Cellular Signaling Mechanisms Underlying the Angiogenic Response to Mycobacterial Infection

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Dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy
in the Department of Molecular Genetics and Microbiology
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ABSTRACT

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Abstract

Pathological angiogenesis is a widespread phenomenon that influences the progression of a variety of diseases, including autoimmune conditions, cancers, and microbial infections. One infection in particular, tuberculosis, induces a potent pro-angiogenic signaling cascade that increases bacterial burden and disease progression, but many of the underlying mechanisms remain unknown. Here, I have delineated a discrete host signaling pathway within responding macrophages that first detects a particular glycolipid on the surface of the bacteria, transduces an intracellular signaling cascade, and drives production of the master regulatory angiogenic chemokine, VEGFA. This signaling pathway is driven by activation of nuclear factor of activated T cells, cytoplasmic 2 (NFATC2) downstream of trehalose 6-6'-dimycolate (TDM) detection. Characterization of this pathway resolves a major unknown factor in the signaling mechanisms underlying this maladaptive host response and may offer opportunities for host-directed therapeutic intervention in mycobacterial infections as well as being potentially generalizable to other disease contexts.

Dedication

For my daily motivation and inspiration; for the person who taught me to read and write, who always believed I could do anything I set my mind to, and who dreamt of this day - my Mamaw Barb.

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Acknowledgements

The number of people who warrant thanks exceeds my capacity to thank them and the space allotted. Suffice it to say that this entire journey would not have been possible without the multifold support systems that got me to where I am today.

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In my darkest days of graduate school, Andy Alspaugh was the shining presence I needed to realize that I could go on and succeed and he has been there every step of the way, encouraging me to pursue my dreams and giving me support in the way that only he is capable. His kindness is an inspiration to all.

Lastly, I want to thank my loving partner, Kristen, who has believed in me and supported me throughout the past 4 years. As is often said of the long-suffering partner, she has tolerated the days, nights, and weeks that I was completely absent and, when I finally had a break, welcomed me home with no (visible) resentment and for that I will be forever thankful.

Contents

Chapter 1

Introduction

1.1 Tuberculosis

Of all the infectious agents to have ever afflicted humankind, *Mycobacterium tuberculosis* is perhaps the most imminently successful. The primary cause of potentially greater than one billion human deaths since 1800 alone (approximately 9% of all deaths in that time period) (citation), this disease has had profound impact on the cultural and political development of the modern world and continues to impact the lives of most people around the world today[1]. Fallaciously considered a disease of antiquity, this disease manifests in active disease in greater than 10 million people each year and has killed greater than one million people per year each year since records or estimates have been available with the case and death burden rising due to health system neglect exposed by the COVID-19 pandemic ongoing at the time of this writing (citation).

Mycobacterium tuberculosis has long been of basic scientific interest on account of the myriad ways in which it undermines host immune responses to establish a replicative niche within the human lung. *M. tuberculosis* infection results in the formation of caseating granulomas encased in a complex network of immune cells within which the bacteria replicate. Over evolutionary time, these bacteria have innovated novel ways of subverting host-protective immune responses while exacerbating maladaptive

[1]For additional reading on this subject of how tuberculosis has impacted the development of human society, see (citation).

ones. This makes the study of tuberculosis not only the study of microbiology and immunology, but a fascinating study in the basic principles of cell biology.

1.1.1 History of Tuberculosis

The overwhelming prevalence of tuberculosis in the 18th and 19th centuries led to an extreme degree of cultural salience for this disease in the daily lives of the people of those times. Responsible for the deaths of many preeminent public figures of these eras[2], it is also a ubiquitous feature of the literature of those times as well. Perhaps most famously, tuberculosis is depicted as the disease that afflicts the Lowood School in Charlotte Brontë's Jane Eyre, among other novels depicting the disease then known as *consumption* for the way in which it leads to cachexia, increasing pallor, hemoptysis, and ultimately death (citation).

This cachexia is a defining feature of tuberculosis across phylogenies; such progressive wasting unable to be ameliorated by improved nutrition is an unusual presentation strongly reminiscent of many cancers and rather dissimilar from most infectious diseases (citation). Indeed, as medical understanding of diseases progressed beyond concepts of humoral imbalance, a prevailing theory was that tuberculosis was a hereditary form of cancer due to the way it spread within families (citation). The functional and consequential similarities between tuberculosis and cancer are replete and will be a subject returned to throughout this document. However, as Louis Pasteur's germ theory came to be more widely accepted in the same period in the Industrial Revo-

[2]The number of such public figures is far too great to list. From the 1840s and 1850s alone, tuberculosis was responsible for the deaths of Andrew Jackson (seventh president of the United States), Henry Clay (Secretary of State, Speaker of the House, three-time presidential candidate for the Whig Party), John C. Calhoun (Vice President, Secretary of State), Alexis de Tocqueville (famed French observer of American culture and author of the classic of political theory, Democracy in America), Henry David Thoreau (naturalist author of Walden), and Emily Brontë (author of Wuthering Heights).

lution as Joseph Lister's antiseptic practices, it became a subject of scientific inquiry to identify the potentially infectious bases for seemingly transmissible diseases[3]

Robert Koch was the first to document the tubercle bacillus as an infectious agent in 1888. *Mycobacterium tuberculosis* proved to be a foundational instrument in the broader development of the field of microbiology and provided Koch with the rationale for the development of what we know as the Koch's postulates[4], a procedure for determining the infectious etiology of a disease. This, along with the identification of the anthrax bacillus (*Bacillus anthracis*) sparked the beginning of the modern era of microbiology. It was only once the causative agent of tuberculosis was identified that it became possible to earnestly pursue curative therapies and vaccines, which came swiftly thereafter to varying degrees of efficacy. By 1921, Albert Calmette and Camille Guérin had attenuated *Mycobacterium bovis* for use as a vaccine, creating the BCG vaccine in use today.

Once thought to have been banished to the annals of history, tuberculosis, after a steady decline in cases throughout the middle of the 20th century[5], came roaring back in the late 20th century with the introduction of HIV into the human population in the 1980s and the corresponding increase in susceptibility to infection, disease, and death

[3]This having the caveat that tuberculosis does not always appear to be transparently transmissible. The disease can take months to years to manifest in infected persons, leading to long time delays between putative exposure and active disease.

[4]The postulates are as follows: the organism should be present in afflicted individuals, but not in healthy individuals; the organism should be able to be grown in pure culture; inoculation of a healthy host should recapitulate the disease; the organism should be able to be harvested back from the new host. Of course, each and every one of these postulates have been broken in pursuit of identifying disease-causing agents, but this remains the basis of identifying new infectious etiologies.

[5]This was coincident with, but likely unrelated to, the development of effective antibiotic therapies. Indeed, the modern disparity between tuberculosis rates between the United States and Western Europe and much of the rest of the world is thought to have more to do with improved living conditions, growing herd immunity, and improved nutrition rather than the use of antibiotics as downward trends actually began 100 years prior to the discovery of streptomycin in 1944 (citation).

from tuberculosis due to the immunocompromising nature of HIV/AIDS (citation).

1.1.2 Pathogenic Features of *Mycobacterium tuberculosis*

In addition to the clear relevance of the study of tuberculosis to human health, the unique biological features of this acid-fast, non-motile, slow-growing mycobacterial species make is a fertile ground for basic scientific studies into the way that both saprophytic and pathogenic species of bacteria adapt to adverse environments and ultimately establish a productive niche (citation). The physiological features of the bacillus – a thick, hydrophobic cell wall, unique export and import systems, and novel mechanisms for cell division and stress tolerance – make this a fascinating case study in the evolutionary processes that drive niche adaptation and, indeed, niche creation (citation). That related members of the same genus of bacteria occupy such diverse infectious niches across a wide spectrum of organisms (from fish and amphibians to birds and mammals and in every major organ system), with many also possessing stages of growth in the environment is a testament to the extent to which these species have evolved structures and responses that can accommodate a wide range of physical and chemical stressors. By contrast, some species, notably *M. tuberculosis* and *M. leprae* are tightly adapted to a more limited range of hosts and have lost the capacity for long-term survival outside of a mammalian host. This diversity within the genus offers abundant opportunity for gene-structure-function discovery to uncover factors both required for maintaining an environmental niche as well as those specifically required for either commensal or pathogenic association with hosts, an approach that has long been fruitful in the discovery of novel virulence factors (citations) but comparatively neglected in the basic bacteriological study of environmental mycobacteria.

When a pathogenic *Mycobacterium* infects a naïve host, there is a unified set of cellular and signaling events that occur at the interface of the host and the bacterium that facilitate either successful clearance or establishment of a productive infection. Taking human tuberculosis infection as the model, an infected person will cough up aerosolized droplets that contain often an individual bacillus. These individual bacilli can then be inhaled by a naïve person nearby, establishing a new cycle of infection. Once that person has inhaled this bacillus, lung-resident macrophages[6] uptake the bacteria and, in an estimated 90% of instances, are able to eradicate the infection at the source. However, in the 10% or so of cases where initial clearance fails, an intricate cascade of events proceeds. After phagocytosis, the macrophage sets in motion signaling processes that should result in fusion between the phagosome and extant lysosomes within the cytosol. However, the bacteria, through a combination of structural features in the cell wall (more on this to come) and secreted effector proteins, blocks phagosomal-lysosomal fusion to establish a productive niche within the macrophage. Subsequently, the outpouring of secreted effectors from the bacteria into the host cytosol results in profound reprogramming that blocks apoptosis, downregulates production of select cytokines and chemokines (while enhancing the expression of others), directs the macrophage to recruit additional macrophages to further the replicative cycle, and directs the macrophage out of the lung proper and into the subpleural space surrounding the lungs, the actual site of primary infection. Due to the slow growth of *M. tuberculosis*, it can take several days before the

[6]Also known as alveolar macrophages, these are one of many, many different types of tissue-resident macrophages. While it is well beyond the scope of this section to explore the distinctions, suffice it to say that macrophages in each tissue niche are functionally distinct from one another and exhibit distinct responses to stimuli. Tissue-resident macrophages are also established from a non-hematopoietic origin from the fetal yolk and are replication-competent, allowing them to self-renew *in situ*. The study of macrophage biology is one of immense challenge and opportunity to uncover novel roles for these multifunctional cells, which are now known to play important roles in processes as diverse as germ cell maturation, metabolism, sleep, cardiovascular disease, and many more.

macrophage has become filled with such a large number of bacteria that it necroses, allowing the bystander macrophages and neutrophils to be infected anew. This cycle continues as more immune cells are recruited by the escalating infection and more extracellular bacteria accumulate. These stages of infection, cell lysis, aggregation, and further recruitment eventually result in the formation of what we know as a granuloma.

Tuberculous granulomas are complex aggregates of (primarily) macrophages that have differentiated into a less inflammatory, epithelioid state that encapsulate a central focus of extracellular bacteria within a necrotic core. These epitheloid macrophages are augmented by the full spectrum of other immune cell types – inflammatory macrophages, neutrophils, basophils, eosinophils, natural killer cells, T cells, B cells, and a range of stromal cells. As a discrete structure, these provide a full immunological nexus integrating essentially every identifiable immune cell population. These granulomas, although extractable intact from their environment, must exist in a tissue environment not of their own design. Although immune cells can be readily recruited, the extrapulmonary space is an existing physical location that can be remodeled to some degree but is inherent in the course of the infection. Mycobacteria are tasked with manipulating these tissues, which they do not directly infect, to further their own lifestyle. One of the ways in which they do this, and which is the focus of much of this work, is by inducing the pathological growth of blood vessels toward the site of infection in a process known as angiogenesis.

The classical description of hemoptysis is a clear clinical manifestation of this vascular involvement with the disease. As the granulomas cavitate and release their contents into the airway, the nearby vasculature is damaged. While such damage could have occurred incidentally by damaging the vessels that service the alveoli, it

is now clear that much of this hemorrhage is the product of the encapsulating vascular web that is developed around the granuloma over the course of the infection. These clinical observations have, however, left unaddressed the role for this vasculature in the development, progression, transmission, and treatment of the disease.

1.1.3 Treatments for Tuberculosis and their Mechanisms of Action

Mycobacterial infections are uniquely integrative biological phenomena that require a careful balance between both the host and the bacteria. The host, seeking to eradicate the bacteria, needs a potent but highly specific immune response capable of sterilizing the invading bacilli while the bacteria, seeking to establish a replicative niche, must evade these host defenses. Historically, treatment for bacterial infections has been through the application of bacteria-targeting antibiotics, despite their mechanism of action rarely being understood at the time of clinical introduction. Modern tuberculosis infections are treated with a four-drug cocktail of antibiotics over the course of six to nine months: isoniazid, ethambutol, pyrazinamide, and rifampin (citation). Should the bacteria exhibit resistance to one or more of these, a state known as multi- or extensive-drug resistance (MDR/XDR), additional drugs with further host toxicity are used: kanamycin, ciprofloxacin, and cycloserine are common choices, although new drugs are slowly coming onto the market (citation). Of these, bedaquiline appears to have the most promise in improving the overall treatment of tuberculosis, but long-term impact remains to be seen (citation).

The first modern, clinically effective treatment for tuberculosis was pioneered by the discovery of streptomycin from *Streptomyces griseus* in 1944 (citation). Unlike its

antibiotic predecessor, penicillin, streptomycin was effective in killing *Mycobacterium tuberculosis* bacilli *in vitro*. However, due to its lack of oral bioavailability, the use of this drug was limited to hospitals and clinics able to deliver the drug intravenously. Additionally, like many of the attempts at drug development for tuberculosis that had preceded it[7], it was not particularly effective at eliminating disease when used alone. Streptomycin is an aminoglycoside antibiotic that acts by interfering with protein biosynthesis by poisoning the 30S subunit of the ribosome as well as by inhibiting peptidoglycan biosynthesis through nucleophilic attack of the glycosidic bonds in peptidoglycan (citation). These mechanisms are common to all of the diverse bacteria against which streptomycin is effective, making it a good general purpose antibiotic, if somewhat limited in the face of the unique features of mycobacterial anatomy.

Thus, the introduction of a mycobacteria-specific antibiotic in the form of isoniazid in 1952 was a major breakthrough in the treatment of this disease. Orally bioavailable and highly effective at killing mycobacteria, it comes with the dose limiting side effects of peripheral neuropathy and occasionally fatal hepatitis that make it a less than perfect therapeutic option (citation). It is still in use today on account of its synergy with other antimycobacterials and independent efficacy. Isoniazid works by targeting mycobacterial cell wall synthesis and targets InhA to block earlier stages of fatty acid biosynthesis. This prevents the synthesis of the mycolic acids that comprise the outermost layer of the cell wall and which are essential for mycobacterial survival and growth (citation).

Recognizing the inherent limitations to isoniazid, additional drugs came into use over the next twenty years. Next on the list of drugs was ethambutol, which entered into use in 1961. Ethambutol, like isoniazid, targets the synthesis of the cell wall, this time

[7]One of these, para-aminosalicylic acid (PAS) is an interesting, if distracting tale in the history of microbiology. For more information, see (citation)

by inhibiting the enzymatic ligation of arabinogalactan to the lower peptidoglycan layer and the outer mycolic acid layer, which destabilizes the cell wall and increases bacterial susceptibility to killing. Interestingly, the precise mechanism of action of ethambutol remains unknown despite over 60 years of extensive study (citation).

Rifampin (1965) was the next addition and has an entirely novel mechanism of action compared to the previous entrants. Targeting multiple simultaneous essential biological pathways is an excellent and repeatedly proven way of killing pathogens and preventing the emergence of resistance to all of them simultaneously (citation). Rifampin targets RpoB, the major subunit of the bacterial DNA-dependent RNA polymerase, which is essential for gene transcription. Although mutations have arisen that confer resistance to rifampin, these have particular fitness costs on the bacteria under conditions lacking antibiotic pressure. Rifampin has proven to be an excellent antimycobacterial drug with a comparatively favorable side effect profile compared to the other commonly used drugs.

To round out the four drug cocktail generally recommended for the first-line treatment of tuberculosis today, pyrazinamide (1972) is the most mechanistically interesting of the drugs commonly used to treat tuberculosis. It appears to work by diffusion into the acidic necrotic center of the granuloma where protons activate the prodrug and allow it to be enzymatically converted into pyrazinoic acid, the active antimicrobial. The low pH maintains the stoichiometry in favor of the protonated pyrazinoic acid form over the conjugate base pyrazinoate, facilitating diffusion into the cytosol of the bacteria. Despite the knowledge that has been ascertained about the precise conditions under which pyrazinamide is active, the mechanism of action remains under hot contention with a variety of different mechanisms proposed and the most recent – that it inhibits the synthesis of the essential fatty acid and metabolic carrier

coenzyme A – still under dispute (citation). Pyrazinamide, entirely by accident, takes advantage of the particular biological environment of the infecting bacteria to specifically target the pathogen. As a relatively innocuous prodrug that is activated at the precise site of infection, it is able to reduce some of the toxic effects that would be associated with direct use of pyrazinoic acid while concentrating active drug where bacteria are actively growing with passive diffusion moving additional prodrug into the granuloma to be activated and trapped inside the necrotic caseum (citation).

Modern antibiotic development generally has been stymied by a lack of incentive for the development of high research and development cost, low profit drugs. As new antibiotics are likely to be reserved for cases with extensive antibiotic resistance and are likely to be cost-prohibitive, few have been developed despite pressing need. One of the success stories is that of bedaquiline, which was first approved in 2012. The development of bedaquiline required $500 million in public investment compared to $100 million in investment by the profiteering corporation, Janssen Biotech (citation). Bedaquiline is a potent and highly effective drug reserved for use in multidrug resistant (MDR) and extensively drug resistant (XDR) cases of tuberculosis and which acts to block ATP synthase and shuts down bacterial metabolism and directly leads to bacterial death (citation).

1.1.4 The Mycobacterial Cell Wall

Given that inhibition of cell wall biosynthesis is a common and highly effective mechanism of action for many antimycobacterial drugs, this structure is of clear importance to the survival and pathogenic success of these bacteria. *In vitro*, mycobacteria are unique microbes that grow in intricate serpentine cords along agar plates. These cords were among the first observations that helped to classify diverse mycobacterial

species together and defining the ontogeny of these cords was of immense concern to early mycobacteriologists (citation). By the 1950s they had identified what they called the cord factor – an isolable molecule required for the cording effect seen in mycobacteria and, indeed, able to replicate key features of cording when isolated, even in the absence of bacteria. The chemical composition of this cord factor was determined and this allowed it to be given a name – trehalose 6-6'-dimycolate or TDM. TDM features a trehalose head group and two long mycolic acid ester tails that can number up to C_{100} in length, creating an incorrigibly hydrophobic molecule that forms an extremely thick amphipathic bilayer at the surface of the mycobacteria with the trehalose moieties facing the outside world and attached to the arabinogalactan layer below with a dimensionally thick[8] interior of interleaved mycolic acid chains. TDM is the predominant mycolic acid species in this cell wall layer and has been studied since its discovery for the ways in which it contributes to mycobacterial fitness in a diverse range of environments.

Mycobacteria do not fit into standard binary classifications of bacteria within the Gram staining system. While Gram-negative bacteria feature both an inner and outer phospholipid membrane, Gram-positive bacteria have only a single plasma membrane but are encased in a thick layer of peptidoglycan. Although evolutionarily derived from the Gram-positive bacterial phylum *Actinomycetota*[9], mycobacteria possess fea-

[8] 40 nm in thickness, representing approximately 30% of the total volume of the bacteria, if we take the size of a single bacillus as 0.2 μm in depth and 2 μm in length

[9]This large phylum of bacteria includes incredible diversity and a number of other important human pathogens with varying degrees of relatedness to *Mycobacterium*. A notable example is *Corynebacterium diphtheriae*, the causative agent of diphtheria, which also produces mycolic acids, albeit shorter in length. The existence of a highly effective vaccination to diphtheria while no effective vaccine exists against tuberculosis is emblematic of the divergent strategies these species use to undermine their hosts *C. diphtheriae* produces a classical toxin, diphtheria toxin, that is responsible for much of the pathology of disease while M. tuberculosis was thought to lack toxins until the discovery of the tuberculosis necrotizing toxin (TNT), although this is only selectively expressed and not thought to be absolutely essential for disease (citation).

tures of both Gram-postive and Gram-negative bacteria; they have a single phospholipid bilayer and a thick peptidoglycan layer, but also have an additional membrane comprised of mycolic acids which is occasionally referred to as the *mycomembrane* (citation).

The mycomembrane and its primary constituent, TDM, have many well-defined roles in providing tolerance to environmental stress, detoxifying reactive oxygen species, providing dehydration resistance, and modulating host immune responses. TDM, for instance, is able to block a key step in phagosomal maturation, which would normally be able to kill the bacteria after uptake into phagocytic immune cells, including macrophages and neutrophils. The broad ability of TDM to mediate mycobacterial interactions with the environment is one of the critical dimensions of the evolution of mycobacteria and the ability to then utilize novel modifications on this same molecular framework to undermine host immune responses appears to have been essential for their transition to a pathogenic or commensalistic[10] lifestyle in association with eukaryotic hosts ranging from amoeba to fish to humans.

1.1.5 Trehalose 6-6'-Dimycolate (TDM)

TDM, in many ways, defines the lifestyle of mycobacteria. As mentioned previously, this remarkably hydrophobic (indeed, wax-like) structure provides bacteria a potent tool in surviving both harsh environmental conditions but also the conditions likely to be encountered in a host. This structure has been thoroughly dissected over the

[10]This notion of commensal mycobacteria warrants a vast degree of additional study. Although the laboratory model of non-pathogenic mycobacteria, *Mycobacterium smegmatis*, was isolated from syphilitic chancres and, later, smegma, very little is known about the niche of these commensal mycobacteria, how they maintain a neutral or neutral-positive relationship with their (often transient) hosts, and how their presence impacts host immunity to future encounters with pathogenic mycobacteria (citation).

past decades of research, and a range of modifications are known that influence both the biochemical properties of the cell wall, but also the ways in which host organisms response to this structure.

Along the length of the mycolic acid tails, there are four main classes of modifications that may be present in two major locations. These modifications include methoxy, methyl, keto, and cyclopropyl groups, which can be located at either proximal or distal locations. Of these, the most research interest has centered on the very unusual cyclopropane modifications, which add a great deal of energetic ring strain to the molecule and is, generally, an unusual biological modification due to its inherent instability and energy investment required to create.

Cyclopropane modification of the proximal modification site has been identified to exist in both *cis* and *trans* isomers, each with distinct immunological properties. The cis modification was described first and is added to TDM by the protein product of the bacterial gene *pcaA*. *M. tuberculosis* deficient in *pcaA* are hypoinflammatory in a mouse model of infection, suggesting that cis-cyclopropane modified TDM is pro-inflammatory. Loss of this gene results in an overall reduction in bacterial survival. This somewhat contradictory result indicates that aspects of the host inflammatory response are important for bacterial survival and replication, findings that have since been replicated in a variety of other contexts in respect to tuberculosis disease. Alternately, trans-cyclopropane modification of TDM is catalyzed by CmaA2 and this orientation was found to be hypoinflammatory. Similar to Δ*pcaA M. tuberculosis*, loss of *cmaA2* resulted in a bacterial growth defect and prompt clearance of the bacteria, but by an alternative mechanism. Instead of a muted inflammatory response, Δ*cmaA2 M. tuberculosis* induced hyperinflammation. This body of work, largely from the Glickman lab, established a variety of important roles for related

but enantiomerically distinct versions of the same biomolecule that differ at only a single chemical site. This specificity is evocative of the high degree to which mycobacterial species have adapted to their hosts by developing novel modifications and mechanisms to perturb the immune response in their favor.

Models of TDM-host cell interactions are often lacking by virtue of the underlying chemistry of TDM. The profound hydrophobicity of TDM limits the avenues by which it can be experimentally presented to cells. On the surface of mycobacteria, TDM is (a) mixed with a range of other co-stimulatory molecules that may be important for the function of TDM, (b) presented along the curved surface of a roughly-cylindrical bacillus, and (c) constantly subject to remodeling as the chemically reactive components are oxidized. *In vitro*, these are difficult aspects to model and two major methods have emerged to agonize cultured cells with TDM: on the surface of polystyrene microbeads and through evaporative monolayers on the surface of tissue culture plastic. Interestingly, these two routes of administration result in profound differences in the overall response from the exposed cells. Surface monolayers of TDM are cytotoxic to cells and trigger a highly inflammatory pyroptotic response; on the other hand, TDM on the surface of beads (although with some variation based on the diameter) tends to drive a more regulated response that still differs in some regards from that induced by whole, metabolically inactive mycobacteria. While whole mycobacteria undoubtedly have other molecular patterns that augment the overall immune response, it is likely that the full breadth of the immune response to TDM has yet to be fully uncovered on account of deficient models to do so. The physiological relevance of these monolayer-like configurations of TDM is up to some debate, but there is some evidence that planes of TDM from dead mycobacteria can form in vivo.

1.1.6 Moonlighting

Pathogenic microorganisms are often constrained by genomic size – too small and too few essential functions can be encoded; too large and the risk of duplication errors and cost of maintenance becomes prohibitory. There is therefore a great deal of evolutionary pressure to economize and multitask – why have two proteins to do two functions if one can do both? That is the precise logic underlying many bacterial toxins, secreted effectors, and structural features. One of the most famous of these multifunctional proteins, often dubbed moonlighting proteins[11], is the alpha-enolase from *Streptococcus pneumoniae*. Enolase is an enzyme critical to glycolysis and converts 2-phosphoglycerate to phosphoenolpyruvate, which is essential for the breakdown of glucose into pyruvate. However, *S. pneumoniae* also secretes this normally cytosolic enzyme onto the surface of the outer membrane, which allows it to interact with host plasminogen and catalyze its conversion into active plasmin. Plasmin degrades host fibrin clots, leading to enhanced tissue invasion and pathogenicity through avoidance of host containment by fibrin and increased dissemination. By evolutionary addition of plasminogen-binding properties, fusion of two unrelated proteins into a single protein, alterations of protein localization, or novel layers of regulation, bacteria can, in a very efficient way, exert multiple essential functions from single biological products.

Similar to protein examples, which tend to be more obvious, the structural features of the bacteria can also serve important "moonlighting" functions in the sense that single elements can play key roles in seemingly unrelated phenomena. TDM is an excellent example of this - it is a conserved feature of non-pathogenic mycobacterial

[11] Conceptually, of course, moonlighting is purely orientational. While the given example is one instance where a historically well-defined enzyme has additional functions based on localization, other multifunctional enzymes that can target both bacterial and host substrates or that have distinct functions when cytosolic or periplasmic or secreted are unlikely to be given this title unless they bear high homology to universally conserved proteins.

species, suggesting that this feature likely emerged to address environmental needs that preceded the need to engage with host immunity. Indeed, TDM serves such a wide array of important functions in the physiology of (especially pathogenic) mycobacteria that to assign it a "major" function would be rather fallacious. As a major structural component of the cell wall, defense from the environment is clearly the overarching theme of this sophisticated glycolipid, but what does that really mean?

Strictly in the context of host immunity, TDM had been generally ascribed a few major roles: blockade of lysosome-phagosome fusion, alteration in expression of major immunoregulatory cytokines, induction of humoral immunity, and mediation of granuloma formation. Delipidation of mycobacteria results in a profound alteration of the overall inflammatory response *in vitro* and results in efficient bacterial killing by macrophages but perturbed expression of IL-1β, TNFα, IL-6, and IL-12. It is now though that many of these functions are mediated by recognition of TDM by surface host receptors, a topic that will be returned to shortly. However, the expression of these critical cytokines (among many others) regulated by TDM results in profound changes in the overall tone and tempo of the inflammatory response that, in aggregate, contribute to granuloma formation, a process we now know to be dependent on both pro- and anti-inflammatory signaling molecules, including IL-4, IL-3, IFNγ, and TNFα. These processes are intimately linked with the phenotype that will be further explored throughout this work: the TDM-dependent induction of VEGFA and resultant angiogenesis.

1.2 C-Type Lectin Signaling

For over 100 years, the cells of the innate immune system were thought to be essentially blind scavengers that existed solely to pick up debris for presentation to and activation of adaptive immune responses. How could cells saddled strictly with their somatic genotype go about "intelligently" identifying pathogens? The first clues came from gene homology between the cloned human IL-1 receptor and a developmental protein from *Drosophila*, Toll. These similarities between IL-1R and Toll first informed the mechanism by which IL-1α/βact on cells and then led to the identification of a range of proteins that shared these homologous domains required to activate NF-$\varkappa$B. These proteins, dubbed the Toll-like receptors (TLRs) proved to be the foundation of the modern understanding of a very sophisticated innate immune system that, in the vast majority of cases, is solely responsible for the prevention of disease. Additionally, these discoveries opened doors into the study of evolutionarily conserved mechanisms of pathogen defense, as TLRs are conserved across both *Animalia* and *Plantae*.

Another notable multipurpose biological product is the lipopolysaccharide (LPS) of Gram-negative bacteria. LPS is a critical component of the outer leaflet of the outer membrane in Gram-negative bacteria and a central interface with their hosts, for host-associated species. As a result, diverse eukaryotes, including both plants and animals, have developed a family of receptors known as Toll-like receptors (TLRs), one of which – TLR4 – induces an inflammatory transcriptional response in many vertebrates. LPS, while often stated as a monolithic entity, is in fact a whole family of diverse lipoglycans that vary widely in saccharide antigen and lipid composition, which has become an active area of study. The precise composition alters the ability for the lipid to bind to TLR4 and induce inflammatory responses. Pathogenic species

of Gram-negative bacteria tend to have six (6) lipid tails on LPS that activate TLR4 while commensal or environmental species have five (5) or fewer lipid tails that do not activate TLR4[12]. Precisely why and how these differences have emerged and evolutionary rationales for the failure of pathogenic species to adopt immune evading tetra- or penta-acyl LPS is the subject of ongoing work, but it seems undoubted that some aspects of the TLR-dependent response pathway must offer benefit to the bacteria and are an avenue for bacterial subversion of the host immune response.

1.2.1 Diversity of Outcomes to Receptor Activation

All the major families of pattern recognition receptors[13] are known to induce the activation of NF-$\varkappa$B, but many of them have specific additional pathways that they are known to induce that drive a particular kind of immune response that depends on the cell type, the particular receptor activated, the specific ligand, the duration of activation, other physiological variables, and more. For instance, RIG-I-like receptor activation after detection of pathogen-derived nucleic acids drives the nuclear translocation of IRF3 and IRF7 to produce type I interferons (IFNα/β), which induces both a paracrine (in neighboring cells) and autocrine (self) response to protect against viruses.

Additionally, particular ligands can have multiple means of detection based on their particular presentation. The canonical example is lipopolysaccharide (LPS) from Gram-negative bacteria. Extracelluarly, detection can occur through cooperation of CD14 and TLR4, which coordinate the activation of MYD88 and subsequent activa-

[12]For instance, the oral opportunistic pathogen *Porphyromonas gingivalis* produces a tetraacylated LPS that actually inhibits TLR4 activation by hexaacylated LPS from *Escherichia coli* (citation).

[13]Those being Toll-like receptor (TLR), NOD-like receptor (NLR), RIG-I-like receptor (RLR), and C-type lectin receptor (CLR) families of receptors.

tion of NF-κB. Intracellarly, detection is mediated by caspases 4 and 5, which drives inflammasome assembly to process pro-IL-1 and pro-IL-18 into their active, secreted forms, which also drives both paracrine and autocrine signaling cascades to defend against intracellular Gram-negative bacterial pathogens.

TDM, at least compared to LPS, is a relatively understudied molecule as far as the precise mechanisms of detection and response. This has led to there remaining a degree of uncertainty in the field over the contributions of either CLR signaling through MCL and MINCLR or TLR signaling through TLR2 and MARCO to the overall effect of TDM detection on the cellular response. Additionally, there is relatively little known about different physiological presentations and their impact on the response to TDM. In vitro, TDM has been demonstrated to adopt different conformational states based on surface composition and geometry. On beads of a small (exact number) diameter, it adopts a bilayer configuration similar to that seen on live bacilli; on larger beads or on a plane, it acts as a monolayer. The monolayer configuration is more inflammatory but was also thought unlikely to exist in vivo. Recent hypotheses have challenged this notion, but what is clear is that TDM must be presented to cells in particular arrangements to have an effect, which is seen in head-to-head comparisons between heat-killed Mtb and gamma-irradiated Mtb. While gamma-irradiated Mtb maintain their shape and structure, heat-killed Mtb are broken down and the presentation of TDM is no longer able to activate CLRs even though it becomes a very potent TLR-mediated vaccine adjuvant. Thus, across different types of bacterial ligands, the context of their presentation to a host is a key determinant of their overall effect on the immune response. This will be a key point in the development of several of our assays in the next chapter.

TLR4 was the first of these to be characterized and remains the most thoroughly

studied. The ostensible ligand (albeit indirectly via CD14) for TLR4 was identified as LPS, a highly conserved pattern common to Gram-negative bacteria. This notion of conserved molecular patterns serving as receptor ligands continues to ignite fields of discovery, which have since identified several major families of pattern recognition receptors (PRRs) that have a diverse set of ligands, commonly known as pathogen associated molecular patterns, or PAMPs. Notably, these receptors do not directly activate NF-$\varkappa$B, they must signal through a series of adaptor and regulatory proteins, the most notable of which is MYD88, which is required for signaling from all of the TLR proteins except TLR3 and a subset of TLR4 responses.

Broadly, these can be classified into nucleotide oligomerization domain (NOD)-like receptors (NLRs), retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), and C-type lectin receptors (CLRs).

NLRs, which are conserved across metazoans, detect cytosolic patterns, most notably peptidoglycan[14] and flagellin[15]. NLRs, like TLRs, activate NF-$\varkappa$B, but also can activate mitogen-activated protein kinase (MAPK) signaling and induce the pro-IL-1$\beta$and pro-IL-18 processing by Caspase-1.

RLRs are another class of cytosolic sensors and detect aberrant cytosolic nucleic acids, including double-stranded RNA and uncapped single-stranded RNA. These sensors then activate IRF3/7, which produce IFNα/β, which activates paracrine and autocrine JAK/STAT signaling.

[14]Peptidoglycan is a major component of the bacterial cell wall and is present in essentially all bacteria.

[15]Flagellin is the monomeric subunit used to generate bacterial flagella in motile strains that use flagella for movement.

1.2.2 Signaling Mechanisms Downstream of C-Type Lectin Receptors

While TLR activation *per se* is a rather monotonal response that is predominantly driven by NF-$\varkappa$B, CLRs terminate in at least two known downstream signaling pathways. In addition to NF-$\varkappa$B, they are capable of activating the nuclear factor of activated T cells (NF-AT or NFAT) pathway. This ability to activate multiple layers of transcriptional regulation either at the same time or under different contexts (length of time, strength of agonism, particular ligand) offers CLRs a powerful additional mechanism of modulating the tone of the immunological response in response to particular insults. CLRs are known to respond primarily to carbohydrate-linked ligands, as they contain lectin domains able to recognize either glucose- or galactose-derived saccharides. Many biomolecules are sugar-modified, from bacteria, fungi, viruses, and eukaryotes (both self and pathogens). This allows CLRs to be a major pathway for the response to host-derived damage-associated molecular patterns (DAMPs) as well as microbe- or pathogen-associated molecular patterns (MAMPs, or more commonly, PAMPs).

CLRs are a diverse class of pattern recognition receptors that are defined by their use of divalent calcium (Ca^{2+}) to coordinate the binding of carbohydrate patterns, generally segregated into two major classes: QPD (glutamine-proline-aspartate) motif lectins, which bind galactose-containing sugars, and EPN (glutamate-proline-asparagine) motif lectins, which bind mannose- or glucose-containing sugars. QPD-containing C-type lectins are, in general soluble or secreted proteins and include the likes of human tetranectin (CLEC3B), an extracellualr matrix-interacting protein, and herring antifreeze protein, which mediates the breakdown of ice crystals in the blood of cold-water fish (citations). By contrast, EPN C-type lectins play a diverse

set of roles and many are the classical members of the CLR family, with many being transmembrane receptors. Most notable among these EPN-containing CLRs is DECTIN-1, the archetypal member of the family which has long been studies for its roles in antifungal immunity, but has now been discovered to have a diverse set of roles in other conditions, including to bacterial pathogens (including mycobacteria) and in autoimmunity.

DECTIN-1 has provided the scientific foundation of much of the knowledge we have about the mechanisms of signaling downstream of CLR activation. DECTIN-1 is a single-pass transmembrane receptor that uses a large C-type lectin domain to engage with various ligands, most notably β-glucans, to stimulate responses in myeloid cells. DECTIN-1 itself possesses an intracellular YxxL/$I_{x_{(6\text{-}8)}}$YxxL/I motif that is then phosphorylated by an adaptor kinase, spleen tyrosine kinase (SYK). This sets off a complex series of signaling events that activate CARD9, ASC, and/or PLCγ2, eventually resulting in NF-κB activation in the former instances and NFAT activation in the latter. For DECTIN-1 specifically, notable roles have been defined for both of these branches in this signaling pathway, but much less is known about these pathways downstream of other, related receptors.

Activation of either TLRs or CLRs can terminate in the activation of NF-κB, a generally pro-inflammatory transcriptional immune pathway. While TLRs are expressed on a rather wide diversity of cell types, CLRs are often specific to myeloid cells, the broad category of innate immune cells that includes macrophages, neutrophils, and dendritic cells. Additionally, the precise outcomes of NF-κB activation can vary based on the particular cell type, the length of stimulation, and other factors. Interestingly, despite commonalities between these pathways, TLR activation follows a highly proscribed set of signaling cascades that, in varying ways and to varying degrees, are

dependent upon NF-κB. For instance, the primary mode of signal transduction depends on MYD88 and/or TRIF, two adaptor proteins, which ultimately lead to the phosphorylation of inhibitor of nuclear factor kappa B (I-κB) and subsequent activation of the NF-κB subunit(s). Often viewed in a monolithic way by those outside the direct field of study, NF-κB is comprised of a family of five independent transcription factors (NF-κB1, NF-κB2, RelA, RelB, and c-Rel) that generally act as heterodimers with one another; this raises the potential for subtle and as yet undescribed levels of regulatory complexity based on the active heterodimers in different contexts. To date, little has been done to fully characterize these distinctions, although some work has found different transcription factor bindings sites to be preferentially bound by some dimers and not others (citations). However, NF-κB activation writ large is able to activate transcription of a full spectrum of cytokines and chemokines, including IL-1β, IL-2, IL-6, IL-12, IL-17, IFN-β, TNFα, and IFN-γ. This robust pro-inflammatory response is essential for effective clearance of many pathogens and also increases the resistance of surrounding tissue to further infection by intracellular pathogens.

CLR activation also result in the activation of ASC-dependent canonical inflammasomes, which process pro-IL-1βand pro-IL-18 for secretion and paracrine and autocrine signaling. This process ultimately depends on a common set of adaptors through IL-1R/MYD88, linking it into common sets of signals that are induced by TLR activation. An additional mode is through the activation of the ASC-dependent inflammasome signaling complex, which processes pro-IL-1βand pro-IL-18 for secretion . However, the IL-1 receptor also induces a MYD88-dependent signaling pathway that terminates in NF-κB. This single pathway thus plays a critical and somewhat circular role in various facets of the host response downstream of TLR activation, which unifies the response tone while potentially limiting response diversity; while TLRs are somewhat broad in their expression pattern, the induction of IL-1βsecretion

activates all neighboring cells that express IL-1R, which is practically ubiquitous in environment-facing tissues. This makes this pathway extremely powerful for increasing the local inflammatory tone to block the replication and spread of (especially intracellular) bacteria, but subject to a unified set of subversive mechanisms utilized by bacteria, fungi, and viruses (citations). Comparatively less work has been done to characterize the activating mechanisms induced by CLRs that result in inflammasome formation, but this could serve as an exacerbatory mechanism to heighten inflammation in response to particular classes of signal. Notably, LPS (the canonical TLR4 ligand) is also capable of activating inflammasomes, albeit by an entirely distinct Caspase-11-dependent mechanism.

Lastly, CLRs can activate NFAT signaling, which is a signaling pathway common across other developmental processes but is thought to be a unique consequence of CLR activation in the context of PRR activation (and is often used as a reporter for their activity). Despite the relative uniqueness of this pathway to CLRs, comparatively less work has been done to characterize it vis-à-vis the CARD9-NF-$\varkappa$B pathway. This pathway can induce the expression of EGR2, EGR3, COX2, IL-2, IL-10, and others, which is a more anti-inflammatory or inflammo-regulatory set of signals than the heavily type I signals induced by NF-$\varkappa$B (IL-1, IFN-γ, etc.) The interplay between these divergent signaling consequences is poorly understood, but is likely important in determining the overall tone and kinetics of the response to infection.

Two additional members of the EPN-containing superfamily of CLRs are MCL and MINCLE. MCL, originally dubbed DECTIN-3[16], is expressed by myeloid cells at baseline and is a comparatively desensitized receptor with low affinity for its primary

[16]And for historical reasons, is still occasionally called this in the modern literature.

known ligand, TDM. MINCLE, on the other hand, is tightly regulated and only induced after cellular priming by some other stimulus, including MCL activation. MINCLE has much higher affinity for TDM and, seemingly, a broader range of agonizing ligands, although the latter discrepancy may be a result of historical scientific focus rather than authentic biological difference.

1.2.3 The TDM Receptor

TDM exerts similarly diverse functions to LPS and is also detected by host pattern recognitions receptors (PRRs), including TLR2 – another member of the Toll-like receptor family – and two C-type lectin receptors (CLRs), MINCLE and MCL. As discussed in Section 1.1.4 and 1.1.5, TDM is a structurally essential component of mycobacteria; the absence of TDM renders the bacteria susceptible to immunological, chemical, and environmental stressors. In addition to the important structural aspects of TDM, it also possesses a number of chemical and biological functions in mycobacterial interactions with their hosts.

Chemically, TDM is radically different from nearly any other biomolecule that an organism is likely to encounter. Comprised of a trehalose head group – an unusual di-glucose that is never synthesized by animals – attached to profoundly hydrophobic, extremely long, and diversely modified branched fatty acid tails, TDM is directly cytotoxic to cells through disruption of plasma membrane integrity. For over 50 years after its initial characterization as an important structural feature of mycobacteria, the host receptor, if any, remained unknown. It was only in 2009 that a trio of publications began to define two distinct signaling pathway families that could be activated by TDM - a TLR2-dependent pathway and a Mincle/FcγR-dependent pathway. Further studies elaborated the latter pathway to incorporate a biphasic and ultimately

heterodimeric model of MCL/MINCLE[17] activation, with MCL being activated first, inducing *MINCLE* expression and then the two acting as a heterodimer over longer courses of signaling.

The TLR2/MARCO/CD14-dependent response axis was first described by David Russell's group in 2009 and established an important role for this complex in regulating part of the expression of TNFα, IL-6, and IL-1β. While this was a very thorough and comprehensive project that demonstrated many cell biological aspects of macrophage TDM exposure, it failed to account for the complete response to TDM and left open the possibility that other pathways may play critical roles in mediating this response. Preliminary results had indicated that a FcγR-SYK-CARD9 signaling axis was important for innate immune activation by TDM. Knockout of Card9 resulted in nearly abolished expression of a far more expansive range of cytokines and chemokines and led to the hypothesis that some co-receptor was actually responsible for directly binding TDM and that this co-receptor likely lacked its own ITAM motif, as it required FcγR to provide one in *trans*.

MINCLE, itself, was viewed with some interest due to its curious regulatory pattern, but until 2008, no ligands had been identified. The Macrophage inducible C-type lectin) was originally identified as a receptor for SAP130, a nuclear protein that is exposed to the extracellular milieu after necrotic cell death, which is then able to activate macrophages to scavenge cellular debris (citation). These early observations were, themselves, clues to the pleiotropic nature of Mincle activation as a strictly inflammatory response to cell death would be inappropriate in tone for the majority of innocuous programmed and incidental cell death events that occur almost constantly

[17]In the literature, these protein products are often listed using mouse-specific nomenclature as Mincle and Mcl for the sake of being more word-legible. MINCLE and Mincle are the protein products of the genes CLEC4E and Clec4e; MCL and Mcl are the protein products of CLEC4D) and Clec4d, from humans and mice respectively.

in the day-to-day lives of organisms comprised of billions of cells. This assigned an initial role to MINCLE, but this broke from the standard pattern of CLRs detecting PAMPs and many of these receptors had already been identified to bind seemingly unrelated ligands.

The intersection of the data on FcγR in TDM detection and MINCLE's known dependence on FcγR for signaling led to a logical hypothesis: that perhaps MINCLE was the sought-after TDM receptor. A seminal paper in 2009 established a direct ligand-receptor binding interaction for TDM and MINCLE, establishing this as the dominant pathway for TDM detection and host signal transduction.

Following this, it remained somewhat unclear what pathways were capable of inducing *CLEC4E* expression in response to pure TDM. Over evolutionary time, an ancestral CLR had undergone a tandem duplication to form the modern *CLEC4E* (MINCLE) and *CLEC4D* (MCL). Based on conserved sequence and differential expression patterns, MCL became a compelling candidate for the basal receptor for TDM. Indeed, although it took four years, MCL was identified as a low-affinity TDM binding receptor that could mediate the upregulation of MINCLE, and later, was identified to act in a heterodimer with MINCLE to mediate signaling when both are present.

1.3 Nuclear Factor of Activated T Cells (NFAT)

NFAT signaling activation results in diverse cell- and context-dependent outcomes. This gene family was first described as a transcription factor that regulated the production of IL-2 in T cells. This pathway could be inhibited by blocking the phosphatase activity of calcineurin, suggesting that NFAT was regulated by calcineurin.

While it was initially studied for its diverse roles in regulating lymphoid biology, it has since had widely diverse roles ascribed to it in nearly all cell types including cardiomyocytes, endothelial cells, skeletal muscle, βislets, oligodendrocytes, keratinocytes, and myeloid cells.

The foundational work on NFAT was done almost entirely in T cells, where it was found to be important not only for intra-T cell differentiation into T_H2 cells, but also in dendritic cells for the initial production of IL-2, suggesting that NFAT is an inducer of anti-inflammatory signaling cascades that drive type II responses. NFAT is also essential for induction of IL-4/IL-13, the archetypal anti-inflammatory (or inflammation-resolving) cytokines. However, a binary type I/II classification for this pathway is ultimately elusive, as it is also critical for regulating the expression of TNFαand IFNγ, critically important pro-inflammatory cytokines. The pleiotropic nature of this pathway makes it of especial interest in the context of host-pathogen interactions where a robust inflammatory response is needed to kill the invading pathogen, but moderation is required to prevent excessive tissue damage.

NFAT was discovered relatively early on to be one of the major and defining responses to CLR activation. Defined by Goodridge et al. in 2007 as an important response mechanism, it has been co-opted over the years as an experimental tool to measure CLR activation because TLRs do not activate NFAT (citations). By using either NFAT proteins fused to fluorescent proteins to monitor nuclear localization or the DNA regulatory elements for NFAT to drive luciferase or GFP from a minimal promoter, it is possible to capture a report of NFAT activation with high sensitivity and with rapid response times. This has been used dozens of times in the literature of define the specificity of a response for a particular receptor and ligand. Despite the ironic ubiquity of this approach as experimental tool, very little additional work

has been done to define the functional consequences of NFAT activation downstream of CLR activation, especially in the specific context of MINCLE or MCL agonism. Given the specificity of the NFAT response, there must be important biological consequences of this pathway being activated during infection, but these have been broadly neglected.

One of the major reasons for this neglect has been a unitary focus on the importance of CARD9-BCL10-MALT1 (CBM) signalosomes as another unique consequence of CLR agonism. Despite this method of activation that has more in common with B cell receptor activation than TLRs, the functional downstream consequence is the same: nuclear translocation of NF-$\varkappa$B and associated induction of immune response genes. Furthermore, the evidence is extremely strong that CBM-dependent signaling is critical for the response to a variety of fungal pathogens and that these generally type I responses are a potent defense against infection. However, numerous datasets have provided evidence of a range of genes that depend on CLR activation but are CARD9-independent. Some of these genes are likely to be NFAT-dependent while others may be activated by as-yet unidentified pathway or through more indirect mechanisms.

NFAT has many features that make it a transcription factor family of broad basic as well as translational interest. The NFAT family is comprised of five members: NFATC1 (also known as NFAT2), NFATC2 (NFAT1), NFATC3 (NFAT4), NFATC4 (NFAT3), and NFAT5. Historical reasons have resulted in a convoluted nomenclature[18], so for the sake of consistency, the NFATCx naming scheme will be used

[18]As often happens in science when multiple independent lab groups discover proteins at the same time, the naming can become a challenge as the field as whole reconciles two distinct naming schema. In this case, no resolution has ever come about. The NFATc subnomenclature was meant to designate that they are calcium-responsive and calcineurin-dependent and distinct from NFAT5, the modern homolog of the ancestral protein with high sequence similarity from humans to sponge. In choosing to maintain the NFATc nomenclature, I take no position on the relative

throughout this document. NFAT5 is a special, and distantly related, member of this family that appears to be important for the transcriptional response to osmotic stress, but unlike all of the other members, is constitutively nuclear and not regulated by changes in cytosolic calcium concentration via calcineurin.

The four calcium responsive members have long been passively assumed to be functionally redundant, with their roles defined by their patterns of tissue expression (citations). All of them are derived from an ancestral single isoform that was duplicated over the course of evolution (although intermediate representatives with greater than one but fewer than four isoforms are unknown among modern species). However, evolution has provided each of these isoforms distinctive biophysical properties that allow them to have non-redundant roles even in cell types where more than one is expressed simultaneously. Most notable is their alterations in sensitivity to changes in calcium: while NFATC2 has a persistent response after strong activation, NFATC3 rapidly traffics in and out of the nucleus in response to small magnitude changes in calcium (citations).

NFAT requires the phosphatase calcineurin for their activation. Upon an increase in calcium, calcineurin dephosphorylates NFAT to expose a nuclear localization sequence (NLS); once in the nucleus, kinases (including GSK3 proteins and protein kinase A) phosphorylate NFAT to drive it back into the cytosol in inactive form. This shuttling behavior allows existing pools of NFAT to rapidly modulate host responses, including developmental, immunological, and pathological responses. This also allows for rapid tuning of the longevity of the response, presumably allowing for the induction of different genes and to different degrees based on the length of activation. Although no work has ever been done to define such distinctions, the

merits of the two systems. Additional, now largely outdated, naming schemes had an additional name for each of the isoforms that I will address only as needed throughout this document.

principles of biochemical affinity dictate that more accessible chromatin with more NFAT binding sites would be activated prior to those in less accessible configurations or with fewer sites more distal from the transcriptional start site, which may require long periods of strong activation to be induced. Defining these different classes of genes in different cell types would provide a far greater depth of understanding for the consequences of NFAT activation and timing of intervention for maximum medical benefit.

Recently, and concurrently with the present work, others have identified *Vegfa*[19] as an NFAT-dependent transcriptional target in myeloid cells downstream of Dectin-1 activation through the use of genetic knockouts of *Card9* in mice and in vitro use of NFAT inhibitors after Dectin-1 agonism. This was among the first published works in over a decade to identify a discrete effect downstream of CLR activation that is NFAT-dependent and Card9-independent. Furthermore, there is somewhat of an NFAT renaissance occurring in the literature at the time of writing. Several new papers have emerged in the past several months identifying novel new roles for NFAT signaling in a variety of (predominantly hematopoietic) tissues, giving new emphasis to this long-neglected pathway. The work discussed in later chapters adds to this body of NFAT-dependent responses and, hopefully, encourages additional future work to define the roles of this important but understudied pathway in the response to not only tuberculosis but the full range of human diseases that engage CLR signaling, especially fungal diseases and additional autoimmune disorders.

[19]In the majority of this document, human gene nomenclature is used when referring to pathways in the abstract. However, when relevant to the literature being discussed, the appropriate model organism's field-appropriate nomenclature will be used. Later, when work specifically done in zebrafish is discussed, the nomenclature will use zebrafish nomenclature.

1.3.1 Review of Known Roles for NFAT

1.3.2 Clinical Utility of NFAT Inhibitors

The imminent importance of NFAT became obvious in the organ transplant era. The recipient immune system will wage immunological war against non-self organs, which requires recipients to undergo life-long immunosuppressive therapy. One of the most successful approaches to preventing organ transplant rejection has been the use of calcineurin inhibitors, including cyclosporine and tacrolimus. These calcineurin inhibitors block the NFAT-mediated transcription of IL-2, IL-4, TNF-α, and IFN-γ, which dampens the adaptive immune response (especially T cell mediated responses) and dramatically extends the useful lifespan of the transplanted organ.

Although many immunosuppressive therapies have markedly increased risks for various opportunistic infections, calcineurin inhibitors are comparatively spared from this disadvantage. For instance, alemtuzumab, which depletes B and T cells, increases the risk of a number of bacterial, viral, and fungal infections, including *Staphylococcus*, Hepatitis B, and *Cryptococcus*. By constrast, tacrolimus has potent antifungal activity and no significant relationship between tacrolimus monotherapy and infectious disease risk. This evidence would suggest compensatory mechanisms are available to fend off many pathogens while maintaining enough targeted immunosuppression to prevent organ rejection. This provides evidence of the potential for the use of NFAT inhibitors in an infectious disease context without overtly inhibiting the overall immune response to the infection.

Tacrolimus especially has found use in other realms of medicine. For atopic dermatitis and psoriasis, it has become a major topical monotherapy to treat these disorders with astonishing results. These inhibitors are site-localized with minimal

skin absorption and exhibit far fewer side effects than comparable use of corticosteroids. Approaches focused on targeted inhibition of misregulated pathways have clear promise in improving overall disease outcomes, both in the context of NFAT inhibition, and many other diseases.

New approaches have begun to be developed for NFAT inhibition. One notable example is the development of a comparatively more selective calcineurin-NFAT inhibitor, INCA-6. While tacrolimus acts through binding of FK506-binding proteins, that then complex with and inactivate calcineurin, INCA-6 selectively blocks the interaction between calcineurin and NFAT, sparing some of the other functions of calcineurin while maintaining potent immunosuppression. While this inhibitor has not been exhaustively trialed, this general theme offers some promise for more targeted therapies that can overcome some of the side effects of traditional calcineurin inhibitors while maintaining most of the benefits.

Another approach that has more recently begun to gain traction is the development of isoform-selective inhibitors. The four NFAT isoforms have selective expression and activation profiles that make them conceivably differentiable biochemically. Select recent work has begun to do exactly that, by taking a structure-guided approach to identifying regions of the proteins unique to particular isoforms and targeting them with small molecules. While no isoform-specific inhibitors are yet available, this will likely change in the coming years.

Beyond small molecule based approaches, the age of personalized medicine opens possibilities for gene therapy approaches to ameliorate pathology caused by one or a combination of NFAT isoforms in particular tissues. For instance, a T cell-targeted NFATC2 mutation may result in comparable graft-sparing immunosuppression to tacrolimus while limiting deleterious consequences to a single cellular compartment.

Alternately, new delivery mechanisms may make it possible to selectively deliver traditional small molecules to particular cell types, perhaps through molecular caging approaches or liposomal delivery to discrete tissues. These possibilities and others foreshadow a future of greater specificity in targeting NFAT signaling, on both the isoform and tissue fronts.

1.3.3 Differentiation of Individual Isoforms

As alluded to previously, the different NFAT isoforms have begun to be assigned discrete functions in different contexts. There is a great deal of interesting biology that derives from the different expression patterns and properties of these proteins, which will be briefly reviewed here.

NFATC1

NFATC2

NFATC3

NFATC4

1.3.4 New Roles for NFAT

The central role of NFAT in the immune system has long been appreciated, albeit in a rather limited context, via the widespread application of NFAT inhibitory drugs in the clinic. Two drugs are widely used to block calcineurin activation and suppress immune responses: cyclosporine A and tacrolimus. These drugs were discovered and developed for clinical use in order to target the T cell response and prevent organ

transplant rejection by blocking the affinity maturation and proliferation of anti-graft T cells. The profound and global immune suppression that accompanies the use of these drugs has prevented their use in other contexts for fear of increase susceptibility to infectious diseases. The weakness of these drugs is that they block all calcineurin activity in all cell types, leading to a vast range of collateral targets – a better approach would be to find a way to locally target only the disease-relevant target of calcineurin (in this case, NFAT). Halfway approaches have emerged using tacrolimus (and derivatives) through its use as a topical ointment for atopic dermatitis, but this is inherently limited to skin conditions. What is needed is a generalizable mechanism to deliver potent and localized cellular inhibition of NFAT. Future efforts toward this end may apply adeno-associated virus (AAV) vectors, liposomes, or other delivery mechanisms to drive the expression of VIVIT or CRISPR/Cas9 in specific tissues at particular times.

In the modern era, further roles have been investigated for NFAT that remain somewhat mysterious in mechanism and ontogeny. NFAT activation alters the behavior of platelets and drives inflammatory cascades during Gram-negative sepsis. Mammalian platelets are anucleated, so it is not clear how NFAT is able to modulate cellular behaviors in the absence of its canonical function as a transcriptional activator. The mechanisms of this are certain to be a fruitful avenue of future investigation and are likely to be applicable to nucleated cells as well – new tools and deeper understandings of NFAT protein topology will be required to differentiate these classes of functions in these cells.

1.4 Host-Microbe Interactions to Study Cell Biological Processes

1.4.1 Host-Directed Therapies: History and Promise

One of these defining characteristics is the formation of caseating granulomas. These granulomas, formerly known as tubercles[20], are the most notable and ubiquitous pathology of human tuberculosis. These granulomas are a highly conserved immunological response to any object pathogen or otherwise that the immune system is unable to clear and are an imminently visible and clinically definitive manifestation of tuberculosis[21]. For reasons that remain poorly understood, but likely related to the inflammatory biases of the C57BL/6 and other mouse models, these mice do not form granulomas[22] after being infected with Mycobacterium tuberculosis and mice do not harbor a strain of Mycobacterium that infects them in the wild. This has set the mouse on an evolutionary trajectory where potentially adaptive or maladaptive responses to mycobacterial infection fail to occur. No matter the relative costs or benefits to the host of granuloma formation, the inability of any as yet known mouse model (with the partial exception of the C3H/FeJ model) to form granulomas compromises their ability to serve as a physiologically relevant model of some, but not all, aspects of human tuberculosis.

[20] Hence, *tubercul*-osis.

[21] A large body of work exists on the mechanisms that Schistosoma eggs use to induce parasite-beneficial granuloma formation. However, even in the absence of active biological induction of granulomas, sterile but indigestible objects will induce granuloma formation, albeit with some distinguishing characteristics.

[22] Strangely, these mice do form granulomas in response to Schistosoma and other stimuli, suggesting something distinguishing about mycobacterial infection and perhaps offering clues as to the unique characteristics of the tuberculous granuloma.

A major challenge has been the specific identification of diseases, stimuli, and biological consequences that drive angiogenic effects. While the angiogenic response to tumors is thought to be mediated strictly through a hypoxia-dependent mechanism, the angiogenic response to other stimuli are far less homogeneous. For instance, in the context of the tuberculous granuloma, these structures initially form in the oxygenated environment of the human lung, which encounters 21% oxygen in air approximately 16 times per minute not an environment that would generally facilitate a hypoxia response. While it is certainly possible in occluded sites to create acute hypoxia, the angiogenic response within the lung would be assumed to rapidly and efficiently alleviate this stressor. No systematic comparison has been done to truly measure the precise oxygen tension in these granulomas from either humans or non-human primates, so it remains difficult to make sweeping assertions. Regardless, the experimental identification of particular mycobacterial components able to induce angiogenesis suggests more sophisticated immunological mechanisms at play than simple hypoxia.

This bacteria-centric approach to treatment of tuberculosis seems logical, as bacteria possess many functions that humans lack entirely that are necessary for their pathogenicity, making these appealing targets for drugs. However, this opens the door to the emergence of resistance when treatment is unable to clear the infecting bacteria and a tolerant or resistant population then expands anew. This makes a compelling niche for a new approach to the treatment of chronic bacterial (and fungal and viral) infections: the host-directed therapy. Host-directed therapies have long been used in cancer. Indeed, anti-angiogenic therapy is one of the earlier examples of a host-directed therapy to cancer. But translating such therapies to infectious disease has, thus far, proven difficult or impractical. One of the reasons is a lack of understanding of the underlying mechanisms that could be targeted to benefit the host to

bacterial detriment; another is the difficulty in interfering with host processes in ways that are specific to the site of infection while minimizing overall toxicity. While host toxicity is generally acceptable collateral damage in cancer treatment, this is often viewed less favorably when treating infectious diseases for which pathogen-targeting therapies are thought superior. Despite these challenges, mycobacterial infections, as a product of the unique intersectionality of host and bacterial biology in the granuloma, offer a spectacular opportunity to develop host-directed therapies that shorten time to cure, abbreviate the current drug regimen, prevent the emergence of antibiotic resistance, and, ultimately, fulfill the World Health Organizations goal of eradicating tuberculosis by 2050[23].

These conflicting responses are indicative of the importance of other factors in determining the overall inflammatory tone of a particular response to a particular insult, a theme that will emerge throughout this dissertation.

Among the guiding themes of this thesis is that immune responses are never solely one thing or the other. There is growing acceptance that biological responses in general are far more complex than has been generally acknowledged in the literature to date. In the context of mycobacterial infection, the balance of inflammatory and anti-inflammatory responses determines the ability of the host to survive infection. Beyond infection, the balance of signals creates human predisposition to allergies, autoimmunity, cancer, heart disease, and many other disorders. A deeper understanding of the ways that individual signal transduction cascades can drive both

[23]Disease eradication has long been a stated goal of many public health campaigns, but has thus far been successful precisely twice: against the scourge of smallpox (in 1977) and against rinderpest (a disease of cattle, in 2011). Current campaigns show promise in the eradication of dracunculiasis (or guinea worm) in the immediate future, with cases down to 14 in 2021. Others, including polio, yaws, and rabies, remain elusive despite all having effective vaccines or treatments, are human-exclusive (or have a known, discrete reservoir), and declining case counts. In the eyes of many, polio is an exceptional disappointment given how close we have come, but the continued need for the use of the oral polio vaccine makes eradication all but impossible in the immediate term.

type I and type II responses is essential for the development of better therapeutics to treat diseases with underlying ontogenies from either type of response.

1.5 Angiogenesis

Tissue perturbations, such as those caused by granulomas, often drive the invasion of blood vessels toward the site as a mechanism to facilitate tissue repair. However, these blood vessels can serve as a maladaptive response in many contexts. Most famous is the context of tumor biology, where these vessels serve as a supply of oxygen and glucose, a route of dissemination to distal sites, and a paradoxical barrier to the effective delivery of curative chemotherapeutics. In the transition toward chemotherapy options with lessened toxicity, a number of kinase inhibitors and monoclonal antibodies were developed that target a specific receptor on those blood vessels required for their growth and maintenance: the vascular endothelial growth factor receptor 2 or VEGFR2. This tyrosine kinase receptor triggers a downstream transcriptional response cascade that results in endothelial proliferation and directed growth toward the source of the ligand: the vascular endothelial growth factor, or VEGF. By inhibiting either the enzymatic activity of the receptor using kinase inhibitors or blocking the interaction between the receptor and the ligand using monoclonal antibodies, effective regression of the vascular webs around tumors can be achieved. This therapy has become standard of care for a subset of tumor types and physiological locations, but the mystery remains why this therapeutic strategy targeting a highly conserved (indeed nearly ubiquitous) feature of tumors is not more broadly applicable and generally successful.

The most common of the anti-angiogenic therapies targeting VEGFR2 is bevacizumab.

Bevacizumab is a humanized monoclonal antibody that very potently (KD =) blocks the interaction between VEGFR2 and VEGF and induces vascular regression. However, the physiological stress that this causes appears to drive a compensatory upregulation of VEGF production by the tumor itself the escalating hypoxia in the local region drives rapid amplification of VEGF production to alleviate such detrimental hypoxia. By this mechanism it is proposed that tumors increase the local VEGF concentration beyond the binding affinity of bevacizumab for VEGFR2 and promote vascular relapse and renewed angiogenesis toward the site.

Thus, despite the initial promise of anti-angiogenic therapy, the current implementations have several shortcomings that need to be addressed before this can be a viable and widespread strategy to treat solid cancers. However, by analogy, the same challenges exist with using anti-angiogenic therapies to treat other vascularized disorders. Given the central role of the hypoxia response driven by HIF1a to the induction of angiogenesis through the regulation of VEGF, efforts at inducing vascular regression inevitably drive a reduction in local oxygen tension and a corresponding increase in HIF1a activity and VEGF production. This has logically led to investigation into HIF1a-targeting therapeutics, despite the many challenges associated with targeting transcription factors.

HIF1a-directed therapeutic options remain limited in 2022. The most promising drug candidates are actually those that agonize HIF1a and drive increased local angiogenesis, which is rather beneficial for a number of disorders, including major burns and diabetes. However, existing inhibitors, through either direct or indirect mechanisms, remain either impotent or excessively toxic in vivo. However, it has long been established that other transcriptional pathways are important for the production of VEGF and these may prove to be a more fertile ground for discovery.

ϕ_c	Before		After	
	Z_c	β	Z_c	β
0.84058	2.390 ± 0.135	0.5166 ± 0.064	1.198 ± 0.310	0.5024 ± 0.093
0.84075	2.512 ± 0.138	0.5472 ± 0.073	1.071 ± 0.359	0.4601 ± 0.090
0.84172	2.632 ± 0.151	0.4935 ± 0.077	0.9747 ± 0.458	0.3631 ± 0.083
0.84204	2.858 ± 0.127	0.5637 ± 0.086	1.183 ± 0.413	0.3665 ± 0.079
0.84236	2.916 ± 0.133	0.5555 ± 0.093	1.744 ± 0.298	0.445 ± 0.088
0.84269	3.003 ± 0.124	0.5627 ± 0.095	1.989 ± 0.267	0.4691 ± 0.092
0.84301	3.075 ± 0.12	0.5603 ± 0.095	2.28 ± 0.235	0.5245 ± 0.108

Table 1.1: Kaj subteno de cxiuj lingvoj kondamnas al formorto la plimulton de la lingvoj de. Ni estas movado por lingvaj rajtoj Lingva;, Z_c and β fitting parameters.

1.5.1 Developmental Angiogenesis

1.5.2 Angiogenesis in Cancer

1.5.3 The Relative Failure of Bevacizumab

1.5.4 Historical Observations of Angiogenesis in Tuberculosis

1.5.5 Modern Studies on Granuloma Angiogenesis

Definitions used here:

- *Naciaj* lingvoj neeviteble starigas barojn al.
- *Starigas* barojn al, cxe granda parto de la monda logxantaro.
- *La lingvo* Ni estas movado por lingvaj rajtoj Lingva diverseco.
- Ni asertas ke la ekskluziva uzado de naciaj lingvoj *hoarder*.

Chapter 2

Scientific Methodology

2.1 Zebrafish as a Model Organism

Laboratory model organisms have been a staple of research since the dawn of the scientific endeavor, but only in the past century has standardization of models allowed for improvements in reproducibility and reliability among experiments. One model in particular, the C57BL/6 Mus musculus mouse model, has become a ubiquitous feature of every major research institution all over the world due to their clonal nature[1], relative ease of use, and minimal expense[2]. However, their genetic homogeneity fails to reproduce many phenotypes seen in human disease, making them an excellent model for some disorders and an insufficient one for others. This is nowhere more true than in developmental biology. Although mouse viviparous development is extremely well defined and stereotyped over the course of gestation, that is precisely the challenge. Gestation is an internal and ongoing process of physiological and anatomical development and while it is possible to catalog the process of development in snapshots in time through vivisection, it is impossible to understand the kinetics and processes of development using a model that does not allow for immediate visual accessibility.

In the 1980s this led researchers in Oregon to seek a model that would allow for the full visual access only possible in oviparous organisms. Although Xenopus frogs

[1]Genetic diversity between individual C57BL/6 mice is in the range of x single nucleotide polymorphisms per individual in a genome of x bases (ref).

[2]A single C57BL/6 mouse from Jackson Laboratories (jax.org) at the time of writing is $USD.

had been in use for some time, their long time to sexual maturity (up to 2 years for Xenopus laevis, the dominant model at the time) and other challenges led researchers to a fish model, considered to be the root of the land-adapted branch of the tree of life. A happenstance purchase at a local pet store led to the establishment of the imminently powerful zebrafish model, which led to seminal and otherwise impossible findings in developmental biology. This model has since found applications in nearly every field of biology for many of the same reasons: optical transparency, extremely rapid development, high fecundity, and genetic tractability. These features make the zebrafish an extremely powerful and robust tool for the study of many different biological processes and, thanks to their intolerance for inbreeding, have remained a genetically diverse outbred[3] model for research that allows for more sophisticated modeling of complex processes with the caveat that it also fuels a need for high n-values due to inherent variation between individuals. Conversely, detectable effects from a high-noise environment are often more robust associations.

Only in the past twenty years has an earnest effort been put forth to develop the zebrafish as a model for immunological studies. Although it has long been known that zebrafish, like all vertebrates, possess the full repertoire of immune cells and responses, little was done with that knowledge until recently, given the perceived benefits of the C57BL/6 model, which more closely resembles some aspects of the human immune system and has a superabundance of useful genetic tools with which to study immune responses in cancer, inflammation, autoimmunity, and infection.

[3]The scale of zebrafish outbreeding is difficult to define, even among strains that are used in research laboratories. For instance, the majority of the work in subsequent chapters is done in the *AB background, a classic wild-type reference strain used around the world. This strain, similar to other strains, has upwards of 6000 copy number variations between individuals (15% of the genome) in addition to approximately 1 single nucleotide polymorphism (SNP) for every 500 bases of genome sequence. Experimentalist anecdotes of the intolerance of the zebrafish for inbreeding are ubiquitous, as this widespread genome-level heterozygosis appears to confer some important advantages to individuals.

The mouse has served as the model for immunology for the past 50 years. It has enables monumental discoveries that have resulted in new medications and therapies to treat nearly every conceivable human disease and is the foundation of every single chemotherapeutic medicine on the market today. The diminutive mouse is an outstanding model for a vast array of human diseases and continues to be the go-to model for many processes. However, classical inbred mouse models, including C57BL/6 and other popular lines, including BALB/c, A/J, and 129S1, fail to replicate defining characteristics of tuberculosis in ways that compromise our ability to apply findings from these models to the kinetics and pathology of human disease.

2.1a Zebrafish and their History in Developmental Biology 2.1b Modern Applications of Laboratory Zebrafish 2.1c Zebrafish as a Model System for the Study of Immunity 2.1d Challenges in the Use of the Zebrafish Model 2.2 Mycobacterium marinum-Zebrafish Model of Tuberculosis

Other popular laboratory models of tuberculosis are able to form granulomas, including rabbits and guinea pigs; the former is highly resistant to tuberculosis while the latter is highly susceptible. However, these tend to require maintenance via outbreeding, are larger mammals with associated higher husbandry costs, and are devoid of most useful genetic tools. This left a clear gap in our ability to understand some of the aspects of this important human disease that required innovative new approaches and a whole new paradigm.

A foundational study in 2002 set the tone for the next two decades of research into host-microbe interactions in the zebrafish. Davis Ramakrishnan took advantage of the optical transparency and manipulative amenability of the zebrafish larvae to infect them with an aquatic pathogen in the Mycobacterium genus Mycobacterium marinum. M. marinum is a globally dispersed pathogen of fish and amphibians that

causes tuberculosis in fish, which tends of manifest in superficial lesions, spinal deformities, and wasting. The use of this heterologous host-pathogen system allowed for the first ever in vivo visualization of the early processes of granuloma formation through the interactions between the invading bacteria and the responding host macrophages, which serve as the first responding innate immune cells to mycobacterial infections.

Further developments over the following years, most notably by Swain et al. in 2006, established the zebrafish as a sophisticated and multifaceted model that allows for both comprehensive live imaging of the early processes of infection and dissection of the later stages of infection using adult zebrafish that form granulomas morphologically similar to those formed by humans in response to both M. tuberculosis and during opportunistic infections by M. marinum. These findings set the stage for the continued development of the zebrafish-M. marinum model of tuberculosis and has enabled the study of processes of human disease that have been long described but previously unable to be evaluated.

2.2a Relevance and Natural History of Mycobacterium marinum 2.2b Deficits of Mouse Models of Tuberculosis

Chapter 3

Macrophage NFATC2 Drives Angiogenic Signaling During Mycobacterial Infection[1]

3.1 Summary

During mycobacterial infections, pathogenic mycobacteria manipulate both host immune and stromal cells to establish and maintain a productive infection. In humans, non-human primates, and zebrafish models of infection, pathogenic mycobacteria produce and modify the specialized lipid trehalose 6-6-dimycolate (TDM) in the bacterial cell envelope to generate host angiogenesis at the site of forming granulomas, leading to enhanced bacterial growth. Here we use the zebrafish-Mycobacterium marinum infection model to define the signaling basis of the host angiogenic response. Through intravital imaging and targeted, cell-specific peptide-mediated inhibition, we identify macrophage-specific activation of NFAT signaling as essential to TDM-mediated angiogenesis in vivo. Exposure of human cells to Mycobacterium tuberculosis results in robust induction of VEGF that is dependent on a signaling pathway downstream of host TDM detection and culminates in NFATC2 activation. As granuloma-associated angiogenesis is known to serve bacterial-beneficial roles, these findings identify potential host targets to improve tuberculosis disease outcomes.

[1]Most of the data in this chapter is from *Cell Reports*, text extensively modified.

3.2 Introduction

The host immune response to infection is driven by an intricately regulated, but occasionally discordant or maladaptive, immune response to pathogenic stimuli at the cell-intrinsic, innate, and adaptive levels (Iwasaki and Medzhitov, 2010). While the contributions of immune cells have been widely studied, there is growing appreciation that non-immune populations such as stromal cells and the endothelium (Honan and Chen, 2021; Worrell and MacLeod, 2021; Amersfoort et al., 2022) are also crucial in shaping the host response in both acute and chronic infections (Mueller and Germain, 2009; Randow et al., 2013; Krishnamurty and Turley, 2020). Just as pathogens have evolved sophisticated mechanisms to hijack signaling pathways in immune cells (Finlay and McFadden, 2006), they have also been shown to manipulate developmental and homeostatic processes to direct them toward pathogen-beneficial host responses (Menzies and Kourteva, 1998; Guichard et al., 2013).

Mycobacterium tuberculosis (Mtb) is among historys most widespread and successful pathogens. It has evolved a range of sophisticated mechanisms to manipulate its human host in order to survive, replicate, and transmit. Upon infection, Mtb induces an intricate immune response wherein innate immune cells, consisting initially of macrophages, congregate at the bacterial focus and then undergo an epithelioid transformation and interdigitate to form an encased granuloma, the hallmark feature of tuberculosis (TB), which provides both the replicative niche and the major host-pathogen interface of TB disease (Cronan et al., 2016; Pagan and Ramakrishnan, 2018; Cronan et al., 2021). Granuloma-associated vasculature has long been noted in human and animal models of TB (Cudkowicz, 1952; Russell et al., 2010) but the mechanisms of induction and precise contributions to infection are not yet fully understood.

Many of the major pathological features of mycobacterial granulomas, including associated vascularization, are conserved from zebrafish to humans (Swaim et al., 2006; Bohrer et al., 2021). Zebrafish can be infected with a natural pathogen, Mycobacterium marinum, which induces a robust angiogenic response during granuloma formation. This process, much like that in humans, non-human primates, and rabbits, is associated with production of the pro-angiogenic chemokine, Vegfaa, at the site of infection (Oehlers et al., 2015). This chemokine is a critical regulator of angiogenesis in both developmental and pathological contexts. Similarly, human granulomas have been shown to express VEGFA and are physically associated with blood vessels that penetrate the outer granulomatous layers (Datta et al., 2015). Subsequent work has demonstrated a role for these vessels in supporting bacterial growth and in dissemination of the bacilli from their primary site of infection (Polena et al., 2016). Recent profiling of human and non-human primate granulomas have confirmed the presence of aberrant vasculature associated with Mtb granulomas (Gideon et al., 2022; McCaffrey et al., 2022) during a non-canonical type II immune response (Cronan et al., 2021).

Pathogenic mycobacteria have evolved specialized mechanisms to promote and accelerate angiogenesis. Notably, the extensively modified and essential outer cell envelope component trehalose 6-6-dimycolate (TDM) is cis-cyclopropanated by the enzyme PcaA (Glickman et al., 2000). Mutation of pcaA results in a reduction in granuloma angiogenesis and reduction in bacterial burden; correspondingly, cyclopropanated TDM alone is sufficient to induce host angiogenesis (Saita et al., 2000; Sakaguchi et al., 2000; Walton et al., 2018). As pcaA-dependent vascularization supports bacterial growth, factors driving this represent potential sites of therapeutic intervention yet the signals that mediate this host process remain unclear.

TDM is an extraordinarily long-chain, hydrophobic (C60-C90) glycolipid that has been shown to be detected in cell culture and murine models by host C-type lectin receptors, most notably MCL (CLEC4D) and MINCLE (CLEC4E), as well as by Toll-like receptor 2 (TLR2) and MARCO (Bowdish et al., 2009; Matsunaga and Moody, 2009; Miyake et al., 2013). Canonically, C-type lectin signaling is transmitted through a CARD9-NF-B signaling pathway that results in the transcription and production of TNF, IL-1, IL-6 and other cytokines (Yamasaki et al., 2008; Goodridge et al., 2009; Lobato-Pascual et al., 2013; Zhao et al., 2014; Deerhake et al., 2021). However, beyond CARD9, a number of other downstream signaling pathways are engaged by C-type lectin activation and likely control discrete aspects of lectin signaling (Goodridge et al., 2007; Deerhake et al., 2021).

Here, we synthesize findings from zebrafish and cell culture models to define the in vivo angiogenic response induced by pathogenic mycobacteria. Contrary to classical models of C-type lectin signaling, we find that cis-cyclopropanated TDM exerts its pro-angiogenic effects through an alternative NFAT-driven pathway rather than canonical CARD9-NF-B signaling. We use peptide-mediated, cell-specific inhibition of NFAT to demonstrate that both early and mature granuloma angiogenesis are dependent upon macrophage-NFAT signaling. We identify Nfatc2a as the predominant isoform mediating vegfaa induction and angiogenesis. These findings define the basis of granuloma-associated angiogenesis during pathogenic mycobacterial infections and suggest new targets for host-directed therapeutic interventions during tuberculosis.

3.3 Results

3.3.1 Macrophage Induction of *vegfaa* and Angiogenesis during Mycobacterial Infection

Injection of live Mycobacterium marinum into the dorsal trunk of the zebrafish larva is sufficient to induce a robust angiogenic response adjacent to nascent granulomas in a macrophage-dependent manner (Oehlers et al., 2015) (Fig. 1A). The stereotyped vasculature along this region of the larva allows facile quantitation of neovascularization during and after granuloma formation or other insult (Lawson and Weinstein, 2002; Jin et al., 2005; Gore et al., 2012; Matsuoka and Stainier, 2018). We demonstrated previously that cis-cyclopropanated trehalose 6-6-dimycolate (TDM) is required for the induction of vegfaa and angiogenesis at the site of infection. Furthermore, we found that genetic blockade of Vegfaa signaling was sufficient to abolish angiogenesis during infection with wildtype mycobacteria (Walton et al., 2018). Taken together, these findings suggest that the failure to induce vegfaa is a major contributor to the loss of angiogenesis in pcaA-deficient granulomas.

To study this phenomenon further, we began by examining the kinetics of vegfaa induction to identify the cellular source of vegfaa during granuloma formation. To test whether macrophages were a significant source of vegfaa, we developed a macrophage-specific reporter using the previously described acod1 promoter (also known as irg1), Tg(irg1:tdTomatoxt40) (from here, irg1:tdTomato). irg1 has been found to be expressed specifically in zebrafish macrophages and is upregulated during infection (Sanderson et al., 2015; Kwon et al., 2022). We then crossed this line with the vegfaa reporter line TgBAC(vegfaa:eGFPpd260) (vegfaa:eGFP throughout) and infected double transgenic irg1:tdTomato; vegfaa:eGFP progeny with M. marinum expressing

eBFP2 (Mm-eBFP2) to simultaneously visualize bacteria, macrophage localization, and vegfaa production in vivo (Takaki et al., 2013).

We began imaging at a time point that preceded robust induction of vegfaa:eGFP but would allow us to capture the maximum time span of these events. We observed an increase in vegfaa reporter signal over time that appeared largely localized to macrophages (Fig. 1B). We observed that bacteria initially grew primarily intracellularly within individual macrophages at 36 hours post infection but began to grow in characteristic extracellular cords by approximately 84 hours post infection with little to no intracellular containment at this site by 96 hours post infection (Fig. 1C). The increase in extracellular growth coincided with the induction of eGFP signal in macrophages at 64 hours (Fig. 1B), suggesting that, at low overall burden, intracellular detection is unable to induce vegfaa expression while extracellular engagement correlates with vegfaa expression during early stages of granuloma formation (Fig. 1B; Supplemental Movie 1).

We next visualized the production of angiogenic vessels throughout infection in parallel to our characterization of vegfaa induction. Due to an inability to separate discrete emission wavelengths using two GFP reporter lines, we were unable to examine all four components (bacteria, vegfaa induction, macrophages, and vasculature) simultaneously. To relate this process directly to the angiogenesis observed in mycobacterial granulomas, we crossed the irg1:tdTomato macrophage reporter to the Tg(kdrl:eGFPs843) (from here, kdrl:eGFP) line, which labels vasculature (irg1:tdTomato; kdrl:eGFP). Under the same conditions and burden at which we infected the vegfaa and macrophage dual reporter line, we observed robust vascularization at approximately 96 hours post-infection, subsequent to initial granuloma formation and vegfaa induction (Fig. 1C, 1D, 1E, Supplemental Movie 2).

3.3.2 Genetic *card9* Deficiency Does Not Compromise Mycobacteria-Induced Angiogenesis

Given these observations suggesting that macrophages engaging extracellular bacteria are an important source of vegfaa expression, we interrogated pattern recognition receptor (PRR) signaling pathways that had been implicated in host responses to TDM, a major external component of the mycobacterial cell envelope. We had previously found that myd88 was dispensable for the induction of angiogenesis in response to TDM in vivo (Bowdish et al., 2009; Walton et al., 2018). This suggested that the described TLR2-mediated responses that function downstream of TDM detection in some contexts were unlikely to be required for this process. Rather, we found that the FcR homologs in zebrafish, fcer1g and fcer1gl, are required for the full angiogenic response to TDM (Walton et al., 2018), implicating C-type lectin receptors signaling in mediating this response (Richardson and Williams, 2014; Zhao et al., 2014).

As many of the downstream activities of C-type lectin receptors have been ascribed to the activation of CARD9-NF-B signaling (Goodridge et al., 2009; Lobato-Pascual et al., 2013; Zhao et al., 2014; Williams, 2017; Deerhake et al., 2021), we assessed what role this pathway might play in angiogenesis during mycobacterial infection. We developed a card9 knockout zebrafish line using CRISPR/Cas9 that carries a 28 bp insertion, resulting in an early stop after 59 amino acids (card9xt31) (Supp Fig. 1A). We then assayed these animals in the kdrl:eGFP transgenic background by incrossing kdrl:eGFP; card9xt31/+ animals and infecting the resulting offspring with tdTomato-fluorescent M. marinum (Mm-tdTomato) at 2 days post fertilization (dpf) (Jin et al., 2005; Oehlers et al., 2015) (Fig. 2A, 2B). We quantitated the resulting aberrant vasculature at 4 days post-infection (dpi) under genotypic blinding and post hoc matched these measurements to genotype. There were no significant differences

between the three genotypes (Supp. Fig. 1B, 1C), suggesting either redundancy between multiple established pathways or the existence of an alternative pathway downstream of TDM detection that was fcer1g/fcer1gl-dependent, but independent of both myd88- and card9.

3.3.3 Pharmacological Inhibition of NFAT Induction Limits Mycobacteria-Induced Angiogenesis

Although many of the physiological consequences of C-type lectin receptor induction are often ascribed to CARD9-NF-B signaling, this PRR class is also known to activate a distinct transcription factor family with known roles in immunity the nuclear factor of activated T cells, or NFAT (Goodridge et al., 2007; Deerhake et al., 2021). This calcium-responsive transcription factor pathway is best described in its role regulating T cell biology, but there are numerous reports describing various roles for the members of this pathway in other cell types, including macrophages (Symes et al., 1998; Jones et al., 2000; Crabtree and Olson, 2002; Horsley and Pavlath, 2002; Elloumi et al., 2012). Given that there are four mammalian members of this pathway and six zebrafish homologs with potentially overlapping functions, we began with a pharmacological approach to globally inhibit NFAT signaling through all six zebrafish isoforms.

We first infected 2 dpf kdrl:eGFP larval zebrafish with Mm-tdTomato in the trunk and treated them with 125 nM FK506, a clinically utilized calcineurin inhibitor that blocks NFAT activation, for the duration of the experiment. This modest dose of FK506 was chosen due to developmental toxicities observed at higher doses. We imaged them at 4 dpi and quantitated the degree of vasculature induced in the

presence and absence of inhibitor. Even with a low dose of FK506, we noted a small, but statistically significant reduction in the mean degree of neovascularization at this time point, consistent with a role for NFAT in controlling angiogenesis in response to M. marinum infection (Fig. 2C, 2D) (Kujawski et al., 2014). To ask whether this effect was specific to recognition of TDM, we injected purified TDM or vehicle (incomplete Freunds adjuvant; IFA) alone into the trunks of 2 dpf larvae. Treatment with FK506 resulted in a statistically significant reduction in the degree of angiogenesis induced at 2 days post-injection (Fig. 2E, 2F), suggesting that this pathway was relevant specifically to TDM-dependent angiogenesis.

3.3.4 The Isoform NFATC2 is Specifically Required for Mycobacteria-Induced Angiogenesis

Combining our observations on the correspondence of granuloma formation and the induction of vegfaa with our data implicating the NFAT pathway, we sought to identify NFAT isoforms that were enriched in granuloma macrophage populations. Aside from investigations made into nfatc1, which is restricted to the endocardium, lymphatic vessels, and the notochord during much of zebrafish development (Pestel et al., 2016; Shin et al., 2019; Bagwell et al., 2020), little is known of the expression patterns of these genes in zebrafish, especially in the context of infection. We first made use of published scRNA-seq datasets from mycobacterial granulomas in zebrafish and non-human primates for nfat transcripts that were robustly expressed in granuloma macrophages and identified both zebrafish nfatc2a and nfatc3a as plausible candidates (Cronan et al., 2021; Gideon et al., 2022).

To examine potential roles for nfatc2a and nfatc3a in granuloma-associated angio-

genesis in vivo, we first screened F0 CRISPR-injected mosaic knockouts (crispants) to rapidly evaluate these candidate genes. Using this approach, similar to that used previously by other groups, we assessed the relative roles of these two isoforms individually and in tandem, measuring the angiogenic response to mycobacterial infection in the kdrl:eGFP background (Jao et al., 2013; Wu et al., 2018; Hoshijima et al., 2019; Kroll et al., 2021). We found that nfatc2a inhibition resulted in a 50-80

We then established stable, germline transmitting indel mutant alleles for both genes to validate our results from mosaic animals. Recapitulating our results in the F0 generation, the nfatc3axt59 mutation carrying a 22 bp deletion (leading to an early stop codon at amino acid 9 in exon 1) had no effect on angiogenesis at 4 dpi (Fig. 3B, Supp. Fig. 2C). We then developed a knockout line of nfatc2a bearing a net 4 bp insertion leading to an early stop codon in the second exon (at amino acid 273, frameshifted after amino acid 247), prior to the DNA-binding domain (nfatc2axt69) (Supp. Fig. 2D). We repeated our angiogenesis assay using larvae from incrosses of kdrl:eGFP; nfatc2axt69/+ animals that produced expected Mendelian ratios of wild-type, heterozygous, and homozygous mutant offspring. Consistent with the results from mosaic animals, homozygous knockout of nfatc2a was sufficient to reduce the degree of angiogenesis present in larval zebrafish at 4 dpi (Fig. 3C, 3D; Supp. Fig. 2E, 2F). Importantly, given the known role of NFAT isoforms in T cell function, these defects emerged prior to the developmental emergence of functional T cells (Trede et al., 2004). However, whole animal knockouts could not address potential roles for other cell types in mediating this process.

3.3.5 NFAT is Essential for Angiogenesis Induction in vivo in a Macrophage-Specific Manner

Given our observations on vegfaa induction in macrophages at the granuloma, we tested whether NFAT signaling was required specifically in macrophages for granuloma-associated angiogenesis. For in vivo inhibition of macrophage NFAT signaling during infection, we applied an approach that takes advantage of the NFAT-inhibitory peptide, VIVIT, which competitively inhibits calcineurin-dependent activation of all the NFATc isoforms (Aramburu et al., 1999). This approach has been successfully used as an exogenous treatment in cell culture (Deerhake et al., 2021) and mice (Noguchi et al., 2004; Elloumi et al., 2012; Rojanathammanee et al., 2015), through ectopic overexpression in cell culture (McCullagh et al., 2004), and, more recently, in mice (Poli et al., 2022). We developed a transgenic zebrafish line in which VIVIT is expressed specifically in macrophages, Tg(irg1:VIVIT-tdTomatoxt38) (from here, simply irg1:VIVIT) (Fig. 4A, 4B) (Sanderson et al., 2015). We assessed whether the macrophage-specific expression of VIVIT would be sufficient to reduce the degree of angiogenesis during infection in the trunk with wildtype M. marinum expressing mCerulean (Mm-mCerulean). We found that macrophage-specific VIVIT expression significantly reduced angiogenesis in response to infection (Fig. 4C, Supp. Fig. 2G, 2H). This suggested a macrophage-specific role for NFAT signaling downstream of mycobacterial detection that was necessary to induce angiogenesis, presumably through the nfatc2a isoform.

To ask more directly whether the decreased angiogenesis observed in the NFAT-deficient macrophages was via the TDM-mediated pathway, we used the TDM injection assay we had developed previously. We injected TDM or the IFA vehicle into the trunk of 2 dpf larval zebrafish (Fig. 4D) and measured the resulting angiogenesis

at 2 dpi (Walton et al., 2018). TDM was sufficient to induce angiogenesis in vivo and this effect was dependent upon functional NFAT signaling, with the degree of TDM-induced angiogenesis reduced to the level of the vehicle alone in irg1:VIVIT animals compared to irg1:tdTomato controls (Fig. 4E, Supp. Fig. 2I, 2J).

3.3.6 NFAT Activation is Essential for Angiogenesis in Adult Granulomas

Adult zebrafish are equipped with both innate and adaptive immunity and form mycobacterial granulomas that histologically mirror epithelioid human tuberculosis granulomas (Swaim et al., 2006), including induction of a surrounding vascular network. To assess whether our findings in the larvae translated to a longer-term context in the presence of adaptive immunity, we infected adult kdrl:eGFP; nfatc2axt69/xt69 zebrafish and kdrl:eGFP; nfatc2a+/+ siblings with Mm-tdTomato and examined their peritoneal organs at 18 dpi after CLARITY-based clearing (Chung and Deisseroth, 2013; Cronan et al., 2015). Cleared organs were then imaged by spinning disk confocal microscopy (Fig. 5A). We measured the total vascular network surrounding the granulomas in a programmatically blinded fashion (Salter, 2016) and found that nfatc2axt69/xt69 fish had a significant reduction (50

3.3.7 Macrophage-specific NFAT Inhibition in Mature Granulomas Reduces Angiogenesis

We next evaluated whether macrophage-specific NFAT inhibition had similar effects on vascularization in adult zebrafish. We infected adult irg1:VIVIT; kdrl:eGFP and irg1:tdTomato; kdrl:eGFP double transgenic zebrafish with Mm-mCerulean and ex-

amined visceral organs at 14 dpi. We used confocal imaging to visualize individual CLARITY-cleared organs and measured the total length of granuloma-proximal vasculature under blinding as above (Salter, 2016). We found that the degree of vascularization was significantly reduced around granulomas from irg1:VIVIT fish as compared to irg1:tdTomato fish (Fig. 5D, 5E, Supp. Fig. 3D, 3E). The extent of the vascular network in the irg1:VIVIT condition was notably restricted in cases or solely comprised of more mature, luminal vessels, suggesting a total failure to induce an angiogenic response (Fig. 5D). These findings, consistent with our previous data from both larval zebrafish infections in the irg1:VIVIT background and in the nfatc2a mutant adult fish, point to a critical role for macrophage-specific NFAT activation in inducing the angiogenic response at mycobacterial granulomas. Furthermore, this establishes that NFAT function is broadly conserved from early larval infection through to the mature necrotic granulomas that characterize adult infection.

3.3.8 Inhibition of NFAT Signaling Results in Decreased Bacterial Burden

We had previously shown that inhibition of granuloma-associated vascularization is associated with decreased bacterial burden. Mycobacterial mutants unable to induce vascularization (pcaA), and either genetic or pharmacological inhibition of VEGF signaling all result in lower bacterial burden, presumably due to functions of the aberrant vasculature promoting bacterial growth and/or inhibiting bacterial killing (Glickman et al. 2000; Oehlers et al. 2015; Walton et al. 2018). To examine the effect on burden of inhibition of NFAT signaling, we performed colony forming unit (CFU) assays at timepoints after the induction of angiogenesis and granuloma maturation. We infected nfatc2a+/+ and nfatc2axt69/xt69 adult zebrafish with Mm-tdTomato

and plated them for CFU at 24 dpi. We found that knockout of nfatc2a resulted in a 50

Finally, we evaluated the impact of macrophage-specific NFAT inhibition on whole organism bacterial burden. We infected adult zebrafish possessing either the irg1:VIVIT or irg1:tdTomato transgenes with Mm-tdTomato and then homogenized and plated these fish at 18 dpi. We found that macrophage expression of the VIVIT peptide resulted in a median reduction of 60

3.3.9 Pharmacological Inhibition of NFAT in Human THP-1 Macrophages Limits VEGFA Induction by Mycobacterium tuberculosis

The zebrafish mycobacterial infection model shares important conserved features with Mtb infection of humans, host response and granuloma angiogenesis (Swaim et al., 2006; Datta et al., 2015; Oehlers et al., 2015; Cronan et al., 2021). In addition, important aspects of the response to cyclopropanated TDM appears to be largely maintained between zebrafish and humans (Walton et al., 2018). We next asked whether our findings discovered in vivo with the zebrafish-M. marinum model were conserved in human cells exposed to Mtb. We developed a cell culture model of macrophage-Mtb interactions using differentiated THP-1 monocytic cells exposed to -irradiated Mycobacterium tuberculosis H37Rv (Mtb), which produces the full spectrum of TDM species, presented to the cell in their native configuration (as compared to heat-killed Mtb, which disrupts cell envelope structure and organization) (Romero et al., 2014; Secanella-Fandos et al., 2014) (Fig. 6A). We found that exposure of differentiated THP-1 macrophages to Mtb was sufficient to induce VEGFA transcription as well

as VEGFA secretion (Fig. 6B, 6C). To examine whether NFAT signaling is required for production and secretion of VEGFA we treated THP-1 macrophages with the small molecule inhibitor INCA-6, which specifically disrupts the interaction between the NFAT family members and their activating phosphatase, calcineurin (Roehrl et al., 2004). Strikingly, treatment of THP1 cells with INCA-6 during Mtb exposure significantly inhibited transcriptional induction of VEGFA (Fig. 6B, Supp. Fig. 4A, 4B), as well as VEGFA secretion (Fig. 6C, Supp. Fig. 4C, 4D). Immunofluorescence revealed robust translocation of NFAT (using an NFATC2 antibody) that was broadly correlated to VEGFA signal (Fig. 6D). Taken together these experiments suggest that human NFAT signaling is required for VEGF production in response to Mtb exposure.

3.3.10 Requirement of human NFATC2 for VEGFA induction

To identify functionally important NFAT human isoforms, we exposed THP-1 macrophages to Mtb and subsequently used the secretion inhibitor brefeldin A to lock VEGF within secreting cells. Simultaneous staining for each of the four human NFATc proteins along with VEGFA allowed us to identify NFAT isoforms that underwent changes in expression and localization and correlate this with VEGFA production (Fig. 7A). While THP-1 macrophages express all of the isoforms to varying degrees, the most intense co-staining with VEGF was found with NFATC2 (Fig. 7B). Additionally, while each of the isoforms showed alterations after Mtb exposure, only NFATC2 showed robust nuclear localization that appeared to correspond to VEGFA induction in individual cells (Supp. Fig. 4E). While some NFAT isoform translocation was observable with at least NFATC1 and NFATC3, this generally had no correspon-

dence to the degree or presence of VEGFA production. Given the strong correlation for NFATC2 with nuclear localization and VEGFA production after Mtb exposure, expression data from zebrafish and non-human primate granulomas, as well as the in vivo zebrafish results implicating macrophage Nfatc2a in Vegfaa production and angiogenesis, we focused on human NFATC2 as a key isoform.

To test a functional role for human NFATC2 in macrophage induction of VEGFA during Mtb exposure, we used a lentivirus-mediated CRISPR/Cas9 approach to introduce high-efficiency disruption of NFATC2. We compared these cells to those transduced with lentiviruses expressing safe-targeting control sgRNAs. (Supp. Fig. 4F-4H) (Kabadi et al., 2014; Sanjana et al., 2014; Morgens et al., 2017; Kitamura and Kaminuma, 2021). We simultaneously expressed four distinct guide RNAs targeting NFATC2 or safe-targeting controls, to maximize the percentage of puromycin-resistant cells possessing complete null mutations (Wu et al., 2018). Due to technical challenges associated with long-term culture of THP-1 cells and to address heterogeneity among cellular responses, we focused these assays on VEGFA induction in these cells by immunofluorescence after Mtb exposure. Because the N-terminal epitope recognized by our NFATC2 antibody was upstream of the targeted sites, we were unable to examine functional protein levels directly and simultaneously in the immunofluorescence images (Supp. Fig. 4I). However, we found that transduced cells targeted by NFATC2 lentivirus generally failed to induce VEGFA while safe-targeting control lentivirus-transduced cells responded normally (Fig. 7D, 7E). Thus, macrophage NFATC2-mediated induction of VEGFA downstream of mycobacterial TDM exposure is conserved from zebrafish to human cells exposed to M. tuberculosis.

3.4 Discussion

This work uncovers an unexpected role for macrophage NFAT activation in immune responses to pathogenic mycobacteria and the maladaptive angiogenic responses that occur during infection. This activation of NFAT is driven through recognition of bacterial cyclopropanated trehalose 6-6-dimycolate, a major constituent of the cell envelope in pathogenic mycobacteria, that we have previously found is necessary and sufficient to drive pathological angiogenesis (Walton et al., 2018). Identifying this unexpected role for NFAT in angiogenesis expands our understanding of the mechanisms governing mycobacterial pathogenesis and offers targets for potential host directed therapeutics. Traditionally, work on TDM-mediated C-type lectin activation has focused on CARD9 and NF-B signaling. Here, in contrast, we describe a specific role for alternative C-type lectin signaling responses through the NFAT pathway to drive VEGFA production and granuloma-associated angiogenesis.

VEGFA induction is a prominent feature of tuberculosis in human disease as well as in a number of animal models, including non-human primates, rabbits, mice, and zebrafish (Datta et al., 2015; Oehlers et al., 2015; Harding et al., 2019; Cronan et al., 2021; Gideon et al., 2022). We found that VEGF was produced specifically within newly arrived macrophages at nascent granulomas. Macrophage populations are critical to VEGF induction, as macrophage-specific inhibition of NFAT signaling as well as deletion of nfatc2a result in reductions in granuloma-associated angiogenesis. Using a human cell culture model, we found that NFATC2 was similarly engaged in human cells as amongst all NFAT isoforms, only NFATC2 underwent robust nuclear translocation in response to M. tuberculosis stimulation. Correspondingly, pharmacological inhibition of NFAT signaling in human cell culture as well as genetic inhibition of NFATC2 resulted in reduced VEGFA production.

Although animal models of tuberculosis generally report high VEGF levels, there are few studies that center on VEGFA induction in cell culture infection models. Through high-resolution time-lapses and reporter lines, we found that vegfaa induction generally does not occur until the formation of initial granulomas and generally correlated with the appearance of extracellular bacteria that could be recognized by incoming, likely uninfected macrophages. This concentration-dependent effect on signaling may reflect key aspects of the disease itself, wherein large masses of extracellular bacteria accumulate in the necrotic core of the granuloma, potentially triggering relatively insensitive and/or chronic C-type lectin signaling in this context.

Consistent with the recognition of extracellular bacteria, exposure of human macrophage-like cells to -irradiated M. tuberculosis rapidly induced VEGF signaling in a NFATC2-dependent manner in a dose dependent manner. Standard cell culture infection models generally eliminate extracellular bacteria using gentamicin treatment and media changes, and so it is possible that engagement of this pathway by extracellular bacteria or TDM stimulation is a key component of this response. A survey of the literature and a variety (Lee et al., 2019; Pisu et al., 2020; Hall et al., 2021; Looney et al., 2021; Pu et al., 2021) of RNA-seq datasets from macrophage-Mtb infection experiments reveal modest or nonexistent induction of VEGFA, further supporting the notion that extracellular exposure to Mtb may be an important element of the angiogenic response and may reflect some aspects of the macrophage-Mtb interface within granulomas.

As its name suggests, the NFAT pathway plays an indispensable role in normal T cell biology. Accordingly, whole animal knockouts of NFAT in standard mouse models of M. tuberculosis infection where granuloma formation itself may be limited may have obscured a role for myeloid-specific effects of NFAT signaling (Via et al., 2012).

The zebrafish model, by looking at early timepoints, uncovered a role both in angiogenesis and, presumably as a consequence, bacterial control. Wholesale, longer-term inactivation of NFAT, which also plays important roles in T cells, would compromise important aspects of a productive adaptive immune response during mycobacterial infection. While genetically manipulable animal models allow for cell-specific separation, any host-based therapeutic approaches might require cell-specific macrophage delivery methods (Hu et al., 2019; Mukhtar et al., 2020), NFATC2-specific targeting (Kitamura and Kaminuma, 2021), and/or contend with the adaptive immune response, an important aspect of host resistance during mycobacterial infection.

It remains unclear why NFATC2, but not any of the other isoforms, is specifically required in macrophages for the induction of VEGFA, given evidence that the others are present in resting macrophages (Fig. 7A). The functional distinctions between the isoforms have long been of basic interest, but relatively few specific differences between them have been identified beyond basal regulation to provide tissue-specificity and more recent findings describing layers of kinetic regulation with isoform-specific stimulation thresholds, nuclear retention, and more (Lyakh et al., 1997; Rao et al., 1997; Kar et al., 2014; Kar and Parekh, 2015; Kar et al., 2016). These novel levels of regulation offer opportunities for uncovering new features of the cell biology of NFAT.

Here, we identify the unique requirement for this single isoform in macrophages to induce angiogenesis in response to mycobacterial infection. One hypothesis is that NFATC2 has binding partner(s) unique among NFAT isoforms required for its effect on the VEGFA promoter. Whether this is HIF1 (the canonical regulator of VEGFA) or one of the many previously described interacting partners is, as yet, unknown, but could be tested either in vitro or in vivo with genetic or chemical approaches.

However, higher order regulatory mechanisms that result in the production of VEGF in the absence of overt hypoxia have been understudied and this work proposes at least one potentially generalizable mechanism whereby NFATC2 activation results in VEGFA transcriptional upregulation, a process that can be inhibited with chemical and genetic intervention. Despite the widespread presence of putative NFAT binding motifs (5-GGAAA-3) (Supp. Fig. 4J) in the proximal VEGFA promoter (Gearing et al., 2019), their influence on VEGFA transcription has been relatively unexplored as this specific effect is generally not seen in T cells or other cell types (Chang et al., 2004). NFAT involvement in the induction of a variety of cytokines is well-documented, but which, if any, are at play in the macrophage-Mtb interaction is a promising subject for future research.

A more comprehensive characterization of NFAT-dependent innate immune responses has begun in recent years (Deerhake et al., 2021; Peuker et al., 2022; Poli et al., 2022), but this pathway has remained unstudied in the context of macrophage signaling during mycobacterial infection. Furthermore, this work draws a connection between the induction of calcium fluctuations which can occur in response to many different developmental, homeostatic, and pathological stimuli, including to mycobacterial infection (Kusner and Barton, 2001; Jayachandran et al., 2007; Matty et al., 2019) to the angiogenic response to that stimulation. Our identification of NFAT regulation of VEGFA offers a novel approach to both pro- and anti-angiogenic intervention in various pathological contexts.

3.4.1 Limitations of the Study

While we have identified interesting macrophage biology mediating an important host immune response during mycobacterial infection, there is no data as to whether

this might translate to other disease contexts, especially those with a prominent role for C-type lectin signaling. Whether or not this mechanism is broadly generalizable is important to understanding key aspects of pro-angiogenic macrophage behavior. Additionally, we have validated important aspects of our observations in the zebrafish with a mammalian cell culture model, but subsequent studies may warrant further integration of mammalian models of tuberculosis infection where angiogenesis is present or human patient samples to better understand certain aspects of the underlying biology.

Chapter 4

Fourth Chapter

Chapter 5

Fifth Chapter

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Figure 5.1: Venn Diagram

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[1]Dume horo centimetro uj jes 1884.

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Biography

Jared was born in the mountains of Appalachia in Barbourville, KY in 1994. After graduating valedictorian from Barbourville High School, he enrolled at Transylvania University in Lexington, KY where he received a Bachelor of Arts degree in Biology and Political Science, *summa cum laude*. In the fall of 2016, he began his Ph.D. at Duke University in the Molecular Genetics and Microbiology department, having been awarded a James B. Duke Fellowship. He then joined the lab of David Tobin in the summer of 2017. In his time at Duke, he was awarded a Ruth L. Kirschstein National Research Service Award F31 fellowship from the National Heart, Lung, and Blood Institute.