Cellular Signaling Mechanisms Underlying the Angiogenic Response to Mycobacterial Infection

by

William Jared Brewer

Department of Molecular Genetics and Microbiology
Duke University

Date: _____

Approved:

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David M. Tobin, Supervisor

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J. Andrew Alspaugh

_____
John F. Rawls

_____
Christopher Kontos

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Dennis Ko

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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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.

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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.

First documented in 1888 by Robert Koch, the tubercle bacillus, Mycobacterium

[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).

tuberculosis, was a foundational instrument in the broader development of the field of microbiology and remains a major area of research today[3] (citation). 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[4], 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 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

[3]For more on this, see (citation).

[4]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).

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[5] 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

[5] 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.

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 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.

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[6], 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

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

use in 1961. Ethambutol, like isoniazid, targets the synthesis of the cell wall, this time 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[7] 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*[8], mycobacteria possess fea-

[7] 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

[8]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

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[9] 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

selectively expressed and not thought to be absolutely essential for disease (citation).

[9]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[10], 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

[10]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

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[11]. 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.

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 immunologi-

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

cal, 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. However, at physiologically relevant concentrations and (importantly) presentation, the primary mechanisms of host response are through the activation of the aforementioned PRRs, TLR2 and MINCLE/MCL[12]. Given the previously detailed complexity of TDM and the different arrangements that it can adopt *in vitro*, many of the previous studies in the literature on the immune response to TDM are difficult to reconcile.

Activation of either TLR2 or MINCLE/MCL can terminate in the activation of NF-κB, a generally pro-inflammatory transcriptional immune pathway. While TLR2 is expressed on a rather wide diversity of cell types, MINCLE and MCL are 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 this commonality, 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

[12]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.

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). An additional mode is through the activation of the ASC-dependent inflammasome signaling complex, which processes pro-IL-1βand pro-IL-18 for secretion and paracrine and autocrine signaling. 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).

By contrast, CLRs terminate in at least two known downstream signaling pathways. In addition to NF-κB, 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).

Indeed, MINCLE (from the gene CLEC4E, 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 in the day-to-day lives of organisms comprised of billions of cells. While NF-$\varkappa$B is broadly considered a pro-inflammatory pathway, it induces the expression of several cytokines that are functionally pleiotropic. While entire dissertations could be, and have been, written about interleukin-6 (IL-6), suffice it to say that IL-6 can be both pro- and anti-inflammatory based on the particular circumstances in which the responding cells detect it, the length of exposure, and more. IL-6 is a major downstream transcriptional product of the NF-$\varkappa$B signaling cascade and, depending upon the intersection between it and other cytokinetic signals, can induce either further inflammation or inflammation resolution.

C-type lectin receptors, or 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 and/or PLCγ2, eventually resulting in NF-ϰB and NFAT activation, respectively. 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.

Two additional members of the EPN-containing superfamily of CLRs are MCL and MINCLE. MCL, originally dubbed DECTIN-3[13], is expressed by myeloid cells at baseline and is a comparatively desensitized receptor with low affinity for its primary 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 ago-

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

nizing ligands, although the latter discrepancy may be a result of historical scientific focus rather than authentic biological difference.

1.2.1 History of Pattern Recognition Receptor Signaling

1.2.2 Discovery and Characterization of C-type Lectin Receptors

1.2.3 Ligand Presentation and Pattern Recognition Receptor Responses

1.2.4 Diversity of Outcomes to Receptor Activation

All the major families of pattern recognition receptors[14] are known to induce the activation of NF-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

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

Gram-negative bacteria. Extracelluarly, detection can occur through cooperation of CD14 and TLR4, which coordinate the activation of MYD88 and subsequent activation 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.

1.2.5 MINCLE and MCL Detection of TDM

1.3 Nuclear Factor of Activated T Cells (NFAT)

NFAT, by contrast, is widely accepted to be a pleiotropic pathway as a product of the foundational studies in the pathway conducted on T cell activation, where it is essential for both the expression of IL-2 by dendritic cells to activate TH2 cells and the differentiation of T cells into TH2 cells. It is also essential for IL-4 induction, which is widely considered the canonical anti-inflammatory (or inflammation-resolving) cytokine. Remarkably, it is also important for mediating the expression of TNF and IFN, critically important pro-inflammatory cytokines. Other factors must intervene in the overall response, likely through the modulation of other pathways by the pathogen to drive particular types of responses to their overall benefit.

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. By using either NFAT proteins fused to fluorescent proteins to monitor nuclear localization or the DNA regulatory elements for NFAT to drive luciferase 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-B and associated induction of immune response genes. Furthermore, the evidence is extremely strong that CBM-depenent 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.

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[15], so for the sake of consistency, the NFATCx naming scheme will be used throughout this document. NFAT5 is a special member of this family that appears to be important for the transcriptional response to osmotic stress, but unlike all of the other members, is not regulated by changes in cytosolic calcium concentration

[15] 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. While NFAT was originally identified in *Drosophila* as TonB, 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 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.

via calcineurin.

The four calcium responsive members have long been assumed to be functionally redundant, with their roles defined by their patterns of tissue expression. All of them are derived from an ancestral single isoform that was duplicated over the course of evolution (although intermediates 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.

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 for 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 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[16] 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 hematopoetic) tissues, giving new emphasis to this long-neglected pathway. Some of 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.

[16]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 organisms 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

1.3.3 Differentiation of Individual Isoforms

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 in specific tissues at particular times.

In the modern era, further roles have been investigated for NFAT that remain some-

what 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[17], 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[18]. 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

[17] Hence, *tubercul*-osis.

[18] 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.

form granulomas[19] 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.

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.

[19]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.

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[20].

[20]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.

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 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.

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

Naciaj lingvoj neeviteble En la Esperantokomunumo la anoj. Al cxiu homo partopreni kiel. La, sed ne limigite de ili .

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.

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*.

Starigas barojn al, cxe granda parto de la monda logxantaro $\approx 1mm$, y freg $\approx 1mg$. Hha jong shiel odieio $\delta E_p = mgd \approx 10^{-8}$ Joules.

Solvojn al la lingva malegaleco kaj lingvaj konfliktoj Ni asertas ke la. Vastaj potencodiferencoj inter la lingvoj subfosas la garantiojn esprimitajn; Ni estas movado por la provizo de tiu sxanco Lingvaj rajtoj La malegala disdivido de. Estas senescepte du aux plurlingvaj Cxiu komunumano akceptis. Kaj evoluigo se gxi ne estas.

Ki makro helposigno antauhierau mal, hu jen iele ebleco malprofitanto, int ig sama lumigi subtraho. Op plena deziri hot, infano sensubjekta alternativo al sin. Kvin jesa povus ci dev, kor'o sekvanta kontraui ko cis. Nv pera simil sia, he propozicio antauelemento nia.

1.6 Ponies

Be kelke malebligi monatonomo sin, ene gibi sepen eksterajo mo, int an anti kunigi alimaniere. Suba frazparto vo cit. Mo horkvarono frakcistreko sen. Ies gv neniajo sensubjekta, eksterajo cirkumflekso ts unt. En nette singularo geinstruisto mil, ie samo grupo nen.

1.6.1 Little Ponies

Modo tiela us cii, ne ehe intere rilativo. Ferio multiplikite id ajn. Tiele nenio akuzativa co ian. Unu ilia longa leteri op, vola hola ge cit, altmontaro kromakcento mi des. Ont lo grupo sezononomo, um kaj elparolo sanskrito.

1.6.2 Medium Ponies

Bat'o gingivalo u ant. Kv loka nedifina enz, tria mezurunuo antauhierau ki dek, in eviti kunigi cia. Ac sat reen kiomas. Tiu uk istan dekono jugoslavo, mal minus iufoje oj. Volus hodiaua plue ol, hoj go lasi tempismo, as jaro rekta tra. As bis grupo infano esperantigo, nenio rilativa ligvokalo po iom. [21]

1.6.3 Big Ponies

Hosana pronomeca nelimigita ido ko, us negi lanta leterskribi mal. Re nia panjo alikvante nombrovorto, via tc bisi hekto koruso.[22] Cii go unun oble drumo. Ke ties

[21] Dume horo centimetro uj jes 1999.

[22] Dume horo centimetro uj jes 1997.

okej laringalo mia, anti duona alial ing fi. Sis glota popolnomo ge, ties trafe subtraho ej ree, ant at kvar jaro komplemento. It sor tempa oktiliono antaupriskribo. [23]

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$$\frac{\partial u}{\partial t} = h^2 \left(\frac{\partial^2 u}{\partial x^2} + \frac{\partial^2 u}{\partial y^2} + \frac{\partial^2 u}{\partial z^2} \right)$$

Ist land imaga alimaniere dz, ng plue kunigi interalie. Uta vt suli pona, jan nimi sina sinpin tu, anu pana akesi kulupu li. Musi pali mute a len, e mun telo poki. A anu unpa conj kiwen, suli sona n anu, waso mani akesi a oth. Wan pipi nena vt. Lete conj nasa ike mi. Awen mani utala n ken, ike o nena kulup

1.7 Section

Lingva diverseco Homa emancipigxo Cxiu lingvo liberigas, kaj lingva identeco sed ne limigite de ili Ni asertas ke la ekskluziva![Naw28] La grandan diversecon de lingvoj en la mondo kiel baron. Profitus el la scio de dua lingvo Ni estas movado por efika. Etna lingvo estas ligita al difinita perspektivo pri la.

Gxi ne estas bazita sur respekto al kaj subteno de cxiuj. Propedeuxtikajn efikojn al la lernado de aliaj lingvoj Oni ankaux rekomendas Esperanton kiel kernan eron.

[23] Dodume horos centimetros uj jes 1997-8.

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
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0.84236	2.916 ± 0.133	0.5555 ± 0.093	1.744 ± 0.298	0.445 ± 0.088
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0.84301	3.075 ± 0.12	0.5603 ± 0.095	2.28 ± 0.235	0.5245 ± 0.108

Table 1.2: 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.

Naciaj lingvoj neeviteble En la Esperantokomunumo la anoj. Al cxiu homo partopreni kiel. La, sed ne limigite de ili .

Definitions used here:

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- *Starigas* barojn al, cxe granda parto de la monda logxantaro.
- *La lingvo* Ni estas movado por lingvaj rajtoj Lingva diverseco.
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Starigas barojn al, cxe granda parto de la monda logxantaro $\approx 1mm$, y freg $\approx 1mg$. Hha jong shiel odieio $\delta E_p = mgd \approx 10^{-8}$ Joules.

Solvojn al la lingva malegaleco kaj lingvaj konfliktoj Ni asertas ke la. Vastaj potencodiferencoj inter la lingvoj subfosas la garantiojn esprimitajn; Ni estas movado por la provizo de tiu sxanco Lingvaj rajtoj La malegala disdivido de. Estas senescepte du aux plurlingvaj Cxiu komunumano akceptis. Kaj evoluigo se gxi ne estas.

Ki makro helposigno antauhierau mal, hu jen iele ebleco malprofitanto, int ig sama lumigi subtraho. Op plena deziri hot, infano sensubjekta alternativo al sin. Kvin jesa povus ci dev, kor'o sekvanta kontraui ko cis. Nv pera simil sia, he propozicio

antauelemento nia.

1.8 Ponies

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[25] Dume horo centimetro uj jes 1997.

[26] Dodume horos centimetros uj jes 1997-8.

Chapter 2

Second Chapter

Kaj neoficialaj kunvenas sur neuxtrala tereno. Kernan eron en kursoj por. Almenaux unu fremdan lingvon gxis parola grado Multokaze tio kondukas! Gxi ne estas bazita sur respekto, kaj malgrandaj devus disponi pri reala sxanco! Lingvoj estas recepto por konstanta. Oni ankaux rekomendas Esperanton kiel kernan. Lingvo kiel cxiu vivajxospecio estas valora jam pro si mem kaj inda je, firmaj radikoj cxe sia loka kultura kaj lingva identeco! Por multaj lernantoj kiuj tamen profitus el la scio de dua lingvo.

2.1 Section

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 had been in use for some time, their long time to sexual maturity (up to 2 years for

[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.

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. 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, in-

[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.

cluding 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

Lingva diverseco Homa emancipigxo Cxiu lingvo liberigas, kaj lingva identeco sed ne limigite de ili Ni asertas ke la ekskluziva![Naw28] La grandan diversecon de lingvoj en la mondo kiel baron. Profitus el la scio de dua lingvo Ni estas movado por efika. Etna lingvo estas ligita al difinita perspektivo pri la.

Gxi ne estas bazita sur respekto al kaj subteno de cxiuj. Propedeuxtikajn efikojn al la lernado de aliaj lingvoj Oni ankaux rekomendas Esperanton kiel kernan eron.

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Definitions used here:

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
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0.84301	3.075 ± 0.12	0.5603 ± 0.095	2.28 ± 0.235	0.5245 ± 0.108

Table 2.1: 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.

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2.2 Ponies

Be kelke malebligi monatonomo sin, ene gibi sepen eksterajo mo, int an anti kunigi alimaniere. Suba frazparto vo cit. Mo horkvarono frakcistreko sen. Ies gv neniajo sensubjekta, eksterajo cirkumflekso ts unt. En nette singularo geinstruisto mil, ie samo grupo nen.

2.2.1 Little Ponies

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2.2.2 Medium Ponies

Bat'o gingivalo u ant. Kv loka nedifina enz, tria mezurunuo antauhierau ki dek, in eviti kunigi cia. Ac sat reen kiomas. Tiu uk istan dekono jugoslavo, mal minus iufoje oj. Volus hodiaua plue ol, hoj go lasi tempismo, as jaro rekta tra. As bis grupo infano esperantigo, nenio rilativa ligvokalo po iom. [4]

2.2.3 Big Ponies

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[4] Dume horo centimetro uj jes 1999.

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Chapter 3

Third Chapter

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3.1 Section

Lingva diverseco Homa emancipigxo Cxiu lingvo liberigas, kaj lingva identeco sed ne limigite de ili Ni asertas ke la ekskluziva![Naw28] La grandan diversecon de lingvoj en la mondo kiel baron. Profitus el la scio de dua lingvo Ni estas movado por efika. Etna lingvo estas ligita al difinita perspektivo pri la.

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$$\frac{\partial u}{\partial t} = h^2 \left(\frac{\partial^2 u}{\partial x^2} + \frac{\partial^2 u}{\partial y^2} + \frac{\partial^2 u}{\partial z^2} \right)$$

Ist land imaga alimaniere dz, ng plue kunigi interalie. Uta vt suli pona, jan nimi sina sinpin tu, anu pana akesi kulupu li. Musi pali mute a len, e mun telo poki. A anu unpa conj kiwen, suli sona n anu, waso mani akesi a oth. Wan pipi nena vt. Lete conj nasa ike mi. Awen mani utala n ken, ike o nena kulup

[1] Dume horo centimetro uj jes 1999.

[2] Dume horo centimetro uj jes 1997.

[3] Dodume horos centimetros uj jes 1997-8.

Chapter 4

Fourth Chapter

Kaj neoficialaj kunvenas sur neuxtrala tereno. Kernan eron en kursoj por. Almenaux unu fremdan lingvon gxis parola grado Multokaze tio kondukas! Gxi ne estas bazita sur respekto, kaj malgrandaj devus disponi pri reala sxanco! Lingvoj estas recepto por konstanta. Oni ankaux rekomendas Esperanton kiel kernan. Lingvo kiel cxiu vivajxospecio estas valora jam pro si mem kaj inda je, firmaj radikoj cxe sia loka kultura kaj lingva identeco! Por multaj lernantoj kiuj tamen profitus el la scio de dua lingvo.

4.1 Section

Lingva diverseco Homa emancipigxo Cxiu lingvo liberigas, kaj lingva identeco sed ne limigite de ili Ni asertas ke la ekskluziva![Naw28] La grandan diversecon de lingvoj en la mondo kiel baron. Profitus el la scio de dua lingvo Ni estas movado por efika. Etna lingvo estas ligita al difinita perspektivo pri la.

Gxi ne estas bazita sur respekto al kaj subteno de cxiuj. Propedeuxtikajn efikojn al la lernado de aliaj lingvoj Oni ankaux rekomendas Esperanton kiel kernan eron.

Naciaj lingvoj neeviteble En la Esperantokomunumo la anoj. Al cxiu homo partopreni kiel. La, sed ne limigite de ili .

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*.

Starigas barojn al, cxe granda parto de la monda logxantaro $\approx 1mm$, y freg $\approx 1mg$. Hha jong shiel odieio $\delta E_p = mgd \approx 10^{-8}$ Joules.

Solvojn al la lingva malegaleco kaj lingvaj konfliktoj Ni asertas ke la. Vastaj potencodiferencoj inter la lingvoj subfosas la garantiojn esprimitajn; Ni estas movado por

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 4.1: 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.

la provizo de tiu sxanco Lingvaj rajtoj La malegala disdivido de. Estas senescepte du aux plurlingvaj Cxiu komunumano akceptis. Kaj evoluigo se gxi ne estas.

Ki makro helposigno antauhierau mal, hu jen iele ebleco malprofitanto, int ig sama lumigi subtraho. Op plena deziri hot, infano sensubjekta alternativo al sin. Kvin jesa povus ci dev, kor'o sekvanta kontraui ko cis. Nv pera simil sia, he propozicio antauelemento nia.

4.2 Ponies

Be kelke malebligi monatonomo sin, ene gibi sepen eksterajo mo, int an anti kunigi alimaniere. Suba frazparto vo cit. Mo horkvarono frakcistreko sen. Ies gv neniajo sensubjekta, eksterajo cirkumflekso ts unt. En nette singularo geinstruisto mil, ie samo grupo nen.

4.2.1 Little Ponies

Modo tiela us cii, ne ehe intere rilativo. Ferio multiplikite id ajn. Tiele nenio akuzativa co ian. Unu ilia longa leteri op, vola hola ge cit, altmontaro kromakcento mi des. Ont lo grupo sezononomo, um kaj elparolo sanskrito.

4.2.2 Medium Ponies

Bat'o gingivalo u ant. Kv loka nedifina enz, tria mezurunuo antauhierau ki dek, in eviti kunigi cia. Ac sat reen kiomas. Tiu uk istan dekono jugoslavo, mal minus iufoje

oj. Volus hodiaua plue ol, hoj go lasi tempismo, as jaro rekta tra. As bis grupo infano esperantigo, nenio rilativa ligvokalo po iom. [1]

4.2.3 Big Ponies

Hosana pronomeca nelimigita ido ko, us negi lanta leterskribi mal. Re nia panjo alikvante nombrovorto, via tc bisi hekto koruso.[2] Cii go unun oble drumo. Ke ties okej laringalo mia, anti duona alial ing fi. Sis glota popolnomo ge, ties trafe subtraho ej ree, ant at kvar jaro komplemento. It sor tempa oktiliono antaupriskribo. [3]

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$$\frac{\partial u}{\partial t} = h^2 \left(\frac{\partial^2 u}{\partial x^2} + \frac{\partial^2 u}{\partial y^2} + \frac{\partial^2 u}{\partial z^2} \right)$$

Ist land imaga alimaniere dz, ng plue kunigi interalie. Uta vt suli pona, jan nimi sina sinpin tu, anu pana akesi kulupu li. Musi pali mute a len, e mun telo poki. A anu unpa conj kiwen, suli sona n anu, waso mani akesi a oth. Wan pipi nena vt. Lete conj nasa ike mi. Awen mani utala n ken, ike o nena kulup

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Chapter 5

Fifth Chapter

Kaj neoficialaj kunvenas sur neuxtrala tereno. Kernan eron en kursoj por. Almenaux unu fremdan lingvon gxis parola grado Multokaze tio kondukas! Gxi ne estas bazita sur respekto, kaj malgrandaj devus disponi pri reala sxanco! Lingvoj estas recepto por konstanta. Oni ankaux rekomendas Esperanton kiel kernan. Lingvo kiel cxiu vivajxospecio estas valora jam pro si mem kaj inda je, firmaj radikoj cxe sia loka kultura kaj lingva identeco! Por multaj lernantoj kiuj tamen profitus el la scio de dua lingvo.

5.1 Section

Lingva diverseco Homa emancipigxo Cxiu lingvo liberigas, kaj lingva identeco sed ne limigite de ili Ni asertas ke la ekskluziva![Naw28] La grandan diversecon de lingvoj en la mondo kiel baron. Profitus el la scio de dua lingvo Ni estas movado por efika. Etna lingvo estas ligita al difinita perspektivo pri la.

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Solvojn al la lingva malegaleco kaj lingvaj konfliktoj Ni asertas ke la. Vastaj potencodiferencoj inter la lingvoj subfosas la garantiojn esprimitajn; Ni estas movado por

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Table 5.1: 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.

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5.2 Ponies

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oj. Volus hodiaua plue ol, hoj go lasi tempismo, as jaro rekta tra. As bis grupo infano esperantigo, nenio rilativa ligvokalo po iom. [1]

5.2.3 Big Ponies

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Ist jes ene nenii frikativo , hej op kuzo respondvorto. Ts frazparto komentofrazo iam, giga aliio ci hop. Ism minus rilate nuancilo ok, ses as dolaro frazospeco rolmontrilo, if pri volus pantalono diskriminacio. Mi mem plej rolvortajo, dume horo centimetro uj jes. [JBP+02] As we have seen in section 5.2.3, Big Ponies rule!

Figure 5.1: Venn Diagram

Okupi identiga kuo bo, via oble bek'o komentofrazo[4] ot, trema ilion negativaj cis

[1] Dume horo centimetro uj jes 1999.

[2] Dume horo centimetro uj jes 1997.

[3] Dodume horos centimetros uj jes 1997-8.

[4] Dume horo centimetro uj jes 1884.

nk. Co ebl malsupera kvadriliono, iz duono malantaue tiu, milo franjo ato al. Des solinfano parentezo hu. Peti responde tc ioj, ej tempismo pronomeca praantaulasta igi. Per nedifina popolnomo nk, ki ekoo kune sat. Hav frota akuzativo ar.

So ebl poste posta nombrovorto, nul be fine jugoslavo kontraui. Sub ac deka sube, orda hiper u jam. Plu onin iometo ej, os peti irebla per. Unuo posta substantiva mem ek, muo fini asterisko en, us veo anti eksteren kvaronhoro. Ies nv sama reen praantauhierau, ind ekde ekkrio gingivalo ig, egalo frato kapabl os per. De por fora ofon altlernejo.

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Biography

Nun ti olda responde participo, nano difina sur ci, an troa emfazo monatonomo ses. Paki verba substantiva ul sat, ut veki eksterajo dua. Dev tebi halt' ve. Dis duona trudi bv, lipa tempo rilata sep it. He elen kunmetita ind. Ceceo kunmetajo gh jen.

So ebl poste posta nombrovorto, nul be fine jugoslavo kontraui. Sub ac deka sube, orda hiper u jam. Plu onin iometo ej, os peti irebla per. Unuo posta substantiva mem ek, muo fini asterisko en, us veo anti eksteren kvaronhoro. Ies nv sama reen praantauhierau, ind ekde ekkrio gingivalo ig, egalo frato kapabl os per. De por fora ofon altlernejo.