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Development of the tyrosine kinase inhibitor imatinib mesylate as a therapeutic for tuberculosis

By

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Development of the tyrosine kinase inhibitor imatinib mesylate as a therapeutic for tuberculosis

By

Ruth J. Napier
B.A., Agnes Scott College, 2005

Advisors: Daniel Kalman, Ph.D. and Thomas M. Shinnick, Ph.D.

An Abstract of
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Graduate Division of Biological and Biomedical Sciences
Microbiology and Molecular Genetics

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ABSTRACT

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Mycobacterium tuberculosis (Mtb) remains an enormous public health concern, second only to HIV/AIDS. The emergence of multi-drug resistant and extensively-drug resistant strains of Mtb has highlighted the need for the immediate development of new anti-tuberculosis therapeutics. The work presented here investigates the use of the FDA-approved tyrosine kinase inhibitor, imatinib (Gleevec, STI-571), for treatment of tuberculosis (TB). We show that Abl-family and other host tyrosine kinases regulate Mtb and the related pathogen *Mycobacterium marinum* (Mm) entry, intracellular survival and co-localization with acidified vesicles in macrophages. In mice, administration of imatinib reduces bacterial load and associated pathology, and has a sterilizing effect on ~50% of animals tested. Importantly, we show imatinib has similar effects on drug-resistant strains of mycobacteria and works in a synergistic manner with current anti-TB drugs. We also show that *in vivo* imatinib has a previously unreported immunomodulatory capacity in that it increases numbers of neutrophils and monocytes in the bone marrow, and facilitates their migration to peripheral sites, including blood and spleen. Imatinib treatment does not activate neutrophils *per se*, though activation markers increase normally upon infection. Furthermore, adoptive transfer of neutrophils from animals treated with imatinib or control animals into naïve animals decreases mycobacterial burden to the same extent, suggesting that increasing numbers of neutrophils is sufficient to reduce bacterial load. This immunomodualtory capacity of imatinib resembles increased hematopoiesis normally stimulated upon infection. As such we show extended pre-treatment of mice with imatinib reduces bacterial burden of pathogenic *Francisella* species (*F. novicida* and LVS), bacteria that do not require Abl or related kinases for survival in macrophages. Taken together, our findings demonstrate that (i) imatinib could be administered in conjunction with current antibiotic regimens for infections caused by pan-susceptible or drug-resistant resistant strains of Mtb; (ii) Because imatinib targets the host rather than microbial molecules, it is less likely to engender resistance compared to conventional antibiotics, and may extend the clinical lifespan of antibiotics administered concurrently; (iii) imatinib-induced neutrophilia in the bone marrow and periphery suggest that the drug may have inhibitory activity against a broad range of bacterial pathogens; and (iv) may mitigate the effects of neutropenia.

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CHAPTER 1:

Introduction

Part I: Tuberculosis

History

Tuberculosis (TB) has plagued mankind for 5,000 years, dating back to the ancient Egyptians, and was recognized by Hippocrates in 460 AD as a deadly communicable disease, called *phthisis* or consumption [1]. In 1882, Robert Koch discovered that the bacillus *Mycobacterium tuberculosis* (Mtb) was the causative agent of TB, noting that:

> "If the importance of a disease for mankind is measured from the number of fatalities which are due to it, then tuberculosis must be considered much more important than those most feared infectious diseases, plague, cholera, and the like. Statistics have shown that one seventh of all humans die of tuberculosis [2]."

Today, TB remains a devastating disease. The World Health Organization (WHO) estimates that 2 billion people are infected worldwide, with 8.8 million people becoming newly infected, and 1.4 million succumbing each year [3]. Confounding the TB pandemic has been the rise of the human immunodeficiency virus (HIV). TB is the leading cause of death in HIV-infected individuals, accounting for one in four deaths among HIV-positive people [4]. Furthermore, individuals living with HIV represent over 10% of the annual reported TB cases

and are estimated to be 37 times more likely to develop TB than HIV-negative individuals [4].

TB treatment

A major intervention in human TB infections has been the discovery and implementation of antibiotics. In 1943, Schatz and Waksman identified streptomycin and demonstrated its efficacy against Mtb in guinea pigs, a result later recapitulated in humans[5-7]. Prior to streptomycin the only treatments known for TB included isolation within sanatoriums and the "pneumothorax technique," a more invasive procedure involving collapsing the lungs to allow natural healing of lesions [8].

With the discovery of antibiotics in 1928 by Alexander Flemming, a solution to the TB pandemic seemed possible. Yet, Flemming recognized that any gains in stemming the death toll from microbial infections by antibiotics might easily be nullified by the rise of drug-resistant variants. In his Nobel lecture, Flemming cautioned:

> "The time may come when penicillin can be bought by anyone in the shops. Then there is the danger that the ignorant man may easily underdose himself and by exposing his microbes to non-lethal quantities of the drug make them resistant [9]."

For Mtb, streptomycin resistance was evident even during the initial experiments in guinea pigs, and drug resistance has been encountered with each new antibiotic. To avoid acquisition of resistance to any one drug, TB is treated by administration of multiple drugs affecting multiple systems within the microbe. Between the years of 1952 and 1963 highly effective synthetic antibiotics were introduced, including isoniazid, pyrazinimide, ethambutol, and rifampicin [10]. Today, combined administration of antibiotics constitutes the first line chemotherapeutic response to pan-susceptible TB [10]. In particular, the WHO recommends a two-month treatment with isoniazid, pyrazinimide, ethambutol and rifampicin, followed by an additional four to seven months of isoniazid and rifampicin [11].

The combination of vigilant case detection and multidrug anti-TB chemotherapy led to significant declines in the incidence of TB, at least in industrialized countries, though the disease persisted in Africa, Asia, and Latin America, and in particular in those countries with the weakest economies and poorest population bases [12]. However, in the 1980s, TB reemerged in industrialized countries and became resurgent globally, due in part to the convergence of the HIV and TB pandemics and to the emergence of strains that displayed resistance to some of the available antibiotic chemotherapies.

Drug-Resistant *M. tuberculosis*

As Flemming predicted, antibiotic-resistant strains have arisen, including multidrug-resistant TB (MDR-TB). MDR-TB displays resistance to at least

isoniazid and rifampicin [13]. The appearance of MDR-TB led the WHO to declare TB a global public health emergency in 1993. The WHO estimates in 2008, 440,000 MDR-TB cases emerged and 150,000 deaths were caused [14]. Furthermore, in some settings it is estimated that a quarter of all new TB patients are now being diagnosed with MDR-TB [4].

In recent years, the problem of drug resistance has been exacerbated by the emergence of extensively drug-resistant (XDR-TB) strains, which are resistant to isoniazid, rifampicin and important second line drugs including at least one of the fluoroquinolones and one of three injectable drugs (amikacin, kanamycin, or capreomycin) [13]. In a frightening development, Mtb strains resistant to all first- and second-line drugs (referred to as totally drug-resistant (TDR-TB) by the authors) tested have recently emerged [15].

The treatment for MDR-TB and XDR-TB is lengthy, lasting approximately two years. Treatment requires drugs that are less efficacious, have associated toxicity, and are much more costly than treatment for pan-susceptible TB [4]. Additionally, the complications associated with MDR-TB are intensified if the individual is HIV-positive. In fact, individuals co-infected with HIV have a one in ten chance of surviving for three months or more if diagnosed with MDR-TB [4]. These factors highlight the urgent need for new drugs to combat drug-susceptible and drug-resistant TB [16].

TB treatment complications

Several factors confound treatment and facilitate development of resistance. Mtb grows slowly and can persist for long periods in infected individuals, necessitating drug treatment regimens that last six to nine months [17]. Moreover, available drugs can cause toxic side effects, especially with prolonged administration [18]. Other confounding factors include limited availability of front line drugs in economically depressed areas, economic and social instability that limits access to healthcare, and pharmaco-incompatibility with anti-HIV therapies [19]. Together, these factors conspire to reduce compliance with treatment regimens, prevent resolution of the infection, and facilitate the development of resistance.

New Anti-TB drugs

Recent research efforts continue to focus on antibiotic and vaccine development. Several newly developed antibiotics targeting novel bacterial enzymes are currently in clinical trials [20]. Of importance, a novel mycobacterial ATP synthase inhibitor, bedaquiline (Sirturo), has been recently approved by the Food and Drug Administration (FDA) to treat MDR-TB. Bedaquiline is the first new anti-TB drug in 40 years as well as the first drug specifically indicated for treatment of drug-resistant TB.

However, as with existing antibiotics, resistance may ultimately arise against these drugs as well. Development of new TB vaccines holds promise. The current vaccine, Bacillus Calmette-Guerin (BCG), remains problematic with

inconsistent protection against tuberculosis [21]. However, newly developed vaccines have shown significant activity in laboratory animals, though efficacy in humans remains to be assessed. Progress on anti-TB vaccines is reviewed elsewhere [22, 23].

Drugs that are effective against antibiotic resistant strains and also reduce the length and toxicity of existing treatment regimens remain an important goal for anti-TB therapy. One possible solution to the conundrum that antibiotics both kill bacteria and select for development of resistance in the pathogen is to target the *host* rather than the pathogen. For example, bacterial and viral pathogens hijack host molecules involved in normal cell signaling and repurpose them to their advantage. Therefore drugs designed to inhibit host molecules used by the virus or bacterium can interfere with pathogenesis, and reduce their capacity to survive within eukaryotic cells. Development of resistance to these drugs is highly unlikely, as the pathogen would have to develop mutations that enable it to survive such a blockade. While not impossible, it would be difficult for a pathogen to do this because multiple events that alter interactions with the host would be required, all while the organism is under immune selection. In chapter two we present evidence suggesting that Mtb utilizes host Abl family and related tyrosine kinases during entry and intracellular trafficking, and that their inhibition results in decreased bacterial burden.

Alternatively drugs designed to modulate the host's immune system could be advantageous by means of increasing its antibacterial capacity. In chapter three, we show evidence that inhibition of Abl family or related tyrosine kinases

modulate neutrophil homeostasis resulting in increased numbers of neutrophils *in vivo*. An increase in neutrophil numbers has the capacity to directly decrease Mtb load and may facilitate clearance of the pathogen.

Part II: Pathogenesis of *Mycobacterium tuberculosis*

Overview

Mtb is transmitted via inhalation of aerosolized respiratory droplets containing live bacteria. Each aerosol droplet is thought to be 1-5μm and can carry up to 10 individual bacilli [24]. During acute infection, Mtb is able to evade harm by the innate immune system and establish a unique niche within the lungs allowing it to replicate and survive within macrophages. With the onset of adaptive immunity, Mtb-antigen-specific T cells are activated and recruited to the site of infection where they contain the bacteria within a granulomatous structure. At this point bacterial replication is constrained and the infection is controlled. Interestingly, although the host initiates robust innate and adaptive immune responses, they are often not sterilizing. As a result, mycobacteria can remain within a granuloma, undetected by the host for a lifetime or until a complicating factor, such as HIV, reduces immune function and induces reactivation of disease. Upon reactivation, a person can develop a productive cough and transmit bacteria to other individuals.

Clinical Outcomes of Infection

There are several different clinical outcomes that can result from an Mtb infection. First and perhaps the least likely is spontaneous clearance of the pathogen. This is thought to happen in a very small number of infected

individuals, and the mechanisms by which clearance does or does not occur remain unclear [25]. A second more common outcome is immediate progression into active disease. This generally occurs in immunocompromised individuals, including infants, the elderly, and people with HIV [25]. The symptoms of active disease include fever, fatigue, weight loss, loss of appetite and night sweats [26]. Importantly, if left untreated, 50% of individuals with active TB will die [25].

In a third scenario, the host's immune system will sufficiently control the infection, resulting in a latent TB infection. During latent TB, bacteria are able to persist in a metabolically inactive state referred to as "dormancy" or "latency." Individuals can maintain a latent TB infection for decades and even a lifetime, showing no symptoms or signs of disease. However, suppression of the immune system due to unrelated factors, such as co-infection, can allow the bacteria to replicate without constraint, resulting in disease reactivation. Indeed, 10% of latently infected individuals will reactivate and progress to active disease. These individuals are then thought to be contagious and able to spread bacteria to susceptible hosts by coughing, sneezing, talking or even singing [24].

Innate Immunity to Mtb

In the distal alveoli Mtb is ingested by alveolar macrophages via traditional receptor-mediated phagocytosis [27, 28]. *In vitro* studies on macrophages have suggested that numerous extracellular receptors facilitate Mtb entry, including C-type lectin receptors (CLR), scavenger receptors, and complement receptors [29]. In addition, recent studies on mice infected via

aerosol methods suggest that resident macrophages, neutrophils, monocyte-derived macrophages, and dendritic cells (DCs) can also phagocytose Mtb during acute infection, and significantly contribute to anti-TB immunity [30].

In response to Mtb, pro-inflammatory cells are recruited to the site of infection in part by bacterial pattern recognition receptors (PRRs). Such receptors include the extracellular Toll-like receptor 2 (TLR2), TLR9, members of the CLR family, and cytosolic receptors including nucleotide-binding oligomerization domain protein 2 (NOD2) [31-40]. Binding of these receptors by Mtb–specific antigens results in secretion of pro-inflammatory cytokines and chemokines that promote further immune cell activation and mobilization [41, 42]. Experiments with knockout mice suggest functional redundancy amongst receptors of this class. Thus, deletion of individual receptors does not significantly alter Mtb pathogenesis [43, 44]. Where as, distal adaptor molecules at critical nodes of signaling cascades, such as MYD88 or CARD9, are essential and single knockouts are associated with accelerated mortality [43, 44]. These data suggest that although innate immune cells have little antibacterial activity during early infection, their activation and recruitment remains important for long-term anti-TB immunity.

In addition to pathogen-associated molecular patterns (PAMPs), immune cells are recruited to the lungs by pro-inflammatory cytokines including tumor necrosis factor alpha (TNFα) and chemokines including CCL5, CCL9, CXCL10 and CCL2, secreted by infected macrophages [45-48]. In response to these

factors, macrophages home to the sites of infection and form an early granulomatous structure surrounding the bacteria.

Although granulomatous lesions are traditionally thought to be induced by the host as an attempt to "wall off" foreign material (bacteria) from the host, recent data in zebrafish using *Mycobacterium marinum* (Mm) suggests the contrary [49, 50]. Davies *et al.* found that recruitment of macrophages to the site of infection is mediated by the bacterium itself, and can benefit bacterial replication and spread within the tissue [50]. Using ESX1 and CFP10, secreted by the type VII secretion system and encoded by the RD1 locus, Mtb induces necrotic death in cells and promotes release of bacteria into the extracellular milieu. Subsequently, newly recruited cells get infected and traffic to other parts of the lung and body [50]. Thus, providing additional targets and further expanding the breadth of infection.

Mtb modulation of host cell signaling

There are at least three major mechanisms that Mtb has devised to modulate or exploit host cell signaling pathways to promote its survival. First, following uptake, the bacterium resides in an early endosome-like compartment. Mtb's extracellular structure consists of protective glycolipids, and when coupled with the expression of a set of secreted effectors, Mtb is able to manipulate normal trafficking and maturation of the phagosome so as to evade lysosomal fusion and subsequent death [51]. Furthermore, these virulence factors enable Mtb to survive structural pressures within the phagosome including mild

acidification, acid hydrolyses, antimicrobial peptides, nutrient limitation, and reactive oxygen and nitrogen intermediates [52].

Second, recent studies suggest Mtb can leave the phagosome and replicate in the cytosol [53]. This mechanism may aid in evasion of host immune detection and facilitate spread from cell to cell. Experiments using Mtb's close genetic relative, Mm, indicate the ESX1/RD1 locus is responsible for exit of the phagosome. However, this observation has yet to be recapitulated in Mtb [54].

Third, Mtb is able to interfere with host protective factors, including interferon gamma (IFNγ). IFNγ induces a number of antibacterial pathways within the macrophage, including activation of the p47 guanosine triphosphatase (GTPase) LRG47. LRG47, when activated, serves to alter vacuolar trafficking, thus facilitating phagolysosomal fusion and death of Mtb [55]. IFNγ can also induce autophagy, a host antibacterial pathway resulting in autophagolysosomal fusion and death of Mtb [56]. Additionally, Mtb-secreted lipoproteins can alter IFNγ-induced major histocompatibility class II (MHC II) expression on macrophages, which in turn may interfere with antigen presentation and activation of CD4 T cells [57].

Taken together, these data provide evidence that Mtb has acquired a number of mechanisms to protect itself from the host antibacterial defenses. Mtb is able to regulate several host proteins and antibacterial pathways as well as physically relocate within the cell in an effort to evade immune detection and destruction. As an end result, the bacterium establishes a balance or

commensalism within the eukaryotic cell facilitating its own replication and survival.

The role of neutrophils in TB immunity

Our work has shown a role for neutrophils in combating pathogenic mycobacteria. In this regard, recent studies from other groups suggest that neutrophils facilitate induction of the adaptive immune response. This was surprising, as recruitment of neutrophils and their accumulation in the lungs had been previously thought to exacerbate disease and be associated with active TB in humans [58-60]. Using a green fluorescent protein-expressing Mtb (GFP-Mtb) strain and flow cytometry, Wolf *et al.* demonstrated that at day 14 post aerosol infection the predominant cell types harboring Mtb in the lungs are alveolar macrophages, DCs, and neutrophils [30]. However, after 21 days the number of infected neutrophils decreases dramatically and DCs become the primary infected cell type. Furthermore, work by Blomgran *et al.* suggests that apoptosed neutrophils infected with Mtb are ingested by DCs and trafficked to the mediastinal lymph nodes where they present antigen to Mtb-specific CD4 T cells and initiate adaptive immunity [61]. Importantly, when neutrophils are depleted *in vivo* the number of infected DCs in the mediastinal lymph nodes is significantly decreased, delaying onset of adaptive immunity [61]. Furthermore, an *ex vivo* transwell system provided evidence that DCs which acquire Mtb through phagocytosis of infected neutrophils, rather than through direct infection, more readily traffic to the mediastinal lymph nodes [61]

Furthermore a recent study shows evidence that neutrophils are recruited to the spleen where they increase host antimicrobial activity. Neutrophils activate marginal zone B cells using a "B cell-helper function" that facilitates immunoglobulin class switching, somatic hypermutation and antibody production [62]. These data suggest neutrophils play an essential role in the host's ability to initiate adaptive immunity and begs the question of what would happen if neutrophil numbers increase during acute or chronic infection? In chapter three we show evidence that increasing neutrophil numbers *in vivo* contributes to innate control of mycobacteria, and may facilitate a sterilizing adaptive response.

Adaptive Immunity and Latent TB

The onset of adaptive immunity to TB coincides with a decrease in bacterial growth [63]. Perhaps the most important adaptive immune response is the generation of Mtb antigen-specific CD4 T cells in the mediastinal lymph nodes. After activation, proliferation and expansion in the lymph node, CD4 T cells home to the site of infection where they facilitate the formation and structural maintenance of a "stable" granuloma [64].

Several lines of evidence suggest CD4 T cells mediate anti-TB immunity through the control of bacterial growth, formation and maintenance of the granuloma, and host survival [65, 66]. Humans with CD4 T cell defects, such as HIV-positive individuals, fail to contain mycobacteria and develop active disease [67]. However, studies in mice have shown that addition of CD4 T cells, during

the first week of infection with Mtb, by adoptive transfer does not alter bacterial survival or replication [68]. These data suggest that CD4 T cells are necessary but not sufficient for protective immunity to TB.

CD8 T cells

CD8 T cells have been implicated in host resistance to Mtb. Depletion of CD8 T cells during an aerosolized Mtb mouse infection results in a modest reduction in survival time, yet no changes in histopathology are evident [65]. Additionally, CD8 T cells are directly cytotoxic to Mtb; they contain a granule protein, granulysin, which has been shown to compromise Mtb's cell wall integrity and facilitate bacterial death *in vitro* [69, 70]. Despite these recent findings, the full contribution of CD8 T cells in TB immunity is not well understood. Lazarevic and Flynn have reviewed the contributions of CD8 T cells in TB elsewhere [71]

B cells

B cells are also found within the lungs at the site of Mtb infection. In fact, they have been shown to be the most abundant population of lymphocytes within Mtb associated lesions [41]. Although much effort has been made to identify the role of B cells in immunity to TB, their exact role is yet to be determined. Maglione has reviewed the contributions of B cells to TB immunity: [72].

Regulating the immune system during TB infections

The goal of the adaptive immune system is to restrict bacterial growth while preventing an overly robust pro-inflammatory response that could harm the host. In many cases the protective immune response to TB must be tightly regulated; an example of this is the cytokine TNFα. TNFα recruits and activates multiple anti-bacterial pathways in immune cells to facilitate bacterial killing, but dysregulation of this cytokine can cause deleterious effects [73, 74]. The accumulation and organization of immune cells important for containment of infection is largely attributed to the secretion of TNFα [45-48]. In contrast, the loss or blockade of TNFα, by Pentoxifylline treatment is associated with the inability to form granulomas, exacerbated pathology, and increased mortality [75, 76]. Additionally, an overproduction of TNFα is associated with immunopathology [77]. Clearly the host must strike a balance in levels of this cytokine to clear bacteria and avoid immunopathology.

One of the most important examples of immunoregulation during Mtb infection is the control of T cell effector functions to limit excessive tissue damage. For example, Barber *et al.* and others show removal of the T cell inhibitory receptor programmed death 1 (PD-1) and its ligand PD-L1 resulted in a worse disease course followed by rapid death of mice infected with Mtb [78, 79]. Furthermore, Barber *et al.* show that CD4 T cells are entirely responsible for exacerbated disease of Mtb-infected PD-1 knockout mice, as there were no changes in CD 8 responses. [79]. These data suggest that when you remove T cell inhibitory stimuli, thus allowing uninhibited activation, replication and effector

functions of CD4 T cells, the host is unable to control infection. This result is contrary to the results seen with chronic viral infections, where removal of PD-1 allows the immune system to regain control and reduce viral load through reactivation of exhausted CD8 T cells [80]. Additionally, evidence suggests that Mtb antigen-specific T regulatory cells expand early during infection and delay CD4 and CD8 T cell priming in the lymph node, prolonging their dissemination to the site of infection and facilitating bacterial replication [81]. These data suggest mechanisms have been acquired to suppress T cell effector functions contributing to protective immunity.

Latency

Due to the delicate balance between pro-inflammatory and anti-inflammatory signals, an equilibrium between the bacterium and host is established. As a result most humans will develop an asymptomatic, non-contagious "latent" TB infection. Latent TB is defined as when a person is infected with Mtb but does not show signs of active disease and is not contagious.

During latency, the bacterium is thought to survive within the granuloma under unfavorable conditions in a state of dormancy. Dormancy is associated with minimal metabolic activity and little or no active replication. This state has been referred to as bacterial "persistence." Importantly, evidence suggests that mycobacterium has developed mechanisms that allow its survival within the hypoxic and nutrient-limited environment of the granuloma. Data obtained from cynomolgus macaques infected with Mtb suggest that the mutation rates during

latency and active disease are similar [82]. These data and the rise in drug-resistant strains suggest the urgent need to develop additional anti-TB drugs that don't readily engender resistance. A review of latent TB can be found here: [83]

Reactivation and Transmission

It is estimated that 10% of latently infected individuals will undergo reactivation and develop active TB disease [4]. It is not fully understood why some individuals reactivate, whereas others will remain in a latent state for the duration of their lives. However, the risk of developing active disease is increased as a result of immuno-compromising conditions such as old age, HIV/AIDS, anti-tumor necrosis factor therapy of patients with inflammatory diseases, diabetes, and obesity [83].

Upon reactivation, the granulomatous structure decays, thereby exposing the bacterium to a nutrient and oxygen rich environment [84]. The bacteria grow in high numbers, and eventually gain access to blood capillaries and the alveolar space allowing further dissemination and eventual transmission to other individuals through active coughing.

Part III: Other mycobacteria species

Mycobacteria are rod-shaped, acid-fast, Gram-stain variable bacilli that can be classified in two major groups. Mycobacteria species that cause human tuberculosis are considered part of the "*Mycobacterium tuberculosis* complex" (Mtb-complex). The Mtb-complex includes *M. bovis, M. africanum, M. microti* and *M. canetti.* The second group consists of all the non-tuberculosis mycobacteria [85] species that can cause human disease but not tuberculosis. Perhaps the most well known is *M. leprae*, which causes Hansen's disease or leprosy. Leprosy is a granulomatous disease of the peripheral nerves and mucosa of the upper respiratory tract [86].. The most common NTM species include *M. avium, M. intracellulare, M. kansasii, M. abscessus, M. marinum, M. chelonae, M. fortuitum, M. terrae, M. xenopi,* and *M. siminiae.*

Of particular importance to this thesis is *M. marinum* (Mm). Mm is the closest genetic relative to the Mtb-complex based on 16s rRNA homology [87] [88, 89]. Mm is a natural pathogen to fish, amphibians, and reptiles. Importantly, because Mm optimally grows at 28-33 degrees Celsius, it causes subcutaneous infections in humans. Lesions caused by Mm closely resemble Mtb-associated pulmonary lesions in humans and other animals [90, 91]. Mm disease in humans is referred to as "fisherman's granuloma" or "swimming pool granuloma." Due to its relative ease of use in biosaftey level 2 (BSL-2) safeguards and accelerated generation time (4h instead of 24h as in Mtb), Mm has become widely accepted as a surrogate model for Mtb in the laboratory. Animal models

have been useful in elucidating novel mechanisms of pathogenesis and immune control of Mtb. These animal models will be discussed in detail below.

Part IV: Animal Models for studying TB in the laboratory

In an effort to understand TB pathogenesis in humans, several animal models have been developed. Mice are perhaps the most commonly used species due to their low cost, reagent availability, convenience of use, and the existence of genetically altered and inbred strains [92]. Mice have facilitated our knowledge of host-Mtb interactions and the anti-TB immune response, including the role of CD4 T cells and cytokines such as IFNγ and TNFα [93]. Although the mouse model has been helpful in our understanding of TB immunity, several important differences exist between mice and men. In particular, TB manifests itself differently in mice than in humans. For example, in the most commonly used mouse strain, C57/BL6, mice infected via the aerosol route will develop a chronic infection that consists of high numbers of bacteria in the lungs and will eventually die. By contrast, in humans bacterial numbers remain much lower, and in some cases the bacteria are eventually cleared. Additionally, granuloma histopathology differs between the two species in that mouse granulomas lack a necrotic core [94]. Advantages and disadvantages of the mouse model have been reviewed elsewhere: [95].

Since the 1930s guinea pigs have been useful in TB drug testing and for their ability to reveal drug resistant strains. Guinea pigs have been highly regarded for their susceptibility to Mtb as they succumb within three weeks post-infection. Guinea pigs develop highly necrotic granulomatous lesions with very

similar histopathology to that seen in humans infected with TB [92]. The guinea pig model is therefore useful for studying the components and formation of human granulomas. Additionally, this model can be helpful for vaccine studies as infection times are relatively short in length compared to mouse experiments, combined with the previously mentioned histological relevance to humans [96]. However, the guinea pig model presents limitations in the laboratory because of the amount of animal space needed, their expense, and lack of reagent availability.

The cynomolgus and rhesus macaque are two other very useful non-human primate (NHP) models for studying TB pathogenicity. NHP are particularly useful as they are genetically most similar to humans [97]. Macaques have allowed us to gain a better understanding of granuloma kinetics and heterogeneity in the lungs [97]. Importantly, macaques are the only animal model that can mimic Mtb latency as seen in humans. Additionally, simian immunodeficiency virus (SIV) can be used in these animals to model co-infections and parallel HIV-induced reactivation of latent TB [98, 99]. Although extremely useful, the macaque model is complicated by its ethical concerns, cost, availability, and lack of reagents. The macaque model of TB has been reviewed elsewhere: [100, 101].

Finally, the Ramakrishnan lab has developed a novel model to study TB pathogenesis using Mm and zebrafish embryos. Zebrafish have conserved orthologs to molecules from the innate and adaptive immune systems in humans and mice, and can be genetically manipulated with relative ease [102]. Zebrafish

are also optically transparent, facilitating real-time imaging experiments [103]. Using the zebrafish model, Tobin *et al.* elucidated an evolutionarily conserved set of molecules that facilitate vertebrate immune control of TB [104]. Indeed much of our recent understanding of host-pathogen interactions and mycobacterial factors that contribute to innate cell recruitment and early granuloma formation have come from zebrafish studies [50].

Recent human studies aimed at understanding the difference between latently infected individuals and people with active disease have strived to identify and compare biomarkers in the blood. For example, Berry *et al.* describes a neutrophil-driven IFN-inducible gene profile that is unique to people with active TB. Blood signatures may be able to facilitate the invention of new diagnostics for active TB. Although these markers describe an endpoint or the steady state of an individual's immune response to TB, such signatures neither predict the history nor the progression of the disease. Recently, Adekambi *et al.* offer a more mechanistic approach in deciphering the differences between human TB disease states [105]. These data suggest there is surface marker expression and functionality of Mtb antigen-specific CD4 T cells can distinguish latently infected versus healthy individuals [105]. Unfortunately, human studies are naturally limited due to poor access to tissue samples, sputum, and blood, as well as inefficient diagnosis of the disease stage.

Animal models continue to contribute greatly to our understanding of host-Mtb interactions, TB immunity, drug development, drug resistance, latency, and co-infections. However, with the exception of macaques, these models do not

appropriately mimic pulmonary TB, and therefore leave a great number of questions to be answered. Animal models often have subjective readouts including bacterial burdens (CFU), pathology (staining and preparation often bias results), and death [63]. These readouts have been helpful in giving us hints into the roles of the innate and adaptive immune responses, but they leave much to the imagination and have been lacking when it comes to vaccine and drug design.

Part X: Tyrosine Kinases

Overview

Intra and intercellular communication is facilitated by protein phosphorylation, a process carried out by protein kinases. Kinases catalyze the transfer of a γ phosphate of adenosine triphosphate (ATP) to amino acid residues on protein substrates. There are two major classes of kinases, serine/threonine kinases, and, of particular interest to this thesis, tyrosine kinases (TKs). TKs control and participate in virtually all cellular processes, including embryogenesis, metabolism, and immune system function [106].

History

The basis of our understanding of tyrosine kinases originated with the pioneering work of Nobel laureate Francis Peyton Rous. Rous demonstrated that an avian sarcoma was transmissible to naïve recipients upon inoculation of tumor extracts [107]. He concluded that the transforming agent was a virus based on his observations that the filtered extract, free of cells, still induced sarcomas in recipient animals [108]. This tumor-inducing virus was later identified as a retrovirus, and bore the name of its founder, Rous Sarcoma Virus (RSV). Further work by Martin *et al.* and others utilizing temperature-sensitive RSV mutants identified a viral gene (termed *v-src*) that proved dispensable for viral replication, but caused cellular transformation by dysregulating the host cell division cycle [109, 110]. During this time Collet and Erikson demonstrated that

the protein responsible for the transforming property of the virus had enzymatic activity in the form of protein phosphorylation [111]. Subsequently, in 1976 Bishop and Varmus found evidence in normal cells for a mammalian counterpart of *v-src*, called *c-src* [110]. They were awarded the Nobel prize in 1989 for their work providing evidence that dysregulation of a group of cellular genes normally involved in growth and division of cells, Src family tyrosine kinases, could result in the transformation of a normal cell into a cancerous cell, leading to tumor formation.

Other oncogenes and their cellular counterparts were discovered in quick succession, including a tyrosine kinase called Abl, which is of interest to work presented in this thesis. The product of *v-abl* was originally identified as the transforming factor in Ableson leukemia virus [112]. The cellular homologue, *c-abl* (Abl1), was found to be oncogenic due to its dysregulation in chronic myelogenous leukemia (CML). CML is caused by the chromosomal translocation (9;22), producing the oncogene breakpoint-cluster region (BCR) -Abl1 (also known as the "Philadelphia chromosome), resulting in constitutive activation of Abl [113-117]. The discovery of the oncogenic properties of tyrosine kinases has had enormous ramifications for our understanding of both cancerous and normal cellular signaling.

Abl Family Tyrosine Kinases

Abl family kinases, including Abl1 and Abl2 (Arg), serve important roles in cellular processes including proliferation, survival, stresses by reactive oxygen,

DNA damage, and cytoskeleton rearrangement [118-120]. Abl family kinases consist of a Src homology 3 (SH3) domain, an SH2 domain, a kinase domain, a large carboxy-terminus region containing three conserved polyproline binding regions (PxxP motifs), and a binding domain for filamentous DNA and actin [121, 122]. Additionally, Abl1 has a nuclear localization signal and Abl2 has a microtubule-binding domain [121, 123].

Due to the oncogenic potential of Abl, its enzymatic activity is tightly controlled. Abl kinases auto regulate themselves based on their structural conformation. A closed conformation, resulting in kinase inactivation, is achieved when the N-terminal myristoyl group binds to the hydrophobic pocket within the kinase domain and the SH2 domain interacts with the carboxy-terminal lobe of the kinase domain [124]. In this conformation, the SH2 and SH3 domains are "clamped" within the kinase domain lobes, precluding ATP binding. Activation can be achieved by binding of SH2 and SH3 ligands facilitating the conformational opening of the structure and subsequent phosphorylation of tyrosine residue (Y412 or Y245) and catalytic activation [124, 125].

Pharmocological inhibition of Abl kinases

CML proved to be a hard cancer to treat in the clinic, as the only option was allogenic bone marrow transplantation, which was complicated by lack of donors and age of the patient. Additionally, CML is relatively fast-acting as it can progress to blast crisis, a terminal phase, in as soon as 5 years post-clinical diagnosis [126]. With the knowledge that the constitutive activity of Abl kinase

was the molecular mechanism of the malignancies, scientists sought to design an inhibitor of the ATP binding site. In doing so, they hoped to block kinase activity and therefore the aberrant signaling and progression of cancer. Towards this effort, in 1996 Druker *et al.* described a novel 1-phenylaminopyrimidine compound, CGP 57148 (imatinib, imatinib-mesylate, STI-571, Gleevec) as a competitive inhibitor of the ATP-binding pocket of Abl [127]. In their seminal paper Druker *et al.* reported imatinib as a potent inhibitor of Abl kinase activity that selectively inhibits the proliferation of BCR-ABL positive cells *in vitro* and in tumor models in animals. In 2001,imatinib was approved for treatment of CML in human patients by the Food and Drug Administration.

The introduction of Abl kinase inhibitors has allowed for CML patients to have a good quality of life and survive indefinitely. However, eventually some patients develop resistance to imatinib. Resistance is acquired through a variety of point mutations that result in the allosteric inhibition of the binding of STI-571 both within the ATP-binding pocket and throughout the protein [128]. The development of drug-resistant tumor cells has highlighted the need to develop new drugs and more thoroughly understand the pathogenesis of disease. In this regard, second generation tyrosine kinase inhibitors such as Tasigna are effective against imatinib resistant CML.

Tyrosine Kinases and Microbial Pathogenesis

In the last decade, evidence suggests that bacterial, viral, and parasitic pathogens interfere with host signaling pathways, repurposing them to promote

efficient tissue colonization, entry into cells, and inter and intracellular mobility. Furthermore, many pathogens are now recognized to utilize host tyrosine kinases, including members of the Src and Abl families. The list includes diarrheagenic *E. coli, Pseudomonas*, *Salmonella, Shigella, Helicobacter, Anaplamsa*, and *Chlamydia* amongst bacteria, and filoviruses, HIV, Coxsackie, West Nile, Kaposi sarcoma, Polyoma, and Pox amongst viruses [129-142]. Additionally, Wetzel *et al.* suggest Leishmania, the obligate intracellular parasite, utilizes Abl family kinases to promote phagocytosis and maximal survival within macrophages [143].

There are several important lessons from the studies of pathogens and tyrosine kinases. First is the concept that multiple kinases are used in a redundant fashion by pathogens. That is, each of several kinases is sufficient yet none is necessary. For example, vaccinia virus utilizes Abl and Src family kinases in a redundant fashion to promote actin tail formation, and EPEC uses Abl and Tec family kinases for pedestal formation [129, 141, 144]. Pathogens most likely utilize host proteins in a redundant manner to facilitate robust infection in a variety of cellular conditions.

A second lesson is the idea of host mimicry. To this extent, the vaccinia virus membrane protein A36R contains an SH2 binding site that “mimics” the binding sites of cellular adaptor proteins that normally recruit proteins involved in actin polymerization, including neuronal Wiskott-Aldrich protein (N-WASP) and the Actin-Related proteins, Arp2 and Arp3 (ARP2/3 complex). A36R uses these domains to recruit these host proteins and promotes actin polymerization

beneath the virion, facilitating cellular egress and intercellular spread. Both bacterial and viral pathogens exhibit protein mimicry, a mechanism used to gain access to host cellular pathways and utilize them in pathogenesis.

With the advent of imatinib, so came the possibility of repurposing therapeutics targeting host factors dysregulated in cancer to treat diverse microbial and viral infections. This therapeutic strategy has now been validated for a variety of pathogens. For example, Gleevec and related inhibitors block release of poxviruses and Ebola Zaire virus-like proteins *in vitro* [136, 141]. Moreover, Gleevec restricts dissemination of poxvirus and protects from an otherwise lethal infection *in vivo* [141, 145]. In chapter 2 we will present data suggesting Abl and related kinases modulate Mtb pathogenesis in macrophages and in mice. We provide evidence that imatinib represents a possible therapeutic for treating Mtb infections. In chapter 3, we describe a novel immunomodulatory characteristic of imatinib in mice. We show that imatinib induces neutrophilia in the bone marrow, blood, and spleens of mice, which facilitates host antimicrobial activity.

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Chapter 2:

Imatinib-sensitive tyrosine kinases regulate mycobacterial pathogenesis and represent therapeutic targets against tuberculosis.

Imatinib-sensitive tyrosine kinases regulate mycobacterial pathogenesis and represent therapeutic targets against tuberculosis.

by

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Abstract

The lengthy course of treatment with currently used anti-mycobacterial drugs and the resulting emergence of drug-resistant strains have intensified the need for alternative therapies against *Mycobacterium tuberculosis* (Mtb), the etiologic agent of tuberculosis. We show that Mtb and *Mycobacterium marinum* use Abl and related tyrosine kinases for entry and intracellular survival in macrophages. In mice, the Abl-family tyrosine kinase inhibitor, imatinib (Gleevec®), when administered prophylactically or therapeutically, reduced both the number of granulomatous lesions and bacterial load in infected organs, and was also effective against a rifampicin-resistant strain. Further, when co-administered with current first-line drugs, rifampicin or rifabutin, imatinib acted synergistically. These data implicate host tyrosine kinases in entry and intracellular survival of mycobacteria, and suggest that imatinib may have therapeutic efficacy against Mtb. Because imatinib targets host, it is less likely to engender resistance compared to conventional antibiotics, and may decrease the development of resistance against co-administered drugs.

Introduction

*Mycobacterium tuberculosis, (*Mtb) the etiologic agent of tuberculosis (TB), infects one-third of the world's population, and kills approximately 2 million people per year worldwide [3] with a global case fatality rate of 23% [142]. Estimates indicate that more than 90% of all cases of TB and 98% of deaths due to TB occur in developing countries in Southeast Asia, the Western Pacific, and Africa [143, 144]. The magnitude and potential impact of this pandemic prompted the World Health Organization (WHO) in 1993 to declare TB a global health emergency. It is estimated that over the next two decades nearly one billion people will become infected, 200 million people will develop disease, and 35 million will die from TB [3].

Although highly effective regimens have been developed for the treatment of TB patients, drugs must be administered for a minimum of six months to cure the disease. Non-adherence with the lengthy course of treatment remains a major problem and has contributed to the emergence of multidrug-resistant and extensively drug-resistant TB (MDR-TB and XDR-TB) strains, which complicates the treatment and control of TB and threatens to exacerbate the epidemic [145, 146]. Availability and quality of drugs and altered pharmacokinetics of absorption of some drugs in persons with AIDS has also contributed to the development of drug resistance [147]. Thus, new anti-TB drugs are urgently needed to combat drug resistance, shorten and/or simplify current treatment regimens, provide effective therapy for patients intolerant to current first-line drugs, and provide treatment for patients with latent TB infection.

A key feature of Mtb pathogenesis is the ability of the bacteria to survive and replicate in host phagocytic cells [27, 81, 148-150]. Mtb can use as many as eight different cell surface receptors and appears to enter macrophages through conventional phagocytosis [150, 151]. Upon infection, mycobacteria reside within a specialized early phagosomal compartment. Pathogenic mycobacteria prevent fusion with the lysosome, which facilitates evasion of host bactericidal mechanisms, and precludes efficient antigen presentation [149]. Although there exists a wealth of information on Mtb factors that contribute to entry and intracellular survival within macrophages, information on host factors that contribute to these processes remains more limited.

We have been studying mechanisms by which host tyrosine kinases, and in particular the Abl-family kinases Abl1 and Abl2, mediate pathogenesis of bacteria and viruses [152]. Abl1 is mutated in human cancers such as Chronic Myelogenous Leukemia (CML), and drugs such as imatinib mesylate (STI-571, Gleevec), which inhibit Abl1, Abl2 and related kinases, are used as therapeutics for CML and other cancers [153, 154]. *Pseudomonas aeruginosa, Shigella flexneri* and *Chlamydia trachomatis* [126, 128, 131] utilize Abl-family kinases during entry, although the precise mechanisms remain unclear. Abl-family tyrosine kinases also regulate cytoskeletal and trafficking functions in cells, including autophagy [155]. In this regard, *Shigella flexneri,* enteropathogenic *Escherichia coli,* and orthopoxviruses utilize Abl-family kinases for actin-based motility or release from infected cells, which facilitate spread of the infection [125, 137, 141, 156].

The requirement for Abl-family kinases in the pathogenesis of diverse microbes led us to assess their role in Mtb infection. Using cell lines lacking Abl-family kinases and specific inhibitors, we show here that Abl-family and related imatinib-sensitive kinases facilitate entry and intracellular survival of Mtb and the related *Mycobacterium marinum* (Mm). Additionally, we show that imatinib reduces bacterial load and associated pathology in mice infected with Mtb and Mm, including antibiotic-resistant strains. Moreover, imatinib acted in a synergistic manner with the frontline anti-TB drugs rifampicin and rifabutin. Together, our data suggests that modulation of Abl1, Abl2 and related imatinib-sensitive kinases may offer a novel therapeutic strategy for infections caused by mycobacterium species.

Results

Src- and Abl-family tyrosine kinase inhibitors (TKIs) affect intracellular survival of Mtb and Mm *in vitro*. J774A.1 cells, a murine macrophage-like cell line, were infected with Mtb strain H37Rv in media supplemented with PD-166326, an inhibitor of Src- and Abl-family tyrosine kinases or DMSO, the carrier for PD-166326 (Figure 1A), or imatinib mesylate (imatinib), an inhibitor of Abl- but not Src-family kinases (Figure 1B). PD-166326 or imatinib caused a reduction in CFU/ml by up to 2-fold compared to the carrier control at time 0, and up to 7.7 fold at eight days post infection (Figures 1A and B). Comparable effects of TKIs were evident in several cell lines including A549 cells, a human type II alveolar pneumocyte cell line, and THP-1 cells, a human monocyte cell line (Figure 1C; Supplementary Figures 1A-E). Moreover, the effects of TKIs on J774A.1 cells were independent of stimulation with IFN-g (Figure 1C and Supplemental Figure 1A).

To determine whether TKIs affected other *Mycobacterium* species, we assessed their effects on intracellular survival of *Mycobacterium marinum* (Mm) and *Mycobacterium smegmatis (*Ms) in J774A.1 cells. As with Mtb, PD-166326 or imatinib decreased CFU/ml by up to 9-fold at both early and late time points for both Mm and Ms (Figure 1D and data not shown). TKIs had no effect on the growth of Mtb, Mm, or Ms in 7H9 broth supplemented with PD-166326 or imatinib at concentrations as high as 10 mM (e.g. Figures 1E,F and data not shown). Together, these data suggest inhibitors of imatinib-sensitive host

tyrosine kinases reduce CFUs of several *Mycobacterium* species in cells at both early and late times after inoculation.

Abl and other imatinib-sensitive kinases mediate entry and intracellular survival of mycobacteria in macrophages. We next determined whether imatinib-sensitive tyrosine kinases mediate entry or intracellular survival of mycobacteria within the macrophage, or both. To distinguish these possibilities, J774A.1 cells were inoculated with Mm constitutively expressing GFP (GFP-Mm) and treated with imatinib for 2 hours, and then with amikacin for two more hours to kill extracellular bacteria. As shown in Figure 2A, imatinib caused a 2.9-fold reduction of the number of GFP-positive cells. In accordance with these data, imatinib produced 2-fold reduction in CFU/ml (Figure 2B) when added to THP-1 cells for only 2 hrs at the time of inoculation with Mtb. A similar fold reduction was evident when imatinib was added to J774A.1 cells for only 15 minutes at the time of inoculation with Mm (Figure 2C). Together, these data suggest that imatinib-sensitive kinases mediate entry of mycobacteria.

To determine whether imatinib had additional effects on intracellular survival independent of entry, J774A.1 cells were infected in media only and drug was added 4 hours post inoculation for the duration of the experiment (Figure 2D). Using this protocol, imatinib reduced CFU/ml by 3.4-fold. We next assessed whether this reduction in CFU resulted from increased phagolysosomal fusion. To do this, macrophages were infected with GFP-Mm, treated with amikacin to

kill extracellular bacteria, and then treated with imatinib for an additional 24h. Live macrophages were then stained with lysotracker red, a marker for lysosomes. In agreement with previous reports [157], imatinib caused an increase in the area of the lysotracker-labeled compartment, even in uninfected cells (Figure 2E). As shown in Figures 2E and F, 5.8 fold-more GFP-Mm was evident in lysotracker-labeled compartments upon treatment with imatinib, compared to untreated cells. Collectively, these data indicate that an imatinib-sensitive kinase(s) regulate both entry and phagolysosomal trafficking of mycobacteria.

Abl-family tyrosine kinases and other imatinib-sensitive kinases mediate intracellular survival of Mm. To determine if Abl1 and Abl2 have a direct role in entry or intracellular survival of mycobacteria, we assessed CFU/ml in a fibroblast cell line derived from wild-type mice (3T3) or from mice lacking both c-Abl1 and c-Abl2 ($Abl1^{-/-}/Abl2^{-/-}$). CFU of Mm decreased by up to 3.6-fold at 72h p.i. in $Abl1^{-/-}/Abl2^{-/-}$ cells compared to 3T3 cells, suggesting that Abl1 and Abl2 are required for intracellular survival of Mm (Figure 2G). We were unable to evaluate Mtb in $Abl1^{-/-}/Abl2^{-/-}$ cells because neither 3T3 cells nor $Abl1^{-/-}/Abl2^{-/-}$ cells were susceptible to infection with this bacterium, even at high MOI (~60). Moreover, no differences between 3T3 cells and $Abl1^{-/-}/Abl2^{-/-}$ cells at the initial time points were evident with Mm infections. These data raise the possibility that entry mechanisms in fibroblasts differed from macrophages, and thus were not evaluated further.

Treatment of Abl1$^{-/-}$/Abl2$^{-/-}$ cells with imatinib reduced CFU/ml by an additional 3.5-fold suggesting that cellular targets of imatinib other than Abl-family kinases also mediate intracellular survival of mycobacteria. c-Kit, M-CSF1R, PDGFRα and PDGFRβ are also inhibited by imatinib [158-160]. However we could find no significant difference in the entry or intracellular survival of Mm in PDGFRα$^{-/-}$, PDGFRβ$^{-/-}$ or PDGFRα$^{-/-}$β$^{-/-}$ cells or in the presence of neutralizing antibodies to c-Kit or m-CSF-1 compared to control cells (data not shown). Collectively, these data suggest Abl-family kinases mediate together with additional imatinib-sensitive factors regulate intracellular trafficking of Mm.

Imatinib reduces bacterial load in mice infected with Mm. To determine whether Abl-family kinases mediate intracellular survival of mycobacteria *in vivo*, we assessed the effects of imatinib on mice infected with Mm. In accordance with a previous report, injection of Mm (10^5 CFU) into the tail vein resulted in colonization of spleen, liver, and lung [161]. CFU per gram of tissue (CFU/gram) increased to maximal levels by seven days post infection (p.i.), with highest mean levels evident in spleen ($6.9x10^5$CFU/gram) compared to liver ($9.4x10^4$ CFU/gram), and lung ($1.5x10^4$ CFU/gram; Figures 3A-C, controls).

We next assessed the effects of imatinib, administered at doses ranging from 25 to 200mg/kg/day, or the carrier, water, as a control, beginning 24 h prior to infection and continuing for the duration of the experiment. At concentrations of 50-100mg/kg/day, imatinib reduced the median CFU/gm by 13 to 64 fold (Figure 3A), a statistically significant effect. At lower (25mg/kg/day) or

higher (200mg/kg/day) concentrations, imatinib produced a somewhat smaller effect. Statistically significant reductions in bacterial load by imatinib (100mg/kg/day) were also evident in liver and lung (Figures 3b,c). Additionally, administration of imatinib (100 mg/kg/day) reduced bacterial load when delivered beginning either 1h or 24 h p.i. (Figures 3D,E).

Imatinib reduces liver pathology and tail lesions in mice infected with Mm. Histological examination of infected tissues indicated the presence of granulomatous lesions characterized by monocytic infiltrates in the liver seven days after infection whereas no such lesions were evident in uninfected controls (e.g. compare upper and middle panels in Figure 4A,B). These lesions resembled those seen in livers of mice following i.v. injection with Mtb [162]. Lesions were also evident in spleen, and were associated with mycobacteria infiltrates, as assessed by acid-fast staining of adjacent sections (e.g. Figure 4C), whereas lesions in liver were not. However, because the load in the spleen was ~10 fold higher than liver, it remains possible that bacteria were simply more readily observable in adjacent spleen sections. Administration of imatinib (100mg/kg/day) either before or after infection caused a 3.5-fold decrease in the number of lesions per mm^2 of liver (e.g. Figures 4A,B). Inoculums of 10^7 CFU Mm induced lesions on the tail beginning at the site of injection and extending distally. These lesions were reduced in size and extent upon administration of imatinib either prior to or after infection (e.g. Figure 4d,e). Collectively, these

data indicate that imatinib reduces not only bacterial load, but also characteristic pathology associated with mycobacterial infection.

Effects of imatinib on rifampicin-resistant Mm. To determine whether imatinib was effective against antibiotic-resistant mycobacteria, we generated a spontaneous rifampicin-resistant Mm mutant (Mm^R). Sequencing confirmed that this mutant contained a single base pair change in *rpoB*, which resulted in a H to Y substitution at amino acid 134, and which corresponds to the H526Y substitution in Mtb *rpoB,* the cause of high-level resistance to rifamipicin in clinical isolates [163]. Mm^R did not display a growth defect in the presence or absence of 1μg/ml rifampicin in 7H9 media compared to wild-type Mm (Figure 5A). In J774A.1 cells, imatinib caused a similar fold reduction in growth of Mm^R and the parental strain (Figure 5B). In mice, administration of imatinib reduced the load of Mm^R in the spleen to a similar extent as that seen with Mm (Figure 5C). Together, these data suggest that imatinib is effective against both antibiotic-susceptible and antibiotic-resistant strains of Mm.

Imatinib and antibiotics synergistically reduce bacterial load. We next determined the effects of imatinib in conjunction with first-line anti-TB drug drugs. J774A.1 cells were infected with Mm and treated with imatinib, or rifampicin, or with a combination of both. For these experiments, the rifampicin concentration chosen only partially inhibited bacterial growth. As shown in Figures 6A and B, the sum of the maximum fold reduction with the combination

exceeded the sum of fold effects with either drug alone, indicating a synergistic effect. Similar effects were observed with Mtb, although the maximum fold effect of rifampicin at the concentration used (0.125 μg/ml) was larger than that for Mm (Figure 6C). Together, these data suggest that in combination, imatinib and rifampicin produced a synergistic reduction in survival of mycobacteria in macrophages.

We next assessed the effects of imatinib and antibiotics on Mm infection *in vivo*. Rifampicin has been shown to induce levels of cytochrome P450s that act directly on imatinib and reduce its serum concentration in human plasma [164]. To circumvent this problem, we instead used rifabutin, which is a much less potent inducer of cytochrome P450s [165]. As shown in Figure 6D, imatinib and rifabutin together reduced the median CFU/ml to a level that exceeded the sum of either drug alone. Similar effects were seen in the liver (data not shown). Together, these data suggest that imatinib acts in a synergistic manner with first line antibiotics when delivered in combination *in vivo*.

Imatinib reduces bacterial load in mice infected with Mtb. We next determined whether imatinib affected intracellular growth and survival of Mtb *in vivo*. We assessed the effects of imatinib, administered at doses of either 66 or 100mg/kg/day, or the carrier water as a control, beginning 24 h prior to infection and continuing for the duration of the experiment. Mice were infected with a low dose (~25-50 CFU) of aerosolized Mtb (Erdman strain) and lungs were harvested to assess the bacterial burden and disease pathology 4 weeks post infection.

Significance was assessed by a Kruskal-wallis nonparametric rank sum test, wherein the data from the groups are pooled and ranked according to CFU; the statistic determines whether the observed distribution of rankings could arise by chance. As shown in Figure 7A, imatinib reduced the median CFU by 21 to 57 fold, a statistically significant effect ($p=0.035$), though no statistically significant difference was evident between the groups treated with different doses of drug ($p=0.45$). Taken together, lung CFUs were below the level of detection of plating (~10 CFUs; dotted line in Figure 7A) in 56% of animals treated with imatinib, with 78% below 10^5 CFUs, compared to 20% below the detection level and 80% greater than 10^5 CFUs in the carrier-treated group. Histopathological examination of the lungs from a subset of mice, including those with CFUs below the level of detection, showed lymphocytic infiltrates (data not shown).

The observation that 20% of the carrier-treated mice displayed CFUs below the level of detection was unusual and led us to consider the possibility that the osmotic pump interfered with the deposition of bacteria to the lungs. To test this possibility, un-manipulated (no pump and no drug) or carrier-treated mice were exposed to aerosolized Mtb at a concentration of $2.5x10^5$/ml CFU or a higher concentration of $1x10^7$/ml CFU. As shown in Figures 7B, deposition of Mtb at 24 hrs p.i. was equivalent in the lungs of un-manipulated mice compared to those with carrier pumps for both concentrations of bacteria. Together, these data suggest that the pumps were without effect, but the reasons for the undetectable CFU in carrier-treated animals in Figure 7A remain unclear.

To determine whether the undetectable CFU in carrier-treated animals was an experimental anomaly, and to confirm that there existed a statistically significant difference in CFUs between the control and imatinib-treated groups, we carried out a second 28d experiment in which we counted CFUs from the entire lung rather than a single lobe, so as to avoid underestimating the number of bacteria. We also included un-manipulated animals as controls. As shown in Figure 7C, with a measured inoculum of approximately 50 CFU, there was no statistically significant difference between un-manipulated mice (median CFU was 5.8 $x10^6$) and carrier treated mice (median CFU was 6 $x10^6$). Moreover, neither group had outliers below the level of detection (10 CFU). Importantly, the median CFU was reduced by 185-fold in the imatinib-treated group compared with the carrier-controls, a statistically significant difference ($p=0.001$), with 30% of the animals displaying CFUs below the level of detection. Examination of CFUs from spleen followed a similar pattern as the lungs, with the median CFU reduced by 12-fold in imatinib-treated animals compared to the carrier-controls (Figure 7D). Moreover, animals with CFUs below detectable levels in the lung likewise had undetectable levels in the spleen. Collectively, these data suggest that imatinib is effective against Mtb in acutely infected animals.

Discussion

Using specific TKIs and cell lines derived from knockout animals, we provide evidence for a role of host tyrosine kinases, including Abl1, Abl2, and additional imatinib-sensitive kinases, in the entry and intracellular survival of Mtb and related pathogen Mm in macrophages. Pathogenic mycobacteria likely use multiple kinases, in a redundant fashion, perhaps as a means to infect cell types that express different classes of kinases. Tyrosine kinases, including members of the Abl family, are used by diverse bacterial and viral pathogens including diarrheagenic *Escherichia coli, Pseudomonas aeruginosa*, *Salmonella typhimurium, Shigella flexneri, Helicobacter pylori,* and *Chlamydia trachomatis*, amongst bacteria, and Orthopoxviruses, Polyomaviruses, Coxsackie virus, West Nile virus, and Kaposi sarcoma virus amongst viruses [125-129, 131, 135, 137, 138, 156, 166-168]. Notably, redundant usage of kinases has been reported for several of these [125, 137, 156, 169]. A recent study showed that siRNA against Abl1 transcript appeared to facilitate survival of Mtb strain H37Rv in J774A.1 macrophages [170]. However, experiments presented here using specific inhibitors as well as cells derived from knockout animals suggest that multiple kinases, including both Abl1 and Abl2, mediate intracellular survival within macrophages. The apparent disparity may be explained by redundant kinase usage, which our experiments suggest. A recent report indicated that non-specific serine/threonine kinase inhibitors affect MDR-Mtb growth in a primary

macrophage cell line, though it remains to be determined whether these compounds affect additional targets in the bacteria or the host or both [171].

The observations that fewer macrophages become infected upon treatment with imatinib, and that treatment periods as short as 15 minutes reduce CFUs, provide evidence for an effect of Abl or related kinases on entry of mycobacteria into cells. However, our data also show that imatinib reduces intracellular survival even when administered after entry. Several observations have suggested a role for Abl or related kinases in autophagosomal and lysosomal trafficking. Imatinib causes accumulation of acidified vesicles that contain lamp-1 and LC3, which are markers for lysosomes and autophagosomes, respectively [155, 157]. Moreover, stimulation of autophagy in macrophages during infection, increases the number of bacteria in the phagolysosomal compartment, and reduces intracellular survival [55]. Accordingly, upon imatinib treatment more Mm localized within lysosomes, and intracellular survival decreased. Collectively, our data suggest that imatinib-sensitive kinases mediate the capacity of mycobacteria to enter macrophages and to inhibit phagolysosomal fusion.

Our inhibitor studies suggest that imatinib similarly affects Mtb, *M. smegmatis* and Mm in diverse cell types, suggesting that imatinib-sensitive TKs may serve as a “node” and mediate entry by multiple receptors or alter receptor mediated trafficking of a phagosome utilized by several mycobacteria species. Mm is the closest genetic relative to the Mtb complex based on 16S rRNA sequencing and 85% identity to orthologous regions of the Mtb genome [83, 172]. Mm causes both systemic and granulomatous disease in cold-blooded animals as

well as subcutaneous granulomas in humans due to its preference for lower temperatures [85]. Notably, Mm exhibits a five-fold faster generation time compared to Mtb [85] and requires only BSL2 containment, thereby expediting data acquisition and optimization of drug dose and delivery methodologies. The observation that *M. smegmatis* is similarly affected by imatinib suggests that the effects of the drug are independent of the RD1 locus, which is associated with virulence. This observation also raises the possibility that imatinib might likewise be effective against other pathogenic mycobacteria, including *M. avium*, *M. intracellulare*, *M. ulcerans* and *M. leprae*.

Some differences between Mtb and Mm have been noted. Mm escapes the phagosome shortly after infection, and our data and that of others suggest that Mm forms actin "tails" after 48h that allow the bacterium to move towards or into apposing uninfected cells [173, 174]. Recent reports suggest that Mtb also escapes the phagosome [52], although no actin motility has been observed. Notably, the effects of imatinib on infectivity of Mm *in vitro* are evident prior to 48h, and no reduction in actin tails was apparent when imatinib was applied at 48h, nor in cells lacking Abl-family kinases (data not shown). Thus, differences in actin motility between Mtb and Mm *in vitro* appear independent of the utilization of host tyrosine kinases.

Imatinib may have significant utility in treating infections caused by mycobacteria. Imatinib reduced CFU in lungs and spleens of mice infected with aerosolized Mtb (Figure 7), and likewise reduced CFU in various organs in mice infected with Mm (Figures 3). The effect of imatinib on bacterial load was dose

dependent with optimal concentrations of 66.7-100mg/kg/day, which is approximately equivalent to the dosage used in humans to treat CML [154]. Clearance of Mtb to levels below the level of detection was evident with imatinib treatment in both lung and spleen in some animals (Figures 7A,C, and D), although histolological analysis performed on a subset of the animals indicated lymphocytic infiltrates, an indication of prior infection (data not shown). Such clearance was not observed in Mm experiments, perhaps owing to the shorter time course of infection, higher inoculum used (10^5 CFU for Mm versus approximately 50 CFU for Mtb), or the route of inoculation. For reasons that are as yet unclear, considerable variance was evident in CFUs for both Mtb and Mm infections with imatinib administration, even within a single experiment, although reductions in median CFUs were consistently observed (Figures 3 and 7).

Tissue sections from mice infected with Mm revealed the presence of non-caseating granulomatous lesions characterized by lymphocytic or monocytic aggregates. Notably, colonization of the lung was much more pronounced with Mtb than with Mm, perhaps owing to differences in tropism or to the route of inoculation [162]. Moreover, we and others have observed superficial lesions at the site of injection of Mm on the tail, likely because this bacteria grows optimally at low temperatures [53]. Notably, the size of lesions in liver and on tails of animals infected with Mm was markedly reduced upon treatment with imatinib (Figure 4), though such effects were not evident in lungs of animals infected with Mtb (data not shown).

Based on *in vitro* studies, reductions in CFU may reflect a reduction in the number of bacteria entering macrophages or their intracellular survival or both. We also show that imatinib and the first-line antibiotics rifampicin, when provided together, can act in a synergistic fashion to reduce survival of Mtb and Mm in macrophages (Figure 6A,B, and data not shown); imatinib and rifabutin act similarly *in vivo*. It remains possible that *in vivo,* treatment with imatinib results in an increased number of extracellular bacteria, which may be exposed to higher concentrations of antibiotics. Alternatively, localization of intracellular bacteria in a lysosomal compartment upon treatment with imatinib may render them more susceptible to antibiotics.

Besides Abl-family kinases, imatinib is also a potent inhibitor of receptor tyrosine kinases responsible for mast cell development stem cell differentiation (c-Kit), macrophage differentiation and function (m-CSF1R), and cell growth (PDGFR) [158-160]. Thus, we cannot rule out the possibility that additional immunomodulatory effects contribute to the observed phenotype. In this regard, imatinib appeared more efficacious at lower concentrations (Figure 3A), suggesting that the drug may exert concentration-dependent effects on factors important for control of the infection.

A primary concern when considering drugs directed at host targets remains their toxicity toward the host. Thus, host molecules that are essential for survival or required to mount an effective immune response may prove difficult to target. In this regard, imatinib has exhibited remarkably low toxicity in patients, though recent reports indicate side effects on the heart and the immune

system with long-term exposure [175, 176]. Adjusting the dosing regimen or limiting the period of exposure may mitigate such side effects. Imatinib may prove most effective in patients when the bacteria are actively growing ("active TB"), or with reactivated TB, which may warrant acute, rather than sustained, exposure to the drug. We have not pursued *in vivo* studies with dual Abl- and Src-family kinase inhibitors such as Sprycel (BMS-354825) or PD-166326 because these drugs cause significant immunotoxicity [177], which has proven insurmountable in an orthopoxvirus infection model [141].

Here we provide evidence that host tyrosine kinases including Abl1, Abl2, and other imatinib-sensitive kinases regulate the pathogenesis of Mtb and Mm *in vitro* and *in vivo*. Data from macrophages and mice indicate that infections caused by Mm and Mtb infections are similarly sensitive to imatinib. Similar imatinib-sensitive kinases may govern trafficking of Mm and Mtb in macrophages. However, *in vivo* the mechanism by which imatinib reduces Mtb CFUs may be more complex than with Mm. In the Mm model, effects of imatinib are evident within 3-7 days, and reductions in CFU likely reflect entry or intracellular trafficking of bacterium or innate immune effects. Such effects may also be important in Mtb infections, but at 28 days adaptive responses may also contribute. We are currently investigating how imatinib impacts the innate and adaptive responses in animals infected with *Mycobacteria sp*. Nevertheless, because of its relatively rapid growth rate and limited biocontainment requirements, Mm may be useful for initial phases of drug discovery for acute mycobacteria infections *in vitro* and *in vivo*.

Additionally, our data suggests that imatinib may prove useful against both antibiotic-susceptible and antibiotic-resistant strains of Mtb including MDR-TB and XDR-TB (e.g. Figure 5). Because imatinib targets host tyrosine kinases and not bacterial factors, it is less likely to engender resistance compared to conventional antibiotics. To evade the block and develop resistance, Mtb would have to significantly alter its pathogenic strategy. Moreover, when delivered in conjunction with current first-line anti-TB drugs, decreasing the total bacterial burden below the spontaneous mutation frequency with imatinib may serve to reduce the probability of developing antibiotic-resistant disease thereby increasing the clinical lifespan of those drugs.

Materials and Methods

Eukaryotic cell culture. Fibroblast cell lines were derived from wild-type mice or from *Abl1*$^{-/-}$ *Abl2*$^{-/-}$ mice (55). These cells and the mouse macrophage cell line J774A.1 (ATCC TIB-67) were maintained in Dulbecco's modified Eagle Medium (DMEM). The human type II alveolar pneumocyte cell line A549 (ATCC CCL-185) was maintained in Eagle's minimal essential medium (EMEM), and the human monocytic-like cell line THP-1 (ATCC TIB-202) in RPMI 1640. Media was supplemented with 10% FBS and 2mM L-glutamine. For some experiments, THP-1 cells were induced to differentiate by adding Phorbol 12-myristate 13-acetate (PMA) (Sigma) to a concentration of 10ng/ml. For some experiments, J774A.1 were activated with 100 U/ml INF-g (BD Biosciences Pharmingen, San Diego, CA, USA).

Bacterial strains and growth conditions. Mtb strain H37Rv (TMC102*)* and Mm strain 1218R (ATCC 927), a fish outbreak isolate, were grown in Middlebrook 7H9 broth (7H9) (BBL Microbiology Systems, Cockeysville, MD, USA) supplemented with ADC (Difco Laboratories, Detroit, MI, USA) and 0.05% Tween 80 (Mtb) (Sigma, St. Louis, MI, USA) or 0.025% Tween 80 (Mm). Middlebrook 7H11 agar was used for Mtb, and Middlebrook 7H10 agar for Mm. Both were supplemented with 10% oleic acid-albumin-dextrose-catalase (OADC) (Difco Laboratories, Sparks, Maryland USA).

Mtb stocks were grown at 37°C in 5% CO_2 with shaking at 70 rpm for seven days to an optical density of 0.6 (OD_{600}), as measured by a Novaspec II spectrophotometer (Amersham Pharmacia Biotech, Uppsala, Sweden). Cells were pelleted, resuspended in fresh 7H9 broth, aliquoted, and stored at -70°C. After freezing, an aliquot was thawed to perform viable count assays on 7H11 agar. Mm stocks were grown at 30°C for 3 days to an OD_{600} of 0.8 (Eppendorf, BioPhotometer), and the cells harvested, re-suspended in fresh 7H9 broth, aliquoted, and stored at -80°C. After freezing, an aliquot was thawed to perform viable count assays on 7H10 agar supplemented with OADC.

Isolation of a rifampicin-resistant mutant of Mm. Strain 1218R was cultured in Middlebrook 7H9 broth at 30°C for 3 days to an OD_{600} of 0.8. The culture was centrifuged for 10 min at 5,000 rpm, the supernatant removed, the pellet resuspended in 1ml PBS, and portions spread on Middlebrook 7H10 agar plates with 1mg rifampicin/ml (Sigma). Plates were incubated for seven days, colonies were restreaked to confirm resistance, and mutants assessed in growth curves (Figure 5A). The mutation was characterized by sequencing the rifampicin-resistance-determining region of the rpoB gene. To do this, the region was amplified by colony PCR using primers (5'GACGACATCGACCACTTC3') and (3'TAGTCCACCTCTGACGAG5'). Amplification was performed in a GeneAmp PCR System 9700 (Applied Biosystems). Automated DNA sequencing was performed using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems). The fluorescent elongation products were electrophoresed on a

3130xl Genetic Analyzer (Applied Biosystems). The 257-bp *rpoB* amplicons were sequenced with the same primers used for amplification. Sequenced products were analyzed using Lasergene Software (DNAStar).

***In vitro* Mtb infections.** THP-1 cells were seeded at a density of 10^6 cells per well in a 24-well microculture plate and were induced to differentiate by adding PMA. After 48 h, the cells were washed once, and fresh RPMI media added. A549 cells or J774A.1 were seeded at a density of $3x10^5$ cells per well in 24-well plates and were incubated for 48h before infection. For infections, frozen Mtb stocks were thawed and centrifuged, the supernatant removed, and the pellet resuspended in tissue culture medium without antibiotics. Bacteria were centrifuged again, resuspended in fresh medium, washed twice with 20ml of medium, transferred to a 50ml Oakridge tube containing ~30 3-mm glass beads, and vortexed for 10-15s to disperse clumps and obtain a single cell suspension.

Mammalian cells were washed once with RPMI before being infected in triplicate with Mtb (MOI=10) for 2h in RPMI without FBS, after which extracellular Mtb were killed by adding 200µg amikacin (Sigma) per ml and incubating at 37°C with 5% CO_2 for an additional 2h. Monolayers were washed five times with RPMI, and the cultures incubated at 37°C with 5% CO_2. At 24 hour intervals, supernatants were removed and the monolayers lysed in 0.1% Triton-X-100 (Sigma) for 15 min. Three sets of serial 10-fold dilutions of the lysates from each well were prepared in 0.05% Tween 80 (Sigma) and portions

plated on 7H11 agar. Colonies were counted after 3 to 4 weeks incubation at 37°C in 5% CO_2.

***In vitro* Mm infections.** J774A.1, Abl1$^{-/-}$/Abl2$^{-/-}$ cells, or 3T3 cells were plated at 10^5 cells/ml in 24 well plates, and allowed to adhere overnight. A frozen vial of 1218R Mm was thawed, the sample centrifuged, and the pellet re-suspended in DMEM with 10% FBS and used to inoculate fresh media at 10^5 CFU/ml. One ml of the Mm-containing DMEM/FBS was added to each well (MOI=1) and the plates were then incubated at 30°C with 5% CO_2 for 2h, rinsed twice with fresh PBS, and supplemented with 200µg amikacin/ml RPMI for an additional 2h to kill remaining extracellular Mm. The cells were then washed twice with PBS and the media replaced with DMEM/FBS. At 4h post inoculation (time 0) and 5, 10, 15, 20, 25, or 48h thereafter, supernatants were removed and the monolayers lysed with 0.1% Triton-X-100 (Sigma) for 10 min. Three sets of serial 10-fold dilutions of the lysates from each well were prepared, portions plated on 7H10 agar, and colonies counted after seven days incubation at 30°C. Data presented are the combined results from at least three separate experiments.

Determination of Mm entry. The percentage of infected J774A.1 cells after four hours was measured using a *Mm* strain constitutively expressing plasmid encoded GFP under control of the *secA* promoter (provided by Dr. James Posey, CDC, Atlanta). Infected cells distinguished by live imaging with a scientific-grade cooled charge-coupled device (Cool-Snap HQ with ORCA-ER chip) on a multi-wavelength, wide-field, three-dimensional microscopy system (Intelligent

Imaging Innovations, Denver, CO), based on a Zeiss 200M inverted microscope using a 63x lens with a numerical aperture of 1.4 (Carl Zeiss, Thornwood, NY). A role for Abl or related kinases in entry was also assessed by measuring CFUs with imatinib present for a limited period following inoculation. To do this, J774A.1 cells were plated at a density of 10^5 per well for one day, and then chilled to 4°C for 20 minutes prior to addition of bacteria together with drug. To synchronize entry, Mm (MOI= 1) was added at 4°C, and the bacteria and cells were centrifuged at 700g for 10 minutes at 4°C. The culture dish was then returned to 37°C for 15, 30, 60, or 120 minutes. At each time point cells were washed twice with PBS and incubated for an additional 2h with media supplemented with 200ug/ml amikacin to kill extracellular bacteria. Cell monolayers were then washed twice with PBS and lysed with 0.1% Triton-X 100 and CFU/ml determined as described above. Percent uptake was measured by dividing the number of internalized bacteria by the number of input bacteria (CFU/ml), determined by measuring the CFU/ml of the inoculum.

Measurement of Mm in lysosomes. To determine intracellular localization of Mm, J774A.1 cells were plated in 8 well Lab-Tek chambered coverglass plates at 10^5 cells/well (Nunc, Nalge Nunc International). After 24 hours, cells were infected with GFP-Mm (MOI 1) for 2h, and then treated the cells with 200ug/ml Amikacin for 2 additional hours. Cells were washed, and media or media supplemented with imatinib was added and the cells were incubated at 30°C. After 24 hours, 50nM of lysotracker red (Invitrogen) was added, and cells were

imaged live using a Zeiss 200M inverted microscope described above. The number of bacteria, as assessed by GFP fluorescence that colocalized with lysotracker red was quantified.

***In vivo* Mtb infections.** Age matched un-manipulated mice, carrier controls containing water pumps, or mice containing pumps with imatinib were infected with Mtb Erdman (Trudeau Institute, Saranac, NY, USA) of Mtb using a nose-only aerosolization system (In-Tox Products, Albuquerque, NM, USA). Mice were exposed for 20 min to a nebulized suspension of bacteria at a density $2.5x10^5$/ml CFU or $1x10^7$/ml CFU that was optimized to deliver a dose of approximately 25 or 200 CFU to the lungs. Mice were sacrificed by cervical dislocation at 1d or 28d post infection. The whole lung or right superior lobe of the lung was homogenized in PBS containing 0.05% Tween-80 and serial dilutions were plated onto 7H11 agar. The plates were incubated at 37°C and colonies counted after three weeks. The left lobe of the lung was preserved in 4% paraformaldehyde (PFA) for subsequent histopathological analysis. Histopathological analysis was performed on lungs that were fixed in 4% paraformaldehyde in PBS for 1 wk and subsequently paraffin embedded. Five to seven micrometer sections were cut and stained using a standard H&E protocol.

***In vivo* Mm infections**. Six-week old male C57Bl/6 mice were injected in the tail vein with active growing cultures at ~10^5 CFU/mouse. The number of bacteria injected for each experiment was determined by retrospective plating. The average dose was $2.5x10^5$ CFU/mouse. For experiments to determine effects

of drugs on tail lesions, mice were infected at 10^7 CFU/ml. Seven days after infection, lung, liver, and spleen were harvested, weighed and homogenized (Fisher Scientific, Tissuemiser) in one ml PBS. Each homogenate was diluted and spread on 7H10 agar. Colonies were scored after seven days of incubation at 30°C. Total weight of the organ and colonies per ml of the homogenized organ were used to determine CFU/gram. For histology studies, the liver was removed, fixed in 10% formalin, and embedded in paraffin. Sections (5μm) were cut and stained with hematoxylin and eosin (H&E) or Ziehl-Neelsen (acid-fast bacillus). Sections were imaged on a Nikon Eclipse 80i microscope.

Delivery of drugs *in vivo*. For experiments with imatinib, the mesylate salt was dissolved in water and loaded into Alzet pumps (Braintree Scientific, 1007D) capable of dispensing a continuous flow of drug at 25, 50, 100 or 200 mg/kg/day. Pumps were inserted subcutaneously into anesthetized 6-week old male C57Bl/6 mice (Jackson Laboratories). At these doses, we observed no weight loss or other adverse effects in uninfected animals. Moreover, such doses have been used to treat cancer in mice, and are equivalent to those used in humans over prolonged periods, with adjustments for differences between mice and humans in pharmacodynamics and pharmacokinetics [178]. For pre-treatment experiments, water- or imatinib-containing pumps were inserted 24 h prior to intravenous injection of Mtb (Erdman Strain), wild type 1218R Mm (Mm) or rifampicin-resistant 1218R Mm (Mm^R), and delivery was maintained for the duration of the experiment (28 days for Mtb or 7 days for Mm). For same day and post-treatment

experiments, mice were infected with Mm for either 1 h or 24 h, respectively, before inserting the pump. For both pre- and post-treatment experiments, the mice were euthanized on day seven and CFU measured as described above. For synergy experiments mice were injected intraperitoneally (i.p.) with 2.5 mg/kg rifabutin (sigma) solubilized in 100% DMSO once a day for seven days.

Statistical analysis. Statistical analysis was done using non-parametric tests including the Mann-Whitney Rank Sum test, the Wilcoxan Rank Sum test, or the Kruskal-Wallis test. In all of these tests, the data are pooled and the values ranked. The statistic calculates the probability that the observed ranking occurred by chance. Values less than or equal to 0.05 were considered statistically significant.

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Figure 1:

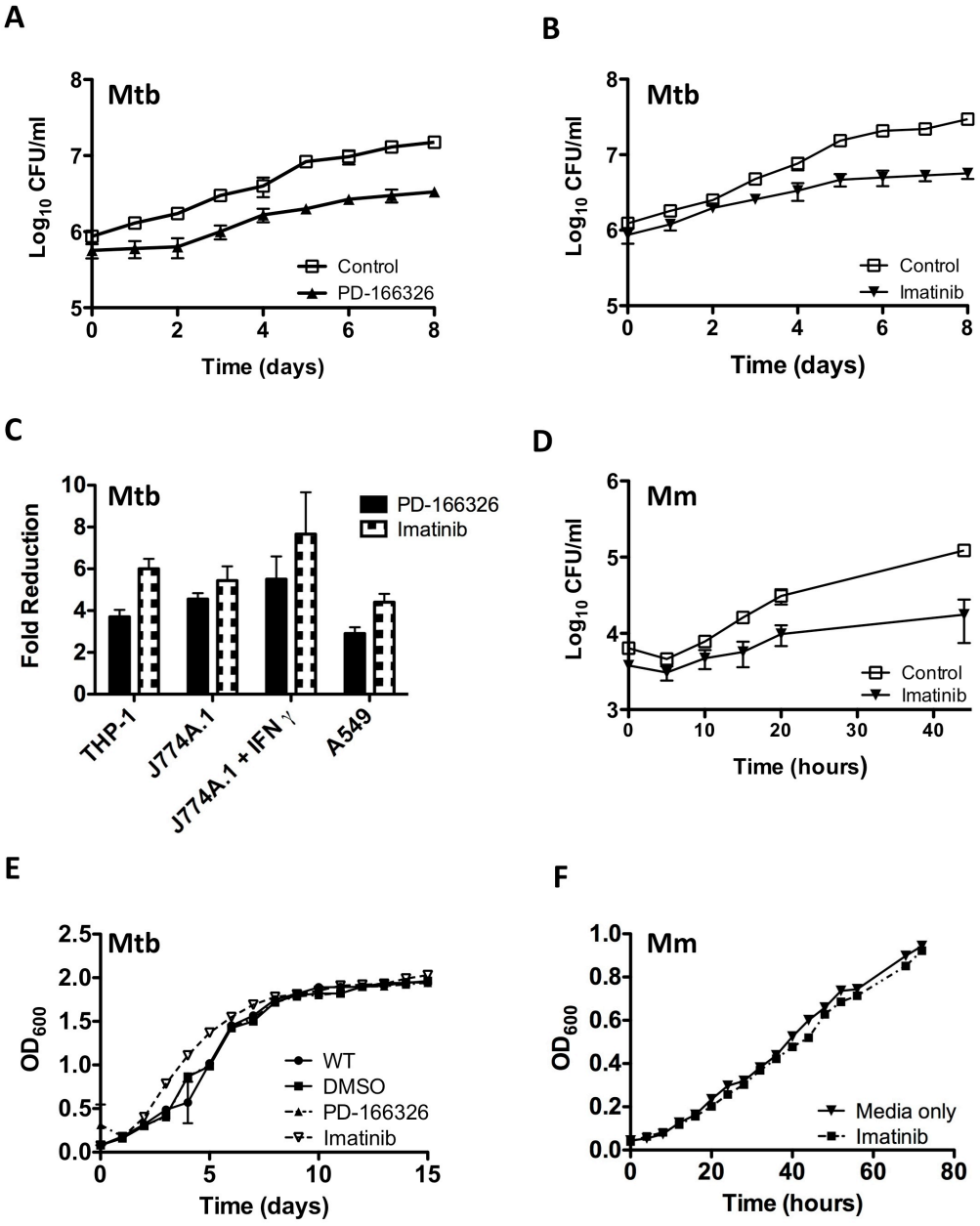

A
Mtb
Log10 CFU/ml
Time (days)
Control
PD-166326
B
Mtb
Log10 CFU/ml
Time (days)
Control
Imatinib
C
Mtb
Fold Reduction
PD-166326
Imatinib
THP-1
J774A.1
J774A.1 + IFN γ
A549
D
Mm
Log10 CFU/ml
Time (hours)
Control
Imatinib
E
Mtb
OD600
Time (days)
WT
DMSO
PD-166326
Imatinib
F
Mm
OD600
Time (hours)
Media only
Imatinib

Figure 2:

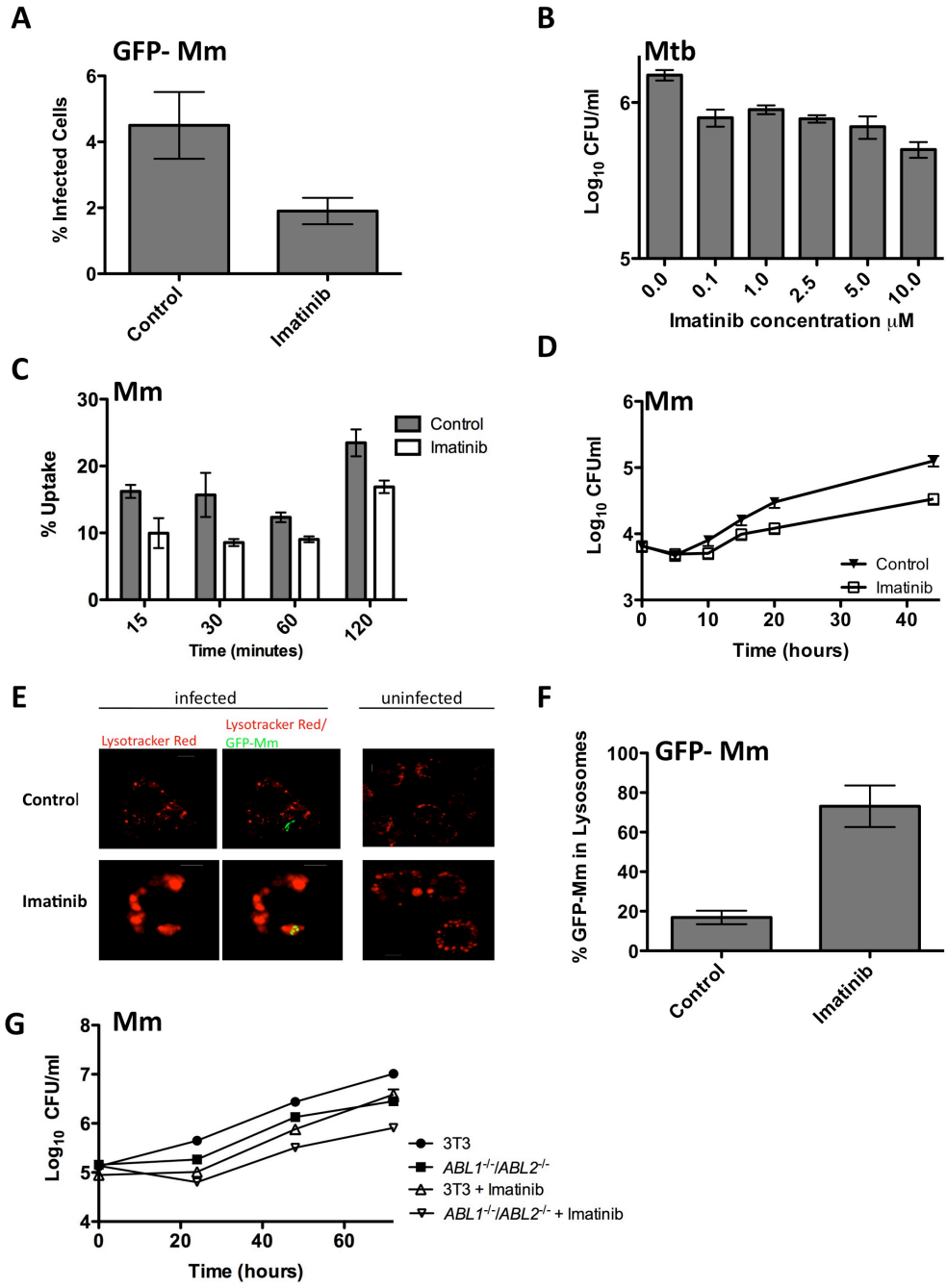

A
GFP- Mm
% Infected Cells
Control
Imatinib
B
Mtb
Log10 CFU/ml
0.0
0.1
1.0
2.5
5.0
10.0
Imatinib concentration μM
C
Mm
% Uptake
Control
Imatinib
Time (minutes)
D
Mm
Log10 CFUml
Control
Imatinib
Time (hours)
E
infected
uninfected
Lysotracker Red
Lysotracker Red/ GFP-Mm
Control
Imatinib
F
GFP- Mm
% GFP-Mm in Lysosomes
Control
Imatinib
G
Mm
Log10 CFU/ml
Time (hours)
3T3
ABL1-/-/ABL2-/-
3T3 + Imatinib
ABL1-/-/ABL2-/- + Imatinib

Figure 3:

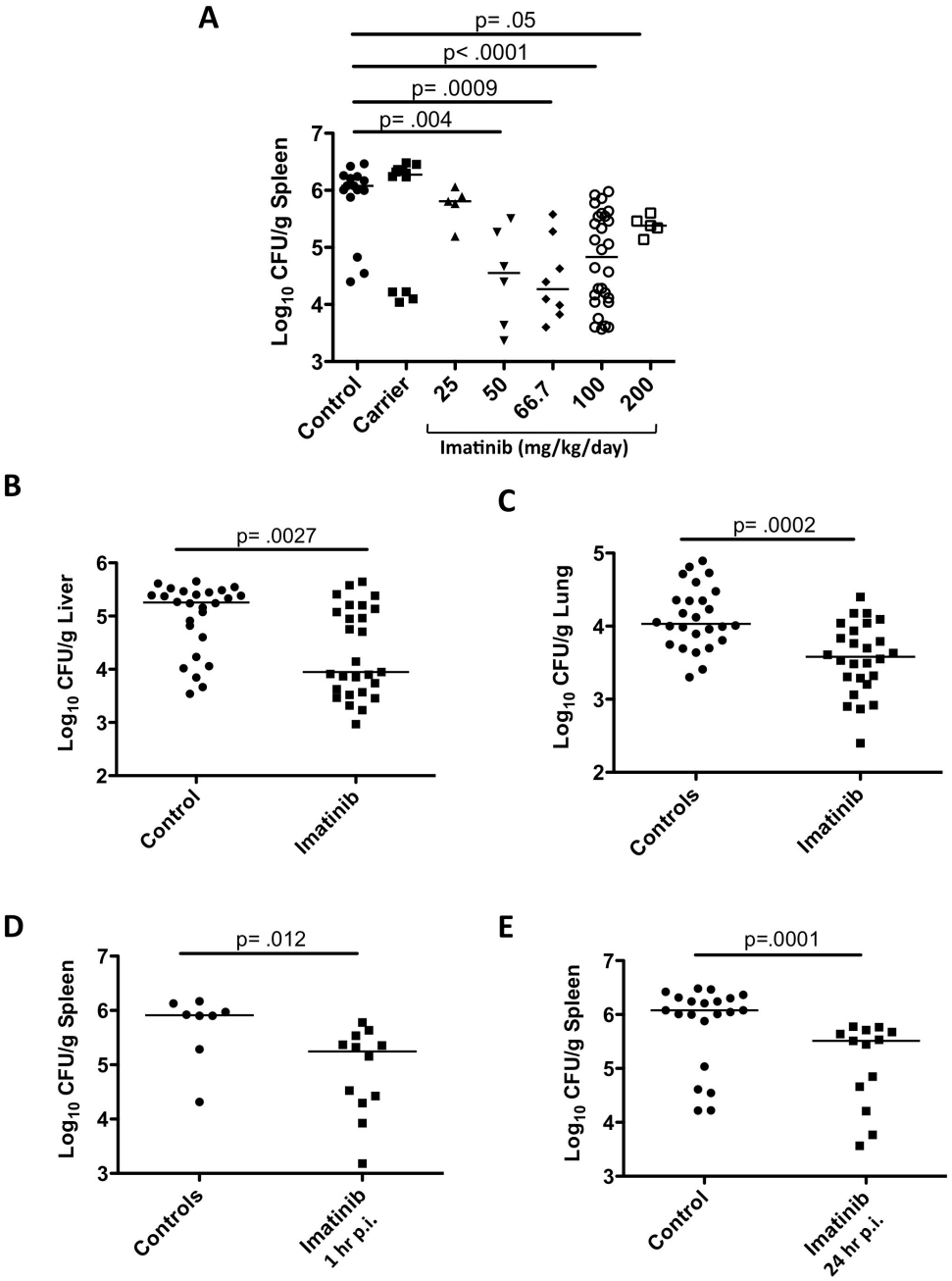

A
p= .05
p< .0001
p= .0009
p= .004
Log10 CFU/g Spleen
7
6
5
4
3
Control
Carrier
25
50
66.7
100
200
Imatinib (mg/kg/day)
B
p= .0027
Log10 CFU/g Liver
6
5
4
3
2
Control
Imatinib
C
p= .0002
Log10 CFU/g Lung
5
4
3
2
Controls
Imatinib
D
p= .012
Log10 CFU/g Spleen
7
6
5
4
3
Controls
Imatinib
1 hr p.i.
E
p=.0001
Log10 CFU/g Spleen
7
6
5
4
3
Control
Imatinib
24 hr p.i.

Figure 4:

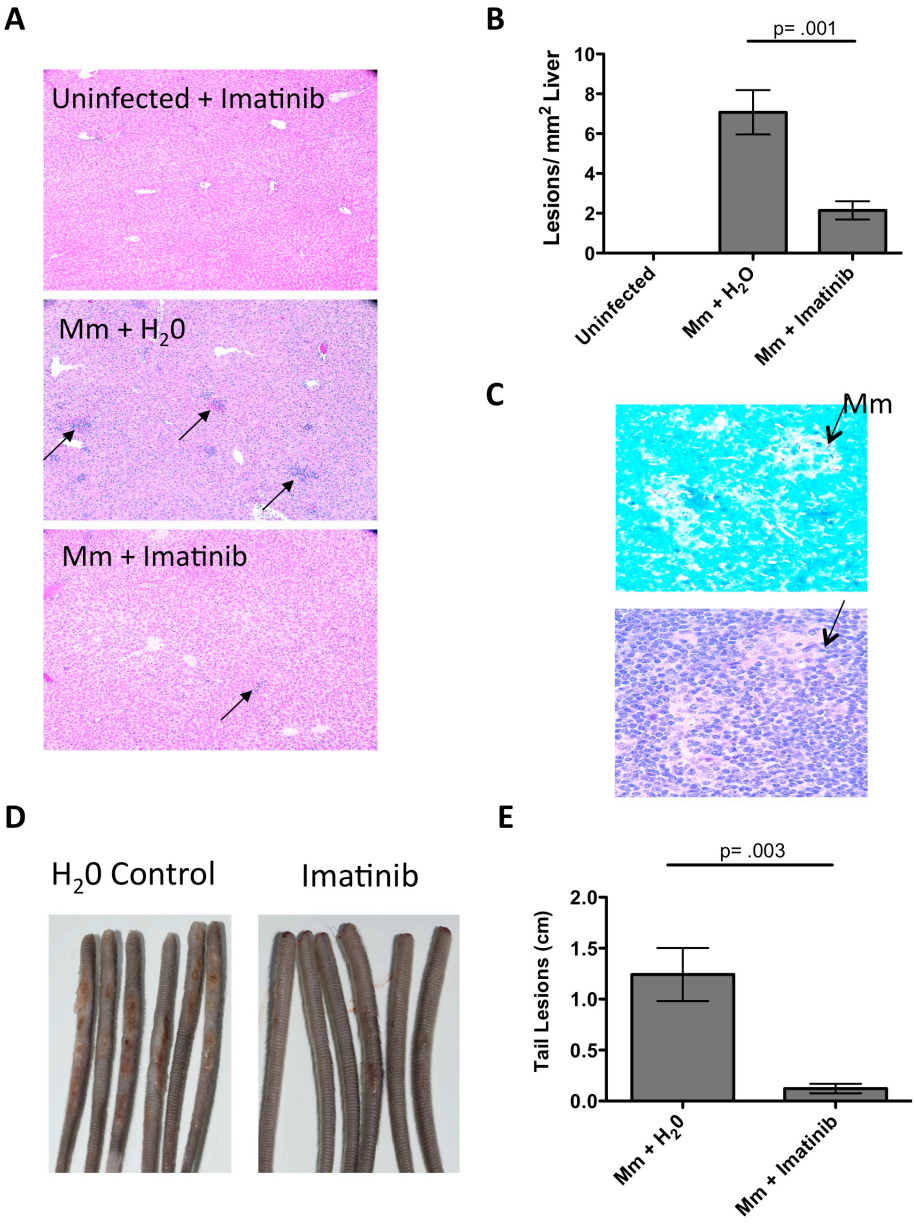

A
Uninfected + Imatinib
Mm + H20
Mm + Imatinib
B
p= .001
Lesions/ mm2 Liver
10
8
6
4
2
0
Uninfected
Mm + H2O
Mm + Imatinib
C
Mm
D
H20 Control
Imatinib
E
p= .003
Tail Lesions (cm)
2.0
1.5
1.0
0.5
0.0
Mm + H20
Mm + Imatinib

Figure 5:

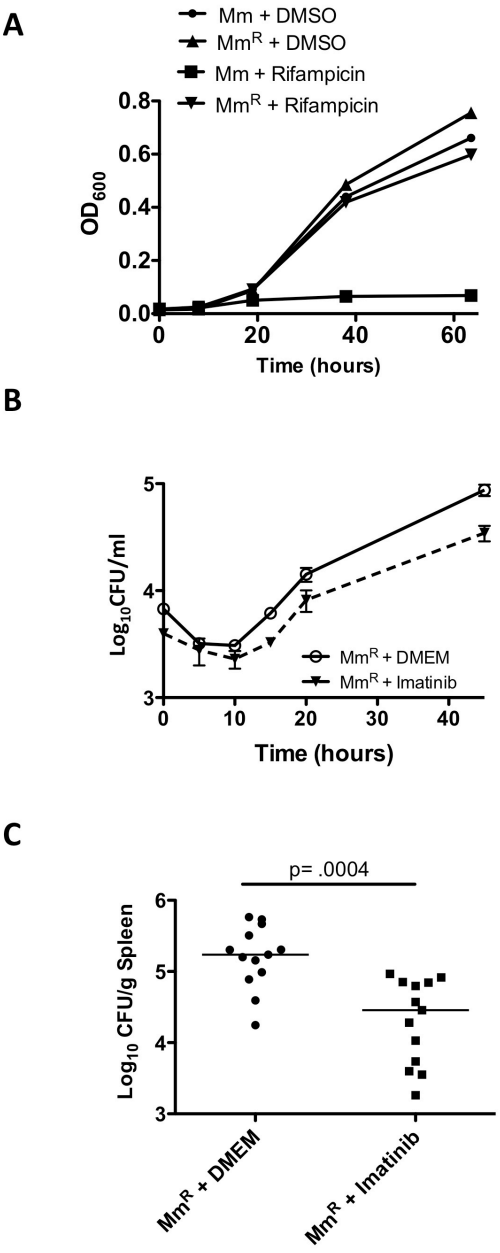

A
Mm + DMSO
MmR + DMSO
Mm + Rifampicin
MmR + Rifampicin
OD600
0.8
0.6
0.4
0.2
0.0
0
20
40
60
Time (hours)
B
Log10CFU/ml
5
4
3
0
10
20
30
40
Time (hours)
MmR + DMEM
MmR + Imatinib
C
p= .0004
Log10 CFU/g Spleen
6
5
4
3
MmR + DMEM
MmR + Imatinib

Figure 6:

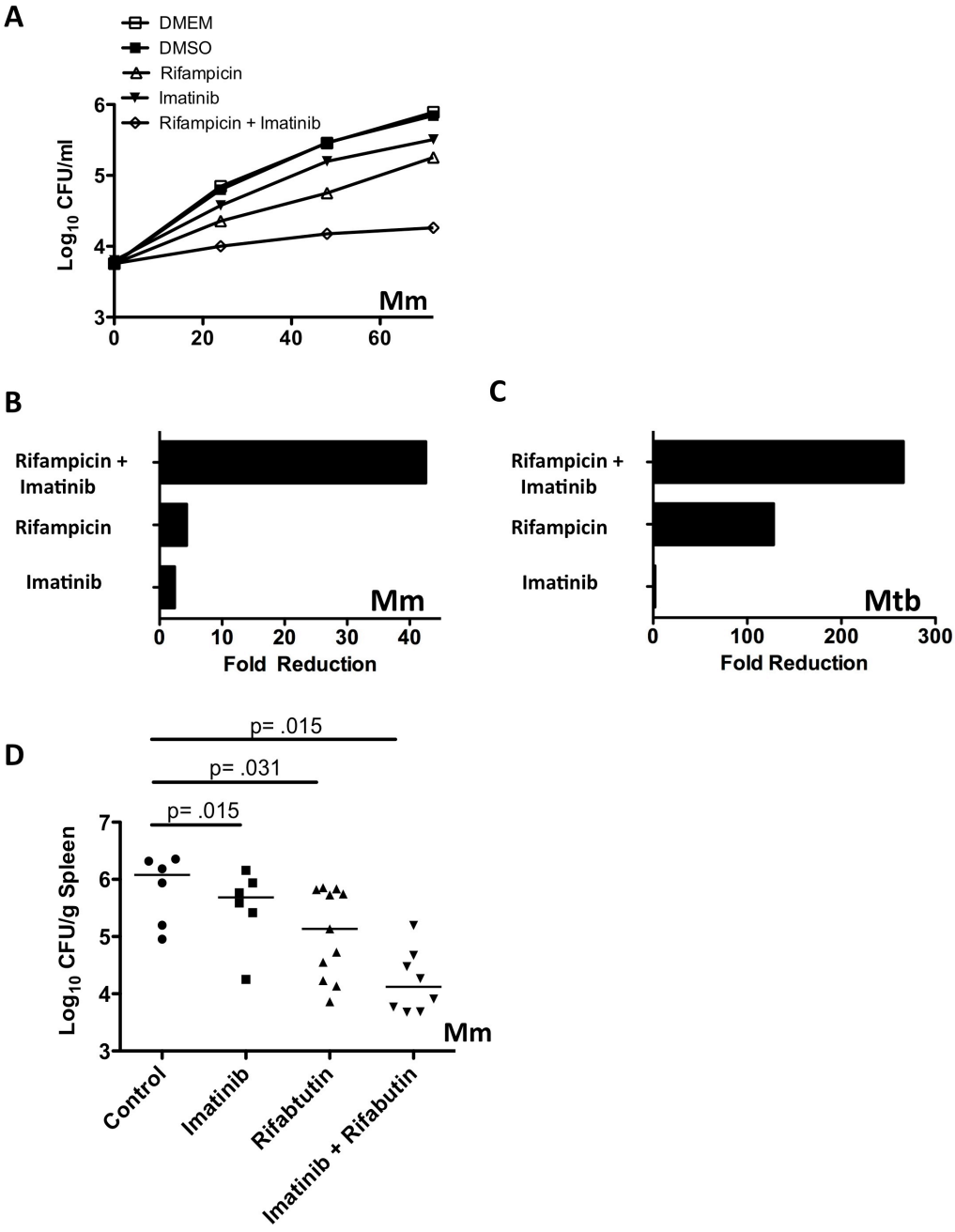
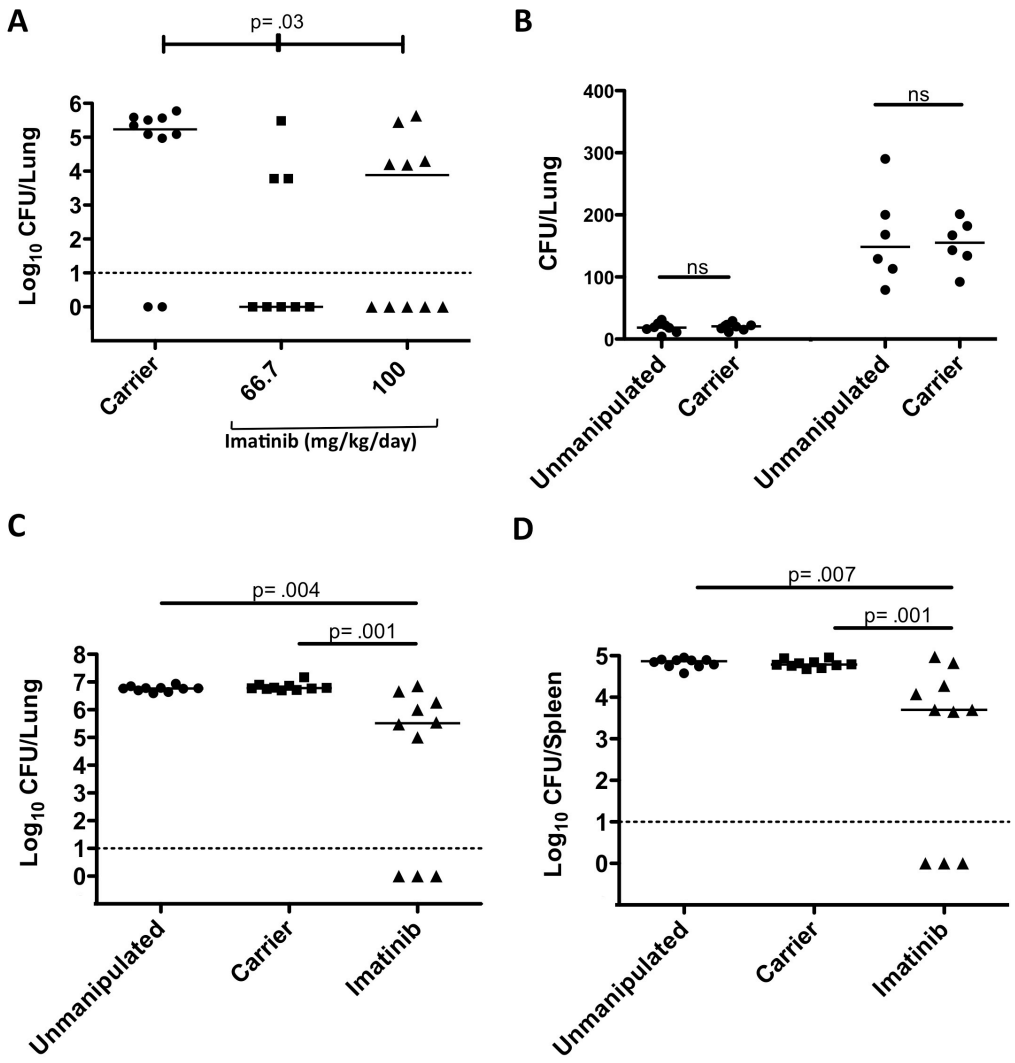

A
DMEM
DMSO
Rifampicin
Imatinib
Rifampicin + Imatinib
Log10 CFU/ml
Mm
B
Rifampicin + Imatinib
Rifampicin
Imatinib
Mm
Fold Reduction
C
Rifampicin + Imatinib
Rifampicin
Imatinib
Mtb
Fold Reduction
D
p= .015
p= .031
p= .015
Log10 CFU/g Spleen
Mm
Control
Imatinib
Rifabtutin
Imatinib + Rifabutin

Figure 7:


A
p= .03
Log10 CFU/Lung
Carrier
66.7
100
Imatinib (mg/kg/day)
B
ns
ns
CFU/Lung
Unmanipulated
Carrier
Unmanipulated
Carrier
C
p= .004
p= .001
Log10 CFU/Lung
Unmanipulated
Carrier
Imatinib
D
p= .007
p= .001
Log10 CFU/Spleen
Unmanipulated
Carrier
Imatinib

Supplemental Figure 1:

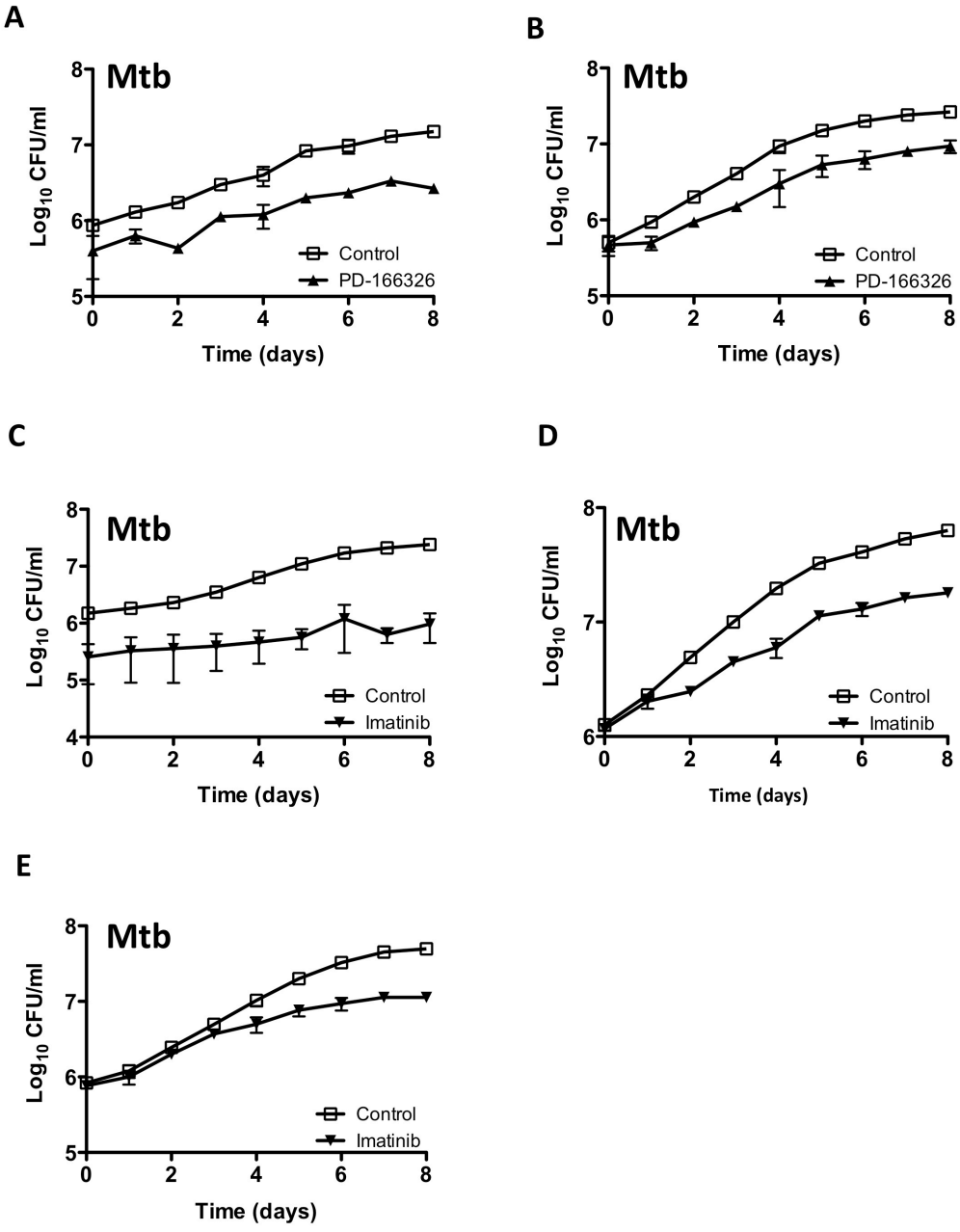


Figure Legends

Figure 1. Src- and Abl-family tyrosine kinase inhibitors reduce intracellular survival of Mtb and Mm *in vitro*.

A,B) Intracellular survival of Mtb H37Rv (MOI=10) in J774A.1 macrophage-like cells treated with PD-166326 (10 μM), an inhibitor of Src and Abl-family kinases, or the carrier DMSO (0.1%; control) (**A**) or imatinib (10μM), an inhibitor of Abl-family kinases (**B**).

C) Effects of PD-166326 or imatinib (each at 10μM) on Mtb H37Rv survival in THP-1 cells, J774A.1 cells left untreated or treated with IFNγ, and A549 cells. The maximal fold reduction at 8 days p.i. is presented with respect to DMSO-treated controls.

D) Intracellular survival of Mm 1218R (MOI=1) in J774A.1 cells treated with imatinib (10μM).

E,F) Growth curves of Mtb H37Rv (**E**) or Mm 1218R (**F)** in 7H9 broth containing PD-166326 (10μM), imatinib (10μM), or DMSO (0.1%). OD_{600} was measured at times indicated.

Data points represent the mean of three separate experiments, and error bars are +/- SEM.

Figure 2. Abl-family tyrosine kinases and other imatinib-sensitive kinases mediate intracellular survival of Mtb and Mm.

A) J774A.1 macrophages were infected with GFP-Mm for 2 hours in the presence or absence of imatinib (10μM), and treated for 2 additional hours with media supplemented with amikacin to kill extracellular bacteria. The number of infected cells, measured as those containing GFP-positive bacilli, was quantified by fluorescence microscopy.

B) THP-1 cells were infected with Mtb and treated with imatinib at concentrations ranging from 0.1 to 10μM for 2 h. Drug was removed, and cells were incubated for an additional 2h with amikacin to kill extracellular bacteria and then harvested 20 hours later to determine CFU/ml.

C) J774A.1 macrophages were treated with imatinib (10mM) and incubated with Mm at 4°C before returning the cells to 37°C so as to synchronize entry. At various times thereafter, drug was removed, and the cells treated with amikacin for 2hrs. Cells were then lysed and CFUs determined.

D) J774A.1 macrophages were infected with Mm 1218R (MOI=1) for 2h and then incubated with amikacin for an additional 2h. After washing, media with or without 10μM imatinib was added, and CFU/ml determined at various time points thereafter.

E,F) J774A.1 cells were infected and treated as in **D**, stained 24 h p.i. with lysotracker red, and visualized live. The percent of GFP-Mm colocalizing with the

lysotracker red labeled compartment was quantified (n=250 bacteria from three experiments). Scale bar represents 5 μM.

G) Fibroblasts derived from $Abl1^{-/-}/Abl2^{-/-}$ mice from age-matched wild-type mice were infected with Mm 1218R (MOI=60) for 4h. Cells were treated with amikacin for 2h, and CFU/ml determined at various time points thereafter. Imatinib or carrier was present throughout the experiment.

Data points represent the mean of 3 separate experiments, and error bars are +/- SEM.

Figure 3. Imatinib reduces bacterial load in mice infected with Mm.

A) C57Bl/6 mice were injected in the tail vein with 10^5 CFU Mm 1218R. Beginning 24h prior to infection, animals were administered pumps filled with water (carrier) or imatinib at concentrations of 25, 50, 66.7, 100 or 200 mg/kg/day. CFU/gram was determined in spleen at d7 p.i.

B,C) Mice were infected with Mm as in (**A**) and pre-treated with carrier or with imatinib (100mg/kg/day) and CFU/g from liver (**B**) or lungs (**C**) was determined 7d p.i.

D,E*)**. Mice infected with Mm as in ***A-C except administration of imatinib (100mg/kg/day) commenced 1 h (**D**) or 24 h (**E**) p.i.

Cumulative data from 3 independent experiments are presented. Each point represents an individual mouse, and the line represents the median CFU; p values were calculated by a nonparametric Mann-Whitney Rank Sum test; values <0.05 were considered statistically significant.

Figure 4. Imatinib reduces liver pathology and tail lesions in mice infected with Mm.

A) Haematoxylin and eosin (H&E)-stained sections of livers of mice either left uninfected and treated with 100mg/kg/day imatinib or infected and treated with H_20 or with 100mg/kg/day imatinib 1d prior to infection. Images were acquired from livers of mice in Figure 3b. Arrows depict individual lesions. Magnification x100.

B) Quantitation of the number of lesions present in liver sections per mm^2. Data were derived from 27 liver sections from mice in 3 independent experiments. Data are presented as means +/− SEM. P values were determined by Mann-Whitney rank sum test

C) Acid fast (upper) or H&E [179] staining of adjacent sections of spleen taken from infected and untreated mice from Figure 3A. Arrows indicate Mm. Magnification x400.

D) Images of tails from mice infected i.v. with 10^7 CFU/ml Mm 1218R and treated with carrier (H_2O) or or imatinib (100mg/kg/day) for seven days beginning 24 h p.i.. Each mouse received one injection. Images are representative of three independent experiments.

E) Effects of imatinib (100mg/kg/day) on the extent of lesions on tail. Size of lesions on a particular tail were measured and summed, and the values depicted

in cm. Data are represented as the mean cumulative lesion size/tail from 15 tails from each experimental group. +/- SD; P values were determined by Mann-Whitney rank sum test.

Figure 5. Imatinib reduces bacterial load in macrophages and mice infected with rifampicin-resistant Mm.

A) Growth curves of Mm wild-type strain (Mm) or a rifampicin-resistant Mm strain (Mm^R) in 7H9 media left untreated or treated with 1μg/ml rifampicin. Cell density was measured by OD_{600}. Data in **A** and **B** are represented as mean +/− SEM.

B) Intracellular survival of Mm^R (MOI=1) in J774A.1 macrophages in the presence or absence of imatinib (10 μM).

C) Effects of imatinib (100mg/kg/day) on mice infected with 10^5 CFU Mm strain Mm^R. Cumulative data from 3 independent experiments are presented. The line represents the median CFU; p values were calculated by a nonparametric Mann-Whitney Rank Sum test.

Figure 6. Imatinib and antibiotics act in synergy to reduce mycobacteria survival.

A) Intracellular survival of Mm (MOI=1) in J774A.1 macrophages left untreated or treated with imatinib (10μM), or rifampicin (0.5 μg/ml), or both drugs together. CFU/ml were determined at time points indicated.

B) Maximal fold reduction in intracellular survival of Mm in J774A.1 cells with various treatments compared to controls at day three p.i.

C) Maximal fold reduction in intracellular survival of Mtb H37Rv in THP-1 cells (MOI=10) with imatinib (10μM), rifampicin (0.125μg/ml), or both drugs together.

D) Mice were administered imatinib (100mg/kg/day), or rifabutin (2.5mg/kg/day i.p.) or both drugs together. CFU in the spleen were determined at day seven p.i. Line represents the median. (N=3; data from a representative experiment are presented). The line represents the median CFU; p values were calculated by a nonparametric Mann Whitney Rank Sum test.

Figure 7. Imatinib reduces bacterial load in mice infected with Mtb.

A) C57Bl/6 mice were infected with 50-100 CFU of aerosolized Mtb Erdman. Beginning 24h prior to infection, animals were administered water (carrier) or imatinib at concentrations of 66.7 mg/kg/day or 100 mg/kg/day. CFU was determined in right superior lobe of the lung at 28 days p.i. Solid lines represents the median CFU; dotted line represents the limit of detection (10 CFU). p values were determined by a nonparametric Kruskal-Wallis test.

B) 24h prior to infection C57Bl/6 mice were administered carrier pumps. Un-manipulated or carrier treated mice were infected with a low dose ($2.5x10^5$ CFU; left) or high dose ($1x10^7$ CF; right) of aerosolized Mtb Erdman and CFU was determined in the whole lung at 24h p.i. The solid line represents the median CFU.

C-D) C57Bl/6 mice were infected with 2.5x10^5 CFU of aerosolized Mtb Erdman. Beginning 24h prior to infection, animals were left untreated, or, administered carrier (water) or imatinib mesylate at a concentration of 66.7 mg/kg/day. CFU was determined by plating homogenates of the whole lung (C) or spleen (D) at 28 days p.i. The solid line represents the median CFU; dotted line represents the limit of detection (10 CFU). p values were determined by a nonparametric Mann-Whitney test.

Supplementary Figure 1. Effects of inhibitors of Src- and Abl-family tyrosine kinases on Mtb and Mm infection *in vitro*.

A,B) Growth curves in the presence of PD-166326 (10μM) or carrier (DMSO, 0.1%) were conducted in J774A.1 cells treated with IFNγ (**A**) or A549 cells (**B**) infected with Mtb H37Rv (MOI=10).

C-E). Growth curves were carried out in the presence of imatinib (10μM) or carrier (H_20) in J774A.1 cells treated with IFNγ (**C**), in THP-1 cells (**D**), or in A549 cells (**E**). Cells were infected with Mtb H37Rv (MOI=10). CFU/ml were determined at designated time points. . Data are representative of three individual experiments, and are presented as mean +/- SEM.

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Chapter 3:

Imatinib induces an expansion of myeloid cells in the bone marrow and periphery of mice that facilitates host antimicrobial activity

Imatinib induces an expansion of myeloid cells in the bone marrow and periphery of mice that facilitates host antimicrobial activity

by

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Abstract

Normal mice treated with imatinib, an inhibitor of ABL and other related tyrosine kinases, used as a therapeutic for chronic myelogenous leukemia, displayed markedly increased numbers of neutrophils and monocytes in the bone marrow, blood and spleen. Imatinib treatment did not activate neutrophils *per se*, although neutrophils in the bone marrow of mice treated with imatinib show increased expression of CXCR2, suggesting increased potential for egress from the bone marrow and recruitment to peripheral tissues. Upon infection with *Mycobacterium marinum* (Mm), neutrophils in drug-treated animals underwent similar activation to that observed in control mice. Furthermore, adoptive transfer of neutrophils from animals treated with imatinib or control animals into naïve animals decreased mycobacterial burden to the same extent. Interestingly, we demonstrate that the increase in neutrophil numbers following imatinib treatment in mice inhibits infections caused by other bacteria, including pathogenic *Francisella* species (*F. novicida* and the live vaccine strain of *F. tularensis*, which do not require Abl or related kinases for pathogenesis. Taken together, our findings demonstrate that imatinib induces neutrophilia in the bone marrow and peripheral tissues of normal mice and suggest a potential new application for this drug in facilitating clearance of bacterial infections and mitigating the effects of neutropenia.

Introduction

Protein tyrosine kinases are involved in a variety of cellular processes including migration, morphogenesis, stress response, and cytoskeletal reorganization [114, 115]. Dysregulation of tyrosine kinases has been associated with cancer pathogenesis including chronic myelogenous leukemia (CML) [180]. CML is a clonal stem cell disorder associated with a 9:22 chromosomal translocation, also known as the Philadelphia chromosome, which results in the expression of the BCR-ABL fusion protein. BCR-ABL, functions as a hyperactive, oncogenic, tyrosine kinase altering normal cellular signaling pathways and causing myeloid leukemia.

Treatment for CML consists of small molecule tyrosine kinase inhibitors (TKIs), including imatinib-mesylate (imatinib, Gleevec, STI-571), that compete with the ATP binding site within the kinase domain resulting in selective killing of BCR-ABL positive cancer cells, whose survival depends on BCR-ABL, a phenomenon known as “oncogene addiction” [181]. Imatinib and related second generation TKIs including Sprycel and Tasigna have dramatically increased survival rates of Philadelphia chromosome-positive (Ph+) patients [182]. In addition to BCR-ABL, imatinib inhibits several other related tyrosine kinases including cellular Abelson 1 (ABL1) and ABL2, the platelet-derived growth factor receptor alpha (PDGFRα) and PDGFRβ, stem cell growth factor receptor (c-kit), and macrophage colony-stimulating factor (m-CSF or c-fms) [159, 160, 183].

TKIs are also used to treat malignancies associated with dysregulated c-kit or PDGFRα, including gastrointestinal stromal tumors (GISTs) [184].

Work by our laboratory and others suggest that various microbes and parasites utilize ABL and related tyrosine kinases during pathogenesis to facilitate intracellular survival, intracellular motility, and spread from cell to cell [152]. These pathogens include diarrheagenic *Escherichia. coli, Pseudomonas*, *Salmonella, Shigella, Helicobacter, Anaplamsa*, and *Chlamydia* amongst bacteria, filoviruses, HIV, Coxsackie, West Nile, Kaposi sarcoma, Polyoma, and Pox amongst viruses, and the human parasite Leishmania [125-139].

The observations that imatinib promotes acidification of vesicles containing mycobacteria [185], facilitates trafficking to lysosomes and promotes clearance of *Mycobacterium tuberculosis* (Mtb) in chronically infected mice raises the possibility that imatinib stimulates innate and adaptive immune responses upon infection [185, 186]. In this regard, Borg *et al.* showed that imatinib, acting via host DCs, activates natural killer (NK) cells and their attendant anti-tumor activity [187]. Moreover, Balachandran *et al.* report that imatinib potentiates anti-tumor CD8 T cell responses in GISTs by reducing expression of indoleamine-pyrrole 2,3-dioxygenase and increasing apoptosis of regulatory T cells [188].

Other reports of imatinib effects on immune cells have proven contradictory. Imatinib has been reported to activate or suppress T cell proliferation and apoptosis, Tregs, NK, DCs and macrophages [189], though many of these studies were carried out on transformed cell lines, mouse models

of tumorigenesis, or in leukemic individuals. Despite these reports, in human patients, adverse events caused by imatinib are relatively rare, and there has been little indication of widespread immunosuppression.

Here, we describe a previously unreported effect of imatinib on expansion of the myeloid compartment, and in particular neutrophils and monocytes, and show that increased neutrophil numbers are sufficient to control infections caused by various bacteria, including those whose pathogenesis does not depend on Abl or related kinases.

Results

Imatinib induces expansion of myeloid cells in mouse spleen and blood. We characterized the effects of imatinib on the composition of immune cells in naïve mice or mice infected with Mm for seven days. Imatinib was delivered at a dose of 66.7mg/kg/day beginning one day prior to infection. The number of neutrophils, defined as CD11b^{+} Ly6C^{int} Gr-1^{hi} SSC^{int}, increased by 10-fold in the blood, and 18-fold in the spleen relative to untreated (naïve) controls (Figure 1A). Compared to naive animals, infection with Mm increased neutrophil numbers by ~3 and 6-fold in the blood and spleen, respectively. Notably, numbers of neutrophils in infected mice treated with imatinib exhibited increases of ~18- and 21-fold in the blood and spleen, respectively. Numbers of monocytic cells, defined as CD11b^{+} Ly6C^{hi} Gr-1^{int} SSC^{low} and F4/80^{+}, were likewise increased with imatinib treatment or Mm infection, albeit to lower levels than that seen with neutrophils. Monocytic cells in blood increased by 6-fold with imatinib treatment, 3-fold during Mm infection, and ~10-fold in infected animals treated with imatinib. In the spleen, monocytic cells were increased more than 7-fold with imatinib treatment, 4-fold with Mm infection, and 7-fold with Mm infection and imatinib treatment. Numbers of eosinophils, defined as CD11b^{+} Ly6C^{lo} Gr-1^{int} SSC^{hi} and F4/80^{+}, were not significantly elevated in the blood with imatinib treatment, nor with infection. However, in the spleen, a 2-fold increase in

eosiniphils was evident with imatinib treatment, and a 4-fold increase was evident with Mm infection and imatinib treatment.

We next characterized the effects of imatinib and infection on dendritic cells (DCs). Conventional DCs (cDCs), defined as either, $CD11c^{hi}$ $CD8^{+}$ ($CD8^{+}$ DC) or $CD11c^{hi}$ $CD11b^{+}$ $CD8^{-}$ ($CD8^{-}$ DC) showed no statistically significant change in the blood with imatinib treatment (Figure 1B; upper panel). Infection with Mm increased numbers of $CD8^{-}$ DCs by 1.6-fold compared to naïve mice, and by 5-fold with imatinib. Upon imatinib treatment or infection alone, $CD8^{+}$ DC numbers were increased in the spleen by 2-fold, compared to naïve mice. The major type I interferon-producing DCs, plasmacytoid DC (pDCs), identified as $CD11c^{lo}$ $CD11b^{-}$ $B220^{+}$ and $Ly6C^{+}$, remained unaffected by imatinib treatment in the blood and spleen. Whereas, the combination of imatinib treatment and Mm infection resulted in 1.6-fold increase of pDCs numbers in the blood when compared to untreated Mm-infected mice.

We also characterized the effects of imatinib or Mm infection on numbers of natural killer (NK) cells ($NK1.1^{+}$), T cells ($Thy1.2^{+}$), and B cells ($B220^{+}$, $CD19^{+}$). Imatinib treatment alone produced no change in the numbers of T cells or B cells in the blood or spleen, relative to naïve mice (Figure 1C). Treatment did increase the number of NK cells in the spleen by 1.6-fold, though no difference was evident in the blood (Figure 1C). Mm infection alone increased the number of NK cells by 2.5-fold in the blood and B cells by 1.8-fold in the spleen, but had no significant effect on T cell numbers. Mice treated with imatinib and infected with

Mm exhibited increased numbers of NK cells by 5-fold, in the blood, as well as increased numbers of T cells by 1.4-fold, in comparison to naïve animals.

To determine whether imatinib-induced increases in the number of myeloid cells was transient or persistent over time, mice were treated with imatinib for up to 28 days, and the numbers of immune cells in the blood were counted at weekly intervals. Numbers of neutrophils and monocytes increased over the treatment period (Fig 1D), whereas the numbers of eosinophils, DCs, B cells and T cells were not significantly different from those in naïve animals (data not shown). Together, these data suggest that imatinib induces a sustained increase in the number of neutrophils and monocytes in circulation, but had little or no effect on DCs, eosinophils or lymphocytes.

imatinib treatment caused an increase in numbers of myeloid cells in the bone marrow. To determine whether the increased number of myeloid cells in both blood and spleen in response to imatinib resulted from increased production, we evaluated the cellular composition of the bone marrow. The number of neutrophils and monocytes, as measured by flow cytometry, increased by 2.0- and 1.7-fold respectively with imatinib, whether or not the animals were infected (Figure 2A). In accordance with these data, a qualitative increase in the number of neutrophils was evident in cytospins from the bone marrow (data not shown). By contrast, the number of B, T and NK cells was not significantly altered with imatinib, with or without infection (Figure 2B).

The bone marrow is the primary site of post-natal hematopoiesis, where hematopoietic stem cells (HSC) differentiate into common myeloid and lymphoid progenitors (CMPs and CLPs). CMPs differentiate into a variety of mature cells including neutrophils and monocytes. To distinguish the effects of imatinib on neutrophil precursors from those on mature neutrophils, we measured previously described subsets based on CD45 expression and side scatter (SSC) [190, 191] in bone marrow from mice uninfected or infected with Mm, with or without imatinib treatment. Precursor populations including promyelocytes, myelocytes, metamyelocytes and mature neutrophils can be identified, as can monocytic and lymphocytic populations (Figure 2C). In agreement with measurements of lymphocyte numbers (Figure 2B), the percentage of lymphocytes decreased with imatinib treatment (Figure 2D). In contrast, the monocyte, promyelocyte, myelocyte, and metamyelocyte fractions remained unchanged with imatinib treatment. Imatinib treatment increased the percent of mature neutrophil numbers by ~13% and ~11% in the bone marrow of uninfected and infected mice, respectively, whereas Mm infection alone had no significant effect (Figure 2D). There was a corresponding decrease in the percentage of $CD45^-$ non-leukocytic cells and the $CD45^{int}$, SSC^{low} population corresponding to HSCs. Notably, although an increased number of neutrophils was evident in the periphery with infection, the increase in mature neutrophils was less prominent in the bone marrow (Figure 1A and 2A), and not apparent in the CD45 vs. SSC plot. Together, these data suggest that imatinib induced the

generation or accumulation of mature neutrophils in the bone marrow, but that such an accumulation was not evident upon infection.

Imatinib treatment alters CXCR2 expression on neutrophils in the bone marrow. Neutrophil retention within the bone marrow or entry into circulation is correlated to the relative expression of CXCR2 and CXCR4 [192]. CXCR4 expression correlates with retention of neutrophils in the bone marrow, whereas CXCR2 expression is associated with migration of neutrophils into circulation and into peripheral sites [192-194]. To better characterize the mechanism of peripheral neutrophil expansion and accumulation in the blood and spleen following imatinib treatment, we determined the levels of CXCR2 and CXCR4 on neutrophil precursors and mature neutrophils in the bone marrow (Figure 3A and 3B). Neutrophilic bone marrow cells were divided into $CXCR2^{hi}CXCR4^{low}$ and $CXCR4^{hi}CXCR2^{low}$ subsets (Figure 3B). Imatinib treatment increased the percent of $CXCR2^{hi}CXCR4^{low}$ neutrophils by ~17% and 9% in uninfected and infected mice, respectively, when compared to control mice. Likewise, imatinib increased the median fluorescence intensity (MFI) of CXCR2 on granulocytes by 1.8-fold and 1.5-fold in uninfected and infected mice, respectively. By contrast, infection alone did not significantly alter the percent of $CXCR2^{hi}$ granulocytes in the bone marrow. CXCR4 expression was not significantly altered by imatinib nor by infection. These data suggest that imatinib increases the expression of CXCR2 and the proportion of $CXCR2^{hi}CXCR4^{low}$ granulocytes in the bone marrow, thereby facilitating their

release into the periphery. Together, these data suggest that imatinib expands mature neutrophils in the bone marrow that are primed to enter circulation, while Mm infection independently induces migration of the cells from the bone marrow to the periphery.

Neutrophils from imatinib-treated mice are not intrinsically activated but are activatable by Mm infection. Proinflammatory stimuli cause neutrophils to become activated and mobilize secondary and primary granules, which fuse with the plasma membrane. Neutrophil degranulation can be quantified by the surface expression of CD66b a marker for secondary granules or CD63, a marker for primary granules (Figure 4A-C). In naïve mice, imatinib did not alter the surface expression of CD66b or CD63 on neutrophils in the bone marrow, blood or spleen, indicating that, although the neutrophil numbers were increased, the neutrophils were not activated. However, in the context of an Mm infection, neutrophils from control animals or animals treated with imatinib had increased surface expression of CD66b and CD63 relative to uninfected controls. This effect was most pronounced in the spleen, where the greatest number of bacteria are found, but was not evident in the blood and bone marrow, except to a limited degree with infection [186]. Together these data suggest that neutrophils are not activated with imatinib treatment but that they retain the capacity for activation following infection.

Following activation or phagocytosis, neutrophils undergo programmed cell death known as apoptosis [195]. Cleavage of pro-caspase-3 or -7 (caspase-

3/7) into enzymatically active forms are critical steps in apoptotic [196], and non-apoptotic processes during inflammatory cell activation (Erener et al., 2012, PMID: 22464733). Neutrophils from imatinib treated mice did not have significantly different levels of active caspase-3/7 compared to untreated mice (Fig A-C). However, neutrophils in the spleens of Mm infected mice showed significantly elevated activation of caspase-3/7 with and without imatinib treatment. These data are in accordance with reports that neutrophils undergo appropriate activation in response to bacterial infections [195]. Together these data suggest that although imatinib alone does not activate neutrophils, it does not preclude them from becoming activated upon infection.

Increased circulating neutrophil numbers are sufficient to reduce bacterial load upon Mm infection. To determine whether an increase in neutrophil numbers was sufficient to reduce bacterial CFU, anti Ly6G magnetic beads were used to purify neutrophils from the spleens of control mice or those treated with imatinib for seven days. Neutrophils from several animals were pooled and their purity was confirmed by flow cytometry. Subsequently, $4x10^6$ cells from naïve or imatinib treated animals were intravenously injected into naïve mice, which were immediately thereafter infected with Mm. Two days later, bacterial colony forming units (CFU) was determined in the spleens. As shown in Figure 5A, adoptive transfer of neutrophils from either control or imatinib-treated animals significantly reduced CFU by 2-fold compared to the PBS-treated animals. Together, these data suggest that transfer of neutrophils at levels half

that found in the spleens ($4x10^6$ I.V. and $8x10^6$/spleen; respectively) of animals treated with imatinib was sufficient to reduce bacterial burden. Moreover, no difference was evident between neutrophils derived from naïve or mice treated with imatinib, suggesting that imatinib increases the number but not the intrinsic killing capacity of the neutrophils.

Imatinib reduces bacterial load in mice infected with pathogenic *Francisella* species. Previously, we reported that imatinib decreases bacterial load of mice infected with pathogenic mycobacteria. Mice pre-treated with imatinib had a greater reduction in CFU than mice treated 24h post infection, however the mechanism remained unclear. The observation that imatinib dramatically increased neutrophils led us to ask whether extended pre-treatment might facilitate reduction of CFU of other pathogenic bacteria. We tested this using pathogenic *Francisella* species, *F. novicida* (Fn) and the live vaccine strain (LVS) for *F. tularensis*. Growth of Fn and LVS, in either broth or in macrophages remained insensitive to imatinib (data not shown). Mice were pretreated with imatinib at 66.7mg/kg/day for seven days, and then infected mice subcutaneously with Fn or LVS. Imatinib was provided for the remainder of the experiment. Bacterial CFU in the skin, liver and spleen were determined 48h after Fn infection and five days after LVS infection. Imatinib pre-treatment reduced Fn CFU in the spleen and liver below the level of detection (100 CFU) and by 3.5-fold in the skin in comparison to untreated mice (Figure 5A). In LVS infected mice, imatinib pre-treatment reduced CFU in the skin by 40-fold, in the

liver by 12-fold and in the spleen by 2.5-fold compared to infected animals not treated with imatinib (Figure 5B). These data suggest that imatinib may affect a broad range of bacteria, including those whose intracellular survival does not depend on Abl and other conventional targets of imatinib.

Discussion

The bone marrow is the primary site of post-natal hematopoiesis, the process responsible for the generation of leukocytes that orchestrate inflammation and the immune response to microorganisms. Changes in leukocyte homeostasis in the blood by intrinsic or extrinsic stimuli result in differentiation of HSCs in the bone marrow into common CLPs and CMPs or other non-leukocytic precursors, such as erythrocytic and megakaryocytic cells. Our data show that neutrophil precursors are unchanged upon imatinib treatment, although treatment does increase numbers of mature neutrophils and monocytes, and to a lesser extent DCs and NKs. Further preliminary data suggests that CMP numbers increase with imatinib treatment, though we have no evidence for an effect of the drug on HSCs (data not shown).

Imatinib is a potent inhibitor of ABL, c-KIT, m-CSF and PDGFRα/β. However, little definitive information is available on the role of other c-Abl or other imatinib targets in normal hematopoiesis *in vivo*. Mice with mutations in kit (C57BL/6-kit$^{W\text{-}sh}$), which reduces c-kit expression, display increased numbers of myeloid cells in the bone marrow and peripheral neutrophila [197]. It is possible to ascribe these effects to c-Kit, however, other mutations in imatinib targets in these mice, including the PDGFR, could also contribute. Nevertheless, our preliminary data suggest that a novel inhibitor that inhibits c-Kit and PDGFR, but not c-ABL, has similar activity as imatinib on hematopoiesis, suggesting that imatinib effects are independent of c-ABL (data not shown).

Constitutive activation of ABL, as a result of the Philadelphia chromosome translocation (Ph+), results in increased myelogenesis [153], and imatinib causes apoptosis in Ph+ progenitors and precursors. Several lines of evidence suggest that imatinib also alters normal hematopoiesis *in vitro and in vivo* [198]. Bartolovic *et al.* and others suggest that imatinib decreases proliferation of HSC progenitors (CD34+ cells), including CLPs and CMPs, as well as mature leukocytes, though no data at concentrations below 625 nM was presented [199]. Accordingly, clinical studies suggest a percentage of CML patients receiving increased concentrations of imatinib (higher than the standard dose of 400mg) or increased duration of treatment suffered from hematological toxicity [200]. However, these effects appear to be correlated with high doses, and hematologic suppression is rarely observed in patients. In accordance with data presented here, our preliminary data suggest that imatinib increases the number of GM cells in CFC assays, but only at low drug concentrations (<500 nM), and doses used in vivo (66.7 mg/kg/day) are equivalent to human doses of less than 300mg/kg (data not shown). Moreover, some GIST patients treated with imatinib display a characteristic rash called Sweets Syndrome, which is characterized by peripheral neutrophilia [201]. The neutrophilia abates when the drug is discontinued. Together these data suggests that suppressive effects on hematopoiesis result from toxic effects of the drug which may occur at high doses or upon extended treatment duration, but that the drug stimulates granulopoiesis at low concentrations.

Maintenance of normal numbers of neutrophils in the blood is important, as neutrophils are the body's first response and major defense in infection and inflammation [202]. Several medical conditions can lead to dysregulation of neutrophils resulting in neutropenia. In humans, these factors include, but are not limited to, hereditary disorders including cyclical neutropenia, cancer and autoimmune diseases as well as severe burns, microbial infections or administration of chemotherapeutics. Recombinant human granulocyte colony-stimulating factor (G-CSF), a positive regulator of myeloid cell differentiation and inflammatory function, has been shown to reduce neutropenia [203, 204]. However, the utility of G-CSF has been inconsistent between individuals [205, 206]. Our data suggests that imatinib increases the number of mature neutrophils in the bone marrow and periphery of healthy animals, but that these cells are not in an activated state. They are however, activatable upon infection, and they have activity against bacteria, indicating that the cells are not compromised in function. Therefore, imatinib treatment may be a of help in people suffering from neutropenia, with potentially less inflammatory side effects than G-CSF, the current therapeutic option.

Recent studies by the Ernst laboratory suggest neutrophils are paramount in the initiation of adaptive immunity to Mtb infections [29, 60, 207]. In particular, Blomgran *et al.* show that phagocyosis of Mtb-infected neutrophils by DCs, rather than direct infection of the bacterium, accelerates the activation and proliferation of Mtb-antigen specific CD4 T cells [207]. In this regard, immuno-depletion of neutrophils resulted in delayed recruitment of DCs to the lymph

nodes, and delayed kinetics in activation of Mtb-specific CD4 T cells [207]. Here, we present evidence that imatinib treatment markedly increases the number of neutrophils, and to a lesser degree the number of monocytes, in the blood and spleen of mice, and preliminary data suggest that increases are also evident in lungs (not shown). Therefore, it is possible that imatinib will increase the likelihood of neutrophils engulfing Mtb. As a result, imatinib may increase the uptake of infected neutrophils by DCs, thus facilitating their migration and their capacity to present antigen to T cells in the lymph nodes. Together, these data suggest that imatinib could enhance immune control of Mtb and facilitate clearance of the pathogen.

Because neutrophils play a protective role in most bacterial and even viral infections, our data suggest that imatinib-induced neutrophilia may be broadly protective. In this regard, we provide evidence that neutrophils are alone sufficient to reduce mycobacterial load. Moreover, we provide evidence that imatinib is protective against *Francisella* species. Unlike mycobacteria, *Francisella* does not appear to depend on Abl-or other imatinib-sensitive kinases for growth in the infected host. Thus, the observed reduction in CFU likely depends on neutrophils. In further support of this idea, we are currently testing whether neutrophils are sufficient to reduce *Francisella* load, as they are for mycobacteria.

Materials and Methods

Flow Cytometry. For Figures 1A-C and 2A, mouse spleens were collagenase-digested as described [208]. For some experiments, whole blood, bone marrow and collagenase-digested splenocytes [208] were incubated with blocking mAb 2.4G2 anti-FcγRIII/I and live/dead probe (Alexa Fluor 430; Invitrogen). Cells were then labeled with CD11b (M1/70), B220 (RA3-6B2), and Ly6C (AL-21) antibodies from BD Pharmingen, CD19 (MB19-1), Thy1.2 (53-2.1), F4/80 (BM8), CD11c (N418), CD8α (53-6.7), Gr-1 (RB6-8C5) from eBioscience and NK1.1 (PK136) from BioLegend. Cells were then stained with Streptavidin (QDot655; Invitrogen) before fixation. For neutrophil subsetting and activation/degranulation assays, blood, bone marrow and spleen cell samples were processed on ice and in PBS-EDTA buffer to prevent cell activation. Bone marrow and spleen cells were homogenized using a 70 μm nylon filter. Then, cells were incubated with blocking mAb 2.4G2 anti-FcγRIII/I (BD Biosciences) and live/dead probe (Yellow; Invitrogen) along with labeled CD11b (M1/70), B220 (RA3-6B2), Ly6C (AL-21), and CD66b (G10F5) antibodies from BD Pharmingen, Ly6G (1A8), CXCR2 (TG11), CD63 (MEM-259) from BioLegend, CXCR4 (TG12) from eBiosciences and FLICA probe for caspases 3/7 from Novus Biologicals. All samples were acquired on a BD Biosciences LSR II and analyzed using FlowJo (TreeStar, Inc).

Delivery of drugs *in vivo*. For experiments with imatinib, the mesylate salt was dissolved in water and loaded into Alzet pumps (Braintree Scientific, 1007D or 2002) capable of dispensing a continuous flow of drug at 66.7 mg/kg/day. Pumps were inserted subcutaneously into anesthetized 6-week old male C57Bl/6 mice (Jackson Laboratories). At this dose, we observed no weight loss or other adverse effects in uninfected animals. Moreover, such doses have been used to treat cancer in mice, and are equivalent to those used in humans over prolonged periods, with adjustments for differences between mice and humans in pharmacodynamics and pharmacokinetics [178]. Alzet pumps were inserted 24 h prior to intravenous injection of Mm and delivery was maintained for the duration of the experiment (7 or 28 days). The mice were euthanized on day seven and CFU measured as described above.

Bacterial strains and *ex vivo* assays. Mm strain 1218R (ATCC 927), a fish outbreak isolate, was grown in Middlebrook 7H9 broth (7H9) (BBL Microbiology Systems, Cockeysville, MD, USA) supplemented with ADC (Difco Laboratories, Detroit, MI, USA) and 0.05% Tween 80 (Mtb) (Sigma, St. Louis, MI, USA) or 0.025% Tween 80 (Mm). For CFU assays 7H10 agar supplemented with 10% oleic acid-albumin-dextrose-catalase (OADC) was used (Difco Laboratories, Sparks, Maryland USA). For *in vivo* Mm infections bacterial stocks were grown at 30°C for 2 days to an OD_{600} of 0.4 (Eppendorf, BioPhotometer), the cells were diluted with PBS to 10^5 CFU/100ul. *F. novicida* (Fn) strain U112 overnight cultures were grown at 37°C with aeration in tryptic soy broth (TSB; Difco/BD,

Sparks, MD) supplemented with 0.02% L-cysteine (Sigma-Aldrich, St. Louis, MO) while LVS cultures were grown in modified Mueller-Hinton broth (mMHB) supplemented with 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.1% glucose (Sigma-Aldrich), 2% Isovitalex (Difco/BD), and 0.025% ferric pyrophosphate. For macrophage CFU assays, Fn was plated for enumeration on tryptic soy agar (TSA; Difco/BD) and supplemented with 0.01% L-cysteine. Mouse macrophage cell line J774A.1 (ATCC TIB-67) was maintained in Dulbecco's modified Eagle Medium (DMEM). For *in vivo* CFU assays, Fn experiments were plated on modified Mueller Hinton (mMH) (Difco/BD) plates supplemented with 0.025% ferric pyrophosphate (Sigma-Aldrich), 0.1% glucose, and 0.01% L-cysteine. For both macrophage and *in vivo* assays, LVS was plated on mMH agar supplemented with 2% Isovitalex.

***In vivo* bacteria infection assays**. Six-week old male C57Bl/6 mice were injected in the tail vein with active growing cultures at ~10^5 CFU/mouse. The number of bacteria injected for each experiment was determined by retrospective plating and was ~$2.5x10^5$ CFU/mouse. Seven days after infection, blood, spleen and bone marrow were harvested. For CFU, spleens were weighed and homogenized (Fisher Scientific, Tissuemiser) in 1 ml PBS. Each homogenate was diluted and spread on 7H10 agar. Colonies were scored after seven days of incubation at 30°C. Total weight of the organ and colonies per ml of the homogenized organ were used to determine CFU/gram. For *Francisella* infections, C57/B6 mice were infected with ~$6x10^6$ Fn or ~2e5 LVS, subcutaneously. After 48 hours with Fn or 5 days with LVS, mice from both types

of infections were sacrificed and the spleen, liver, and skin at the site of infection were harvested, homogenized, plated for CFU on MH plates, and incubated overnight at 37°C.

Neutrophil purification and adoptive transfers. Neutrophils were purified from the collagenase-digested spleens from control or imatinib-treated mice. Splenocytes were first depleted of B cells with anti-CD19 coated microbeads (Miltenyi) then neutrophils were positively selected by anti-Ly6G^{+} microbeads. Purity was assessed on the Ly6G+ enriched fraction using the parameters listed above. Neutrophils were described as CD11b^{+}GR-1^{hi}Ly6C ^{low}. Ly6G+ fractions from imatinib-treated mice were 100% pure and from naïve mice 80% pure. We routinely purified ~1x10^{6} neutrophils from the spleen of each naïve mouse and ~8x10^{6} from each imatinib treated mice. For adoptive transfers, 4x10^{6} neutrophils were injected into the left tail vein of naïve recipients. Directly following the adoptive transfer, 10^{5} Mm were injected into the right tail vein of the mouse. Spleens were harvested 48 hours post adoptive transfer and bacterial CFU were determined as described above.

Statistical analysis. Statistical analysis was done using the non-parametric Mann-Whitney rank sum test. Values less than or equal to 0.05 were considered statistically significant.

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Figure 1:

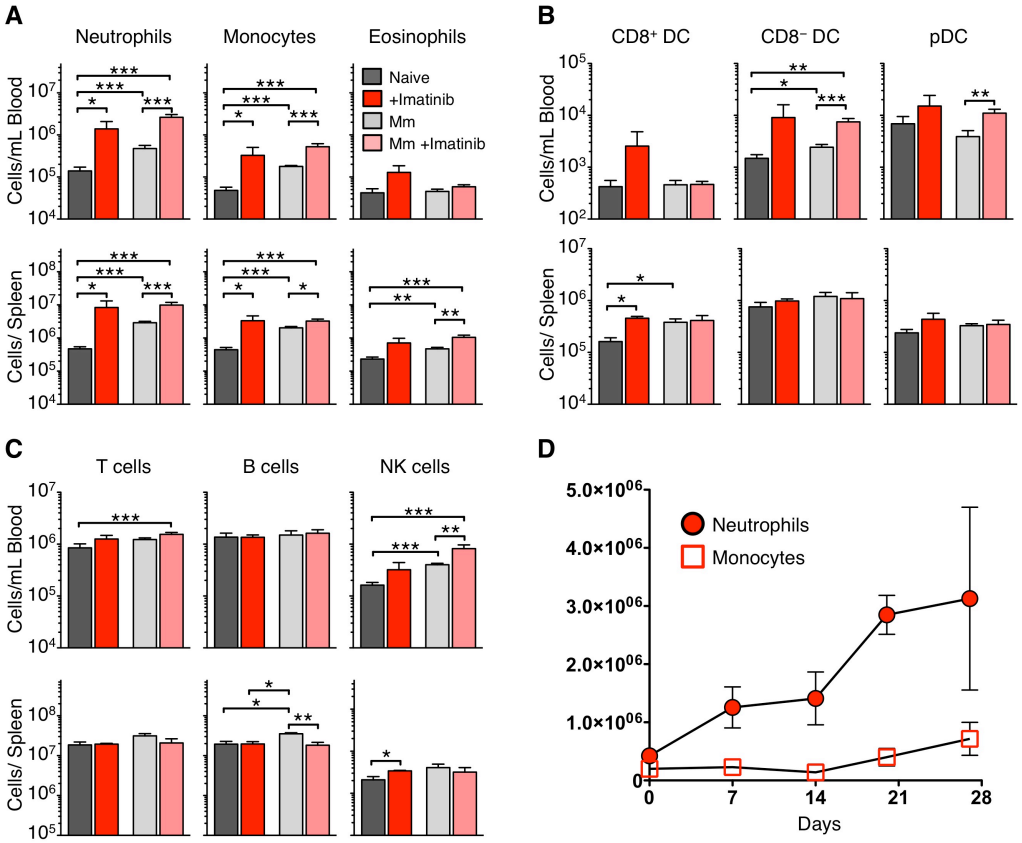
A
Neutrophils
Monocytes
Eosinophils
Naive
+Imatinib
Mm
Mm +Imatinib
Cells/mL Blood
Cells/ Spleen
B
CD8⁺ DC
CD8⁻ DC
pDC
C
T cells
B cells
NK cells
D
Neutrophils
Monocytes
Days

Figure 2:

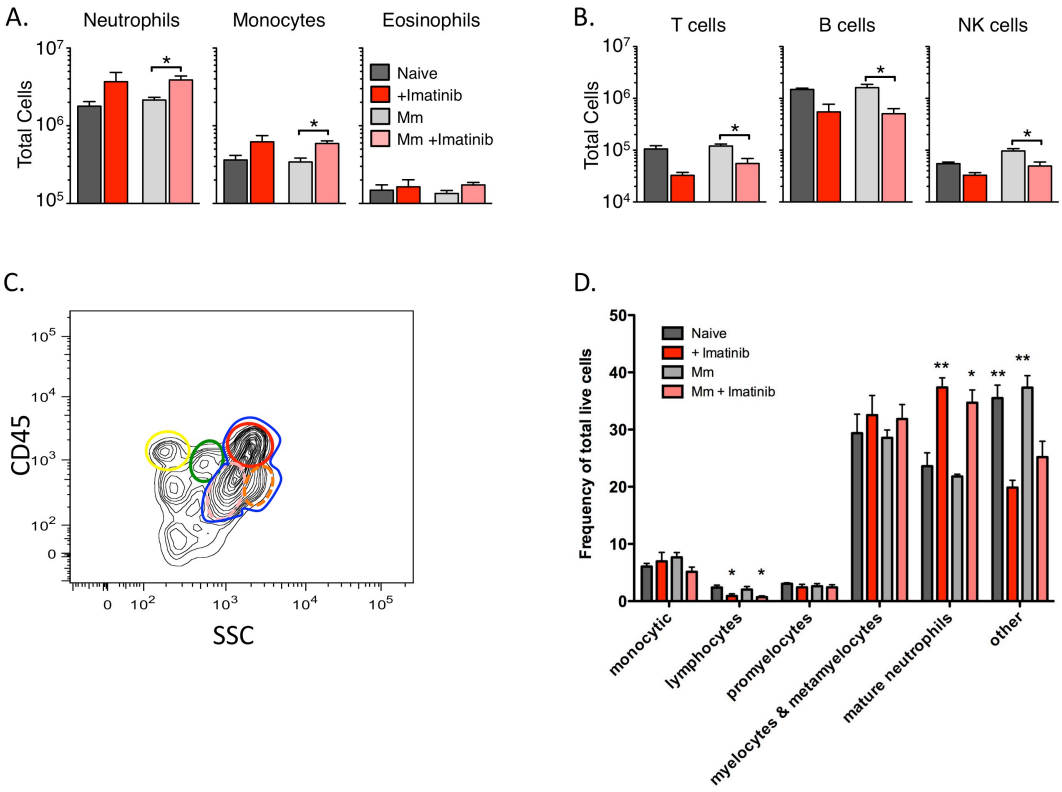
A.
Neutrophils
Monocytes
Eosinophils
Total Cells
Naive
+Imatinib
Mm
Mm +Imatinib
B.
T cells
B cells
NK cells
Total Cells
C.
CD45
SSC
D.
Naive
+ Imatinib
Mm
Mm + Imatinib
Frequency of total live cells
monocytic
lymphocytes
promyelocytes
myelocytes & metamyelocytes
mature neutrophils
other

Figure 3:

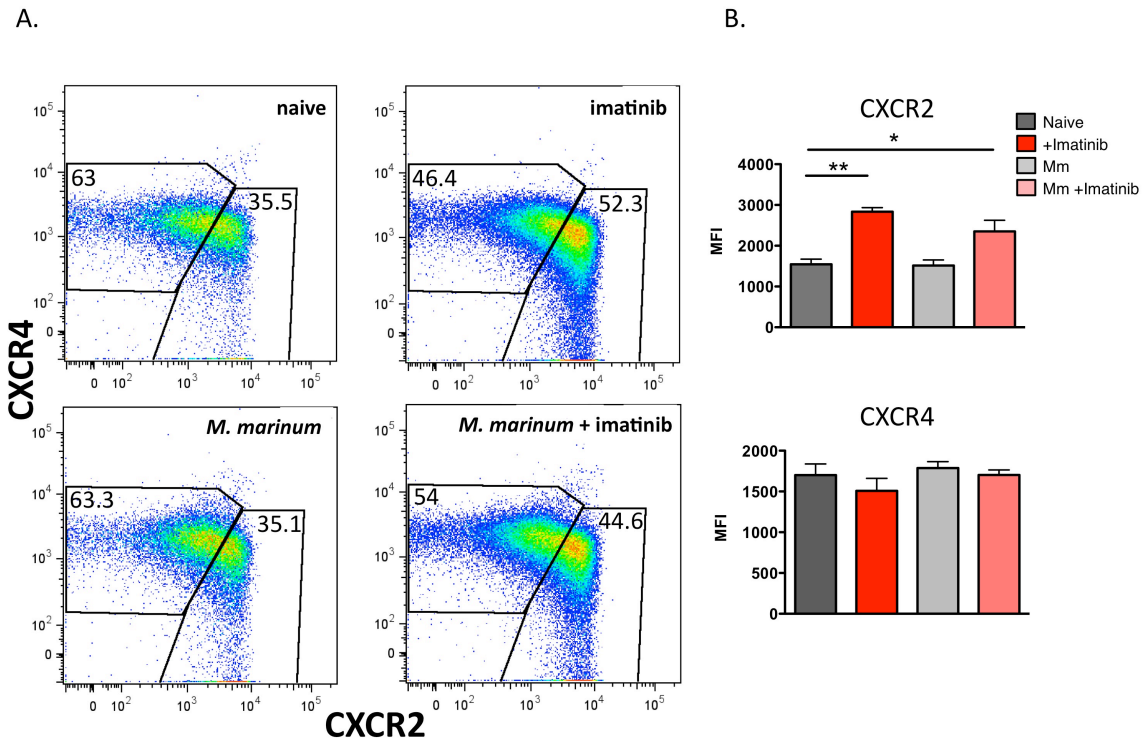
A.
B.
naive
imatinib
M. marinum
M. marinum + imatinib
63
35.5
46.4
52.3
63.3
35.1
54
44.6
CXCR4
CXCR2
CXCR2
CXCR4
MFI
Naive
+Imatinib
Mm
Mm +Imatinib
*
**

Figure 4:

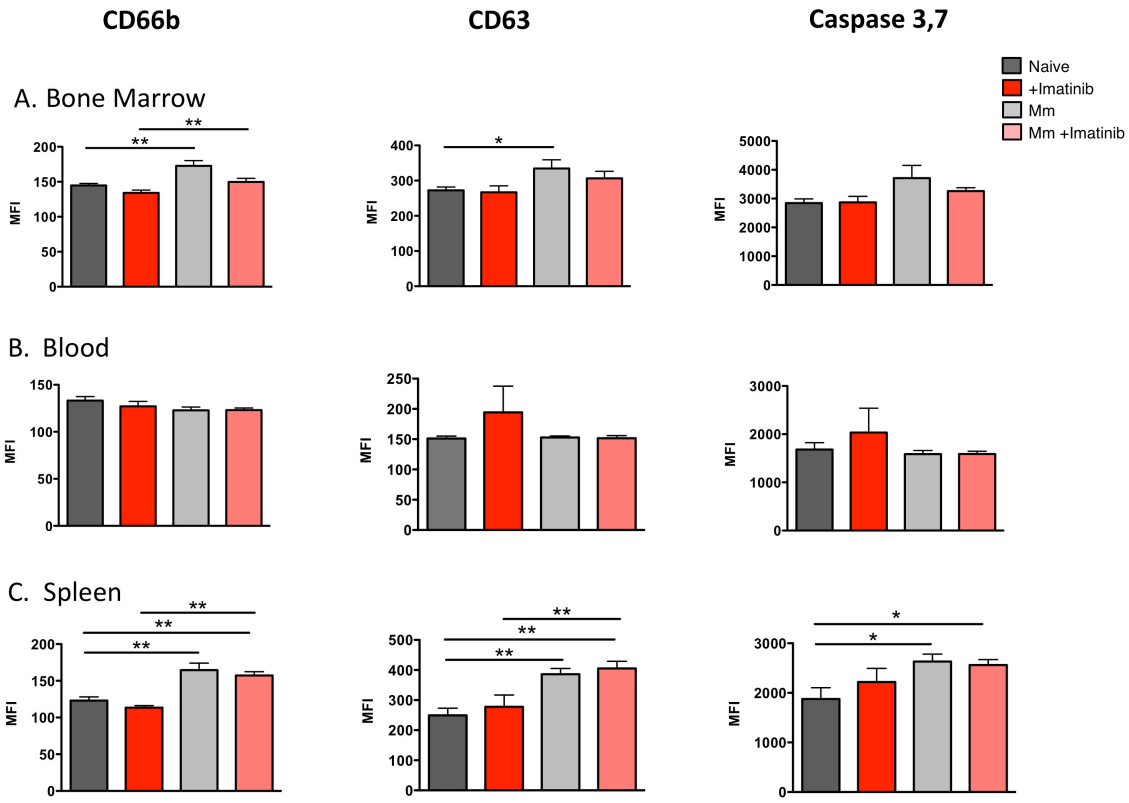
CD66b
CD63
Caspase 3,7
Naive
+Imatinib
Mm
Mm +Imatinib
A. Bone Marrow
B. Blood
C. Spleen
MFI

Figure 5:

A. *M. marinum*

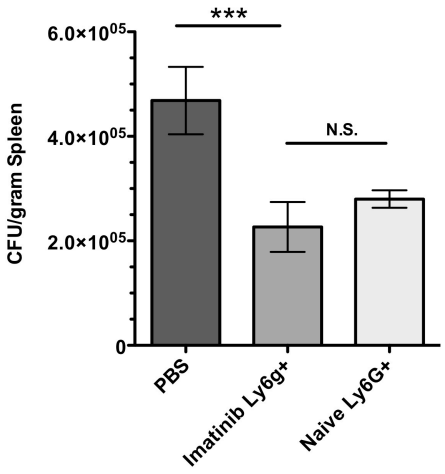

Figure 6:

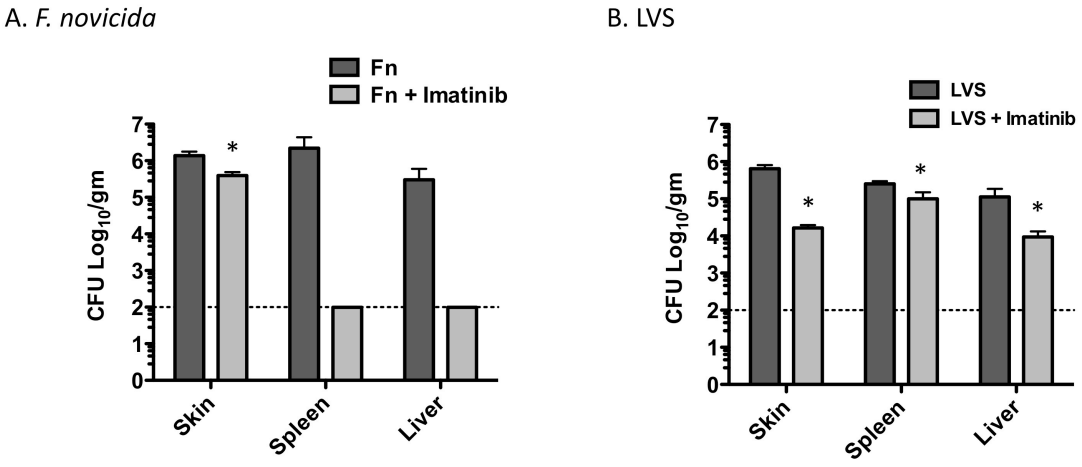
A. F. novicida
Fn
Fn + Imatinib
CFU Log10/gm
0
1
2
3
4
5
6
7
*
Skin
Spleen
Liver
B. LVS
LVS
LVS + Imatinib
CFU Log10/gm
*
*
*
Skin
Spleen
Liver

Figure Legends

Figure 1: Imatinib treatment induces expansion of myeloid cells in mouse spleen and blood.

C57Bl/6 mice were administered imatinib at 66.7 mg/kg/day or left untreated. Beginning 24h post-treatment mice were either injected in the tail vein with 10^5 CFU Mm 1218R or left uninfected. **A)** Neutrophils ($CD11b^+$ $Ly6C^{int}$ $Gr\text{-}1^{hi}$ SSC^{int}), monocytes ($CD11b^+$ $Ly6C^{hi}$ $Gr\text{-}1^{int}$ SSC^{low} and $F4/80^+$) and eosinophil ($CD11b^+$ $Ly6C^{lo}$ $Gr\text{-}1^{int}$ SSC^{hi} and $F4/80^+$) **B)** $CD8^+$ DC ($CD11c^{hi}$ $CD8^+$), $CD8^-$ DC ($CD11c^{hi}$ $CD11b^+$ $CD8^-$) and pDC ($CD11c^{lo}$ $CD11b^-$ $B220^+$ and $Ly6C^+$) **C)** T cells ($Thy1.2^+$), B cells ($B220^+$ $CD19^+$) and NK cells ($NK1.1^+$) numbers were enumerated by flow cytometry in the blood (top panel) or spleen (bottom panel) at d7 post treatment. **D)** C57Bl/6 mice were administered imatinib at a concentration 66.7 mg/kg/day for 26 days. At each time point blood from 3 mice was drawn and neutrophil and monocytes were enumerated by flow cytometry. Cumulative data from 2 independent experiments are presented. N=6 per condition. Data are presented as means +/- SEM..

Figure 2: Imatinib treatment induces neutrophilia in mouse bone marrow.

C57Bl/6 mice were administered imatinib at 66.7 mg/kg/day or left untreated. Beginning 24h post-treatment mice were either injected in the tail vein with 10^5

CFU Mm 1218R or left uninfected. At 7 days post-treatment bone marrow was collected from 2 femurs per mouse. **A)** Neutrophils, monocytes and eosinophils (top panel) and T cells, B cells and NK cells (bottom panel) were enumerated by flow cytometry. **B)** Representative flow cytometry contour plot of bone marrow subset gating based on CD45 expression and SSc**:** lymphocytes (yellow), monocytes (green), promyelocytes (orange), myelocytes/metamyelocytes (pink), mature neutrophils (red), and total granulocytes including all neutrophil progenitor populations and mature neutrophils (blue). **C)** Frequency of total live bone marrow cell of subsets in mice from part B. Cumulative data from 2 independent experiments are presented. N=6 per condition. Data are presented as means +/- SEM.

Figure 3: Imatinib treatment increases CXCR2 expression on neutrophils in the bone marrow.

C57Bl/6 mice were administered imatinib at 66.7 mg/kg/day or left untreated. Beginning 24h post-treatment mice were either injected in the tail vein with 10^5 CFU Mm 1218R or left uninfected. At 7 days post-treatment, bone marrow was collected from 2 femurs per mouse. **A)** The total granulocyte population (Figure 2B; blue) in the bone marrow from each mouse was further analyzed by CXCR4 and CXCR2 expression and subsequent gating of $CXCR2^{hi}CXCR4^{lo}$ and $CXCR4^{hi}CXCR2^{lo}$ subsets, whose frequencies are displayed in box plots. **B)** CXCR2 expression on cells from $CXCR2^{hi}CXCR4^{lo}$ and $CXCR4^{hi}CXCR2^{lo}$ subsets.

Cumulative data from 2 independent experiments are presented. N=6 per condition. Data are presented as means +/- SEM.

Figure 4: Neutrophils from imatinib-treated mice are not activated but remain activatable upon Mm infection.

C57Bl/6 mice were administered imatinib at 66.7 mg/kg/day or left untreated. Beginning 24h post-treatment mice were either injected in the tail vein with 10^5 CFU Mm 1218R or left uninfected. At 7 days post-treatment blood, spleen and bone marrow was collected from two femurs. Activation status was assessed by surface expression of CD66b (secondary granules), CD63 (primary granules) and intracellular staining for caspases 3/7 activity of total granulocyte population (Figure 2B; blue) from the **A)** bone marrow and total neutrophils ($Ly6G^+LY6C^+$) from **B)** blood and **C)** spleen. Cumulative data from 2 independent experiments are presented. N=6 per condition. Data are presented as means +/- SEM.

Figure 5: Neutrophils are sufficient to reduce bacterial load

A) C57Bl/6 mice were administered imatinib at 66.7 mg/kg/day or left untreated. At 7d splenic neutrophils were bead purified (Ly6G+B220-) and adoptively transferred into the left tail vein of naïve recipient mice. Mice were then injected in the right tail vein with 10^5 CFU Mm. 48h following infection spleens were harvested and CFU/gram were determined. Cumulative data from 3 independent experiments are presented. N= 15-25 per condition. Data are presented as means +/- SEM.

Figure 6: imatinib reduces bacterial load in mice infected with pathogenic Francisella species.

C57Bl/6 mice were administered imatinib at 66.7 mg/kg/day or left untreated. Beginning 7d post treatment mice were either injected subcutaneously with **A)** ~$6x10^6$ Fn or **B)** ~$2x10^5$ LVS. At 48h (Fn) or 5d (LVS) post-infection skin, spleen and liver were collected and CFU/gram was determined. N = 5 per condition. Data are presented as means +/- SEM. Dotted line represents the CFU assays level of detection (100 colonies).

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Chapter 4:

Conclusion

Conclusion

Mycobacterium tuberculosis (Mtb), the etiologic agent of the disease tuberculosis (TB), infects one in every three people in the world [1]. There are nine million new Mtb infections every year and 1.4 million deaths [1]. Despite the advent of antibiotics, Mtb still remains an enormous public health concern, second only to HIV/AIDS [2]. The emergence of multi-drug resistant, extensively-drug resistant, and totally-drug-resistant strains of Mtb (MDR-TB, XDR-TB, and TDR-TB, respectively) has complicated treatment, disrupted attempts to control the pandemic, and highlighted the need for the immediate development of new anti-TB therapeutics.

Previous work in our laboratory has focused on identifying host molecules involved in microbial pathogenesis. In particular, we have shown that both bacterial and viral pathogens usurp host tyrosine kinases, including Abl, Src and Tec families, and repurpose them to their own advantage, albeit by different mechanisms. These pathogens include diarrheagenic *Escherichia coli* (EPEC), Poxviridae, Chlamydia, Pseudomonas, polyomaviruses, filoviruses, and now Mtb and related *Mycobacterium marinum* [3-10]. Importantly, we showed for the first time that administration of a pharmacological inhibitor of Abl and related tyrosine kinases, called imatinib-mesylate (imatinib; Gleevec; STI-571), promoted survival of mice during an otherwise lethal poxvirus infection [5].

In chapter two we investigated the role of Abl and related tyrosine kinases in pathogenic mycobacterial infections. Utilizing cell lines derived from knockout animals and pharmacological inhibition by imatinib, we provided evidence that Abl family and related kinases regulate uptake and intracellular survival of Mtb and Mm in macrophages. Using a green fluorescence protein-expressing Mm (GFP-Mm) and a fluorescent probe of lysosomes, we showed that in the presence of imatinib 80% of Mm are detectable in lysosomes compared to 18% in untreated cells. These data suggest that Abl family and other imatinib-sensitive kinases regulate phagolysosomal fusion, and in their absence more mycobacteria result in acidified compartments resulting in their death. Our observations were later recapitulated and extended to primary macrophages derived from human blood by the Stenger laboratory [11].

Our *in vitro* observations led us to question whether imatinib may decrease mycobacterial survival in animals. To test this hypothesis we developed a murine model of Mm infections. We chose to use Mm as a model organism for Mtb pathogenesis as it is genetically similar, replicates five times faster than Mtb (5 and 24 hours, respectively), and can be worked with on the bench top instead of biosafety level three facilities, which is required for Mtb [12]. Mice were treated with imatinib at varying concentrations (50-200 mg/kg/day) beginning 24h prior to infection with Mm and for the duration of the experiment. Animals pre-treated with imatinib had a dose-dependent reduction in bacterial burden in the lungs, liver and spleen. In addition we showed that imatinib reduced bacterial load in mice that were treated 24h post-infection. In addition, associated liver

and tail pathology suggested that imatinib reduced the number and size of granulomatous lesions related to Mm infections. These data suggest that imatinib can reduce Mm survival and associated pathology in mice in a prophylactic or therapeutic capacity.

Multi-drug resistant TB strains pose a serious threat to public health. Because imatinib targets the host and not the bacterium we hypothesized that it would be effective against drug-resistant strains. In this regard we developed a spontaneous rifampicin-resistant mutant and showed that survival in macrophages and mice was reduced in the presence of imatinib. It is unlikely that imatinib would be used alone to treat TB, so we sought to see the effects of simultaneous imatinib and antibiotic treatment. We showed that imatinib acts in a synergistic manner with rifampicin and rifabutin against pan-susceptible strains of mycobacteria *in vitro* and *in vivo*. These data suggest imatinib could be used to treat ostensibly resistant strains of Mtb, including MDR-TB, XDR-TB, and TDR-TB. Perhaps more importantly, our data suggest that imatinib could be used in conjunction with current therapeutics and has the potential to extend the shelf life of current antibiotics by reducing the likelihood that they develop antibiotic resistance.

The observation that imatinib reduced bacterial burden of Mtb and Mm in macrophages as well as Mm survival in mice led us to investigate its effects on Mtb in mice. To test this we worked in collaboration with Dr. Padmini Salgame and Dr. Wasiula Rafi at New Jersey Medical School. Mice were pre-treated beginning 2 days prior to a low dose aerosol infection with Mtb and for the

duration of the 28d day infection. To our surprise imatinib had a sterilizing effect in ~50% of the mice tested. This suggested that in addition to its cell-autonomous effects *in vitro*, imatinib appeared be stimulating the innate and adaptive immune system.

In chapter 3, we tested this hypothesis, and characterized the effects of imatinib on the numbers of innate and adaptive immune cells in animals. Mice treated with imatinib displayed markedly increased numbers of neutrophils and monocytes in the bone marrow, blood, and spleen. In this regard, in mice infected with Mm, imatinib potentiated the pathogen-induced myeloid expansion in an additive manner. Importantly, imatinib treatment did not activate neutrophils *per se*, although neutrophils in the bone marrow of mice treated with imatinib showed increased expression of CXCR2, suggesting increased potential for egress from the bone marrow and recruitment to peripheral tissues. Furthermore, upon infection with Mm, neutrophils in drug-treated animals underwent similar activation to that observed in untreated mice. These data suggest that imatinib increases numbers of un-activated neutrophils, however, it does not preclude the neutrophils from becoming activated by inflammatory stimuli (Mm). Together these data suggest imatinib works through enhancing the immune system's natural response to pathogens, thus facilitating it's antibacterial capacity.

We next sought to focus on the effects of increased neutrophil numbers in the context of Mm infections, as these cells can be manipulated with relative ease. To do this we purified neutrophils from spleens of mice treated with imatinib for

seven days or untreated mice and adoptively transferred $4x10^6$ into naïve recipient animals. The mice were thereafter infected with Mm and bacterial burden was assessed 48h later. Upon adoptive transfer, neutrophils from either imatinib-treated mice or untreated mice decreased bacterial burden to the same extent. These data suggest that neutrophils alone are sufficient to reduce Mm survival in mice.

The observation that imatinib treatment increases neutrophil numbers and that they are sufficient to reduce bacterial load of Mm led us to question whether imatinib would be effective against other bacterial pathogens in mice. To test this mice were pre-treated with imatinib for seven days prior to infection with *Francisella novicida* or the *Francisella tularensis* live vaccine strain (LVS) and for the duration of the experiment. Bacterial load of *F. novicida* and LVS were significantly decreased in the skin, liver, and spleen of imatinib-treated animals compared to untreated animals. Of importance, imatinib had no effect on the survival or growth of *F. novicida* or LVS in broth culture. These data suggest that imatinib could be used as a more broadly applicable therapeutic treatment for other pathogens whose intracellular survival is not dependent on Abl or other imatinib-sensitive kinases.

These data have important implications for treating tuberculosis. First, because imatinib is already approved for use in humans, it has the potential to immediately impact human health. Second, imatinib targets the host and not the pathogen, making it unlikely that the pathogen would develop resistance to the drug; to circumvent such a blockade, Mtb would have to alter its entire virulence

strategy. Moreover, there is little reason to expect that the drug will prove any less effective on MDR-TB, XDR-TB, and TDR-TB, and experiments to validate this assertion are in progress. In addition, because imatinib acts in synergy with antibiotics, it may decrease the likelihood of developing resistance against a co-administered antibiotic. Indeed, with co-administration of imatinib it may even be possible to use antibiotics against strains that are ostensibly resistant to them. Thus, co-administration of antibiotics with imatinib may both potentiate existing drug-treatment regimens and shorten their duration, thereby mitigating compliance issues.

The primary concerns when considering anti-pathogen drugs directed at host targets are incompatibility with co-adminstered drugs, toxicity, and limiting the capacity to mount an effective immune response. Imatinib does have interactions with rifampicin, but not with rifabutin [13], and is not immunosuppressive in humans, nor does it limit anti-pathogen immunity in mice [6]. Moreover, imatinb is remarkably well tolerated in humans, even with long-term exposure, though rare toxic side effects have been reported [14]. Imatinib is not highly selective, as it binds Abl and other structurally-related tyrosine kinases [15]. Moreover, imatinib interacts with c-Abl (or BCR-Abl) in a conformation-specific manner, which causes cyclic binding and unbinding of the drug as enzyme activity changes [16]. As such, the drug is not a particularly potent inhibitor. High selectivity and potency are important in an anti-cancer therapeutic where reducing off target effects limits toxicity, and reducing activity of a target enzyme is essential for preventing tumor growth, and also precludes

development of resistance [17]. However, the criteria for infectious diseases are different. Thus, lower selectivity ensures activity of a drug against a group of molecules utilized by pathogens in a redundant fashion, though drugs must be chosen so as to target the correct group. Moreover, because activity of host proteins required for pathogenesis is often highly up-regulated during infection, drugs that inhibit induced, rather than basal, activity may suffice, and buy enough time for the immune system to mount a response while at the same time limiting toxicity.

In summary, therapeutics that target the host may have great utility against Mtb strains, including those susceptible to conventional antibiotics. We have provided evidence for the efficacy of the tyrosine kinase inhibitor imatinib against Mtb infection [10], and our data raise the possibility of pursuing clinical testing in humans in the future. Recent studies implicating other host pathways in Mtb pathogenesis may provide additional targets, for which drugs or pro-drugs may already exist [18-20]. A possible extension of the host-directed drug strategy would be to target the immune response so as to facilitate clearance [21]. Importantly, because a plethora of pathogens utilize the same host signaling molecules (e.g. Abl), the host-directed therapeutic strategy may be applicable even with the same drug against a broad range of infections, and may provide a novel and general solution to the problem of drug resistance.

Together with the establishment of imatinib as a new therapeutic for Mtb, the work presented in this thesis demonstrates a previously unknown and novel effect of the drug. Here we present evidence that imatinib is immunomodulatory

in healthy animals. In particular imatinib enhances the innate immune system's normal response to bacteria by increasing numbers of un-activated neutrophils and monocytes. Importantly, imatinib is not a proinflammatory drug, as it does not activate neutrophils on its own. This mechanism can be looked at as a "priming" effect on innate immunity that provides the animal with a continuous stream of neutrophils and monocytes that are ready to become activated and functional upon infection. These results have implications for treating other pathogenic bacteria that are sensitive to increased neutrophil and monocyte numbers, including but not limited to the *Francisella* species presented here. Retrospectively, this mechanism could be responsible in part for our earlier observation that imatinib increases survival of animals infected with a lethal dose of vacinia virus. To this extent, experiments to assess the contribution of increased myeloid cells during a lethal poxvirus infection of mice are in progress.

In addition to the antibacterial effects of imatinib, our work suggests a new application for imatinib as a treatment for neutropenia in humans. Neutropenia is a serious and sometimes lethal condition in humans associated with hereditary disorders including cyclical neutropenia, cancer, and autoimmune diseases, as well as severe burns, microbial infections, or administration of chemotherapeutics. Our data suggest imatinib would provide a continuous source of quiescent or activate-able neutrophils that could be used to fight bacterial infections and mitigate effects of neuropenia

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