact with cellular factors in order to induce similar pedestal formation as pox viruses (Swimm, Bommarius, Reeves, et al., 2004). These interactions also utilize host Abl-family tyrosine kinases such as Abl and Arg in the recruitment of Nck, N-WASP, and Arp2/3 allowing for pedestal formation (Swimm, Bommarius, Li, et al., 2004; Swimm, Bommarius, Reeves, et al., 2004; Swimm & Kalman, 2008). Utilizing pyrido[2,3-d]pyrimidine (PD) compounds which inhibit Abl, Arg and related kinases is also able to stop the pedestal formation with EPEC (Swimm, Bommarius, Li, et al., 2004).

Pathogenic mycobacteria reside inside phagocytic cells by preventing the phagosome from fusing with the lysosome. Though many factors are involved in the process of arresting phagosome maturation, Abl family tyrosine kinases have been implicated as important host factors in preventing the phagosome from fusing with the lysosome (Bruns et al., 2012; Napier et al., 2011). *M.* tuberculosis produces proteins, SapM and PknG, which limit PI3P-

phosphorylation, dissociation of early-endosomal protein Rab5 and inhibits recruitment of late-endosomal protein Rab7 (Ankley et al., 2020). Recruitment of V-ATPase, a proton pump required for phagosome acidification, is also prevented by *M. tuberculosis* (Wong et al., 2011). Abl family tyrosine kinases have shown to be involved actin cytoskeletal rearrangements within the cells and vesicle trafficking throughout the cell, including bacterial uptake and phagolysosome fusion. Utilizing PD compounds on *Mycobacterium mariunum* or *Mycobacterium tuberculosis* infected macrophages is able to push the bacteria from the phagosome into the lysosome thus reducing bacterial numbers and decreasing dissemination (Bruns et al., 2012; Napier et al., 2011). In mouse model systems with either *M. marinum* and *M. tuberculosis* providing a well-tolerated PD compound, Imatinib mesylate (Gleevec), bacterial burdens were reduced with no other treatment (Napier et al., 2011).

Targeting the host systems utilized by bacteria such as Abl- and Arg-family kinases is one potential way to moderate host disease and decrease pathogenicity of a variety of different viruses and bacteria. Though not all pathogens are candidates for treatment with Abl-family kinase inhibitors, some diseases may benefit from such treatment. Pathogenic mycobacteria are particularly interesting candidates for treatment with PD compounds as survival and pathogenesis of these bacteria depends on modulating the host immune system by limiting bacterial recognition and activation of host cells. One way in which pathogenic mycobacteria can avoid detection is by delaying phagolysosome fusion. By utilizing PD compounds such as Imatinib, there is a potential to undo pathogen related immune modulation and “fix” a pathogen beneficial dysregulated immune response.

Imatinib and mycobacteria

Imatinib mesylate, Gleevec, is a revolutionary cancer drug developed in the early 1990's to treat chronic myelogenous leukemia (CML), a blood cancer characterized by an expansion of a hematopoietic population carrying the Philadelphia chromosome. When imatinib was introduced as the frontline therapy for CML patient five-year survival rate was raised from 31% to nearly 90% when 400 mg of imatinib was taken daily (Druker et al., 2006). This type of cancer was associated with the presence of a mutation that resulted in the Philadelphia chromosome, a translocation between chromosome 9 and 22. This results in adding an additional region, the breakpoint cluster region (Bcr), to the c-Abl tyrosine kinase resulting in a constitutively active Bcr-Abl protein allowing the cancerous cells to survive and proliferate. Imatinib competitively binds the active site of the Bcr-Abl protein lowering kinase activity and reducing the proliferating population of cancer cells. Imatinib is thought to be selective, only targeting Abl, c-kit, and PDGF-R (platelet derived growth factor receptor). Though other cell types express and utilize Abl-family tyrosine kinases, non-cancerous cells will use tyrosine kinases in a redundant fashion, meaning that there are few off target effects and imatinib is well long term. Along with CML, imatinib is now used to treat c-kit positive gastral intestinal stromal tumors (GISTs). Research into imatinib as a cancer drug has also revealed some interesting immunomodulatory activities of the drug. Tumors often have an immune suppressive environment to reduce immune system recognition. This immune suppressive environment is associated with an upregulation of IDO in some tumors that induces the production of T regulatory cells (Tregs). Research has shown that imatinib is able to reduce the population of Tregs in the tumor and thereby increase the population of T effector cells in the tumor (Larmonier et al., 2008). As a result, imatinib might potentially be useful on

other immune structures that result in localized immune suppression such as the immune environment seen in mycobacterial granuloma. To date, imatinib has two defined roles proven beneficial against mycobacterial infection: 1) inducing phagolysosome fusion, and 2) inducing myelopoiesis.

Many bacteria utilize host tyrosine kinases to cause cytoskeletal rearrangements. Blocking host tyrosine kinases using cancer drugs such as imatinib can supplement a host response in order to induce a more favorable state. Utilizing *in vitro* and *in vivo* model systems, imatinib was shown to induce phagolysosome fusion in macrophages infected with *M. marinum* or *M. tuberculosis* as well as in mouse model systems using *M. marinum* and *M. tuberculosis* infections as well (Napier et al., 2011). During an infection with pathogenic mycobacteria innate immune cells struggle to kill the bacteria due to phagosome arrest, however with the addition of imatinib induced phagosome maturation thus inhibiting a critical component of mycobacterial survival strategies. Trafficking the bacteria into the lysosome also increases immune recognition of the pathogen by breaking down and deactivating the bacteria which generates peptides to be loaded into major histocompatibility complex (MCH) to be used to activate the adaptive immune system (Hu et al., 2015). Likewise, TLRs and TLR signaling machinery can be found in the lysosome after infection, however it has yet to be determined whether these proteins are functional or in the process of being recycled (Gao et al., 2017). Thus, by inducing phagosome maturation, imatinib could potentially spur a more robust and directed immune response by preventing the bacteria from limiting immune recognition and skewing the immune response.

At low doses, imatinib has also been shown to directly modify the cellular basis of the potential immune response by inducing myelopoiesis in the bone marrow and increasing the

numbers of granulocytes (Napier et al., 2015). This increase in neutrophils was shown to reduce disease severity by reducing the bacteria load during the early phase of *M. marinum* infection in mice. Though increase in neutrophils has been associated with increased inflammation and poor outcome during Tb infection (Eruslanov et al., 2005; Keller et al., 2006; Ong et al., 2015; Ong et al., 2014), a certain amount of inflammation and neutrophils are necessary in order to maintain immune control (Blomgran & Ernst, 2011; Fletcher et al., 2016). Imatinib however is not just mildly boosting the number of neutrophils but causing a 1-2 log increase. This huge increase in neutrophils could theoretically cause a dramatic increase in inflammation. However, excessive information was not seen in the early phases of *M. tuberculosis* or *M. marinum* infection in mice with increased number of neutrophils in the blood or imatinib induced myelopoiesis.

Neutrophils during *M. tuberculosis* infection have been studied in two situations. Early during the infection *M. tuberculosis* infection in mice, an influx of neutrophils into the tissues has been associated with poor outcomes (Eruslanov et al., 2005; Keller et al., 2006). This is caused an increase in inflammation leading to higher tissue pathology caused by the neutrophils. Neutrophils and neutrophil derived proteins have also been studied in the context of *M. tuberculosis* in human patients. In humans, the increase in neutrophils resulted in increase of neutrophil myeloperoxidase (MPO) and neutrophil gelatinase associated lipocalin (NGAL), which were shown *in vitro* to degrade important structural components of granulomas and were associated with reactivation of disease (Ong et al., 2015). This suggests that neutrophils are involved in granuloma breakdown. Preliminary unpublished data with imatinib suggests that the neutrophils induced from the bone marrow on mice during treatment are different from endogenous neutrophils, as chromatin appears more highly condensed and globular, a state seen

more often in aged neutrophils. Experiments with imatinib have yet to look at the function of the neutrophils induced by the drug from the bone marrow to see if these neutrophils are activated in the same way and contribute to granuloma deconstruction.

The clinical trial to test imatinib in patients with Tb disease has not begun yet, however to date there have been reported cases of patients reactivating latent Tb infection while taking imatinib to treat CML (Daniels et al., 2009). Three patients were observed to reactivate their tuberculosis 1, 2 or 4 year after initiating treatment with imatinib for CML. The doses for each patient were 800 or 400 mg/day which is much higher than mouse model systems suggest is required for imatinib effects against mycobacterial infections. Inflammation as determined by CRP levels was increased at the time the patients were diagnosed with Tb likely resulting from the activation of the chronic infection. These observations could suggest that dosing is very important when utilizing imatinib to treat Tb and other complicating factors, such as CML, which causes immune dysregulation, must be considered before treatment of Tb with imatinib. Ideally, markers need be established in patients responding well imatinib therapy during Tb infection in order to optimize treatment for each patient, as it is possible that imatinib therapy would not be ideal for Tb in every case. Since immune balance is very important in the context of Tb infection, these markers should look at inflammation and tolerance factors in order to find the balance that can help in bacterial clearance.

While taken individually the direct effects of imatinib might not be minimal, but together it is possible that an immune response can be induced that will allow the host to clear an infection more efficaciously. Maintenance of a latent Tb infection requires a very strict balance of immune activation, however more research needs to be done on how the host immune system might be

able to clear infection on its own. Imatinib treatment may provide critical clues into how to induce an immune response to mycobacterial infection that is capable of subverting mycobacterial defense mechanisms to give the host immune response an advantage against the bacteria. This may even provide insight into how to better induce an effective adaptive immune response to mycobacterial infections that will allow for better vaccinations to be developed.

Thesis Overview

In my thesis, I focus on how imatinib may be used as a host directed therapy to treat specific mycobacterial infections. While previous work has focused the intercellular trafficking and myeloproliferative aspects of imatinib treatment on mycobacterial infection, I focus my work on how these changes effect global immune changes in a way that might benefit the host immune response on a whole. Ruth Napier noted in 2011 (Napier et al., 2011) that the lesions that developed on the tails of the mice with imatinib treatment were notably smaller than lesions without imatinib treatment. As I continued experiments, I also took note of this and noted that lesions were smaller at every timepoint even when imatinib had no effect on the bacteria load. Initial unpublished data from non-human primates also showed a similar effect of imatinib limiting the pathology associated with infection even when an effect on the bacterial burden was nebulous. Focusing on the reduction of overall pathology, we hypothesized that by inducing phagolysosome fusion and/or inducing myelopoiesis a more directed immune response to the bacteria would be achieved, thus limiting unintended tissue damage and limiting pathology. Using RNA-seq, mouse models, and cell culture we address the changes imatinib induces in the infected tissue.

This work will examine how a host directed therapeutic can induce small changes in an immune response that disrupt how an infection develops, resulting in a more favorable outcome to the host. Though much is known about immune responses to *M. tuberculosis* infection, scientists are still looking for ways to utilize what we know to alter the host pathogen interaction. While many host directed therapeutics are directed at very specific molecules and functions, in a biological system, no single event is isolated and changing one aspect of an immune response

will lead to a cascade of downstream changes. In this thesis I focus on the overall changes induced by imatinib to the immune response and propose factors that contribute the reduction of lesions size, including better activation of macrophages as measured by cytokine production, and increased cell survival.

Chapter 2:

The host-directed therapeutic imatinib mesylate accelerates immune responses to *Mycobacterium marinum* infection and limits pathology associated with granulomas

By

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Abstract

Mycobacterial infections, including those caused by members of the mycobacterium tuberculosis complex [MTC] and Nontuberculous mycobacteria [NTM], can induce widespread morbidity and mortality in people. Mycobacterial infections cause both a delayed immune response, which limits rate of bacterial clearance, and formation of granulomas, which contain bacterial spread, but also contribute to lung damage, fibrosis, and morbidity. Granulomas also limit access of antibiotics to bacteria, which may facilitate development of resistance. MTC members resistant to some or all antibiotics are estimated to account for a third of deaths from tuberculosis [TB], and newly developed antibiotics have already engendered resistance, pointing to the need for new therapeutic approaches. Imatinib mesylate, a cancer drug used to treat chronic myelogenous leukemia [CML] that targets Abl and related tyrosine kinases, is a possible host-directed therapeutic [HDT] for mycobacterial infections, including TB. Here, we use the murine *Mycobacterium marinum* [Mm] infection model, which forms quantifiable granulomas on the tails, in conjunction with transcriptomic analysis of the tail lesions. The data indicate that imatinib induces gene signatures indicative of immune activation at early time points post infection that resemble those seen at later ones, suggesting that imatinib accelerates but does not substantially alter anti-mycobacterial immune responses. Moreover, focusing on the TNFα pathway, which is induced by imatinib, we show that imatinib promotes cell survival in infected bone marrow-derived macrophages [BMDMs] in a manner that depends on caspase 8. Moreover, imatinib limits formation and growth of granulomas, an effect abrogated in mice lacking caspase 8. These data provide evidence for the utility of imatinib as an HDT for mycobacterial infections

in accelerating immune responses, and limiting pathology associated with granulomas, and thus mitigating post-treatment morbidity.

Introduction

Pathogenic mycobacteria have developed numerous strategies to manipulate and evade host immune responses. Notable members of this genus include *Mycobacterium tuberculosis* [Mtb], the causative agent of tuberculosis [TB], a leading cause of morbidity and mortality that claims 1.5 million lives each year (WHO, 2021, October 14), *Mycobacterium leprae*, which causes Hansen's disease (leprosy), *Mycobacterium avium- intracellulare*, an opportunistic pathogen affecting immunocompromised patients and those with severe lung disease such as cystic fibrosis, and *Mycobacterium marinum* [Mm], a human pathogen acquired from contaminated aqueous environments or infected fish that causes skin lesions called "fish tank granulomas"(Aubry et al., 2017). All these bacteria are either naturally resistant to antibiotics or readily acquire resistance (Jarlier & Nikaido, 1994; Saxena et al., 2021). The standard of care for TB, for example, is a multi-drug antibiotic regimen given over four to nine months. Importantly, Mtb strains resistant to some or all available antibiotics have emerged (Udwadia et al., 2012; Velayati et al., 2009), including stains resistant to newly developed antibiotics such as bedaquiline, delamanid, and pretomanid (Bloemberg et al., 2015; Peterson et al., 2016), highlighting the need for novel treatment strategies for TB.

Mycobacteria subvert the host immune response in a variety of ways. Infection with Mtb attracts macrophages and other innate cells to the site of infection (Kang et al., 2011) but limits activation and cytokine production of antigen presenting cells (Wolf et al., 2007). Within macrophages, Mm and Mtb prevent phagolysosomal fusion (Ehrt & Schnappinger, 2009; Gao et al., 2004; Stamm et al., 2003), which both precludes activation of macrophages and limits antigen presentation (Armstrong & Hart, 1971; Houben et al., 2012; Russell et al., 2002). Mtb has also

been shown to limit maturation of dendritic cells (DCs) and their migration to lymph nodes (Khader et al., 2006; Wolf et al., 2008), thereby delaying the onset of adaptive responses (Chackerian et al., 2002; Ernst, 2012; Reiley et al., 2008). Accordingly, in humans adaptive immune responses to Mtb emerge approximately 42 days after exposure (Poulsen, 1950; Wallgren, 1948). Notably mycobacterial infections are accompanied by chronic inflammation, possibly facilitated by secretion of antigenic decoy proteins such as Antigen 85 (Ernst et al., 2019). Yet the immune response to mycobacteria remains highly effective in most people, and in TB, it is estimated that 90% of those infected with Mtb maintain control of the infection and do not develop active disease (Behr et al., 2019; WHO, 2021, October 14). However, for all mycobacterial infections, those with immunocompromising conditions remain far more susceptible(Wolinsky, 1992). An important question concerning mycobacteria treatment strategies for people with chronic disease remains how to facilitate a more efficient or efficacious immune response (Ernst et al., 2019).

With a delay in immune responses and chronic inflammation, the infected host forms granulomatous lesions that appear to contain the bacteria and thereby limit its spread. Granuloma formation is in part mediated by tumor necrosis factor α [TNFα](Bean et al., 1999; Gómez-Reino et al., 2003; Kaneko et al., 1999; Keane et al., 2001; Park et al., 2022) and involves congregation of macrophages that enlarge and have more cytoplasm, known as epithelioid macrophages, which surround and ingest infected cells (Cronan et al., 2016; Ramakrishnan, 2012). Granulomas also contain neutrophils, dendritic cells, B cells, T cells, and natural killer cells, together with fibroblasts that produce extracellular matrix [ECM](Ramakrishnan, 2012). Epithelialioid macrophages and fibroblasts encase the infected cells and limit bacterial dissemination. Many

granulomas exhibit necrosis (Flynn et al., 2011; Hunter et al., 2007), which may contribute to chronic inflammation that damages surrounding lung tissue and impairs respiratory function (Ravimohan et al., 2018). Moreover, the structure of the granuloma reduces penetrance of antibiotics, resulting in suboptimal antibiotic concentrations within the granuloma, thereby facilitating development of resistance (Sarathy & Dartois, 2020; Sarathy et al., 2018). Chronic inflammation results in tissue destruction that contributes to the development of fibrosis and reduces elasticity of lung tissue. As a result, half the patients successfully treated for TB exhibit lasting respiratory impairment (Hnizdo et al., 2000; Meghji et al., 2020; Plit et al., 1998), further exacerbating the economic burden of the disease (Meghji et al., 2021). While much is known about structure and formation of granulomas, less is known about how to resolve granulomas and restore lung function in TB patients following treatment, or whether resolution would allow bacteria to escape, thereby exacerbating disease.

To address the need for novel therapeutics for mycobacterial disease, we have been developing imatinib as an adjunctive host-directed-therapeutic (HDT). Imatinib is a well-tolerated cancer drug that inhibits tyrosine kinase activity of c-Abl, c-Kit, and platelet derived growth factor receptor [PDGFR], and is the frontline therapy for chronic myelogenous leukemia [CML] and gastrointestinal stromal tumors [GISTs]. As an HDT for infections, imatinib is less likely to engender resistance compared to conventional antimicrobial drugs (Hawn et al., 2015). Upon infection with Mtb or Mm infection, imatinib induces phagolysosomal fusion in mouse monocytes (Napier et al., 2011) and in human macrophages (Bruns et al., 2012), an effect evident at micromolar concentrations. At low doses in mice, imatinib also induces myelopoiesis (Napier et al., 2015). Finally, prophylactic administration of imatinib also limits development of

granulomas induced by Mm in mice (Napier et al., 2011). Notably, fibrosis may contribute to bacterial persistence and tissue dysfunction even after discontinuation of antibiotic chemotherapy (Hnizdo et al., 2000; Malherbe et al., 2016; Plit et al., 1998; Ravimohan et al., 2018). Fibrosis in lung, skin and other tissues associated with noninfectious causes is mediated by PDGFR, also a target of imatinib, and imatinib has shown efficacy in these indications (Akhmetshina et al., 2008; Akhmetshina et al., 2009; Distler et al., 2007; Li et al., 2009; Wang et al., 2010; Yoshiji et al., 2005), raising the possibility that the drug may likewise limit fibrosis and granuloma-associated pathology.

The observation that imatinib limits Mm infections and granuloma formation, and induces myelopoiesis, led us to hypothesize that the drug might accelerate the formation of anti-mycobacteria immune responses, and in so doing might limit formation and/or promote resolution of granulomas. To test this idea, we chose the Mm mouse model of mycobacterial infection, in which granulomas develop on the tail within two weeks after infection. We defined gene expression signatures in tail granulomas and asked how imatinib impacts such signatures at different time points post infection. We found that imatinib accelerates appearance of gene signatures associated with immune cell activation in response to infection, including, for example, the macrophage activation marker TNFα. Finally, we found that imatinib effects on granulomas are abrogated in mice lacking caspase 8, a component of TNFα signaling pathways.

Results

Imatinib limits formation of granulomas in mice infected with Mm. Imatinib mesylate, administered to mice starting one day prior to IV infection with low inoculums of Mm (~5 Log_{10} CFU/ mouse), reduced CFUs in the tail, spleen, or lung, whereas no reduction was evident in control animals provided with water as a control (Napier et al., 2011). At higher inoculums [>6 Log_{10} CFU/mouse), the drug was without effect on CFUs in any tissue at 6-, 14-, or 21-days post infection [p.i.] (Figures 1A-C). Notably, such high inoculums induce more rapid formation of granulomatous lesions on the tail, usually within 4-12 days (Figure 1D), which continue to increase in size before reaching their maximal extent by 14-21 p.i. (Figure 1E). Lesion size was quantified over time by measuring the length of each lesion on the surface of the tail to determine the change in lesion size over the treatment period (Figures 1F and G). With imatinib treatment beginning one day prior to infection with 10^7CFU/ mouse, development of granulomatous lesions was restricted as reported (Napier et al., 2011). In an experiment using a lower inoculum ($2x10^6$ CFU/mouse, Figure 1H) only 20% of imatinib-treated animals developing lesions during the first week compared to 40% of animals treated with water (Figure 1I). To determine whether imatinib limited development of actively growing lesions, the drug was administered starting at day seven p.i. and continuing until day 14 (Figure 1H). Over this time period, lesions from water-treated mice grew an average of 13mm, whereas lesions from imatinib-treated animals grew on average 6.5mm (Figure 1J, F and G). To determine effects of imatinib on established lesions, lesions were allowed to develop for 14 days prior to administration of imatinib for an additional seven days (Figure 1H). Whereas lesions on control mice grew by an average of 6.6mm during this period, no net growth was evident in mice treated

with imatinib, with lesion size increasing in some animals (6 of 13 animals), but either not changing (3 of 13 animals) or decreasing (4 of 13 animals) in others (Figure 1K). Together, these data suggest that imatinib limits formation and growth of granulomas in a manner that does not depend on bacterial load.

We next examined histopathologically the tail lesions using hematoxylin and eosin (H&E) stained tail sections of the granulomas taken at day 14 with or without imatinib treatment for 7 days (Figure 2A and B). Pathology scoring of the sections was blinded to treatment received. Scores were assigned for the number of ulcers, inflammation and necrosis in the epidermis and dermis, muscle and bone (Supplemental Figure 1A-N). We did not observe significant differences between the two groups with respect to the inflammation and necrosis in epidermis and dermis (Figure 2C), muscle (Figure 2D) or bone (Figure 2E). However, the percentage of tissue displaying inflammation was significantly reduced in the mice receiving imatinib (Figures 2G-I). Acid fast staining organisms were seen within the necrotic regions of lesions regardless of treatment (Supplemental Figure 1O). Together, these data suggest imatinib does not induce changes in microscopic composition of granulomatous lesions but does reduce the amount of inflammation.

Imatinib upregulates immune genes and downregulates ECM genes in uninfected animals. To determine how imatinib affects gene expressions profiles within granulomas, RNA-seq was performed on tail sections from uninfected animals or from those infected with Mm for one or three weeks (accession no. **GSE215176**). Infected groups included mice treated with water or imatinib beginning one day prior to Mm infection for 7 days [“1 week infection” group (Figure 3A)] and mice in which lesions were allowed to develop for 14 days and then treated with water

or imatinib for 7 days ["3-week infection" group (Figure 3A)]. RNA-seq profiles from tails of uninfected mice treated with water or imatinib served as a baseline with which to determine differentially expressed genes associated with infection based on a false discovery rate [FDR] of 0.05.

In the absence of infection, imatinib differentially regulated 514 genes compared to water controls, of which 373 were upregulated and 141 were downregulated. GO analysis of upregulated genes indicated that imatinib regulated diverse cellular processes, many of which were associated with the function, activation, or regulation of the immune response (Supplemental Figure 2A). Imatinib has been reported to induce myelopoiesis in mice at the dose used (Napier et al., 2015); accordingly, GO terms associated with neutrophil function (granulocyte activation and migration) and myeloid cell differentiation were upregulated with imatinib. GO processes significantly downregulated with imatinib include skin-epidermis development and ECM organization, which was ascribed to a significant reduction of 17 collagen subunits including *col3a1*, c*ol1a2*, *col1a1*, *col5a1*, and *col8a2* (Supplemental Figure 2B). Thus, without infection, imatinib upregulated genes and processes associated with immune function, and downregulated genes associated with ECM.

Imatinib rapidly induces expression of infection genes. Genes regulated by infection ("infection genes") were identified as those differentially expressed at one week or three week time points following infection as compared to expression levels in uninfected mice treated with water (controls). We identified 903 genes upregulated at the one-week time-point, of which 577 remained upregulated at three weeks (Figures 3B & C). GO analysis of the infection genes

upregulated at one week indicated processes involved in immune responses and cytokine production, including responses to interferon-gamma and positive regulation of TNF, IL6, and IL1β production (Supplemental Figure 2C). At the three-week time point, 99 additional upregulated genes were identified as infection genes, for a total of 676 genes at this time point. GO analysis of upregulated infection genes at three weeks indicated immune-related processes, including regulation of the cytokines IL6, IL1, IL12, IL10, IL8 and TNF, as well as macrophage activation, and responses to wounding (Supplemental Figure 2D). GO analysis of genes downregulated by infection at either the one- or three-week timepoints did not identify any processes.

We next examined how imatinib impacted expression of infection genes. To do this, the variance of infection genes without or with imatinib was analyzed by principal component analysis ([PCA]; Figure 3D). Uninfected mice clustered together in terms of PC1 (pink box), and imatinib treatment for one week shifted the variance, though only in PC2 (compare pink and blue boxes; Figure 3D). Infection alone for one week shifted the variance primarily along the PC1 axis (gold box; Figure 3D), and imatinib treatment again shifted the variance only in PC2 (compare gold and red boxes; Figure 3D). Infection for three weeks shifted the variance in PC2 (compare gold and teal boxes; Figure 3D) to a position near that seen with imatinib treatment at one week (compare red and teal boxes; Figure 3D). Imatinib treatment at this time point produced little additional shift (compare green and teal boxes), however, less variance in expression was apparent, as indicated by the reduced area of the green box. Overall, imatinib altered the pattern of variance in infection genes such that the variance at the one-week time-point with imatinib

resembled that seen at three weeks without imatinib, and at three weeks, imatinib further restricted the variance.

A similar pattern was evident when expression levels for the infection genes were displayed on a heatmap with unsupervised hierarchical clustering (Figure 3E). Three main clusters were evident. Uninfected mice displayed low expression levels for most infection genes, and imatinib induced a marginal increase in expression of some of these genes (Cluster I). A second cluster included the one-week infection water-treated group together with a few mice from the three-week infection time-point, which displayed a low bacterial burden (Cluster 2). A third cluster included all the one-week infection imatinib-treated mice, together with the remaining three-week infection mice (Cluster 3). As in the PCA plot, four of the five imatinib-treated mice in the three-week infection group displayed similar gene expression profiles, whereas expression levels of infection genes in water-treated mice exhibited more variance between animals. Taken together, these data indicate that treatment with imatinib caused the pattern of gene expression at 1-week of infection to resemble that seen at three weeks, and imatinib at three weeks of infection resulted in more uniform expression of infection genes.

Imatinib augments the infection gene signature. We next identified genes differentially regulated by imatinib. When directly comparing differences in gene expression with infection and with or without imatinib at the one- and three-week timepoints, no genes were identified as differentially expressed with imatinib at either timepoint as defined by an FDR of less than 0.05. Thus, treatment with imatinib did not cause significant changes in genes expressed during infection.

We next compared genes expressed with imatinib and infection at the one- and three-week time points to that of water-treated uninfected controls [called imatinib genes]. At one-week infection time point, imatinib upregulates 1,418 genes compared to 903 infection genes in infected mice treated with water alone (compare blue and red circles in Figure 3B). Of the 1,418 genes, 874 were shared amongst the infection and infection plus imatinib groups, and 569 were core infection genes that were upregulated at both one and three weeks of infection (Figure 3B). At the three- week timepoint, imatinib upregulated 1,490 genes, including all 676 three-week infection genes upregulated with water, as well as 229 additional genes also upregulated at the one-week time point (Figure 3C). Thus, at each timepoint, most infection genes are induced by imatinib, together with an additional 544 genes at one week and 585 genes at three weeks. Of the additional genes, ~300 genes are specific to each timepoint, and 227 genes are upregulated by imatinib at both timepoints (Supplemental Figure 2E). Thus, imatinib induced some 902 additional genes in infected animals, not originally identified as infection genes ("imatinib-infection genes").

When expression of the imatinib-infection genes differently regulated with imatinib during infection but not with infection alone are displayed in a heat map with unsupervised hierarchical clustering, three clusters were evident (Figure 3F). Cluster 1 contained all the uninfected mice together with 3 of 5 one-week infection mice with no treatment. The second cluster (Cluster 2) contained the rest of the mice infected for one week, including all the one-week infection mice treated with imatinib, and a few of the three-week infection mice that had lower bacterial CFUs in the spleen and tail (*, Figure 3F). The third cluster contained all remaining three-week infection mice, including those treated with imatinib or water. Taken together, these

data indicate that imatinib treatment augments the infection gene signature to include additional imatinib-infection genes at both the one-week and three-week time points, which are similarly regulated without imatinib treatment, but not as reliably expressed.

We surmised that the imatinib-infection gene signature might correlate with changes in granuloma formation. To test this possibility, we further characterized the imatinib-infection gene signature, focusing on the one-week time point, where differences between the imatinib- and water-treated animals were most apparent. At this timepoint, imatinib upregulated 626 more genes than infection alone and downregulated 219 genes (Figure 4A). GO analysis of the upregulated genes indicated many immune system processes, including cell activation, response to and regulation of cytokines (Il1b, Il12, Il6, TNFα, Il8), wound healing, and cell death (Figure 4B). Notably, imatinib downregulated processes similar to those downregulated by imatinib in uninfected mice (Supplemental Figure 2B), including skin development as well as ECM and collagen organization (Figure 4C). At the three-week infection timepoint, imatinib upregulated 814 more genes than infection alone and downregulated 372 genes (Supplemental Figure 2F). GO analysis indicated the upregulated genes at three weeks are involved in similar processes as those upregulated with imatinib at the one-week timepoint. This included response to lipopolysaccharide, regulation of TNFα production, and apoptotic processes (Supplemental Figure 2G). The downregulated genes at the three-week timepoint were associated with lipid metabolism, tissue development, and supramolecular fiber organization (Supplemental Figure 2H). Thus, processes up- and down-regulated with imatinib at three weeks were similar to those evident at the one-week timepoint. Taken together, GO analysis indicated that imatinib

upregulated processes associated with immune responses and wound healing, but downregulated those associated with development of epidermis and connective tissue.

Imatinib treatment enhances macrophage activation and induces production of TNFα. The observations that imatinib enhances myelopoiesis and phagolysosomal fusion in infected macrophages (Napier et al., 2015; Napier et al., 2011), and upregulates GO processes associated with immune activation (Figure 4B), led us to hypothesize that imatinib facilitates the capacity of macrophages to detect and respond to mycobacterial infection. To test this possibility, we quantified cytokine expression in granulomas. In the RNAseq analysis, infection increased levels of *tnf* at the one-week timepoint, an effect slightly augmented with imatinib, and levels increased further by three weeks, where imatinib effects were still more apparent, though these differences were not statistically significant (Figure 4D). Levels of *tnf* RNA by qPCR were likewise not significantly different with imatinib plus infection compared to infection alone at one week (Figure 4E); however, levels of TNFα protein were significantly higher with imatinib plus infection compared to infection alone (Figure 4F). Changes in the levels of *il12b* RNA, which encodes a subunit of IL12p70, were similar in both groups (Figure 4G). However, protein levels of IL12p70 were strongly upregulated with imatinib at one week of infection (Figure 4H). Levels of *il10,* which encodes the immune regulatory cytokine IL-10, significantly increased with infection plus imatinib (Figure 4I) compared to infection alone, though this effect was not recapitulated in measurements of IL10 protein levels (Figure 4J). Other factors upregulated in Mtb granulomas and associated with protection, such as *nos2,* were unchanged with imatinib (Figure 4K).

Together, these data indicate that imatinib treatment activates pro-inflammatory signaling in granulomas, but also induces transcription of cytokines that resolve inflammation.

Macrophages are among the first cells infected by mycobacteria and a source of TNFα (Harris et al., 2008; Keane et al., 2002). To test the hypothesis that imatinib induces TNFα or other cytokines in macrophages, bone marrow derived macrophages (BMDM) from WT C57Bl/6J mice were infected with Mm at an MOI of 10, exposed to imatinib, and cytokine levels in the media measured 24 hours later. Compared to untreated cells, imatinib induced expression of *tnf* mRNA (Figure 4L) and enhanced release of TNFα into the media (Figure 4M) compared to infection alone. Notably, *tnf* mRNA levels evident with imatinib returned to baseline levels similar to those of control infected cells by 44 hours p.i.(Figure 4N). Il12p70 protein was not produced in measurable levels from the BMDMs after Mm infection though levels of *il12b* mRNA were increased with imatinib treatment (Figure 4O). Imatinib also induced secretion of IL10 from BMDMs (Figure 4P), contrary to *in vivo* results, though *il10* mRNA levels remained unchanged in these cells (Figure 4Q). *nos2* mRNA levels were strongly induced with infection in BMDMs over the first 24 hours and continued to increase thereafter, with imatinib limiting *nos2* mRNA production (Supplemental Figure 3A and B). Despite imatinib reducing levels of *nos2*, a marker of M1 macrophage activation, M2 macrophage activation markers *chil3l3* (YM1) and *arg1* were not changed (Supplemental Figure 3C and D). These data indicate that upon infection of BMDMs, imatinib augments production of TNFα, a marker of macrophage activation, mirroring effects seen in infected tissues in the mice (Figure 4F), though *in vivo* regulation by imatinib of other factors, such as *nos2* and *il10*, was not recapitulated in BMDMs.

Imatinib increases cellular survival and reduces necrosis in infected BMDMs. TNFα signaling coordinates cell death and survival (Laster et al., 1988; Webster & Vucic, 2020), and regulates Mtb infections and granulomas, including in humans (Bean et al., 1999; Gómez-Reino et al., 2003; Kaneko et al., 1999; Keane et al., 2001). The observation that imatinib augments TNFα production in infected cells raised the possibility that the drug might also regulate cell death or cell survival within lesions. To determine whether imatinib regulates apoptosis in granulomas, the level of cleaved caspase 3 was quantified by western analysis in the tails of the mice infected with Mm and treated for 7 days with water or imatinib. Levels of cleaved caspase 3 were evident with infection, with some animals showing higher levels than others; however, on average, imatinib treatment did not affect levels of cleaved caspase 3 (Figure 5A), nor, by inference, the level of apoptosis. Although necrosis was evident in most tissue sections (Supplemental Figure 1A), no differences were evident with imatinib treatment (Supplemental Figure 1C, H, and M).

Induction of NFKb signaling by TNFα upregulates expression of factors that promote cell survival (Schlicher et al., 2016; Webster & Vucic, 2020), raising the possibility that imatinib might promote cell survival. To test whether imatinib increased cell viability, mitochondrial redox potential was assessed in WT BMDMs. To do this, cells were treated with imatinib with or without infection with Mm at an MOI of 5, and mitochondrial redox potential measured 24 hours later. Imatinib treatment of uninfected BMDMs increased redox potential by ~15%, and no changes in were evident upon infection with Mm compared to uninfected cells. However, imatinib treatment increased redox potential by 8% in infected cells (Figure 5B), indicating a survival benefit with the drug.

To determine whether imatinib affected necrosis, WT BMDMs were cultured with or without imatinib and infected with Mm at an MOI of 1 or left uninfected. Cells were imaged over a 36-hour period in the presence of Cytotox reporter, which labels cells that have lost membrane integrity, an indication of necrosis. With or without imatinib, some necrotic cell death was evident after four hours p.i., which plateaued after 24 hours. Imatinib reduced the amount of necrosis between 16 and 24 hours (compare black and green lines; Figure 5C). Upon infection with Mm at an MOI of 1, the percentage of cells undergoing necrosis increased over time (blue line, Figure 5C). With imatinib, the levels of necrosis in infected cells initially increased over 20 hours at a rate similar to that of untreated cells. After 24 hours, levels of necrosis in untreated cells continued to increase whereas necrosis in imatinib-treated cells declined to levels seen in uninfected cells (compare red and blue lines; Figure 5C). By 30 hours p.i., the level of necrosis with infection was 2-fold lower in imatinib-treated cells compared to controls (Figure 5D). Taken together, these data indicate that imatinib increases survival of BMDMs and limits necrosis.

Imatinib effects on necrotic cell death in BMDMs depend upon caspase 8. To test the possibility that imatinib might regulate cell death or survival, imatinib effects were assessed in BMDMs derived from mice with deficiencies in TNFα signaling. Cells from mice lacking RIP3 kinase but heterozygous for caspase 8 ($Ripk3^{-/-}$ $Casp8^{-/+}$) do not undergo TNFα-mediated necroptosis, whereas cells from mice lacking both RIP3 kinase and caspase 8 ($Ripk3^{-/-}$ $Casp8^{-/-}$) can neither undergo necroptosis nor extrinsic apoptosis in response to TNFα (Kaiser et al., 2011; Moerke et al., 2019). Without infection, little necrotic cell death was evident in BMDMs derived from either $Ripk3^{-/-}$ $Casp8^{-/+}$ mice or $Ripk3^{-/-}$ $Casp8^{-/-}$ mice, and no additional effect of imatinib was

discernable (Figures 5E and F; Black and green lines). Induction of discernable levels of cell death in BMDMs from Ripk3$^{-/-}$ Casp8$^{-/+}$ or Ripk3$^{-/-}$ Casp8$^{-/-}$ required infection with Mm at an MOI of 10 rather than an MOI of 1 used in WT BMDMs, though even at this MOI, the percentage of cells undergoing necrosis was still lower than in WT cells (compare Figure 5C, E, and F). With infection, BMDMs from Ripk3$^{-/-}$ Casp8$^{-/+}$ and Ripk3$^{-/-}$ Casp8$^{-/-}$ showed similar initial levels of necrosis without imatinib, which increased over 24hrs before plateauing (Figures 5E and F). With imatinib treatment, Ripk3$^{-/-}$ Casp8$^{-/+}$ showed statistically significant reductions in the amount of necrosis by 30 hrs. p.i. (Figures 5E and G; compare blue and red line). However, imatinib did not affect the level of necrosis in Ripk3$^{-/-}$ Casp8$^{-/-}$ cells (Figure 5F and H, compare blue and red lines). Together, these data indicate that infection with Mm induces necrosis in BMDMs, and that imatinib limits necrosis in a manner that depends upon caspase 8.

Imatinib-mediated reduction in pathology of granulomatous lesions is abrogated in mice lacking caspase 8. We next determined effects of imatinib on granuloma growth in Ripk3$^{-/-}$ Casp8$^{-/+}$ and Ripk3$^{-/-}$ Casp8$^{-/-}$ mice infected with Mm and exposed to imatinib or water seven days later (Figure 6A). Lesions in Ripk3$^{-/-}$ Casp8$^{-/+}$ mice treated with water grew an average of 13.5mm from day seven to fourteen p.i, whereas lesions from mice treated with imatinib grew on average by 4mm over the same period (Figure 6B). Lesion growth in Ripk3$^{-/-}$ Casp8$^{-/+}$ mice was not significantly different from that seen in C57Bl/6J mice, and imatinib treatment reduced lesion growth by a similar amount in both the C57Bl/6J and Ripk3$^{-/-}$ Casp8$^{-/+}$ animals (Figures 6B and C). Lesions in infected Ripk3$^{-/-}$ Casp8$^{-/-}$ mice grew by 23mm on average from day seven and fourteen, with half the animals developing severe and extensive lesions measuring >30mm. Treatment of

the Ripk3$^{-/-}$ Casp8$^{-/-}$ mice with imatinib did not significantly reduce lesion growth (Figures 6B and D). The bacterial burden in the tails of both Ripk3$^{-/-}$ Casp8$^{-/+}$ and the Ripk3$^{-/-}$ Casp8$^{-/-}$ mice was ~7 Log_{10} CFU gram^{-1}tissue, and imatinib treatment resulted in a 1 log_{10} (Figure 6E) increase in both genotypes. In the Ripk3$^{-/-}$ Casp8$^{-/+}$ mice, this increase in CFUs in the tail was seen despite the reduction in lesion size. Notably, bacteria load in spleen was similar between C57BL/6J, Ripk3$^{-/-}$ Casp8$^{-/+}$, and the Ripk3$^{-/-}$ Casp8$^{-/-}$ mice, and imatinib did not cause a detectable change (Figure 6F). These data indicate that deletion of RIP3 kinase does not preclude imatinib from limiting the pathology associated with granulomatous lesions; moreover, the capacity of imatinib to limit pathology of granulomatous lesions resulting from Mm infection was abrogated in mice lacking caspase 8.

Discussion

Imatinib augments anti-mycobacterial immune responses. Imatinib both induces myelopoiesis and promotes phagolysosome fusion (Napier et al., 2015; Napier et al., 2011). Data presented here indicate that many untreated mice develop transcriptional responses associated with immune activation after three weeks of infection that resemble those induced in infected animals after one week with imatinib (Figure 3). Moreover, imatinib-induced transcriptional responses at three weeks appear more uniform owing to an increased proportion of mice displaying similar responses at that time point. In short, the response to infection with imatinib is more rapid and more efficient, but not substantially different from that developing at three weeks without drug. One possible explanation for the more rapid response to infection with imatinib is an increased capacity for phagolysosomal fusion in infected macrophages (Bruns et al., 2012; Napier et al., 2011), which precludes the capacity of mycobacteria to limit macrophage activation and antigen presentation. Additionally, increased numbers of innate immune cells resulting from enhanced myelopoiesis with imatinib could facilitate a more robust innate response (Napier et al., 2015).

TNFα appears to be a key marker of macrophage activation in response to imatinib (Figure 4). However, granuloma formation and maintenance are also in part mediated by TNFα. Thus, mice deficient in TNFα exhibit disorganized granulomas (Bean et al., 1999; Kaneko et al., 1999), and patients administered drugs that neutralize or inhibit TNFα are at increased risk of reactivating TB (Gómez-Reino et al., 2003; Keane et al., 2001; Park et al., 2022). Other cytokines, including interleukin-12 (Il12) and interferon-γ (INF-γ), also contribute to development and organization of granulomas, and activate innate immune cells that combat Mtb (Cooper et al.,

1993; Cooper et al., 1997; Flynn et al., 1993; Harris et al., 2008; Ramakrishnan, 2012). In line with TNFα as a cellular activation marker, imatinib initially increases TNFα production in infected BMDMs. However, induction of TNFα is not sustained, and declines to levels seen in untreated cells (Figure 4). Notably, sustained increases in TNFα are associated with chronic inflammation and tissue damage in inflammatory bowel disease and rheumatoid arthritis (Webster & Vucic, 2020). Likewise, excess TNFα is associated with ROS-mediated tissue damage in Mm infections in zebrafish (Roca & Ramakrishnan, 2013; Tobin et al., 2010). Taken together, these data support the hypothesis that imatinib subtly modulates TNFα production to augment anti-mycobacterial immune responses but may also regulate granuloma growth.

Imatinib limits granuloma formation and growth. Granulomas have long been considered a beneficial response against mycobacterial infection as they limit bacterial dissemination. However, during Mtb infection, these structures have also been shown to promote chronic infection by limiting access of immune cells to bacteria(Gautam et al., 2018; McCaffrey et al., 2022). Granulomas also limit access of antibiotics to the bacteria, resulting in suboptimal concentrations in the caseum, where most bacteria reside (Sarathy & Dartois, 2020; Sarathy et al., 2018). Bacterial persistence can also induce chronic inflammation, which can scar and damage lung tissue. Moreover, attempts to heal damaged tissue results in deposition of collagen, which causes fibrosis and restricts pulmonary elasticity (Wilson & Wynn, 2009). In mice infected with Mm, where granulomas are induced by a high inoculum (Figure 1), significant reductions in lesion growth are evident with imatinib. This effect does not depend on bacterial load, which is unaffected by imatinib at this inoculum. Microscopic analysis indicated that imatinib reduced the

amount of inflammation but not the composition as necrosis, calcification, edema, ulceration, thombi, acute inflammation, and chronic inflammation were observed in both groups (Figure 2). Taken together, these data indicate that imatinib reduces the growth of the lesions and the extent of inflammation but does not otherwise alter histological features of the lesions.

Imatinib has been shown to limit collagen deposition by inhibiting PDGFR and fibrosis in non-infectious indications (Akhmetshina et al., 2008; Akhmetshina et al., 2009; Distler et al., 2007; Li et al., 2009; Wang et al., 2010; Yoshiji et al., 2005). Accordingly, reductions in collagen are suggested by the GO analysis of gene expression data from imatinib-treated mice (Supplemental Figure 2). These data raise the possibility that imatinib may facilitate wound healing in a way that is less likely to induce fibrosis, which in turn may limit scaring and long-term tissue damage. This finding has important implications for lung health during Mtb infection. Chronic inflammation has been associated with increased fibrosis and lung dysfunction in TB patients (Hnizdo et al., 2000; Malherbe et al., 2016; Plit et al., 1998; Ravimohan et al., 2018), which results in increased economic burden of patients who have successfully completed antibiotic therapy(Meghji et al., 2021). We postulate that imatinib may be an effective treatment for patients with active TB by reducing fibrosis and lung dysfunction. The structure of the granuloma has also been shown to limit antibiotic penetrance into the granuloma(Sarathy & Dartois, 2020; Sarathy et al., 2018). By limiting formation and/or promoting resolution of granulomas, imatinib may increase access of antibiotics to the bacteria and thereby shorten duration of treatment.

Imatinib promotes cell survival and limits necrosis. Cell death is a major contributing factor to bacterial spread and tissue pathology (Behar et al., 2010; Pan et al., 2005; Ramakrishnan, 2012; Srinivasan et al., 2014). Apoptosis has been shown to limit spread and replication of Mtb by encapsulating the bacteria in apoptotic bodies and allowing macrophages to efferocytose apoptotic bodies containing Mtb, and transfer them into the lysosome (Martin et al., 2012). By contrast, necrosis facilitates spread of bacteria, promotes inflammation, and contributes to tissue damage by releasing ROS (Amaral et al., 2019; Behar et al., 2010; Ramakrishnan, 2012). Ferroptosis, an iron-dependent form of necrosis, has recently been identified as a major contributor to pathology during Mtb infection (Amaral et al., 2019). Several lines of evidence indicate that altering the type of death that a cell undergoes can impact Mtb infection. For example, a mutation in the Lpr1 gene, which encodes LDL receptor, can shift macrophages from necrosis to apoptosis, thereby limiting replication and spread of the bacteria (Pan et al., 2005). In this regard, imatinib has been shown to increase apoptotic cell death in BCR-Abl^{+} transformed cells but is generally without effect on non-transformed cells (Puissant et al., 2010). Tails in mice infected with Mm display reduced gross pathology and more focused areas of tissue damage with imatinib, which is consistent with reduced necrosis and/or increased survival though not changes in necrosis at a gross level were evident (Supplemental Figure 1). Accordingly, in cultured macrophages, imatinib has little effect on uninfected cells, but reduces necrosis in infected ones (Figure 5).

Our data with the Ripk3$^{-/-}$ Casp8$^{-/-}$ mice suggest a possible role for caspase 8 in imatinib effects on granulomas (Figure 5 and 6), possibly by regulating necrosis, or cell survival, or both. In this regard, caspase 8 promotes extrinsic apoptosis in response to signaling via death receptors such

as TNF receptor 1 and CD95 (Moujalled et al., 2013; Webster & Vucic, 2020), though no evidence of caspase 8-mediated apoptosis was evident in our analysis (Figure 5A). However, caspase 8 also promotes cell survival by complexing with c-Flip, which inhibits apoptosis, and precludes activation of RipK3, which prevents necroptosis (Fritsch et al., 2019; Oberst & Green, 2011). Finally, caspase 8 regulates transcriptional responses that dampen inflammation (Philip et al., 2016). Any of these activities could contribute to imatinib's effect on reducing granuloma growth or limiting inflammation *in vivo*. Further characterization of cellular mechanisms associated with enhanced survival and/or reduced necrosis with imatinib and caspase 8 are needed to elucidate how the drug facilitates resolution of granulomas.

Interestingly, an increase in CFUs was observed in the mouse tail but not spleen with imatinib treatment in both mice strains lacking RipK3. In mice infected with Mtb, it has been suggested that RipK3 facilitates the spread of bacteria (Zhao et al., 2017) by mediating necrotic death, and mice lacking RipK3 developed lower bacterial CFUs in their lungs. In our model system, mice deficient in RipK3 did not show significant differences in bacterial colonization of the tails compared to control C57BL/6J mice. While imatinib treatment increased the bacteria load in the tail, the bacteria load induced by imatinib was not significantly different from that in C57BL/6J mice. Based on our analysis to date, a role for RipK3 in imatinib effects on Mm is not apparent. However, we cannot rule out that possible secondary alterations in immune responses evident in these mutant animals that could mask an addition role for this enzyme during infection.

Imatinib as an HDT. Recent efforts by us and others (Napier et al., 2012) have raised the possibility that HDTs may be effective against TB. Because HDTs do not directly select against the

bacteria, they are less likely to engender resistance compared to antibiotics. Some HDTs interfere with the capacity of Mtb to subvert host systems, whereas others regulate immune responses, so as to induce novel responses or target deficiencies in individuals with active disease (Wallis et al., 2022). Drug discovery efforts have traditionally focused on drugs targeting specific processes with minimal off-target effects, though recent efforts, for example with COVID-19, have targeted hyperinflammatory pathways as a means to limit tissue damage(Wallis et al., 2022). Such approaches can be complex to implement, requiring proper timing and dosing to limit tissue damage while still permitting requisite inflammatory responses that contain the infection. Our studies suggest that imatinib likewise affects multiple aspects of the host immune response to mycobacteria, but it does so in ways that do not fundamentally alter the response, but rather subtly tune it to favor the host.

Imatinib remains a promising candidate HDT to treat mycobacterial infections such as TB by not only activating innate immune responses but also reducing pathology. Imatinib is currently being tested in the IMPACT-TB clinical trial to determine effective dosing in healthy humans as measured by the capacity of human blood to eliminate mycobacteria. Data presented here indicate that imatinib does not fundamentally alter the immune response to mycobacterial infection, but rather augments the rate and efficiency at which it develops. This work also shows how an HDT can limit granuloma formation. Such an effect has important implications for therapeutic strategies to control TB as the granuloma has been shown to limit antibiotic and immune cell access to the bacteria, leading to bacterial persistence and contributing to lasting tissue damage and scaring.

Acknowledgments: The authors thank Rustom Antia, Greg Bisson, Robert Wallis, Deepak Kaushal, Ruth Napier, Alyson Swimm, Bernie Weiss, and Robert Sonowal for helpful discussions or for reviewing the manuscript. *Research reported in this publication was supported in part by the Cancer Tissue and Pathology shared resource of Winship Cancer Institute of Emory University and NIH/NCI under award number P30CA138292. Research reported in this publication was supported by the Emory NPRC Genomics Core in part by NIH P51 OD011132. Sequencing data was acquired on an Illumina NovaSeq 6000 funded by NIH S10 OD026799*.

Materials and Methods

Mice. 8–12-week-old C57Bl/6J mice were purchased from The Jackson Laboratory. Ripk3$^{-/-}$Casp8$^{-/-}$, or Ripk3$^{-/-}$Casp8$^{-/+}$ were bred at Emory University by crossing Ripk3$^{-/-}$ Casp8$^{-/+}$ females to Ripk3$^{-/-}$Casp8$^{-/-}$ males (Kaiser et al., 2011). PCR genotyping of *Casp8*$^{-/-}$ and Casp8$^{-/+}$ mice was performed with primers 5'-TTGAGAACAAGACCTGGGGACTG and 5'-GGATGTCCAGGAAAAGATTTGTGTC. PCR amplification allele produces a 750-bp band (*Casp8*$^{+}$), or a 200-bp band (*Casp8*$^{-}$). Mice were bred and maintained by Emory University Division of Animal Resources where all procedures were approved by the Emory University Institutional Animal Care and Use Committee.

Imatinib administration. Imatinib mesylate salt was dissolved in water and loaded into Alzet pumps (Braintree Scientific, 1007D; Cupertino, CA) capable of dispensing a continuous flow of drug at 100mg/kg/day. Pumps were inserted subcutaneously into anesthetized 8-12-week old mice. Alzet pumps were inserted 24 h prior to infection or 7-21 days post infection depending on experiment time course.

Bacterial strains. *M. marinum (*Mm*)* strain 1218R (ATCC 927) was grown in Middlebrook 7H9 broth (7H9) (BBL Microbiology Systems, Cockeysville, MD) supplemented with ADC (Difco Laboratories, Detroit, MI,) and 0.05% Tween 80 (Sigma-Aldrich, St. Louis, MO) or 0.025% Tween 80. For CFU assays, 7H10 agar supplemented with 10% oleic acid-albumin-dextrose-catalase (OADC) was used (Difco Laboratories, Sparks, MD). For Mm infection in mice and infections of BMDMs, bacterial stocks were grown at 30°C for 2 days to an OD_{600} of 0.4 approximately $6.3x10^6$CFU/mL (Eppendorf, BioPhotometer; Hamburg, Germany). Bacteria were washed with

sterile PBS and aspirated through a 27G needle against the side of the tube multiple times to break up bacterial clumps. Bacteria was then diluted in sterile phosphate buffered saline [PBS] for mouse infection or complete cell culture media for the BMDM infections.

Mouse Mm infection. Mice were infected with actively growing Mm (ATCC 927) via the tail vein to induce a systemic infection that results in the development of mycobacterial granulomas on the tail of the mouse. The inoculum, determined by retrospective plating, was ~2x10^6CFU in each experiment. In C57BL/6J mice tail lesions appear on the mouse tail about 1 week after infection and can be monitored over time by measuring the length of each lesion from top to bottom using a ruler, to get a measure of the total lesion lengths per mouse tail. After 7 days of treatment, tail, spleen, and lung were harvested. For CFU, spleens, and lung, were weigh and homogenized in the Bullet Blender Tissue homogenizer (Next Advance, Troy, NY) in 1 ml PBS. ~3-5 mm of tail was cut into small pieces using sterile scissors before being homogenized in the same way as lung and spleen. Each homogenate was diluted and spread on 7H10 agar plates. Colonies were scored after 7 days at 30°C. Colonies per ml were normalized to the initial weight of the tissue, to determine CFU/g tissue.

Tail protein isolation for ELISA and Western analysis. For soluble protein isolation for ELISA, 3-5mm sections of tail were cut fresh from the tail and immediately placed in liquid nitrogen. Samples were stored at -80°C until processed. Samples were weighted and put in PBS, scissors were used to break apart the tissue before the tissue was homogenized in the Bullet Blender Tissue homogenizer (Next Advance, Troy, NY). Il12P70 (Invitrogen), TNFα(Invitrogen), and IL10

(Invitrogen) ELISAs were run according to manufacture directions. For western analysis of tail protein content, 3-5 mm of flash frozen tail samples were weighted and put in ice cold RIPA buffer supplemented with a protease inhibitor cocktail (Roche). Scissors were used to break apart the tissue before the tissue was homogenized in the Bullet Blender Tissue homogenizer (Next Advance, Troy, NY). Protein concentration was determined using BCA assay kit (ThermoFisher), before the sampled were run on 15% SDS-PAGE gel and blotted onto a PVDF membrane. Cleaved Caspase 3 (ASP175) antibody (1:1000, Cell Signaling) and GAPDH (D16H11) antibody (1:1000, Cell Signaling) were used sequentially on the same membrane to quantify and normalize protein content.

***Mouse tail RNA isolation*.** 3-5mm sections of tail were cut fresh from the tail from areas containing granulomatous lesions and immediately placed in Trizol (Invitrogen). Tissue was cut with sterile scissors and then homogenized in the Bullet Blender Tissue homogenizer (Next Advance, Troy, NY). RNA extraction was performed in accordance with manufacture instructions.

Histology. Tails were removed from the mouse and a clean razor used to slice tails into 3-5 mm lengths that were placed in a cassette and submerged in 10% neutral buffered formalin for 24-48 hours. Tail sections were washed and decalcified in Immunocal decalcifier (StatLab, McKinney, Tx) for 48 hrs before being moved to 70% ethanol for storage until the sections could be embedded in paraffin, and sectioned. Sections were stained by H&E (abcam) and acid-fast bacillus [AFB] stain (abcam).

***Histology scoring*.** Histology was obtained from the tails of mice infected for 14 days and treated with control or 100 mg/kg/day imatinib for the last 7 days of infection. Slides of H&E-stained tail sections containing 2-9 individual tail tissue cross sections/mouse were deidentified and randomized before they were submitted to a pathologist for scoring. The pathologist examined slides first to create a rubric for characteristics, then blindly scored all the sections based on the noted characteristics. The percent of inflammation was determined in the section with highest amount of inflammation.

BMDM generation. L929 cells were maintained in DMEM (Corning) with 10% fetal bovine serum (FBS, Gibco), 100U/ml penicillin and 100 U/ml streptomycin (Invitrogen). At confluency, fresh media was added to the L929 cells and collected and sterile filtered after 3 days. L929 conditioned media was stored at -20°C until use. For BMDM culture, tibias and femurs were collected from C57BL/6J, Ripk3$^{-/-}$Casp8$^{-/-}$, or Ripk3$^{-/-}$Casp8$^{-/+}$ mice. The ends of the bones were removed with a clean razor before the bones were placed in a sterile 0.7 ml Eppendorf tube with the bottom tip cut off inside a 1.5 mL sterile Eppendorf tube. Tubes were centrifuged at 13,000 RPM for 3 minutes to extrude the bone marrow cells. Cells were washed and filtered through a 70μM mesh filter with sterile PBS, before being plated in 10 cm petri dishes with 20% L929 conditioned media in fresh DMEM + 10% FBS + 100U/ml penicillin and 100 U/ml streptomycin. Cells were differentiated for 7-9 days before collection in ice cold PBS containing 0.5M EDTA.

BMDM RNA and protein collection. For RNA and protein collection, BMDMs were plated at 1x10^6 cells/ well in a 6 well plate. Cells were allowed to adhere overnight before being infected with

Mm at an MOI of 10 and treated with 1μM imatinib. Infection commenced for 24 hrs, at which time media was removed and stored at -80°C for cytokine quantification. TNFα (Invitrogen), and IL10 (Invitrogen) ELISAs were performed according to manufacturer instructions on undiluted media. Cells were lysed and RNA was collected using the RNeasy Mini Kit (Qiagen), accordioning to manufacture instructions.

BMDM cell viability assay. For cell viability assays, 10,000 cells/well were plated into a 96-well plate and allowed to adhere overnight. Cells were infected with actively growing Mm at an MOI of 5 and treated with 1 μM Imatinib in DMEM + 10% FBS and placed in an incubator at 37°C with 5% CO2. After 20 hours of infection, Cell Titer-Blue (Promega, Madison, WI) was added to the media and cells were incubated for 4 hrs at 37°C with 5% CO2 before fluorescence was measured using a plate reader.

BMDM Time course experiments. For time course experiments, 1-3x10^5 cells/well were plated into a 24 well plate and allowed to adhere overnight. BMDMs from C57Bl/6J mice were infected with actively growing Mm at an MOI of 1 of while BMDMs from Ripk3$^{-/-}$Casp8$^{-/-}$ and Ripk3$^{-/-}$Casp8$^{-/+}$ mice were infected with actively growing Mm at an MOI of 10 for 2 hrs in DMEM + 10% FBS. Wells were washed with PBS and 200 μg/ml amikacin in DMEM + 10% FBS was added for 2 hrs. Cells were washed again with PBS before DMEM + 10% FBS was added with 250nM Incucyte® Cytotox Green (Sartorius) +/- 1 μM Imatinib. Trays were then placed in the IncuCyte® ZOOM (Saritorius Essen) at 37°C with 5% CO2. Phase images and Green Fluorescence Images were taken at 20x every 2 hrs in. The area of phase objects and the area of green objects was quantified to

determine the green area per phase area for each image, indicating the amount of necrosis indicated by the green stain per the area of cells.

RNA-seq analysis. RNA was isolated from mouse tails from uninfected mice +/- 100mg/kg/day imatinib, infected mice at 6 days post infection +/- 100mg/kg/day imatinib, or infected mice 21 days post infection +/- 100mg/kg/day imatinib. RNA from the tails of 5 individual mice were used per group for a total of 30 samples. RNA libraries for RNA-seq were prepared using Clontech SmartER Stranded Total RNA-seq Kit- Pico Input Mammalian + rRNA depletion following manufacturer's protocols. Sequencing was performed at Yerkes Nonhuman Primates Genomics Core, Emory University, using Illumina NovaSeq 6000. Quality control was performed on the raw reads using FastQC and the remaining analysis was done using R. Adaptors were trimmed from the ends of reads using QuasR. Hisat2 was used for alignment of the reads to the GRCm38.p6 mouse genome with 66-80% of reads mapped to a single gene. 6-25% of the mapped reads were successfully assigned to gene alignments resulting in 5.5 million-14million assigned alignments per sample. Genes with low expression levels (>20 copies per million) in at least 5 samples filtered out. Data was normalized using the Trimmed Mean of M-values [TMM] method. Differential expression analysis was performed using edgeR, genes were determined by comparison of each group to the uninfected mice not treated with imatinib. Raw reads and processed gene counts in this paper have been deposited in the Gene Expression Omnibus (GEO) database, https://www.ncbi.nlm.nih.gov/geo/ (accession no. **GSE215176**).

QPCR. RNA was isolated from mouse tail or BMDMs as previously described. Reverse transcription was performed using the RevertAid First-Strand cDNA Synthesis Kit (Thermo Fisher Scientific) with the oligo(dT)18 primer. SYBR® Green Supermix (Bio-Rad) according to manufacturer instructions using a MyiQ real-time PCR system (Bio-Rad). The ΔΔCT method was used to determine relative gene expression using *Gapdh* as internal controls. Primers used were: *Gapdh*-forward 5' AGGTCGGTGTGAACGGATTTG3', *Gapdh*-reverse 5'TGTAGACCATGTAGTTGAGGTCA3', *il12b*-forward 5'TGGTTTGCCATCGTTTTGCTG3', *il12b*-reverse 5'ACAGGTGAGGTTCACTGTTTCT3', *tnf*-forward 5'CCCTCACACTCAGATCATCTTCT3', *tnf*-reverse 5' GCTACGACGTGGGCTACAG3', *il10*- forward 5'GCTCTTACTGACTGGCATGAG3', *il10*-reverse 5'CGCAGCTCTAGGAGCATGTG3', *nos2*-forward 5' GTTCTCAGCCCAACAATACAAGA3', *nos2*-reverse 5' GTGGACGGGTCGATGTCAC3', *chil3l3*-forward 5'CAGGTCTGGCAATTCTTCTGAA3', *chil3l3*-reverse 5'GTCTTGCTCATGTGTGTAAGTGA3', *arg1*-forward 5'CTCCAAGCCAAAGTCCTTAGAG3', *arg1*-reverse 5'AGGAGCTGTCATTAGGGACATC3'. The date generated was normalized to the lowest value.

***Statistical analysis*.** Statistical analysis was done using either Mann-Whitney U test to compare two groups or a one-way ANOVA to compare multiple groups. Values less than or equal to 0.05 were considered statistically significant. For RNA-seq analysis the false discovery rate (FDR) of less than 0.05 was used to determine differentially expressed genes and GO processes.

Figures

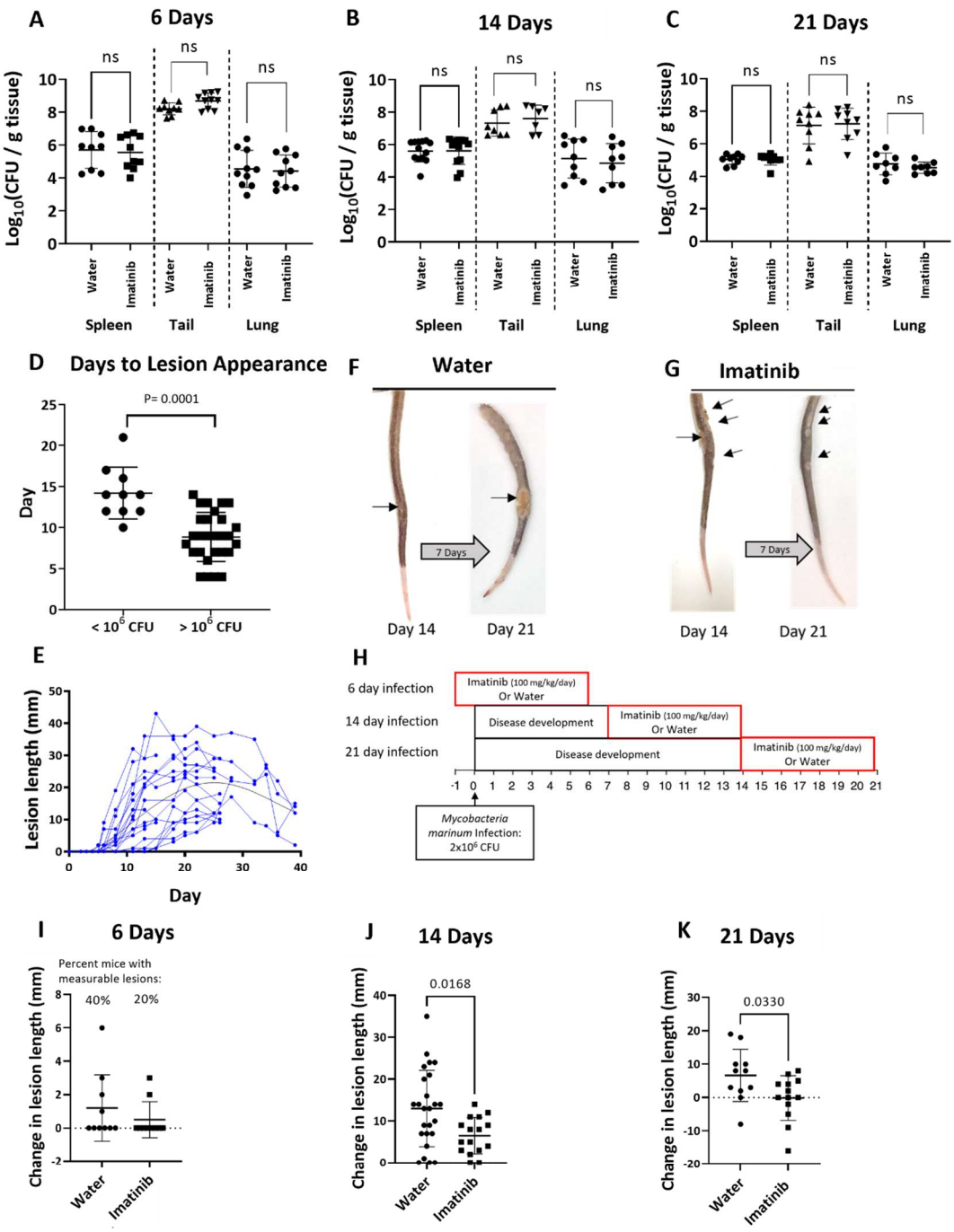


Figure 1: Imatinib causes a reduction in the size of granulomatous lesions in Mm-infected mice.

Figure 1: Imatinib causes a reduction in the size of granulomatous lesions in Mm-infected mice. **A-C.** Bacterial load in the spleen, tail, and lung of mice infected with $2x10^6$ CFU of Mm and treated with 100mg/kg/day imatinib via osmotic pump starting day -1, day 7, or day 14 for at total infection period of 6 days **(A)**, 14 days **(B)**, and 21 days **(C)**. **D.** Relationship between the number of days post infection that lesions are first visible on the tails as a function of the inoculum. High inoculum (>10^6 CFU/mouse) or a low inoculum (<10^6 CFU/mouse) of Mm were used (n=10-28 mice/group). **E.** Time course of lesion size increases and decreases for individua mice following inoculation with $2x10^6$ CFU of *Mm.* Tail lesion size was measured (mm) every 1-2 days for each mouse for up to 5.5 weeks post infection. **F, G.** Representative images of changes in tail lesions from day 14 to day 21 in mice treated with water (**F**) or imatinib (**G**) beginning at day 14. **H.** C57BL/6J mice were infected with $2x10^6$ CFU of *Mm*. Mice were then treated with 100mg/kg/day imatinib via osmotic pump starting day -1, day 7, or day 14. Treatment was administered for 7 days before mice were sacrificed for a total infection of 6 day (n= 10 mice/group), 14 days (n= 16-26 mice/group), or 21 days (n= 11-16 mice/group). **H.** Schema of administration of imatinib or water for 6, 14 and 21 days. **I-K.** Change in lesions size during imatinib or water treatment for 6 days **(I)**, 14 days **(J)**, or 21 days **(K).** Each data point represents one individual mouse in 2-5 experiments at each time point. Statistical tests used for comparisons in **A-D** and **I-K** was a two tailed Mann-Whitney U test, with $p \leq 0.05$ judged as significant; ns, not significant at the p=0.05 level. The mean +/- SD, for each group is presented to show the variance in the data.

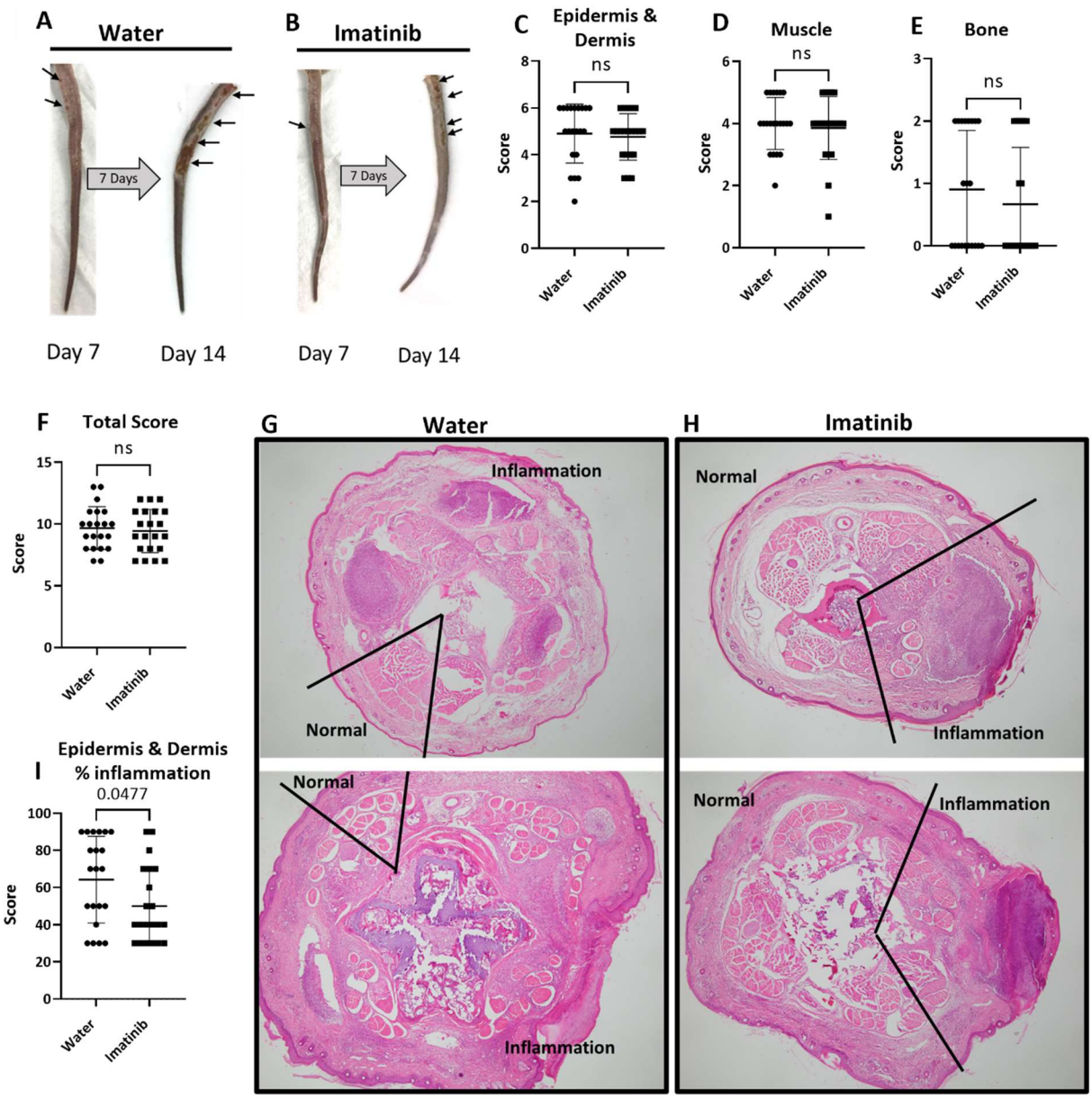


Figure 2. Imatinib reduces area of inflammation.

Figure 2. Imatinib reduces area of inflammation. Representative images of changes in tail lesions from day 7 to day 14 in mice treated with water (**A**) or imatinib (100mg/kg/day; **B**) beginning at day 7. Following sacrifice on day 14, cross sections of the tail were cut and stained with H&E. **C.** Composite pathology scores for the epidermis and dermis accounting for ulceration, necrosis, calcification, edema, acute inflammation, and chronic inflammation. **D.** Composite pathology scores for the muscle accounting for necrosis, edema, thrombi, acute inflammation, and chronic inflammation. **E.** Composite pathology scores for the bone accounting for necrosis and inflammation. **F.** Total composite pathology scores based on scores from epidermis and dermis, muscle, and bone. **G,H.** Representative images of whole tail sections at 14 days post infection from mice treated with water **(G)** or imatinib (100 mg/kg/day**; H)** demonstrating percent of the tissue section showing inflammation. Magnification: 40X. **I.** Quantification of the area of the tissue sections showing inflammation. Scoring in **C-F** and **I** is based on mice from 5 separate experiments (n=21 in each treatment group). Comparisons analyzed by a two tailed Mann-Whitney U test with p values ≤ 0.05 judged as significant; ns, not significant. The mean +/- SD, for each group was graphed to show the variance in the data.

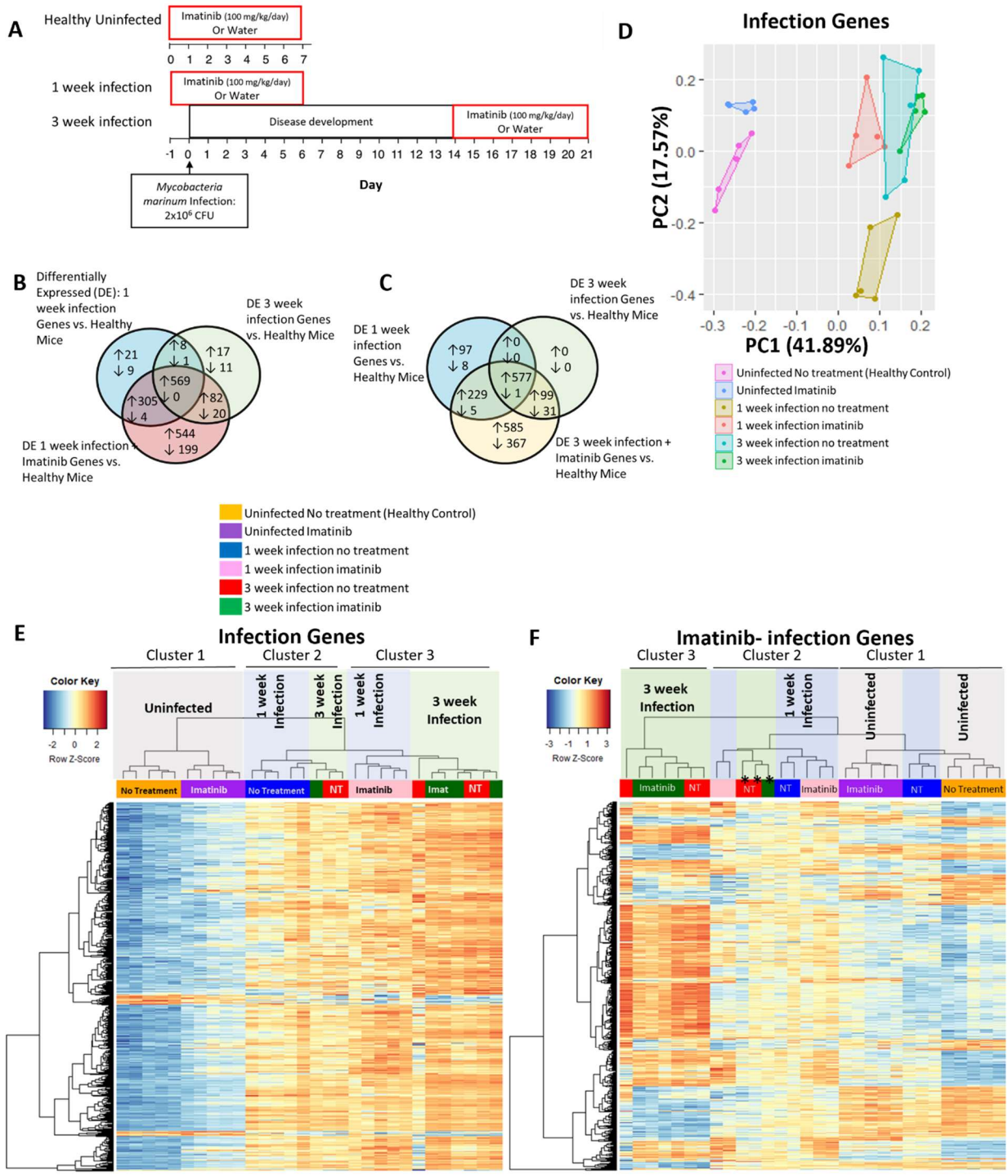


Figure 3. Imatinib upregulates a similar but larger number of genes with infection and at an earlier time point and with greater reliability than infection alone.

Figure 3. Imatinib upregulates a similar but larger number of genes with infection and at an earlier time point and with greater reliability than infection alone. A. Schema of administration of imatinib or water to C57BL/6J mice left uninfected or infected with $2x10^6$ CFU of *Mm*. Mice were treated with imatinib (100mg/kg/day) or water for 7 days starting day -1 or day 14 in infected group, or starting at day 0 in uninfected group, and then sacrificed seven days later (n=5 mice/group). **B. "**Infection genes" were identified by determining differentially expressed genes between the uninfected water-treated group, the one week infection water-treated group (917 genes, False discovery rate [FDR]< 0.05; blue circle), and the 3 week infection water-treated group (708 genes, FDR < 0.05; green circle) for a total of 1047 unique "infection genes" (578 genes were present at both time points). Imatinib upregulates most infection genes. Here, genes induced by imatinib at 1 week infection were identified by determining differentially expressed genes between the uninfected water treated group and 1 week infection plus imatinib group (1723 genes, FDR < 0.05; red circle). **C.** Genes induced by imatinib at 3 weeks post infection were identified by determining differentially expressed genes between the uninfected water-treated group to 3 week infection plus imatinib group (1894 genes, FDR < 0.05; yellow circle). This group was then compared to Infection genes at 1 week (blue circle) and 3 weeks (green circle) previously defined in **B. D.** The top two principal components for the variance of expression levels in all 6 of our treatment/infection groups of the 1047 "Infection Genes" identified in **B** are presented. **E.** Infection genes identified in **B** were graphed in a heat map with unsupervised hierarchical clustering. **F.** Genes specific to imatinib treatment at both time points identified in **B** and **C,** defined as "Imatinib-infection Genes," were graphed in a heat map with unsupervised hierarchical clustering.

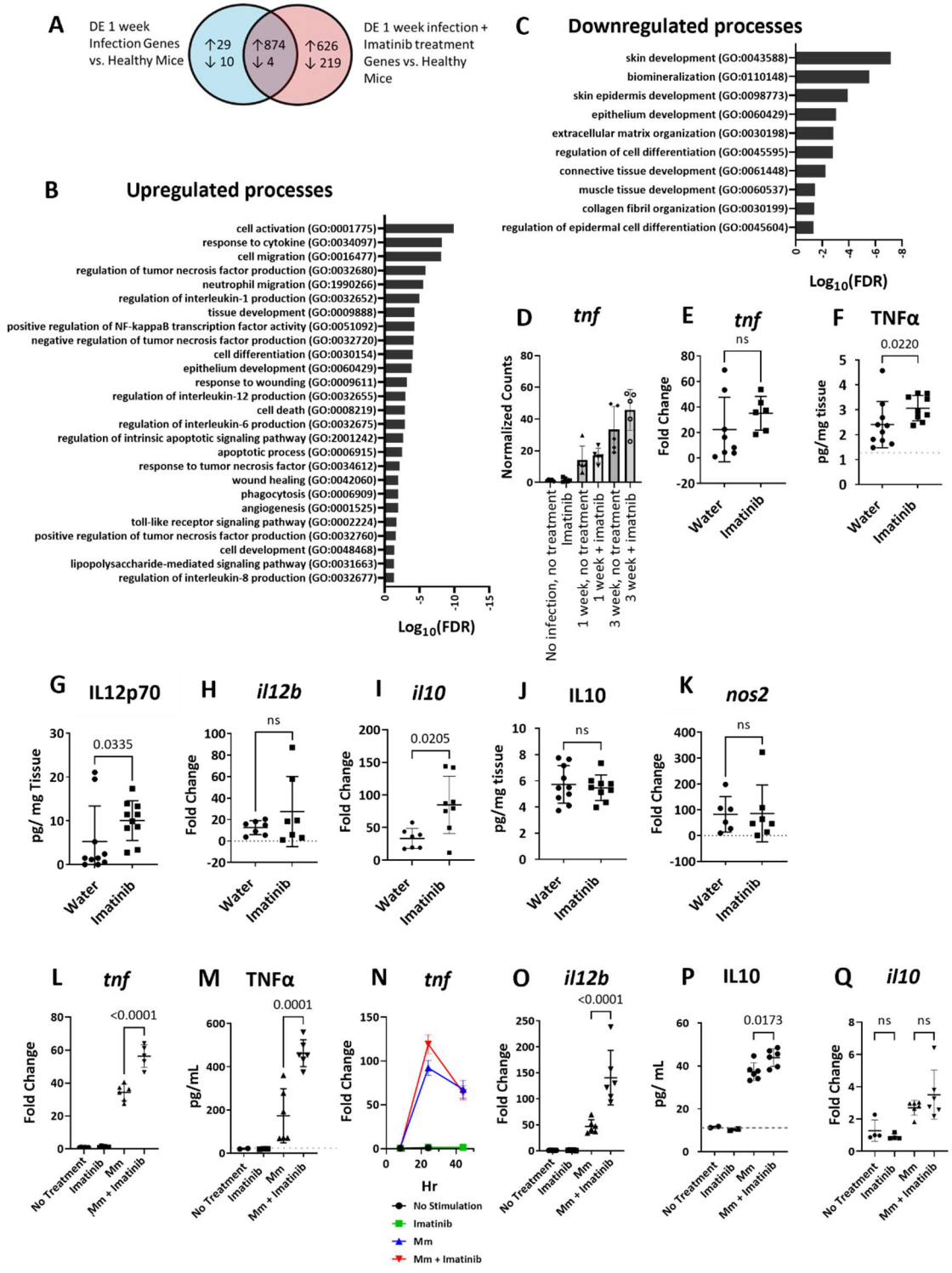


Figure 4. Imatinib treatment increases cell activation and cytokine production.

Figure 4. Imatinib treatment increases cell activation and cytokine production. A. Comparison of 1 week infection related genes and 1 week infection plus imatinib genes as described in Figure 3B**. B.** Selection of GO terms identified by analysis of 626 upregulated genes from **A** unique to imatinib treatment at 1 week infection. **C.** Selection of GO terms identified by analysis of 219 downregulated genes from **A** unique to imatinib treatment at 1 week infection. **D.** Normalized gene counts from RNAseq mapped to the *tnf* gene. **E-K.** RNA and protein were isolated from sections of the mouse tail at the 1 week infection time point (6 days infection) with water or imatinib treatment (100 mg/kg/day)**.** qPCR was used to measure mRNA levels of *tnf* **(E),** *il12b* **(H),** *il10* **(I),** and *nos2***(K)** (n=7 mice/group). Cytokine content was measured via ELISA for TNFα **(F)**, Il12p70 **(G)**, and Il10 **(J)** on the protein isolated from the mouse tails (n=10 mice/group). Statistical test used in **E-K** was a two-tailed Mann-Whitney U test, with p values indicated. A value of ≤ 0.05 was considered significant. **L-Q.** BMDMs derived from C57BL/6J mice infected with Mm at an MOI of 10 for 8, 24 or 44 hrs at 37◦C. ELISAs were used to measure TNFα **(M)**, and Il10 **(P)** in the media at 24 hrs (n=2-6 wells/group). RNA was isolated from the cells and qPCR was used to measure mRNA levels of *tnf* **(L),** *il12b* **(O),** and *il10* **(Q)** at 24 hrs (n=5-6wells/ group)**.** qPCR was used to measure levels of *tnf* in the cells 8,24, and 44hrs (**N;** n= 2 wells/ group; data are representative of 2 separate experiments). Statistical test used **in L-Q** was one-way ANOVA, with p values indicated. The mean +/- SD, for each group was graphed to show variance of data.

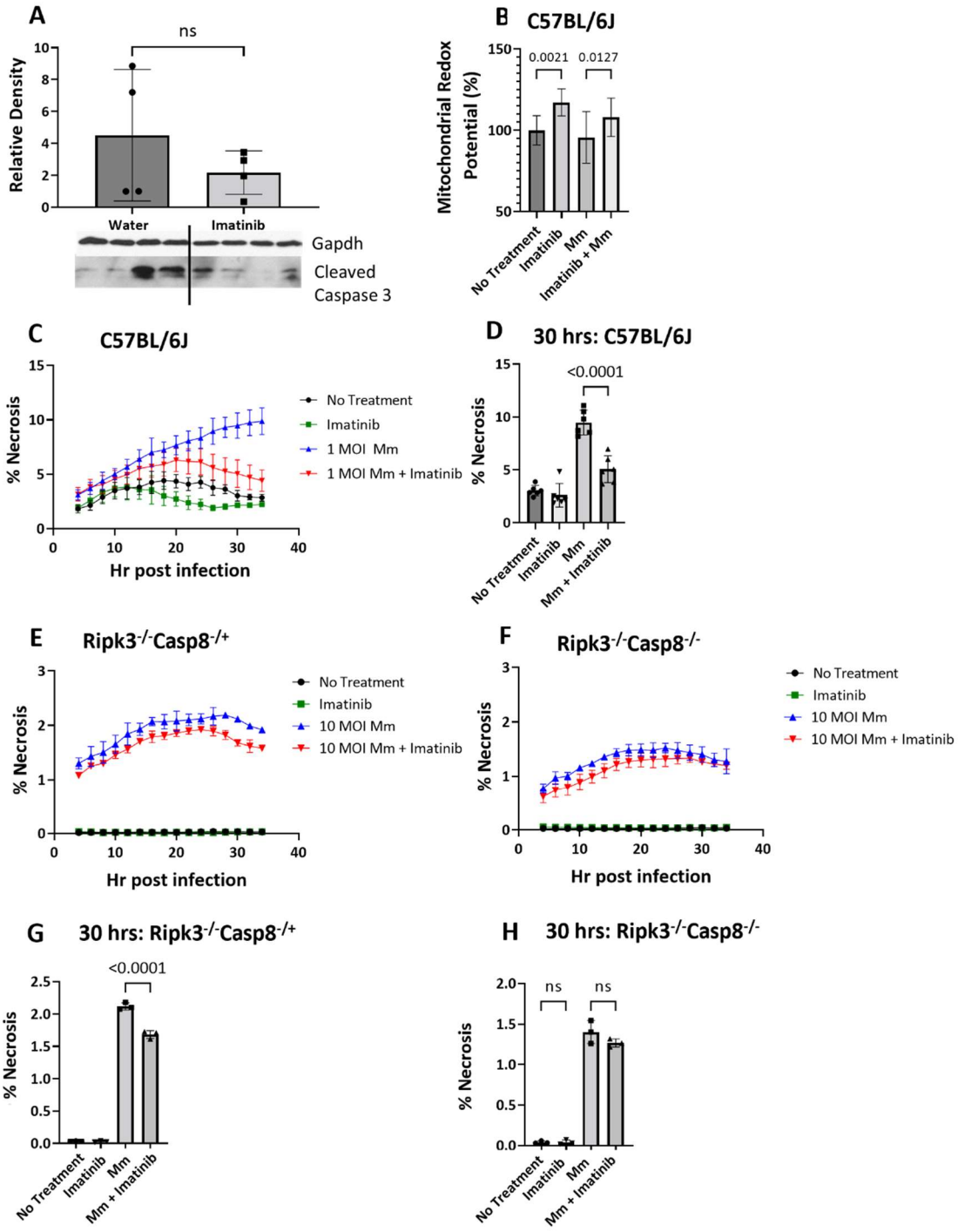


Figure 5. Imatinib limits necrosis in BMDMs, which depends upon caspase 8.

Figure 5. Imatinib limits necrosis in BMDMs, which depends upon caspase 8. A. Western blot analysis of cleaved caspase 3 was performed on protein isolated from sections of the mouse tail at 6 days post infection with water or imatinib treatment (100 mg/kg/day) for 7 days**. B.** BMDMs derived from C57BL/6J mice were infected with Mm at an MOI of 5 and left untreated or treated with 1μM imatinib. Cell viability was assessed 20 hrs post infection by measuring fluoresecence of resorufin, and indicator of redox potential. All groups were normalized to the average of the no infection/no treatment group (n= 10 wells/group; data shown are representative of 3 separate experiments). **C,D.** BMDMs derived from C57BL/6J mice were infected with Mm at an MOI of 1 and left untreated or treated with 1μM imatinib. Cells were imaged every 2 hrs with Cytotox dye (n= 6 wells/group, representative of 3 separate experiments), an indicator of necrosis. Percent (%) necrosis was quantified at each time point and graphed as a function of time post infection (**C**). Statistical analysis was performed on data at 30 hrs post infection, and graphed in **D**. **E-H.** BMDMs derived from Ripk3$^{-/-}$ Casp8$^{+/-}$ or Ripk3 $^{-/-}$ Casp8$^{-/-}$ mice were infected with Mm at an MOI of 10 and left untreated or treated with 1μM imatinib. Cells were imaged every 2 hrs with Cytotox dye (n=3 wells/ group, representative of 3 separate experiments). Data is show as a function of time **(E,F)**, and at the 30 hr timepoint **(G,H)** for the Ripk3$^{-/-}$ Casp8$^{+/-}$ or Ripk3 $^{-/-}$ Casp8$^{-/-}$ BMDMs, respectively. Statistical test used in **A** was two-tailed Mann-Whitney U test. Statistical test used in **B, D, G, & H** was one-way ANOVA. p values are indicated in graphs**.** The mean +/- SD for each group was graphed to indicate variance of data.

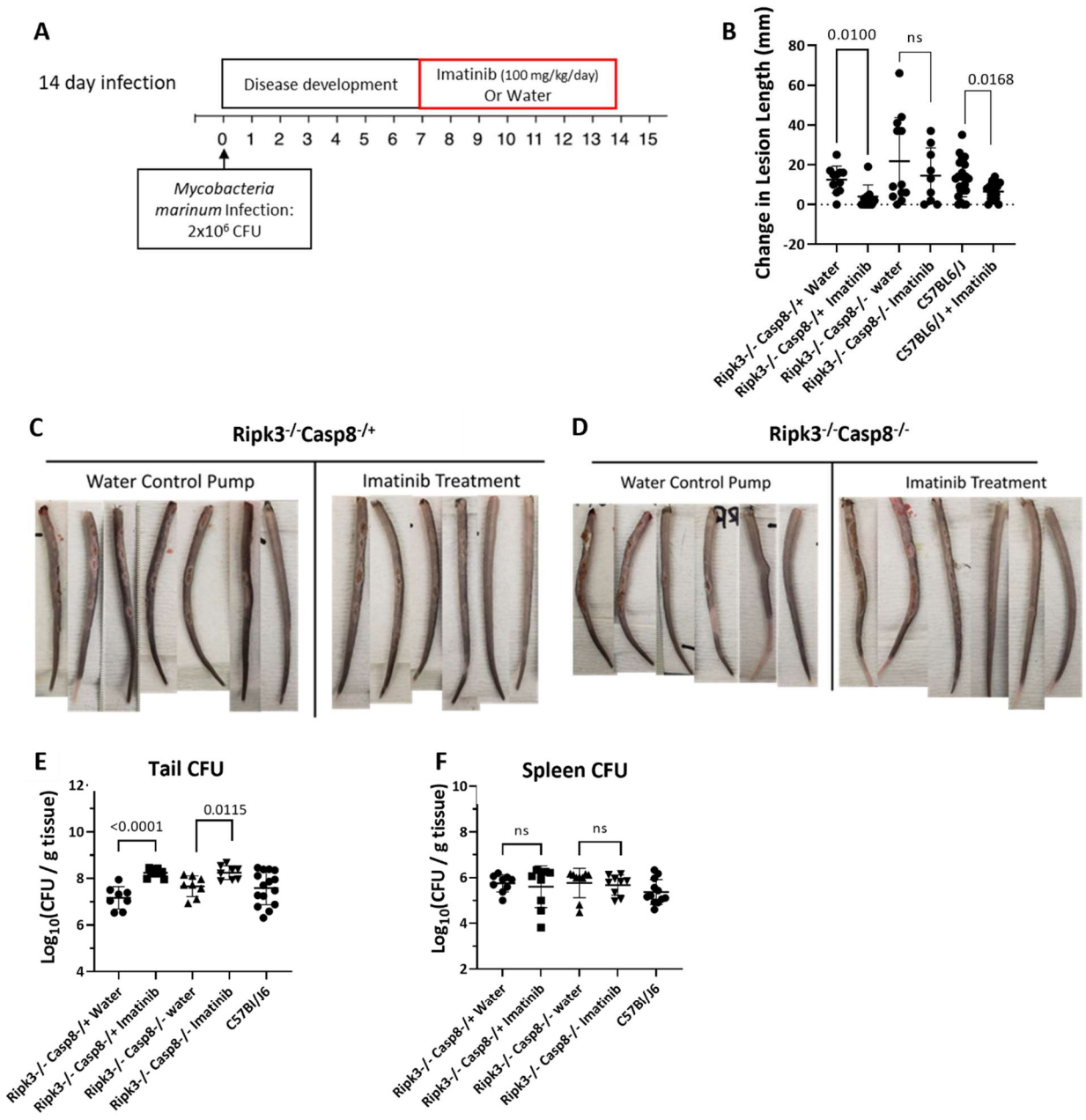
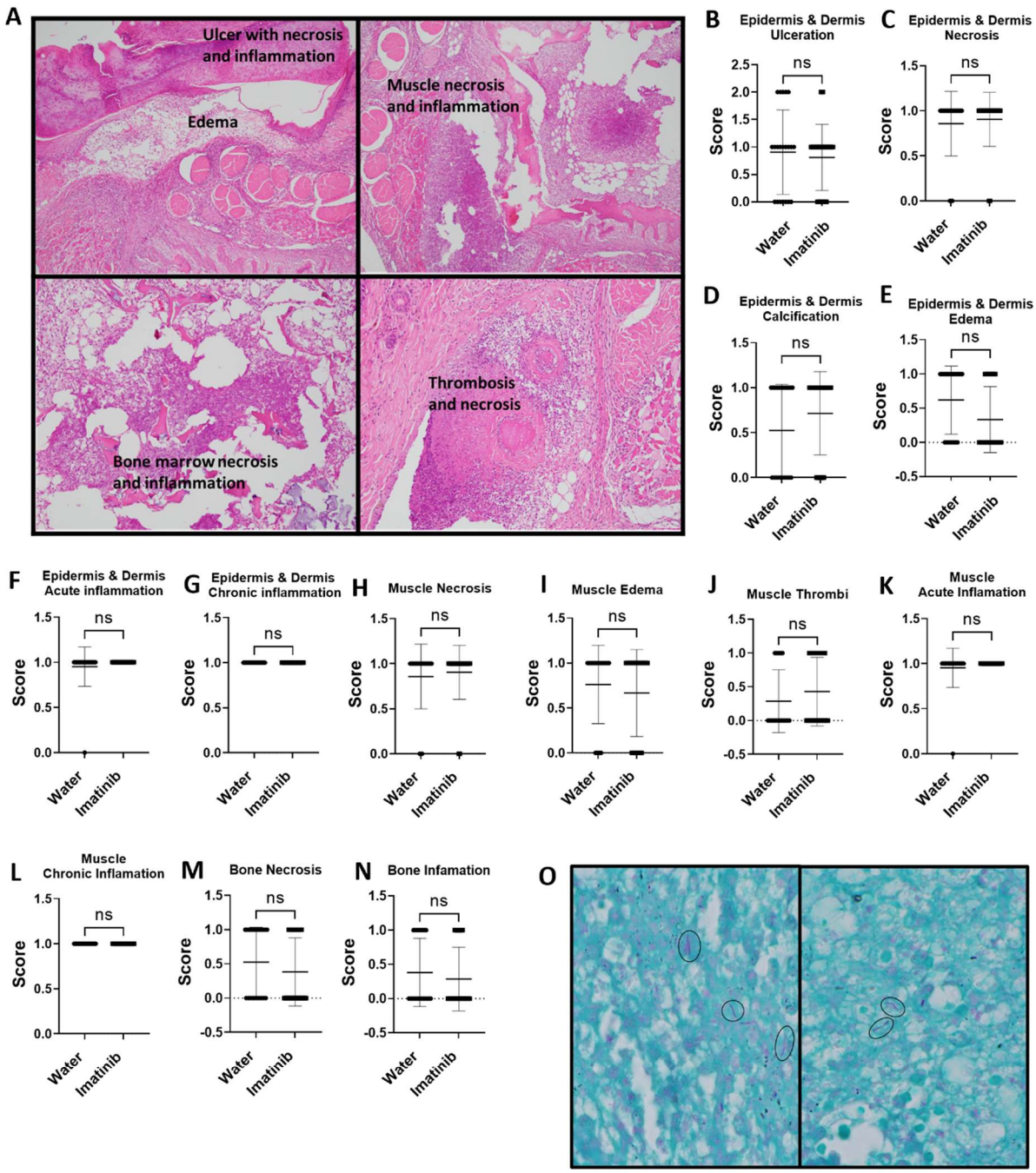


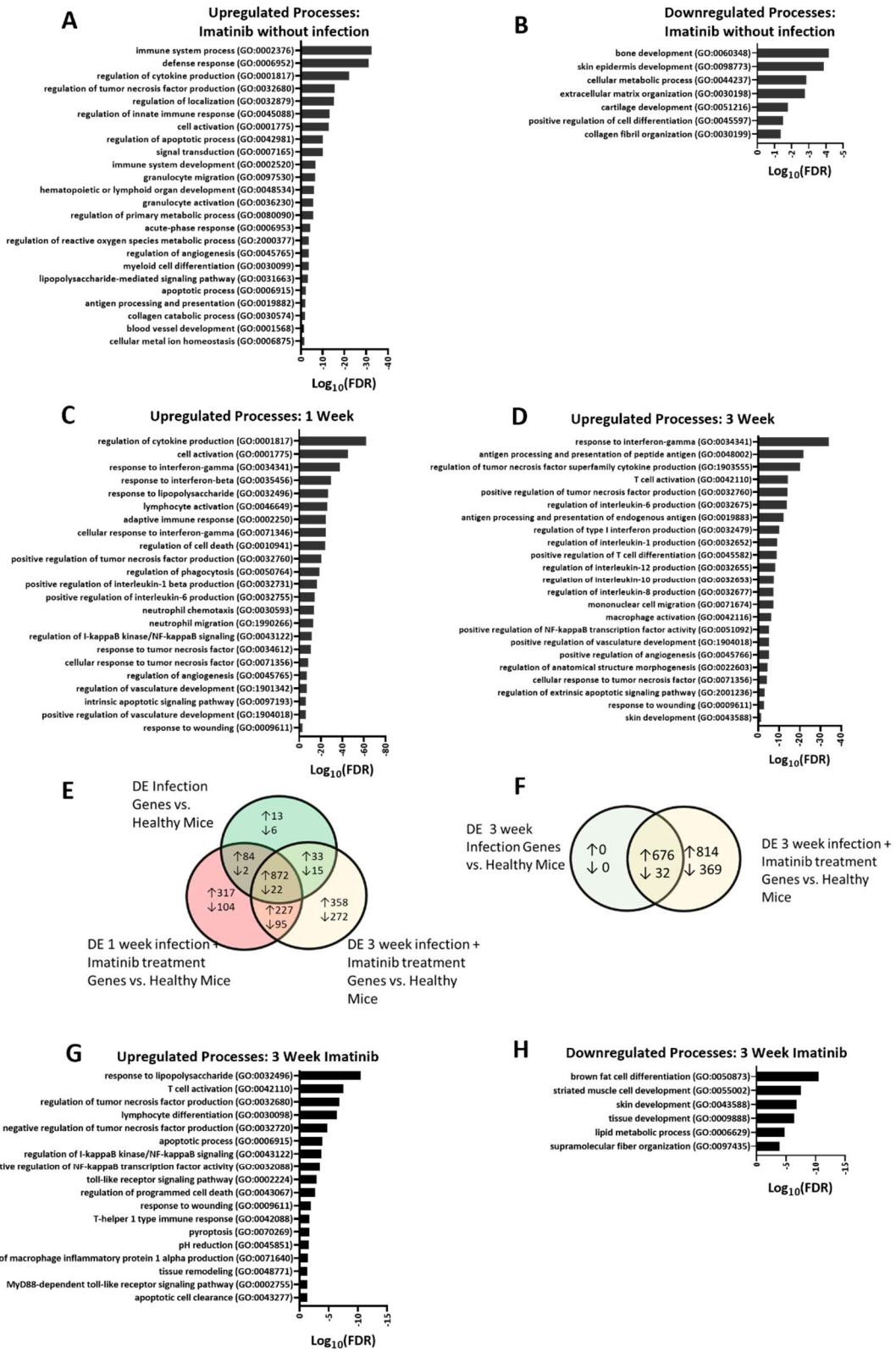
Figure 6. Imatinib-mediated reduction in pathology of granulomatous lesions is abrogated in mice lacking caspase 8.

Figure 6. Imatinib-mediated reduction in pathology of granulomatous lesions is abrogated in mice lacking caspase 8. **A.** Ripk3$^{-/-}$ Casp8$^{-/-}$ mice or Ripk3$^{-/-}$ Casp8$^{-/+}$ mice were infected with 2x10^{6} CFU of *Mm*, for 7 days. Mice were then treated with imatinib (100mg/kg/day) or water starting day 7 post infection. for an additional 7 days before mice were sacrificed at day 14 (n= 9-12 mice/group). **B.** Lesions were measured at the beginning of treatment and at the end of treatment to determine the change in lesion size over the treatment period. Historical data from Mm-infected C57/Bl6/J mice treated with water or imatinib (100 mg/kg/day) was included for comparison. **C,D.** Representative images of the tail lesions from Ripk3$^{-/-}$ Casp8$^{-/+}$ **(C)** and Ripk3$^{-/-}$ Casp8$^{-/-}$ **(D)** mice. **E.** Tail CFUs from the Ripk3$^{-/-}$ Casp8$^{-/+}$ and Ripk3$^{-/-}$ Casp8$^{-/-}$ treated with water or imatinib and historical control from C57BL/6J mice infected as above. **F.** Spleen CFUs from the Ripk3$^{-/-}$ Casp8$^{-/+}$ and Ripk3$^{-/-}$ Casp8$^{-/-}$ with no treatment or with imatinib treatment and historical control from C57BL/6J mice infected as above. Statistical test used in **B,E,** and **F** was two-tailed Mann-Whitney U test, with p values indicated. The mean +/- SD, for each group was graphed to show variance of data.



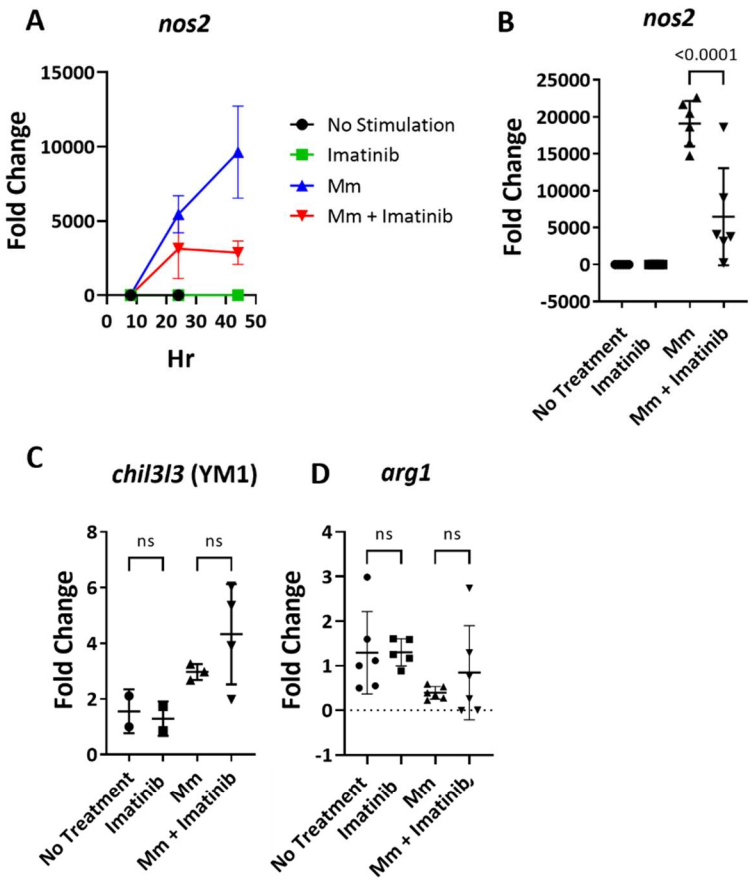
Supplemental Figure 1. Histology measurements of mouse tails.

Supplemental Figure 1. **Histology measurements of mouse tails.** C57BL/6J mice were infected with $2x10^6$ CFU of *Mm*. Beginning at day 7 post infection, mice were treated with imatinib (100mg/kg/day) or water for 7 days. Cross sections of the tail were cut and stained with H&E (A-N) or acid-fast bacillus (AFB) stain. **A.** Representative H&E images showing various features of the tail lesions scored in **B-N**. The upper two panels are at a magnification of 100x, and the lower two panels are at a magnification of 200x. **B-G.** Pathology scores for the epidermis and dermis for ulceration **(B)**, necrosis **(C)**, calcification **(D)**, edema **(E)**, acute inflammation **(F)**, and chronic inflammation**(G)**. **H-L.** Pathology scores for the muscle for necrosis **(H)**, edema **(I)**, thrombi **(J)**, acute inflammation **(K)**, and chronic inflammation **(L)**. **M,N.** Pathology scores for the bone for necrosis **(M)**, and inflammation **(N)**. **O.** Representative images of AFB stain in the mouse tail lesions with micro-organisms identified in circles at a magnification of 1000x. Each data point represents scoring from one individual mouse in 5 separate experiments (n=21/group). Statistical test used was two-tailed Mann-Whitney U test, with p values did not reach significance (ns, not significant). The mean +/- SD for each group was graphed to show variance of data.



Supplemental Figure 2. GO analysis of imatinib induced and infection related genes.

Supplemental Figure 2. **GO analysis of imatinib induced and infection related genes. A,B.** Genes differentially expressed with imatinib treatment in the absence of infection were identified by comparing differentially expressed genes from the uninfected water group, to imatinib-treated mice (514 genes, FDR < 0.05). Selection of GO terms identified by GO analysis of 373 genes upregulated with imatinib treatment (**A).** Selection of GO terms identified by GO analysis of 141 genes downregulated with imatinib treatment (**B). C,D.** Selection of GO terms identified by GO analysis of the 903 genes upregulated at the 1 week infection timepoint **(C)** and the 676 genes upregulated at the 3 week infection timepoint **(D)** identified in Figure 3b. **E.** Comparison of **"**Infection Genes" (green circle; genes identified in Figure 3B), imatinib genes at 1 week infection (red circle; genes identified in Figure 3B), and imatinib genes at 3 weeks of infection (yellow circle; genes identified in Figure 3C). **F-H.** Comparison of 3 week infection genes identified in Figure 3B, to 3 week infection with imatinib genes identified in Figure 3C **(F)**. Selection of GO terms identified by GO analysis of the 814 upregulated genes **(G)**, and the 369 downregulated genes **(H)** specific to imatinib treatment at the 3 week infection time point.



Supplemental Figure 3. Imatinib effects on BMDM during Mm infection.

Supplemental Figure 3. **Imatinib effects on BMDM during Mm infection. A.** RNA was collected from BMDMs derived from C57BL/6J mice infected with Mm at an MOI of 10 and left untreated or treated with 1μM imatinib for 8hrs, 24 hrs, or 44hrs. qPCR was used to measure levels of *nos2* in the cells at each time point (n= 2 wells/ group; data are representative of 3 separate experiments). **B-D.** BMDMs derived from C57BL/6J mice infected with Mm at an MOI of 10 and left untreated or treated with 1μM imatinib for 24 hrs. RNA was isolated from the cells and qPCR was used to measure levels of *nos2* **(B)**, *chil3l3* **(C)**, and *arg1* **(D;** n= 6 wells/ group)**.** Statistical test used was one-way ANOVA, with p values indicated. The mean +/- SD, for each group was graphed.

Chapter 3:

Discussion: Imatinib as a host directed therapeutic

Imatinib as a host directed therapeutic

Imatinib is known to induce phagolysosome fusion in macrophages as well as to induce myelopoiesis in mouse bone marrow. Both effects have shown efficacy against mycobacterial infection. In cellular culture systems imatinib was shown to increase the percentage of either *M. marinum* or *M. tuberculosis* bacteria that are found in the lysosome of cell culture macrophage-like cell lines (Napier et al., 2011). Imatinib has also been shown to traffic *M. tubercoulosis* or *Mycobacterium bovis* Bacille Calmette-Guerin (BCG) into the lysosome of human macrophages (Bruns et al., 2012; Steiger et al., 2016). During the early phase of *M. marinum* infection in mice, imatinib-induced myelopoiesis increased the number of circulating neutrophils, which was shown to be protective and limit bacterial colonization of various organs (Napier et al., 2015). These effects have only been measured during acute timepoints and outcomes have been limited to disseminated bacterial burden. This work looks more in depth into how imatinib effects ongoing immune responses and the microenvironment within the infected tissue. During these early infections, we had observed imatinib induced reductions of the overall size of the granulomas in the absence of an effect on the bacterial burden (Napier et al., 2011). The aims of this work were to 1) determine if imatinib limited lesion growth only during the initial development phase of the infection or during any stage of infection, and 2) determine what changes imatinib was inducing to the immune response to limit lesion size.

Mycobacteria have developed many immune evasion strategies and tools to survive within the macrophage. They do this my inducing phagosome arrest (Bruns et al., 2012; Chackerian et al., 2002; Napier et al., 2011), and escaping the phagosome (Houben et al., 2012; Simeone et al., 2012; Smith et al., 2008). Other work has shown *M. tuberculosis* utilizes proteins

in order to survive the harsh environment within the lysosome (Levitte et al., 2016) leading to debate whether inducing phagolysosome fusion alone would be sufficient to kill bacteria. However, imatinib effects multiple aspects of the innate immune system including both phagolysosome fusion, and the availability of circulating innate immune cells by inducing myelopoiesis. One major immune evasion strategy of mycobacteria is delaying the adaptive immune response (Clay et al., 2008; Flynn et al., 1995; Poulsen, 1950; Wallgren, 1948) by altering how innate immune cells see and react the bacteria (Khader et al., 2006; Wolf et al., 2008). Together the effects of imatinib have the potential to overcome a critical factor of mycobacterial immune evasion by changing how and what cells interact with mycobacteria.

In past work it was observed that, when given prophylactically, imatinib was able to reduce the lesion size during *M. marinum* infection in mice when a large dose of mycobacteria is given despite no effect on bacterial burden (Napier et al., 2011). Likewise in nonhuman primates infected with *M. tuberculosis*, lung pathology appeared to be more consolidated in imatinib treated animals than in animals that did not receive imatinib treatment (unpublished data). To further investigate this, *M. marinum* infection was allowed to develop in mice for 0, 7 or 14 days before imatinib treatment began. We had seen previously that lesions begin to appear on the mouse tail around 7 days of infection. From day 7 to 14 the lesions are growing the fastest as the infection develops. Between day 14 and 21 the growth of the lesions levels off as the host gains control of the infection. After about 3-4 weeks on infection the lesions will begin to get smaller while retaining culturable bacteria. We found that, imatinib treatment at any point during the infection works to limit lesion size and results in sooner recession of lesions.

When we observed these lesions histologically, there was no definitive changes in the features of the lesions, but there were changes in the overall area of inflamed tissue. This could suggest that despite the increase in myeloid cell numbers, the immune response is more directed to infected tissues and there is less off target tissue damage. It could also suggest that the imatinib induced immune response is better able to limit disseminated bacteria and bacterial proteins without requiring the formation of granulomas. We were unable to confirm changes in localization of the bacteria as few bacteria could be found via acid-fast bacillus staining in imatinib treated animals despite cultures from the tissue showing similar levels of bacteria (data not published). This could indicate relocalization of the bacteria to an unknown compartment within the tissue or changes to the structure of the bacterial cell wall that might affect staining efficacy. Though imatinib treatment in not thought to directly affect bacteria viability (Napier et al., 2011), imatinib induced effects on bacterial protein production and cell wall have not been studied. It is also likely that indirect effects of imatinib on the host immune system could stress the bacteria in a way to change components of the cell wall. Dispersed antigens produced by mycobacteria are also known to induce inflammation even in areas of the lung that do not seem to contain mycobacteria (Hunter, 2020). One possible explanation for the reduction of the area of inflamed tissue with imatinib could be related to less dispersed mycobacterial antigens throughout the tissue. This could work either through the imatinib induced immune response to the bacteria or be related to an effect of imatinib directly on the bacteria that has yet to be understood. Further research should be done to determine localization of the bacteria and bacterial antigens as well as to look more closely at whether imatinib directly effects cell wall and proteins produced by the bacteria.

The granuloma is considered a beneficial structure that limits bacterial dissemination during *M. tuberculosis* infection. However these structures also limits access of immune cells to the bacteria (Gautam et al., 2018; McCaffrey et al., 2022) and limits the concentration of antibiotics that are able to reach the center of the granulomas where the bacteria are (Sarathy & Dartois, 2020; Sarathy et al., 2018). Bacterial persistence and the secreted proteins that they produce lead to chronic inflammation of the lung tissue(Hunter, 2020), which over time, lead to permanent damage, fibrosis, and scarring (Hnizdo et al., 2000; Malherbe et al., 2016; Plit et al., 1998; Ravimohan et al., 2018; Wilson & Wynn, 2009). The ability of imatinib to reduce the size of the lesions and inflammation of the surrounding tissues could have important implications for treatment of TB. Much of the economic burden of tuberculosis (TB) is due to lasting lung damage after TB patients have completed antibiotic therapy (Meghji et al., 2021). Imatinib treatment may be able to be used in conjunction with antibiotics to limit inflammation and lung damage while supporting the immune system in the clearance of the bacteria.

Transcriptomic analysis was performed on the infected tail tissue from the mice infected for 7 or 21 days with *M. marinum* and treated with imatinib for the last 7 days of infection in order to better understand the how imatinib changed the immune response in the tissue. We found that at an early time point imatinib treatment made the infection gene signature look more like that of the gene signature at a later time point in the infection. At the later time point, imatinib was inducing a response that had less variability between mice. Notably imatinib did not differentially regulate any genes compared to the infection alone groups at either time point. One major issue with the immune response to mycobacteria is that the bacteria are experts at modulating the immune response in order to delay recognition (Blomgran & Ernst, 2011; Ernst,

2012), and development of the adaptive immune system (Clay et al., 2008; Flynn et al., 1995). In a biological system this can be overcome at varying rates with each mouse developing a response at slightly different times. As the infection carries on subsequent stages of an infection will be initiated at various times, so the longer an infection has been going the more diversity will be seen in the immune response between individuals. Imatinib treatment seems to spur this immune response to activate at an early stage of infection. At a later stage of the infection, imatinib seems to induce a more uniform response in the mice.

When we looked specifically at macrophages, we saw a possible explanation for a faster induction of immune response without a change in the overall nature of the immune response. When macrophages are infected with *M. tuberculosis* cellular activation and production of cytokines such as TNFα and IL12p40, one subunit of IL12P70, is limited compared to when a macrophage is infected with other gram-positive bacteria (Nau et al., 2002). When imatinib is used on macrophages infected with *M. marinum* we see that both levels of the mRNA encoding the cytokine TNFα and the protein are induced further with imatinib and the mRNA *il12b*, which codes for Il12P40, is also induced, though levels of Il12P70 were not measurable a this timepoint. This increase in *tnf* mRNA is not sustained over time, but only a transient effect. When we look at the 7 day timepoint in the imatinib treated mice, we also see that TNFα and Il12p70 are slightly upregulated compared to the untreated animals. While RNA-seq data suggest the levels of TNFα are slightly upregulated in the mice at 21 days post infection with imatinib treatment, this is not significant as some of the mice have developed "high" levels of *tnf* mRNA production without imatinib treatment. These observations seem to support the idea that imatinib treatment augments an immune response but does not change the nature of the response. Imatinib is

known to induce phagolysosome fusion in mycobacteria infected macrophages (Bruns et al., 2012; Napier et al., 2011), this in turn could increase recognition and response to the bacteria resulting in the more robust activation of the macrophages. The induction of myelopoiesis with imatinib (Napier et al., 2015) could also facilitate the initial robust immune response or further augment the immune response at a later stage of infection.

However, we only looked at one cell type, in the tissue many cell types would be responding to the bacterial infection and feedback between cells would be affecting the overall course of the immune response. During *M. tuberculosis* infection, dendritic cell activation and maturation is delayed resulting in a delayed adaptive immune response (Ernst, 2012; Khader et al., 2006; Wolf et al., 2008; Wolf et al., 2007). Imatinib may work on dendritic cells is a similar manner as macrophages and increase activation and maturation of dendritic cells. The effects we see in our RNA-seq data may reflect a more robust adaptive immune response as well as changes to the innate immune response.

TNFα is a marker of macrophage activation. This cytokine has also been shown to be critical in the immune response against mycobacterial infections. Mice deficient in TNFα exhibit disorganized granulomas (Bean et al., 1999; Kaneko et al., 1999), this has been attributed to loss of control of the infection and necrotic death of macrophages rather than direct effects on the structure of the granuloma (Clay et al., 2008; Lin et al., 2010). Likewise, patients that are on drugs to neutralize or inhibit TNFα, such as adalimumab or infliximab to treat autoimmune disorders, are at increased risk of developing TB (Gómez-Reino et al., 2003; Keane et al., 2001; Park et al., 2022). We see TNFα and other immune activation marker such as Il12p70 increased at an earlier timepoint *in vivo* with imatinib treatment. *In vitro* this effect was notably seen in TNFα protein

and mRNA production, while Il12p70 was not measurable in the media at 24hrs post infection, though *il12b* mRNA coding for one subunit of Il12p70 was greatly increased with imatinib. The induction of TNFα was not sustained over long periods of time, and after 44 hrs the levels of *tnf* mRNA were returned to levels seen in infected, but not imatinib treated cells. Excess TNFα has been shown to be associated with ROS-mediated tissue damage in *M. marinum* infection zebrafish (Roca & Ramakrishnan, 2013; Tobin et al., 2010). This seems to indicate that though TNFα is essential for control of mycobacterial infection, too much can do just as much damage. TNFα needs to be well balanced during the infection. It does not seem that imatinib is directly inducing TNFα production in an uncontrolled manner, but instead inducing activation of the cells to react to the bacteria in a burst. To further investigate this point mutant mycobacterial stains with deficiencies in limiting phagolysosome fusion could be used in macrophage cell culture to determine if the induction of phagolysosome fusion by imatinib is sufficient to skew the macrophages to produce higher levels of cytokine after infection.

One possible explanation for the reduction in inflamed tissue and size of the lesions could be related to cell death pathways. Various forms of cell death have been implicated in mycobacterial infections and contribute to tissue pathology (Behar et al., 2010; Pan et al., 2005; Ramakrishnan, 2012; Srinivasan et al., 2014). When cells go through apoptosis, bacteria are encased in apoptotic bodies that are then taken up by subsequent macrophages for trafficking into the lysosome. This process has been shown to limit the replication and spread of *M. tuberculosis* (Martin et al., 2012). Alternatively, necrosis, a broad category encompassing many forms of inflammatory cell death in which the cell membrane is compromised releasing cellular components into the environment, has been shown to contribute to bacterial spread, and

promotes tissue damage though the release of ROS (Amaral et al., 2019; Behar et al., 2010; Ramakrishnan, 2012). One type of necrosis, ferroptosis, has been identified as a major contributing factor to pathology during *M. tuberculosis* infection (Amaral et al., 2019). Altering cell death pathways during *M. tuberculosis* infection has been shown to limit pathology. For example, a mutation in the Lpr1 gene, which encodes LDL receptor, can shift macrophages from necrosis to apoptosis when infected with *M. tuberculosis*, thereby limiting replication and spread of the bacteria (Pan et al., 2005). In our data we see imatinib has no effect *in vivo* on activated caspase 3, indicating that we not altering the amount of apoptosis within the tissue. However, our cell culture experiments show a reduction of necrosis with imatinib treatment of *M. marinum* infected BMDMs. A reduction of necrosis could limit inflammation *in vivo* and potentially reduce the granuloma associated pathology and tissue damage.

Upon activation and differentiation of macrophages, they become resistant to cell death (Müller-Sienerth et al., 2011). This is due to signaling through toll-like receptors that induces translocation of NFκB into the nucleus to induce pro-inflammatory factors, such as Il6, Il12, TNFα, and Il8 (Liu et al., 2017), and pro survival factors such as FLIP, BCL-xL, c-IAP, XIAP, TRAF1, and TRAF2 (Karin & Lin, 2002; Kucharczak et al., 2003). The gene ontology analysis from our RNA-seq data pointed to upregulation of many of these NFκB regulated pro-inflammatory factors as well as suggested changes in pro-survival factors that we were unable to define with the limited number of mice we had avalible. When we looked more closely at the pro-survival factors in our macrophage assays, imatinib treatment tended to increase these factors, but not to a significant level at the time points we observed. Imatinib also has the potential to effect NFκB signaling. The BCR-Abl mutation triggers NFκB and imatinib treatment has the potential to limit NFκB activation

in these mutant cells (Ciarcia et al., 2012). Imatinib also increases apoptotic cell death in these BCR-Abl^{+} transformed cells though imatinib does not cause apoptosis in hematopoietic cells that do not contain this mutation (Puissant et al., 2010). In human islet cells, imatinib promotes NFκB activation but dampens response to cytokines over time (Mokhtari et al., 2011), suggesting imatinib has the potential to modulate NFκB signaling, but the effects are not yet understood and may vary based on cell type and duration of exposure to imatinib. For our work, we focused our analysis on cell death pathways related to TNFα signaling downstream of NFκB activation as this effect was suggested in the RNA-seq data, measurable in the mouse tails, and recapitulated in the BMDMs. However, it is likely that NFκB is the mediator behind the effects we see *in vivo* with imatinib, either through increases in toll-like receptor signaling related to more efficient phagolysosome fusion or effects of imatinib on Abl signaling.

TNFα signaling goes through a few different pathways. Signaling can lead to NFκB activation and induction of pro-survival and pro-inflammatory cytokines (Scheidereit, 2006) and can also lead to cell death via apoptosis or necroptosis. TNFα signaling promotes apoptosis by activating enzymatic activity of caspase 8 or necroptosis via RipK3. However, caspase 8 has also been shown to promote cell survival by complexing with c-Flip, inhibiting apoptosis, or by blocking activation of RipK3, which prevents necroptosis (Fritsch et al., 2019; Oberst & Green, 2011). Caspase 8 has also been shown to play a role in regulating transcriptional responses that dampen inflammation (Philip et al., 2016). When we infect BMDMs lacking Ripk3 and either heterozygous for Caspase 8 (Ripk3$^{-/-}$ Casp8$^{+/-}$) or without either copy of caspase 8(Ripk3$^{-/-}$ Casp8$^{-/-}$), we see a marked reduction in the amount of necrosis in the BMDMs. These cells are unable to undergo TNFα induced necroptosis and therefore the lower level of necrosis seen in both

groups can be attributed to the lack of necroptosis in these cells. When one copy of caspase 8 is present (Ripk3$^{-/-}$ Casp8$^{+/-}$), imatinib has mild effects on reducing remaining levels of necrosis in these cells. Without caspase 8 (Ripk3$^{-/-}$ Casp8$^{-/-}$), imatinib has no effect on reducing levels of necrosis. The effects seen in the cultured macrophages was relatively small though. However other studies looking at cell death during mycobacteria infection (Pan et al., 2005) have shown that even relatively small effects on cell death can potentially effect disease outcome.

When mice deficient in both Ripk3 and caspase 8 (Ripk3$^{-/-}$ Casp8$^{-/-}$) are infected with *M. marinum* we see two populations of in terms of visible pathology on the surface of the tail. About half of the mice develop terrible pathology (>30 mm) by day 14 of infection, while the other half of the mice develop mild pathology similar to that seen in the wild type C57Bl6/J mice. Imatinib treatment obscures these two populations into one, however we are unable to reduce the overall pathology with imatinib in these mice. In the mice heterozygous for Caspase 8 (Ripk3$^{-/-}$ Casp8$^{+/-}$) we see pathology similar to that seen in the C57BL6/J mice, and imatinib is able to reduce the overall lesion size in these mice. This reduction in does not appear to be related necroptosis, as cells from either of these mice are unable to undergo necroptosis without RipK3. However, without caspase 8, the cells from these mice cannot undergo TNFα induced apoptosis, or benefit from caspase 8 mediated effects to dampen inflammation (Philip et al., 2016). Our *in vitro* BMDM assays also suggest that imatinib is unable to reduce necrosis in infected macrophages within these mice, however this is difficult to test for *in vivo* as we would be limited to looking at necrosis cells at single timepoint at a time during the infection. Though we can conclude from our data that imatinib needs caspase 8 to limit the pathology during mycobacterial infection, we cannot directly correlate this to a reduction in necrosis *in vivo* at this time. It is likely multiple roles of

caspase 8 come to play in dampening inflammation and reducing the pathology in the mice such as limiting necrosis, inducing apoptosis, and transcriptional effects of caspase 8.

Notably CFUs in the tails of all the mice lacking Rip3K were increased with imatinib treatment. During *M. tuberculosis* infection in mice, mice lacking RipK3 develop lower bacterial CFUS in their lungs, suggesting that RipK3 plays a role in spreading the infection (Zhao et al., 2017). In our model system Ripk3 deficiency alone did not alter bacterial colonization of the tail tissue. Despite imatinib increasing the bacterial burden in the tails of the RipK3 deficient mice, the lesions size was reduced with imatinib. While more research would need to be done in order to delineate why this may be, one possible hypothesis is that imatinib effects on the cells without necroptosis limits the activation of the immune system and leads to unchecked bacterial growth. As in any infection an effective immune response to *M. tuberculosis* is a balancing act between inducing inflammation to activate the immune system and dampening inflammation to limit tissue damage. While it seems that imatinib spurs the immune system to response to mycobacteria, too much response would also not benefit the host, without further characterization of the immune response in the RipK3 deficient mice, it is difficult to determine why differences in bacteria load was seen with imatinib treatment.

Imatinib has potential as a host directed therapeutic to mycobacterial infections. Drug discovery efforts have focused on developing drugs to block specific functions of the host or bacteria with limited off-target effects, for example COVID-19 host directed therapeutics target hyperinflammatory pathways to limit tissue damage (Wallis et al., 2022). However, such approaches can be difficult, often requiring a vast knowledge of how the host and pathogen interact leading to possible theories on how to “fix” the interaction in favor of the host. Often

the timing of the proposed therapy is critical. Introducing a host directed therapeutic too early or late may compromise the ability of the therapeutic to work as desired, for example limiting inflammatory pathways may be beneficial at a later stage of infection, early during an infection limitation on the inflammatory pathways may limit the immune response. One benefit of imatinib treatment is that it does not seem to induce extreme effects on the host, instead imatinib induced mild effects on bacterial trafficking within the cell that seem to augment the immune system to respond in a more effective way with less off-target tissue damage. In this work we have been able to identify an effect of imatinib on augmenting the development of the host immune response and limiting tissue damage, though more would be needed to fully understand the mechanisms behind the reduction in tissue pathology. This work has implications for therapeutic strategies to control TB as the granuloma has been shown to limit antibiotic and immune cell access to the bacteria, leading to bacterial persistence and contributing to lasting tissue damage and scaring. By reducing these structures imatinib has the potential to enhance effectiveness of antibiotic therapy and potential even reduce treatment times. Imatinib could also lead to less long-term lung dysfunction after resolution of TB infection by limiting mycobacteria associated tissue damage and long-term lung scaring. Though much more work is needed before imatinib will be of use in human patients, these early studies in mice can provided insights into how to determine treatment efficacy in future clinical trials.

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