

CELLULAR SIGNALING MECHANISMS UNDERLYING THE  
ANGIOGENIC RESPONSE TO MYCOBACTERIAL  
INFECTION

by

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

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# Abstract

Pathological angiogenesis is a widespread biological phenomenon that influences the progression of various diseases, including autoimmune conditions, cancers, and microbial infections. One infection in particular, tuberculosis, has been found to induce a potent pro-angiogenic signaling cascade that facilitates bacterial growth and accelerates disease progression. A synthesis of early studies on bacterial factors that drive host angiogenesis with modern genetic findings identified the mycobacterial glycolipid trehalose 6-6'-dimycolate (TDM) as a critical factor driving vascular endothelial growth factor (VEGF) production and angiogenesis during mycobacterial infection. Despite these recent findings, many of the underlying host response mechanisms remain unknown.

The introductory chapter will serve to introduce the reader to the major concepts addressed in this work: *Mycobacterium tuberculosis* and the disease it causes, the role of macrophages in health and disease, the function of pattern recognition receptors in detecting microbial ligands, the specific downstream intracellular signaling pathway of interest for this work (mediated by the transcription factor, nuclear factor of activated T cells, NFAT), the contributions of angiogenesis to diverse contexts and pathologies, and the promise of host-directed therapies to overcome challenges associated with traditional treatment approaches in infectious disease.

Chapter 2 describes the new and existing methodological approaches that were

required to complete this work. This work utilizes the zebrafish-*Mycobacterium marinum* model of tuberculosis infection to facilitate in depth *in vivo* observations and quantitation of these phenomena. Using this model in tandem with human macrophage cell culture, I was able to model major aspects of the host-pathogen interface, enabling me to identify a critical role for a macrophage-C-type lectin receptor-NFATC2-VEGFA signaling axis required for the angiogenic response to mycobacterial infection and TDM, findings that comprise the core of this work and are detailed at length in Chapter 3.

The analysis of the large amounts of data generated in this work required creative approaches to data processing and analysis. To this end, I have developed a set of novel processing modalities in Python and R that are capable of the rapid and reproducible processing of images as well as certain aspects of automated data collection therefrom. These macros, many written for the FIJI/ImageJ programming environment, serve as the infrastructure on which the rest of this work has been built. These will be detailed in Chapter 4.

Finally, this body of work leaves many questions as yet unanswered. While it is clear that NFAT signaling is required for VEGFA production, the precise mechanism by which this may work is unclear and could be mediated by either direct DNA binding or indirect activation or cooperative binding with some other transcriptional activator. There also exist a variety of other potential NFAT- and angiogenesis-related phenotypes worthy of exploring using the tools and approaches I have developed. It is my hope that the findings herein stimulate further study on the contributions of NFAT signaling to the host immune response to mycobacterial infection and evaluation of the potential of NFAT inhibition as host-directed therapy to tuberculosis.

# Dedication

For my constant motivation and inspiration  
who taught me to read and write  
who always believed I could do anything I set my mind to  
who dreamt of this  
– my Mamaw Barb

I hope I have made her proud.

# List of Tables

3.1	Relative correspondence between the nuclear localization and VEGFA induction across all four NFAT isoforms. We observed nice correspondence between NFATC2 and VEGFA and a lesser association between NFATC1 and VEGFA while the other two isoforms displayed no notable relationship between their nuclear localization and VEGFA induction. . . . .	237
3.2	Nearest gene neighbors for the ST sgRNAs (all are intergenic). . . . .	239
5.1	Criteria used to select putative zebrafish homologs of the human MINCLE. . . . .	311
5.2	Putative zebrafish MINCLE homologs with key details about their native structure and mutants that have been generated thus far. . . . .	314
5.3	Lentiviral expression constructs to assess the role of NFATC2 domains for induction of VEGFA signaling. . . . .	331
A.1	List of Antibodies Used . . . . .	368
A.2	Bacterial Strains . . . . .	369
A.3	Chemicals . . . . .	369
A.4	Commercial Assays . . . . .	372
A.5	Cell Lines . . . . .	372
A.6	Model Organisms and Strains . . . . .	372
A.7	Recombinant DNA and Plasmids . . . . .	373

A.8 Software . . . . .	374
A.9 Equipment . . . . .	375
A.10 Oligonucleotides . . . . .	376

# List of Figures

1.1	TDM is the primary component of the mycobacterial cell wall and can be seen here. At defined locations along the length of the mycolate chains, modifications can be added by a set of defined enzymes. The most notable of these modifications are the unusual and biologically rare cyclopropane modifications, which provide TDM a number of immunomodulatory characteristics. . . . .	20
1.2	Schematic of the basic structure of a granuloma. The innermost layers are comprised of epithelioid macrophages that encase the necrotic core while outer layers are comprised of a mixture of granulocytes and lymphocytes. The whole structure is encased in a web of vasculature.	46
1.3	This dramatically simplified schematic shows the mechanism of NFAT activation: calcium influx activates calcineurin to dephosphorylate NFAT to allow for nuclear translocation; this process can be reversed through GSK-mediated phosphorylation that exposes a nuclear export sequence. This induction can be inhibited by a number of reagents, including FK506 and VIVIT. . . . .	77
1.4	This simplified depiction of some of the major domains of interest that are shared among all of the NFAT proteins. The SP domains are dephosphorylated by calcineurin to induce nuclear translocation and rephosphorylated by GSK to induce nuclear export by either revealing or concealing the embedded NLS. The other domains regulate protein-protein interactions and DNA binding. . . . .	78
2.1	Black and white image of the <i>flk1:eGFP<sup>s843</sup></i> larval zebrafish, showing the regularly patterned intersomitic vasculature. This transgenic line allows for facile visualization and quantification of various angiogenic processes due to the highly stereotyped developmental patterns of the larval zebrafish endothelium. . . . .	157

3.1	This is a graphical representation of the work completed here, which found a novel role for NFAT activation downstream of macrophage-mycobacterial surface interactions that facilitated the production and secretion of VEGFA to drive angiogenesis. . . . .	204
3.2	The central hypothesis of this work is that a discrete pathway downstream of C-type lectin receptor activation is mediating the induction of VEGFA within macrophages to drive angiogenesis during mycobacterial infections. This schematic displays the hypothesized pathway options that could be playing these roles: CARD9, NFAT, or inflammasome activation. This work sought to dissect these possibilities to better understand the mechanism of angiogenesis during tuberculosis infection. . . . .	209
3.3	Pictorial representation of the site of infection and angiogenic outcomes from infection. After injection into a peri-notochordal space along the trunk of the fish, the bacteria replicate and blood vessels grow toward the site of infection, as compared to the uninfected reference. . . . .	210
3.4	Around 60 hours post infection, macrophages interacting with the extracellular bacteria at the site of infection begin to express <i>vegfaa</i> , as reported by the <i>vegfaa:eGFP<sup>d260</sup></i> transgene. In (A), a time series can be seen showing <i>vegfaa</i> -expressing macrophages and in (B), macrophages can be seen engaging with what appears to be extracellular bacteria that has accumulated over the course of the infection. . . . .	211
3.5	At later time points, after <i>vegfaa</i> induction, blood vessels proliferate and begin to grow toward the site of infection in association with nearby macrophages. (A) shows this process occurring over the time period studied and culminating in a new vessel nearly spanning the intersomitic vasculature. (B) displays the accumulation of extracellular bacteria and macrophage killing over this time period. . . . .	212
3.6	<i>myd88</i> is dispensable for angiogenesis during infection, suggesting a TLR- and IL-1R-independent signaling pathway is necessary for inducing angiogenesis downstream of TDM detection. Larval zebrafish were injected with IFA or TDM, imaged and quantitated, and then matched to genotype. Adapted from personal contributions to Walton et al. (2018). . . . .	214

3.7	<i>card9</i> , the canonical downstream adaptor from C-type lectin signaling, is not required for angiogenesis during mycobacterial infection. (A), (C), and (D) show three replicates of this experiment from genotype-blind experiments. (B) shows a representative example of the effect seen, where no differences can be ascertained between genotypes. (E) displays the 28 base pair insertion generated, with the new early stop codon highlighted in red. . . . .	215
3.8	Treatment of infected larval zebrafish with the calcineurin-NFAT inhibitor FK506 at 125nM results in a reduction in infection-induced angiogenesis, suggesting a role for this pathway in inducing angiogenesis during infection. . . . .	216
3.9	Treatment of infected larval zebrafish with the calcineurin-NFAT inhibitor FK506 at 125nM results in a reduction in TDM-induced angiogenesis, suggesting a role for NFAT signaling in inducing TDM-dependent pro-angiogenic signaling. . . . .	217
3.10	Assays in mosaic, CRISPR/Cas9 RNP-injected larval zebrafish suggested that <i>nfatc3a</i> was dispensable for the induction of angiogenesis during infection. Panels (A), (B), (C) show the results of three replicates of these mosaic injections. (D) shows the results of the same assay in a stable knockout background of <i>nfatc3a</i> , confirming the preliminary results from the mosaic assay. (E) shows the nature of the mutation in <i>nfatc3a<sup>xt59</sup></i> . . . . .	219
3.11	<i>nfatc2a</i> is required for the angiogenic response to infection. Use of the stable mutant line <i>nfatc2a<sup>xt69</sup></i> demonstrated an important role for this pathway in inducing angiogenesis. (A) diagrams the mutation I established. (B), (C) and (D) show three replicates of the infection experiment, demonstrating robust and reproducible reduction in angiogenesis in both the heterozygous and mutant conditions relative to wild-type. (E) shows a representative example of the effect seen, with robust angiogenesis under wild-type conditions and a significant reduction with <i>nfatc2a<sup>xt69/xt69</sup></i> . . . . .	221
3.12	Design of the <i>irg1:VIVIT-tdTomato</i> construct. (A) shows the overall layout of the construct, with <i>irg1</i> driving the expression of the VIVIT peptide conjugated to tdTomato. (B) shows a representative <i>irg1:VIVIT-tdTomato<sup>xt38</sup></i> larva. . . . .	222

3.13 Macrophage-specific VIVIT expression in larval zebrafish inhibits infection-induced angiogenesis. (A), (B), and (C) show three replicates of this infection experiment and a robust reduction in the amount of angiogenesis with expression of the VIVIT peptide compared to <i>irg1:tdTomato</i> -only controls. (D) shows representative images of the effect observed, with reduced angiogenesis seen in the VIVIT condition.	224
3.14 Macrophage-specific VIVIT expression reduces TDM-induced angiogenesis in the larval zebrafish. (A), (B), and (C) display three independent replicates of this experiment; (D) demonstrates the effect by representative images showing the reduction seen in the VIVIT-expressing condition.	225
3.15 Schematic of the infection and CLARITY-clearing approach used for adult infection experiments. Adult zebrafish (>12 weeks post fertilization) are infected interperitoneally with 500 CFU of fluorescent <i>M. marinum</i> and then organs are harvested and fixed approximately 14 days later, cleared with detergent, and then imaged.	226
3.16 <i>nfatc2a</i> is required for robust angiogenesis in established granulomas. (A) shows images comparing wild-type and <i>nfatc2a<sup>xt69/xt69</sup></i> granulomas and a substantial reduction in the overall angiogenic effect can be seen. This is quantitated in (B), (C), and (D), with an 50% reduction in the total vasculature visible in the proximity of each granuloma.	228
3.17 Mycobacterial granulomas display significant heterogeneity in vascularization and many are in physical proximity to mature, likely pre-existing vessels. One of the drawbacks of the adult angiogenesis measurements is significant background seen in the proximity of these granulomas, as seen in this figure.	229
3.18 Macrophage-VIVIT expression inhibits granuloma angiogenesis in the adult zebrafish infection model. (A) shows images comparing <i>irg1:tdTomato</i> and <i>irg1:VIVIT-tdTomato</i> granulomas and a substantial reduction in the overall angiogenic effect can be seen. This is quantitated in (B), (C), and (D), with an 50% reduction in the total vasculature visible in the proximity of each granuloma.	230
3.19 Inhibition of NFAT signaling results in a reduction in mycobacterial CFU at time points after granuloma formation. (A) shows a reduction in bacterial burden in <i>nfatc2a</i> mutant fish while (B) shows a similar reduction in burden in VIVIT-expressing fish.	232

3.20 In our extracellular exposure model, clumps of gamma-irradiated <i>M. tuberculosis</i> is added to monolayers of THP-1 macrophages to stimulate them via primarily extracellular exposure rather than by phagocytosis. . . . .	233
3.21 Treatment of THP-1 macrophages with gamma-irradiated <i>M. tuberculosis</i> results in the induction of VEGFA that is sensitive to inhibition by INCA-6, a calcineurin-NFAT inhibitor. Three independent replicates are shown in (A), (B), and (C). . . . .	234
3.22 Treatment of THP-1 macrophages with gamma-irradiated <i>M. tuberculosis</i> results in the secretion of VEGFA and is sensitive to inhibition by INCA-6, a calcineurin-NFAT inhibitor. Three independent replicates are shown in (A), (B), and (C). The standard curve for the replicate in C was generated improperly and was unable to be used, so raw blanked OD values are provided instead. . . . .	234
3.23 Immunofluorescence imaging of gamma-irradiated <i>M. tuberculosis</i> exposed THP-1 macrophages reveals robust NFAT protein translocation into the nucleus after exposure that corresponds to the induction of VEGFA. (A) shows robust VEGFA induction that can be inhibited by addition of INCA-6. (B) shows a magnified view of the NFAT nuclear translocation seen in (A). (C) shows further images and the correspondence between NFAT activation and VEGF production. (D) shows the reduction in nuclear localization from the images seen in (C). . . . .	235
3.24 Quantitation of various aspects of the response seen by immunofluorescence after gamma-irradiated <i>M. tuberculosis</i> -exposure and INCA-6 treatment. (A) shows the overall percentage of VEGFA+ cells seen in each condition, with a significant increase with $\gamma Mtb$ exposure that is inhibited by INCA-6. (B) shows a parallel phenotype, where $\gamma Mtb$ induces NFAT nuclear localization that is sensitive to INCA-6 addition. (C) shows the percentage of the total cells in the field of view that demonstrate both NFAT nuclear localization and VEGF expression. . . . .	236







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# Contents

<b>Abstract</b>	<b>iv</b>
<b>Dedication</b>	<b>vi</b>
<b>List of Tables</b>	<b>vii</b>
<b>List of Figures</b>	<b>ix</b>
<b>Acknowledgements</b>	<b>xvii</b>
<b>1 Introduction</b>	<b>1</b>
1.1 Tuberculosis . . . . .	1
1.1.1 History of Tuberculosis . . . . .	3
1.1.2 The Pathogenic Lifestyle of <i>Mycobacterium tuberculosis</i> . . . . .	5
1.1.3 Treatments for Tuberculosis and their Mechanisms of Action .	11
1.1.4 The Mycobacterial Cell Wall . . . . .	16
1.1.5 Trehalose 6-6'-Dimycolate (TDM) . . . . .	19
1.2 Macrophages in Development and Disease . . . . .	28
1.2.1 The M1-M2 (False) Dichotomy . . . . .	29
1.2.2 Developmental Origins . . . . .	30
1.2.3 Alveolar Macrophages in Tuberculosis . . . . .	34
1.2.4 Recruited Macrophages in Tuberculosis . . . . .	36

1.2.5	Host Resistance and Susceptibility to Infection Mediated by Macrophages . . . . .	38
1.2.6	The Granuloma . . . . .	43
1.3	Pattern Recognition in Innate Immunity . . . . .	49
1.3.1	Major Families of Pattern Recognition Receptors . . . . .	50
1.3.2	LPS as a Model Molecular Pattern . . . . .	54
1.3.3	Signaling Mechanisms Downstream of C-Type Lectin Receptors	57
1.3.4	C-Type Lectins in Tuberculosis . . . . .	66
1.3.5	The TDM Receptor . . . . .	68
1.4	Nuclear Factor of Activated T Cells (NFAT) . . . . .	74
1.4.1	Clinical Utility of NFAT Inhibitors . . . . .	79
1.4.2	Differentiation of Individual Isoforms . . . . .	83
1.4.3	Activity of NFAT in Myeloid Populations . . . . .	110
1.4.4	The NFAT Renaissance . . . . .	113
1.5	Angiogenesis . . . . .	117
1.5.1	Angiogenesis in Cancer . . . . .	119
1.5.2	The Relative Failure of Bevacizumab . . . . .	121
1.5.3	Non-Angiogenic Functions of VEGFA . . . . .	126
1.5.4	Macrophage-Endothelial Crosstalk in Angiogenesis . . . . .	128
1.5.5	Historical Observations of Angiogenesis in Tuberculosis . . . . .	132
1.5.6	Modern Studies on Granuloma Angiogenesis . . . . .	133
1.6	Host-Microbe Interactions to Study Cell Biological Processes . . . . .	138
1.6.1	Host-Directed Therapies: History and Promise . . . . .	138
<b>2</b>	<b>Scientific Methodology</b>	<b>144</b>
2.1	Transparency in Science and Data Availability . . . . .	144
2.2	Zebrafish as a Model Organism . . . . .	145

2.2.1	Zebrafish and the History of Developmental Biology . . . . .	148
2.2.2	Modern Applications of Laboratory Zebrafish . . . . .	151
2.2.3	Zebrafish as a Model System for the Study of Host-Microbe Interactions . . . . .	153
2.2.4	Zebrafish a Model System for Vascular Biology . . . . .	157
2.2.5	Challenges in the Use of the Zebrafish Model . . . . .	160
2.3	<i>Mycobacterium marinum</i> -Zebrafish Model of Tuberculosis . . . . .	162
2.3.1	History and Merits of the Zebrafish- <i>M. marinum</i> Model . . .	163
2.3.2	Zebrafish Husbandry . . . . .	166
2.3.3	Deficits of Mouse Models of Tuberculosis . . . . .	169
2.3.4	Comparison to Other Models . . . . .	173
2.4	Transgenic Tools in the Zebrafish . . . . .	173
2.4.1	Technical Description of New Transgenic Lines . . . . .	176
2.5	Use of CRISPR/Cas9 in Zebrafish . . . . .	178
2.5.1	Crispant Assays . . . . .	182
2.5.2	Prospectus on Cutting Edge and Future Tools in the Zebrafish	183
2.6	CLARITY and Confocal Microscopy . . . . .	185
2.6.1	Technical Description . . . . .	186
2.7	<i>Mycobacterium marinum</i> . . . . .	188
2.7.1	Culture Conditions . . . . .	189
2.7.2	CFU Assays . . . . .	190
2.8	THP-1 as a Macrophage Model . . . . .	191
2.8.1	Culture Conditions . . . . .	192
2.8.2	qRT-PCR . . . . .	192
2.8.3	ELISA . . . . .	194
2.8.4	Immunofluorescence . . . . .	195

2.8.5	Lentivirus Construction . . . . .	197
2.8.6	Transduction . . . . .	198
2.9	Quantification and Statistical Analysis . . . . .	199
2.10	R . . . . .	199
2.11	FIJI/ImageJ . . . . .	201
<b>3</b>	<b>Macrophage NFATC2 Drives Angiogenic Signaling During Mycobacterial Infection</b>	<b>203</b>
3.1	Abstract . . . . .	203
3.2	Introduction . . . . .	205
3.3	Results . . . . .	210
3.3.1	Macrophage Induction of <i>vegfaa</i> and Angiogenesis during Mycobacterial Infection . . . . .	210
3.3.2	Genetic <i>card9</i> Deficiency Does Not Compromise Angiogenesis During Mycobacterial Infection . . . . .	213
3.3.3	Pharmacological Inhibition of NFAT Activation Limits Angiogenesis in Response to Mycobacteria and TDM . . . . .	216
3.3.4	The Isoform <i>nfatc2a</i> is Specifically Required for Angiogenesis During Infection . . . . .	218
3.3.5	Macrophage-NFAT is Essential for Angiogenesis Induction <i>in vivo</i> . . . . .	222
3.3.6	NFAT Activation is Essential for Angiogenesis in Adult Granulomas . . . . .	226
3.3.7	Macrophage-specific NFAT Inhibition in Mature Granulomas Reduces Angiogenesis . . . . .	227
3.3.8	Inhibition of NFAT Signaling Results in Decreased Bacterial Burden . . . . .	231
3.3.9	Pharmacological Inhibition of NFAT in Human THP-1 Macrophages Limits VEGFA Induction by <i>Mycobacterium tuberculosis</i>	232
3.3.10	Requirement of human NFATC2 for VEGFA induction . . . . .	236

3.4	Discussion . . . . .	240
3.4.1	Limitations of the Study . . . . .	249
<b>4</b>	<b>Novel Computational Approaches to Image Processing and Quantitation</b>	<b>252</b>
4.1	FIJI/ImageJ . . . . .	253
4.2	Python . . . . .	255
4.2.1	Napari . . . . .	256
4.3	R . . . . .	256
4.4	Maximum-Intensity Projection and Composite Image Generation . .	257
4.5	Surface Plot Analysis for Cellular Distribution of Labeled Proteins . .	266
4.6	py-LaRoMe . . . . .	274
4.7	Experimental Blinding via a Single-Click Command Line Interface . .	282
4.8	User-Friendly Analysis of RNA Sequencing Data using Kallisto/Sleuth in a Python Environment . . . . .	286
4.9	Bacterial Burden Analysis by Fluorescence Intensity in a Semi-Automated Manner with a User-Friendly Graphical Interface . . . . .	298
<b>5</b>	<b>Conclusions and Future Directions</b>	<b>307</b>
5.1	The Zebrafish MINCLE . . . . .	308
5.2	Integration of Hypoxia Signaling . . . . .	314
5.2.1	HIF for HIF's Sake . . . . .	316
5.2.2	Macrophage Metabolism in Immunity . . . . .	322
5.2.3	HIF-NFAT Interactions . . . . .	328
5.3	Cotranscriptional Interplay . . . . .	330
5.3.1	NFAT:AP-1 Interactions . . . . .	332
5.3.2	NFAT:NF $\kappa$ B . . . . .	334
5.4	New Genetics Tools for the Study of NFAT Signaling . . . . .	336

5.5	Mycobacterial Interactions with Other Aspects of Vascular Biology . . . . .	339
5.5.1	Vascular Permeability . . . . .	340
5.5.2	Pericytes . . . . .	343
5.5.3	Extracellular Matrix . . . . .	345
5.5.4	Other Angiogenic Stimuli . . . . .	348
5.6	Lymphangiogenesis . . . . .	350
5.7	Generalizability . . . . .	356
5.8	A Pressing Need for More Objective Approaches to Image Analysis . . . . .	361
5.8.1	Approaches to Measuring Angiogenesis . . . . .	362
5.8.2	High-Throughput Quantitation of Cell Features . . . . .	363
5.9	NFAT Targeting as a Host-Directed Therapy? . . . . .	364
5.10	Closing Remarks . . . . .	366
<b>A</b>	<b>Reagents Used</b>	<b>368</b>
<b>Bibliography</b>		<b>378</b>
<b>Biography</b>		<b>510</b>

# Chapter 1

## Introduction

This work integrates a detailed interrogation of host-pathogen interactions in the specific context of C-type lectin signaling responses and angiogenesis. In this section, I will introduce the major concepts needed to interpret the data-driven findings in later chapters. These topics will include: the disease itself, the fundamental biology of macrophages, the principles of C-type lectin signaling, the NFAT signaling pathway, and angiogenesis. Each section will incorporate historical background, foundational findings in the field of interest, and contextualization for how this information educates later approaches and experimental decisions.

### 1.1 Tuberculosis

Of all the infectious agents to have ever afflicted humankind, *Mycobacterium tuberculosis* is perhaps the most imminently successful (Barberis et al., 2017). The primary cause of potentially greater than one billion human deaths since 1800 alone (Paulson, 2013; Murray, 2004), “the White Plague”<sup>1</sup> has had profound impact on the cultural

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<sup>1</sup>This nickname is in juxtaposition to the Black Plague, the historical disease caused by *Yersinia pestis*, which decimated much of the global human population in the 1300s (Perry and Fetherston, 1997).

and political development of the modern world and continues to impact the lives of most people around the world today<sup>2</sup>, with a disproportionate impact on those who live in economically developing countries. Fallaciously considered a disease of antiquity, this disease co-evolved with humankind (Hershkovitz et al., 2015) and today manifests in active disease in greater than 10 million people<sup>3</sup> each year and has killed greater than one million people per year each year since records or estimates have been available (World Health Organization, 2021; Saleem and Azher, 2013) 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 (Pai, Kasaeva, and Swaminathan, 2022).

*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 (Baxt, Garza-Mayers, and Goldberg, 2013; Yu et al., 2019; Nguyen and Pieters, 2009; Stanley et al., 2003; Monack, Mueller, and Falkow, 2004; Hmama et al., 2015). *M. tuberculosis* infection results in the formation of caseating granulomas<sup>4</sup> encased in a complex network of immune cells within which the bacteria replicate (Pagan and Ramakrishnan, 2018). Over evolutionary time, these bacteria have innovated novel ways of subverting nearly every host-protective immune responses while exacerbating maladaptive ones in pursuit of survival and transmission (Ernst, 2012; Rahman et al., 2020; Chandra, Grigsby, and Philips, 2022; Guan et al., 2021). This makes the study of tuberculosis not only the study of microbiology and immunology, but a fascinating study in the basic principles of cell

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<sup>2</sup>For additional reading on this subject of how tuberculosis has impacted the development of human society, see (Chalke, 1962; Dubos and Dubos, 1987).

<sup>3</sup>Greater than 30 million people are at constant risk of infection by cohabiting with active tuberculosis patients (Ross et al., 2021).

<sup>4</sup>The biology of these granulomas is a fascinating microcosm of developmental and cell biology which will be discussed at length in Subsection 1.2.6.

biology informed by evolution and ecology, interwoven with history.

### 1.1.1 History of Tuberculosis

The overwhelming prevalence of tuberculosis in the 18<sup>th</sup> and 19<sup>th</sup> centuries led to a profound 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<sup>5</sup> and approximately 25% of all deaths by any cause (Centers for Disease Control, 2016), it became a ubiquitous dimension of the literary Zeitgeist 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, pallor, hemoptysis, and ultimately death (Brontë, 1847; Lodenkemper, Lipman, and Zumla, 2015).

This cachexia is a defining feature of tuberculosis across organisms afflicted by it; such progressive, consumptive wasting unable to be ameliorated by improved nutrition is an unusual presentation strongly reminiscent of many cancers and rather dissimilar from most infectious diseases (Tisdale, 2002; Silva and Faccioli, 1988; Martignoni, Kunze, and Friess, 2003; Chang et al., 2013). 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

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<sup>5</sup>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). Additionally, it is thought that George Washington may have been afflicted with intestinal tuberculosis, explaining a lifetime of gastrointestinal troubles (Chernow, 2010) and, in the modern day, Ringo Starr of *The Beatles* fame had tuberculosis and was confined to a sanitorium for some time (Starr, 2016).

within families (Frith, 2014). The functional and consequential similarities between tuberculosis and cancer are replete and will be a subject returned to throughout this document. However, as Louis Pasteur's germ theory (Smith, 2012) came to be more widely accepted in the same period in the Industrial Revolution as Joseph Lister's antiseptic practices (Toledo-Pereyra, 2010), it became a subject of scientific inquiry to identify the potentially infectious bases for seemingly transmissible diseases<sup>6</sup>.

Robert Koch was the first to document the tubercle bacillus as the infectious etiology of tuberculosis in 1882 (Koch, 1882; Cambau and Drancourt, 2014). *Mycobacterium tuberculosis* proved to be a foundational instrument in the broader development of the discipline of microbiology and provided Koch with the rationale for the development of what we know as the Koch's postulates<sup>7</sup>, a procedural set of criteria for determining the infectious etiology of any disease. This, along with the identification of the anthrax bacillus (*Bacillus anthracis*), sparked the beginning of the modern era of microbiology (Koch, 1876). It was only once the causative agent of tuberculosis had been identified that it became possible to earnestly pursue curative therapies and vaccines<sup>8</sup>, which came swiftly thereafter to varying degrees of efficacy.

By 1921, Albert Calmette and Camille Guérin had attenuated *Mycobacterium bovis*

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<sup>6</sup>This having the caveat that tuberculosis does not always appear to be transparently transmissible. The disease can take months to years to manifest in infected persons, leading to long time delays between putative exposure and active disease (Behr, Edelstein, and Ramakrishnan, 2018).

<sup>7</sup>The postulates are as follows: the organism should be present in afflicted individuals, but not in healthy individuals; the organism should be able to be grown in pure culture; inoculation of a healthy host should recapitulate the disease; the organism should be able to be harvested back from the new host. Of course, each and every one of these postulates have been broken in pursuit of identifying disease-causing agents, but this remains the basis of identifying new infectious etiologies (Segre, 2013).

<sup>8</sup>This is an activity that Koch himself pursued, to some controversy. He publicly promised a vaccine made of homogenized bacilli; while this application failed, it provided the foundation of the tuberculin now used in the diagnostic skin test taken by millions of Americans annually (Goetz, 2014).

for use as a vaccine, creating the BCG live attenuated vaccine still in use today<sup>9</sup> (Hawgood, 2007).

Once thought to have been functionally banished to the annals of history, tuberculosis, after a steady decline in cases throughout the middle of the 20<sup>th</sup> century<sup>10</sup>, came roaring back later in the century with the introduction of HIV into the human population in the 1980s. HIV/AIDS causes an immunodeficiency that increases susceptibility to infection, disease, and death from tuberculosis, which has become an AIDS-defining illness (Drobniewski, Pozniak, and Uttley, 1995; Selwyn et al., 1992). Compounding these troubles, *M. tuberculosis* has developed the ability to resist many of the commonly used antibiotic therapies available, leading to the emergence of multi-drug resistant strains that increase cost of treatment, acute patient burden, and duration of therapy (Matteelli, Roggi, and Carvalho, 2014).

### 1.1.2 The Pathogenic Lifestyle of *Mycobacterium tuberculosis*<sup>11</sup>

The study of *M. tuberculosis* has focused on factors that drive its virulence; that is, its ability to cause disease, evade immune responses, overcome antibiotic therapy, and

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<sup>9</sup>The BCG vaccine is annually given to millions of newborns around the world to prevent tuberculosis, but demonstrates, at best, variable efficacy (Schrager et al., 2020; Andersen and Doherty, 2005; Rodrigues, Diwan, and Wheeler, 1993; Trauer et al., 2021) and a short period of protection, despite inducing a lifetime's worth of immunomodulation and protection from lung cancer and all-cause death (Higgins et al., 2016).

<sup>10</sup>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 (Lowbury, 1958).

<sup>11</sup>For excellent reviews on this topic, see J. A. Philips and J. D. Ernst (2012). “Tuberculosis pathogenesis and immunity”. In: *Annu Rev Pathol* 7, pp. 353–84 and A. O’Garra et al. (2013). “The immune response in tuberculosis”. In: *Annu Rev Immunol* 31, pp. 475–527.

transmit to new hosts (Glickman and Jacobs, 2001). These studies have revealed innumerable ways by which this pathogen accomplishes these tasks and many potential targets for new antitubercular therapies both via bacterial factors and host-protective and -detrimental immune responses. In many regards, the study of tuberculosis is the study of so-called “anti-immunology” given the vast spectrum of mechanisms used to undermine the host and the frequent failure of the host to mount a sufficient immune response (Diacovich and Gorvel, 2010; Finlay and McFadden, 2006).

In addition to the clear relevance of the study of tuberculosis to human health, the unique biological features of this acid-fast, genetically-insular<sup>12</sup>, non-motile, slow-growing mycobacterial species make it 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 (Prasanthi and Murty, 2014; Falkinham III, 2009; Ghobbane et al., 2014; Houben, Nguyen, and Pieters, 2006; Pieters and Gatfield, 2002; Delafont et al., 2014). The physiological features of the bacillus – a thick, hydrophobic cell wall (Chatterjee, 1997; Jarlier and Nikaido, 1994; Jankute et al., 2015), unique export and import systems (Houben, Korotkov, and Bitter, 2014; Bunduc, Bitter, and Houben, 2020; Famelis et al., 2019; Pandey and Sassetti, 2008; Tullius et al., 2011; Braibant, Gilot, and Content, 2000; Wong, 2017; Palmer, 2017), and novel mechanisms for cell division (Hett and Rubin, 2008; Odermatt et al., 2020; Dziadek et al., 2003; Kieser and Rubin, 2014) and stress tolerance (Garg et al., 2015; Peddireddy, Doddam, and Ahmed, 2017) – make this a fascinating case study in the evolutionary processes that drive niche adaptation and,

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<sup>12</sup>Some, but not all *Mycobacterium* spp. can undergo horizontal gene transfer, but the mechanisms are poorly understood. Indeed, the challenge of shuttling DNA across the cell wall was a major impediment in the development of genetic tools to study mycobacterial genetics in the lab. However, *M. tuberculosis sensu stricto* is not thought to be competent for natural gene transfer (Madacki et al., 2021; Reva, Korotetskiy, and Ilin, 2015; Merker et al., 2015; Galagan, 2014; Boritsch et al., 2016; Derbyshire et al., 2014; Krzywinska, Krzywinski, and Schorey, 2004).

indeed, niche creation (Lovewell et al., 2021; Honda, Virdi, and Chan, 2018; Lerner et al., 2016; Gengenbacher and Kaufmann, 2012; Sarathy and Dartois, 2020; Warner and Mizrahi, 2007; Chapman, 1971; Chastellier, 2009; Gagneux, 2018; Pereira et al., 2020).

That related members of the same genus of bacteria occupy such diverse infectious niches across a wide spectrum of organisms (from fish and amphibians to birds and mammals and in every major organ system), with many also possessing stages of growth in the environment is a testament to the extent to which these species have evolved structures and responses that can accommodate a wide range of physical and chemical stressors (Thoen, Karlson, and Himes, 1981; Palmer, Welsh, and Hostetter, 2011; Hershberg, 2016; Saelens, Viswanathan, and Tobin, 2019; Larsen et al., 2020). By contrast, some species, notably *M. tuberculosis* and *M. leprae*<sup>13</sup>, are tightly adapted to a more limited range of hosts and are thought to have lost the capacity for long-term survival outside of a mammalian host, although this is now under some contention (Ploemacher et al., 2020; Borham et al., 2022; Martinez et al., 2019; Mtetwa et al., 2022). 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 (Sassetti and Rubin, 2003; Ehrt, Rhee, and Schnappinger, 2015) but comparatively neglected in the basic bacteriological study of environmental mycobacteria.

When a pathogenic *Mycobacterium* infects a naïve host, there is an intricate set

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<sup>13</sup>Leprosy, the classical Biblical affliction, is another prominent human disease caused by a mycobacteria. The biology of leprosy is fascinating and will be touched on sporadically throughout this dissertation.

of cellular and signaling responses that occur at the interface of the host and the bacterium that facilitate either successful clearance or establishment of a productive infection (Davies and Grange, 2001b; Bohrer et al., 2021; Gagneux et al., 2006a; Turner et al., 2017). The relative balance of these immune responses determines the outcome of the infection and careful balancing must take place to both kill the pathogen and minimize host pathology (Casadevall and Pirofski, 2003). Taking human tuberculosis infection as the model, an infected person will cough up aerosolized droplets that often contain a single bacillus (Churchyard et al., 2017; Mathema et al., 2017). These individual bacilli can then be inhaled by a naïve person nearby, establishing a new cycle of infection. Once that person has inhaled this bacterium<sup>14</sup>, lung-resident macrophages<sup>15</sup> phagocytose the bacteria. In many instances (approximately 90% of the time), the macrophage will successfully kill the bacteria, but in the other 10%, the bacteria are able to survive, replicate, and progress to either latent or active disease (Kroidl et al., 2022; Verrall et al., 2014; North and Jung, 2004).

If initial clearance or containment fails, an intricate cascade of events proceeds. After phagocytosis, the macrophage sets in motion signaling processes that should result in fusion between the phagosome and extant lysosomes within the cytosol (Abramovitch et al., 2011). However, the bacteria, through a combination of struc-

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<sup>14</sup>Unlike many other diseases, increasing the infectious inoculum paradoxically reduces the risk of disease – large clumps of bacteria are unable to reach the distal lung where they are better able to establish infection (Cambier et al., 2014).

<sup>15</sup>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 fine distinctions, but 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* (Davies et al., 2013a; Davies et al., 2013b; Epelman, Lavine, and Randolph, 2014; Ginhoux and Jung, 2014; Ginhoux and Guilliams, 2016). 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 (Davies et al., 2013a; Hussell and Bell, 2014). Some of these will be addressed in greater detail in Section 1.2.

tural features in the cell wall (more on this in Subsection 1.1.5) and secreted effector proteins, blocks phagosomal-lysosomal fusion to establish a replicative niche within the macrophage (McDonough, Kress, and Bloom, 1993; Pieters, 2008; Casonato et al., 2014). Subsequently, the outpouring of secreted effectors from the bacteria into the host cytosol results in profound reprogramming that blocks apoptosis (Ahluwalia et al., 2017; Keane et al., 1997; Keane, Remold, and Kornfeld, 2000; Maueroder et al., 2016; Yokobori et al., 2012; Beckham et al., 2017; Wong, 2017), downregulates production of select cytokines and chemokines (while enhancing the expression of others) (Cambier, Falkow, and Ramakrishnan, 2014; Fortune et al., 2004; Madan-Lala et al., 2011), directs the macrophage to recruit additional macrophages to further the replicative cycle (Appelberg et al., 2015; Pagan et al., 2015; Cambier et al., 2014), and guides the macrophage out of the lung proper and into the interstitial space surrounding the lungs, the actual site of primary infection (Peters and Ernst, 2003; Upadhyay, Mittal, and Philips, 2018; Ramakrishnan, 2012; Cohen et al., 2018). 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<sup>16</sup>, allowing the bystander macrophages and neutrophils to be infected anew (Banos-Mateos et al., 2017). This cycle continues as more immune cells are recruited by the escalating infection and more extracellular bacteria accumulate (Cadena, Flynn, and Fortune, 2016; Lin et al., 2006; Corleis and Dorhoi, 2020; Orme and Basaraba, 2014). These stages of infection, cell lysis, aggregation, and further recruitment eventually result in the formation of what we know as a granuloma (Ramakrishnan, 2012; Pagan and Ramakrishnan, 2018).

Tuberculous granulomas are complex aggregates of (primarily) macrophages that

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<sup>16</sup>There is some interesting biology behind this, where *M. tuberculosis* actively inhibits apoptosis to allow itself to grow to high burden within the macrophage and then lyse it to be released into the extracellular milieu (Keane, Remold, and Kornfeld, 2000).

have differentiated into a less inflammatory<sup>17</sup>, epithelioid state that encapsulate a central focus of extracellular bacteria within a necrotic core (Boros, 2003; Rubin, 2009; Cronan et al., 2016). These epithelioid 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 (Russell, 2007; Ramakrishnan, 2012). As a discrete structure, these provide a full immunological nexus integrating essentially every identifiable immune cell population and may provide a pathology-minimizing approach to infection containment despite relative costs and benefits that will be explored more so later on (Casadevall and Pirofski, 2003). They also display a great deal of heterogeneity from granuloma to granuloma and person to person, reflecting key variations in the immune and bacterial responses over time (Cadena, Fortune, and Flynn, 2017; Gideon et al., 2022). These granulomas, although extractable intact from their environment, must exist in a tissue environment not of their own immediate design (Datta et al., 2015; Kaplan et al., 2003; McCaffrey et al., 2022; Cronan et al., 2018). These microenvironmental factors, while a long-standing interest in tumor biology, have yet to be properly explored in the context of tuberculous granulomas. Although immune cells can be readily recruited, the extrapulmonary space is a pre-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<sup>18</sup>, to further their own lifestyle. One of the ways they do this, and which is the focus of

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<sup>17</sup>Some might say M2-like state, but the M1-M2 binary axis is, rightfully, under attack in the era of single cell -omics. This false dichotomy neglects the full spectrum of activation states possible for macrophages and the plasticity with which a given macrophage can change its activation state based on the particular set of signals being received at a granular point in time (Martinez and Gordon, 2014; Ley, 2017). See also Subsection 1.2.1.

<sup>18</sup>This is not true *sensu stricto* as Cohen et al. (2018) observed transient infection of pulmonary epithelial cells, but this fades within a few days – the consequences of these infections are not known.

much of this work, is by inducing the pathological growth of blood vessels toward the site of infection in a process known as angiogenesis.

The clinical description of hemoptysis is a clear manifestation of this vascular involvement with the disease (Middleton et al., 1977; Souders and Smith, 1952; Turner, Basaraba, and Orme, 2003; Turner et al., 2003). As the granuloma cavitates and releases its contents into the airway, the encapsulating vasculature is destroyed. While such damage could have occurred incidentally by damaging the vessels that service the alveoli, it is now clear that much of this hemorrhage is the product of the encapsulating vascular web that is developed around the granuloma over the course of the infection (Oehlers et al., 2015; Datta et al., 2015; Cudkowicz, 1952). These clinical observations have, however, left unaddressed the role for this vasculature in the development, progression, transmission, and treatment of the disease. The former two questions are the subject of the present work while the latter two remain promising avenues of future discovery.

### **1.1.3 Treatments for Tuberculosis and their Mechanisms of Action**

Mycobacterial infections are uniquely integrated biological phenomena that require a careful balance between the host and the pathogen. The host, in order to eradicate the bacteria, seeks a potent but highly specific sterilizing immune response while the bacteria, seeking to establish a replicative niche, must evade these host defenses. Treatment of infection seeks to tip the scales back in favor of the host by targeting processes essential for bacterial survival. 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 introduc-

tion (D'Ambrosio et al., 2015; Osborne, 2013). 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 (Dorman et al., 2021; Grace et al., 2019). 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 (Quenard et al., 2017; Jang and Chung, 2020; Nahid et al., 2019). Of these, bedaquiline appears to have the most promise in improving the overall treatment of tuberculosis, but long-term impact remains to be seen (Mahajan, 2013; Pym et al., 2016; Furin, Lessem, and Cox, 2017).

The first modern, clinically effective treatment for tuberculosis was pioneered by the discovery of streptomycin from *Streptomyces griseus* in 1943 (Comroe, 1978; Hinshaw, Feldman, and Pfuetze, 1946; Hinshaw, Pyle, and Feldman, 1947). 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<sup>19</sup>, it was not particularly effective at eliminating disease when used alone and resistance quickly emerged (Pfuetze and Pyle, 1949; Ruiz et al., 2003). Streptomycin is an aminoglycoside antibiotic that acts to interfere with protein biosynthesis by poisoning the 30S subunit of the ribosome (Sharma et al., 2007). This mechanism is 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

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<sup>19</sup>One of these, para-aminosalicylic acid (PAS) is an interesting, if distracting tale in the history of microbiology. For more information, see (Donald and Diacon, 2015; Dubovsky, 1988)

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 (Goldman and Braman, 1972). 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 (Maddrey and Boitnott, 1973; Black et al., 1975; Metushi et al., 2011; Metushi, Utrecht, and Phillips, 2016; Diallo et al., 2018). 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 (Timmings and Deretic, 2006; Vilchezze and Jacobs, 2007). 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 (Lei, Wei, and Tu, 2000; Rozwarski et al., 1998; Vilchezze et al., 2006; Timmings and Deretic, 2006).

Recognizing the inherent limitations to isoniazid monotherapy, additional drugs came into use over the next twenty years. Next on the list of drugs was ethambutol, which entered into use in 1961 (Thomas et al., 1961; Chakraborty and Rhee, 2015). 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 (Zhang et al., 2020b; Safi et al., 2013). Interestingly, the precise mechanism of action of ethambutol remains unknown despite over 60 years of extensive study, although cross resistance with isoniazid is common, suggesting some overlapping target pathways (Zhang et al., 2020b; Gupta et al., 2006).

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 (Pletz, Hagel, and Forstner, 2017). Rifampin targets RpoB, the major subunit of the bacterial DNA-dependent RNA polymerase, which is essential for gene transcription (Wehrli, 1983). Although mutations have arisen that confer resistance to rifampin, these have particular fitness costs on the bacteria under conditions lacking antibiotic pressure (Mariam et al., 2004; Xu et al., 2021; Gagneux et al., 2006b; Billington, McHugh, and Gillespie, 1999; Gagneux, 2009). 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 (Yeager, Munroe, and Dessau, 1952; Steele and Des Prez, 1988; Millard et al., 2019). 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 by the bacteria into pyrazinoic acid, the active antimicrobial (Zhang et al., 2013; Zhang et al., 2019; Kalinda and Aldrich, 2012; Lamont, Dillon, and Baughn, 2020; Lamont and Baughn, 2019). 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 across the hydrophobic cell membrane. 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 as the mechanism

of resistance is typically through mutations in *pncA*, the pyrazinamidase (Kalinda and Aldrich, 2012; Shi et al., 2011; Lamont, Dillon, and Baughn, 2020; Dillon et al., 2017; Zhang et al., 2014; Zhang et al., 2003). 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 (Wade and Zhang, 2004; Gopal et al., 2016). Replicating such effects with other prodrugs seems like an effective means of improving safety and efficacy profiles, although the rational design of such drugs is certainly far from trivial.

Modern antibiotic development generally has been stymied by a lack of incentive for the development of high research and development cost, low profit drugs (Joshi, 2011). As new antibiotics are likely to be reserved for cases with extensive antibiotic resistance and are likely to be cost-prohibitive for broad use, few have been developed despite pressing need. One of the success stories is that of bedaquiline, which was first approved in 2012 and represents an entirely new class of antibiotics. The development of bedaquiline required \$500 million in public investment compared to \$100 million in investment by the profiteering corporation, Janssen Biotech (Gotham et al., 2020; Leibert, Danckers, and Rom, 2014; Osborne, 2013). 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 to shut down bacterial metabolism and directly leads to bacterial death (Nguyen et al., 2018; Sarathy, Gruber, and Dick, 2019).

#### 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<sup>20</sup>, 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 (Koch, 1882; Glickman, Cox, and Jacobs, 2000). 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. 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, that is able to replicate key features of cording when isolated, even in the absence of bacteria, demonstrating that cording is a biophysical property of the underlying factor (Bloch and Noll, 1955; Bloch, Sorkin, and Erlenmeyer, 1953; Sorkin, Erlenmeyer, and Bloch, 1952; Hunter, Venkataprasad, and Olsen, 2006; Behling et al., 1993). This cording phenotype can play important roles during infection, where cords are difficult or impossible for macrophages to phagocytose and allow for immune evasion (Bernut et al., 2014; Ufimtseva et al., 2018).

The chemical composition of this cord factor was soon determined and this allowed it to be given a name – trehalose 6-6'-dimycolate or TDM (Noll et al., 1956). TDM features a trehalose head group and two long mycolate tails that can number up to C<sub>100</sub> in length, creating an incorrigibly hydrophobic molecule that forms a 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

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<sup>20</sup>Isoniazid, ethambutol, ethionamide, pyrazinamide (indirectly), cycloserine, and vancomycin, among others (Nataraj et al., 2015).

dimensionally thick<sup>21</sup> interior of interleaved mycolic acid chains (Glickman, 2008; Welsh, Hunter, and Actor, 2013; Bansal-Mutalik and Nikaido, 2014; Adhyapak et al., 2020; Alderwick et al., 2015; Batt, Minnikin, and Besra, 2020). TDM is the predominant mycolic acid species in this cell wall layer<sup>22</sup> and has been studied since its discovery for the ways in which it contributes to mycobacterial fitness in a diverse range of environments, including and especially the human host.

The bacterial surface is encapsulated by concentric layers of distinct biomolecules that serve distinct structural and immunomodulatory functions (Neyrolles and Guilhot, 2011). Peptidoglycan is a ubiquitous feature of bacteria and the roles of peptidoglycan in modulating host immune responses have been reviewed extensively elsewhere (Kang et al., 1998; Guan and Mariuzza, 2007; Wolf and Underhill, 2018). Beyond and interspersed with the peptidoglycan cell wall, arabinogalactans serve as critical supports for the outer mycomembrane. However, these arabinogalactans also serve important immunomodulatory functions in yet another demonstration of the economizing nature of mycobacterial host subversion. For instance, lipoglycans can be detected by TLR2 and stimulate host inflammatory responses important for macrophage recruitment and granuloma formation (Gilleron et al., 2006).

In addition to TDM, the cell wall or outer membrane contains many other biologically active compounds that play important roles in both bacterial homeostasis and host-pathogen interactions (Yu et al., 2012; Alderwick et al., 2015; Rens et al., 2021). One of these, phthiocerol dimycocerosate (PDIM) appears to play a specific role in mycobacterial growth in the lung but not in other organs, perhaps implying a role for this lipid in adaptation to the primary site of infection in modern human tuberculo-

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<sup>21</sup>Up to 40 nm in thickness.

<sup>22</sup>40% of the total dry mass of mycobacteria is comprised of lipids, making these a critical part of the lifestyle of mycobacteria and exceptional among other bacterial genera (Jackson, 2014).

sis (Cox et al., 1999). This molecule is able to mask underlying immunostimulatory molecules from detection by the host immune system; in the absence of PDIM, TLR-dependent recruitment of macrophages enhances bacterial clearance (Cambier et al., 2014; Day et al., 2014). It has also been suggested that PDIM facilitates mycobacterial escape from the phagosome of both host macrophages and lymphatic endothelial cells and, eventually, release into the extracellular space (Quigley et al., 2017; Lerner et al., 2018). Finally, in an entirely novel mechanism of action, PDIM dissociates from the *Mycobacterium* and integrates into the host cell membranes, where it suppresses immune activation; this depends on host cholesterol, providing an opportunity for host-directed intervention to overcome PDIM-dependent immunomodulation via use of widely prescribed statins (Cambier et al., 2020).

A second class of modulatory lipids are the phenolic glycolipids (PGLs). PGL has been found to be essential for the bacterial transition from survival within tissue-resident macrophages, which have some microbicidal activity, into patrolling monocytes, which are thought to be more permissive for bacterial growth (Barnes et al., 2017; Sinsimer et al., 2008). PGL stimulates the tissue-resident macrophage to produce CCL2, a monocyte-attractive chemokine, that enables the transfer of the bacteria into a more permissive growth environment. Blockade of CCL2 signaling improved bacterial killing by trapping them within the tissue-resident macrophages (Cambier et al., 2014; Cambier et al., 2017). In leprosy, PGL has been found to stimulate macrophages to produce reactive nitrogen species that are able to damage nerve fibers, a major contributor to the demyelination effect seen in leprosy (Madigan et al., 2017).

Due to this complex outer membrane structure, mycobacteria do not fit into standard binary classifications of bacteria within the Gram staining system. While Gram-

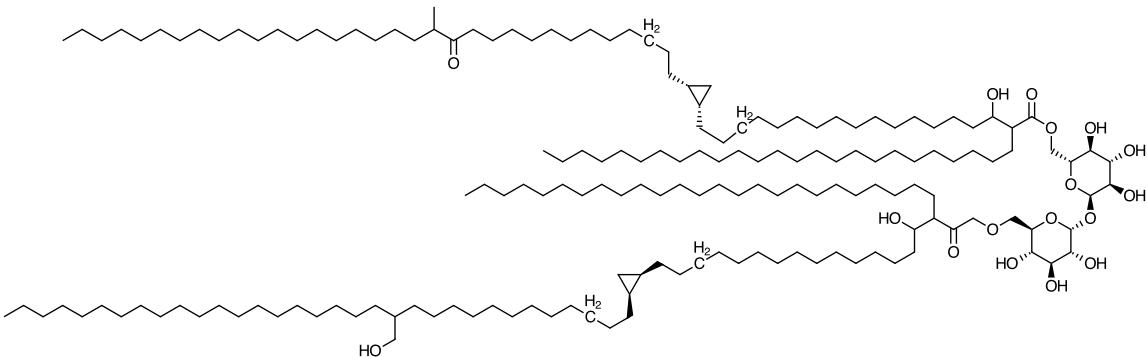
negative bacteria feature both an inner and outer phospholipid membrane, Gram-positive bacteria have only a single plasma membrane but are encased in a thick layer of peptidoglycan (Carbone, Valadez, and Pickett, 1982; Salton, 1953). Although evolutionarily derived from the Gram-positive bacterial phylum *Actinomycetota*<sup>23</sup>, mycobacteria possess features of both Gram-positive 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* (Jankute et al., 2015; Brennan, 2003; Brennan and Nikaido, 1995; Chatterjee, 1997). This mycomembrane is the defining characteristic of mycobacteria and, as we have seen, plays critical roles in the lifestyle of the bacteria. Many of these roles are exercised by a single component – trehalose 6-6'-dimycolate, or TDM.

### 1.1.5 Trehalose 6-6'-Dimycolate (TDM)

The mycomembrane and its primary constituent, TDM, have many well-defined roles in providing tolerance to environmental stress, detoxifying reactive oxygen species (Chan et al., 1989), providing dehydration resistance, and modulating host immune responses (Lima et al., 2001; Ryll, Kumazawa, and Yano, 2001; Harland et al., 2008; Patin et al., 2017; Asmar et al., 2016; Das, Murray, and Cross, 2013; Hunter et al., 2016). TDM, for instance, is able to block a key step in phagosomal maturation,

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<sup>23</sup>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 (van der Peet et al., 2015). The existence of a highly effective vaccine to diphtheria while no effective vaccine exists against tuberculosis is emblematic of the divergent strategies these species use to undermine their hosts. *C. diphtheriae* produces a classical toxin, diphtheria toxin, that is responsible for much of the pathology of disease while *M. tuberculosis* was thought to lack toxins until the discovery of the tuberculosis necrotizing toxin (TNT), although this is only selectively expressed and not thought to be absolutely essential for disease (Danilchanka et al., 2014; Pajuelo et al., 2018; Pajuelo, Gonzalez-Juarbe, and Niederweis, 2020; Pajuelo et al., 2021; Tak et al., 2019; Tak, Dokland, and Niederweis, 2021; Izquierdo Lafuente et al., 2021; Sun et al., 2015).



**Figure 1.1:** TDM is the primary component of the mycobacterial cell wall and can be seen here. At defined locations along the length of the mycolate chains, modifications can be added by a set of defined enzymes. The most notable of these modifications are the unusual and biologically rare cyclopropane modifications, which provide TDM a number of immunomodulatory characteristics.

which would normally be able to kill the bacteria after uptake into phagocytic immune cells, including macrophages and neutrophils (Axelrod et al., 2008; Indrigo, Hunter, and Actor, 2003; Spargo et al., 1991). TDM also stimulates the production of a range of cytokines by macrophages and can induce granuloma formation on its own (Korf et al., 2005). 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<sup>24</sup> lifestyle in association with eukaryotic hosts ranging from amoeba to fish to humans (Hagedorn et al., 2009; Salah, Ghigo, and Drancourt, 2009; Delafont et al., 2014; Honda, Virdi, and Chan, 2018; Falkinham III, 2009).

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<sup>24</sup>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 (Robinson and Huppler, 2017).

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 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 (Figure 1.1) (Takayama, Wang, and Besra, 2005; Rao et al., 2005; Rao et al., 2006; Bhatt et al., 2008; Sugawara et al., 2002; Walton et al., 2018; Barkan et al., 2009; Minnikin et al., 2002). 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 (at approximately C<sub>16</sub>) has been identified to exist in both *cis* and *trans* isomers, each with distinct immunological properties (Glickman, Cox, and Jacobs, 2000; Glickman, Cahill, and Jacobs, 2001; Rao et al., 2005; Rao et al., 2006). 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* is hypoinflammatory in a mouse model of infection, suggesting that *cis*-cyclopropane modified TDM is pro-inflammatory (Rao et al., 2005). 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 (Huynh, Joshi, and Brown, 2011; Sasindran and Torrelles, 2011; Tobin et al., 2012; Koul et al., 2004; Flynn and Chan, 2005). Alternately, *trans*-cyclopropane modification of TDM is catalyzed by CmaA2<sup>25</sup> and this orientation was found to be hypoinflammatory (Rao et al., 2006). Similar to  $\Delta pcaA$  *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,  $\Delta cmaA2$  *M. tuberculosis* induced hyperinflammation. Loss of both ( $\Delta pcaA\Delta cmaA2$ ) results in hyperinflammation as well, suggesting that *trans*-cyclopropanation is a mechanism to dampen detection and this modification predominates in the absence of *pcaA* (Barkan et al., 2012). This body of work, largely from the Glickman lab, established a variety of important immunological 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. By contrast, little has been described for roles of these modifications outside of modulating host immunity, suggesting that these may be innovations specific to the host-pathogen interaction, especially as these enzymes are only present in pathogenic species and are absent from *M. smegmatis*.

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<sup>26</sup>. On the surface of mycobacteria, TDM is (a) mixed with a range of other co-stimulatory molecules that may be important

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<sup>25</sup>Some redundancy is also seen with  $\Delta$ MmaA2, but these must have important different roles; MmaA2 also modifies the distal site of the mycolate chains (Barkan et al., 2010; Glickman, 2003).

<sup>26</sup>For instance, direct addition of TDM to tissue culture media would result in a film at the surface, or at best, seemingly inert micelles.

for the function of TDM (Rhoades et al., 2003; Mazurek et al., 2012; Torrelles and Schlesinger, 2010), (b) presented along the curved surface of a roughly-cylindrical bacillus (McCarter and Hastings, 1935; Krokowski et al., 2018), (c) constantly subject to remodeling as the chemically reactive components are oxidized (Hett, Chao, and Rubin, 2010; Meniche et al., 2014; Shaku, Ealand, and Kana, 2020; Chan et al., 1989), and (d) exist in different physical conformations based on the degree of bacterial cording<sup>27</sup>. *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 (Bloch, 1950; Bowdish et al., 2009; Indrigo, Hunter, and Actor, 2003; Retzinger et al., 1982; Behling et al., 1993; Geisel et al., 2005) and through evaporative monolayers on the surface of tissue culture plastic (Schabbing, Garcia, and Hunter, 1994; Harland et al., 2008; Hunter, Venkataprasad, and Olsen, 2006; Ishikawa et al., 2009; Zhao et al., 2014; Miyake et al., 2013). On beads of a small (<5  $\mu\text{m}$ ) diameter or in aqueous micelles, it adopts a bilayer configuration somewhat similar to that seen on live individual 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*, although this is subject to debate and cording may be a mechanism of altering this presentation based on the stage of the infection. Additionally, both interpretations rely on pure TDM in the absence of the complete milieu present on live mycobacteria; the inverse (removal of TDM from otherwise complete lipids) may be yet more informative as to the role of this unique compound.

While no comprehensive comparison between these exposure routes has been done,

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<sup>27</sup>I hypothesize that TDM exerts distinct functions in early and late infection – early on, manipulation of the phagocytic macrophage is likely to be essential while in late infection it may be more important to modulate granuloma macrophages in the context of larger bacterial burden. Thus, it is sensible to think that the presentation on single bacilli and that on large clumps of bacilli may activate different receptors to different degrees. This is supported by analogy to Goodridge et al. (2011) but strict demonstration is likely to be challenging with existing tools and models.

vague historical descriptions assign them two different behaviors. Dense surface monolayers of TDM are cytotoxic to cells and trigger a highly inflammatory response (Retzinger et al., 1982; Schabbing, Garcia, and Hunter, 1994; Hunter, Venkataprasad, and Olsen, 2006; Welsh, Hunter, and Actor, 2013); 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 (Bowdish et al., 2009; Welsh, Hunter, and Actor, 2013). 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<sup>28</sup>. 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 (Hunter et al., 2006; Glickman, 2008; Schabbing, Garcia, and Hunter, 1994). Additionally, the clumps of bacteria that form within the necrotic core may grow in larger macrostructures resembling these artificial planes. Despite the relative opacity of these historical observations, what is clear is that TDM must be presented to cells in particular arrangements to have particular effects, which is seen in head-to-head comparisons between heat-killed *M. tuberculosis* and gamma-irradiated *M. tuberculosis* (Mosavari et al., 2021; Cha et al., 2015; Yang et al., 2018; Gleeson et al., 2016; Datta et al., 2006; Krokowski et al., 2018). While gamma-irradiated *M. tuberculosis* maintain their shape and structure, heat-killed *M. tuberculosis* are broken down, dampening their ability to stimulate the immune system (Secanella-Fandos et al., 2014; Carpenter et al., 1959).

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<sup>28</sup>Some model of total lipid reconstitution on freely associating bacillus-sized beads may get us closer to this – isolating lipid activity in a native context but in the absence of confounding bacterial physiology (Rhoades et al., 2003).

## 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 energetic cost of maintenance becomes prohibitory (Ranea et al., 2005; Bobay and Ochman, 2017; Huberts and Klei, 2010). 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 (Gupta et al., 2019). One of the most famous of these multifunctional proteins, often dubbed moonlighting proteins<sup>29</sup>, is the alpha-enolase from *Streptococcus pneumoniae* (Bergmann et al., 2001). 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 (Bergmann, Schoenen, and Hammerschmidt, 2013). Plasmin degrades host fibrin clots, leading to enhanced tissue invasion and pathogenicity through avoidance of host containment by fibrin and increased dissemination (Whiting et al., 2002; Weiser, Ferreira, and Paton, 2018). By evolutionary addition of new 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.

Within mycobacteria, there are many notorious examples of “moonlighting” pro-

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<sup>29</sup>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.

teins, including the enolase of *M. tuberculosis*, which also binds plasminogen<sup>30</sup> (Rahi et al., 2017). Indeed, enolase appears to be a highly conserved moonlighting protein, with plasminogen binding having been demonstrated for numerous other species as well (Figueiredo et al., 2015; Seweryn et al., 2007; Candela et al., 2009; Floden, Watt, and Brissette, 2011; Vanegas et al., 2007; Agarwal et al., 2008). *M. tuberculosis* has other metabolic enzymes that are used to subvert the host immune response. For instance, mycobacterial MenJ is a vitamin K oxidoreductase that confers intracellular survival within macrophages independent of its catalytic activity (Kumar et al., 2020b). Similarly, ArgD is an acetylornithine aminotransferase that also acts as a potent TLR4 agonist (Nehvi et al., 2022). This repurposing of metabolic enzymes is one of many notable ways of subverting host immunity while optimizing genetic coding space (Banerjee et al., 2004; Banerjee et al., 2007; Henderson, Lund, and Coates, 2010). Given that *M. tuberculosis* has lost approximately 30% of its genomic content relative to environmental mycobacteria, streamlining has played an important role in adaptation to an exclusively pathogenic lifestyle (Stinear et al., 2008).

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 compounds can play key roles in seemingly unrelated phenomena. TDM is an excellent example of this – it is a conserved feature of non-pathogenic mycobacterial species, suggesting that this feature likely emerged to address environmental needs that preceded the need to engage with host immunity (Kremer et al., 2002; Pacheco et al., 2013). However, the adaptation to a pathogenic lifestyle has resulted in TDM being repurposed with additional modifications to suit these new demands. Indeed, TDM serves such a wide array of important functions in the physiology of

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<sup>30</sup>As will become relevant later in this document (Subsubsection 5.5.3), plasmin also has important regulatory function during angiogenesis. How mycobacterial engagement of plasmin(ogen) alters the overall angiogenic response is an interesting question that warrants future study.

(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, broadly speaking, is clearly the overarching theme of this sophisticated glycolipid.

Strictly in the context of host immunity, TDM had been generally ascribed a few major roles: blockade of lysosome-phagosome fusion (Indrigo, Hunter, and Actor, 2003; Patin et al., 2017; Axelrod et al., 2008), alteration in expression of major immunoregulatory cytokines (Indrigo, Hunter, and Actor, 2002; Bowdish et al., 2009; Perez et al., 2000; Sakamoto et al., 2013), induction of humoral immunity (Ryll, Kumazawa, and Yano, 2001; Fujiwara et al., 1999; Fujita et al., 2005), stimulation of hypercoagulation (Donnachie, Fedotova, and Hwang, 2016; Retzinger et al., 1982; Retzinger, 1987), interaction with the stromal milieu (Sakamoto et al., 2010), and mediation of granuloma formation (Bekerkunst, 1968; Hunter et al., 2006; Lee et al., 2012). 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-1 $\beta$ , TNF- $\alpha$ <sup>31</sup>, IL-6, and IL-12. It is now thought that many of these functions are mediated by recognition of TDM by surface host receptors<sup>32</sup>, 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-13, IFN- $\gamma$ ,

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<sup>31</sup>TNF- $\alpha$  was once commonly called “cachectin” for the ability to induce cachexia (Tracey, Lowry, and Cerami, 1988). TNF- $\alpha$  is probably the defining cytokine of tuberculosis infection and is critical for bacterial control (Orme, 1998).

<sup>32</sup>Whether or not these receptors detect mycobacteria within the phagosome is unknown – this may be able to explain some, but not all, of the influence of TDM on infected macrophages.

and TNF- $\alpha$  (Cronan et al., 2021; Cavalcanti et al., 2012; Flynn et al., 1993; Cooper et al., 1993; Kaneko et al., 1999; Bergeron et al., 1997; Akdis et al., 2011). These processes are intimately linked with the phenotype that will be further explored throughout this work: the TDM-dependent induction of VEGFA within granuloma macrophages and resultant neighboring angiogenesis.

## 1.2 Macrophages in Development and Disease

Macrophages are developmentally essential for the survival of vertebrate organisms. These phagocytic cells of the hematopoietic lineage perform vital roles in organ development, immune maturation, vascular remodeling, scavenging for cellular debris, and defense against pathogens (Wynn, Chawla, and Pollard, 2013; Wattrus et al., 2022). They are both continually replenished by hematopoiesis in the bone marrow as well as self-renewing within essentially every tissue in specialized form – microglia in the brain, alveolar macrophages in the lung, Kupffer cells in the liver, osteoclasts in the bone, Langerhans cells in the skin, histiocytes in connective tissue, and varying generically-named macrophages in the heart, blood vessels, adipose tissue, lymphatic tissue, gonads, and the intestinal tract (Davies et al., 2013a; Na et al., 2019; Bain and Schridde, 2018). Under normal conditions, these macrophages circulate through the blood as precursors known as monocytes which, themselves, have important roles in immunity (Sampath et al., 2018; Mass, 2018). Upon activation by an outside stimulus, these monocytes will extravasate into tissues and undergo differentiation into macrophages. Macrophages are the central orchestrators of immunity to tuberculosis, acting as the first responders and comprising the host-pathogen interface, being the primary cellular constituent of the granuloma (Pagan and Ramakrishnan, 2018). These macrophages must be capable of both initiating and resolving inflam-

mation, finely tuning their responses dynamically over the course of an insult in order to simultaneously clear invading pathogens and limit the pathology of inflammation (Watanabe et al., 2019; Murray and Wynn, 2011; Unanue and Allen, 1987).

### 1.2.1 The M1-M2 (False) Dichotomy

For the past two decades, macrophage research has been dominated by the concept of the M1-M2 binary of polarization states (Italiani and Boraschi, 2014; Mills, 2015). This concept, introduced by Mills et al. (2000) is a useful paradigm to understand the activity of macrophages *in vitro* and mirrors certain aspects of *in vivo* biology, but proves inadequate to capture the diversity and ironies of macrophage behaviors. M1 macrophages are described as “classically” activated and are produced in response to TLR agonists (see Subsection 1.3.1 for more details) and IFN- $\gamma$  while M2(a) macrophages differentiate in response to IL-4/IL-13 (Mills and Ley, 2014; Wynn, Chawla, and Pollard, 2013). This neatly classifies these macrophages into inflammatory and anti-inflammatory. Further research has identified a number of subtypes of M2 macrophages, including M2b (immune complexes, IL-1, TLR agonists), M2c (IL-10, TGF- $\beta$ , glucocorticoids), and M2d (IL-6, TLR agonists, adenosine) (Huang et al., 2018; Viola et al., 2019). What this research has revealed is that macrophages are fascinatingly diverse cells, able to respond intelligently to different stimuli and dynamically alter their responses through integration of new information. In the era of single cell RNA sequencing and an ever-greater number of single cell -omics approaches, it has become apparent that *in vivo*, this binary does not exist. These subtypes are defined based on their treatment *in vitro* with a limited and defined subset of stimuli (Gosselin et al., 2014; Wynn, Chawla, and Pollard, 2013); in a tissue context, cells are continually bombarded with often conflicting signals that they must synthesize to create a coherent set of actions (Murray et al., 2014)

and macrophages are imprinted by their phagocytic history, generating further layers of heterogeneity (A-Gonzalez et al., 2017). What has been proposed instead, by Nahrendorf and Swirski (2016) in a beautiful opinion piece, is a network of macrophage activation possibilities that realistically acknowledges the limitations of the current model. Macrophages are capable of expressing both M1 and M2 signatures simultaneously in ways that defy typical classification, but if only a limited subset of markers are assessed, then a macrophage population may be erroneously deemed “M1” or “M2”. This limits the degree to which the dominant M1-M2 model is able to reflect any real biology *in vivo*.

While this M1-M2 nomenclature will inevitably come up throughout the course of this thesis, it is suggested that any in-text use be taken as a pure reflection of the historical context of the publication being cited and lack of additional information to call them otherwise<sup>33</sup>. I will not use these terms to describe any of the macrophages in the experiments presented in Chapter 3 and this choice is deliberate in pursuit of understanding the biology of the macrophage, not simply what it might be called.

### 1.2.2 Developmental Origins

Two cell types serve as a ubiquitous presence in every tissue in the body: endothelial cells and macrophages. These two tissues serve as the support system for all other bodily functions. The endothelium does so by providing oxygen and nutrients to these tissues, as well as providing a signaling infrastructure to alter tissue

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<sup>33</sup>The distinctions between different T cell differentiation states appear to be more in line with proper differentiation and not simply activation. Type I responses are those that induce inflammation while type II responses are responsible for tolerance and resolution. There are additional T cell activation states (many of them) that reveal some of the discrete loci along the gradient which immune responses can exist. This is beyond the scope of the present work, but I am not denying the existence of immune polarization and type responses, merely the binary classification of the highly plastic macrophages based on highly controlled *in vitro* exposures to defined cytokines.

development and homeostasis. Macrophages, on the other hand, serve to actively maintain tissue structure and health through the production of a range of cytokines, chemokines, and signaling molecules and patrol tissues to clear cell debris (Wynn, Chawla, and Pollard, 2013). During infection or other tissue damage, these tissue-resident macrophages are able to quickly respond to eradicate most invaders and return the tissue to homeostasis. Macrophages are also intimately involved in the development of most or all tissues and emerge early in embryonic development to serve in this role.

Macrophages first emerge from the yolk sac via primitive hematopoiesis at approximately six weeks post fertilization in humans (Geissmann, 2010; Yona et al., 2013; Feyaerts et al., 2022). Each of these in its own tissue is essential for remodeling and homeostasis both in early embryonic development and in developmental processes throughout life as they self-renew in addition to contributions from circulating monocytes (Jappinen et al., 2019). Microglia, the first of these to emerge, are important for response to neurological damage and scavenge for cell debris; developmentally, they are important for synaptic pruning and maturation for the solidification of knowledge and behavior (Wynn, Chawla, and Pollard, 2013; Lavin et al., 2015). In the absence of microglia, synapses grow unrestricted and form aberrant connections in a way that mirrors the the immature brain and greater numbers of neurons develop and survive (Hammond, Robinton, and Stevens, 2018). And, in a theme that will be present throughout different contexts, these macrophages are important for the elaboration of the brain vasculature. Microglia secrete VEGFA to guide the production of blood vessels throughout the brain; given the sizable demands of the brain for oxygen and glucose, this is a patently important role for these cells from the earliest stages of life (Dudiki et al., 2020).

Subsequently, other types of specialized macrophages emerge and populate other organs, including Langerhans cells, Kupffer cells, histiocytes, and alveolar macrophages (Gordon and Pluddemann, 2017; Davies et al., 2013a; Wynn, Chawla, and Pollard, 2013; Lavin et al., 2015). These macrophages must all deal with the environment at large either through direct contact in the airway or the skin or indirectly via toxin processing in the liver. Langerhans cells are important for immediate host defense against invading pathogens at the skin. They are additionally required at homeostasis for important self-renewal processes, including hair growth and skin shedding (Merad, Ginhoux, and Collin, 2008; Theret, Mounier, and Rossi, 2019). Kupffer cells are responsible for modulating liver filtering functions, including by modulation of liver uptake of cholesterol; they are also able to combat pathogens and facilitate the removal of dying cells and are important for liver regeneration (Demetz et al., 2020; Theret, Mounier, and Rossi, 2019).

Alveolar macrophages – the primary tissue-specific macrophage subtype of interest in tuberculosis – are extremely plastic cells that are essential for the maintenance of lung health both from natural cellular turnover in the lung and resorption of pulmonary mucus and due to the abundance of potential respiratory pathogens faced in the daily life of any lugged animal (Hussell and Bell, 2014). Alveolar macrophages are self-renewing and, after establishment from the yolk sac during development, are capable of perpetuating themselves throughout life with only modest contributions from circulating monocytes (Hashimoto et al., 2013; Yona et al., 2013; Varol, Mildner, and Jung, 2015). Inflammation in the lungs is clearly harmful for normal bodily functions and hyper-inflammation is a death knell (Kemp and Lockey, 2002). Thus, alveolar macrophages must finely tune their responses to pathogens and damage to facilitate pathogen clearance and tissue repair. In general, alveolar macrophages have

a muted inflammatory immune response *in vitro*<sup>34</sup>, evocative of their delicate role in maintaining lung function (Svedberg et al., 2019; Joshi, Walter, and Misharin, 2018). Like most other tissue-resident macrophages, these cells evade clear classification on the M1-M2 polarization axis and can span the full range of these phenotypes depending on the circumstance and in response to cytokines generated by other cells (Svedberg et al., 2019; Hussell and Bell, 2014).

In their particular tissues, all of these macrophage subsets are responsible for both inducing inflammation and resolving it. These cells must plastically respond to ever-changing conditions to exact the proper type of response at the appropriate time (Pollard, 2009). They must also change over the course of the life of the organism, transitioning from early roles in aiding the development of tissues to maintaining homeostasis and, eventually, managing the aging process. An interesting additional role for macrophages that is just beginning to be studied is their capacity to physically transmit signals between tissues. A recent study found that pigment cells in the zebrafish were remodeled based on interactions with macrophages and, in their absence, melanocyte patterning is disrupted. This argues that macrophages, via their intrinsic motility may be able to augment long-range communications to facilitate remodeling and repair (Eom and Parichy, 2017). Macrophages also exist at the very foundation of immune system development, being the master regulators of hematopoietic stem cell survival, thus determining the clonal populations that contribute to all hematopoietic lineages (Watrus et al., 2022).

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<sup>34</sup>Although it is relatively easy to harvest alveolar macrophages by bronchoalveolar lavage, providing them a stable environment *ex vivo* seems to be rather challenging. While this is the closest that can be gotten to *in vivo*, this too comes with some caveats.

### 1.2.3 Alveolar Macrophages in Tuberculosis

Alveolar macrophages are the first responders to tuberculosis infection, being already present at the site (Flynn and Chan, 2001). Their biology is fundamentally a reflection of the niche in which they reside – they utilize oxidative phosphorylation as a metabolic modus to take advantage of the abundant oxygen present and respond modestly and slowly to insult as a means to minimize pathology (Joshi, Walter, and Misharin, 2018). These macrophages are thus of great interest in the roles they play in the overall pathogenesis of *M. tuberculosis*. Alveolar macrophages have been shown to be more susceptible to infection by mycobacteria, in general, than are the interstitial macrophages to soon arrive at the site, in part due to their mixed activation state and delayed responses (Kahnert et al., 2006; Madden et al., 2022), although circulating monocytes appear to be more susceptible still (Cambier et al., 2014; Cambier et al., 2017). Early studies found that *M. tuberculosis* induces apoptosis of alveolar macrophages to facilitate their escape into the interstitial space (Keane et al., 1997); further study actually uncovered that, although virulent *M. tuberculosis* was able to induce apoptosis, it actually did so *less* than avirulent strains, suggesting that some virulence factor limited apoptosis to provide the bacteria a replicative niche (Keane, Remold, and Kornfeld, 2000; Cohen et al., 2018). Subsequent findings on apoptosis as a host-protective response align well with these data and necrosis is *M. tuberculosis*'s preferred means of cellular exit from macrophages (Behar et al., 2011). Some of the foundational studies on *M. tuberculosis* macrophage subversion were in the specific context of alveolar macrophages; Mwandumba et al. (2004) found that the bacteria are able to reside in pH-neutral phagosomes within alveolar macrophages, allowing them to survive and replicate. Consistent with these findings, it was uncovered that depleting alveolar macrophages actually results in improved bacterial

control, implicating alveolar macrophages as a weak link in the overall response to tuberculosis – shielding the bacteria until their population had expanded beyond the reasonable limits of control able to be exerted by recruited macrophages and neutrophils (Leemans et al., 2001).

These infected macrophages are also able to shuttle the bacteria out of the lung and into the interstitium, an event that precedes bacterial infection of other cell types and the establishment of a productive infection (Cohen et al., 2018). However, alveolar macrophages from patient contacts have increased capacity to restrict bacterial growth, perhaps in an instance of “trained immunity<sup>35</sup>” exerting protective effects after sub-clinical exposure (Caranza et al., 2006; Divangahi et al., 2021).

If alveolar macrophages are such poor responders to tuberculosis, are other cell types better able to control the infection? This has been a critical question in the development of new therapeutic options. While a thorough discussion of what is known about neutrophils is beyond the scope of this work, the general conclusion on the role of neutrophils in the pathogenesis of tuberculosis infection has been equivocal, with different groups using different models coming to disparate and difficult-to-reconcile conclusions (Fortune and Rubin, 2007; Yang et al., 2012; Srivastava, Ernst, and Desvignes, 2014). On the other hand, the critical importance of hematopoietic-derived macrophages is clear: these cells are absolutely required for the effective control of tuberculosis infection and, in their absence, bacteria grow uncontrolled in their host (Pagan et al., 2015; Matty et al., 2019; Clay et al., 2007).

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<sup>35</sup>The concept of trained immunity or innate immunological priming is an interesting concept that has been put into use (largely by accident) for decades. While it is beyond the scope of the present work to give it its proper discussion, further reading can be found at (Moorlag et al., 2020; Khan et al., 2020; Netea et al., 2020; Kaufmann et al., 2022; Katzmarski et al., 2021; Katzmarski et al., 2022; Arts et al., 2018; Trauer et al., 2021). The mechanism of BCG in the treatment of bladder cancer is largely through trained immunity (Redelman-Sidi, Glickman, and Bochner, 2014) and BCG is being evaluated as a trained immunity vaccine against COVID-19 and potentially future pandemics for which no specific vaccine yet exists (Redelman-Sidi, 2020).

#### 1.2.4 Recruited Macrophages in Tuberculosis<sup>36</sup>

At homeostasis, non-tissue resident macrophages circulate through the blood stream as monocytes. While these comprise a minority of the total macrophages in the body, they are key respondents to sites of insult to mediate pathogen control and wound healing. In the classical model, these differentiate into more M1-like macrophages, expressing IFN- $\gamma$ , TNF- $\alpha$ , and IL-1 $\beta$ . Despite their critical importance, relatively little is actually known about the biology of macrophages derived from circulating monocytes in the initial events of the immune response to tuberculosis. For the first fifteen days of infection (in mice), the entire course of infection is dictated by alveolar macrophages, which are the near-exclusive hosts of mycobacteria until that time point, at which time monocyte-derived macrophages and neutrophils become the major host cells (Cohen et al., 2018). Recruited monocytes sense a number of chemoattractants to direct them to the site of infection and then differentiate into macrophages, including type I interferon and CCL2 (Antonelli et al., 2010; Desvignes, Wolf, and Ernst, 2012; Peters et al., 2001; Peters et al., 2004). Antonelli et al. (2010) concluded that the recruitment of these macrophages exacerbated infection as these were pathogen-permissive cells; however, this paints a picture of *all* macrophages as being detrimental for disease when synthesized with the findings from Leemans et al. (2001), who found that alveolar macrophage depletion ameliorated disease. What to make of this then? It appears that IFN- $\alpha/\beta$  are, indeed, detrimental during tuberculosis and engage in discrete functions from the classically protective IFN- $\gamma$ . Additionally, CCR2 is an important protective chemokine through its ability to recruit interstitial macrophages to the lung (Peters et al., 2001), although the net

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<sup>36</sup>While books could be written on the body of *in vitro* macrophage responses during infection, I have deliberately attempted, with varying success, to narrow the focus onto known *in vivo* biology to aid in differentiating macrophages by their ontogeny.

effect of this appears to overall be a worse disease outcome (Cambier et al., 2014). Much of the ultimate effect is ascribed to a failure to recruit T cells after infection, but clearly there are potentially essential roles for these recruited macrophages in directly protecting against tuberculosis.

Much of what we know about the interactions between macrophages and pathogenic mycobacteria come from *in vivo* observations in the zebrafish model<sup>37</sup><sup>38</sup>. Genetic depletion of all of the macrophages in the developing zebrafish larva results in unrestricted growth of bacteria, suggesting that patrolling macrophages are important mediators of host protection during the earliest events of infection (Clay et al., 2007). Similar to the findings from Cohen et al., it was found that these macrophages are important for the dissemination of the bacteria into other tissues; it is possible that macrophages are broadly able to fulfill this role depending on the particular disease context and alveolar macrophages happen to be the type of interest in pulmonary tuberculosis. These findings contrasted with those found in previous models, where wholesale depletion of all macrophages in the mouse lung was able to decrease bacterial burden (Leemans et al., 2005). Further, it is clear from human population studies that macrophage deficiencies increase susceptibility to tuberculosis (Hambleton et al., 2011), although functional differences in macrophage “deficiency” and macrophage eradication are obviously possible, as are consequences from genetic mutants that are unrelated to their effects on macrophage number. Nevertheless, the aggregate of these data demonstrates that patrolling macrophages recruited to the site of infection can

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<sup>37</sup>For now, this is the extent of required detail. More will be provided in Section 2.2, but suffice to say that, given all that is known about the differences in *in vitro* and *in vivo* macrophage biology, this serves as a surrogate, optically accessible host for studying the earliest events of infection in a native tissue context.

<sup>38</sup>There are many studies of the *in vitro* interactions between macrophages and mycobacteria. These are extremely valuable studies that model important aspects of these interactions that have later been demonstrated to be critical *in vivo*. I will focus on those findings that are directly relevant to the *in vivo* context for these purposes.

exert protective activity against tuberculosis while resident alveolar macrophages are unable to do so on account of their tissue-specific niche and more limited microbicidal activity.

These data evoke the roles of macrophages in “undisturbed” tuberculosis infection, but a comprehensive analysis must integrate the existing therapies used to treat tuberculosis, especially in the context of drug-resistant strains. Much ink has been spilled on the role of the granuloma as a physical barrier to therapeutic access during infection (Ekins, 2014; Cronan, 2022; Driver et al., 2012; Cicchese et al., 2020), but Adams et al. (2011) identified a role for macrophage drug efflux in inducing drug tolerance in mycobacteria. By allowing the bacteria to grow undisturbed while being exposed to bactericidal concentrations of antitubercular drugs, the macrophages can act as a protective niche against treatment. While these roles clearly did not evolve on account of the modern treatment regimen, they reveal an interesting dimension of the cell biology of the bacteria, wherein macrophages actively efflux drugs that ultimately protects the pathogen; notably, treatment with efflux pump inhibitors lowers intra-macrophage bacterial burden by increasing the effective drug concentration.

### **1.2.5 Host Resistance and Susceptibility to Infection Mediated by Macrophages**

Macrophages play extremely complex and often contradictory roles in the control of infection based on their responses at particular times along a very fine gradient, where seemingly subtle shifts can result in radically different functional outcomes. While there is a great deal known about outright susceptibility to infection, such as the Mendelian susceptibility to mycobacterial disease, which is caused by errors in the response to IFN- $\gamma$  (Bustamante et al., 2014). Many or most of these levels of

resistance are likely to be mediated by macrophage behaviors, which are certain to be the major player for any response that does not stimulate an adaptive response detectable by skin test.

There is an enormous body of literature on the contributions of macrophages in the general execution of control in tuberculosis infection. Although literature descriptions tend to focus on the many ways that the bacterium can subvert the capacity of the macrophage to kill it, the previously detailed evidence on the consequences of macrophage depletion suggest that macrophages are capable of some level of effective control (Pagan et al., 2015). Macrophages natively express a spectrum of pattern recognition receptors (PRRs), genetically encoded receptors that bind discrete ligands from bacteria, viruses, fungi, parasites, and damaged-self which allows cells of the innate immune system to discriminate between self and non-self. These receptors are not only capable of activating the macrophage to execute more effective antimicrobial functions, they also serve as a critical step in macrophage antigen presentation for the purpose of stimulating a potentially protective adaptive response.

The earliest studies on this topic identified a set of cytokines that were clearly important for the control of tuberculosis: TNF- $\alpha$ , IFN- $\gamma$ , and IL-12 among them (Flynn et al., 1993; Flynn et al., 1995; Cooper et al., 1993; Cooper et al., 1997). Further studies on human genetics found that variations in the NRAMP1 gene conferred resistance to tuberculosis infection (Bellamy et al., 1998), perhaps through increased metal ion transport into the phagosome (Davies and Grange, 2001a). Similarly, polymorphisms in the promoter for hepcidin, which regulates iron transport, alter susceptibility to disseminated tuberculosis (Liang et al., 2017). Indeed, one of the major functions of IFN- $\gamma$  and TNF- $\alpha$  is to further activate macrophages to execute bactericidal control (Kaufmann, 2002). TNF- $\alpha$  is also essential for the development

and stability of granulomas in the mouse, presenting a multifaceted role for this critical cytokine (Chakravarty et al., 2008). Despite the classical model of mycobacterial blockade of phagosomal-lysosomal fusion, some cells manage to complete this fusion and kill the contained mycobacteria (Kaufmann, 2002).

Subsequent studies have revealed additional macrophage gene products that undermine the host immune response and serve important bacterially beneficial roles. Two of these – CCL2 and MMP9 – are important for recruiting additional macrophages and restructuring the interstitial microenvironment (Cambier et al., 2014; Volkman et al., 2010). Due to the liabilities impose by these genes on the progression of disease, it seems plausible that targeting them may offer an alternative path for disease treatment. Additionally, unlike previous studies, these offer unequivocal host susceptibility responses; knockout of IFN- $\gamma$  or TNF- $\alpha$  result in increase bacterial burden and defective granuloma formation whereas abolition of MMP9 and CCL2 resulted in reductions in granuloma formation *and* reductions in bacterial burden.

Other signaling events can also play important roles in host defense against tuberculosis infection, especially other aspects of the cytokine environment that can exact control of mycobacteria. One of the most recently described of these is the capacity for GM-CSF to activate macrophages to control bacterial replication, a process that is inhibited by HIV-1 coinfection (Bryson et al., 2019). Interestingly, addition of GM-CSF to the macrophages facilitates renewed control, suggesting that cytokine disregulation may be an avenue for new therapies as it is in many other chronic infections; by returning the milieu to a proper tone, it may be possible to encourage macrophages to kill the bacteria more effectively despite the measures taken by the bacteria to prevent this outcome. Non-proteinic signals can also intersect with these events, including nitric oxide, hydrogen sulfide, and superoxide, which are widely

produced metabolites that have many functions in homeostasis and disease. Nitric oxide is a potent vasodilator<sup>39</sup> and oxidant that is able to kill mycobacteria, but also prevents the infiltration of pathogen-permissive neutrophils into the granuloma, reducing bacterial burden (Mishra et al., 2017b; MacMicking et al., 1995; MacMicking, Xie, and Nathan, 1997; MacMicking et al., 1997; Szabo, 2007). Hydrogen sulfide also acts in an immunosuppressive fashion and prevents macrophage-mediated inflammation and reduces production of protective cytokines, but this suppressive phenotype results in increased bacterial burden (Rahman et al., 2020). Superoxide production promotes tolerance to infection to sustain host survival even at the same bacterial burden, a new and more complex role for these simple compounds on determining host outcomes (Olive et al., 2018).

Macrophage cell death pathways can play important roles in priming later steps of protection. Through the process of necrosis or necroptosis, macrophages release bacteria into the tissue to infect new macrophages, a process that the bacteria actively exploits through production of TNT<sup>40</sup> (Guirado, Schlesinger, and Kaplan, 2013; Pajuelo et al., 2018). However, not all macrophages succumb to death by this mechanism and many will undergo apoptosis, a protective form of cell death associated

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<sup>39</sup>Nitric oxide release is the primary mechanism of action for nitroglycerin treatment during infarction.

<sup>40</sup>For many years, tuberculosis was thought to lack traditional toxins that define the pathogenicity of many other bacterial pathogens, such as *Shigella* and *Vibrio cholerae* (Gyles, 2007; Tesh and O'Brien, 1991; Holmgren, 1981; Guichard et al., 2013). However, recent findings have identified the existence of the tuberculosis necrotizing toxin, or TNT, a subunit of the outer membrane protein CpnT that can be cleaved off and exported into the host macrophage where it induces an immunologically blunted necrosis, allowing the bacteria to escape and infect new host cells (Danilchanka et al., 2014; Pajuelo et al., 2021; Tak, Dokland, and Niederweis, 2021; Izquierdo Lafuente et al., 2021). TNT acts as an NAD<sup>+</sup> glycohydrolase, depleting host cell stores of NAD<sup>+</sup> and inducing cell death (Sun et al., 2015; Tak et al., 2019; Pajuelo, Gonzalez-Juarbe, and Niederweis, 2020). *M. tuberculosis* itself encodes an anti-toxin that prevents bactericidal activity while maintaining effective control of host cells (Sun et al., 2015). Although only recently more appreciated, this toxin serves as a major mechanism of host cell killing by *M. tuberculosis* that is far more “traditional” than the major mechanisms of immune subversion that will be discussed throughout much of the remainder of this thesis.

with enhanced bacterial clearance and nitric oxide production (Divangahi, Behar, and Remold, 2013; Herbst, Schaible, and Schneider, 2011). Trapping the bacteria within apoptotic bodies allows other macrophages and neutrophils to undergo proper phagosomal-lysosomal fusion to destroy the contents (Dallenga et al., 2017; Molloy, Laochumroonvorapong, and Kaplan, 1994; Mahamed et al., 2017; Behar et al., 2011).

Less, by comparison, is known from human genetic studies on host resistance to tuberculosis, despite the likelihood of such factors existing. This model has been proposed as a means of heterozygous advantage<sup>41</sup> for diseases like Tay Sachs without rigorous experimental validation (Spyropoulos et al., 1981). One notable finding from Das et al. (2013) is that human genotype at the *MIF* locus is a strong determinant of susceptibility to infection, with high-expressing genotypes conferring protection through the upregulation of DECTIN-1 expression on macrophages. More subtle gene-gene interactions or gene-environment interactions might underly relative resistance to tuberculosis infection, which can be epidemiologically observed among many people who are chronically exposed but never tuberculin skin test convert and among people who become latently infected but never develop active disease (Flynn, Chan, and Lin, 2011; Orme, Robinson, and Cooper, 2015).

Particular genetic disorders are known to lead to increased susceptibility to mycobacterial infections, including normally opportunistic mycobacteria. For instance, lysosomal storage disorders alter macrophage behavior and migration to increase patient susceptibility to tuberculosis (Berg et al., 2016). More general approaches have also been taken to identify macrophage genes that mediate susceptibility to tuberculosis (Thuong et al., 2008), but this remains a field worth additional exploration.

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<sup>41</sup>Such analogies rely on the widely known model of heterozygous advantage against malaria infection among sickle cell trait carriers. It is tempting to think that such a phenomenon could exist for tuberculosis, but it is unclear whether it does.

Additionally, host resistance genes may be an alternative direction for these sorts of studies – alteration in the function of genes that decrease likelihood of infection or increase clearance may be more difficult to isolate from human population studies, but may be addressable with enough statistical power or with *in vitro* approaches (Bourgeois, Smith, and Ko, 2021).

### 1.2.6 The Granuloma

After many rounds of bacterial replication and reinfection of macrophages, the nodus of infection begins to coalesce into a structure known as a granuloma, the true center of tuberculosis infection. These granulomas are sophisticated immunological foci that contain the entire spectrum of immune cells within this confined structure, all seeking to eradicate the invading mycobacteria but finding themselves incapable of doing so. As alluded to previously (Subsection 1.1.2), these structures are comprised of uniquely activated macrophages that span the traditional M1-M2 axis as well as granulocytes of all varieties, lymphoid cells, and novel cell types including foamy macrophages<sup>42</sup> and multinucleated giant cells. However, as has been reviewed comprehensively by (Pagan and Ramakrishnan, 2018)<sup>43</sup>, the macrophage is the central driver of the structure and function of the granuloma – they arrive at the site of infection and initiate the cascade of events that ultimately defines the course of tuberculosis disease.

The granuloma has been long proposed to have both host-protective and host-detrimental effects and the precise balance between them likely varies from granuloma to granuloma. However, the invading mycobacteria have extensively evolved to survive

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<sup>42</sup>As will be more interesting later, it has been demonstrated that NFAT signaling is important for the development of foam cells (Du et al., 2021); whether the macrophage-specific VIVIT approach used later (Chapter 3) might alter foamy macrophage formation is unknown and difficult to test with present tools.

<sup>43</sup>A. J. Pagan and L. Ramakrishnan (2018). “The Formation and Function of Granulomas”. In: *Annu Rev Immunol* 36, pp. 639–665

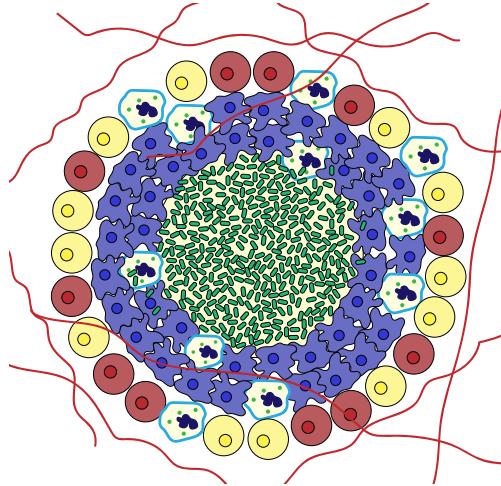
within the granuloma and it has thus become a known quantity in their evolution and they respond extensively to the unique physiology of that environment (Gagneux, 2018; Ramakrishnan, Federspiel, and Falkow, 2000); indeed, they appear to home to existing granulomas and integrate into them unscathed when introduced as a superinfection (Cosma, Humbert, and Ramakrishnan, 2004). Pathogenic mycobacteria also secrete effectors into the macrophage that encourage granuloma formation, providing addition evidence of the bacterial-beneficial roles of this structure (Volkman et al., 2004; Volkman et al., 2010).

As the nexus of immunological activity in tuberculosis, it is also the source of a dizzying array of cytokines and chemokines that comprise the overall response to infection, many of which are produced by macrophages. Macrophage-derived TNF- $\alpha$  and IFN- $\gamma$ <sup>44</sup> are important for control of infection and, in their absence, the bacteria grow unrestricted (Fenton et al., 1997; Flynn et al., 1993; Flynn et al., 1995; Algood, Lin, and Flynn, 2005). However, the bacteria also modulate the expression and downstream responses of these cytokines to further their own lifestyle in what has often been referred to as an evolutionary arms race (Ting et al., 1999). Others have roles that are just beginning to be uncovered. All of these signals are largely induced by engagement of the macrophage with bacterial- or host-derived ligands within the granuloma and serve to modulate critical aspects of the granuloma microenvironment. Much like the tumor microenvironment (see Subsection 1.5.1), the granuloma microenvironment is a key aspect of the progression of the disease, but these features are only now beginning to be dissected properly to understand how neighboring stromal cells impact the immune response, in the broadest sense.

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<sup>44</sup>Although T cells produce most of the total IFN- $\gamma$ , macrophages remain a potentially physiologically relevant source of this critical cytokine (which is regulated by NFAT) (Darwiche et al., 2009; Robinson et al., 2010). As a caveat, no known studies have utilized a *LysM-Cre*; *Ifng*<sup>f/f</sup> approach to begin to assess the functional contributions of macrophage IFN- $\gamma$  even in the presence of T cell-derived interferon.

Granuloma formation is a process that remains poorly understood. While initial findings in the 1990s found that critical host-protective cytokines were required for granuloma development, these did not necessarily explain the full expanse of the phenotype and were unable to differentiate granuloma formation from gross differences in mycobacterial burden (Flynn et al., 1993; Flynn et al., 1995). TNF- $\alpha$  especially has important roles in inducing macrophage microbicidal activity, so it is difficult to disentangle these roles from any contribution to granuloma formation *per se* (Raman Krishnan, 2013a). More recent efforts have focused on other signaling events that might be more limited in scope but have more granuloma-specific effects. Torracca et al. (2017) found that CXCR4 signaling is an important aspect of granuloma formation and did so by sustaining granuloma angiogenesis. In the absence of CXCR4, granulomas developed a lessened degree of vasculature and correspondingly lower bacterial burden, potentially through signaling to vascular pericytes (Pollard, 2009). This left other questions unaddressed, but set the stage for future studies on particular host determinants. Disrupting the structure of the granuloma via inhibition of protective host immunity leads to increased bacterial burden (Flynn et al., 1993; Flynn et al., 1995; Juffermans et al., 2000; McElvania Tekippe et al., 2010), but disruption through inhibition of the epithelialization event itself results in prolonged survival and improved bacterial killing (Cronan et al., 2016). This work reconciles aspects of the unending argument over the host-beneficial and host-detrimental nature of the granuloma, which is what it was always going to be: a structure that under some circumstances and in some regards is host-beneficial but in others prevents the execution of an effective immune response. The underlying distinctions depend on the immune status of the host and, likely, bacterial genetic background and environmental factors, such as smoking (Glickman and Schluger, 2016).



**Figure 1.2:** Schematic of the basic structure of a granuloma. The innermost layers are comprised of epithelioid macrophages that encase the necrotic core while outer layers are comprised of a mixture of granulocytes and lymphocytes. The whole structure is encased in a web of vasculature.

## Heterogeneity

Granulomas, like the cells that comprise them, are incredibly heterogeneous structures, varying between individual granulomas within the same person and from person to person (Matty et al., 2015; Lenaerts, Barry, and Dartois, 2015). This heterogeneity is a reflection of the complex states in which macrophages can exist and their ontologies (Kiss et al., 2018). Within individual humans, the dynamics of individual granulomas showcase the diverse possible outcomes for a given infectious focus, a pattern reflected in some infection models (Lenaerts, Barry, and Dartois, 2015; Lin et al., 2014). The granuloma by the grossest histological observation contains epithelioid macrophages, motile inflammatory macrophages, multinucleated giant cells, foamy macrophages, and macrophages in various stages of various types of cell death in addition to all the other immune cell populations in the granuloma. However, even among neighboring granulomas, these relative proportions can vary widely and one can achieve effective sterilization while the other cavitates.

This diversity within individuals in rate of progression and outcome provides a full spectrum of possibilities for each granuloma that has largely escaped our language to describe them except in sweeping intervals. Such language, including “centroacinar<sup>45</sup>, perifocal, tree-in-bud<sup>46</sup>, metabolically active, exsudative<sup>47</sup>, suppurative<sup>48</sup>, miliary<sup>49</sup>, caseous, circumscribed, and fibro-calcified<sup>50</sup>” is but a sampling of the possible terms that pathologists can use to describe these lesions (Ehlers and Schaible, 2012). Such striking variation can be readily seen in the images that accompany Subsection 3.3.6, where the size and shape of these structures can vary widely even within an individual.

One of the many sources of this heterogeneity lies not in the genetics but in the life history of the person. Alveolar macrophages are long-lived cells that must handle a vast diversity of insults on a daily basis and it has been shown that these insults alter their biology in a permanent fashion and likely are propagated to progeny of these mitotic cells (A-Gonzalez et al., 2017; Duan et al., 2017; Berg et al., 2016; Glickman and Schluger, 2016; Woodruff et al., 2005; Hodge et al., 2007). Behaviors such as smoking are clearly inhibitory of alveolar macrophage responses and are known to exacerbate tuberculosis. What is not as well known is how these environmental factors alter the structure of the granuloma over the long term, especially once the granuloma has established in the interstitium and has a larger proportion of the cell mass comprised of recruited macrophages.

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<sup>45</sup>Within the alveolar space.

<sup>46</sup>Bronchiolar spread leading to fluid filling the cavity, which radiologically resembles a budding tree branch (Gosset, Bankier, and Eisenberg, 2009).

<sup>47</sup>filled with circulatory fluid, but not necessarily red blood cells.

<sup>48</sup>purulent, pus-filled.

<sup>49</sup>Literally, millet seed-like lesions along the lung.

<sup>50</sup>Encased in fibrotic tissue that has undergone calcification and partial ossification.

Even macrophages within otherwise unperturbed organs can display remarkable morphological and expressive heterogeneity (Gordon and Pluddemann, 2017; Gordon and Taylor, 2005). This intrinsic variation from macrophage to macrophage would suggest that the outcome of tuberculosis exposure *could* plausibly be determined based on which individual in a population of macrophages is first infected (Verrall et al., 2014). While this would be challenging or impossible to test experimentally, hopefully some future approach will allow this level of micro-scale dissection of individual macrophage heterogeneity in their interactions with *M. tuberculosis* *in vivo* or *ex vivo*. Studies from the zebrafish had displayed such heterogeneity clearly – regardless of what the initial phagocytic cell is, the bacteria subvert host responses to drive the recruitment of more permissive monocytes and macrophages (Cambier et al., 2014). This manipulation allows the bacteria to “select” their future host cells by specifically recruiting those least likely to have bactericidal capacity. This baseline heterogeneity within the host presents an exploitation schema for the bacteria that may begin at the very first encounter (Ramakrishnan, 2013b).

The initial events of infection remain difficult to study in most models, but insights from the zebrafish have allowed new studies on the heterogeneous outcomes of these early events and, more importantly, potential sources of this heterogeneity. The experimenter is burdened by entropy – there is a great deal of intractable noise in every system and one of these sources of noise is the microbiota of the host. While no comprehensive study has yet been done on particular commensal species and their potentially protective role against tuberculosis infection, it is known that commensal species can undermine mycobacterial defenses by triggering TLR-dependent sensing mechanisms that negate the shielding exercised by the infecting mycobacteria (Ramakrishnan, 2013b; Cambier et al., 2014).

In the granuloma, there exists an essentially random (Orme and Basaraba, 2014) distribution of lymphocytes around the periphery of the structure. While it is clear that the innermost macrophage layers have some reasonable degree of “organization” as a product of their epithelial junctions with one another, other portions of the granuloma can vary substantially (Cronan et al., 2016). Even within the epithelioid layers, the macrophages remain respectably motile and are able to move throughout the granuloma (Cronan et al., 2018). These features likely contribute to the diverse outcomes seen within individuals.

### 1.3 Pattern Recognition in Innate Immunity

For over 100 years, the cells of the innate immune system were thought to be essentially blind scavengers that existed solely to pick up debris for presentation to and activation of adaptive immune responses (Iwasaki and Medzhitov, 2010). How could cells saddled strictly with their somatic genotype go about “intelligently” identifying pathogens? The first clues came from gene homology between the cloned human IL-1 receptor and a developmental protein from *Drosophila*, Toll. These similarities between IL-1R and Toll first informed the mechanism by which IL-1 $\alpha/\beta$  act on cells and then led to the identification of a range of proteins that shared these homologous domains required to activate NF- $\kappa$ B (O’Neill, Golenbock, and Bowie, 2013; Rock et al., 1998). These proteins, dubbed the Toll-like receptors (TLRs) proved to be the foundation of the modern understanding of a very sophisticated innate immune system that, in the vast majority of cases, is solely responsible for the prevention of disease (Janeway, 2005). Additionally, these discoveries opened doors into the study of evolutionarily conserved mechanisms of pathogen defense, as TLRs are conserved

across both *Animalia* and *Plantae*<sup>51</sup> (Armant and Fenton, 2002; Ausubel, 2005). Since that time, numerous other families of receptors, known as pattern recognition receptors (PRRs), have been identified that are able to detect and respond to various pathogen-derived ligands, commonly referred to pathogen<sup>52</sup>-associated molecular patterns. These PAMPs serve as the foundation of the innate immune system and give it an intrinsic ability to detect and respond to threats, both from pathogens and from internal signals indicative of inflammation or damage. To varying degrees, all of these pathways have been implicated in the host response to tuberculosis, although TLR- and CLR-mediated responses stand out as the most well-studied in this regard and seemingly the most prominent during infection (Stamm, Collins, and Shiloh, 2015).

### 1.3.1 Major Families of Pattern Recognition Receptors

Decades of research have revealed a number of families of pattern recognition receptors that are responsible for detecting diverse sets of widely conserved ligands from bacteria, viruses, fungi, and parasites. All of these have members that can respond, in some degree, to mycobacterial infection, making all of them relevant to the study of tuberculosis (Ishikawa, Mori, and Yamasaki, 2017). As alluded to previously, the Toll-like receptor family was the first to be identified. There are 13 known TLRs in the human genome, each of which detects a number of ligands ranging from lipopolysaccharide from Gram-negative bacteria (TLR4) and flagellin<sup>53</sup> (TLR5) to

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<sup>51</sup>This is mostly true – plants have structurally similar proteins that recognize similar ligands, but this is thought to have arisen by convergent evolution rather than a single shared origin (Johnson et al., 2003b).

<sup>52</sup>These are occasionally referred to as microbe-associated molecular patterns; this nomenclature successfully captures the reality that these ligands are not restricted to pathogenic species, but has not become widely used.

<sup>53</sup>Flagellin is the monomeric subunit used to generate bacterial flagella in motile strains that use flagella for movement.

peptidoglycan<sup>54</sup> (TLR2) and RNA (TLR7) (Kawai and Akira, 2007). In addition to the TLR family, there are nucleotide oligomerization domain (NOD)-like receptors (NLRs), retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), absent in melanoma 2-like receptors (ALRs) and C-type lectin receptors (CLRs). All of these families can activate NF-κB signaling, highlighting the central importance of this pathway in activating immunoresponsive genetic programs and executive host defense against pathogens. However, most of these receptors also engage more specific immune pathways downstream that provide them with unique responses tailored to the classes of pathogens to which they are responding.

TLR activation has long been the model for understanding the host innate immune response to pathogens. TLR activation induces a somewhat monotone response dominated by NF-κB (Arbibe et al., 2000; Kawai and Akira, 2007; Brandt et al., 2013; Kawai and Akira, 2009). Most of the TLRs utilize a downstream adaptor protein, MYD88, while others (TLR3 and some TLR4 responses) utilize a different adaptor, TRIF (Yamamoto et al., 2003), to transduce signals which ultimately lead to the phosphorylation of inhibitor of nuclear factor kappa B (I-κB) by the IκB kinase (IKK), which releases NF-κB for translocation into the nucleus (Wright et al., 1990; Triantafilou and Triantafilou, 2002; Kawai et al., 1999; Yamamoto et al., 2003; Akira and Takeda, 2004; Kawasaki and Kawai, 2014; Takeda and Akira, 2004). This transcription factor family is often viewed in a somewhat monolithic manner, but is comprised of five independent transcription factors (NF-κB1, NF-κB2, RelA, RelB, and c-Rel) that generally act as heterodimers with one another; this raises the potential for subtle and as yet undescribed levels of regulatory complexity based on the active heterodimers in different contexts (Baeuerle and Henkel, 1994; Finco and Bald-

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<sup>54</sup>Peptidoglycan is a major component of the bacterial cell wall and is present in essentially all bacteria.

win, 1995; Rice, MacKichan, and Israel, 1992; Oeckinghaus and Ghosh, 2009; Ghosh et al., 2012; Albensi, 2019; Liu et al., 2017). To date, little has been done to fully characterize these distinctions, although some work has found different transcription factor binding sites to be preferentially bound by some dimers and not others (Siggers et al., 2011; Ramsey et al., 2019; Florio et al., 2022). Nonetheless, NF- $\kappa$ B activation writ large is able to activate transcription of a full spectrum of cytokines and chemokines, including IL-1 $\beta$ , IL-2, IL-6, IL-12, IL-17, IFN- $\alpha$ / $\beta$ , TNF- $\alpha$ , and IFN- $\gamma$  (Pahl, 1999; Liu et al., 2017; Gilmore, 2006). This robust pro-inflammatory response is essential for effective clearance of many pathogens and also increases the resistance of surrounding tissue to further infection by intracellular pathogens. As the TLRs can recognize such a diverse array of ligands, this makes them a critical inducer of potent host-protective responses through the induction of microbicidal pathways and recruitment of additional immune cells (Kawai and Akira, 2007; Kawasaki and Kawai, 2014).

NLRs are conserved across metazoans and detect cytosolic PAMPs to defend against intracellular pathogens (Creagh and O'Neill, 2006; Clarke, 2014; Motta et al., 2015; Martinon and Tschopp, 2005). NLRs, like TLRs, activate NF- $\kappa$ B, but also can activate mitogen-activated protein kinase (MAPK) signaling, including p38 and JNK (Shaw et al., 2008; Franchi et al., 2009; Saxena and Yeretssian, 2014; Platnich and Muruve, 2019; Zhong, Kinio, and Saleh, 2013; Velloso et al., 2019). NLRs detect a range of primarily bacterial ligands, including gamma-D-glutamyl-meso-diaminopimelic acid (iE-DAP) from Gram-negative bacteria, muramyl dipeptide from pan-bacterial peptidoglycan, *Legionella* flagellin, and bacterial RNA (Saxena and Yeretssian, 2014; Franchi et al., 2009; Zhong, Kinio, and Saleh, 2013). NLRs initiate the formation of inflammasomes, large, cytosolic, punctate structures that process pro-IL-1 $\beta$  and pro-IL-18 for secretion and paracrine signaling. Within the respond-

ing cell itself, however, the combination of MAPK and NF- $\kappa$ B signaling provides a different signaling tone to that induced by TLRs. MAPKs are known to induce the expression of IL-1 $\alpha$ / $\beta$ , IL-10, IL-12, and TNF- $\alpha$  (Dong, Davis, and Flavell, 2002; Arthur and Ley, 2013; Soares-Silva et al., 2016), which are potent immunmodulators, with TNF- $\alpha$  inducing cell death and inflammation while IL-10 is a potent anti-inflammatory chemokine (Soares-Silva et al., 2016; Couper, Blount, and Riley, 2008). Work remains to be done to more thoroughly define the specific MAPK-dependent responses downstream of NLR activation, which may inform more targeted approaches to immunotherapy in the context of infection.

RLRs are an additional class of cytosolic sensors, most commonly implicated in the antiviral immune response as they detect aberrant nucleic acids, usually those with abnormal features that would not typically be found in eukaryotic cells, including double-stranded RNA and uncapped single-stranded RNA of varying lengths (Loo and Gale, 2011; Jia, Fu, and Tang, 2021; Rehwinkel and Gack, 2020; Kawai and Akira, 2010). The RLR family is composed of RIG-I itself as well as MDA5; these have distinct affinity for different ligands, but ultimately activate IRF3/7, which are transcriptional regulators of IFN- $\alpha$ / $\beta$ , classic type I interferons that activate autocrine and paracrine JAK/STAT signaling to induce antiviral responses in neighboring tissues (Loo and Gale, 2011). These responses are notable in the history of hepatitis C treatment, as the previous standard of care treatment for hepatitis C was treatment with IFN- $\alpha$ , which facilitated viral clearance in many cases (Rong and Perelson, 2010). Interestingly, RIG-I signaling is also able to mediate some immune responses against *M. tuberculosis* and drives the production of IFN- $\beta$  through MAVS to exacerbate pathology; *Mavs* mutant mice had dramatically improved survival after challenge (Cheng and Schorey, 2018).

ALRs are the newest addition to the class of PRRs and serve to detect cytosolic DNA – a potent signal of some sort of pathogenic invasion, either by DNA viruses or some bacteria, or cellular damage. These direct sensors of cytosolic DNA appears to be largely redundant with the existing cGAS pathway as both of these pathways activate STING, which induces the interferon response cascade (Gray et al., 2016). However, an alternative hypothesis suggested that ALRs antagonize the interferon response by inhibiting cGAS-mediated activation of STING (Nakaya et al., 2017). These genes are important for promoting the pathogenesis of tuberculosis, as in the absence of IFI16 and the effector IRF3, *M. tuberculosis* is attenuated for growth in a mouse model (Manzanillo et al., 2012). It is hypothesized, although seemingly untested, that each of the 13 ALR genes (in mice) have distinct families of targets and exerts these modulating effects in distinct contexts in cooperation and antagonism with STING (Nakaya et al., 2017). As this pathway is relatively new to study, there is still a great deal to be learned about how it mediates type I interferon responses and how those interact with engagement of other PRR signaling pathways. However, early studies have already begun to identify consequences of misregulation of these genes, including in the development of autoimmunity (Caneparo et al., 2018).

### 1.3.2 LPS as a Model Molecular Pattern

Lipopolysaccharide (LPS) from Gram-negative bacteria has long been the model for understanding ligand-receptor interactions and signal transduction in the context of PAMP-PRR pairs. On the cell surface, LPS is detected by a complex of CD14, MD2, and TLR4, making it a rather unusual mechanism of detection in comparison to other TLRs, which can either independently detect ligands or act in heterodimers with other members of the TLR family. This complex coordinates the activation of MYD88 and TRIF, which activate NF- $\kappa$ B signaling, as previously described (Miller, Ernst, and

Bader, 2005; Beutler, 2000). For intracellular LPS, which might be encountered in the context of a cytosolic bacterial infection, detection by Caspase-11 mediates the formation of inflammasomes to process IL-1 $\beta$  and IL-18 to defend against these intracellular Gram-negative bacterial pathogens (Wright et al., 1990; Triantafilou and Triantafilou, 2002; Kawai et al., 1999; Vasudevan et al., 2022; Kayagaki et al., 2013; Rathinam, Zhao, and Shao, 2019; Shi et al., 2014). LPS-TLR4 interactions mediate key aspects of sepsis – a systemic inflammatory disorder set on by infection – as well as normal immune responses (Freudenberg et al., 2008).

TLR4 was the first of the TLR family to be characterized and remains the most thoroughly studied (Lu, Yeh, and Ohashi, 2008). The ostensible ligand (albeit indirectly via CD14) for TLR4 was identified as LPS, a highly conserved pattern on the outer leaflet of the outer membrane of Gram-negative bacteria (Wright et al., 1990; Guha and Mackman, 2001; Shi et al., 2014; Kayagaki et al., 2013). 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. LPS, as the name suggests, is comprised of a lipid moiety that docks into the outer leaflet of the outer membrane of Gram negative bacteria and is connected to a series of well-defined and highly diverse glycans in different physical locations along the molecule; the outermost of these is the O-antigen, which in some species can antagonize adaptive immune responses (Dominguez-Medina et al., 2020). In addition to the highly variable O-antigen, other locations within the molecule can vary both within a single bacterium and across species.

The precise composition of the lipid tails of LPS alters its ability to bind 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<sup>55</sup> (Dixon and Darveau, 2005; Ueda et al., 2010; Steimle, Autenrieth, and Frick, 2016; Gyorfy, Duda, and Vizler, 2013; Tan et al., 2015; Simpson and Trent, 2019; Lebeer, Vanderleyden, and De Keersmaecker, 2010). 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, although this remains understudied in non-tuberculous bacterial infections (Park et al., 2013; McGuire and Arthur, 2015).

The ability for variations of a single structure to modulate such diverse host immune responses establishes a model for pathogens to intricately manipulate the features of these widely shared and physiologically essential patterns to alter the capacity for the host to respond and, potentially, drive bacterially-beneficial responses through perversion of the “intended” role of these host receptors. The molecule of interest for my purposes, TDM, is comparatively less studied in the context of host immunity, driving some uncertainty around the direct mediators of this response. Additionally, the specific presentation and conformation of TDM seems to impact the immune response generated to this ligand. *In vitro*, TDM has been demonstrated to adopt different conformational states based on surface composition (for instance, at the air-water interface) and geometry, although the *in vivo* relevance of these different states is contentious (Hunter et al., 2006; Behling et al., 1993). While this will be detailed in Subsection 1.3.5, the major receptors known to detect TDM are within the C-type lectin receptor family and are known as MINCLE and MCL.

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<sup>55</sup>For instance, the oral opportunistic pathogen *Porphyromonas gingivalis* produces a tetraacylated LPS that actually inhibits TLR4 activation by hexaacylated LPS from *Escherichia coli* (Zhang et al., 2008b; Darveau et al., 2004; Herath et al., 2013).

### 1.3.3 Signaling Mechanisms Downstream of C-Type Lectin Receptors

While TLR activation *per se* is a rather monotonous response that is predominantly driven by NF-κB, CLRs terminate in at least two known downstream signaling pathways. In addition to NF-κB (via a pathway to be discussed shortly), they are capable of activating the nuclear factor of activated T cells (NF-AT or NFAT) pathway (Goodridge, Simmons, and Underhill, 2007; Dambuza and Brown, 2015). 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 (Brown, Willment, and Whitehead, 2018).

CLRs are a diverse class of pattern recognition receptors that are defined by their use of divalent calcium ( $\text{Ca}^{2+}$ ) to coordinate the binding of carbohydrate patterns (Hosoi, Imai, and Irimura, 1998; Dodd and Drickamer, 2001), 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 (Furukawa et al., 2013; Alenton et al., 2017; Zelensky and Gready, 2005; Holtet et al., 1997). QPD-containing C-type lectins are, in general soluble or secreted proteins and include the likes of human tetranectin (CLEC3B), an extracellular matrix-interacting protein, and herring antifreeze protein, which mediates the breakdown of ice crystals in the blood of cold-water fish (Liu et al., 2007; Ewart et al., 1998; Ewart and Fletcher, 1993; Graversen et al., 1998; Nielsen et al., 1997).

These receptors are critical mediators of antibacterial, antiviral, and antifungal im-

munity and have evolved to encompass a vast range of different receptors with unique ligand specificities and expression patterns (Hoving, Wilson, and Brown, 2014; Tang et al., 2018). CLRs are known to respond primarily to these carbohydrate-linked ligands via their lectin domains (McGreal, Miller, and Gordon, 2005; Dodd and Drickamer, 2001). Many biomolecules are sugar-modified, from bacteria, fungi, viruses, and eukaryotes (both self and pathogens) (Rudd et al., 2001; Ohtsubo and Marth, 2006). This allows CLRs to be a major pathway for the response to host-derived damage-associated molecular patterns (DAMPs) as well as PAMPs (Garcia-Vallejo and Kooyk, 2009). 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 (Sancho and Sousa, 2012). Most notable among these CLRs is DECTIN-1, the archetypal member of the family which has long been studied for its roles in anti-fungal immunity, but has now been discovered to have a diverse set of roles in other conditions, including responses to bacterial pathogens (including mycobacteria) and in autoimmunity (Brown et al., 2002; Brown et al., 2003; Brown, 2006; Reid, Gow, and Brown, 2009; Drummond and Brown, 2011; Schorey and Lawrence, 2008; Yadav and Schorey, 2006; Wagener et al., 2018; Deerhake et al., 2021).

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-mediated signaling responses are known to be protective against fungal infections, making this a centerpiece of defense against common fungal pathogens, including *Malassezia* and *Candida* (Shiokawa, Yamasaki, and Saijo, 2017). Although not the focus of the present work, DECTIN-1 also has a number of important roles in antimycobacterial defense, which established the importance for this class of C-type lectin receptors in tuberculosis (Yadav and Schorey, 2006). DECTIN-1 is a single-pass transmembrane receptor that uses a large C-type lectin domain to engage with vari-

ous ligands, most notably  $\beta$ -glucans, to stimulate responses in myeloid cells (Brown et al., 2007). DECTIN-1 itself possesses an intracellular YxxL/I (hemITAM) motif that is then phosphorylated by an adaptor kinase, spleen tyrosine kinase<sup>56</sup> (SYK) (Getahun and Cambier, 2015; Kerrigan and Brown, 2011; Bauer and Steinle, 2017). It has been shown that stabilization of SYK facilitates stronger immunological processes and generates protection against fungal infections; cells actively degrade this kinase to limit inflammation, sometimes to the detriment of effective immune clearance (Wirnsberger et al., 2016; Zhu et al., 2016; Sohn, Gu, and Pierce, 2003). The ITAM motif is critical for the binding and activation of downstream process from a range of tyrosine kinase-dependent receptors, including B cell receptor activation (Monroe, 2006; Bauer and Steinle, 2017). This sets off a complex series of signaling events that activate CARD9, ASC, and/or PLC $\gamma$ 2, eventually resulting in NF- $\kappa$ B activation in the former two instances and NFAT activation in the latter (Geijtenbeek and Gringhuis, 2009; Drummond and Brown, 2013). 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.

## CARD9

CARD9-mediated signaling has long been considered to be the dominant modality of signaling downstream of C-type lectin receptors and is important for transcriptional responses to and defense against many intracellular pathogens (Hsu et al., 2007; Hara et al., 2007). This pathway was identified as a critical mediator of TLR-independent antifungal immunity through DECTIN-1, establishing the prototype for C-type lectin signaling responses (Gross et al., 2006). Human CARD9 deficiencies have a very spe-

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<sup>56</sup>SYK itself has many important roles in macrophage and B cell biology, but to spare the distraction, see Mocsai, Ruland, and Tybulewicz (2010) for a nice review of these roles. This also makes SYK a poor genetic target as disruption is embryonic lethal in mice (Yanagi et al., 2001).

cific susceptibility to fungal infections, but interestingly not other types of infections (Drummond, Franco, and Lionakis, 2018; Drummond and Lionakis, 2016).

One of the major focuses has been on the importance of CARD9-BCL10-MALT1 (CBM) signalosomes as a unique consequence of CLR agonism (Drummond et al., 2011; Drummond and Lionakis, 2016; Drummond, Franco, and Lionakis, 2018; Marakalala, Graham, and Brown, 2010; Marakalala and Ndlovu, 2017). Despite utilizing a method of activation that has more in common with B cell receptor activation than TLRs (Monroe, 2006), the functional downstream consequence is the same: nuclear translocation of NF- $\kappa$ B through IKK and associated induction of immune response genes including IL-2<sup>57</sup>, IL-10, TNF- $\alpha$ , and more (Sancho and Sousa, 2012). Furthermore, the evidence is extremely strong that CBM-dependent signaling is critical for the response to a variety of fungal pathogens and that these generally type I responses are a potent defense against infection (Drummond, Franco, and Lionakis, 2018; Hardison and Brown, 2012; Willment and Brown, 2008). Despite this, early studies found that unprimed macrophages do not utilize the CBM signalosome and require priming in order to active CARD9-dependent transcriptional responses (Goodridge et al., 2009). These variations in the ability for DECTIN-1 to induce CARD9 activation may be useful in discriminating between different types of downstream responses; this study specifically found that bone-marrow derived macrophages exhibited DECTIN-1 responses that were essentially independent of CARD9 while dendritic cells promoted CARD9-dependent signaling. The responses downstream of C-type lectin activation through CARD9 tend to activate T<sub>H</sub>1 and T<sub>H</sub>17 responses, which are important for effective fungal control and defense against mycobacterial infections (Drummond et

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<sup>57</sup>While all of these genes are confusing from most perspectives, this one is extremely confusing and requires a bit of gymnastics to come to – why would it be assumed that NF- $\kappa$ B signaling is mediating IL-2 expression in the context of the body of literature stating that NFAT is the almost exclusive regulator of IL-2 with no affirmative evidence of that being the case?

al., 2011; Lyadova and Panteleev, 2015; Zanoni et al., 2005; Zenaro, Donini, and Dusi, 2009). However, numerous datasets have provided evidence of a range of genes that depend on CLR activation but are CARD9-independent (Deerhake et al., 2021). Some of these genes are likely to be NFAT-dependent while others may be activated by as-yet unidentified pathway or through more indirect mechanisms.

Specifically in tuberculosis immunity, CARD9 is important for limiting bacterial growth; in its absence, mice develop severe pneumonia and rapidly succumb to infection. Several different C-type lectin receptors can activate CARD9, so mutation of this adaptor protein compromises signaling through several distinct receptors that recognize a range of ligands (Wagener et al., 2018). Some of these effects may be mediated by loss of DECTIN-1-dependent responses, but much is likely to be mediated by other receptors, as will be discussed in Subsection 1.3.5 (Marakalala, Graham, and Brown, 2010; Marakalala and Ndlovu, 2017). CARD9 is not only essential for transcriptional responses downstream of C-type lectin receptors, it is also required for the transcriptional induction of MINCLE downstream of MCL. This provides a priming step that depends on CARD9 and then feeds-forward with additional CARD9-dependent MINCLE expression for the duration of agonism (Zhao et al., 2014).

This pathway also has known roles in exacerbating autoimmunity. A definitive study on the matter by Deerhake et al. (2021) demonstrated that Card9<sup>58</sup> activation during experimental autoimmune encephalomyelitis drove pathology despite the overall protective effects of Dectin-1 activity. This study generated a comprehensive dataset of the Card9-dependent signaling events in myeloid cells after exposure to

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<sup>58</sup>Throughout this document, whenever I am synthesizing data from different sources or speaking of a pathway in the abstract, I will typically use human gene nomenclature (Tweedie et al., 2021), however, when discussing particular studies in depth, I will adopt the nomenclature of the model organism used (in this case, the mouse). I hope to minimize any confusion on this topic by using species-specific nomenclature only sparingly and when relevant to the topic at hand.

the Dectin-1 agonist curdlan. This will provide a great deal of value to future experimenters attempting to isolate the contributions of distinct signaling pathways to an observed C-type lectin-dependent phenotype.

As will be discussed more exhaustively in Subsubsection 1.5.4, macrophages associated with tumors exert particular levels of regulation over the biology of the tumor itself. In this context, CARD9 is able to be activated by tumors to drive increased tumor metastasis (Yang et al., 2014c). In the context of wound healing, CARD9 is required for wound closure; this offers a different side to CARD9 signaling, which is often viewed as strictly inflammatory, but is evidently important for wound healing processes as well although the mechanism remains largely unknown (Kanno et al., 2017).

## Inflammasomes

CLR activation also result in the activation of ASC-dependent canonical inflammasomes, which process pro-IL-1 $\beta$  and pro-IL-18 for secretion and paracrine and autocrine signaling (Gross et al., 2011) and play critical roles during both development and pathogen responses (Tyrkalska et al., 2019). This occurs through the formation of an intracellular signaling complex with many distinct subunits that, together, mediate the activation of inflammatory programs and pyroptosis (Pandey et al., 2021). This process ultimately depends on a common set of adaptors through IL-1R/MYD88, linking it into common sets of signals that are induced by TLR activation. 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 restricting response diversity; while TLRs are somewhat broad in their expression pattern, the induction of IL-1 $\beta$  secretion activates all neighboring cells that express IL-1R, which is practically ubiquitous in environ-

ment-facing tissues (Deyerle et al., 1992; Malik and Kanneganti, 2018). This makes this pathway extremely powerful for increasing the local inflammatory tone to block the replication and spread of (especially intracellular) pathogens, but subject to a unified set of subversive mechanisms utilized by bacteria, fungi, and viruses (Wein and Sorek, 2022; MacMicking, 2012; Poeck and Ruland, 2010; Tavares, Burgel, and Bocca, 2015). Comparatively less work has been done to characterize the activating mechanisms induced by CLRs that result in inflammasome formation, but this could serve as an exacerbatory mechanism to heighten inflammation in response to particular classes of signal. Notably, LPS (the canonical TLR4 ligand) is also capable of activating inflammasomes, albeit by an entirely distinct Caspase-11-dependent mechanism (Hagar et al., 2013; Vanaja et al., 2016; Pilla et al., 2014; Finethy et al., 2020).

Inflammasome activation has many described roles in the control of mycobacterial infections and is actively manipulated by the bacteria, suggesting that C-type lectin mediated activation of this pathway may play a role in host defense against mycobacteria as it does against fungi (Hardison and Brown, 2012; Wassermann et al., 2015). Knockout of the mediator caspases results in increased bacterial burden in a strictly innate immune context (Kenyon et al., 2017). However, it has been demonstrated that *M. tuberculosis* actively blocks activation of the inflammasome, suggesting that this pathway may be of variable efficacy in defense against mycobacteria, or that inflammasome-mediated activation of gasdermin D is a more important mediator of these effects than is pro-IL-1 $\beta$  processing (Master et al., 2008; Qu et al., 2020). Yet another plausible alternative incorporates non-canonical functions of ASC, as ASC knockout mice are demonstrate increased susceptibility to infection while NLRP3 and Caspase-1 are both dispensable (McElvania Tekippe et al., 2010). There are also a number of tools that can be utilized to study this process, including a fluorescent

ASC knock-in line that reports of inflammasome activation through formation of the characteristic cytosolic specks (Kuri et al., 2017). Such controversy within the field makes this a prime area for future clarifying study although it may be a technical challenge to disentangle some of these complex factors. However, the presumed primary activation mechanism for inflammasomes in mycobacterial infection is via plasma membrane damage, although this does not rule out contributions from other mechanisms (Beckwith et al., 2020).

## NFAT

Lastly, CLRs can activate NFAT signaling, which is a signaling pathway common across other developmental processes but is thought to be a unique consequence of CLR agonism in the context of PRR activation (and is often used as a reporter for CLR activity) (Wilkins et al., 2004; Chow, Rincon, and Davis, 1999; Jauliac et al., 2002; Aramburu et al., 1998; Hannanta-Anan and Chow, 2016; Hooijberg et al., 2000; Goodridge, Simmons, and Underhill, 2007; Goodridge and Underhill, 2008; Bendickova et al., 2020; Fuller et al., 2007; Zhao et al., 2014). Despite the relative uniqueness of this pathway to CLRs, comparatively less work has been done to characterize it vis-à-vis the CARD9-NF- $\kappa$ B pathway (Goodridge, Simmons, and Underhill, 2007; Goodridge et al., 2009; Deerhake et al., 2021). This pathway can induce the expression of EGR2, EGR3, COX2, IL-2, IL-10, and others, which is a more anti-inflammatory or immunoregulatory set of signals than the heavily type I signals induced by NF- $\kappa$ B (IL-1, IFN- $\gamma$ , etc.) The interplay between these divergent signaling consequences is poorly understood, but is likely important in determining the overall tone and kinetics of the response to infection. This pathway will be given a much more extensive dissection in Section 1.4, but suffice to say that the consequences of this pathway's activity downstream of CLR activation are critical

but poorly understood mediators of the overall response in tandem with NF- $\kappa$ B activation.

## Phagocytosis

One of the key integration mechanisms between C-type lectin receptors and NFAT pathway activation is the contribution that C-type lectins make to myeloid cell phagocytosis. These cells are widespread phagocytic receptors and are critical for the uptake of fungi (Robinson et al., 2006). Could this be the case during mycobacterial infections? Seemingly so. The mannose receptor (CD206) is thought to be a major uptake receptor for mycobacteria, as ectopic expression drives the internalization of mycobacteria in other cell types (Schlesinger, 1993). While this is somewhat redundant with other mechanisms of uptake, this suggests a fundamental role for C-type lectins in driving mycobacterial phagocytosis (Ernst, 1998). One of the central consequences of the phagocytosis of any particle is the activation of the NFAT signaling pathway – tying this pathway intimately to the response to diverse infections and activation of adaptive immunity (Khameneh et al., 2017).

An excellent study by Goodridge et al. (2011) found that the activation of DECTIN-1 was dependent upon immobilized binding. Soluble  $\beta$ -glucan particles were unable to activate DECTIN-1 while those same particles immobilized on plastic were sufficient to do so, providing a model wherein C-type lectin receptors can discriminate between happenstance binding of soluble ligands and direct microbial recognition to only induce phagocytosis when bound by a sufficiently large particle (Elder et al., 2017). This prevents microbes from shedding small decoy ligands to distract the innate immune system. This phagocytosis is intimately linked to the activation of NFAT, where internalization of particles through phagocytic receptors (primarily C-type lectins) facilitates increased signaling activity (Fric, Zelante, and Ricciardi-

Castagnoli, 2014). Whether this is the case for every conceivable ligand is unknown and unlikely, but it, at minimum, offers yet another mechanism for host detection and potential microbial subversion.

These phagocytic activities require an intricate (and often physical) association of the inducing receptor with calcium ion channels, calmodulin, calcineurin, and mTOR. It has been found that STIM1 physically links phagosomes to the endoplasmic reticulum to enhance phagocytosis; this release of calcium via STIM1 is then able to activate NFAT to drive further transcriptional responses meant to address the contents of the phagocytic vesicle, to varying degrees of success (Nunes et al., 2012).

#### 1.3.4 C-Type Lectins in Tuberculosis

In addition to DECTIN-1, there are a number of other C-type lectin receptors that have come under study for their contributions to host immunity. DECTIN-1 itself is thought to bind to mycobacteria, although the ligand remains unknown and DECTIN-1 is dispensable for effective antimycobacterial responses (Yadav and Schorey, 2006; Schorey and Lawrence, 2008; Rothfuchs et al., 2007; Marakalala et al., 2011). One of these other receptors largely predates acknowledgement of C-type lectins as a major pattern recognition signaling pathway but is critical for bacterial uptake. As previously mentioned, the mannose receptor is a major contributor to mycobacterial phagocytosis in a manner partially redundant with complement opsonization and uptake (Schlesinger, 1993; Kang and Schlesinger, 1998). However, this mode of uptake does not induce lysosomal fusion and is thus a pro-bacterial mode of cell entry (Astarie-Dequeker et al., 1999; Goyal, Klassert, and Slevogt, 2016; Rajaram et al., 2017).

DC-SIGN was the first C-type lectin receptor to have described roles in host pro-

tection against *M. tuberculosis*. It was initially described as the major receptor for mycobacteria on the surface of dendritic cells, although it is now known to also be expressed by macrophages, especially alveolar macrophages (Geijtenbeek, Engering, and Van Kooyk, 2002; Tailleux et al., 2003; Tailleux et al., 2005). The general consensus was that DC-SIGN expression was a mechanism for host subversion by mycobacteria, but that does not adequately explain the function of DC-SIGN *in primo loco* (Kooyk and Geijtenbeek, 2003), especially as the mycobacteria specifically target DC-SIGN to inhibit host immunity (Geijtenbeek et al., 2003). In this context, binding of *M. tuberculosis* mannose-capped lipoarabinomannan (ManLAM) (Koppel et al., 2004; Maeda et al., 2003; Pitarque et al., 2005) to DC-SIGN induced IL-6, IL-10 and IL-12 expression by dendritic cells; similar effects were seen with binding of HIV-1 and *Helicobacter pylori*, making DC-SIGN a rather promiscuous receptor of pan-kingdom ligands ranging from bacteria to fungi to viruses (Gringhuis et al., 2009; Dunnen, Gringhuis, and Geijtenbeek, 2009). Interestingly, this gene is all but impossible to study in the murine model because mice have no fewer than eight DC-SIGN duplicates, evidence of the evolutionary pressures likely to have necessitated such diversification of function, which may be common to C-type lectin receptors more generally (Tanne et al., 2009; Garcia-Vallejo and Kooyk, 2013).

There are other C-type lectin receptors that engage distinct aspects of host immunity to tuberculosis (Mishra et al., 2017a). DECTIN-2 has been shown to detect mannose-capped lipoarabinomannans and induce the expression of the familiar cast of cytokines – IL-2, IL-6, IL-10, and TNF- $\alpha$ . This ligand is another component of the mycobacterial cell wall and is likely to be present after bacterial killing or other loss of cell wall integrity, so is unlikely to be a phagocytic receptor for mycobacteria, but may be an important mediator of bystander cell activation (Marakalala and Ndlovu, 2017). All of these receptors have major downstream pathways that run through

CARD9-NF- $\kappa$ B, which has been shown to be important for mycobacterial control and limitation of inflammatory responses through IL-10 (Dorhoi et al., 2010).

Two additional members of the EPN-containing superfamily of CLRs are MCL and MINCLE<sup>59</sup>. MCL, originally dubbed DECTIN-3<sup>60</sup>, is expressed by myeloid cells at baseline and is a comparatively desensitized receptor with low affinity for its primary known ligand, TDM (Zhu et al., 2013; Miyake et al., 2013; Zhao et al., 2014). MINCLE, on the other hand, is tightly regulated and only induced after cellular priming by some other stimulus, including MCL activation (Wells et al., 2008; Patin, Orr, and Schaible, 2017). MINCLE has much higher affinity for TDM and, seemingly, a broader range of agonizing ligands, although the latter discrepancy may be a result of historical scientific focus rather than authentic biological difference (Hansen et al., 2019; Richardson and Williams, 2014; Feinberg et al., 2016).

### 1.3.5 The TDM Receptor

TDM exerts similarly diverse functions as 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 Subsection 1.1.4 and Subsection 1.1.5, TDM is a structurally essential component of mycobacteria; the absence of TDM renders the bacteria susceptible to immunological, chemical, and environmental stressors (Moliva et al., 2019; Kan-Sutton, Jagannath, and Hunter, 2009; Rao et al., 2005). In addition to the important structural aspects of TDM, it also possesses a number of chemical and biological

<sup>59</sup>Interestingly, engagement of MINCLE by fungi suppresses the protective DECTIN-1-mediated responses. How these receptors can be manipulated by pathogens to be either active or repressive is an interesting biochemical and biophysical question that has yet to be fully addressed (Wevers et al., 2014).

<sup>60</sup>And for historical reasons, is still occasionally called this in the modern literature.

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 (Elbein et al., 2003) – 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 (Figure 1.1). For over 50 years after its initial characterization as an important structural feature of mycobacteria, the host receptor, if any, remained unknown. It was only in 2009 that a trio of publications began to define two distinct signaling pathway families that could be activated by TDM – a TLR2-dependent pathway and a Macrophage inducible C-type lectin (MINCLE)/Fc $\gamma$ R-dependent pathway (Werninghaus et al., 2009; Ishikawa et al., 2009; Bowdish et al., 2009). The TLR2/MARCO/CD14<sup>61</sup>-dependent response axis was first described by David Russell's group in 2009 and established an important role for this complex in regulating part of the expression of TNF $\alpha$ , IL-6, and IL-1 $\beta$  (Bowdish et al., 2009; Manukyan et al., 2005). While this was a very thorough and comprehensive project that demonstrated many cell biological aspects of macrophage TDM exposure, it failed to account for the complete response to TDM and left open the possibility that other pathways may play critical roles in mediating this response.

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<sup>61</sup>Interestingly, CD14 has a variety of MYD88-independent roles and can induce NFAT activation (see Subsection 1.4.3) which may be one mechanic by which these conflicting pathways can be resolved (Jiang et al., 2005; Nakata et al., 2006). Alternately, the pathways could play complimentary or antagonistic roles in particular contexts over the course of infection; all of these seem like reasonable hypotheses, but research on the TLR2-pathway has largely stagnated in favor of the MCL/MINCLE pathway. Additionally, these MYD88-independent signaling functions predominate in previously unstimulated monocytes and macrophages (Bjorkbacka et al., 2004)

## MYD88

MYD88 is a critically important adaptor protein downstream of most TLR-dependent responses<sup>62</sup>. In addition, MYD88 is essential for transcriptional responses downstream of IL-1R activation, making it essential for both the generation and transduction of important pro-inflammatory signals. MYD88 executes these functions by forming what is known as the myddosome, which is a complex oligomeric structure comprised of MYD88, which directly binds TLRs, and IRAK4 (Latty et al., 2018). This complex, through a series of intermediate steps, activates IKK to induce NF-κB. This is then essential for transcription of many pro-inflammatory cytokines including TNF-α, IL-1β, and IL-6. These responses aid in the eradication of various pathogens and prime an effective adaptive immune response (Balka and De Nardo, 2019).

As would be expected, MYD88 is critically important for restricting mycobacterial growth during infection, although all the receptor-mediated contributions to this control have not been fully addressed (Berod et al., 2014; Shi et al., 2003; Scanga et al., 2004; Holscher et al., 2008; Sugawara et al., 2003; Cervantes, 2017; Underhill et al., 1999; Hosseini et al., 2021). Much of this response may be mediated by IL-1R-dependent responses rather than TLRs, suggesting a relatively minor role for TLRs in host defense to mycobacteria (Fremond et al., 2007; Holscher et al., 2008; Cambier et al., 2014). Adaptive responses are able to develop even in the absence of MYD88, implicating innate response failure in the overall increase in mortality (Fremond et al., 2004). Despite these important roles in host defense, it was previously unknown whether MYD88 played a role in important granuloma processes, such as angiogenesis. MYD88 has known roles in contributing to angiogenesis in tumors and wound healing (Macedo et al., 2007; Zhang et al., 2020a), but our work has found

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<sup>62</sup>Aside from TLR3 and some signals from TLR4, MYD88 is essential for TLR-mediated responses (Takeda and Akira, 2004; Kawasaki and Kawai, 2014).

that it is dispensable for angiogenesis in response to TDM, suggesting the existence of an alternative, TLR- and IL-1R-independent signaling pathway for inducing VEGF expression (see Figure 3.6) (Walton et al., 2018).

## MINCLE/MCL

Preliminary results had indicated that a Fc $\gamma$ R-SYK-CARD9 signaling axis was important for innate immune activation by TDM (Werninghaus et al., 2009). Knockout of *Card9* resulted in nearly abolished expression of a far more expansive range of cytokines and chemokines and led to the hypothesis that some co-receptor was actually responsible for directly binding TDM and that this co-receptor likely lacked its own ITAM motif, as it required Fc $\gamma$ R to provide one in *trans*. The intersection of the data on Fc $\gamma$ R in TDM detection (Werninghaus et al., 2009) and MINCLE's known dependence on Fc $\gamma$ R to provide an ITAM (YxxL/I<sub>x(6-8)</sub> YxxL/I) motif in *trans* for signaling (Yamasaki et al., 2008) led to a logical hypothesis: that perhaps MINCLE was the sought-after TDM receptor.

MINCLE, itself, was viewed with some interest due to its curious regulatory pattern after having been identified as a macrophage-specific gene induced by C-EBP $\beta$ , but until 2008, no ligands had been identified (Balch et al., 1998; Akira et al., 1990). The first identified ligand, SAP130, is 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 (Yamasaki et al., 2008). 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. This assigned an initial role to MINCLE, but this broke from the standard pattern of CLRs detecting

PAMPs and many of these receptors had already been identified to bind seemingly unrelated ligands. Ultimately, a seminal paper in 2009 established a direct ligand-receptor binding interaction for TDM and MINCLE, establishing this as the dominant pathway for TDM detection and host signal transduction (Ishikawa et al., 2009).

Following this, it remained somewhat unclear what pathways were capable of inducing *CLEC4E* expression in response to pure TDM. Over evolutionary time, an ancestral CLR had undergone a tandem duplication to form the modern *CLEC4E* (MINCLE) and *CLEC4D* (MCL). Based on conserved sequence and differential expression patterns, MCL became a compelling candidate for the basal receptor for TDM. Indeed, although it took four years, MCL was identified as a low-affinity TDM binding receptor that could mediate the upregulation of MINCLE<sup>63</sup> (Miyake et al., 2013), and later, was identified to act in a heterodimer with MINCLE to mediate signaling when both are present (Lobato-Pascual et al., 2013; Kerscher et al., 2016b; Yamasaki, 2013; Furukawa et al., 2013). Additionally, it has been found that this heterodimer is able to mediate phagocytosis, offering yet another potential route of entry into phagocytes for mycobacteria (Yamasaki, 2013; Lobato-Pascual et al., 2013). Further studies elaborated the latter pathway to incorporate a biphasic and ultimately heterodimeric model of MCL/MINCLE<sup>64</sup> activation, with MCL being activated first, inducing *MINCLE* expression and then the two acting as a heterodimer over longer courses of signaling (Lobato-Pascual et al., 2013; Yamasaki, 2013; Furukawa et al., 2013).

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<sup>63</sup>Many inflammatory stimuli are able to induce the expression of MINCLE. It was originally identified as an NF-IL6 target gene, which can also be induced by MYD88 signaling and other danger signals (Kerscher et al., 2016a).

<sup>64</sup>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.

While the mechanisms of detecting TDM are, themselves, biochemically interesting as an organism evolves to detect this rather unusual compound, the functional consequences of MINCLE-TDM interactions remained unknown and, to this day, remain conflictual. Lee et al. (2012) found that MINCLE expression on neutrophils was protective against infection with *M. tuberculosis* Erdman while Behler et al. (2012) found that MINCLE expression in alveolar macrophages was important for responses to BCG and in the absence of MINCLE, the mice exhibited increased bacillary load. However, Heitmann et al. (2013) found that *Clec4e*<sup>-/-</sup> mice had no defect in bacterial control after infection with either *M. tuberculosis* H37Rv or BCG. These results are difficult to reconcile, but each uses different strains, routes of infection, and infectious doses, leading to further difficulty in analysis<sup>65</sup>. A subsequent study found that MINCLE on dendritic cells was also important for control of disseminated tuberculosis of the spleen (Behler et al., 2015). Human genetic studies have investigated polymorphisms in the human MINCLE gene and have not yet found any association with tuberculosis, although none of the investigated mutations were loss of function (Bowker et al., 2016). By contrast, the inducing receptor MCL seems to have a stronger body of evidence supporting its role in mediating protective responses, with knockout mice having higher bacterial burden and mortality despite having functional MINCLE<sup>66</sup> (Wilson et al., 2015). While the picture as a whole comes out somewhat murky, it may be the case, similar to responses downstream of DECTIN-1 in experimental autoimmune encephalomyelitis, that the net effect of MINCLE is protective, but other factors can change the relative benefit by antagonizing other

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<sup>65</sup>As suggested by Lang (2013), culture conditions and mouse colony hygiene may also underly these differences. In retrospect it is impossible to know, but this set of papers has stymied future research designed to clarify the importance of this ligand-receptor interaction during infection.

<sup>66</sup>As will be discussed later, result of full-locus deletion mutants across both MCL and MINCLE would be an interesting addition to our knowledge of the role of TDM detection *per se* to antimycobacterial immunity rather than this gene-centric model.

immunological pathways.

In addition to binding to mycobacterial TDM, MINCLE has a number of other ligands, including fungal PAMPs, such as those from *Malassezia* and, perhaps, *Candida* (Yamasaki et al., 2009; Wells et al., 2008; Ishikawa et al., 2013). In response to *Malassezia*, MINCLE induces IL-6 and TNF- $\alpha$ , aiding in host defense against this opportunistic fungal pathogen (Lu, Nagata, and Yamasaki, 2018). MINCLE can also bind related mycolic acids from other species, including *Corynebacterium* mycolic acids, which are much shorter than the comparable species in *Mycobacterium* (van der Peet et al., 2015). This suggests that MINCLE is a versatile receptor with a broad set of ligands, the specificity of which is probably determined by higher orders of regulation, including potential heterodimers with other receptors and conformational changes (Jegouzo et al., 2014). MINCLE, like similar C-type lectin receptors, is able to induce a variety of downstream signaling cascades, including the one which is the focus of this work, NFAT.

## 1.4 Nuclear Factor of Activated T Cells (NFAT)

NFAT signaling activation results in diverse cell- and context-dependent outcomes. This gene family was first described as a transcription factor that regulated the production of IL-2 in T cells (Shaw et al., 1988; Jain et al., 1993; Northrop et al., 1994). This pathway could be inhibited by blocking the phosphatase activity of calcineurin, suggesting that NFAT was regulated by calcineurin (Jain et al., 1993; Loh et al., 1996). While it was initially studied for its diverse roles in regulating lymphoid biology, it has since had widely diverse roles ascribed to it in nearly all cell types including cardiomyocytes, endothelial cells, skeletal muscle,  $\beta$  islets, oligodendrocytes, keratinocytes, and myeloid cells (Horsley and Pavlath, 2002; Crabtree and Olson,

2002; Fric et al., 2012a; Kegley et al., 2001; Stevenson et al., 2001; Weider et al., 2018; Al-Daraji et al., 2002; Muller and Rao, 2010).

This pathway has had a vast range of roles ascribed in the context of infectious disease (as already briefly discussed in Subsubsection 1.3.3), cancer (Muller and Rao, 2010), autoimmunity (Park et al., 2020), and development (Horsley and Pavlath, 2002; Crabtree and Olson, 2002). In this section, I will review some of these roles and provide a more comprehensive review of the unique roles of the different isoforms as a means of informing the later results.

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 (Rao, Luo, and Hogan, 1997). Historical reasons have resulted in a convoluted nomenclature<sup>67</sup>, so for the sake of consistency, the NFATCx naming scheme will be used throughout this document. NFAT5 is a special, and distantly related, member of this family that appears to be important for the transcriptional response to osmotic stress, but unlike all of the other members, is constitutively nuclear and not regulated by changes in cytosolic calcium concentration via calcineurin (Go et al., 2004; Kumar et al., 2020a).

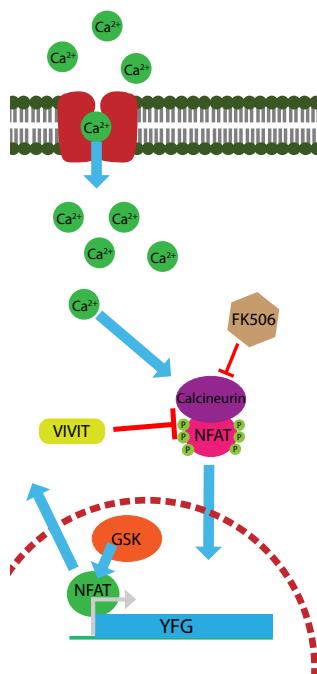
The four calcium responsive members have long been passively assumed to be functionally redundant, with their roles defined by their patterns of tissue expression

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<sup>67</sup>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 or more distinct naming schema. In this case, no resolution has ever come about. The NFATc subnomenclature was meant to designate that they are calcium-responsive and calcineurin-dependent and distinct from NFAT5, the modern homolog of the ancestral protein with high sequence similarity from humans to sponge. In choosing to maintain the NFATc nomenclature, I take no position on the relative 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. Typically, the text will obfuscate the use of these alternative names and translate them into the desired nomenclature.

(Klein et al., 2006; Mancini and Toker, 2009; Macian, 2005; Chow, Rincon, and Davis, 1999). All of them seem to be derived from an ancestral single isoform that was duplicated over the course of evolution (although intermediate representatives with greater than one but fewer than four isoforms seem to be unknown among modern species) (Muller and Rao, 2010). 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 near the plasma membrane and endomembranes, NFATC3 rapidly traffics in and out of the nucleus in response to small magnitude changes in calcium and requires high levels of nuclear calcium to maintain activity (Ulrich et al., 2012; Yissachar et al., 2013; Kar and Parekh, 2015; Kar et al., 2016).

NFAT proteins require 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) are able to phosphorylate NFAT to drive it back into the cytosol in inactive form; the balance of these effects determine the overall transcriptional response (Figure 1.3) (Crabtree and Olson, 2002). This shuttling behavior allows existing pools of NFAT to rapidly modulate host responses, including developmental, immunological, and pathological responses. This also allows for rapid tuning of the longevity of the response, presumably allowing for the induction of different genes and to different degrees based on the length of activation. Although no work has ever been done to define such distinctions, the 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



**Figure 1.3:** This dramatically simplified schematic shows the mechanism of NFAT activation: calcium influx activates calcineurin to dephosphorylate NFAT to allow for nuclear translocation; this process can be reversed through GSK-mediated phosphorylation that exposes a nuclear export sequence. This induction can be inhibited by a number of reagents, including FK506 and VIVIT.



**Figure 1.4:** This simplified depiction of some of the major domains of interest that are shared among all of the NFAT proteins. The SP domains are dephosphorylated by calcineurin to induce nuclear translocation and rephosphorylated by GSK to induce nuclear export by either revealing or concealing the embedded NLS. The other domains regulate protein-protein interactions and DNA binding.

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.

NFAT proteins are by and large structurally similar and are comprised of three broad domains: the N-terminal domain, the DNA binding domain, and the C-terminal domain. The DNA binding domain shares strong homology with other REL-like proteins, placing NFAT on a branch of the NF- $\kappa$ B evolutionary tree. While the C-terminal domains are relatively understudied, much is known about the N-terminal domain, which is important for interacting with a range of binding partners, including AP-1 transcription factors (Boise et al., 1993; Martinez et al., 2015). The N-terminal domain also contains the calcineurin binding motif (SPRIEIT) and the serine-proline domains that determine the phosphorylation status of the protein and exposure of the sequestered NLS for importin trafficking (Rao, Luo, and Hogan, 1997). A simplified diagram of this is shown in Figure 1.4.

The foundational work on NFAT was done almost entirely in T cells, where it was

found to be important not only for intra-T cell differentiation into T<sub>H</sub>2 cells, but also in dendritic cells for the initial production of IL-2, suggesting that NFAT is an inducer of anti-inflammatory signaling cascades that drive type II responses to facilitate tolerance (Granucci et al., 2001; Granucci et al., 2003; Malek and Bayer, 2004). NFAT is also essential for induction of IL-4/IL-13, the archetypal anti-inflammatory (or inflammation-resolving) cytokines (Monticelli, Solymar, and Rao, 2004; Agarwal, Avni, and Rao, 2000; Monticelli and Rao, 2002; Wierenga and Messer, 2000). NFAT activity regulates aspects of the T cell lifecycle from proliferation to exhaustion, making it central to the lives of these cells (Crabtree and Olson, 2002; Martinez et al., 2015; Malek and Bayer, 2004; Ranger et al., 1998b; Yahia-Cherbal et al., 2019). However, a binary type I/II classification for this pathway is ultimately elusive, as it is also critical for regulating the expression of TNF- $\alpha$  and IFN- $\gamma$ , critically important pro-inflammatory cytokines (Kiani et al., 2001; Sica et al., 1997; Kaminuma et al., 2008; Ke et al., 2006; Fric et al., 2012b; Nathan et al., 1983). The pleiotropic nature of this pathway makes it of especial interest in the context of host-pathogen interactions where a robust inflammatory response is needed to kill the invading pathogen, but moderation is required to prevent excessive tissue damage (Casadevall and Pirofski, 2003). These distinct effects of NFAT depend on both the cell type in which it is acting and the particular isoform activated, as these proteins have diverse but differentiable roles depending on such variables, among others.

#### 1.4.1 Clinical Utility of NFAT Inhibitors

The central role of NFAT in the immune system has long been appreciated, albeit in a rather focused 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 (Mihatsch et al., 1998). These

drugs were discovered and developed for clinical use 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 (Sandwijk, Bemelman, and Ten Berge, 2013). The weakness of these drugs is that they block all calcineurin activity in all cell types, leading to a vast range of collateral targets that manifests in renal toxicity and neurological side effects – 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 (Cheer and Plosker, 2001; Al-Daraji et al., 2002; Gupta, Adamiak, and Chow, 2002; Gutfreund et al., 2013).

Topical tacrolimus is site-localized with minimal skin absorption and exhibits far fewer side effects than comparable use of corticosteroids. Approaches focused on targeted inhibition of misregulated pathways have clear promise in improving overall disease outcomes, including in the context of NFAT inhibition, but 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<sup>68</sup> or CRISPR/Cas9 in specific tissues at particular times.

The imminent importance of NFAT became obvious in the organ transplant era. The recipient immune system will wage immunological war against non-self organs,

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<sup>68</sup>VIVIT will be discussed more extensively in later sections, but is a peptide-based inhibitor of the calcineurin-NFAT interface that acts in a functionally dominant-negative fashion by utilizing a higher-affinity version of the SPRIEIT (or PxIxIT) sequence natively found in the NFAT proteins.

which requires recipients to undergo life-long immunosuppressive therapy. One of the most successful approaches to preventing organ transplant rejection has been the use of calcineurin inhibitors, including cyclosporine and tacrolimus (Ellis, 1995; Scott et al., 2003; Mihatsch et al., 1998; Lerut et al., 2008). These calcineurin inhibitors block the NFAT-mediated transcription of IL-2, IL-4, TNF- $\alpha$ , and IFN- $\gamma$ , which dampens the adaptive immune response (especially T cell mediated responses) and dramatically extends the useful lifespan of the transplanted organ, most notably in the case of liver transplants (McCaffrey et al., 1993; Moench et al., 2007).

Although many immunosuppressive therapies have markedly increased risks for various opportunistic infections, calcineurin inhibitors are comparatively spared from this disadvantage. For instance, alemtuzumab, which depletes B and T cells, increases the risk of a number of bacterial, viral, and fungal infections, including *Staphylococcus*, Hepatitis B, and *Cryptococcus* (Harris et al., 2021; Fishman, 2007). By contrast, tacrolimus has potent antifungal activity and few infectious disease risks have been noted with tacrolimus monotherapy (Singh et al., 2016b; Gong et al., 2021; Papon et al., 2021; McAlister et al., 2006; Emal et al., 2019). This evidence would suggest compensatory mechanisms are available to fend off many pathogens while maintaining enough targeted immunosuppression to prevent organ rejection. This provides evidence of the potential for the use of NFAT inhibitors in an infectious disease context without overtly inhibiting the overall immune response to the infection.

New approaches have begun to be developed for NFAT inhibition. One notable example is the development of a comparatively more selective calcineurin-NFAT inhibitor, INCA-6 (Roehrl, Wang, and Wagner, 2004; Roehrl et al., 2004). While tacrolimus acts through binding of FK506-binding proteins that then complex with and inactivate calcineurin, INCA-6 selectively blocks the interaction between cal-

cineurin and NFAT, sparing some or most of the other functions of calcineurin while maintaining potent immunosuppression. While this inhibitor has not been exhaustively trialed, this general theme offers some promise for more targeted therapies that can overcome some of the side effects of traditional calcineurin inhibitors while maintaining most of the benefits.

Another approach that has more recently begun to gain traction is the development of isoform-selective inhibitors. The four NFAT isoforms have selective expression and activation profiles that make them conceivably differentiable biochemically. Select recent work has begun to do exactly that, by taking a structure-guided approach to identifying regions of the proteins unique to particular isoforms and targeting them with small molecules (Kitamura and Kaminuma, 2021). While no isoform-specific inhibitors are yet available, this will likely change in the coming years.

Beyond small molecule based approaches, the age of personalized medicine opens possibilities for gene therapy approaches to ameliorate pathology caused by one or a combination of NFAT isoforms in particular tissues. For instance, a T cell-targeted NFATC2 mutation may result in comparable graft-sparing immunosuppression to tacrolimus while limiting deleterious consequences to a single cellular compartment. With the advent of chimeric antigen receptor T cells (CAR T cells), the use of autologous donation for genetic modification has both clinical approval and potential additional practical applications (June, 2007). Alternately, new delivery mechanisms may make it possible to selectively deliver traditional small molecules to particular cell types, perhaps through molecular caging approaches or liposomal delivery to discrete tissues (Mukhtar et al., 2020; Hu et al., 2019). These possibilities and others foreshadow a future of greater specificity in targeting NFAT signaling, on both the isoform and tissue fronts.

### 1.4.2 Differentiation of Individual Isoforms

As alluded to previously, the different NFAT isoforms have begun to be assigned discrete functions in different contexts. As part of the present work sought to define isoform-specific roles for NFATC2, it may be of utility to dissect the known differentiable functions of these four isoforms, which may further inform future studies. There is a great deal of interesting biology that derives from the different expression patterns and properties of these proteins, which will be briefly reviewed here. While an early publication (Masuda et al., 1995) evaluated the expression of the then-three known isoforms of NFAT across a range of tissues, little immediate work was done to evaluate if this expression carried any functional consequences but this set off a flurry of study on the roles of these isoforms in many different tissues, which is the focus of this section.

#### NFATC1

Like most<sup>69</sup> of the NFAT isoforms, NFATC1 was first investigated in the context of lymphocyte biology, having been isolated from a bovine thymus and from human Jurkat T cells (Northrop et al., 1994). This protein was initially found to be important for T cell activation and relatively restricted in expression pattern based on the cDNA library hybridization approaches used at the time. NFATC1 was found to bind and activate the IL-2 promoter, suggesting an important role in the differentiation of T cells into CD4<sup>+</sup> effector T cells (including both T<sub>H</sub>1 and T<sub>H</sub>2 cells) and, later, memory T cells (Martinez et al., 2015; Monticelli and Rao, 2002; Yahia-Cherbal et al., 2019; Torgerson et al., 2009; Klein-Hessling et al., 2017; Oestreich et al., 2008). Regulation of this essential pathway in T cell biology made this isoform of especial biological

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<sup>69</sup>Really, all but NFATC4.

interest going forward. It was subsequently found that NFATC1 is an important host factor for HIV-1 replication; this is tied to the importance of NFATC1 in T cell activation as HIV-1 requires T cell activation for efficient replication (Kinoshita et al., 1997). Further evidence of this conflicted nature of NFATC1 was evinced by findings that mice doubly deficient in NFATC1 and NFATC2 displayed both effector CD4<sup>+</sup> T cell differentiation defects and B cell hyperinflammation and hyperproliferation, ascribing these two NFAT isoforms key roles in not only lymphocyte activation, but in the regulation of tonally appropriate response to both pathogens and at homeostasis (Peng et al., 2001). In addition to its roles in regulating IL-2, further studies found that NFATC1 (again in collaboration with NFATC2) regulates IL-4 transcription. Specifically, NFATC1 deficiency led to diminished production of IL-4, demonstrating that NFATC1 is a positive regulator of IL-4 production and T<sub>H</sub>2 responses (Monticelli and Rao, 2002). A further study found that, conversely to the effect of NFATC1 and NFATC2 on the IL-4 promoter, NFATC1 was incapable of ectopically inducing *TNF* transcription in a manner that depended upon the C-terminal activation domain of NFATC2, providing compelling evidence at one level at which NFAT isoforms can be differentially regulated (Kaminuma et al., 2008).

While work has largely slowed on this pathway in the context of T cells, additional work built on this foundation to further dissect specific aspects of T cell biology regulated by NFATC1. In the modern era of cancer immunotherapy, factors regulating PD-1 expression became of interest and it was found that NFATC1 is required for PD-1 expression after T cell activation, which gives this pathway an important role in regulating anti-cancer responses, especially in the context of PD-L1/PD-1 checkpoint inhibitor treatment (Oestreich et al., 2008). NFATC1 was also found to control the cytotoxicity of CD8<sup>+</sup> T cells, giving it key roles on both arms of T cell differentiation. In the absence of NFATC1, T cells have reductions in cytotoxicity and fail to control

infection by bacterial pathogens (Klein-Hessling et al., 2017). NFATC1 activity in some ways defines this differentiation process as FOXP3, a major regulator of T<sub>reg</sub> development, downregulates the transcription-level expression of *NFATC1* to block the expression of T<sub>eff</sub> cytokines (Torgerson et al., 2009). This was an early indicator of the ways in which the NFAT isoforms can be dynamically regulated at the transcription level, a phenomenon worthy of further discussion later on.

Recently, far more work has been done to define the role of NFATC1 in other realms of biology; most notably, important roles for NFATC1 have been uncovered in osteoclast differentiation, muscle cells, (lymphatic) endothelial cell biology, cardiac development, podocytes, other immune cell types, and various aspects of stem cell and cancer biology.

Osteoclasts are complex bone-degrading cells ontologically derived from macrophages after M-CSF and RANKL stimulation<sup>70</sup>. In this realm, NFATC1 has been found to be the master regulator of osteoclast differentiation, constituting perhaps the single most important biological role for this isoform. While individual isoform mutants tend to have mild effects in T cells and combinatorial mutation (as we have seen) is necessary for complete disruption of function, in osteoclasts NFATC1 is the sole necessary member in this differentiation process from macrophages into osteoclasts (Kim and Kim, 2014). The regulatory mechanisms of the NFAT family logically fit into the overall kinetics of bone biology; the high calcium environment primes the calcineurin-NFAT axis and this environment is required for osteoclastogenesis (Negishi-Koga and Takayanagi, 2009). Study of osteoclast biology has also revealed interesting and potentially informative roles in the interplay of NF-κB and NFATC1

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<sup>70</sup>These cells work in tandem with osteoblasts to regulate bone mass. Interestingly, NFAT is important on both sides, playing important regulatory roles in bone deposition as well (Winslow et al., 2006).

transcriptional activation, with NF- $\kappa$ B having been shown to bind the *NFATC1* promoter (Muhammad et al., 2014). A further study found that the NF- $\kappa$ B protein RelB was able to inhibit the transcription of *NFATC1*, adding yet more nuance to the interaction between these proteins (Zhao et al., 2015). Whether or not NF- $\kappa$ B can bind other NFAT promoters to drive increased transcription remains unknown. These studies also identified auto regulatory functions of NFATC1, which binds its own promoter to drive transcriptional upregulation during differentiation (Asagiri et al., 2005). NFATC1 is initially activated through the combinatorial effects of TRAF6, c-Fos, STAT3, and p38 signaling after RANKL binding to RANK; ectopic expression of NFATC1 in development facilitates osteoclast differentiation in the absence of RANKL (Takayanagi et al., 2002; Huang et al., 2006; Yang et al., 2019; Matsumoto et al., 2004; Huang et al., 2020)<sup>71</sup>. Additional signals from ITAM-linked receptors through NFATC1 are also important for inducing these responses in a non-redundant fashion with RANK signaling (Koga et al., 2004). The absence of functional osteoclasts leads to progressive osteopetrosis in the mouse (Takayanagi et al., 2002; Kim and Kim, 2014). This paper led to a series of follow-ups defining some of the previously mentioned regulatory mechanisms. Particular inflammatory stimuli, including activation of DECTIN-1 is able to inhibit osteoclastogenesis through inhibition of NFATC1 via *MAFB* promoter occupancy (Zhu et al., 2017). These studies have revealed in remarkable detail the absolutely indispensable role for NFATC1 signaling in the process of osteoclastogenesis and homeostasis by integrating environmental factors with a series of intersecting pathways. One notable feature of osteoclasts is that they are multinucleated cells and their fusion is dependent on NFATC1 activity (Kim et al., 2008); another major multinucleated cell type with important roles for

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<sup>71</sup>The text of (Huang et al., 2020) (presumably) erroneously refers to NFATC2 and, given the abundance of evidence against NFATC2 playing a role in osteoclastogenesis, I will assume that NFATC1 was intended here.

NFATC1 is that of muscle cells.

Muscle cells allowed for a more thorough exploration of NFATC1 as an electrical and mechanical sensor. Skeletal muscle is another context in which multiple NFAT isoforms are playing important roles, with NFATC1, NFATC2, and NFATC3 all having described roles (Ehlers, Celona, and Black, 2014; Tothova et al., 2006; McCullagh et al., 2004; Delling et al., 2000; Amberg et al., 2004; Layne et al., 2008; Stevenson et al., 2001). For NFATC1 specifically, others have found that NFATC1 can detect nerve activity as exercise stimulation induces the nuclear translocation of NFATC1 while extensive rest results in shuttling back to the cytoplasm (Tothova et al., 2006). NFATC1 also plays a key role in muscle regeneration by regulating expression of MYOD; after muscle damage, NFATC1 activity is correlated with increased *MYOD* expression (Sakuma et al., 2003), but during muscle fiber type differentiation, NFATC1 negatively regulates MYOD protein by binding and blocking MYOD interaction with p300. The net effect of this is the NFATC1<sup>hi</sup> myocytes become slow-twitch muscle fibers while NFATC1<sup>low</sup> myocytes become fast-twitch fibers (Ehlers, Celona, and Black, 2014).

An early histological observation of *Nfatc1*<sup>-/-</sup> mice was that they possessed a heart defect leading to pulmonary and aortic valve abnormalities that resulted in congenital death by E15 (Pompa et al., 1998; Ranger et al., 1998a). These defects gave an important role to NFATC1 in regulating endothelial development and lead to speculation that NFATC1 may regulate some aspect of VEGFR signaling, as VEGFR is the master regulator of endothelial biology. Indeed, VEGF stimulation of human pulmonary valve endothelial cells (HPVECs) led to NFATC1 activation (and not that of any other isoforms) through VEGFR2, which induces proliferation of the HPVECs (Johnson et al., 2003a). NFATC1 was also activated in the endocardium by RANKL, the same

ligand that drives myeloid differentiation into osteoclasts; this shared pathway suggests that RANKL may broadly activate NFATC1 in tissues where the receptor is present (Combs and Yutzey, 2009). This was among the first mechanistic descriptions of a role for NFAT in the endothelium, where it is now known to be an important transcription factor mediating angiogenesis, but in a manner that is somewhat limited by the particular endothelial subtype and microenvironment (specifically in the endocardium) (Jang et al., 2010; Wu et al., 2012; Wu, Baldwin, and Zhou, 2013; Gunawan et al., 2020; Courtwright et al., 2009). This work *in toto* establishes a developmentally essential role for endocardial NFATC1 expression in the formation of pulmonary and aortic valves and a potentially broader role of NFATC1 in the regulation of particular aspects of endothelial cell biology. A slightly broader study looked at this phenomenon from a pan-NFAT perspective and found similar effects, where interactions between endocardial and myocardial NFAT responses determined the development of the heart valve (Chang et al., 2004).

In contrast to the rather specific role for NFATC1 in endocardial development, NFATC1 is broadly essential to lymphatic endothelial specification and elaboration. NFATC1 plays an essential and non-redundant role in regulating the formation of lymph sacs and valves, for which it also serves as a definitive genetic marker (Shin et al., 2019). *Nfatc1<sup>-/-</sup>* mice have a normal vascular system but a reduction in lymphatic proliferation and morphogenesis (Kulkarni, Greenberg, and Akeson, 2009; Norrmen et al., 2009). Similar to previous findings in the endocardium, the activation of NFATC1 depended on VEGFR2 activity, providing further evidence of the unique relationship between this receptor and transcription factor in mediating major processes in endothelial biology.

The activity of NFATC1 in immune cell types other than lymphocytes is relatively

unclear. There is evidence that TNF-TNFR signaling on macrophages activates macrophage NFATC1, driving increased osteoclastogenesis. However, this was studied primarily in the context of bone homeostasis (as discussed previously) and not in the context of infection. Whether macrophage TNFR signaling through NFATC1 contributes to the biology of infection in any way remains, as yet, unknown (Yarilina et al., 2011). TNF- $\alpha$  also contributes to podocyte<sup>72</sup> damage through an NFATC1-dependent mechanism. TNF induces alterations in cholesterol metabolism that allows NFATC1 to induce apoptosis, which in turn compromises kidney function (Pedigo et al., 2016; Zhang et al., 2013).

Lastly, NFATC1 has been found to have variable, but influential roles in the biology of stem cells and during particular cancers, notably hepatocellular carcinoma. However, the general theme of NFATC1 activation in the context of cancer is the induction of an epithelial-mesenchymal transition that promotes dissemination and invasion. This was first described in 2006 in a publication that found that NFATC1 is over expressed in pancreatic carcinoma and drives increased expression of c-myc, a classical oncogene (Buchholz et al., 2006; Eerola et al., 2019). As is common with c-myc activation, this drives increased proliferation and reduced contact inhibition. This set a foundation for exploration of this pathway in other cell types and suggested that inhibition of NFATC1 may be a promising therapeutic target in carcinoma. This pathway was then translated to hepatocellular carcinoma, in which c-myc, once again, increases proliferation of the cancer (Wang et al., 2012). Notably, ectopic expression of NFATC1 in non-expressing cancers increases cell invasion and motility through repression of *CDH1*<sup>73</sup> expression (Oikawa et al., 2013). This model was later inte-

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<sup>72</sup>Podocytes are epithelial cells that reside within the glomerulus of the kidney and aid in filtering blood.

<sup>73</sup>Epithelial cadherin

grated with potential cancer initiating stimuli through evaluation of NFATC1-STAT3 transcriptional complexes<sup>74</sup> that can be induced by chronic inflammation to promote KRAS<sup>G12D</sup>-driven carcinogenesis using a pancreatitis model. This raises an obvious question: can pharmacological inhibition of NFATC1 improve cancer prognosis, given that we have two highly efficacious calcineurin-NFAT inhibitors with FDA approval – tacrolimus and cyclosporine A. Indeed, treatment of mice bearing prostate carcinomas with either drug improved disease outcome by reducing proliferation and metastasis (Kawahara et al., 2015a). Identical outcomes were found by the same group in the context of bladder cancer<sup>75</sup> (Kawahara et al., 2015b). From these results, it is reasonable to conclude that NFATC1 can act as an oncogene across a range of different oncological contexts through driving increased c-myc expression (Buchholz et al., 2006; Flockhart et al., 2009; Seifert et al., 2009; Oikawa et al., 2013; Kawahara et al., 2015a; Kawahara et al., 2015b; Liu et al., 2021a). Other oncogenic targets of NFATC1 have been proposed including BMI1 (Wu et al., 2019) and ITGA5 (Eerola et al., 2019).

However, the literature is not unidimensional on this topic. A more recent study reevaluated NFATC1 in hepatocellular carcinoma and found that *low* NFATC1 expression was correlated with increased tumor progression and size and that NFATC1 activates *FASL* to drive apoptosis (Xu et al., 2018). Conversely, NFATC1 overexpression suppressed proliferation via an EGR2-dependent response pathway (Wang et al., 2020). This would suggest that NFATC1 is a tumor suppressor in hepatocellular car-

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<sup>74</sup>This same NFATC1-STAT3 transcriptional complex has been shown to be important for osteoclastogenesis, continuing on the theme that context is essential for interpreting consequences of pathway activation (Baumgart et al., 2014)

<sup>75</sup>The bladder cancer finding is interesting and little has been done to study the intersectional benefit of the standard bladder cancer treatment – BCG inoculation – and some of these other drugs. It would be intriguing if NFAT inhibition altered BCG-dependent cytotoxicity against bladder cancer, as this could reveal further mechanisms of NFAT in mycobacterial responses more generally.

cinoma. While this is an outlier in the overall schema, it is probable that NFATC1 can act as either pro- or anti-tumor depending on additional, as-yet-unknown factors.

A variety of minor roles have been uncovered for NFATC1 in regulating particular populations of stem cells. In the hair follicle, NFATC1 activity is related to quiescence while downregulation of NFATC1 spurs proliferation (Horsley et al., 2008; Keyes et al., 2013). This finding explained a known clinical phenomenon wherein patients receiving cyclosporine A display accelerated hair growth and established an important regulatory role for this protein in cycles of growth and quiescence in this tissue. In contrast to its roles in cancer, here NFATC1 activity correlates with inhibition of growth. Similar effects are seen in lung stem cells, where these cells are induced toward terminal differentiation during regeneration and replacement of alveolar epithelia (Lee et al., 2014).

While comprehensive profiling of the presence of NFAT isoforms in particular immune cell subtypes and under different conditions is broadly lacking, one cell type in which NFATC1 is known to be active is neutrophils. Exposure of neutrophils to allergic antigens<sup>76</sup> or immunoglobulin E activated them to express *COX2* and secrete prostaglandin E2<sup>77</sup> in a NFAT-dependent manner (Vega et al., 2007). Subsequent work investigated this pathway in the context of mast cell biology and, although the approaches are somewhat discordant<sup>78</sup>, found an important role for NFATC1 in reg-

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<sup>76</sup>These neutrophils were purified from patients with known allergies and compared to “normal” neutrophils from patients who lacked the allergy.

<sup>77</sup>There is much that can be said about the role of prostaglandin E2 ( $PGE_2$ ) in tuberculosis immunity (Serhan and Chiang, 2008). One of the major roles that has been uncovered is as a protective lipid able to modulate the balance between IL-1 and IFN- $\alpha/\beta$  to improve disease outcomes (Mayer-Barber et al., 2014). The study of host lipid signaling molecules is an interesting field of tuberculosis biology but is beyond the present scope of this work.

<sup>78</sup>For instance, they precipitated the HIF-1 $\alpha$  promoter with NFATC1 but utilized NFATC4 over-expression to demonstrate increased HIF-1 $\alpha$  expression. Under a model where all of the NFAT isoforms are equivalent, this is a reasonable enough approach, but lacks the subtlety that both more modern and more classical approaches might take.

ulating the transcript-level expression of HIF-1 $\alpha$  under hypoxia, potentially linking NFAT activity to the induction of hypoxic responses and, importantly for this work, VEGFA expression and angiogenesis (Walczak-Drzewiecka et al., 2008). No further work has been done to evaluate these results on the NFAT-HIF-1 $\alpha$  interaction, but this is one potential pathway by which the results given in Chapter 3 might operate, although with a different isoform – NFATC2.

## NFATC2

NFATC2 was actually the first of the NFAT proteins to be identified, in 1988 by Gerald Crabtree. This protein was found to be required for the T cell activation cascade and bound the IL-2 promoter as well as the LTR of HIV-1 (Shaw et al., 1988). Anjana Rao subsequently found that NFATC2 was activated by calcineurin and could be inhibited by clinical calcineurin inhibitors and that NFATC2 engaged in cooperative binding with members of the AP-1 transcription factor family to induce transcription of IL-2 (Jain et al., 1993). These two studies provided us the general model of NFAT activity that has been repeatedly validated into the present. Subsequent results added some subtlety to this described transcriptional effect through the discovery that NFATC2 acted in an immunoinhibitory fashion, with *Nfatc2*<sup>-/-</sup> mice demonstrating increased B cell proliferation in response to *Leishmania* and exacerbated allergic responses to ovalbumin (Xanthoudakis et al., 1996).

As might be expected, this founding member of the NFAT family was also the first to have described roles beyond the adaptive immune system with Aramburu et al. (1995) finding that NFATC2 was activated in natural killer cells<sup>79</sup> after stimulation of Fc $\gamma$ RIII. Intriguingly and in an observation rarely noted in the literature, this

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<sup>79</sup>While this is not a dramatic deviation from the theme, as natural killer cells are derived from the lymphoid compartment, it was an early indication that T cell activation was not the whole tale of NFAT.

activation of NFATC2 drove the transcription of *NFATC1*, suggesting NFAT cross-family regulatory patterns and the potential for NFAT transcriptional induction to be an additional layer of regulation within this pathway.

But by 2000, it was found that NFATC2 was an important repressive determinant of chondrogenesis as mice lacking NFATC2 exhibited increased chondrocyte<sup>80</sup> development and ossification and appeared to act as a tumor suppressor (Ranger et al., 2000). This tumor suppressor hypothesis introduced by the authors would be more thoroughly explored some time later by comparison of the effect of activation of NFATC1 and NFATC2 in fibroblasts. A constitutively active NFATC2 induced apoptosis in these cells while a similarly modified NFATC1 induced proliferation and transformation; conversely, *Nfatc2*<sup>-/-</sup> mice were more susceptible to tumor formation (Robbs et al., 2008). However, together, these two proteins repress the development of osteoarthritis (Greenblatt et al., 2013). This mechanistic differentiation between these closely related isoforms was a strong indication that, despite having a common DNA binding motif and overlapping gene expression, that these isoforms were differentiable in function and, likely, act in a balancing manner in normal cells. Other studies found that NFATC2 inhibits the expression of *CDK4*, a kinase important for cell division and often misregulated in cancer (Baksh et al., 2002; O’Leary, Finn, and Turner, 2016) and cyclin A2, a key cell cycle protein (Carvalho et al., 2007).

Similar to the previous description for NFATC1, NFATC2 is a critical regulator of T and B cell activation, but primarily in concert with NFATC1 (Peng et al., 2001). Individual roles for NFATC2 came later, with a notable publication finding that IL-6 detection by T cells drives increased NFATC2 expression and subsequent T<sub>H</sub>2 differentiation and colitis, as this signaling pathway drives NFATC2-dependent tran-

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<sup>80</sup>Chondrocytes are cells that produce and maintain cartilage between bones and are essential for healthy skeletogenesis.

scription of IL-4 (Diehl et al., 2002; Weigmann et al., 2008). Unlike NFATC1, which appears to more broadly induce the expression of both pro- and anti-inflammatory cytokines, NFATC2 generally regulates the expression of anti-inflammatory signaling cascades; NFATC2-deficient mice have reduced expression of IL-10, one of the classical, anti-inflammatory, type II cytokines<sup>81</sup> (Lee et al., 2009). Further contributing to this is that while constitutively active versions of both NFATC1 and NFATC2 are capable of activating transcription of the IL-4 promoter, knockout mice for NFATC2 demonstrate dramatically reduced IL-4 production while NFATC1 knockout mice show *increased* IL-4 production (Monticelli and Rao, 2002) and delays in IL-4 resolution, conferring a resistance defect to *Leishmania* (Kiani et al., 1997). Conversely, NFATC2 alone appears to regulate TNF- $\alpha$  and IFN- $\gamma$  expression in T cells as expression of NFATC2 was able to induce TNF- $\alpha$  while expression of NFATC1 was unable to do so, an ability conferred by the C-terminal activation domain (Kaminuma et al., 2008; Teixeira et al., 2005). TNF- $\alpha$ , although considered a classical pyrogenic cytokine, is highly pleiotropic and induces context dependent protection from bacterial infections and cancers but can induce autoimmunity and allergy. Signaling from this single molecule can induce a spectrum of phenotypes from proliferation to necrosis (Gough and Myles, 2020). Notable is the essential role of TNF- $\alpha$  in host defense to tuberculosis, implicating by proxy a potential role for NFATC2 in this process<sup>82</sup>.

For the purposes of this document, one of the most impactful publications on NFATC2 came through an investigation on the contribution of NFATC2 to tuberculosis pathogenesis in mice (Via et al., 2012). While a more comprehensive analysis

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<sup>81</sup>Along with IL-4 and IL-13, IL-10 is a primary stimulant of inflammation-resolution after insult.

<sup>82</sup>Given the positive and negative roles that TNF- $\alpha$  has in the pathology of tuberculosis, NFAT may offer some mechanism for modulating expression without complete blockade, which may be one way that TNF- $\alpha$ -targeted therapies could go about shifting the balance of host immunity and host damage (Mootoo et al., 2009).

of how these findings integrate with the findings I will present in Chapter 3 is well warranted and will be addressed in Section 3.4, this was one of, if not the first study of NFAT signaling as a contributor to tuberculosis pathogenesis and/or host defense. *Nfatc2*<sup>-/-</sup> mice have impaired adaptive immune responses to *M. tuberculosis* infection, with CD4<sup>+</sup> T cells having especially notable defects. This study used the tuberculosis-resistant BALB/c background and infected with *M. tuberculosis* HN878, a more recent clinical isolate<sup>83</sup>. *Nfatc2* null mice had 10<sup>3</sup> greater bacterial burden and greatly disseminated infection at the terminal time point, although they had equivalent burden up to approximately 6 weeks post infection. Previous studies had identified that NFATC2 was required for production of both IFN- $\gamma$  and TNF- $\alpha$  by T cells, but the findings here displayed an unusual mispatterning, with greater levels of TNF- $\alpha$  at 4 weeks post infections, a phenotype specific to CD4<sup>+</sup> T cells. This collection of data suggested that NFATC2 was important for tuberculosis disease control over the entire course of infection, although the precise immunological mechanism remained unknown, with the majority of the phenotype attributed to these misregulated CD4<sup>+</sup> cells.

NFATC2 has also been demonstrated to have important roles in antifungal responses with mesenchymal stromal cells within the adipose tissue, an unusual finding that suggests an important role for this pathway in various aspects of cell-autonomous defense as well as innate immunity (Tidu et al., 2021). Such findings may inform the use of calcineurin inhibitors in graft-versus-host disease, as the immune responses of stromal cells may be important in mediating antifungal control both from the intestine as well as invasive skin infections.

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<sup>83</sup>Both are somewhat unconventional choices, especially for the time. BALB/c mice are more T<sub>H</sub>2 skewed than C57BL/6 mice, which are very T<sub>H</sub>1 skewed. Mutation of NFATC2 on the BALB/c background should make the overall immune response more inflammatory, based on all of the findings already detailed.

One of the innovations of the new millennium in the study of NFAT was more thorough characterization of double knockout phenotypes. We have already seen studies conducted with NFATC1 and NFATC2 double knockout animals<sup>84</sup>, but none with other isoform combinations. Later studies investigated NFATC2 and NFATC3 combinatorial effects and uncovered evidence that double knockout of these isoforms renders CD4<sup>+</sup> T cells unresponsive to suppression by T<sub>regs</sub>, causing systemic hyperinflammation (Bopp et al., 2005) while NFATC2 mutation alone has no effect on development (Cante-Barrett, Winslow, and Crabtree, 2007). This effect also causes intrinsic differentiation into hyperproliferative T<sub>H</sub>2 cells, which may explain aspects of the inflammation seen in this context (Rengarajan, Tang, and Glimcher, 2002; Ranger et al., 1998a). Both of these had been previously seen to be immunosuppressive isoforms, so their combinatorial knockout appears to exacerbate the inflammatory phenotypes of the single knockout (Oukka et al., 1998; Hodge et al., 1996) while allowing NFATC1 unrestricted access to promoter elements rather than the wild-type counterbalancing that likely occurs (Ranger et al., 1998a).

The capacity for NFATC2 to regulate growth became important for the development of muscle cells, which must undergo syncytiation to become multinucleated. It was noted that *Nfatc2*<sup>-/-</sup> mice had reduced muscle size compared to wild-type mice. These mice are capable of forming normal numbers of muscle fibers but are incapable of muscle hypertrophy, endomitosis, and myoblast fusion required for standard musculature (Horsley et al., 2001; Pavlath and Horsley, 2003; Schulze et al., 2005). Keeping with this theme, NFATC2 is also required for cardiac hypertrophy. NFATC2 is the dominant isoform in the heart and myocardial-specific constitutively active calcineurin-induced hypertrophy was abrogated in the *Nfatc2* null background. In this

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<sup>84</sup>These have high overlapping expression in T cells, were the first to be discovered, and are most similar to one another, providing a solid basis to begin study with these.

context, NFATC2 is clearly detrimental as knockout of NFATC2 protected mice from various pathologies with no impact on exercise-induced cardiac enlargement (Bourajjaj et al., 2008). In  $\beta$  islets in the pancreas, NFATC2 activation is required to induce proliferation, which depends on the transcriptional target *NR4A1* (Simonett et al., 2021). This develops an important theme for NFATC2, which generally appears to regulate important growth and cell division phenomena in contrast to the role of the other isoforms.

NFATC2 was also found to contribute to tumor-associated macrophage biology by promoting further macrophage infiltration into melanomas, which corresponded to increased growth and metastasis. Notably, this phenomenon was present in response to overexpression of NFATC2 in either the melanoma itself or the tumor-associated macrophages (Liu et al., 2018). It had previously been found that NFATC2 transcriptionally inhibits *MITF* expression, inducing dedifferentiation which facilitates immune evasion (Perotti et al., 2016). Further publications on melanoma found that, through the induction of IL-8 and MMP3, NFATC2 increases tumor growth and metastasis (Shoshan et al., 2016). Similar effects were seen in lung adenocarcinoma, with more exhaustive work investigating the influence of NFATC2 on recurrence and survival, where high expression increased the likelihood of recurrence (Xiao et al., 2017); depletion of NFATC2 conversely decreased invasion and migration (Liu, Zhao, and Wu, 2013). While this phenotype is more reminiscent of those seen with NFATC1, these isoforms can clearly display some elements of redundancy and inappropriate activity, especially in a context like cancer. An interesting paper found an NFATC2 translocation event fusing EWSR1 (the classic Ewing sarcoma RNA binding protein) with NFATC2 through a chromosome 20:chromosome 22 fusion event that drove Ewing sarcoma. In these translocations, the N-terminal domains of NFATC2 were replaced by the N-terminal region of EWSR1, driving a constitutively active

NFATC2 linked to the transactivating segment of EWSR1 (Szuhai et al., 2009).

Relevant for this work, roles for NFATC2 in myeloid cells are relatively under-studied and much of what is known is derived from studies in dendritic cells<sup>85</sup>. As previously mentioned, important pro-tumor roles for NFATC2 in tumor-associated macrophages have been identified (Liu et al., 2018). An earlier study examined NFATC2 in the context of myeloid development and found that myeloid progenitors expressed high levels of NFATC2 that diminished over the course of development, however this did not address any changes that might happen with further differentiation or functional stimulation in the context of disease (Kiani et al., 2004). A study on the underlying ontogeny of inflammatory bowel disease found that NFATC2 nuclear translocation in macrophages was essential for the development of the disease and could be regulated by the NFAT kinase, LRRK2 (Liu et al., 2011). A follow-up study (which did not look at the isoform-specific level) used the VIVIT peptide to inhibit NFAT activation in macrophages and found that this provided a benefit in the context of ulcerative colitis in a mouse model (Elloumi et al., 2012). This inhibition blocked the expression of several major cytokines implicated in this disease, including TNF- $\alpha$ , IFN- $\gamma$ , and IL-12. Unlike NFATC1, NFATC2 is thought to be absent from neutrophils, the other major cell type in the initial-early response to tuberculosis infection, suggesting that any primary role for NFATC2 in the immune response to tuberculosis is most likely to be restricted to the responding macrophages (Greenblatt et al., 2010). NFATC2 in mast cells has recently been identified to be important for the mast cell-mediated production of IL-3 and, indirectly, IL-9, which are key cytokines that regulate macrophage and T cell biology (Sabbaghi et al., 2021). While

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<sup>85</sup>Dendritic cells can derive from either the myeloid or lymphocytic compartments. Despite this caveat, for most purposes, most of the circulating dendritic cells are “conventional” dendritic cells from a myeloid origin while a minority are plasmacytoid (Shortman and Liu, 2002; Collin and Bigley, 2018).

not a crystalline picture, this establishes some generally known roles for NFATC2 in myeloid cell biology, an important foundation for my work presented in Chapter 3.

### NFATC3

One of the major roles that have been described for NFATC3 is in vascular biology, especially as it pertains to pulmonary hypertension (Nieves-Cintron et al., 2007; Nieves-Cintron et al., 2008). The first indication in this direction was that NFATC3 in tandem with NFATC4 is important for vascular patterning during mouse development, where mutation of either both of these isoforms or of calcineurin causes a lethal failure of angiogenesis, rendering the fetus unable to develop properly organized vasculature (Graef et al., 2001). Additional characterization of these double mutants revealed profound defects in the cardiac tissue itself, exhibiting mitochondrial dysfunction (Kegley et al., 2001; Bushdid et al., 2003). Later, it was discovered that NFATC3 alone was responsible for cardiac hypertrophy in response to hypertension, giving this isoform a key role in cardiovascular responses more broadly (Wilkins et al., 2002). Under a condition described as “ $\text{Ca}^{2+}$  sparklets,” it was uncovered that these calcium signals were able to stimulate cardiac calcineurin to drive the activation of NFATC3, which induces a variety of hypertensive phenotypes, including increases in cytosolic calcium and increased muscle tone (Nieves-Cintron et al., 2007). A pair of papers from the same group found that a set of micro RNAs were able to regulate the activity of NFATC3 in the context of cardiac hypertrophy, adding a novel layer of regulation to the NFAT family of proteins and suggesting that other isoforms may be regulated by miRNAs under specific circumstances (Lin et al., 2009; Wang et al., 2010).

Shortly thereafter, a new player in the regulation of NFATC3 was introduced – hypoxia. Under conditions of hypertrophy, the cardiac muscle experiences increased

hypoxia and it was a question of whether or not these previously described NFATC3 phenotypes may be integrated with, independent of, or in opposition to that hypoxia. A study found that hypoxia increased the expression of endothelin-1 (a vasoconstrictive signaling peptide), which activated arterial NFATC3 in a way facilitated by changes in actin polymerization (Frutos et al., 2011). These somewhat discordant observations set the stage for further studies on the interactions of hypoxia, a classic stimulus of angiogenesis, and the NFAT signaling pathway. Similar to other hypoxia-responsive pathways, this one is also responsive to superoxide and other reactive oxygen species, suggesting that there may be some connection between the activation of NFATC3 and HIF-1 $\alpha$  either directly or indirectly (Ramiro-Diaz et al., 2014). Outside of the heart proper, NFATC3 is required for muscle development; knockout results in decreased muscle size in adult mice via alterations in myosin heavy chain expression (Kegley et al., 2001; Delling et al., 2000). Other alterations are seen other arterial smooth muscle, reducing the expression of potassium channels after angiotensin treatment (Stevenson et al., 2001; Amberg et al., 2004); a similar effect is seen in bladder smooth muscle cells (Layne et al., 2008).

More minor roles for NFATC3 have been identified in astrocytes, which are support cells within the brain. NFATC3 is activated by glutamate receptors, which induce influxes of calcium. Despite uncovering this activity, no particular role was ascribed to NFATC3 in these cells (Jones et al., 2003). The role for NFATC3 in liver regeneration has also been assessed and this isoform encourages hepatocyte proliferation (Pierre et al., 2009). These anecdotal roles for NFATC3 suggest broader roles in other tissues that have not been fully explored. In the days of single cell transcriptomics, it is likely worthwhile to explore the role of this isoform and others in tissues with clearly enriched expression of single isoforms.

By intriguing contrast to the previously discussed findings on NFATC2, NFATC3 was found to be an important *positive* determinant of chondrogenesis (Ranger et al., 2000; Tomita et al., 2002). NFATC3 activity induces *BMP2*, which drives differentiation of chondrocytes from mesenchymal cells. This suggests an interesting potential interplay between NFAT isoforms where they may be able to competitively inhibit particular binding motifs and repress or activate transcription based on the activity of other transcriptional coregulators exclusive to one or a subset of the isoforms. A more comprehensive assessment of these sort of interactions would certainly provide an invaluable resource in interpreting some of these polarized results found with distinct isoforms.

NFATC3, more than any of the other isoforms, has been found to have a number of roles in macrophages, courtesy of the observation that NFATC3 nuclear localization was required for TLR-mediated inflammatory responses (Minematsu et al., 2011) but that NFATC3 nuclear localization was inhibited by NF- $\kappa$ B as well (Conboy et al., 1999). As has been seen previously, NFAT protein can have antagonistic roles with NF- $\kappa$ B and this is the explanation provided here. These unusual results (both necessary and inhibitory) are somewhat at conflict with the remainder of the literature, however, as TLR agonists were incapable of directly activating NFAT and this effect was mediated by existing pools of nuclear NFATC3 in the cells they were using that appeared to mediate this phenotype. This suggested a new level of transcriptional regulation that depended on some layer of interaction between NFATC3 and NF- $\kappa$ B that prevented excessive NF- $\kappa$ B activity. A complementary study evaluated the contribution of NFATC3 in Gram-negative sepsis and found that NFATC3 was important for the release of pro-inflammatory cytokines and compounds, specifically nitric oxide<sup>86</sup>. Again, these results argue against a direct role for TLRs in inducing

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<sup>86</sup>Nitric oxide has many described roles in both modulating vascular dilation and in host defense

NFAT, but suggest that existing nuclear NFATC3 or some other stimulus facilitate transcriptional responses in cooperation with TLR-induced signals (Ranjan et al., 2014). During lung injury, NFATC3 was found to exacerbate lung pathology due to regulation of CCR2<sup>87</sup> and TNF- $\alpha$  production by macrophages (Karpurapu et al., 2018). These NFATC3-dependent macrophage activities drove neutrophil recruitment and vascular damage; genetic removal of NFATC3 accelerated lung repair and diminished inflammatory responses. Finally, in 2019, an answer was uncovered as to what, if any stimulus, was causing NFAT activation in macrophages during sepsis. TRPV4, a calcium channel responsive to vanilloid activation, was found to increase Ca<sup>2+</sup> influx in macrophages to exacerbate the production of reactive oxygen species and cytokines; inhibition of this channel dampened these effects and improved mouse survival after injury (Li et al., 2019). Other studies have identified an important role for NFATC3 in inhibiting the formation of foam cells<sup>88</sup> in atherosclerosis. Transgenic overexpression of NFATC3 was protective against atherosclerosis and prevented the formation of foam cells via miR-204, making NFAT activation potentially of therapeutic use in this disease (Liu et al., 2021b).

Additional studies have identified important roles for NFATC3 in concert with NFATC1 in antifungal responses in neutrophils. Greenblatt et al. (2010) found that cyclosporine treatment of mice increased their susceptibility to *Candida* infection

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against mycobacteria. Early studies found an essential role for the inducible nitric oxide synthase in protection against *M. tuberculosis*, adding yet another key pathway that is regulated, at least in part, by NFAT (MacMicking et al., 1995; MacMicking, Xie, and Nathan, 1997; MacMicking et al., 1997).

<sup>87</sup>CCR2 is a chemokine of two minds: essential for the recruitment of macrophages in response to mycobacterial infection and ultimately detrimental to the outcome of disease by recruiting the wrong kinds of monocytes and macrophages. This will be detailed in Subsection 1.6.1, but for now, CCR2 induction can be considered an incidental pro-bacterial response during tuberculosis although it is likely protective in most other disease contexts (Cambier et al., 2014).

<sup>88</sup>These lipid-laden cells also develop during granuloma formation; it would be interesting to see if the NFAT approaches in Chapter 3 alter the formation of these in the granuloma and, if so, the consequences of those changes.

through blockade of calcineurin activity. Through a genetic approach, they found that NFATC1 and NFATC3 were redundantly required for the induction of IL-10, but were dispensable for fungal killing and overall murine survival, suggesting that calcineurin is executing NFAT-dependent and -independent functions within neutrophils during fungal infection – dissection of these contributions and any alterations resulting from NFATC1/NFATC3 knockout in a more granular way would certainly add to our understanding of this pathway more generally.

None of these sections would be complete without a description of the role of NFATC3 T cell biology, where, once again, it plays an important role in thymocyte positive selection and  $T_{reg}$  activity (Ho et al., 1995; Bopp et al., 2005). In the absence of NFATC2 and NFATC3,  $T_{regs}$  are unable to inhibit CD4 $^{+}$  T cells, assigning these isoforms inhibitory roles toward the helper activity of CD4 $^{+}$  T cells. NFATC3 is also specifically required for positive selection and null mice are unable to generate CD4 $^{+}$ /CD8 $^{+}$  double-positive thymocytes; indeed, this is an effect specific to NFATC3 as rescue experiments with other isoforms failed to restore the generation of double positive cells (Cante-Barrett, Winslow, and Crabtree, 2007). NFATC3 is also capable of inducing expression of IFN- $\gamma$  and TNF- $\alpha$  while downregulating IL-4 and IL-5 expression; these findings are gain-of-function experiments as no role was able to be identified with mutation of NFATC3 alone (Chen et al., 2003). Aside from these roles, relatively little else is known about this isoform in T cells, unlike the extensive roles already described for NFATC1 and NFATC2.

## NFATC4

As mentioned in the previous section, NFATC3 and NFATC4 have a number of cooperative roles, including in the developing vasculature and in cardiac development (Graef et al., 2001; Bushdid et al., 2003). However, NFATC4 has additional roles that

are somewhat unusual for the NFAT family of proteins, as it is minimally expressed in the immune system and has found a variety of roles in diverse somatic tissues, including adipocytes, hepatocytes, and neurons. However, this is likely the least studied of this family, so further roles are likely to be discovered in the coming years.

NFATC4 has been of some interest in the pathology of diabetes due to its ability to regulate the expression of genes in adipocytes, including by inhibition of the transcription of adiponectin, an important biomarker for diabetes and obesity. While the ability for NFATC4 to inhibit expression of adiponectin was demonstrated *in vitro*, whether this pathway was important for the expression *in vivo* is unknown (Kim et al., 2006). Conversely, active NFATC4-mediated transcription in adipocytes is associated with the expression of a variety of pro-inflammatory cytokines, including TNF- $\alpha$  (Kim et al., 2008). Adipocyte Lipin 1 is able to repress NFATC4 activation to mute these inflammatory responses, making Lipin 1 a compelling anti-inflammatory target in obesity (Kim et al., 2010). This provides a somewhat unified model, as chronic inflammation and obesity are known to be correlated and adipocyte NFATC4 activity may contribute to obesity-induced inflammation.

NFATC4 has also been ascribed important roles in mediating liver damage; this is in contrast to the role NFATC3 plays in facilitating liver repair and furthers the arguments for more targeted, isoform-level approaches to understanding the functions of these proteins. In non-alcoholic steatohepatitis (NASH), knockdown of *NFATC4* liberated PPAR $\alpha$ , which enabled renewed fatty acid metabolism to reduce the burden of lipids in hepatocytes; the activity of NFATC4 also increased macrophage recruitment via inflammatory signaling from the hepatocytes, further contributing to the inflammation seen in NASH (Du et al., 2020). Later studies also revealed a role for NFATC4 in ethanol-induced cell death via inhibition of PPAR $\gamma$  (Wu et al., 2021).

In both cases, inhibition of NFATC4 resulted in improvements in disease and argues that this pathway is an key regulator of liver damage that is serving a maladaptive role in this context.

NFATC4 also has a number of described roles within neurons and neural progenitors. The earliest study found that NFATC4 was required for regulating the expression of the vasoactive intestinal peptide, an important neurotransmitter, but did not look at this activity in neuronal cells *per se* (Symes, Gearan, and Fink, 1998). The first description of NFATC4 specifically in neurons found it to be responsive to L-type calcium-channels and to induce ITPR1 expression, implicating it in hippocampal neurons and memory formation (Graef et al., 1999). Further studies have expanded upon this initial finding and found important roles for NFATC4 in regulating apoptosis in the cochlea (Luoma and Zirpel, 2008; Benedito et al., 2005), cortical neurons (Vashishta et al., 2009), and adult-born neurons (Quadrato et al., 2012). Moreno et al. (2015) found that hypoxia in neural stem cells activated NFATC4 and that this pathway is a major contributor to stem cell proliferation, differentiation, and self-renewal through regulation of ID2. Another study found that NFATC4 inhibits  $\beta$ -catenin signaling to drive neuronal proliferation and induce cell cycle exit (Huang et al., 2011). Together, these studies identify an important role for NFATC4 in regulating neuronal development and behavior; further studies in a knockout mouse should explore the behavioral and intellectual consequences of this disruption.

An older study also found that NFATC4 *inhibits* breast cancer motility (Fougere et al., 2010). This study found that inhibition of NFATC4 increased motility by releasing inhibition of lipocalin 2 gene expression. This is in converse to a previous study that had found that NFATC4 was an interacting partner for the estrogen receptor

and facilitated the expression of estrogen-responsive genes; knockdown of NFATC4 diminished breast cancer cell growth and was proposed as a potential therapeutic target (Zhang et al., 2005). This is a further demonstration of the pleiotropic nature of the NFAT pathway, but may also reflect differences in the biology of breast cancer given the dizzying array of subtypes and subtype-specific treatments now available.

NFATC4 has other miscellaneous described roles that warrant further investigation. During cardiac hypertrophy, overexpression of SIRT6 is able to inhibit the transcription and activation of NFATC4, which prevents excessive hypertrophy. This study was done primarily in the context of overexpression, so it would have been interesting to see if any physiological stimuli could activate SIRT6 to exert these effects as well (Li et al., 2018b). In the endothelium, NFATC4 was described as an important mediator of vascular inflammation; activated ORAI1 calcium channels drove NFATC4 activation and inhibition of these channels resulted in reduced inflammatory signatures in the vasculature (Yu et al., 2018). Both of these suggest that NFATC4 acts as a pro-inflammatory transcription factor during diverse cardiovascular responses.

### **Distinct Regulatory Mechanisms**

As has become apparent in the previous section, one of the notable roles of NFAT is not only its ability to induce transcription, but the many contexts in which it inhibits transcriptional responses, especially those induced by NF- $\kappa$ B. While transcription factors are generally thought of as *inducers* of transcriptional responses, the complex interactions between this family and other families of transcription factors as well as between NFAT isoforms is an interesting subject for future discovery. For instance, in the context of angiogenesis, it is thought that NF- $\kappa$ B induction of the Fas ligand increased endothelial apoptosis and inhibits angiogenesis while NFAT-mediated activation of cFLIP, a pro-survival gene. Thus, the functional antagonism between

these two transcription factors determines the overall degree and timing of angiogenesis within the endothelium (Aurora et al., 2010). Previously, Conboy et al. (1999) had found antagonism between NFAT and NF- $\kappa$ B in the process of macrophage activation, where NF- $\kappa$ B induces TNF- $\alpha$  while NFATC3 opposes that transcriptional induction. In the opposite direction, in osteoclasts, NF- $\kappa$ B induces transcription of NFATC1, a key aspect of the differentiation of osteoclasts from macrophages (Asagiri et al., 2005). Future models of NFAT activation need to reconcile the tension between this and the NF- $\kappa$ B signaling pathway in different contexts given their ability to mutually inhibit one another; it is even likely that these tensions have some level of isoform-specific regulation that is, to date, completely unknown.

Of course, one of the major mechanisms of differentiating the members of the NFAT family is in their expression profile. Isoforms that are exclusive to particular tissues are likely to exert specific roles in those tissues. However, in the presence of overlapping expression, there must be additional signals or distinctions that allow them to exert distinct phenotypes rather than functioning strictly redundantly. While the breadth of these differences are not yet known, one of the major aspects that have been studied is in the dynamics of nuclear import and export across the different isoforms (Chow et al., 1997; Yissachar et al., 2013; Kar and Parekh, 2015; Kar et al., 2016; Ulrich et al., 2012). Each isoform also has a range of different spliceoforms that can modulate activity (Vihma, Pruuksild, and Timmus, 2008; Mancini and Toker, 2009). For instance, NFATC2 can be spliced into at least three functionally distinct isoforms with different abilities to activate transcription with one being inhibitory toward the others (Chuvpilo et al., 1999). These levels of regulation are sure to be more complex than we presently understand and offer another avenue of fruitful future study.

The NFAT isoforms share a common DNA binding motif (5'-GGAAA-3'), which enables them to exhibit the redundancies previously described (Rao, Luo, and Hogan, 1997; Chen et al., 1998). However, these isoforms may also have distinct DNA binding motifs to which they bind more efficiency than others, which can offer an additional layer of regulation. For instance, NFATC2 can bind to the sequence 5'-C<sup>m</sup>GGAA-3', which will comprise some, but not all of the consensus sequences (Ray et al., 2021). Differential binding affinity for the other isoforms has not yet been exhaustively detailed, but future studies are likely to uncover preferences among them for neighboring nucleotides around the consensus sequence. An additional layer of regulation is via posttranscriptional modifications, including SUMOylation, which has been noted for NFATC1 and ubiquitination, which has been described for NFATC4 (Fan et al., 2008; Xiao et al., 2021).

A further layer of relatively understudied biology is the aggregation of NFAT around RNA scaffolds in the cytosol, making them RNA binding proteins. This description is consistent with our immunofluorescence observations (in Subsection 3.3.10) and makes for an interesting additional layer of potentially variable regulation (Sharma et al., 2011). Knowledge as to whether or not different NFAT isoforms bind to different RNA scaffolds or are differentially regulated by that binding would add to our understanding of the complex mechanisms that allow some to be activated while others remain inactive within the same cells.

A quartet of foundational studies set the stage for some of the mechanisms by which the different NFAT isoforms can be regulated by distinct mechanisms. Ulrich et al. (2012) observed that NFATC3 and NFATC4 displayed distinct activation kinetics in hippocampal neurons, with NFATC3 rapidly activating while NFATC4 slowly activated over a longer period of time. This interestingly made NFATC3 the func-

tionally dominant isoform and knockdown of this single isoform abrogated most of the responses even in the presence of NFATC4. This effect was thought to be mediated by different sensitivity of the isoforms to rephosphorylation by GSK3 $\beta$ . Yissachar et al. (2013) compared the kinetics and dynamics of NFATC2 and NFATC3 and found that, while NFATC3 exhibited rapid nuclear localization and rapidly shuttles in and out of the nucleus over time, NFATC2 exhibits a slower response that steadily increases over time with increasing signal duration. While these distinctions had been grossly noted for some time (Chow et al., 1997; Rinne, Banach, and Blatter, 2009), the precise mechanisms were unclear. A later study by Kar and Parekh (2015) found that the precise cellular location of the Ca<sup>2+</sup> flux resulted in different effects; NFATC2 depended on a close association with ORAI1 channels while NFATC3 also required high nuclear calcium for sustained activity; this rise in nuclear calcium depends on inositol triphosphate receptors on the nucleus (Kar et al., 2016). These interesting layers of regulation are thought to be mediated by differences in the SP3<sup>89</sup> motif that governs nuclear import and export and which is the target of both calcineurin and NFAT kinases. As a layer of additional subtlety, it appears that these differences in kinetics can vary between different cell types, as NFATC2 translocates quickly in neurons while NFATC4 exhibits relatively slow translocation (Vihma et al., 2016). These effects may, in part, be regulated by different cytosolic compartments utilized by the resting NFAT isoforms and their proximity to various calcium channels and calcineurin (Ulengin-Talkish and Cyert, 2022). The degree to which findings in particular tissues can be generalized to others is not yet known and may be difficult to assess until further studies have done more exhaustive comparisons (perhaps in T cells or uterine epithelia, where all of them are expressed to some degree).

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<sup>89</sup>NFAT proteins have a series of serine-proline (SP) phosphorylation sites in the N-terminal domain that regulate its nuclear localization. One of these, the SP3 site, seems to be of additional importance in this process. See Figure 1.4.

### 1.4.3 Activity of NFAT in Myeloid Populations<sup>90</sup>

NFAT was discovered relatively early on to be one of the major and defining responses to CLR activation. Early studies had identified a novel SYK-dependent pathway that was required for transcriptional responses to zymosan through DECTIN-1 (Rogers et al., 2005). This work set the stage for the discovery of the signaling mechanisms downstream of SYK activation that were required to induce immune responses. CARD9 was discovered first as a master regulator of CLR-dependent anti-fungal signaling (Subsubsection 1.3.3) (Gross et al., 2006; Hara et al., 2007), but this was not long to be the only pathway engaged by DECTIN-1. Defined by Goodridge, Simmons, and Underhill in 2007 as an important response mechanism, NFAT activation has been co-opted over the years as an experimental tool to measure CLR activation because TLRs do not<sup>91</sup> activate NFAT (Yamasaki et al., 2009; Richardson and Williams, 2014; Ishikawa et al., 2013; Furukawa et al., 2013; Hattori et al., 2014).

By using either NFAT proteins fused to fluorescent proteins to monitor nuclear localization or the DNA regulatory elements for NFAT to drive luciferase or GFP from a minimal promoter, it is possible to capture a report of NFAT activation with high sensitivity or with rapid response times (Colella et al., 2008; Gwack et al.,

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<sup>90</sup>For additional reviews on this subject, see J. Fric et al. (2012b). “NFAT control of innate immunity”. In: *Blood* 120.7, pp. 1380–9 and K. Bendickova, F. Tidu, and J. Fric (2017). “Calcineurin-NFAT signalling in myeloid leucocytes: new prospects and pitfalls in immunosuppressive therapy”. In: *EMBO Mol Med* 9.8, pp. 990–999

<sup>91</sup>This is presented as the dogma in the literature although it warrants some degree of elaboration. Zanoni et al. found that CD14, the TLR4 co-receptor for LPS, induced the activation of NFAT in dendritic cells that was independent of TLR4, MYD88, and TRIF, suggesting TLR-independent signaling mechanisms govern NFAT activation in this context while Minematsu et al. found that NFATC3 and NFATC4 were required for TLR responses in macrophages. Notably, both CD14 and DECTIN-1 localize to lipid rafts and recruit PLC- $\gamma$ 2, offering a potential unifying model on the mechanisms of action at play here (Xu et al., 2009b). On the other hand Kang et al. found that NFAT activation was a negative regulator of TLR responses. As previously summarized, the bulk of the evidence is that TLRs themselves cannot directly activate NFAT although co-receptors and other effects may be able to engage this pathway (Zanoni and Granucci, 2012).

2006; Kar and Parekh, 2015; Kar et al., 2016; Jauliac et al., 2002; Wilkins et al., 2004; Chow, Rincon, and Davis, 1999; Aramburu et al., 1998). 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 subsequent 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.

An early study found that NFAT was required for the induction of EGR2<sup>92</sup>, EGR3, M-CSF, COX2, IL-2, IL-10, IL-12, and myeloperoxidase transcriptional responses and prostaglandin E2 production in response to zymosan through DECTIN-1 (Goodridge, Simmons, and Underhill, 2007). Conversely, TNF- $\alpha$  and IL-6 were independent of NFAT in this context and instead dependent on TLR2 and MYD88. These functional distinctions between the different pathways that could be engaged by a single ligand are important for determining these relative contributions and more selective therapeutic targeting of particular pathways. Activation of NFAT is critical for control of various fungal infections through both direct myeloid activation as well as important priming of the adaptive immune system through induction of TNF- $\alpha$ , IL-2, IL-10, and IL-12. Interestingly, it seems that genetic inhibition of NFAT is sensitizing to a greater degree than pharmacological treatment, likely due to the antifungal activity of calcineurin inhibitors (Herbst et al., 2015; Xu et al., 2009c; Zelante et al., 2017; Sugita et al., 2005).

Classically, the TNF-TNFR signaling pathway induces transcriptional responses

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<sup>92</sup>EGR2 is required for the development of natural killer T cells, so this is another mechanism whereby myeloid NFAT responses can drive adaptive immunity (Lazarevic et al., 2009).

primarily through NF-κB, although it is understood that other transcription factors can mediate downstream responses through TNFR. One of these, intriguingly, is the NFAT signaling pathway, which has been shown to be induced in macrophages (Yarilina et al., 2011). This offers the opportunity for TNF-α to feed-forward by driving its own transcription through NFAT. Given that these experiments were conducted under chronic TNF-α stimulation, this may be uniquely relevant to the biology of the granuloma where TNF-α is a major component of the cytokine milieu and is critical to host defense against mycobacteria.

A recent review has highlighted some of the known roles for NFAT activity in neutrophils in mediating important protective immune responses (Vymazal et al., 2021). This review posits that neutrophil NFAT signaling is a master regulator of neutrophil responses through the detection of various microbial ligands and subsequent induction of protective responses, most notably during fungal infection and after exposure to LPS through CD14. This review focused on the consequences of NFAT inhibition via calcineurin inhibitors on neutrophil function and concluded that suppression of neutrophil functions may increase patient susceptibility to various opportunistic infections, although these models struggle to completely differentiate the impact on different cell populations (Herbst et al., 2013). This is nonetheless an important consideration and makes NFAT targeting perhaps most appealing in the context of excessive inflammation. The study of these pathways within opportunistic fungal pathogens also in some ways limits the scope of these studies; while these are important and relevant pathogens in people receiving these immunosuppressive therapies after solid organ transplant, this may not reflect the behavior that would occur at lower doses or in otherwise healthy people.

Furthermore, there is somewhat of an NFAT renaissance occurring in the litera-

ture at the time of writing. Several new papers have emerged in the past several months identifying novel new roles for NFAT signaling in a variety of (predominantly hematopoietic) tissues, giving new emphasis to this long-neglected pathway (Deerhake et al., 2021; Poli et al., 2022; Peuker et al., 2022). This work will be more thoroughly reviewed in the next section, Subsection 1.4.4. My 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. This pathway has been neglected for some time due to a focus on CARD9-dependent signaling, but has now come back into the spotlight for its important roles in mediating diverse aspects of immunity.

#### 1.4.4 The NFAT Renaissance

As previously alluded to, a number of major papers have been published in the past few months assigning new roles to NFAT, with many of them specifically in cells of the hematopoietic lineage – neutrophils, macrophages, and platelets. The last major review of the role of NFAT in these cells was published by Fric et al. in 2012 and, compared to now, the picture was rather broad in the roles that NFAT plays in mediating response to various conditions. At the time it was understood that NFAT plays an important role in the development of myeloid cells, where it inhibits their differentiation; inhibition of NFAT signaling increased the percentage of cells that differentiate into the monocyte/dendritic cell lineage vis-à-vis neutrophils. While these roles for NFAT in development have important implications for the clinical use of NFAT inhibitors, they do not address the role of NFAT in these cells after development. Seminal early findings connected the NFAT signaling pathway to the

signaling response to different PAMPs, including  $\beta$ -glucan and zymosan via DECTIN-1 in macrophages and dendritic cells and LPS via CD14 exclusively in dendritic cells (Goodridge, Simmons, and Underhill, 2007; Zanoni et al., 2009). NFAT also has a number of role in the microbicidal activities of neutrophils and phagocytosis in macrophages. NFAT inhibition in the absence of adaptive immunity was known to increase susceptibility to fungal infections via blockade of the expression of anti-inflammatory cytokines.

Probably the first truly modern, comprehensive investigation of the role of NFAT in the innate immune response was published in late 2021 by Deerhake et al. This study, concurrent with the present work, evaluated the role of DECTIN-1 signaling in the pathogenesis of experimental autoimmune encephalomyelitis (Deerhake et al., 2021) here at Duke. DECTIN-1 expression was protective from the disease, but CARD9-dependent signaling exacerbated disease progression, demonstrating important roles for NFAT signaling in protection. Use of both genetic and pharmacological methods in a range of cell types found that myeloid cells stimulated with DECTIN-1 agonists drive activation of NFAT to produce neuroprotective cytokines, including oncostatin M and, importantly for this work, VEGFA. This modern, dataset-driven analysis is a key resource for understanding the spectrum of NFAT-dependent phenotypes induced by C-type lectin receptors and, while focused on one particular ligand, remains evocative of the potential for C-type lectin receptors to induce pro-angiogenic signaling. In their context, neutrophils were far more responsive to curdlan stimulation than were bone-marrow derived macrophages, although as has been discussed in Subsubsection 1.3.3, unprimed macrophages have overall dampened responses and different priming approaches may have provided differing results.

The study by Poli et al. (2022) is exceptionally interesting as it studied the role

of NFAT in platelets which, in mammals, are anucleated. Thus, this work studies the influence of NFAT on mediating cellular responses in the absence of transcription. Inhibition of NFAT in platelets using transgenic VIVIT expression exacerbated Gram-negative sepsis by inducing hypercoagulation, in part through interactions with neutrophils to induce neutrophil extracellular trap release. NFATC2 is dephosphorylated by activation of the thrombin receptor, which then inhibits activation of integrin  $\alpha$ IIb $\beta$ 3. Inhibition of NFAT leads to hyperactivation of integrin  $\alpha$ IIb $\beta$ 3 and increased coagulation of platelets. This increased integrin activity also increases platelet binding to circulating neutrophils; NFAT inhibition also leads to increased platelet degranulation, a marker of coagulative activity and inflammation and drives the recruitment of additional leukocytes. Use of a platelet-specific VIVIT mouse demonstrated a localized effect specific to platelets that drove increased inflammation in a model of Gram-negative sepsis. This work is interesting in the context of previous findings on the role of ITAM-linked receptors on platelets, which activate SYK, a known upstream activator of NFAT (Manne et al., 2015). The precise mechanism here remains unknown – what interacting partners could an NFAT protein have in an anuclear cell to mediate these activities? An exploration of the protein-protein interaction network of NFAT proteins could reveal interesting new biology applicable to nucleated cells as well; the function of this ostensible transcription factor in other realms of biology offers interesting opportunities to more specifically target those aspects of NFAT to modulate its activity. Clearly, new tools and deeper understandings of NFAT protein topology will be required to differentiate these classes of functions in these cells. This work highlights the potentially *pro*-inflammatory nature of NFAT inhibition (a potential benefit for those taking NFAT-inhibitory drugs for organ transplants) and a potential means to increase blood clotting responses for those with clotting disorders.

Peuker et al. (2022) found that deletion of calcineurin in myeloid cells was able to reduce the size of colorectal tumors in a mouse model in a manner dependent on microbiota-mediated activation of NFAT. NFATC1-dependent expression of IL-6 from macrophages induced the tumoral expression of two genes that negatively regulate T cell activation, B7H3 and B7H4; treatment of antibiotic-treated mice with LPS or myeloid-VIVIT expressing mice with IL-6 is able to restore the expression of these proteins within the tumor and promote tumor growth. Through selective ablation of particular immune cell populations, this effect was isolated to the response to neutrophils. However, this publication utilized some evidence of a role for MYD88 in contributing to this phenotype to attempt to link TLR4-MYD88 activation to NFAT activation, despite no other publication having ever made this argument and repeated historical demonstrations that TLRs could not activate NFAT and actually antagonized NFAT activity<sup>93</sup>. The reality in this situation is likely cooperative roles for NFAT and MYD88-mediated NF-κB pathways rather than a common signaling origin; this is especially notable for the studies testing various TLR agonists on the effect on IL-6 expression, which may be additionally sensitive to these sorts of combinatorial effects where both LPS (TLR4 ligand) and Pam3CSK4 (TLR1/2 ligand) induce IL-6 in an NFAT-dependent manner. Both LPS and Pam3CSK4 could be mediating these effects directly through CD14-NFAT signaling, but this hypothesis was not tested in this instance; it would certainly be of some interest to investigate whether these described phenotypes are dependent specifically on CD14 or if there are yet further mechanisms at play.

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<sup>93</sup>One study, Liu et al. (2008a) argued for an NFAT-dependent cardiac response downstream of LPS, but did not explore a precise mechanism; CD14-mediated signaling seems likely but further study would need to be done.

## 1.5 Angiogenesis

Angiogenesis is a fundamental process in the development of vertebrate animals, which require the elaboration of blood vessels into all of the corporeal tissues in order to supply them with oxygen (Adams and Alitalo, 2007; Carmeliet and Jain, 2011). This process is an elaborate coordination between the endothelium itself, stimulating cells (fibroblasts, somites, macrophages, and cancer cells among them), the surrounding connective tissue, and mural cells that maintain the vasculature (Armulik, Genove, and Betsholtz, 2011; Stratman et al., 2017). This process also requires an intricate series of intracellular signaling events within endothelial cells to direct these processes of growth (but not too much) and directionality (Simons, Gordon, and Claesson-Welsh, 2016).

Much of what is known about angiogenesis was first established in the optically transparent and genetically tractable zebrafish model, which allows for very fine observation of the very earliest processes of blood vessel formation (Chan et al., 2002; Chavez et al., 2016). The initial stages of this are known as vasculogenesis, in which endothelial cells as such differentiate from hemangioblasts early in development (Vogeli et al., 2006; Bertrand and Traver, 2009). Once a basic vasculature has been established, it can be remodeled and extended upon by the process of angiogenesis (Koch and Claesson-Welsh, 2012). Much of this process is regulated by a single pathway – the vascular endothelial growth factor A/vascular endothelial growth factor receptor 2 (VEGFA/VEGFR2) signaling axis (Olsson et al., 2006; Chung and Ferrara, 2011; Villanueva, 2016). Despite the seeming simplicity in this, there are additional layers of regulation at every conceivable level. The detection of VEGFA by an endothelial cell sets off signals that drive proliferation and growth toward the

source of the chemokine, which can be a variety of cell types, including macrophages (Olsson et al., 2006). These tip cells sprout along the VEGFA gradient and regulate the cells behind them as a mechanism to prevent excessive branching<sup>94</sup> (Sakabe et al., 2017; Tammela et al., 2011). In the absence of both of the *VEGFA* homologs in the fish, *vegfaa* and *vegfab*, the somitic vasculature is completely absent and only the dorsal aorta, the caudal vein, and other vasculogenic vessels are able to form through VEGFA-independent processes. Notably, these phenomena are profoundly difficult to study in the mouse, which must overcome tissue hypoxia as an environmental stressor from very early stages of embryonic development while the externally developing larval zebrafish are perfused with oxygen until many days post fertilization. To complete the growth of normal blood vessels, the stalk of the angiogenic vessel must open a lumen, a process that is still not well understood but is known to be mediated, at least in part, by macrophages (Gerri et al., 2017).

This entire process must be finely regulated to maintain vascular homeostasis. Too much angiogenesis is clearly bad, as seen in cancer, but even in normal tissues, vascular hyperproliferation can result in retinal disorders and psoriasis (Bisht, Dhasmana, and Bist, 2010; Malecic and Young, 2017; Gupta and Zhang, 2005). Endothelial cells utilize a variety of downregulatory mechanisms to block excessive angiogenesis, including apoptosis and transcriptional repressors (Ben Shoham et al., 2012; Chavakis and Dimmeler, 2002; Garcia-Barros et al., 2003; Duval et al., 2003; Dimmeler, Haen-del, and Zeiher, 2002; Stefanec, 2000). Thus, the weight of pro-angiogenic factors must outweigh the inhibitory factors in order to induce a productive angiogenic response overall.

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<sup>94</sup>This is highly analogous to the ways in which plants regulate their growth. In plants, the growth tip secretes hormones along a gradient that prevents the activation of new growth points until the hormone concentration drops sufficiently low. This facilitates directional growth until the plant is large enough to warrant further branching.

These same fundamental processes are at play during pathological angiogenesis as these processes are similarly regulated by VEGFA/VEGFR2, but, depending on the cause of the pathology are notably disordered. While developmental angiogenesis is orderly and ultimately results in the formation of “normal” blood vessels, pathological angiogenesis often lacks the fine-tuning needed to form stable vessels with high integrity and are thus leaky, highly branched, and lack lumen formation. During these events, a much more complex and chaotic set of signals are at play which influence the endothelial biology of the region; ideally, the course of the response to the perturbation would restore homeostasis and facilitate vascular normalization and, eventually, regression. Additionally, the endothelium itself is a major source of signaling that support tissue homeostasis and responses, a role only recently appreciated (Amersfoort, Eelen, and Carmeliet, 2022). The vast degree of specification which the endothelium undergoes based on its surroundings is a key aspect of endothelial cell biology which can be difficult to study, much like modeling tissue-resident macrophages can be nearly impossible *in vitro*. Nevertheless, these roles have been increasingly dissected through the use of single cell transcriptomics to evaluate the diversity among inter- and intra-tissue endothelium, revealing so-called unexpected levels of heterogeneity in these tissues (Chavkin and Hirschi, 2020).

### 1.5.1 Angiogenesis in Cancer

Tissue perturbations, such as those caused by wounds, often drive the invasion of blood vessels toward the site of insult as a mechanism to facilitate tissue repair. However, angiogenesis can serve as a maladaptive response in many contexts. Most famous of these is in 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 (Park et al., 2016; Yoncu et al.,

2017; Fujita and Akita, 2017). One of the foundational observation in solid tumors was that they were often encased in vascular webs that differed substantially from those seen in neighboring, unaffected tissues (Chung and Ferrara, 2011). The size and cellular density of these tumors created a hypoxic environment at the center of the tumor that drove the activation of HIF-1 $\alpha$  and subsequent transcriptional responses geared toward the alleviation of this hypoxia; this pathway is also key in the metabolic switch in cancer that drives the Warburg effect (Mesange et al., 2014; Masoud and Li, 2015; Courtney et al., 2015). One of the most efficient means of alleviating cellular hypoxia is to shorten the distance between the cell and arteries, which are able to supply the cell with oxygenated blood. Under homeostatic conditions, no cell is more than 1 millimeter from the nearest blood vessel (Weber, Ju, and Borner, 2020). Tumors are thus under profound selective pressure to increase the availability of oxygen or perish and do so via upregulation of VEGF signaling both endogenously and via tumor-associated macrophages (Napione, Alvaro, and Bussolino, 2017; Riabov et al., 2014). However, the deregulated transcriptional responses of these tumors drive the production of new blood vessels that fail to properly mature and remain highly permeable to blood solutes while adapting to and influencing the tumor microenvironment (Qian et al., 2009; Carlson et al., 2021). This vascular permeability is advantageous to the tumor by allowing glucose and other nutrients to diffuse out of the vessels and into the tumor microenvironment (Park et al., 2016). Such leakiness would logically appear to be a boon to chemotherapeutic delivery, but actually serves as a monumental barrier to achieving sufficiently high drug concentrations at the site of the tumor to reach an effective dose (Datta et al., 2015; Goel, Wong, and Jain, 2012; Yoncu et al., 2017).

In the transition toward chemotherapeutic 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 (Potente, Gerhardt, and Carmeliet, 2011; Ranieri et al., 2014; Shibuya, 2011; Welti et al., 2013). This tyrosine kinase receptor triggers a downstream transcriptional response cascade that results in endothelial proliferation and directed growth toward the source of the ligand, VEGFA<sup>95</sup>. 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 and normalization of the vascular webs around tumors can be achieved.

### 1.5.2 The Relative Failure of Bevacizumab

These factors and others led to active development of vascular targeting therapies throughout the 1990s and early 2000s, which culminated in the approval of bevacizumab to treat metastatic colon cancer in 2004, only 15 years after the discovery of VEGF (Ferrara et al., 2004; Leung et al., 1989; Gordon and Cunningham, 2005; McCormack and Keam, 2008; Kabbinavar et al., 2005; Muhsin, Graham, and Kirkpatrick, 2004). Bevacizumab is a humanized monoclonal antibody targeting VEGFA, blocking ligand-receptor binding, VEGFR2 activation, and resultant angiogenesis (Keating, 2014). The promise of bevacizumab was to adjunctively enhance the efficacy of other drugs while starving the tumor of needed oxygen – a theoretical one-two punch at the cell biology of these tumors. However, the story ultimately proved more complicated and bevacizumab has been only a limited success and other

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<sup>95</sup>VEGF, or VEGFA, is the “canonical” VEGFR2 ligand, but there are four independent VEGF genes (VEGFA-D), each with different affinity for different VEGFRs (VEGFR1-3) and with different properties. VEGFB modulates VEGFA signaling in angiogenesis, while VEGFC and VEGFD are lymphangiogenic chemokines, which stimulate the growth of lymphatic vessels. This process remains relatively unstudied in the context of mycobacterial infection (Harding et al., 2015), but seems likely to play important roles in regulating the course of infection.

vascular-targeting therapies have fared little better. Bevacizumab is a humanized monoclonal antibody that very potently ( $K_D=1.1\text{nM}$ ) blocks the interaction between VEGFR2 and VEGF and induces vascular regression by binding and sequestering VEGFA (Papadopoulos et al., 2012; Yang et al., 2014b). This therapy has become standard of care for a subset of tumor types<sup>96</sup> and physiological locations, but the mystery remains why this therapeutic strategy targeting a highly conserved, indeed nearly ubiquitous (Donnem et al., 2018), feature of tumors is not more broadly applicable and generally successful (Van Meter and Kim, 2010). Such a therapy could, in theory, have been a universally beneficial adjunct through metabolic attack on the tumor coupled with improved drug delivery but proved to be comparatively limited. Initially approved only for metastatic colorectal cancer, it began to accumulate other indications for some time, until further trials in certain cancers found limited efficacy over the long term and relapsed cancers often demonstrating resistance and increased aggressiveness (Itatani et al., 2018; Rivera and Bergers, 2015).

It seems that the physiological stress causes by hypoxia appears to drive upregulation of compensatory pathways within the tumor itself – the escalating hypoxia in the local region drives rapid amplification of VEGF and PIGF production to alleviate such detrimental hypoxia (Alidzanovic et al., 2016; Gardner, Madu, and Lu, 2017). By this mechanism it is proposed that tumors increase the local concentration of VEGF beyond the stoichiometry between bevacizumab and VEGF or induce other growth factors (FGF-β and VEGFC among them) to promote vascular relapse and renewed angiogenesis toward the site in a return to *status quo ante* (Haibe et al., 2020; Montemagno and Pages, 2020; Zhao et al., 2017; Zahra, Sajib, and Mikelis, 2021; Michaelsen et al., 2018). These therapies can also induce extracellular matrix

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<sup>96</sup>Colorectal cancer, non-small cell lung cancer, breast cancer, ovarian cancer, and cervical seem to be susceptible to inhibition (Kazazi-Hyseni, Beijnen, and Schellens, 2010; Garcia and Singh, 2013; Baraniskin et al., 2019; Botrel et al., 2016; Gridelli et al., 2018; Tewari et al., 2014).

remodeling in ways that actually block effective drug delivery (Rahbari et al., 2016). Tumors, through the secretion of such a range of different factors, also coopts the vasculature itself and turns the vasculature into a “malignant” state where it will grow even in the absence of stimulus due to the environment of the tumor itself (Frentzas et al., 2016). Another facet of VEGF signaling in cancer that has become more recently appreciated is the autocrine role of VEGF production in stimulating tumor cell proliferation; just as endothelial cells proliferate in response to VEGF, so too do some cancer cells (Goel and Mercurio, 2013; Ntellias et al., 2020). While this should add yet another putatively beneficial aspect to VEGF-targeting therapies, the reality is much more complex.

Perhaps the most interesting of the stories of attempted use of bevacizumab is in glioblastoma. Despite having been given FDA approval for use in recurrent glioblastoma and initial studies having shown some promise (Segerstrom et al., 2006), there was little evidence of long-term efficacy until further meta-analyses were conducted enabling greater understanding – it was found that treatment was only mildly successful and that withdrawal led to an increase in tumor vasculature and may alter certain aspects of the biology of the tumor (Iwamoto et al., 2009). Later cell biological studies found that treatment of tumors with bevacizumab decreased tumor-associated vasculature but actually increased tumor invasiveness into the parenchyma, much of which is mediated by increased HIF-1 $\alpha$  activity (Keunen et al., 2011). A truly proper double-blind clinical assessment of the potential use of bevacizumab in glioblastoma was finally conducted in 2014 found either no improvement (Gilbert et al., 2014) or a mild benefit (Chinot et al., 2014). The general conclusion is that tumors are initially responsive to bevacizumab but most will progress within months with potential increases in invasiveness and dissemination at that point – whether bevacizumab *per se* alters the tumor in such a way to make it more aggressive is unclear (Li et al.,

2017). One potential is through induction of an epithelial-to-mesenchymal transition that encourages proliferation and cellular motility to other sites. Additionally, the tumors express other pro-angiogenic factors able to compensate for the loss of VEGF signaling, including PIGF, angiopoietins, CXCR4 (Xu et al., 2009a), FGF (Tamura et al., 2017), and VEGFC (Michaelsen et al., 2018; Villefranc et al., 2013; Zhao et al., 2017). Overall, bevacizumab treatment of glioblastoma seems to offer a slight advantage to progression-free survival but no advantage whatsoever to long-term survival; the progression-free survival is likely through the immediate improvements seen radiologically after initiation but recurrence then compensates for any temporary advantage (Wick et al., 2017; Zhan et al., 2019).

New approaches to targeting tumor-associated vasculature are clearly needed (Cesca et al., 2013; Hara et al., 2016). Vascular targeting as a theme has clear potential as an adjunct to other therapies, but the current approaches are limited in their utility (Neri and Bicknell, 2005). As mentioned previously, one approach would be to target VEGF production at the root – by blocking transcription factors needed to induce VEGF. Additional approaches would be to target axillary pathways also important for vascular endothelial cell biology in tumors – the interaction between angiopoietins and the TIE receptors is one primary possibility (Hato, Tabata, and Oike, 2008; Fujita and Akita, 2017). Other options include somehow artificially mimicking normoxia (with a hydroxyl donor?) for the tumor to block HIF-1 $\alpha$  activation, if HIF-1 $\alpha$  is truly the dominant means of inducing VEGF in this context. Lastly, one of the major hazards of this tumor vasculature is the leakiness, which strongly contributes to tumor growth. By inducing vascular normalization, rather than regression, through a combination of VEGFR- and TIE2-targeting therapies, it may be possible to simultaneously improve chemotherapeutic delivery without inducing compensatory responses in the tumor. Alternatively, inducing vascular perfusion

may also offer another anti-tumor strategy that improves chemotherapeutic delivery although the utility of this approach remains unclear (Rivera and Bergers, 2015).

Given the challenges associated with targeting the vasculature directly and compensatory pathways, many of which are induced by HIF-1 $\alpha$ , alternative approaches are needed to more effectively target vascular growth in tumors. HIF-1 $\alpha$ -directed therapeutic options remain functionally non-existent in 2022, with none having been approved for broad use and none directly targeting the activity of the protein itself (Sharma, Sinha, and Srivastava, 2022). All existing approaches impact HIF-1 $\alpha$  activity indirectly, by altering the activity of upstream regulatory factors or major downstream response pathways. HIF-1 $\alpha$ , aside from its role in inducing angiogenesis, exerts a panoply of pro-tumor effects, including promoting the epithelial-mesenchymal transition, which enhances the metastatic capacity of the cells (Sharma, Sinha, and Srivastava, 2022). The most functionally promising drug candidates are actually those that *agonize* HIF-1 $\alpha$ <sup>97</sup> and drive *increased* local angiogenesis, which is rather beneficial for a number of disorders, including major burns and diabetes (Dor et al., 2002; De Rosa, Di Stasi, and D'Andrea, 2018; Atluri and Woo, 2008). 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<sup>98</sup> are important for the production of VEGF and these may prove to be a more fertile ground for discovery of anti-angiogenic therapies that target required upstream factors.

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<sup>97</sup>This is typically by inhibiting the upstream prolyl hydroxylase enzymes that hydroxylate HIF-1 $\alpha$  in the oxygen-dependent degradation domain, resulting in its proteosomal degradation. In the absence of effective activity of these oxygen-dependent hydroxylases, HIF-1 $\alpha$  is able to remain active.

<sup>98</sup>Including SMAD3/4, TGF- $\beta$ , p300, and a series of AP-1 transcription factors including c-Fos and Jun-D (Jeon et al., 2007; Nam, Park, and Kim, 2010; Gray et al., 2005; Kwon et al., 2012; Thangarajah et al., 2009; Marconcini et al., 1999; Schmidt et al., 2007; Catar et al., 2013; Yoshitomi et al., 2021).

A final point worthy of consideration on this topic is the nature of the vasculature itself in the periphery of the tumor. While much has been done to *physically* characterize these vessels, a comprehensive look at the biological ways in which these vessels differ from normal vasculature and how that might impact the spectrum of therapeutic options is lacking (Jambusaria et al., 2020). To my knowledge, there are no resources available that have characterized the vascular endothelium of solid tumors from humans and certainly nothing on the scale of the many genomic and transcriptomic atlases of various tumors and cell lines (Kahn et al., 2021; Carlson et al., 2021; Pepin et al., 2012). The lessons learned from such an effort would be of immense benefit the study of these tumors, developing more targeted therapies, and likely inform our understanding of the granuloma-associated vasculature as well.

### 1.5.3 Non-Angiogenic Functions of VEGFA

VEGFA is also able to exert a number of roles in regulating non-endothelial biology, a set of roles somewhat unappreciated in the literature at large. These early roles were first identified in hematopoietic stem cells, where VEGFA signaling is important for inducing proliferation and preventing apoptosis among this population (Gerber et al., 2002). Other roles for VEGFA have been identified in regulating cancer growth, as briefly discussed in Subsection 1.5.1 (Goel and Mercurio, 2013). This phenomenon of VEGFA-VEGFR signaling inducing cancer cell survival and proliferation is widespread and may represent a general mechanism of cell survival by coopting the pro-growth, pro-survival signals normally used by the endothelium (Wiszniak and Schwarz, 2021; Mercurio, Lipscomb, and Bachelder, 2005).

A key role of VEGFA is in the recruitment of myeloid cells, which has been demonstrated in a number of contexts. Acting through these myeloid cells, further angi-

genic effects can be exacted on the vasculature that are independent of vascular sensing of VEGFA directly (Cursiefen et al., 2004). There are an number of pro-angiogenic therapies in the works for disorders like myocardial infarction, burns, and diabetes, and these have been seen to induce the recruitment of inflammatory types of macrophages whereas anti-inflammatory macrophages can be recruited by FGF and produce VEGFA *in situ* (Lucerna et al., 2007). A model wherein VEGFA produced by “M2” macrophages recruits “M1” macrophages could present a feedback cycle that limits excessive angiogenesis through macrophage-macrophage interactions (Barbay et al., 2015). Macrophages have also been noted to enhance VEGFA-driven angiogenesis by tumor xenografts, suggesting potential roles for either VEGFA-mediated induction of VEGFA within macrophages in a feed-forward loop or direct macrophage-endothelial interactions (Britto et al., 2018).

Lastly, in the context of mycobacterial infections, VEGFA has been found to have important roles in regulating granuloma inflammation independent of its ability to induce vascularization. Harding et al. (2019) found that VEGFA is induced in granulomas in mice and that blockade reduces inflammation without compromising bacterial containment, even in this model system that lacks robust angiogenic responses to so-called granulomas. This is mediated by reduced macrophage recruitment to the site of infection and potentially through P2RX7 activation (Matty et al., 2019). This study provides evidence of the importance of VEGFA in recruiting immune cells to the site of infection, although this recruitment does not manifest as alterations in bacterial burden. It is possible or probable that these effects may interact with alterations in vasculature that are able to be observed in other models of mycobacterial infection and should be accounted for in future studies.

#### 1.5.4 Macrophage-Endothelial Crosstalk in Angiogenesis

As previously alluded to, macrophages are essential mediators of angiogenesis during development and in the context of cancer and various vascular disorders (Sunderkotter et al., 1994; Ribatti et al., 2007; Chung and Ferrara, 2011; Weis and Cheresh, 2011). This role has been widely appreciated for over fifty years and early studies demonstrated that tumor angiogenesis was inhibited by monocyte depletion, which also resulted in a decrease in tumor size (Sunderkotter et al., 1994; Polverini et al., 1977).

While much has been studied regarding the contributions of macrophages to angiogenesis during cancer, less has been established in the realm of macrophage contributions to developmental angiogenesis. So-called “M2” macrophages are known to contribute to angiogenesis as these reparative macrophages produce VEGF during wound healing and in response to other insults (Jetten et al., 2014). This macrophage-mediated angiogenesis also appears to depend on IL-10 and TNF- $\alpha$  signaling, although the contributions of different cytokines is unclear (Nakamura et al., 2015; Leibovich et al., 1987). One area of interest is the ability for macrophages to physically engage the endothelium to mediate angiogenesis, similar to some of the previous descriptions of microglia stimulating and pruning neuronal synapses. In the developmental absence of macrophages, fewer vessels are able to sprout and connect in the brain (Fantin et al., 2010). Macrophages physically bring together sprouting tips of endothelial cells to facilitate new connections between tip cells and create new vascular connections<sup>99</sup> (Liu et al., 2016).

These processes can actually be mediated by macrophages of many different phe-

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<sup>99</sup>This process, called anastomosis, is interesting in development, but beyond the scope of the present discussion.

notypes, including so-called M1 macrophages. However, these different states of these macrophages induce distinct transcriptional changes in the endothelium itself, which would likely alter further macrophage behavior in the context of a native tissue. However, in engineered tissues, M1 macrophages induce early neovascularization while the other cell types were unable to do so (Graney et al., 2020). Unfortunately, this study was focused on the biology of the endothelium and did not profile the responses of the macrophages themselves, leading to important questions about what macrophage-derived factors were modulating these responses. In any case, this study suggests that it is not simply M2d macrophages that are able to induce angiogenesis within tissues.

The endothelium is a critical moderator of macrophage behavior and does so through the expression of important integrins and chemokines that constrain and activate macrophages. During insult (pathogenic, mechanical, thermal, or otherwise), the endothelium becomes an adherent surface for myeloid cells that drives their recruitment (Kalucka et al., 2017). Some of these junction proteins can be specifically induced by inflammatory signals, which allow circulating monocytes to bind and extravasate into the tissue. These proteins include a family of selectins as well as CDH5, PCAM, and ICAM (Imhof and Aurrand-Lions, 2004). The endothelium is also a source of CCL2, a potent myeloattractive chemokine, which, as we have seen, serves a detrimental role in host defense against tuberculosis (Martin et al., 2007; Cambier et al., 2014).

One of the ways that the endothelium and macrophages are known to physically associate is through TIE2-expressing macrophages remaining in contact with angiopoietin-2-expressing endothelial cells (Baer et al., 2013; De Palma et al., 2007; De Palma and Naldini, 2011). TIE2-expressing macrophages are pro-angiogenic cells

that interact with the endothelium directly to provide paracrine signaling to the endothelium to facilitate angiogenesis. While these cells have been extensively explored in the context of tumors, where, as a subset of tumor-associated macrophages, they contribute to both angiogenesis and dissemination<sup>100</sup>, they likely exist at homeostasis and in more transient conditions as well and play important roles in regulating endothelial remodeling (Duran et al., 2021; Lewis, De Palma, and Naldini, 2007).

These physical associations are now known to be mediated by the activation of HIF-1 $\alpha$  in macrophages. HIF-1 $\alpha$  is essential for vascular development, but the underlying rationale was unknown. Gerri et al. (2017) found that macrophage expression of HIF-1 $\alpha$  mediated physical interactions between macrophages and the endothelium that enabled critical aspects of developmental angiogenesis and angiogenesis during wound repair; in the absence of HIF-1 $\alpha$ , macrophages are unable to interact with the endothelium and repair breaks in the vasculature. There is much fertile ground left to be explored on the HIF-1 $\alpha$  signaling targets that enable macrophages to engage with the vasculature, but this sets the foundation for these activities to exist during both developmental and pathological angiogenesis, a pattern long repeated in the history of the field.

## Tumor-Associated Macrophages

Macrophages initially respond to growing tumors and act as antigen-presenting cells to present tumor antigens to the adaptive immune system that stimulate B cells to produce antibodies and T cells to develop into cytotoxic CD8 $^{+}$  T cells. While macrophages are initially protective against tumors, they transition into a more im-

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<sup>100</sup>This is now, interestingly, under dispute as it has been observed that in the absence of TIE2-expressing macrophages, angiogenesis and dissemination can both still occur. Thus, the role for these interesting macrophage subsets remains more obscure than ever; further studies will be required to reconcile these observations from the body of literature with these newer findings (Jakab et al., 2022).

munosuppressive, pro-tumor state after the initial failure to induce effective adaptive immunity and manipulation by the tumor microenvironment (Noy and Pollard, 2014). Tumor-associated macrophages are classically thought to be “M2-like” macrophages on account of their seeming indisposition toward killing the cancerous cells; they migrate into hypoxic regions of the tumor and, upon activation of HIF-1 $\alpha$ , begin to secrete VEGF and drive angiogenesis toward the tumor. While tumors can directly produce these pro-angiogenic factors, they are greatly aided by the complementary activity of these macrophages.

One of the means by which tumor-associated macrophages can facilitate changes in the tissues surrounding the tumor to facilitate invasion is by the production of matrix metalloproteases, which have well-known roles in degrading the extracellular matrix and facilitating angiogenesis. This loosening of the extracellular matrix enables tumor growth, increased macrophage recruitment, and the growth of endothelial cells (Lamagna, Aurrand-Lions, and Imhof, 2006). This sets the stage for further pro-angiogenic factors to stimulate the extensive vascularization seen in many tumors.

The most notable of these pro-angiogenic effectors is, of course, VEGFA. The process of tumor growth eventually requires the neovascularization of the tumor in order to continue growing and this process can be mediated by tumor-associated macrophages. However, it is not simply that tumor-associated macrophages express VEGF, they also express a whole panoply of cytokines that, in a feed-forward loop, drive increased VEGF expression and angiogenesis. These cytokines include IL-1 $\beta$ , TGF- $\beta$ , and M-CSF, all of which can induce further HIF-1 $\alpha$  activation in additional macrophages (Nicholas, Oniku, and Sumbayev, 2010; Guo et al., 2016).

### 1.5.5 Historical Observations of Angiogenesis in Tuberculosis

Histopathological characterization of tuberculous granulomas have served as the foundation of our knowledge of the structural features of tuberculosis disease. All of the major features were described on the basis of this histological analysis, including the necrotic core, the epithelioid macrophage layer<sup>101</sup>, the lymphocytic cuff, and – important for this work – a surrounding web of vasculature.

In contrast to the normal lung, tuberculous lungs demonstrated dramatic aberrant vascularization in the proximity of the tubercle, arguing for some role of the disease in inducing this process; these vessels had even been observed to derive from the aorta, which is a remarkable subversion of the central circulation of the whole body (Cudkowicz, 1952). Such vascular re- and dis-organization was a notable radiological and histological feature of the disease, but what role it may play was, as yet, unknown.

There was rife speculation on the role of this vasculature in the pathology of the disease. Bloody sputum had long been recognized as a primary differentiating factor between tuberculosis and other potential sources of lung disease, suggesting that vascular damage was a major consequence of cavitation and transition to active disease. While some viewed the angiogenesis as circumstantial – granulomas at sterile wounds surrounding foreign objects also become vascularized – others speculated that this may be a host-protective effect, allowing for the recruitment of additional immune cells to the site of the infection (Miller, 1938; Cudkowicz, 1952; Gale, 1957; Oehlers et al., 2015). There lingered the third possibility that rather than being either inci-

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<sup>101</sup>This layer was known as such for decades prior to a proper characterization in Cronan et al. (2016). These cells pseudo- (or properly) differentiate into macrophages possessing notable epithelial characteristics.

dental or host-protective, that this effect may be a maladaptive response, serving to benefit the bacteria to the detriment of the host (Cudkowicz, 1952). Answers to this question would have to wait for quite some time as the tools to properly dissect the contributions of pathological angiogenesis to tuberculosis disease outcome remained many years in the future.

### 1.5.6 Modern Studies on Granuloma Angiogenesis

After a decades-long period of relative inactivity, a series of papers in the early-2000s began to explore mechanisms underlying the granuloma angiogenesis, although these early studies lacked effective tools to dissect this biology in the context of infection *sensu stricto*. A pair of early papers investigated the contribution of TDM to the induction of angiogenesis. The chronological first to be published, Sakaguchi et al. (2000) did extremely thorough work to dissect the chemokines required for angiogenesis and the cellular source of these factors using an air pouch model in the mouse along with complementary work *in vitro*. This study was interested primarily in the potential for these purified mycolic acids to be used for pro-angiogenic effects, potentially for treatment of burns or diabetes, although little discussion is provided on this front. Saita et al. (2000) developed a rat corneal model of angiogenesis and applied purified TDM to induce an effective angiogenic response that depended on VEGF and there was some speculation that TDM may contribute to the neovascularization effect seen in the proximity of tuberculosis granulomas in human patient samples, but little follow-up work was able to be done at that time. These efforts combined a targeted characterization of the cytokine response and piecewise inhibition of these factors to identify both VEGF and IL-8 (CXCL8) as important pro-angiogenic factors caused by TDM. With relatively limited tools and the lack of an effective model, no work was able to be done to thoroughly characterize this angiogenesis phenotype

in the context of infection or the consequences of blocking angiogenesis on disease progression.

Intermittent subsequent work had found important roles for angiogenesis-relevant host genes in tuberculosis pathogenesis, but the connection had not yet been drawn in full. For instance, a study found that endothelin signaling was important for host control of tuberculosis infection, pointing to a specific role of the vascular responses in the control of infection, even in the background of a C57BL/6 mouse (Correa et al., 2014). Because there is no obvious angiogenic effect in this background, these effects are likely mediated by physiological changes in the lung vasculature. Their contributions to vasoconstriction may, in some way, limit nutrient access to the site of infection although these mechanisms may be worthy of future exploration.

The need for a tractable model required innovative new approaches to studying this phenomenon. Classical mouse models fail to form epitheloid granulomas with associated vasculature and many other mammalian models lack the exhaustive set of tools required for a thorough mechanistic dissection of this process. While a more comprehensive discussion of the zebrafish-*Mycobacterium marinum* model will wait for Section 2.2, this model offered a set of unique advantages – most notably in regards to genetic tractability and optical transparency – that enabled pioneering studies on the mechanisms underlying the angiogenic response and the functional consequences of inhibiting this process. This study from Oehlers et al. (2015) identified that growth of vasculature toward the site of infection increased bacterial burden and that inhibition of this process may serve as a useful host-directed therapeutic approach. Concurrently, Datta et al. (2015) developed a rabbit model of infection, which is a model in which granulomas form along with associated vasculature. This work sought to comprehensively characterize the vasculature and identified notable

commonalities between the heterogeneous and structurally abnormal vessels seen in tuberculosis and those seen in solid tumor cancers. The structural deficits of these vessels serve as a barrier to the effective delivery of small molecules because they leak out of the vessels and form a hazy corona around the granuloma without effectively penetrating it. Normalization and regression of these vessels with bevacizumab was able to improve effective small molecule delivery to the inner layers of the granuloma, suggesting that this may be an option for adjunctive therapy to improve drug delivery.

Shortly thereafter, Polena et al. (2016) interrogated granuloma angiogenesis as a potential mechanism of dissemination and, using Matrigel plugs impregnated with *M. tuberculosis*, identified an angiogenic response to infection in SCID<sup>102</sup> mice that depended on macrophage-derived VEGF and which facilitated spread from the plug to distal organs. One of the challenges with these latter studies is that they only observe the functional consequences of angiogenesis inhibition for a limited period of time (three or eight days in the Datta et al. (2015) study and two weeks in Polena et al. (2016)) and Polena et al. (2016) utilize a severely immunocompromised mouse model, where granulomas fail to mimic the human pathology and any protective role for the adaptive immune system is neutralized. More recently, Torraca et al. (2017) found a critical role for CXCR4, an important chemokine receptor, in driving angiogenesis in a manner that was independent of VEGFA production. While VEGFA is clearly a major determinant of angiogenesis, it is not the only possible determinant and its interaction with other signaling molecules may be important for the overall phenotype.

These studies also raised several important questions:

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<sup>102</sup>Severe combined immunodeficiency mice lack functional lymphocytes.

- How does VEGF inhibition interact with the use of existing therapies? Can an additive or synergistic effect be achieved by combining the bacterial burden reduction effects seen in Oehlers et al. (2015) and Polena et al. (2016) with the improved drug delivery identified by Datta et al. (2015) in the context of standard-of-care anti-tuberculosis therapy?
- Does this vascularization effect extend to other granulomatous diseases? For instance, it is known that *Schistosoma* interacts directly with the endothelium, but does the granuloma response itself facilitate growth or transmission via modulation of the surrounding vasculature (Chaves, 1966; Shariati et al., 2011; Pereira et al., 2013)? Similarly, the fungal pathogens *Cryptococcus* and *Histoplasma* induce granuloma formation in the course of disease and the role for angiogenesis in these contexts remains unknown <sup>103</sup> (Coenjaerts et al., 2004; Martidis et al., 1999). Conversely, sarcoidosis granulomas are avascular; what functional distinctions might exist that discriminate between whether or not a granuloma sub-type is heavily vascularized or avascular given that macrophages play the defining role in all cases (Kambouchner et al., 2011).
- Pathogens must actively subvert host immunity in order to survive and transmit and have evolved sophisticated mechanisms for doing so. Given our knowledge that mycobacteria benefit from host angiogenesis, could it be the case that they have developed specific mechanisms for inducing this process?

This last point is notable given the previously discussed foundational findings on the ability of TDM to induce angiogenesis. Could the bacteria be actively modifying TDM in order to enhance the degree of vascularization? Observations from Sakaguchi

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<sup>103</sup>Given that both of these pathogens can disseminate hematogenously, there is reason to believe that blood vessel biology plays some role in the development of these diseases, although how it might be doing so is largely unknown (Kauffman, 2007).

et al. attempted to dissect some elements of this using TDM from *Rhodococcus* spp., but was unable to functionally differentiate these configurations. Additionally, Polena et al. utilized heat killed *M. tuberculosis* and, while their thesis was that VEGF upregulation was dependent on ESX-1, found a substantial upregulation of VEGF in the absence of active metabolism or structural integrity. These findings suggested a heat-stable factor independent of secreted effectors could mediate at least some percentage of the overall angiogenesis phenotype and that it might depend on TDM.

To this end, Walton et al. genetically and biochemically interrogated the mechanisms underlying the TDM-dependent angiogenesis phenotype. This work identified TDM as sufficient to induce angiogenesis using a novel application of the zebrafish model, wherein TDM emulsified in incomplete Freund's adjuvant is injected into the trunk of the larval zebrafish, mirroring the approach used in Oehlers et al. for live mycobacterial infection. Furthermore, this work identified cis-cyclopropyl modification of TDM as necessary for the induction of this phenotype, a process mediated by the bacterial enzyme PcaA.  $\Delta pcaA$  *M. marinum* is attenuated for *in vivo* growth due to deficient angiogenesis, a process that can be complemented in *trans* by co-infection with wild-type *M. marinum*. This work serves as the foundation of the subsequent work presented here as it left a major question as yet unaddressed: **what signal transduction pathways within responding macrophages are capable of detecting and responding to TDM to drive VEGF production and downstream angiogenesis?**

## 1.6 Host-Microbe Interactions to Study Cell Biological Processes

Pathogens are nature's born cell biologists. For their survival, they depend on the coordinated subversion of host responses to infection, having to overcome the restrictive force of 1,000 interferon-stimulated genes, a fully equipped innate and adaptive immune system, and the physical architecture of the infectious focus (MacMicking, 2014; Byrne, Lehmann, and Landry, 1986). The literature is replete with examples how the study of the mechanisms that pathogens use to undermine the host sheds light on normal, homeostatic host processes including DNA replication, RNA splicing and modification, protein biosynthesis, and organelle regulation to cell-cell communication, critical cell receptors, secretion pathways, and post-translational modifications (Compans and Roberts, 1994). While much of the early work was conducted using viruses, modern understanding of cell biology synthesizes findings from across pathogens, including bacteria and fungi (Welch, 2015). This deepened understanding of cell biology can lead to the development of more efficacious treatments of infection through modulation of host responses, an approach known as host-directed therapy.

### 1.6.1 Host-Directed Therapies: History and Promise

One of the defining characteristics of tuberculosis infection is the formation of caseating granulomas. These granulomas, formerly known as tubercles<sup>104</sup>, 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

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<sup>104</sup>Hence, *tubercul*-osis.

definitive manifestation of tuberculosis<sup>105</sup>. For reasons that remain poorly understood, but likely related to the inflammatory biases of the C57BL/6 and other mouse models, these mice do not form granulomas<sup>106</sup> after being infected with *Mycobacterium tuberculosis* and mice do not harbor a species 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 primarily through a hypoxia-dependent mechanism, the angiogenic response to other stimuli is 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 or fluid-filled 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

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<sup>105</sup>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 (Boros, 2003).

<sup>106</sup>Strangely, these mice do form granulomas in response to *Schistosoma* and other stimuli, suggesting something distinguishing about mycobacterial infection or the production of central necrosis and perhaps offering clues as to the unique characteristics of the tuberculous granuloma (Fu et al., 2012).

either humans or non-human primates, so it remains difficult to make sweeping assertions<sup>107</sup>. Coarse assessments with tools like hypoxyprobe have been performed, but these are non-quantitative by their nature and impossible to effectively calibrate *in vivo* (Cousins et al., 2016). Regardless, the experimental identification of particular mycobacterial components able to induce angiogenesis suggests more sophisticated immunological mechanisms at play than simple hypoxia.

This bacteria-centric approach to treatment of tuberculosis seems logical, as bacteria possess many functions that humans lack entirely that are necessary for their pathogenicity, making these appealing targets for drugs. However, this opens the door to the emergence of resistance when treatment is unable to clear the infecting bacteria and a tolerant or resistant population then expands anew. This makes a compelling niche for a new approach to the treatment of chronic bacterial (and fungal and viral) infections: 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 life-or-death 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 in-

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<sup>107</sup>This is partially due to lack of tools to do so. HIF-1 $\alpha$  itself is often used as an endogenous measure of oxygen tension, but this is unreliable as HIF can be regulated at multiple other levels and could only be conceivably used at known identical transcription and translation rates, which is impractical.

tersectionality 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 Organization's goal of eradicating tuberculosis by 2050<sup>108</sup> (Schwegmann and Brombacher, 2008).

Host-directed therapeutic approaches can take many forms: antibodies, repurposed drugs, cell therapies, and more (Zumla et al., 2016). Many have been suggested, some of which have been reviewed by Wallis and Hafner (2015). They work by a number of different mechanisms, but can be categorized into a few main classes: enhancing antimicrobial responses (Khan et al., 2016), modulating inflammation, and altering stromal cell behavior. Of these, the most attention has been given to enhancing antimycobacterial responses as there are a number of useful autophagy activators that may be able to more effectively kill intracellular bacteria (Wallis and Hafner, 2015). Modulation of the expression of known deleterious responses, like the production of CCL2 and MMP9 offer additional avenues of drug discovery (Volkman et al., 2010; Cambier et al., 2014). However, these all come with important limitations in that they may only be effective at particular stages of disease – how prominent is the role of autophagy in later stages of disease? On the other hand, by attempting to modify the stromal microenvironment, it may be possible to address the granuloma *in medias res* and enhance responses through these mechanisms. This is distinct from but analogous to the approach taken checkpoint inhibitors in cancer: nothing about

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<sup>108</sup>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) (Hopkins, 2013; Mariner et al., 2012). Current campaigns show promise in the eradication of dracunculiasis (or guinea worm) in the immediate future, with cases down to 14 in 2021 (Kreier, 2022). Others, including polio, yaws, and rabies, remain elusive despite all having effective vaccines or treatments, are human-exclusive (or have a known, discrete, treatable 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.

the tumor itself has changed, but the way the immune system is engaging with it changes fundamentally.

The most successful currently utilized host-directed therapy against tuberculosis is the use of corticosteroids to inhibit excessive inflammation during disease, a phenomenon that is able to be genetically predicted based on the individuals genotype at the *LTA4H* locus (Tobin et al., 2012; Tobin, 2015). Additional therapies may be as trivial as the addition of excess vitamin D<sub>3</sub>, which may act to enhance antimicrobial responses in granuloma macrophages (Tobin, 2015). Another proposed approach is through the modulation of IL-1 and IFN- $\alpha/\beta$  signaling. While IL-1 mediated responses can be protective against tuberculosis, type I interferon responses are thought to be host-deleterious; both pathways can be address simultaneously through the administration of prostaglandin E2 and zileuton to improve host responses through the eicosanoid pathway (Mayer-Barber et al., 2014; Kaufmann et al., 2014).

This work and the foundational work that preceded it present angiogenesis as a potential target of host-directed therapy against tuberculosis (Oehlers et al., 2015; Datta et al., 2015; Oehlers et al., 2017; Polena et al., 2016; Walton et al., 2018). This approach in broad strokes has already demonstrated clinical value through the use of thalidomide to treat juvenile tuberculous meningitis as thalidomide<sup>109</sup> is both a VEGF and TNF- $\alpha$  inhibitor (Kaufmann et al., 2014; Toorn et al., 2021). I propose that this strategy can be improved or augmented through the use of more targeted approaches that undermine upstream events required for the production of VEGF in such a manner that the granuloma is left few options for developing resistance to anti-angiogenic therapy (Kiran et al., 2016). Additional work will need to identify

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<sup>109</sup>Thalidomide is famous for the tragedy of severe congenital birth defects among mothers who took the drug during pregnancy, which left a severe stigma on the drug, despite more recent findings that it is a safe and effective treatment for a variety of disorders, including cancer and infectious diseases (McBride, 1961; D'Amato et al., 1994; Franks, Macpherson, and Figg, 2004).

the comparative benefits of anti-angiogenic versus normalization therapy in directing host-beneficial responses that are favorable for drug delivery, which should be a major focus of near-future work.

The work presented here focuses on a few critical themes: the diversity of factors that can contribute to the net effect of a particular insult on the immune response, the networked nature of immune responses beyond simple binaries, and the vast and poorly understood complexity beyond the simple dogmas we accept today. 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, these balances 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 pro- and anti-inflammatory responses is essential for the development of better therapeutics disorders of the immune system in the broadest sense.

# Chapter 2

## Scientific Methodology

Detailed explanation of scientific methodology is the foundation of scientific reproducibility. In this chapter, I will introduce the major model organism used for this work – *Danio rerio*, the zebrafish – along with the aquatic pathogen used to model human tuberculosis, *Mycobacterium marinum*. Additional descriptions of tissue culture, molecular biology, genetic, and biochemical approaches are also provided<sup>1</sup>.

### 2.1 Transparency in Science and Data Availability

In the absence of reproducibility and open access to both the results and the raw data from which the results are derived, science is a fruitless venture. A fundamentally human enterprise, science is subjected to the limits of human understanding and human perfectability. The only way to hedge against our base failings as scientists is to make both the product and the process as transparent as possible. To that end, all of the raw images and quantitation used in this manuscript have been made publicly available at Brewer et al. (2022a) along with all of the R scripts used to analyze the data and any processing macros or other analysis scripts written in Python.

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<sup>1</sup>Portions of this methodology section are extracted from Brewer et al. 2022 and are incorporated into this chapter *in lieu* of direct inclusion in Chapter 3, where the results can be found.

Presentation of major uses of these languages can be found in Chapter 4. Any raw images that were unable to be made available due to size limitations can be requested from the author.

Additionally, the raw data to generate this dissertation is available on Github at <https://github.com/jaredbrewer/dissertation>, including source EndNote files (without PDF attachments) and an extended bibliography with additional links and information (generated automatically by EndNote and presented unedited).

## 2.2 Zebrafish as a Model Organism

Laboratory model organisms have been a staple of research since the dawn of the scientific endeavor, but only in the past century has model standardization allowed for improvements in reproducibility and reliability among experiments. In the 1970s and into the 1980s George Streisinger in Oregon sought a model that would allow for the full visual access to development only possible in oviparous organisms (Streisinger et al., 1981). Although *Xenopus* frogs had been in use for some time, their long time to sexual maturity (up to 2 years for the tragically tetraploid *Xenopus laevis*, the dominant model at the time) and other challenges led researchers to a fish model, at the root of the land-adapted branch of the tree of life. A 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 (Bradford et al., 2022). 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 (Grunwald and Eisen, 2002; Eisen, 2020). These features make the zebrafish a potent and robust tool for the study of many different biological processes and, thanks to their intolerance for inbreeding, have remained

a genetically diverse outbred<sup>2</sup> 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 in a high-noise environment may be robust associations than those found in a more monoclonal environment representative of only a single instance in a vast range of genomic possibilities.

*Danio rerio* is a small (1-2 cm in length) freshwater fish natively found in the Ganges River and tributaries in India (Engeszer et al., 2007; Arunachalam et al., 2013; Parichy, 2015). A fetching and robust fish in the pet trade, they possess reflective stripes that play a role in predator evasion, as well as contributing to their mating cycle. They spawn freely, releasing individual eggs into the water where they can be fertilized by an engaged male<sup>3</sup>. The larvae consume waterborne microorganisms for food as they undergo metamorphosis, changing key elements of the body plan to develop the adult anatomy. The fish develop into sexual maturity over the course of approximately 5-8 weeks and are typically fully grown within 3 months, allowing for rapid generation times. Their small size facilitates rearing large numbers of them at once, making it possible to do experiments with large numbers of replicates to overcome some of the heterogeneity present in the model.

Only in the past twenty years has an earnest effort been put forth to develop the zebrafish as a model for immunological studies (Hsu et al., 2004; Davis et al., 2002;

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<sup>2</sup>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 (Guryev et al., 2006; Balik-Meisner et al., 2018; Brown et al., 2012). Experimentalist anecdotes of the intolerance of the zebrafish for inbreeding are ubiquitous, as this widespread genome-level heterozygosity appears to confer some important advantages to individuals, especially in regard to fertility.

<sup>3</sup>Or some bystander male.

Langenau et al., 2003). Although it has long been known that zebrafish, like all vertebrates, possess the full repertoire of immune cells and responses (Lugo-Villarino et al., 2010; Liongue et al., 2009), little was done with that knowledge until more recently, given the perceived benefits of other models<sup>4</sup>. Now zebrafish have become a widely used model in the study of diseases of the immune system ranging from cancer to neurological disorders to tuberculosis, thanks to pioneering work by Leonard Zon, Lalita Ramakrishnan, David Langenau, and others. The features that made the zebrafish beloved for studies of developmental and cell biology also translate into this context, facilitating optical, genetic, and biochemical dissection of the immune system during disease.

Coinciding with their divergence from the other clades of fish, the teleost fish experienced a whole genome duplication that has left them with redundant copies of many proteins, with substantive consequences for their evolution (Amores et al., 2011; Glasauer and Neuhauss, 2014; Howe et al., 2013; Meyer and Schartl, 1999). Such a duplication enables diversification of function, increased mutational tolerance, and transcriptional inactivation of one or the other copies with little ability to *a priori* predict which is expressed (Opazo et al., 2013; Voldoire et al., 2017). This duplication has led to much headache for zebrafish researchers, but has opened up interesting avenues of discovery in the realm of genetic compensation and protein evolution (Rossi et al., 2015; El-Brolosy and Stainier, 2017; El-Brolosy et al., 2019; Sztal and Stainier, 2020; Stainier, Kontarakis, and Rossi, 2015; Moleri et al., 2011; Boudinot et al., 2011; Stainier et al., 2017; Kontarakis and Stainier, 2020). This is one of the features of the model that must be considered in its application as it can increase the difficulty in determining genetic mechanisms at play in a given

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<sup>4</sup>More on this will be provided in Subsection 2.3.3, but for now we will allow the zebrafish to stand on its own merits.

phenotype; new approaches using F<sub>0</sub> crisprants have allowed for more rapid phenotypic assessment. Notable for this work, the zebrafish possess 6 NFATc isoforms: *nfatc1*, *nfatc2a*, *nfatc2b*, *nfatc3a*, *nfatc3b*, and *nfatc4*.

### 2.2.1 Zebrafish and the History of Developmental Biology

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Based on the known features of the zebrafish, it was clear that the zebrafish had potential to be a powerful model to study the development of many major organ systems and tissues in a small, amenable vertebrate, but the tools to do so were lacking (Bakkers, 2011). After the proper establishment of the zebrafish model by Streisinger et al., work was done to finely map the earliest events of development and compare these to those known from other models, including *Xenopus* and the mouse (Kimmel, Sepich, and Trevarrow, 1988; Kimmel, 1989; Kimmel et al., 1995). One of the major needs in the field was the development of genetic tools with which to assess the contribution of different genes to distinct aspect of vertebrate development (Driever et al., 1994; Mullins et al., 1994), an initiative spearheaded by Wolfgang Driever and Mary Mullins that resulted in the famous "Zebrafish Issue" of Development in December 1996, featuring 37 articles all related to the results of these enormous genome-wide mutagenesis screens (Mullins et al., 2021; Nusslein-Volhard, 2012; Haffter et al., 1996; Driever et al., 1996; Knapik et al., 1996). These parallel screens uncovered factors impacting every conceivable aspect of vertebrate development, including brain (Schier et al., 1996; Heisenberg et al., 1996; Jiang et al., 1996; Brand et al., 1996a; Stemple et al., 1996; Odenthal et al., 1996a), craniofacial (Whitfield et al., 1996; Malicki et al., 1996b; Schilling et al., 1996; Piotrowski et al., 1996; Neuhauss et al., 1996), neurological (Malicki et al., 1996a; Furutani-Seiki et al., 1996; Abdelilah et al., 1996;

Baier et al., 1996; Karlstrom et al., 1996; Trowe et al., 1996), early events (Kane et al., 1996a; Kane et al., 1996b), body morphogenesis (Brand et al., 1996b; van Eeden et al., 1996b; van Eeden et al., 1996a; Hammerschmidt et al., 1996b; Mullins et al., 1996; Hammerschmidt et al., 1996a), cardiovascular (Stainier et al., 1996; Chen et al., 1996), hematopoiesis (Weinstein et al., 1996; Ransom et al., 1996), pigmentation (Kelsh et al., 1996; Odenthal et al., 1996b), and more (Granato et al., 1996; Pack et al., 1996). These tools set the foundation for decades of research in developmental biology as the precise genes underlying these effects remained largely mysterious even after their chromosomal location had been, in many cases, mapped – such was the nature of the pre-genomics era.

Although not a part of this monumental screen, a single example of the power of early zebrafish embryonic development studies lies in the *cloche*<sup>m39</sup> mutation. This spontaneous mutant displayed total failure of vasculogenesis and hematopoiesis, providing evidence of the common developmental origin of these two processes and implying the existence of the so-called hemangioblast<sup>5</sup> (Stainier et al., 1995). While further studies were conducted on this gene to characterize its activity in determining endothelial and hematopoietic fate; its first described function was as an upstream regulator of VEGFR2 (*flk1*) expression and that this effected was mediated cell-autonomously (Liao et al., 1997; Parker and Stainier, 1999). Some additional insight on regulated gene networks was revealed with the gene underlying *cloche* having cross-regulatory functions with two other genes, *scl* and *hhx*, where *cloche* mutants have disrupted expression of *hhx* and *scl* (Liao et al., 2000). While sporadic subsequent studies were conducted, without a genetic identity, it became more difficult to generate novel insights on this gene (Qian et al., 2005). Finally, in 2016, Reischauer

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<sup>5</sup>This since-confirmed multipotent cell type that can become either hematopoietic stem cells or endothelium was long hypothesized (Murray, 1932; Sabin, 1920) but took until the early 2000s to confirm their existence (Xiong, 2008).

et al. identified *npas4l* as the gene responsible for *cloche*, which has resulted in renewed interest in this gene, where it has been found to be important for regulation of a range of critical hematopoietic genes (Marass et al., 2019) as well as part of a gene network that determines endothelial and pronephron fate (Mattonet et al., 2022). *npas4l*, while absent from mammalian genomes, provides insight into the evolution of hemangioblasts and shares many functional overlaps with ETV2, a transcription factor in mammals and fish that is absent in birds and reptiles (Weng et al., 2020; Vogeli et al., 2006).

While the precise story of *cloche* is not essential to the work presented here, the general theme is key to an appreciation for the zebrafish as a model organism – general findings at the foundation of developmental biology can feed forward into ever more sophisticated understandings as the technologies develop that enable these clearer perspectives. The zebrafish also enables study of developmentally lethal genes in ways that other vertebrate models cannot; the *cloche* mutant fish dies by 7 days post fertilization, which would make study within the living mouse possible only (partially) by vivisection or at terminal time points<sup>6</sup>. Additionally, the range of available tools in the zebrafish that developed over the course of the study on *cloche* serves as a microcosm for progress in the field at large, where finer tools allowed for more detailed examinations of particular phenotypes – first through transgenic tools and *in situ* hybridizations, then through microarrays and RNA-seq, and ultimately through CRISPR/Cas9 and whole-genome sequencing. As more tools become available, including knock-in technologies and more affordable single cell -omics, more and more of these gross phenotype-driven historical observations will be able to be studied in deep molecular detail.

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<sup>6</sup>The analogous mutation in mammals, of *ETV2*, results in completely non-viable embryos and the precise moment of developmental failure is difficult to define; all embryos perish by E9.0 and lack both hematopoietic and endothelial cell lineages (Garry, 2016).

## **2.2.2 Modern Applications of Laboratory Zebrafish**

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Zebrafish are a widely applicable model to many fields of biology, including developmental biology, cell biology, cancer biology, immunology, neurology, animal behavior, toxicology, pharmacology, and more. As mentioned above, zebrafish are a classical model in developmental biology, but their utility persists into the present, where they are widely used to study many of the important early events in vertebrate development at high optical and temporal resolution. Beyond this, zebrafish are among the most amenable models in use for the study of toxicology and pharmacology on account of their facile breeding and ease of manipulation. They have also become a powerful model in animal behavior, where work by Randall Peterson and others has established the zebrafish as a useful model for drug discovery and profiling – zebrafish can be exposed to various pharmaceutical agents and then, using known responses, quantitatively profiled for behavioral changes (MacRae and Peterson, 2003; MacRae and Peterson, 2015; Peterson and Macrae, 2012; Rihel et al., 2010; Kokel et al., 2010; Kokel and Peterson, 2011; Bruni et al., 2016; Zon and Peterson, 2005; Patton, Zon, and Langenau, 2021). Like all models, the zebrafish had benefitted in recent years from the development of a variety of new technologies that have enabled ever-finer examination of interesting biology.

Leonard Zon has also served as a major force in the advancement of the zebrafish model, this time in the realm of cancer immunology. Zon and colleagues were at the forefront of developing transgenic tools that would facilitate study of the interactions between the immune system and xenograft tumors and the utilizing these newly possible findings to advance the field's knowledge of host-cancer interactions Cagan, Zon, and White (2019), Amatruda et al. (2002), Trede et al. (2004), and McConnell

and Zon (2021). Langenau et al. (2003) developed a novel zebrafish model that drove the expression of the myc oncogene within the lymphoid compartment, which was able to rapidly generate leukemia in the fish. Such a platform is useful for being able to generate genetically-defined cancer-bearing animals that can then be subjected to medium- to high-throughput screening approaches for drugs, drug targets, and other genes that contribute to the biology of these cancers in humans.

Through integrated study of the immune system with these tumors in the context of new adult zebrafish models (including the optically transparent *casper* and *crystal* models), interesting new approaches have been developed, enabling new genetic, optical, and therapeutic approaches to the study of cancer (Yan et al., 2021; Yan et al., 2019; Stern and Zon, 2003; Gomez-Abenza et al., 2019; Hason and Bartunek, 2019; White, Rose, and Zon, 2013) and hematopoietic disorders (Jong et al., 2011; Tang et al., 2014). Through novel genetic barcoding, it is now possible to analyze these cell on the level of individual cell dynamics, an unprecedented look at the biology of these tumors (Sankaran, Weissman, and Zon, 2022). These approaches have been especially powerful in the study of melanoma, for which the zebrafish provide an excellent genetic model (Kaufman et al., 2016). This work has led to clinical trials for new cancer treatments, streamlining the bench-to-bedside pipeline (Hanna et al., 2021). Zebrafish are now being proposed as “patient avatars,” enabling laboratory screening of therapeutics against the patient’s particular tumor in zebrafish to predict patient responses to chemotherapy (Li et al., 2012; Yan et al., 2019; Fazio et al., 2020). This is but a limited sampling of the diverse roles that zebrafish have found in the laboratory, but sets a model for how linear developments over time can shape it into a powerful model even in unexpected contexts.

## 2.2.3 Zebrafish as a Model System for the Study of Host-Microbe Interactions

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In the same time frame as the Zon lab’s foundational discoveries in the study of cancer, the Ramakrishnan lab was developing the zebrafish to study host-pathogen interactions. While much of the understanding of tuberculosis had derived from either *in vitro* models or from the mouse, both lacked some important features that would enable a clearer understanding of these interactions (Davis et al., 2002). While this set the stage for two decades of intensive study of mycobacteria (see Section 2.3), it also set off a cascade of study on various pathogens – including other bacteria, fungi, and viruses – utilizing the zebrafish as a surrogate host and taking advantage of what makes the zebrafish scientifically compelling in other fields: gene and response conservation with humans, optical transparency, and genetic amenability (Kanther and Rawls, 2010; Angosto and Mulero, 2014; Levraud et al., 2014).

While the precise details often vary somewhat<sup>7</sup>, the sweep of zebrafish immunology broadly parallels that of mammalian immunology (Zou and Secombes, 2016; Renshaw and Trede, 2012). Additionally, the high degree of genetic conservation between zebrafish and humans allows for functional interrogation of conserved processes using the zebrafish as an amenable and optically accessible host (Gomes and Mostowy, 2020). They share all of the same major subtypes of immune cells across both the innate and adaptive compartments, allowing for one-to-one comparisons across systems (van der Sar et al., 2004; Thisse and Zon, 2002). More specifically, the major subtypes

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<sup>7</sup>While zebrafish and mammals share many cytokines and receptors, others are difficult to divine. For instance, zebrafish lack GM-CSF and its receptor, as well as IL-3 and IL-5, among other genes (Pazhakh and Lieschke, 2018). The diversity in the C-type lectin receptor lineage is of especial trouble for this work.

of macrophages are also conserved, with microglia and Kupffer cells being present through development (Oosterhof, Boddeke, and Ham, 2015; Shwartz, Goessling, and Yin, 2019). Importantly, both zebrafish and mammals have the major cytokines and cytokine receptors required for an effective immune response against pathogens, allowing findings from the fish to translate into a human context. And useful for the experimenter, the developmental timeline of the zebrafish allows for the temporal segregation of innate and adaptive responses in an otherwise immunocompetent host (Sullivan et al., 2017; Masud, Torraca, and Meijer, 2017).

The zebrafish has become a useful model for the study of a range of pathogens beyond the one used in this work, *Mycobacterium marinum* (Benard et al., 2012; Brannon et al., 2009; Briolat et al., 2014) (discussed further in Section 2.3). Other bacterial pathogens have been used in the zebrafish to study the immune response in detail, including *Salmonella* (van der Sar et al., 2003), *Pseudomonas aeruginosa* (Pont and Blanc-Potard, 2021), *Staphylococcus aureus* (Prajsnar et al., 2008), and *Vibrio cholerae* (Runft et al., 2014). Zebrafish have even found use as useful model of sepsis, allowing for rapid screening of relevant host determinants through live visualization of the cascade of inflammatory events that drive this complex immune system hyperreaction (Barber, Fleming, and Mulvey, 2016; Philip et al., 2017; Ruyra et al., 2014).

Probably the most extensively studied zebrafish-pathogen interaction outside of *M. marinum*, various elements of *Pseudomonas* biology have been dissected using the larval zebrafish. *Pseudomonas* is a prevalent infection among cystic fibrosis patients and the particular virulence factors and host determinants of infection remain under active study. First used in 2009 by Clatworthy et al., they were readily able to validate the *in vivo* attenuation of known virulence mutants, allowing further study on novel

determinants of virulence and their mechanism of action in the interaction with both host macrophages and neutrophils in the larval model. Indeed, the zebrafish allowed for the study of the contributions of the gene underlying cystic fibrosis (CFTR) in the innate immune response and found that *cftr* morphant zebrafish neutrophils were deficient in their ability to kill *Pseudomonas*, implicating altered immune responses in part of cystic fibrosis susceptibility to infection (Phennicie et al., 2010). Subsequent work sought to address the known biofilm interactions between *Candida* and *Pseudomonas*. While *in vitro* studies had assumed that these organisms were antagonistic, this *in vivo* study using the larval zebrafish found that they synergistically enhanced one another's growth and drove increased zebrafish mortality. Such understandings of microbe-microbe interactions in the context of a host expand our ability to model the human disease condition, where cystic fibrosis patients are often afflicted with polymicrobial infections (Bergeron et al., 2017).

Recently the Huttenlocher and Perfect labs have utilized the zebrafish as a model host to study host-fungal interactions, both in *Cryptococcus* and *Candida* (Johnson et al., 2018; Gratacap and Wheeler, 2014). Use of this model revealed new dimensions of the hematogenous dissemination of *Cryptococcus* and the role of macrophages in both controlling the infection and providing a replicative niche (Tenor et al., 2015; Davis et al., 2016). This model has the potential to expand for the study of other fungal pathogens and partially supplant the *Galleria mellonella* model with one that has more advanced genetic tools, more straightforward husbandry, and vertebrate immunity (Rosowski et al., 2018). In the standard mouse model, the hematogenous dissemination effect seen in human cryptococcosis patients is difficult to observe in real-time, so this approach allows for much deeper analysis of macrophage-yeast interactions in a native tissue context.

Zebrafish are also useful in other aspects of host-microbe interactions, including in the microbiome. Pioneering work from John Rawls and Jeff Gordon established the use of gnotobiotic zebrafish to study conserved and unique host responses to microbial colonization and composition (Rawls, Samuel, and Gordon, 2004; Rawls et al., 2006). This work has expanded dramatically over time and has also contributed valuable tools back into the zebrafish toolkit that allow for the study of immune responses in diverse contexts (Kanther et al., 2011). The zebrafish has become a powerful host for studying the otherwise impossible-to-directly-observe immune-microbe interactions in the gut, shedding light on the role of these interactions in health and disease with great precision (Park et al., 2019; Murdoch and Rawls, 2019; Murdoch et al., 2019; Espenschied et al., 2019).

In all instances, the presence of a rich *in situ* innate immune system coupled with live observation has allowed researchers to rapidly screen for novel virulence factors involved in manipulating the host immune response, through bacterial subversion of both macrophages and neutrophils (Torraca and Mostowy, 2018). The tractability and genetic conservation between zebrafish and humans also enables the study of host determinants and the use of the zebrafish as a heterologous host will likely expand as the need for ever-higher-throughput screen grows to find novel dimensions of the host-pathogen response. As the zebrafish develops the initiation of the adaptive immune system also allows the zebrafish to function as a platform to evaluate vaccine candidates, perhaps cutting costs and increasing the rapidity with which new approaches can be tested (Myllymaki et al., 2017).



**Figure 2.1:** Black and white image of the *flk1:eGFP<sup>s843</sup>* larval zebrafish, showing the regularly patterned intersomitic vasculature. This transgenic line allows for facile visualization and quantification of various angiogenic processes due to the highly stereotyped developmental patterns of the larval zebrafish endothelium.

#### 2.2.4 Zebrafish a Model System for Vascular Biology

The zebrafish is a powerful model for studying various aspects of vascular development and endothelial cell biology (Wilkinson and van Eeden, 2014). As we have already seen with the tale of the *cloche* mutation, the zebrafish allows for sophisticated genetic and optical analysis of the fine details of vascular development. The zebrafish offers an array of benefits for the study of vascular processes including strong conservation of underlying genetic pathways, optical accessibility from the earliest events of development, oxygen perfusion by passive diffusion for most of the larval stages (Ellertsdottir et al., 2010), a superabundance of genetic tools (Figure 2.1), and a highly stereotyped pattern of vascularization, from which even minor perturbations can be detected. These features and more make the zebrafish perhaps the gold-standard model for studies of vascular patterning.

The biology of the zebrafish opens the possibility of studying processes in vascular development that are impossible to do in a viviparous animal. External fertilization and development makes it possible to precisely determine the stage at which vascular malformations induce developmental failure. These things are possible to varying degrees in the mouse, for instance, but the number of replicates and the resolution

of observation are unmatched.

Even the earliest events of vascular specification can be modeled in the zebrafish and translated essentially one-to-one into humans and zebrafish have been instrumental in identifying these cellular differentiation events that give rise to the earliest hemangioblasts and the consequences thereof. These hemangioblasts function as a bipotent lineage able to differentiate into both angioblasts and hematopoietic stem cells, generating the blood and lymphatic vasculature as well as all of the hematopoietic lineages (Vogeli et al., 2006). Angioblasts migrate individually toward the midline of the zebrafish and coalesce to form the dorsal aorta, a process that can be watched in real time with fluorescent imaging (Lawson and Weinstein, 2002). This early process is mediated by a short peptide and functions independently of VEGFA despite VEGFA being expressed by this point in development (Nasevicius, Larson, and Ekker, 2000; Liang et al., 2001; Hogan and Schulte-Merker, 2017). At later points in development, cells along the dorsal aorta of the zebrafish larvae dedifferentiate and become the hemogenic endothelium, from which hematopoietic stem cells arise to see the development of myeloid and erythroid populations by approximately 24 hours post fertilization (Gore et al., 2012).

Later steps depend on angiogenesis to form new blood vessels, a process intimately dependent on VEGFR signaling. In the absence of VEGFR, vascular sprouting is profoundly reduced and subsequent vasculature is malformed; this also suggests that there are some non-VEGF cues that initiate angiogenesis but are unable to properly regulate it to generate a mature endothelial system (Hogan and Schulte-Merker, 2017). These may be aberrant vasculogenesis efforts or angiogenesis induction by other mechanisms that will be discussed at greater length in further sections.

These developmental angiogenic processes have a great deal in common with the

mechanisms underlying pathological angiogenesis, as highlighted in Section 1.5. During development, the somites of the zebrafish express Vegfaa to induce the formation of the stereotypic intersomitic vasculature that comprises the vascular patterning along the trunk; such developmental *vegfaa* expression can be seen clearly in transgenic approaches to visualizing this pathway (Karra et al., 2018; Walton et al., 2018). Despite the central role of VEGFA in angiogenesis, there are other auxiliary factors that are also capable of inducing angiogenesis, including BMPs, plexins, Notch, and CXCR4. The role of CXCR4 has been discussed in Subsection 1.2.6, but in development this chemokine can guide endothelial migration to ensure proper vascular connections and proper physical differentiation of the blood and lymphatic vascular systems. This role for CXCR4 is thought to be in mediating migration of already active angiogenesis and not in the initiation stage (Schuermann, Helker, and Herzog, 2014).

An interesting aspect of vascular development that remains somewhat mysterious while also being a critical dimension of vascular-targeting therapeutic strategies is the process of lumen formation within the growing vasculature. While the events of tip cell initiation, migration, and guidance have been detailed in some depth, the process of opening a lumen through which blood can flow is less well detailed, partially for lack of effective *ex vivo* complementary strategies for study. One known mechanism for this is through vacuolar fusion between neighboring cells, making the entire vessel one cell in thickness with a hollow center running along the length of the structure (Kamei et al., 2006). Such a process might be expected to predominate in the long, thin vascular spindles seen in our model of zebrafish granuloma formation, although detailed analysis of the structural features of these vessels remains lacking.

The regularly patterned intersomitic vasculature of the larval zebrafish makes it an

excellent platform to study the angiogenic potential of a vast range of possible stimuli. In my work, I have used this patterned region to measure perturbations in a highly sensitive manner, as has been done by other previously (Oehlers et al., 2015; Walton et al., 2018). The developmental regularity of this location offers many advantages for studying these processes and the area generally has only sparse macrophage residency at early points in development, allowing for some degree of macrophage recruitment and then angiogenesis to be experimentally studied. This location seems like a prime potentiator of future studies on pro- and anti-angiogenic drug screening given the ease with which stimuli can be implanted into the trunk of the larval zebrafish.

### **2.2.5 Challenges in the Use of the Zebrafish Model**

While extremely powerful, the zebrafish is still not a perfect model; the model chosen to address any question must be the one that most clearly mirrors the process being examined (and, for the zebrafish in particular, the tools to address the question must exist within the model). Challenges with zebrafish can be summarized in three major issues: fish-specific differences in biology, the aforementioned whole genome duplication, lack of true inbred models, and a lack of biochemical reagents to study zebrafish proteins.

The focus of this work is the study of tuberculosis disease, which in humans is a predominantly pulmonary infection that spreads from person to person via aerosolized droplets from the respiratory tract. Zebrafish, being fish, do not have lungs and are thus rendered impotent when it comes to the study of mycobacteria-lung interactions, including those with alveolar macrophages. While the zebrafish faithfully recapitulates many aspects of tuberculosis disease and the shared genetic programs that drive macrophage immune responses, granuloma formation, angiogenesis, and

host protection, any aspects that are specific to a given tissue may not be able to be captured with this model and other models should be employed in a complementary fashion to improve overall biological understanding.

The ancestral whole genome duplication has resulted in both a great deal of redundancy within the zebrafish genome that requires much more exhaustive genetic work than the often one-to-one gene pairs in humans and mice. While this does not inhibit the potential for discovery, it increases experimenter labor and can slow progress as the "functional" ortholog is sought out of a potential set of partially homologous genes. On the other hand, the inverse can often occur – unexpected roles for zebrafish proteins then translate into a shared function in the mammalian counterpart, which can typically only be discovered either incidentally or through unbiased screening approaches.

While the outbred nature of common laboratory zebrafish lines is an advantage in many respects, the lack of inbreeding fuels two issues: a poor quality reference genome and high degrees of heterogeneity within the population regarding particular responses, especially immune responses. The reference genome was a monumental effort and resulted in reasonable coverage across 1.4 gigabases (Howe et al., 2013). However, long regions of repeats and ambiguous calls along many introns have left it inferior to comparable mouse and human genomes, which were constructed from extremely laborious but precise bacterial artificial chromosome cloning Sanger sequencing (Osoegawa et al., 2000). While the reference strains were derived from doubled haploid founders, there remains substantial genetic diversity within these so-called strains<sup>8</sup> (Suurvali et al., 2020; Holden and Brown, 2018; Deng et al., 2022;

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<sup>8</sup>As detailed at <https://zfin.org/ZDB-GENO-960809-7>, the \*AB zebrafish lineage is derived from an isolated but widely intercrossed set of lines without a dramatic series of bottlenecks to force true homozygosity (likely because this is impossible). The large numbers of offsprings and parents for each new generation ensures a relative degree of outbreeding that becomes obvious with further

Bradford et al., 2022).

Lastly, the lack of antibodies available for the fish is a ubiquitous complaint among zebrafish researchers. While mice have >5000 antibodies available from Abcam and humans have nearly 20,000, zebrafish have 105 at the time of this writing in October 2022. While obviously not the full breadth of all available antibodies, it demonstrates the relative lack of these tools in the zebrafish, which inhibits protein localization studies in fixed tissue as well as a more trial-and-error approach to studying genes of interest. While the situation is steadily improving, what is needed is a community-wide initiative to generate and share (ideally sequence-defined, monoclonal) antibodies from a variety of host species against a range of proteins, ideally at highly conserved locations for use in other models. Such a resource would greatly accelerate discovery in the fish, but seems to be many years off, by which time, perhaps, new computational methods to antibody-target binding will make the laborious process of host immunization and then affinity purification a moot point and enable a genetics-first approach to antibody discovery. Libraries of camelid single domain antibodies are pushing ever-closer to this elusive goal.

## 2.3 *Mycobacterium marinum*-Zebrafish Model of Tuberculosis

Work by the Ramakrishnan group, began in part in Stanley Falkow's lab, led to the development of a heterologous model system for the study of tuberculosis. *M. tuberculosis* is an extremely slow-growing pathogen<sup>9</sup> that must be handled under

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genomic studies (Deng et al., 2022).

<sup>9</sup>The doubling time is approximately 24 hours and the time to visualizable colonies is on the scale of three or four weeks.

biosafety level 3 (BSL-3) conditions. These requirements make working with *M. tuberculosis* challenging; deficiencies in the mouse model compound the issue and lead to difficulty in studying specific aspects of the host-pathogen interface. Thus, the development of a complementary model able to facilitate visual access and that reproduces key aspects of the human disease had the potential to unlock new insights into these interactions and, over the past twenty years, have done precisely that (Myllymaki, Bauerlein, and Ramet, 2016).

### **2.3.1 History and Merits of the Zebrafish-*M. marinum* Model**

Studies began in the mid-1990s set the stage for the emergence of a new model of tuberculosis infection. Ramakrishnan and Falkow utilized a closely related pathogenic species of mycobacteria, *Mycobacterium marinum* to demonstrate a temperature-dependent persistence in cultured cells (Tobin and Ramakrishnan, 2008). A subsequent study found that *M. marinum* was able to infect and cause consumptive disease in *Rana pipiens* frogs as well as infect human extremities (Ramakrishnan et al., 1997; Ramakrishnan, 1997; Cosma et al., 2006). A new model was also developed using carp leukocytes, allowing macrophage-mycobacterial interactions to be studied at BSL-2 conditions (El-Etr, Yan, and Cirillo, 2001). A foundational study in 2002 set the tone for the next two decades of research into host-microbe interactions in the zebrafish. Davis et al. took advantage of the optical transparency and manipulative amenability of the zebrafish larvae to infect them with *M. marinum*. *M. marinum* is a globally dispersed pathogen of fish and amphibians that causes tuberculosis in fish, which tends to manifest in superficial lesions, spinal deformities, and wasting and which can occasionally infect humans, typically on the superficial extremities where

the temperature is within the growth range of the bacterium (Hashish et al., 2018; Aronson, 1926; Gray et al., 1990; Parisot, 1958). 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 (Davis et al., 2002; Davis and Ramakrishnan, 2009). This ability to dissect the relative contributions of the innate immune system in an unmodified organism has enabled many studies on the specific roles of macrophages and neutrophils in host immune control and has highlighted the imminent importance of these early responses in infection control that had been ignored by the IFN- $\gamma$  and T cell-biased control seen in C57BL/6 mouse models (Lesley and Ramakrishnan, 2008).

Further developments over the following years, most notably by Swaim 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.

While a great deal was done to develop the model over time, there remained important questions about the ability for these findings to be translated into humans; mice are the preeminent preclinical model and to translate findings in the fish directly into humans could, in certain instances, dramatically cut the bench-to-bedside timeline. A

pair of seminal papers (Tobin et al., 2010; Tobin et al., 2012) established the *LTA4H* locus as a major determinant of the course of tuberculous meningitis – individuals with either homozygous allele were at increased risk of poor outcomes and treatment of one of the homozygous populations with dexamethasone was proposed as a host-directed therapy to improve outcomes in this deadliest of tuberculosis manifestations. Indeed, subsequent studies found that this patient stratification strategy was able to determine patient response to dexamethasone in tuberculous meningitis (Thuong et al., 2017; Thwaites, Toorn, and Schoeman, 2013; Wilkinson et al., 2017; Davis, Meintjes, and Wilkinson, 2018; Prasad, Singh, and Ryan, 2016), although there may be additional factors worth considering beyond just *LTA4H* genotype (Siddiqi et al., 2021).

Today, the zebrafish-*M. marinum* model continues to proliferate in utility and acceptance. Indeed, as the field of zebrafish research as a whole advances technologically, it allows the advantages of the fish to shine through even more so in the study of host-pathogen interactions. New technologies for spatial transcriptomics will be greatly enhanced by our knowledge base in zebrafish development and the existing optical transparency of the larval fish; the day is likely not far distant when a comprehensive, spatially-aware transcriptional atlas of the developing zebrafish larva will be available, which will only accelerate our knowledge of host-tuberculosis dynamics through added dimensional awareness.

As alluded to, one of the most useful incidental features of the fish is the temporal segregation of innate and adaptive immune responses (Myllymaki, Bauerlein, and Ramet, 2016). While the adult zebrafish possesses the complete vertebrate immune system, including both myeloid and lymphoid compartments, the larval zebrafish is limited to innate immune responses (Cronan and Tobin, 2014). This allows the

researcher to differentiate the contributions of these two distinct systems to the overall phenotype and to, in an unmodified host, ascribe discrete roles to the innate immune system. This is one of the major reasons why so much has been learned about macrophage-mycobacterial interactions using the larval zebrafish – it allows for analysis of these interactions in the context of a native, immunocompetent host but without confounding intervention from adaptive immunity.

### 2.3.2 Zebrafish Husbandry

Zebrafish husbandry serves as one of the major advantages of the model. Social fish, they can be maintained at a reasonably high density per unit volume as adults<sup>10</sup>, which can then spawn ~200 eggs per week per female. Our fish are kept on a constantly recirculating rack system from Aquaneering, which replaces 90%+ of the volume of each of ~1,000 tanks once per hour, 24 hours a day. The fish are diurnal animals and photoperiodic breeders, which greatly eases experimenter access, as they will reproduce reliably only in the morning, which can be artificially set to the convenience of the laboratory. The fish are kept at 28.5°C on a 14hr-10hr light-dark cycle and kept in either 3 or 6 L tanks. Reverse osmosis water is maintained at 600-700 µS conductivity by addition of Instant Ocean Sea Salt (#SS15-10) and a pH between 7.0 and 7.4 (buffered by automated addition of sodium bicarbonate; Arm & Hammer Pure Baking Soda [#426292]). All of this work were performed in accordance and compliance with policies approved by the Duke University Institutional Animal Care and Use Committee (protocol #A091-20-04).

Infected adult zebrafish are maintained at an identical 14hr-10hr light cycle at 28.5°C in an isolated incubator (ThermoFisher #PR505755L) physically separated

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<sup>10</sup>Approximately 250 mL of water per fish is more than sufficient for rapid growth to adulthood and breeding success.

from the primary fish system. Fish are kept at no greater than 1 fish/100 mL of water in Aquaneering crossing cages (Aquaneering #ZHCT100) and are fed daily with the standard fish diet in our lab (Skretting #GEMMA Micro 500). Water is changed daily using water taken from the primary fish system. While sex is not a standard factor we account for in the analysis of our experiments, approximately equal numbers of fish of each sex are used for infection experiments. Any adult fish exhibiting signs of imminent distress or morbidity<sup>11</sup> are euthanized. Standard adult experiments are conducted for a minimum of 14 days, although infection will continue to progress over the course of approximately four to six weeks if needed. Fish are humanely euthanized at the conclusion of experimentation by tricaine overdose and decapitation.

For infection, fish were anesthetized in 120 µg/mL tricaine. Single-use aliquots of single cell suspensions of *M. marinum* were thawed and diluted in sterile PBS and zebrafish were injected with 10 µL of a solution containing 200-1000<sup>12</sup> fluorescent bacteria using a back-loaded insulin syringe (BD #08290-3284-38). Injection is done into the peritoneal cavity of the fish, achieving a systemic infection within the cavity and which spreads to all major organ systems<sup>13</sup>.

The larval zebrafish, on account of the high fecundity of the model, is a key tool in these studies. We can readily collect >1,000 embryos at once and can reasonably

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<sup>11</sup>inability to right, flared scales, labored breathing, obvious open wounds

<sup>12</sup>While the precise number is kept consistent within each experiment and subsequent replicates, different initial inocula are chosen based on the desired kinetics of disease. Lower initial doses are likely to be superior for identifying factors regulating bacterial control while higher doses are better if the desired outcome is to study aspects of granuloma biology, as high starting doses tend to result in a greater number of granulomas forming earlier in disease. This often appears to balance out as the disease progresses and some functional upper limit on bacillary load is reached.

<sup>13</sup>Although brain involvement is only rarely seen. It is not a standard tissue for us to analyze in the lab, but previous analysis has generally failed to identify meningitis as a manifestation of *M. marinum* infection in the zebrafish.

infect ~200 per hour, allowing for high n-value screening approaches. This, in addition to their remarkable ability to recover and highly stereotyped developmental patterning, facilitates reproducible studies in many fields of biology including the work described herein. Embryos are collected in the early- to mid-afternoon and allowed to develop in the presence of 0.001% methylene blue in E3 medium (5 mM NaCl (Fisher Scientific #S271), 178 µM KCl (VWR #BDH9258), 328 µM CaCl<sub>2</sub> (VWR #BDH9224), 400 µM MgCl<sub>2</sub> (Ward's Scientific #470301)) at no more than 150 larvae per dish. At 24 hours post fertilization, they are transferred into E3 supplemented with 1-phenyl-2-thiourea<sup>14</sup> (PTU, Sigma-Aldrich #P7629) at a final concentration of 45 µg/mL to prevent melanization if they are to be used for imaging studies. Otherwise, they are allowed to develop in unmodified E3 medium until 3-4 days post fertilization, when they are put into the nursery system to be raised to adulthood. Infected larval zebrafish are euthanized prior to 8 days post fertilization in all instances. Zebrafish are of indeterminate sex<sup>15</sup> until they reach the juvenile stage of development, so no distinctions are or can be made on the basis of sex in larval zebrafish studies.

For ease of manipulation and the minimization of distress, larval zebrafish are anesthetized in approximately 160 µg/mL of tricaine (MS-222 or Tricaine-S<sup>16</sup>, Syn-del #ANADA 200-226) prior to injection. Injection of *M. marinum* is done by in-

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<sup>14</sup>The ability to taste phenylthiourea (also known as phenylthiocarbamide) is an interesting genetic trait in humans that can predispose individuals to enjoying the taste of various cruciferous vegetables (Kim et al., 2003).

<sup>15</sup>This is only strictly true for laboratory strains of zebrafish (Kochakpour and Moens, 2008). Wild zebrafish have a sex determining region on chromosome 4 that has been repeatedly lost under laboratory culture conditions, for reasons that remain mostly unknown (Howe et al., 2013; Parichy, 2015; Wilson et al., 2014). In the lab, a variety of factors seem to influence the eventual sex determination of the fish, including rearing density and caloric allotment, in addition to various genetic factors (Kossack and Draper, 2019). Despite the lack of strict genetic determinants of sex, the standard 1:1 female:male ratio is generally maintained under normal conditions.

<sup>16</sup>Now known as “Syncaleine”

jecting larvae into a developmentally undefined peri-notochordal space between the somitic muscle layers along the trunk at 2 days post fertilization. Approximately 50-150 fluorescent bacteria are injected, spreading allong the anterior-posterior length of the fish and establishing a largely localized infection along the avascular trunk. This infection location allows for facile quantitation of vascular aberrations, an essential prerequisite for this study. Injection of TDM is conducted similary. Trehalose 6-6'-dimycolate from *Mycobacterium bovis* (TDM, Sigma-Aldrich #T3034) was resuspended in 2:1 v/v chloroform:methanol at 1 mg/mL and stored at -80°C. Prior to use, the liquid is evaporated under vacuum and emulsified in incomplete Freund's adjuvant (IFA, Sigma-Aldrich #F5506) at 2 mg/mL. Larvae are then anesthetized and injected with approximately 10-20 nL of TDM/IFA or IFA alone along the trunk in this same undefined peri-notochordal space. The droplets coalesce into spheres within 10-15 minutes and remain in place for the entire experimental duration. Larvae are allowed to recover in E3 medium supplemented with PTU and raised in a 28.5°C incubator.

### **2.3.3 Deficits of Mouse Models of Tuberculosis**

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<sup>17</sup>, relative ease of use, and minimal expense<sup>18</sup>. 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. Such loss of heterozygosity far from models the human condition, where there are an estimated

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<sup>17</sup>Genetic diversity between individual C57BL/6 mice is in the range of 10-20 single nucleotide polymorphisms per individual in a genome of 2.5 gigabases - a remarkable degree of isogenicity (Bryant, 2011; Sarsani et al., 2019).

<sup>18</sup>A single C57BL/6 mouse from Jackson Laboratories (jax.org) at the time of writing is \$24 USD.

20 million base pairs of difference from person to person (The 1000 Genomes Project Consortium et al., 2015). The difficulties of the mouse model are nowhere more apparent 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. While mice faithfully recapitulate certain aspects of human development, it is far from perfect and other approaches are able to access different types of knowledge in greater detail.

The mouse has served as the model for immunology for the past 50 years. It has enabled 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 and disorders. 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. For instance, the C57BL/6 mouse is highly resistant to acute tuberculosis disease; these mice can be infected with standard laboratory strains of *M. tuberculosis* and succumb approximately 300 days later<sup>19</sup>.

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<sup>19</sup>Bacterial strain variations and dosage can alter this somewhat, but infection with the reference strain H37Rv will result in a highly reproducible and sudden series of death almost a year after initial infection.

Historically, the C57BL/6 mouse model of tuberculosis has served as the gold standard for studies on host-pathogen interactions in tuberculosis and has been used to identify major host-protective factors as well as bacterial virulence factors. However, even among other mouse models, the C57BL/6 model has clear challenges in the field of tuberculosis biology. For one, C57BL/6 does not form necrotic granulomas under standard laboratory doses of laboratory strains of *M. tuberculosis* (Orme, 1998). This discordance between the observed human phenotype and the mouse model leaves an abundance of room for misinterpretation of data that may or may not be translatable to the human disease context. The loci of infection in this mice becomes a granulocytic infiltrate, with abundant cellular involvement and engagement with the bacteria, including the presence of lymphocytes. One of the standard arguments that the granuloma has host-detrimental aspects is that the physical structure blocks T cell-mycobacteria interactions, which may be able to serve an important host-protective role. That this is incapable of being modeled in the C57BL/6 model leaves room for competing models to model these elements of granuloma biology.

More recently, the C3H/FeJ "Kramnik" mouse model has come into widespread use. This model is able to model granuloma biology, as ~50% of infected mice will form granulomas (Harper et al., 2012; Lenaerts, Barry, and Dartois, 2015). However, this is also a hypersusceptible model of infection, with the average mouse succumbing to infection within approximately 3 months. In humans, disease progression can be of either very rapid progression over the course of weeks or develop in severity over the course of many years (Tiemersma et al., 2011). Thus, there remains an unmet need for a model that (a) forms granulomas and mirrors other important aspects of human disease and (b) exhibits a spectrum of disease presentation similar to that seen in humans.

Other mouse models could potentially fulfill some of these demands, if appropriately chosen. One approach has been to more precisely mimic the infectious trajectory of human disease by instilling mice with ultra-low doses of *M. tuberculosis* by aerosol, which seems to facilitate a more necrotic granuloma phenotype, perhaps suggesting that the previously used larger doses (~300 CFU) were inducing a distinct immune response that led to more granulocytic infiltration (Plumlee et al., 2021). Recently, work by Clare Smith from the Sassetti lab has established the collaborative cross mouse collection as a fruitful tool for the discovery of novel aspects of host immunity to tuberculosis, including IFN- $\gamma$ -independent defense, the role of important T cell integrins, and more across approximately 50 inbred-to-homozygosity mouse strains (Smith et al., 2016; Smith et al., 2022). Further and ongoing dissection of these strains will not only reveal further determinants of host immunity and bacterial susceptibility, but may provide the elusive granuloma mouse model, although what factors render most mice incapable of forming human-like caseating granulomas remains unknown; these host determinants of granuloma formation are also intriguing aspects of this axis that may reveal new contributions from genes of previously unknown function. However, the process of granuloma formation is one of synthetic tone – the overall response itself may be more important than the contribution of any one factor, aside from major basal determinants, like STAT6 signaling (Cronan et al., 2021) and some modicum of host defense through major immunostimulatory cytokines (Flynn et al., 1993; Flynn et al., 1995). In the long term, as understandings of the host determinants of granuloma formation are better understood, knock-in and humanized models may provide an excellent means of modeling granulomas in the mouse but this seems like a far distant development that will require a great deal of work in the meanwhile to clarify the contributions of these diverse factors.

### **2.3.4 Comparison to Other Models**

Other popular laboratory models of tuberculosis are able to form granulomas, including rabbits and guinea pigs; the former is relatively resistant to tuberculosis while the latter is highly susceptible (Clark, Hall, and Williams, 2014; Dorman et al., 2004; Heppleston, 1949). However, these tend to require maintenance via relative 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. Additionally, these models do not escape the challenges of working in a BSL-3 environment with *M. tuberculosis*. While the cynomolgous macaque model of tuberculosis is an excellent representation of human tuberculosis, the costs associated with the use of macaques is enormous (Pena and Ho, 2015). A cost-efficient, genetically amenable small vertebrate model of infection was a clear gap in the resources available in the late 1990s and the zebrafish was able to fill some of these gaps in our ability to model these infectious processes. As time has progressed, our ability to use the zebrafish to model different aspects of mycobacterial infection has expanded, with the zebrafish now able to be used for studies in *M. leprae*, an incorrigibly difficult organism to study due to its peculiar lifestyle and agonizing doubling time ( 12 days) (Madigan et al., 2017) and *M. abscessus*, a close relative of *M. marinum* (Halloum et al., 2016; Stinear et al., 2008).

## **2.4 Transgenic Tools in the Zebrafish**

Since that first monumental forward genetic screen in 1996, the arsenal of tools available to the zebrafish researcher has expanded dramatically. This expansion began

rather soon thereafter, with a major *insertional* mutagenesis screen being published, which allows for more rapid barcoding and identification of the disrupted gene than earlier ethylnitrosourea mutagenesis approaches (Amsterdam et al., 1999). Additionally, the ability to inject plasmid DNA and express exogenous constructs in the fish was discovered relatively early on (Stuart, McMurray, and Westerfield, 1988; Lele and Krone, 1996), but was extremely laborious in both time investment and effort required. The race was on for a technology that would be able to introduce DNA into the zebrafish embryo that would efficiently transmit across generations. The results of these labors were two complementary technologies: I-SceI meganuclease-mediated transgenesis (Thermes et al., 2002) and *tol2*-mediated transgenesis (Kwan et al., 2007). I-SceI transgenesis utilizes a “meganuclease” that recognizes and cleaves a specific, 18 base pair sequence only known to exist in the *Saccharomyces* mitochondria. This, coupled with some useful biophysical properties, allows it to protect foreign DNA within cells until it can interact and integrate with the genome (Soroldoni, Hogan, and Oates, 2009; Grabher, Joly, and Wittbrodt, 2004). While other approaches were attempted, including the use of retroviruses (Kurita, Burgess, and Sakai, 2004), nothing else matched this system for ease of use and efficacy. However, research in medaka had begun utilizing transposons to mediate transgenesis to great effect and this was viewed as the future. Sleeping Beauty was an early attempt, but has largely fallen into disuse (Davidson et al., 2003), while *tol2*, with its large cargo capacity (Balciunas et al., 2006), has become the dominant mode, largely thanks to the tol2kit, which is a Gateway-cloning compatible kit of promoter, gene, and 3' UTR elements that can be used to rapidly clone components to generate transgenic zebrafish – conceptualization to injection can be streamlined into ten days or less if needed (Kwan et al., 2007). This method was used in this work to generate the major new transgenic lines that enabled the work in Chapter 3.

Newer technologies are making the zebrafish ever-more amenable to genetic manipulations and optical control. For a more in-depth analysis of the state and future of genetic tools in the zebrafish, see Subsection 2.5.2. The current range of tools available in the zebrafish is vast and able to specifically dissect key aspects of the host-pathogen interface and new tools are released all the time. For instance, it is possible to drive the expression of multiple individual proteins off of a single polycistronic transcript through the use of 2A peptides (Kim et al., 2011). Relevant to the studies to come is the ability to visualize changes in calcium, which can be seen using calmodulin-modified green and red fluorescent proteins known as genetically encoded calcium indicators, the most notable of which being GCaMP and its many variants (Nakai, Ohkura, and Imoto, 2001; Dana et al., 2016; Zhang et al., 2020c). These have been used to both measure and manipulate calcium flux in cells to alter their movement and immune responses (Beerman et al., 2015). The zebrafish offers the possibility to do calcium imaging across the entire larva at high spatial resolution, especially through use of newer microscopy techniques like LightSheet (Reynaud et al., 2008; Kim et al., 2017).

An additional tool is the use of gene trapping techniques, that insert either transcriptional repressors or polyadenylation sequences into genes and allow for these to be rescued through use of Cre or other methods. These have allowed for various tagging approaches and have generated libraries of strains with fluorescence driven off endogenous promoters. Conversely, by placing native genes under the control of inducible promoters, it is possible to determine temporal effects of particular genes during development (Ma et al., 2017). These tools and others to be discussed later offer the opportunity to study the mechanisms of NFAT activation and the biology of these proteins in their native context. An *nfatc2a* gene trap line would be ideal, but none has yet been identified (Ichino et al., 2020; Clark et al., 2012).

### 2.4.1 Technical Description of New Transgenic Lines

The p5e *irg1* construct was generated by restriction digestion of *irg1*-pTol2linkerswitch (Sanderson et al., 2015) (a gift from Christopher Hall) with FseI and XmaI and then blunted using T4 DNA polymerase (NEB #M0203S) per the manufacturer's instructions. Simultaneously, p5e MCS (Kwan et al., 2007) was PCR linearized using inverted T3 and T7 promoter primers (5-CCCTATAGTGAGTCGTATTAC-3', 5-T CCCTTAGTGAGGGTTAAT-3), digested with DpnI and PCR purified. These fragments were then ligated using T4 DNA ligase (NEB #M0202S) to generate p5e *irg1*. This plasmid was then recombined with pME tdTomato (Addgene #135202), p3e ubb pA (Addgene #188702), and pDEST tol2 ubb pA (Addgene #188701) by Gateway cloning (ThermoFisher #12538120) to generate the pTol2 *irg1*:tdTomato construct that was then injected into single cell embryos alongside 15 ng/ $\mu$ L tol2 mRNA (Balciunas et al., 2006) in 1x Tango buffer (ThermoScientific #BY5). Candidate founders were selected based on fluorescence at 3 dpf, raised to adulthood, and outcrossed to \*AB to establish the line, which transmits at 50% frequency, suggesting a single insertion locus and has exhibited stable expression over ~6 generations.

Tg(*irg1*:VIVIT-*tdTomato*<sup>xt38</sup>), in which the inhibitory peptide VIVIT conjugated to the fluorescent protein tdTomato is expressed strictly in macrophages, was constructed by recombination of p5E *irg1* (Addgene #188698), pME VIVIT NS (Addgene #188699), p3E tdTomato (Addgene #188700), and pDEST tol2 Ubb pA (Addgene #188701). Reactions were incubated at equimolar ratios overnight in a 25°C thermocycler with heated lid, with volumes calculated using the "LR Ratios Calculator" Excel document (provided at Brewer et al. (2022a)). The *irg1* promoter was first described by Sanderson et al. as a macrophage-specific inducible promoter, but our lab has found that this element often drives basal expression in macrophages as well,

likely in an insertion-site-dependent manner. Tg(*irg1:tdTomato*<sup>xt40</sup>) was similarly generated by recombination of p5E *irg1*, pME tdTomato, p3E Ubb pA, and pDEST tol2 Ubb pA. Both of these lines express tdTomato at baseline within macrophages by 48 hours post fertilization.

The middle element, pME VIVIT NS was constructed by a synthetic templated PCR after annealing. Two oligonucleotides from Integrated DNA Technologies (IDT) were annealed by heating to 95°C and then slowly cooled to room temperature (sense: 5'-GCCATCATGGCAGGACCACACCCGGTGATTGTTATCACTGGACCACA TGAGGAG-3', anti-sense: 5'-CTCCTCATGTGGTCCAGTGATAACAATCACC GGGTGTGGTCCTGCCATGATGGC-3'). This was then used as a template for PCR using two primers to add the *attB1* and *attB2* sites required for Gateway recombination into pDONR 221 (forward: 5'-GGGGACAAGTTGTACAAAAAAAG CAGGCTGCCATCATGGCAGGACC-3', reverse: 5'-GGGGACCACTTGTACA AGAAAGCTGGGTACTCCTCATGTGGTCCAGTG-3'). This PCR product was then column purified and recombined into pDONR 221 (ThermoFisher #12536017) using BP Clonase II (ThermoFisher #11789020) to generate pME VIVIT NS (no stop) (Addgene #188699). Constructs were verified by either Sanger sequencing or whole plasmid sequencing from Plasmidsaurus and have been submitted to Addgene, which provides additional whole plasmid sequencing verification.

Genotyping to differentiate the *irg1:tdTomato*<sup>xt40</sup> and *irg1:VIVIT-tdTomato*<sup>xt38</sup> lines can be performed where necessary (either for intentional experimental blinding or due to incidental mixing of fish during husbandry or experimentation) by PCR and gel electrophoresis. Primers (5'-GATTAGGTGACACTATAGATTCA GAGCTCGCACAGG-3', 5'-ATCTCGAACTCGTGGCC-3') amplify across the 3' end of the *irg1* promoter and into the 5' end of the tdTomato insert. VIVIT+ fish

display a 236 bp band while tdTomato-only fish display a 163 bp band. No band is seen in sibling fish lacking an *irg1* transgene.

## 2.5 Use of CRISPR/Cas9 in Zebrafish

One of the major advantages of the zebrafish model is that, unique among vertebrate model organisms, it is trivial to generate targeted knockouts of essentially any gene using CRISPR/Cas9, which can be performed by even new researchers in the course of a couple of hours. Compare this to the mouse where it takes highly technical single cell injection and implantation to screen for new lines, which can often cost thousands of dollars to outsource. Extensive work has been done to establish different applications of this technology to generate F<sub>0</sub> individuals for high-throughput screening, knock-in technology to use endogenous promoters and tag genes, whole-segment deletions for total gene removal, and more.

One of the huge advantages of CRISPR/Cas9 in zebrafish is the ability to use “crisprants” to assess phenotypes, which is very rapid and allows for efficient screening of different genes than the standard injection, rearing, and isolating single heritable mutations pipeline. This approach has been extensively studied and described elsewhere, but with the correct approach, these results often faithfully recapitulate phenotypes seen in stable mutants (Zhang et al., 2017; Wu et al., 2018). This approach transforms six months of waiting into as little as a week from injection to preliminary data – a huge time savings that allows for more informed experimentation.

Generation of mutants in *card9*, *nfatc2a*, and *nfatc3a* was performed as described previously. Alleles were identified by outcrossing of mosaic adults to wild-type \*AB and Sanger sequencing of F1 adults. DNA extraction was conducted by cellular lysis

in 50 mM sodium hydroxide as described previously (Meeker et al., 2007). Briefly, either adult zebrafish tail fins or whole larvae were collected in 50 mM NaOH in H<sub>2</sub>O and lysed at 98°C for 12 minutes in a thermocycler and then neutralized by 1:10 addition of a solution of 1M Tris-HCl (pH 8) in 10x TE (100 mM Tris, 10 mM EDTA). This solution was then directly used as the template for downstream PCR reactions.

Once a mutation has been established, efficient and cost-effective genotyping is essential for differentiating the different genotypes. High-throughput and researcher blinding are also important dimensions in this and one major method of blinded genotyping at scale is via high-resolution melt analysis, or HRMA. HRMA utilizes a qPCR-based amplification with a super-saturating concentration of dsDNA-binding dyes to sensitively detect changes in the thermal melting profile of DNA (Reed, Kent, and Wittwer, 2007; Thomas et al., 2014); changes in the base-pair composition will alter the melting profile of the DNA. While larger mutations are generally easier to differentiate, this assay can theoretically detect changes as small as a A/G or C/T transition, but is often limited by the detection precision of the instrument. We have thus employed a streamlined combination of the described rapid and efficient DNA extraction via alkaline lysis (Meeker et al., 2007) with HRMA for the purposes of genotyping at every possibility. Other methods are only utilized if HRMA has failed, thus the use of classical PCR and restriction digestion to genotype *nfatc2a* mutants.

The allele *card9<sup>xt31</sup>* was generated by injection of a single guide RNA into single-cell embryos (guide sequence: 5'-TAATACGACTCACTATAGGGCAAGGTGCTGAGCAGCGGTTTAGAGCTAGAA-3'). We identified an allele containing a 28 bp insertion, resulting in an immediate downstream frameshift leading to a premature termination codon at amino acid 59 (with missense mutations beginning at amino acid

47). Genotyping was performed using high-resolution melt analysis (HRMA) using the MeltDoctor Master Mix (Applied Biosystems #4415450) with primers flanking the sgRNA site (5'-CCTTATCTGAGACAGTGCAAGGTGC-3', 5''-TTACCAACT TTGCGGCGTCTG-3''). Amplification for Sanger sequencing was performed using primers (5'-GTTTCCCAGTCACGACCGAATGCTTCTCATCAAGACC-3', 5'-C GAATGCTTCTCATCAAGACC-3'). Sequencing was performed with the "M13F(-40)" primer from Eton Biosciences.

The allele *nfatc2a<sup>xt69</sup>* was generated by simultaneous injection of two neighboring guide RNAs to increase odds of a larger intervening deletion (guide sequences: 5'-T AATACGACTCACTATAGGGCTGCGAGAACGGGCCACGTTTAGAGCTAG AA-3', 5'-TAATACGACTCACTATAGGCAGCCGTCGCCCCACGGGTTTA GAGCTAGAA-3'). While this strategy failed in its initial purpose of generating a guide-spanning deletion, we identified a mutation consisting of a complex, bipartite insertion/deletion resulting from independent sgRNA activity leading to a net 4 bp insertion and frameshift leading to a premature termination codon at amino acid 272 (of 894, prior to the DNA binding domain). Genotyping can be performed by one of two distinct restriction digest-based methods. The original method was performed by restriction digest of the 500 bp PCR product produced by the listed sequencing primers (5'-TAGAAGGCACAGTCGAGGCTCGAGGCTTCTGGAGACCTCTG TCC-3', 5'-TGACACACATTCCACAGGGCTCTAGAGGTTGCCCTTCATA TCCTGC-3', underlined portion base pairs with the genomic sequence); digestion was with PflMI (NEB #R0509) directly in the PCR reaction mixture. PCR was performed using LongAmp Taq (NEB #M0323) strictly for reasons of buffer compatibility with the restriction enzyme. Digestion was carried out for 3 hours at 37° in the presence of rSAP (NEB #M0371) to minimize background. Sanger sequencing was conducted on undigested PCR products using the vendor (Eton Biosciences) sup-

plied "BGH Reverse" primer (5'-TAGAAGGCACAGTCGAGG-3') corresponding to the appended 5' tail of the forward PCR primer.

The second method utilizes a separate set of primers (5'-CCTCTATGCAAACGCA CCTACG-3', 5'-GTGATGCTCCTTGTGGCAC-3') to generate a 102-106 bp PCR product spanning the mutation site. This PCR is performed in 20 µL reaction volumes using Taq polymerase (NEB #M0285L) (again, for reasons of buffer compatibility) and 1 µL MwoI (NEB #R0573L) is added directly to the reaction mixture after thermocycling, which is then incubated at 60°C for 1 hour. The reaction is then visualized on a 2-3% agarose gel impregnated with SYBR Safe dye. In our hands, this second method is faster, easier, more robust, and more cost-effective. In both cases, the wild-type product is unable to be cut (single larger band) while the mutant is cleaved into two similarly sized smaller bands (a slightly hazy single lower band); the heterozygotes are differentiated by the presence of both bands. Confirmatory Sanger sequencing was performed as needed.

The allele *nfatc3a<sup>xt59</sup>* was generated using an individual sgRNA (5'-TAATAC GACTCACTATAAGGGCAGTTGCAGTAGTCATGTTTAGAGCTAGAA-3') and a mutation was identified containing a 22 bp deletion leading to a premature termination codon at the 8th amino acid (of 1074). The allele was identified by PCR amplification and Sanger sequencing using F: 5'-GTTTCCCAGTCACGA CCAGAAGGTCGAGCAGTTGG-3' and R: 5'-AACGTGTTCGCCTTGC-3'. Sequencing used the "M13F(-40)" primer supplied by the vendor (Eton Biosciences) (5'-GTTTCCCAGTCACGAC-3'). Genotyping was routinely conducted by high-resolution melt analysis (HRMA) using the MeltDoctor Master Mix (ThermoFisher #4415450) with primers flanking the sgRNA site (5'-AAAGAGTCGGTGTACATA GACGGG-3', 5'-CGAAGATCAGTCTGAAGTCCAGC-3').

### 2.5.1 Crispant Assays

To generate mosaic knockouts in genes of interest, we synthesized sgRNAs targeting the first exon of the respective genes. For *nfatc2a* we used 5'-TAATACGACTCACT ATAGGTCAAGTCAGGTGAACTGTCGTTTAGAGCTAGAA-3' and for *nfatc3a* we used 5'-TAATACGACTCACTATAGGTAGAGGCAGTGACCTGCGGTTTTAGAGCTAGAA-3'. For prospective genotyping of these alleles, we used HRMA to assess approximate editing efficiency; this can only act as a rough proxy due to limitations and feasibility of exhausting genetic analysis of these mosaic larvae. For *nfatc2a*, we used the following primers: 5'-CTCTTTTACGGCGAAAAAGCTGC-3', 5'-GAAACAAACCTTGAAGTCCTGTTGG-3'. For *nfatc3a* we used: 5'-AAAGAGTCGGTGTACATAGACGGG-3', 5'-CGAAGATCAGTCTGAAGTCCAGC-3'. We had already begun generating the future stable alleles *nfatc2a*<sup>xt69</sup> and *nfatc3a*<sup>xt59</sup> and used these listed sgRNAs to increase our likelihood of introducing a functional mutation in these genes and to normalize target location and sgRNA number.

For my contributions to Cronan et al. (2021), I designed and generated crisprants in the IL4R receptor homologs in the zebrafish using the strategy put forth by Wu et al. (2018). The two orthologs are sufficiently similar at the nucleotide level to allow for simultaneous targeting by the same guide RNAs, which may imply a misannotation or some level of total functional redundancy – more targeted deep sequencing approaches would be required to determine which may be the case. These guide RNAs were synthesized as previously described and injected into embryos at the one-cell stage. Four pooled guide RNAs were used with the following sequences: 5'-TAATACGACTCACTATAGGGCTTGGCAGACGAGTGTGGTTTAGAGCTAGAAATAGC-3', 5'-TAATACGACTCACTATAGGTGATCGGATGTCTGCACGTTTAGAGCTAGAAATAGC-3', 5'-TAATACGACTCACTATAGGGAAACTTCATGTT

ACCTGTTTAGAGCTAGAAATAGC-3', and 5'-TAATACGACTCACTATAGG  
CCAGGCCGTCTGTGATTAGCTTAGAGCTAGAAATAGC-3'.

## 2.5.2 Prospectus on Cutting Edge and Future Tools in the Zebrafish

Targeted gene editing with CRISPR/Cas9 has unlocked boundless opportunities to develop new genetic tools in the zebrafish. A large need in the community is greater access to tools that allow for cell-type specific gene ablation on the model of Cre-*loxP* in the mouse (Housden et al., 2017). Indeed, Cre drivers for the zebrafish exist, but incorporating *loxP* sites into genes is a particular challenge due to technical limitations. This has led to some alternative approaches being developed, although the ultimate use of directed Cre-*loxP* gene disruption likely remains the goal. For instance, it is possible to specifically express sgRNAs in cell-types of interest using ribozyme-mediated cleavage, allowing for tissue-restricted gene ablation (Wang et al., 2021; Yin et al., 2015). While the effects are more challenging to validate, this offers a transgenic-based approach to gene ablation that is readily incorporated into existing workflows and is able to be flexibly used through use of the Tg(*ubb:Cas9<sup>xt48</sup>*) fish that I founded during my rotation.

Site-specific addition of new genetic elements is a pressing need and it may be that tools are finally arriving to accomplish these goals. The new GeneWeld approach allows for microhomology mediated end-joining to specifically integrate exogenous genetic elements with reasonable efficiency, which should add new approaches to endogenous tagging and *loxP* incorporation, although this system has yet to come into widespread use (Wierson et al., 2020). This strategy has been used to knock-in fluorescent proteins, tags, Cre recombinase, and more, with utility sure to expand

further (Almeida et al., 2021; Liu et al., 2022). Others have taken slightly older approaches through gene traps to use Cre recombination to turn genes on and off in particular cell types. Gene traps, fundamentally, required luck and perseverance to establish as their integration loci are basically random (Sugimoto et al., 2017). The ability to target particular genes with CRISPR/Cas9 to mediate the specific integration of *loxP* sites remains somewhat elusive. Part of the challenge is the extremely rapid cell generation time of zebrafish embryos, which makes technologies developed for the much slower mouse embryo difficult or impossible to use thanks to cell cycle stage-specific DNA repair strategies (Hustedt and Durocher, 2016; Prill and Dawson, 2020).

In the future, these knock-in strategies need to be exhaustively optimized to allow for trivial generation by researchers in the same way that tol2-mediated transgenesis has been optimized. It is difficult to know what variables warrant the most optimization, but pharmacological treatment of zebrafish embryos to alter the balance of DNA repair outcomes seems to be promising (Nakade et al., 2014; Luo et al., 2018). Relatedly, the ability to efficiently generate precise edits in the zebrafish genome will allow for more effective zebrafish disease modeling that mirror human mutations; base editors have shown some promise but are limited in their scope and precise knock-in strategies are likely to be more extensively utilized.

Optogenetics have also been used in the zebrafish in the past to exercise fine control over the timing of gene induction, but these have been limited by unusual toxicity. Recent developments have allowed for a more robust system of optogenetics, which in the background of tissue-specific expression, has the opportunity to allow intra-granuloma manipulation of gene expression to study the specific effects of gene induction or disruption within a localized structure (Deisseroth, 2015). These will

certainly complement existing transgenic approaches and can likely be integrated into these more advanced knock-in based strategies (Reade et al., 2017).

Lastly, the future of knockouts in the zebrafish is clearly through locus-spanning deletions even if these approaches come with some downsides. The abundance of evidence on the topic of genetic compensation has led to fears that conclusions drawn about the roles of particular genes are the product of transcriptional changes in related genes through an as-yet unknown RNA-dependent mechanism (Rossi et al., 2015; El-Brolosy et al., 2019). While this has the potential to disrupt other genes via removal of trans-acting elements within gene introns, the risks of genetic compensation via mutant mRNA degradation seem too high to simply ignore. An alternate and perhaps easier strategy would be to selectively mutate critical domains in one's protein of interest to generate in-frame mutations that disrupt protein function without generating early stop codons. This is, however, not always possible and may not reveal sufficient information about the function of diverse domains along the length of a gene. Ultimately, this seems to require additional labor on the part of the researcher to effectively characterize their gene of interest through multiple complementary strategies – not necessarily an enticing prospect, but one that will improve the reliability of the literature going forward.

## 2.6 CLARITY and Confocal Microscopy

One of the central advantages of the larval zebrafish is its optical transparency, which allows for intravital imaging through every tissue. This advantage is lost in the adult due to the development of skin pigmentation and other factors. In other animals, this lack of optical access is a severe impediment to understanding the fundamental biological processes. Karl Deisseroth thus created the CLARITY technique, which

uses a custom fixation method to immobilize proteins and nucleic acids within a tissue and then wash away birefringent lipids, generating an optically clear tissue that can be imaged at high depth and resolution (Chung and Deisseroth, 2013; Yang et al., 2014a). This method was adapted for use in the zebrafish by Cronan et al. (2015) and is used to study the biology of granulomas in the adult zebrafish in this work.

### 2.6.1 Technical Description

CLARITY fixation and clearing was conducted as previously described (Cronan et al., 2015). In brief, adult zebrafish were euthanized in tricaine, decapitated, and disemboweled. Visceral organs were immersed in an ice-cold A1P4 CLARITY solution (4% paraformaldehyde (EMS #15710), 1% acrylamide (Bio-Rad #1610140), 0.05% bis acrylamide (Bio-Rad #1610142), 0.0025 g/ml radical initiator (Wako Chemical #VA-044) in 1x final concentration PBS (Corning #46013CM) and nutated at 4°C for 2-3 days prior to overlay with mineral oil (Fisher Scientific #BP2629) and polymerized at 37°C for 3 hours. Hydrogel samples were collected, washed in 1x PBS, and then immersed in clearing solution at 37°C (8% sodium dodecyl sulfate (Bio-Basic #SD8119) in 200 mM boric acid (Sigma-Aldrich #B0394), pH 8.5), which was changed every 2-3 days until samples were optically clear. These samples were washed in 1x PBS supplemented to 0.1% Triton-X (Fisher Scientific #BP151) for two days at 37°C with daily solution changes to remove excess SDS from the tissue. These tissues were then individually placed into black, opaque microcentrifuge tubes (Argos Technologies #EW-06333-80) and immersed in refractive index matching solution (RIMS) (40 g, Histodenz (Sigma-Aldrich #D2158), 30 mL 20 mM phosphate buffer (4.043 g Na<sub>2</sub>HPO<sub>4</sub> (VWR #BDH9296), 678.7 mg NaH<sub>2</sub>PO<sub>4</sub> (Sigma-Aldrich #S9638), 1 L diH<sub>2</sub>O), 0.01% sodium azide (Sigma-Aldrich #71290)) with rotation for at least 24 hours prior to imaging (Yang et al., 2014a).

Imaging was conducted on a spinning disk microscope (Zeiss AxioObserver Z1 connected to an XCite 120 LED Boost with an XLight 2TP, 89North LDI, Hamamatsu C13440 and captured on a Dell Precision Tower 5810 running Windows 10 Enterprise with Metamorph 7.10.5.476) in a MatTek dish (#P35G-1.5-14-C) with optical bottom. Additional RIMS was added to the dish to cover the sample and minimize refraction during imaging. We panned across the proximal surface of the organ bundles to identify granulomas in each individual sample and captured Z-stack images of each of the identifiable granulomas at the maximum possible optical depth in the fish. This is able to capture the majority (but perhaps not all) of the granulomas present in a given fish due to inherent limitations in lens working distance.

All image processing was conducted in FIJI/ImageJ (Schneider et al., 2012). In-focus Z planes were identified and processed with the Maximum Intensity Projection function using a Jython macro (see Section 4.4). These files were saved and then subjected to cropping where the frame was cropped to the vasculature immediately surrounding each granuloma. This distance was unable to be precisely normalized across granulomas due to the heterogeneity in size and shape of the granulomas themselves as well as the nature of their varying physiological locations. Cropped images were then blinded using the blindrename.pl script (Salter, 2016). Images were opened in ImageJ and vessels were traced using the segmented line tool, added to the Region of Interest (ROI) Manager tool and then measured for distance in pixels. The ROI files were saved and have also been provided in the Zenodo archive. Total length was then converted to microns based on the conversion factor provided by the microscope ( $1 \text{ px} = 0.6552 \mu\text{m}$ ). Resulting .csv files were processed in Excel to remove unnecessary tag information from file names and then all subsequent analysis was performed in R using RStudio (R Core Team, 2022; RStudio Team, 2022).

## 2.7 *Mycobacterium marinum*

As previously discussed, *Mycobacterium marinum* is a powerful tool to study host-mycobacterial interactions in the zebrafish, but there are several critical elements of its biology that make it so useful. It was originally discovered in a salt water fish and later described as the cause of an outbreak in humans from a public swimming pool (Aronson, 1926; Linell and Norden, 1954). This bacterial species has a super-sized genome of 6.6 megabases, which is fully 50% larger than the genome of *M. tuberculosis*, reflecting adaptation of *M. tuberculosis* to a human-restricted niche. *M. marinum* possesses orthologs of >80% of *M. tuberculosis* genes with extremely high amino acid identity (Stinear et al., 2008). This allows *M. marinum* to model many aspects of *M. tuberculosis* pathogenesis, which is my primary utility for this species in my work. This extensive genetic conservation allows us to investigate conserved pathogenic mechanisms using the heterologous zebrafish-*M. marinum* system, which offers the benefits of the zebrafish with a natural pathogen in a BSL-2 environment (Tobin and Ramakrishnan, 2008).

However, this excess coding space offers the opportunity to uncover novel aspects of mycobacterial biology through an interrogation of the genes unique to *M. marinum* that may allow it to either survive in the environment for extended periods or facilitate infection of its natural hosts, which are mostly fish, amphibians, and reptiles. These extra genes, of which there are approximately 2,500 have largely unassigned functions and an ecology-centered approach to the autotelic study of *M. marinum* would certainly reveal interesting adaptations to these diverse environments which can range from hot to cold, wet to dry, and transitory stages through various hosts spanning multiple different phylogenetic classes (Stinear et al., 2000; Stamm and

Brown, 2004). Further studies on the genetic diversity that exists within *M. marinum* may offer further hints if comparative genomics/pathogenicity approaches were taken to the study of virulence within *M. marinum* in diverse model hosts (Das et al., 2018).

### 2.7.1 Culture Conditions

All strains are derived from *M. marinum* strain M (ATCC #BAA-535) (Ramakrishnan and Falkow, 1994). Hygromycin-resistant fluorescent strains expressing the tdTomato (Oehlers et al., 2015), mCerulean, or EBFP2 fluorescent proteins have been described previously (Takaki et al., 2013). Bacterial culture was carried out on either 7H10 agar (Difco #262710) plates supplemented with Middlebrook OADC growth supplement (10% v/v; Sigma-Aldrich #M0678) and 50 µg/mL Hygromycin B (ThermoFisher #10687010) or liquid 7H9 media (Difco #271310) supplemented with Middlebrook OADC growth supplement (10% v/v), 0.05% Tween 80 (Sigma-Aldrich #P1754), and 50 µg/mL Hygromycin B.

Single cell preparations of these bacteria were prepared and stored as single-use aliquots at -80°C. Briefly, bacteria were grown at 33°C in 50 mL 7H9 supplemented with 10% OADC (Sigma-Aldrich #M0678), 0.05% Tween-80 (Sigma-Aldrich #P1754), and 50 µg/ml hygromycin B (Invitrogen #10687010) (7H9 Complete). Once cultures reach OD<sub>600</sub> 0.55-0.8, they are spun down at 4600 rcf for 15 minutes and resuspended in 5 mL PBS-T (1x PBS with 0.05% tyloxapol (Sigma-Aldrich #T8761)) and bring to 25 mL total in PBS-T. They are spun and washed 2x in 25 mL PBS-T each time and then resuspend in 2 mL of 7H9 with 10% OADC (Freezing 7H9) and split into 250 µL aliquots and homogenized 10x using a 1 mL syringe and 27G needle (BD #309623). Next, a soft spin at 770 rcf for 1 minute is done to pellet

larger clumps and the supernatants are collected and then push the pooled supernatants through a 5  $\mu\text{m}$  filter (Millipore #SLSV025LS) using a 10 mL syringe. The suspension is collected in 1.5 mL microfuge tubes and spun at 10000 rcf for 5 minutes. Final resuspension of pellet is done in freezing 7H9 and aliquoted into single use aliquots and concentration is calculated by fluorescent bacteria on a hemocytometer and by colony forming units on selective media.

### 2.7.2 CFU Assays

Colony forming unit assays were conducted by complete homogenization of whole adult zebrafish after euthanasia by tricaine overdose and external cleansing of the skin using 70% ethanol. A single 6.5 mm ceramic bead (Omni #19-682) was added to in a pre-filled bead mill tube containing 2.8 mm stainless steel beads (Sigma-Aldrich #Z763829-50EA) and then the fish was homogenized on a bead mill (MP Bio #116004500) for a single 25 second interval at 5 meters/second. Lysate was plated on 7H10 plates supplemented with 10% OADC, hygromycin B (50  $\mu\text{g}/\text{mL}$ ), amphotericin B (Gibco #15290-026) (10  $\mu\text{g}/\text{mL}$ ), and polymyxin B (Cayman Chemical #14157) (25  $\mu\text{g}/\text{mL}$ ). Lysate was plated in serial 1:10 dilutions up to  $10^{-5}$ . Cultures were grown for 10-14 days prior to counting visible colonies. Where possible (due to contamination inherent to the assay), confirmatory counting was performed at 21 days after plating to capture slow-growing colonies, which are quite rare in our hands. Plates displaying overt contamination that occluded colony growth were excluded from further analysis.

## 2.8 THP-1 as a Macrophage Model

THP-1 (ATCC TIB-202) cells are a human monocytic cell culture line derived from a 1-year-old 46XY patient suffering from acute myelogenous leukemia in 1980 (Tsuchiya et al., 1980). They have become a widespread and useful model to study certain aspects of macrophage biology *in vitro* as they can be differentiated into macrophage-like cells by addition of phorbol 12-myristate-13-acetate (PMA) to the media. This makes them a facile model – replicating in suspension at baseline, but becoming adherent, non-replicating, and terminally differentiated after treatment with PMA. While they are widely used, they are an imperfect model and cannot model certain aspects of tissue macrophage biology and are only exposed to the defined set of stimuli provided in the media. Additionally, they are variably aneuploid, with reports providing evidence of pseudo-diploid cells up to tetraploidy and beyond (Odero et al., 2000; Adati et al., 2009; Kasai et al., 2022). Despite these disadvantages, they remain among the most faithful models and typically align well with studies conducted on mouse bone marrow-derived macrophages and human peripheral blood monocyte-derived macrophages, arguably the two best models for macrophage biology *in vitro* (Mendoza-Coronel and Castanon-Arreola, 2016).

Here, we use them in order to model particular aspects of the host interaction with *M. tuberculosis* not only to complement our findings in the fish but also to enable new avenues of discovery that are impossible in the complex *in vivo* setting. The relative ease of genetic and pharmacological manipulation of these cells allows for screening of macrophage phenotypes with relative ease that can then inform later approaches to be taken in the zebrafish. Going forward, using these model systems together is likely to be a potent means of honing in on interesting phenotypes that can then be

dissected in parallel across both systems.

### 2.8.1 Culture Conditions

The cells used for these experiments were sourced from the Duke Cell Culture Facility and tested for mycoplasma prior to receipt. Cells are cultured in RPMI-1640 (Sigma-Aldrich #R8758) supplemented with glucose (Sigma-Aldrich #G8769), HEPES (Gibco #15630), sodium pyruvate (Gibco #11360) and 10% non-heat inactivated FBS (Sigma-Aldrich #F2442) in T-75 flasks (CellStar #658170) in a 37°C incubator with 5% CO<sub>2</sub>. Cells were cultured for no greater than 10-12 passages prior to use and were then disposed of. Extended passage generated strange aberrations in the responsiveness of the cells and seemed to, generally, result in a loss of responsiveness and increase in baseline VEGF production. I found this effect rather inexplicable and unusual, but has been occasionally commented on in the literature (An et al., 2009; Chanput, Peters, and Wickers, 2015; Gazova et al., 2020; Lund et al., 2016; Stokes and Doxsee, 1999); I have no other rational basis for these observed effects, but it is certainly one challenge of working with these cells. Several hypotheses are possible, but none strike one as especially compelling – freezing may offer some sort of “reset” or rejuvenation mechanism or different culture conditions may alter their phenotype in a semi-permanent manner. If this is to be the case, then a more robust system may be required going forward or single clone expansion and testing for sensitivity may be useful.

### 2.8.2 qRT-PCR

qRT-PCR is a powerful and highly precise method for quantitating the amount of a transcript present in a sample. This utilizes RNA extraction followed by quantitative

reverse transcription to turn the RNA into complementary DNA, which can then be measured by standard PCR amplification in the presence of a dsDNA-binding dye. Under optimal conditions, this reaction proceeds by doubling the amount of product present each cycle, which can then be directly translated into a relative quantity by base 2 transformation relative to a control transcript. These data are then analyzed by the  $2^{-\Delta\Delta C_t}$  method, which makes some important but not always valid assumptions about the raw data (Livak and Schmittgen, 2001; Cikos, Bukovska, and Koppel, 2007). For instance, this method assumes that the PCR efficiency for all of the primer sets is 100%, which is not always true. However, this is the gold standard for comparing the relative quantities of a transcript across two samples and also allows for comparisons of the quantities of two or more different transcripts. This method is ubiquitously used for its relative ease and resource economy while remaining highly accurate (Dvinge and Bertone, 2009; Perkins et al., 2012; Pabinger et al., 2014).

## Technical Details

THP-1 cells were transdifferentiated into macrophage-like cells using 50 ng/mL PMA (phorbol 12-myristate-13-acetate) (Sigma-Aldrich #P148), seeded in 24 well cell culture treated plates at a concentration of  $5 \times 10^5$  cells/ml and incubated at 37°C/5%CO<sub>2</sub> for 48hr. After that the PMA media was changed using complete RPMI 1640 media and incubated at 37°C/5%CO<sub>2</sub> for 24hr (rest day). Then the cells were exposed to 0.5 mL of gamma-irradiated Mtb (BEI #NR-49098) in 25% glycerol (Sigma-Aldrich #G7757) diluted in RPMI-1640 at a final concentration of 1 mg/mL. Cells were spun at 100 rcf for 5 min and incubated at 37°C/5%CO<sub>2</sub> for 8hr.

Cells then had media removed and 300 μL of Trizol was added and cells were vigorously resuspended and moved into 1.5 mL microfuge tubes. RNA extraction

was conducted by addition of 0.7 volumes of 1x TE (Sigma-Aldrich #T9285) and 100  $\mu$ L of chloroform (EMD Millipore #CX1055). After spinning at 17,000 rcf for 30 minutes at 4°C, the upper aqueous layer was transferred to another tube, and 100  $\mu$ L of 24:1 chloroform:isoamyl alcohol (Sigma-Aldrich #25666) was added. The tubes were then shaken by hand and spun for another 30 minutes at 17,000 rcf at 4°C. The top aqueous layer was removed and final cleanup was done using the RNA Cleanup Kit (NEB #T2040L) per the manufacturer's instructions.

cDNA synthesis was performed using the LunaScript RT SuperMix Kit (NEB #E3010L) by the manufacturer's instructions. RT-PCR was performed using the Luna Universal qPCR Master Mix (NEB #M3003L) in an Applied Biosystems 7500 Fast (ThermoFisher #4351106) per the manufacturer's instructions. Final calculations were conducted in R.

### 2.8.3 ELISA

An enzyme-linked immunosorbent assay (ELISA) is an effective means of quantifying the amount of protein present in a supernatant and is often used to quantify cytokine production in a variety of contexts. For my purposes, I am most interested in the production of VEGFA by THP-1 cells. The sandwich ELISA we use coats the plate with one antibody, which then fixes the protein of interest in place and is then detected by binding of another antibody at another location on the same protein. While these kits are widely manufactured, it is principally possible to use any two well-defined antibodies to measure any protein whose expression might change due to experimentation. These are somewhat targeted by nature, but reward the experimenter with highly quantitative and standardized data able to be converted into the concentration of the protein in the media.

## Technical Details

Cells were cultured identically to previous, except they were plated in 96 well cell culture treated plates and exposed to gamma-irradiated *M. tuberculosis* for a total of 24 hours to facilitate VEGF production and secretion. Supernatants were collected and spun down and then the upper layer was collected for further analysis. ELISA was performed according to the manufacturer's instructions (R&D Systems #DY293B). Absorbance was read on an Agilent Synergy LX plate reader. For one of the replicates shown in Chapter 3, the standard curve failed and so the normalized OD values are shown instead.

### 2.8.4 Immunofluorescence

As extensively detailed for the zebrafish, the ability to see something happen is extremely impactful in one's ability to understand it. While live cell imaging is possible in tissue culture, we have utilized fixed cells and immunostaining to capture a static point in time so that we can build a relationship between NFAT activation and VEGF production on the cellular level.

THP-1 cells were plated on 4-well chamber slides (MatTek #CCS-4) and differentiated with PMA at 50 ng/mL for 48 hours. Media was then replaced with fresh RPMI-1640 and cells were allowed to rest for 24 hours prior to further stimulation. Cells were then treated by addition of 1 mg/mL final concentration gamma-irradiated *M. tuberculosis*, 40 µM INCA-6 (Cayman Chemicals #21812), and/or vehicle controls (25% glycerol in PBS or DMSO (Fisher Scientific #BP337), respectively). Cells were then incubated at 37°C, 5% CO<sub>2</sub> for 8 hours and then fixed in 4% PFA in 1x PBS for 20 minutes. Cells were then washed twice in 0.25% NH<sub>4</sub>Cl (Sigma-Aldrich #254134) or 0.15M glycine (to neutralize), rinsed in PBS, blocked in 2.5% donkey serum (Fisher

Scientific #50413253) in 1x PBS and 0.01% NaN<sub>3</sub> for at least 20 minutes, and then incubated in primary antibody overnight at 4°C. Cells were then rinsed, secondary antibody was added and cells were again incubated overnight at 4°C. After 5x rinses in PBS, cells were dipped in diH<sub>2</sub>O and mounted in DAPI Fluoromount-G (SouthernBiotech #0100-20), which was allowed to set overnight at RT in the dark. Slides were either stored at 4°C in the dark prior to visualization or visualized immediately.

Images shown in the figures were digitally adjusted for brightness and contrast in FIJI/ImageJ (Schindelin et al., 2012) and all adjustments were applied uniformly across the images within an experiment. All quantitation was performed based on the unadjusted brightness and contrast values and thresholded to better capture positive signal and eliminate the background fluorescence ubiquitous in these images.

To capture differences in VEGF expression across different experimental conditions, we programmatically blinded a subset of images from each experimental condition using blindrename.pl (Salter, 2016) and, using the *Cell Counter* plugin in FIJI/ImageJ, we marked each nucleus (as a proxy for cell number), each cell that visually expressed VEGFA at a minimum/maximum bit value of 100/1500, cells that had nuclear translocation of NFATC2, cells at the intersection of these two factors, and, when applicable, Cas9 expression. These values were exported and subsequently processed in R.

Many of the antibodies used in this study were an extremely kind gift from Aviva Symes at the Uniformed Services University. She had kept them in cold storage for greater than 20 years before passing them on to me and they have proven indispensable to many of these studies, allowing us to profile all of the different NFAT isoforms as well as all of them at once using the pan-NFAT antibody, which desperately needs to be recreated, preferably as a monoclonal antibody. This would certainly make

studies on NFAT more approachable to many other groups around the world.

Zeiss filter sets used were:

- Filter Set 50 (Cy5, Alexa Fluor 647)
- Filter Set 47 (CFP)
- Filter Set 38 (GFP, Alexa Fluor 488)
- Filter Set 43HE (tdTomato, Alexa Fluor 555)
- Filter Set 46 (YFP)
- Filter Set 49 (DAPI)

## 2.8.5 Lentivirus Construction

We sought to generate lentiviruses able to target multiple single guide RNAs to the same gene to maximize overall mutation rate and allow us to conduct experiments in mixed pools of heterogeneous cells, to minimize functional passage number. We therefore adopted a hybrid approach, inserting the sgRNA targeting array and hUbC promoter from Kabadi et al. (2014) (Addgene #53190, a kind gift from Charles Gersbach) into the NotI/XbaI site of the lentiCRISPRv2 plasmid from Sanjana, Shalem, and Zhang (2014) (Addgene #52961, a kind gift from Feng Zhang), creating a hybrid plasmid that simultaneously expressed Cas9, the puromycin resistance marker, and up to 4 single guide RNAs from a single plasmid.

This resulting transfer empty vector (pLV hUbC-Cas9-P2A-Puro\_BsmBI-sgRNA-BsmBI, Addgene #188703) was digested with Esp3I FastDigest (ThermoFisher #FD0454) precisely as previously described (Kabadi et al., 2014) in the presence of equal masses ( 200 ng each) of constituent sgRNA expression plasmids driven from mU6, hU6, 7SK, or hH1 RNA pol III promoters, ligated with T4 ligase (NEB #M0202S), and cloned into NEB Stable (NEB #C3040H) cells. Resulting plasmids were screened by restriction digestion and full plasmid sequencing.

Single guide RNA expression plasmids were cloned from phU6-gRNA, pmU6-gRNA, ph7SK-gRNA, and phH1-gRNA as described previous (Kabadi et al., 2014). The guide sequences for both NFATC2 and the safe targeting loci were chosen from the a database of available guides and safe loci in the human genome to model the DNA damage response from sgRNA targeting without overt toxicity or phenotypic changes (Morgens et al., 2017). These plasmids were purified and used in subsequent steps.

The appropriate lentivirus transfer plasmid was transfected into HEK293T cells alongside pMD2.G (Addgene #12259) and psPAX2 (Addgene #12260) (both kind gifts from Didier Trono) (plus sfGFP-C1 to mark transfected cells, Addgene #54579, a kind gift of Michael Davidson & Geoffrey Waldo) in a 4:3:1(:0.5) mass ratio using TransIT-Lenti reagents (Mirus Bio #MIR-6603) (Pedelacq et al., 2006). Supernatants were collected 48 hours post transfection and immediately used to transduce THP-1 cells in the presence of 8 µg/mL polybrene (Sigma-Aldrich #TR-1003-G). Approximate titer was determined by infecting additional HEK293T cells with varying dilutions of the supernatant.

## 2.8.6 Transduction

THP-1 cells were seeded in complete RPMI-1640 media supplemented with 8 µg/mL polybrene (Sigma-Aldrich #TR-1003-G) in two non-treated six-well plates at a concentration of  $1 \times 10^6$  cells/ml in each well. One six-well plate was infected with 1mL of pLV-ST and the other with pLV-NFATC2. The lentivirus infected THP-1 cells were spun at 1500 rcf/2 hr/22°C, gently resuspended and incubated at 37°C/5%CO<sub>2</sub> for 72hr. Transduced cells were selected with 2 µg/mL puromycin (Sigma-Aldrich #P4512) for 48 hours and then kept in complete RPMI-1640 with 1 µg/mL puromycin

until time of assay.

## 2.9 Quantification and Statistical Analysis

All assays were performed under experimental blinding. For all assays where the genotype or experimental condition of the fish was apparent to the experimenter during data gathering (for instance, experiments in adults and the VIVIT assays in larvae), the resulting images were computationally blinded prior to analysis with either blindrename.pl (Salter, 2016) or an in-house Python translation (available at [urldoi://10.5281/zenodo.6816429](https://doi.org/10.5281/zenodo.6816429) or Section 4.7). For assays where the genotype is unknown (in-cross of heterozygotes experiments for *card9*, *nfatc2a*, *nfatc3a* in the larvae), blinding was inherent in the design of the experiment and genotypes were matched to the individual fish *post hoc*.

Statistical analysis was performed using R 4.2.2 *Innocent and Trusting* within the latest version of RStudio IDE, 2022.12 *Elsbeth Geranium* on macOS Monterey 12.6.1 (R Core Team, 2022; RStudio Team, 2022). Graphing was performed using ggplot2 (Wickham, 2016; Wickham et al., 2022a). All statistical tests performed and the resulting significance values are indicated in figures and figure legends.

## 2.10 R

The R statistical environment began as a competing/complementary/intercompatible alternative to the existing S statistical programming language, but has become nearly ubiquitous in the life sciences over the past fifteen years or so. Offering new users an accessible environment in which to learn programming and with a large community, this language allows for reproducible, open-source analysis and visualization

of nearly any scientific data. R has become immensely flexible and libraries have been developed to accommodate any and every kind of numerical data. This also allow for community reanalysis and reinterpretation of existing data by facilitating the direct sharing of analysis methods and raw data.

R would not be where it is today without RStudio (soon to be renamed “Posit”), which offers a feature-complete integrated development environment complete with in-line output and a user-friendly interface with various easy-to-navigate panels. This interface has allowed much of the work I have completed to occur when I was less knowledgeable about the underlying mechanics of R as a language. New users almost uniformly use RStudio as their entrance to R and it is powerful enough for more advanced users as well.

These features allowed me to develop a comprehensive set of infinitely reproducible analysis scripts to analyze all of the data presented here. These scripts, along with the raw data, are available on Zenodo at doi://10.5281/zenodo.6816429. R (version 4.2.2, *Innocent and Trusting*) was accessed via RStudio (*Elsbeth Geranium* version 2022.12.0) on macOS Monterey 12.6.1 (R Core Team, 2022; RStudio Team, 2022). The libraries used to complete this work were dplyr (Wickham et al., 2022b), reshape (Wickham, 2022), and FSA (Ogle et al., 2022). Graphs utilized ggplot2 (Wickham, 2009; Wickham, 2016; Wickham et al., 2022a), gghighlight (Yutani, 2022), ggbeeswarm (Clarke and Sherrill-Mix, 2017), ggsignif (Ahlmann-Eltze and Patil, 2021), scales (Wickham and Seidel, 2022), extrafont (Chang, 2022), and RColorBrewer (Neuwirth, 2022).

## 2.11 FIJI/ImageJ

FIJI/ImageJ is an extremely powerful image processing application created initially by Wayne Rasband and now largely maintained by Curtis Reuden with support from the National Institutes of Health (Schneider, Rasband, and Eliceiri, 2012). Developed primarily in Java, this portable application has been instrumental in image analysis pipelines for over 20 years. Completely free and open-source, ImageJ as well as its successor ImageJ2 and the bundled plugins included with FIJI, as well as the broader community of plugins, are the standard against which any competing applications are compared. This imminently accessible and, obviously, cost-effective means of analyzing images opens this process to labs all over the world (Girish and Vijayalakshmi, 2004). Through the interpretation of images as numerical pixel values, ImageJ is able to perform nearly every conceivable operation on images to facilitate their revisualization and interpretation. Due to the numerical infrastructure of scientific imaging data, various aspects of these images can be distilled into various quantitative measurements, which is used throughout this work to analysis the angiogenesis phenotype. While the built-in capabilities of ImageJ have evolved over time, the primary advantage of using ImageJ is access to a robust programming environment based on Java in which to create plugins and macros in a range of Java-compatible programming languages, including Groovy, R, and Python as well as a beginner-friendly IJ1 macro language that, while limited, is able to be written trivially by new users, thanks in part to the excellent macro recorder that will translate GUI-based interactions into a set of executable macro commands. For instance, a basic script to measure thresholded pixel intensity within a user-defined area and record it to a ResultsTable is a simple as:

```
setThreshold(600, 4096);  
// setTool("freehand");  
run("Measure");  
close();
```

This work has necessitated the development of a range of these scripts and plugins, which have serviced the analysis of these images in the background both through automating repetitive processing tasks (Z projections, channel splitting, channel merging, file saving, lookup table application, etc.) and extracting quantitative data from these images in a high-throughput fashion. These can all be found in Chapter 4. In a number of instances, I was forced to translate several Java plugins into Python to allow for streamlined access to the underlying API; these are also described in Chapter 4.

New, user-friendly RNA-seq analysis pipelines were developed to facilitate analysis and visualization of the data in Saelens et al. (2022). This analysis pipeline is presented in Section 4.8.

Image analysis was conducted using the FIJI (Schindelin et al., 2012; Rueden et al., 2017) expansion of ImageJ (Schneider, Rasband, and Eliceiri, 2012). Analysis pipelines were written in Jython (v.2.7.18) (van Rossum, 1995) and executed within the ImageJ Jython interpreter. All scripts are provided via Zenodo at the doi listed above. Additional description of the use of FIJI/ImageJ can be found in Section 4.1.

# Chapter 3

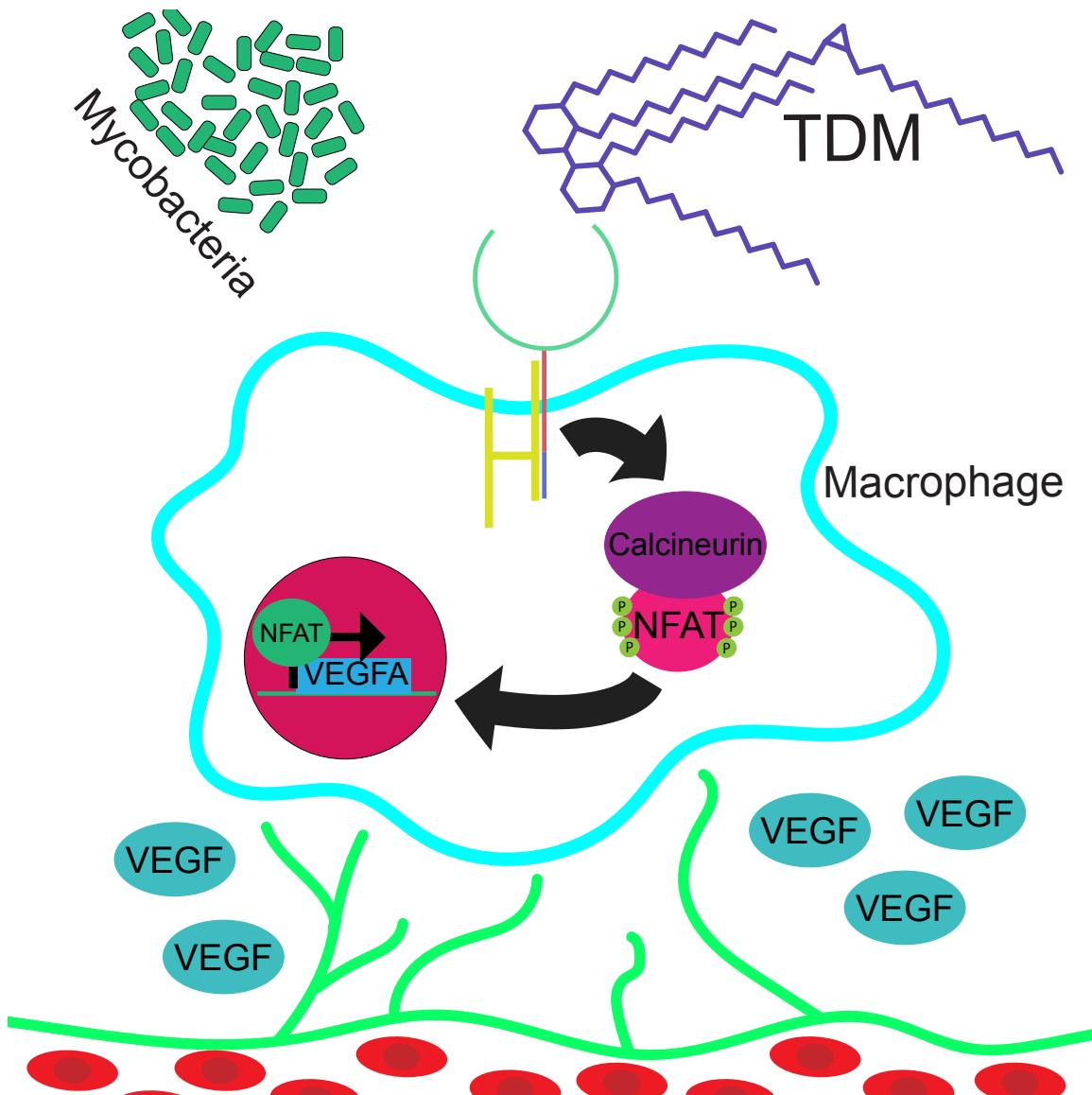
## Macrophage NFATC2 Drives Angiogenic Signaling During Mycobacterial Infection<sup>1</sup>

### 3.1 Abstract

As introduced in the previous chapters, pathogenic mycobacteria manipulate the host immune response to generate a productive infection that enables them to transmit to new hosts. To do so, they must both subvert protective immune responses and agonize the pathways that provide them benefit in the way of nutrients, susceptible host cells, or routes of transmission. This necessitates the engagement of both the immune cells themselves and the surrounding stromal cells, all of which must be subverted to generate the optimal pro-bacterial response within the limits of bacterial control of host physiology. One of the mechanisms by which this has been found to occur is through a specific modification of the specialized cell wall lipid, trehalose 6-6'-dimycolate (TDM). This mycobacterial glycolipid has been found to

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<sup>1</sup>Most of the data in this chapter is from W. Jared Brewer et al. (2022b). “Macrophage NFATC2 Drives Angiogenic Signaling During Mycobacterial Infection”. In: *Cell Rep*, text extensively modified.



**Figure 3.1:** This is a graphical representation of the work completed here, which found a novel role for NFAT activation downstream of macrophage-mycobacterial surface interactions that facilitated the production and secretion of VEGFA to drive angiogenesis.

direct angiogenesis toward nascent granulomas to enhance overall bacterial burden. The present study utilizes the zebrafish-*Mycobacterium marinum* infection model to define the signaling basis of the host angiogenic response. Through intravital imaging and targeted, cell-specific peptide-based inhibition, we identify macrophage-specific activation of NFAT signaling as essential to TDM-mediated angiogenesis *in vivo*. Exposure of human cells to *Mycobacterium tuberculosis* results in robust induction of VEGFA that is dependent on a signaling pathway downstream of host TDM detection and culminates in NFATC2 activation. As granuloma-associated angiogenesis is known to serve bacterial-beneficial roles, these findings identify potential host targets to improve tuberculosis disease outcomes.

## 3.2 Introduction

The host rejoinder to infection is driven by an intricately regulated, but occasionally discordant or maladaptive, immune response to pathogenic stimuli at the cell-intrinsic, innate, and adaptive levels (Iwasaki and Medzhitov, 2010; Finlay and McFadden, 2006; Haldar et al., 2015; MacMicking, 2004; MacMicking, 2012; Kim et al., 2012). Although an inflammatory immune response is essential to host survival and pathogen killing, an overly robust response is clearly deleterious to the host, as seen in sepsis (Finethy et al., 2020; Casadevall and Pirofski, 2003). However, as we have been in previous chapters, the inflammatory response can also serve pro-bacterial purposes, rendering the immune response impotent. The contributions of immune cells to host defense have been widely studied, but there is growing appreciation for the contributions non-immune populations, including stromal cells and the endothelium (Honan and Chen, 2021; Worrell and MacLeod, 2021; Amersfoort, Eelen, and Carmeliet, 2022; Honan and Chen, 2021) make in shaping the host response to both acute and

chronic infections (Mueller and Germain, 2009; Rando, MacMicking, and James, 2013; Krishnamurty and Turley, 2020). Pathogens have long been known to possess sophisticated mechanisms to undermine signaling pathways in immune cells and, more recently, have been shown to manipulate development and homeostatic tissue processes to force them toward pathogen-beneficial states (Menzies and Kourteva, 1998; Guichard et al., 2013).

*Mycobacterium tuberculosis* is among history's most widespread and successful pathogens. It has evolved an array of sophisticated mechanisms that manipulate its human host to enable bacterial survival, replication, and transmission. Upon infection, *M. tuberculosis* sets in motion an intricate immune response wherein innate immune cells, consisting initially of macrophages, congregate at the bacterial focus and then undergo an epithelioid transformation and interdigitate to form an encased granuloma, the hallmark feature of tuberculosis (Subsection 1.2.6), which provides both the replicative niche and the major host-pathogen interface of tuberculosis disease (Cronan et al., 2016; Pagan and Ramakrishnan, 2018; Cronan et al., 2021). Granuloma-associated vasculature has long been noted in human and animal models of TB (Cudkowicz, 1952; Russell, Barry, and Flynn, 2010) but the mechanisms of induction and precise contributions to infection are not yet fully understood (Subsection 1.5.6).

Many of the major pathological features of mycobacterial granulomas, including associated vascularization, are conserved from zebrafish to humans (Swaim et al., 2006; Bohrer et al., 2021). Zebrafish can be infected with a natural pathogen, *Mycobacterium marinum*, which induces a robust angiogenic response during granuloma formation in both the larva and adult (Section 2.3). This process, much like that in humans, non-human primates, and rabbits, is associated with production of a pro-

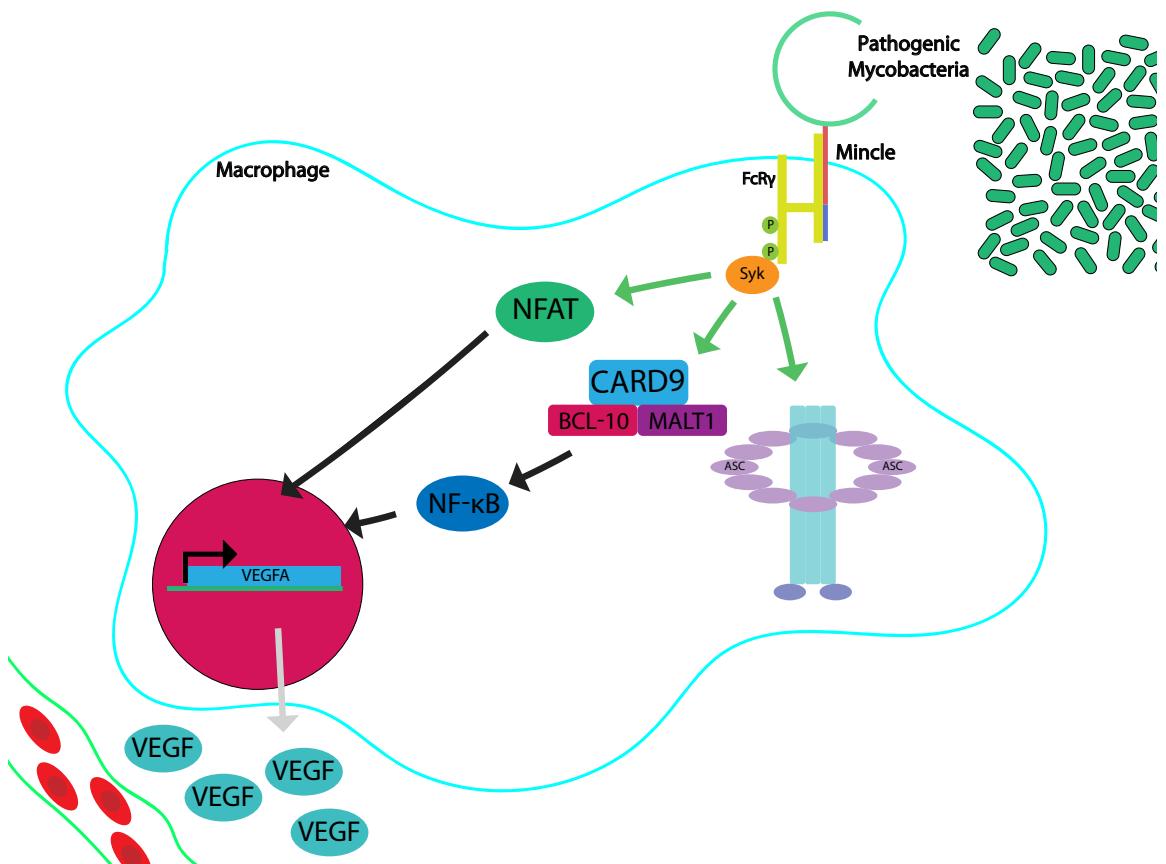
angiogenic chemokine, Vegfaa, at the site of infection (Oehlers et al., 2015). This chemokine has long been known to be a critical regulator of angiogenesis in both developmental and pathological contexts (Chung and Ferrara, 2011; Leung et al., 1989; Adams and Alitalo, 2007). Similarly, human granulomas have been shown to express VEGFA and are physically associated with blood vessels that penetrate the outer granulomatous layers (Datta et al., 2015). Subsequent work has demonstrated a role for these vessels in supporting bacterial growth and in dissemination of the bacilli from their primary site of infection (Polena et al., 2016). Additional roles for VEGFA in non-angiogenic processes have also been noted, suggesting that angiogenic signaling cascades can also alter the biology of granuloma macrophages to exacerbate disease (Harding et al., 2019). Additional roles have been proposed for the related lymphangiogenesis (Alitalo, Tammela, and Petrova, 2005; Duong, Koopman, and Francois, 2012; Lerner et al., 2020), where pharmacological blockade of these vessels of the lymphatic system also offer host-protective benefits (Harding et al., 2015). Recent profiling of human and non-human primate granulomas have confirmed the presence of aberrant vasculature associated with *M. tuberculosis* granulomas (Gideon et al., 2022; McCaffrey et al., 2022; Cronan et al., 2021), although more complete characterization of the endothelium itself is lacking.

Pathogenic mycobacteria have evolved specialized mechanisms to promote and accelerate angiogenesis and modulate other aspects of endothelial biology (Oehlers et al., 2017). Notably, the extensively modified and essential outer cell envelope component trehalose 6-6'-dimycolate (TDM) is cis-cyclopropanated by the enzyme PcaA (Glickman, Cox, and Jacobs, 2000; Rao et al., 2005). Mutation of pcaA results in a reduction in granuloma angiogenesis and concomitant reduction in bacterial burden; correspondingly, cyclopropanated TDM alone is sufficient to induce host angiogenesis (Saita et al., 2000; Sakaguchi et al., 2000; Walton et al., 2018) (Subsection 1.5.6). As

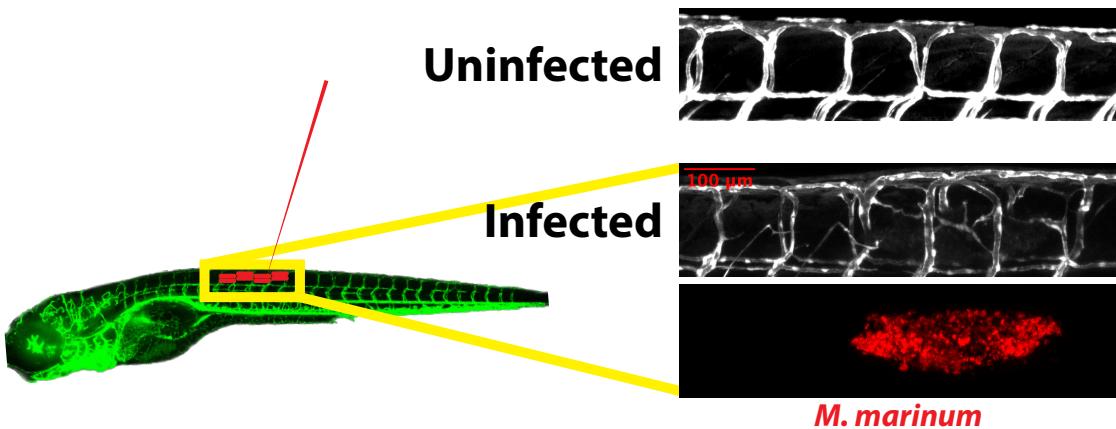
*pcaA*-dependent vascularization supports bacterial growth, factors driving angiogenesis represent potential sites of therapeutic intervention yet the signals that mediate this host process remain unclear.

TDM is an extraordinarily long-chain, hydrophobic ( $C_{60}$ - $C_{100}$ ) glycolipid (Noll et al., 1956; Noll, 1956; Hunter, Venkataprasad, and Olsen, 2006; Behling et al., 1993) that has been shown to be detected in cell culture and murine models by host C-type lectin receptors, most notably MCL (CLEC4D) and MINCLE (CLEC4E), as well as by Toll-like receptor 2 (TLR2), CD14, and MARCO (Bowdish et al., 2009; Matsunaga and Moody, 2009; Miyake et al., 2013; Ishikawa et al., 2009) (see also Subsection 1.3.5). Canonically, C-type lectin signaling is transmitted through a CARD9-NF- $\kappa$ B signaling pathway that results in the transcription and production of TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and other cytokines (Yamasaki et al., 2008; Goodridge et al., 2009; Lobato-Pascual et al., 2013; Zhao et al., 2014; Deerhake et al., 2021) (Subsubsection 1.3.3, Section 1.4). However, beyond CARD9, a number of other downstream signaling pathways are engaged by C-type lectin activation and likely control discrete aspects of signaling (Goodridge, Simmons, and Underhill, 2007; Deerhake et al., 2021).

Here, we synthesize findings from zebrafish and cell culture models to define the *in vivo* angiogenic response induced by pathogenic mycobacteria. Contrary to classical models of C-type lectin signaling, we find that cis-cyclopropanated TDM exerts its pro-angiogenic effects through an alternative NFAT-driven pathway rather than canonical CARD9-NF- $\kappa$ B signaling. We use peptide-mediated, cell-specific inhibition of NFAT to demonstrate that both early and mature granuloma angiogenesis are dependent upon macrophage-NFAT signaling. We identify *NFATC2* as the predominant isoform mediating *VEGFA* induction and angiogenesis *in vitro* and *in vivo*. These



**Figure 3.2:** The central hypothesis of this work is that a discrete pathway downstream of C-type lectin receptor activation is mediating the induction of VEGFA within macrophages to drive angiogenesis during mycobacterial infections. This schematic displays the hypothesized pathway options that could be playing these roles: CARD9, NFAT, or inflammasome activation. This work sought to dissect these possibilities to better understand the mechanism of angiogenesis during tuberculosis infection.



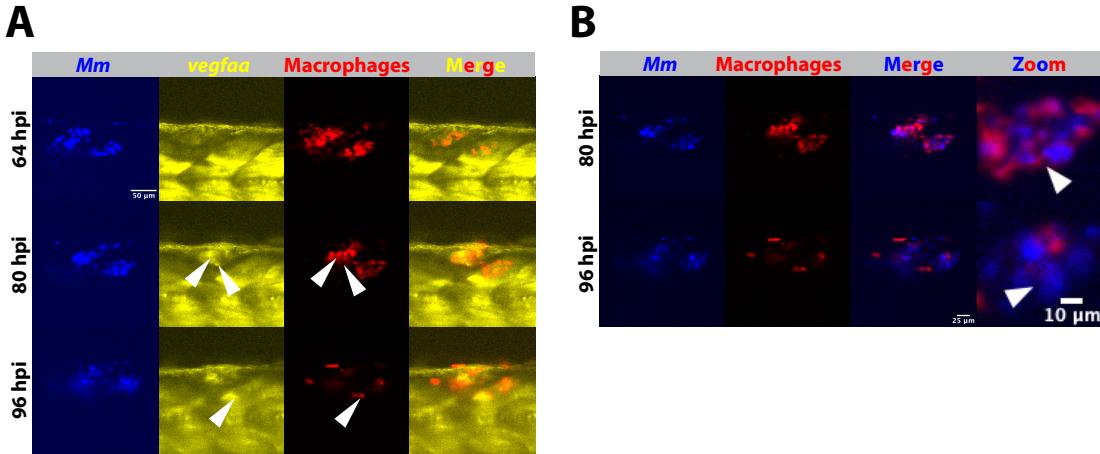
**Figure 3.3:** Pictorial representation of the site of infection and angiogenic outcomes from infection. After injection into a peri-notochordal space along the trunk of the fish, the bacteria replicate and blood vessels grow toward the site of infection, as compared to the uninfected reference.

findings define the basis of granuloma-associated angiogenesis during pathogenic mycobacterial infections and suggest new targets for host-directed therapeutic interventions during tuberculosis.

### 3.3 Results

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

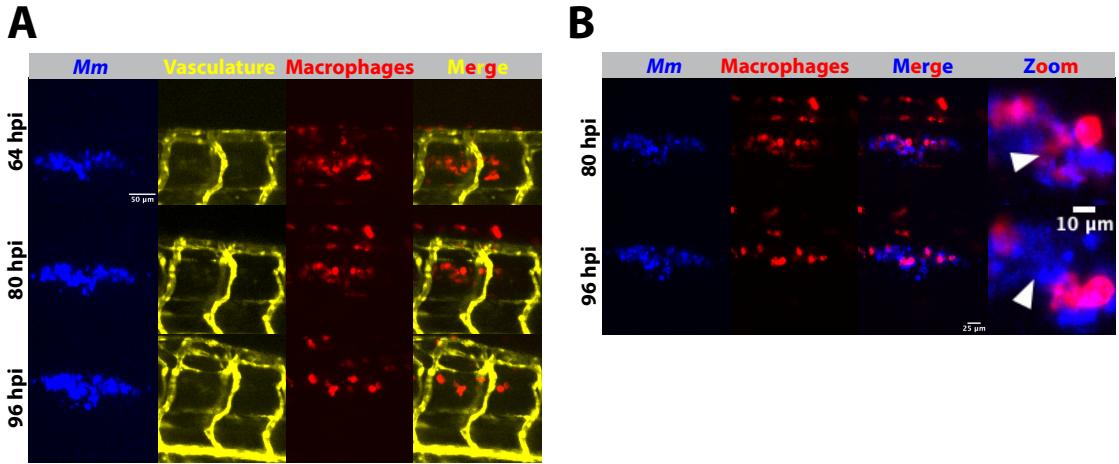
Injection of live *Mycobacterium marinum* into the dorsal trunk of the zebrafish larva is sufficient to induce a robust angiogenic response adjacent to nascent granulomas in a macrophage-dependent manner (Oehlers et al., 2015) (Figure 3.3). The stereotyped vasculature along this region of the larva allows facile quantitation of neovascularization during and after granuloma formation or other insult (Lawson and Weinstein, 2002; Jin et al., 2005; Gore et al., 2012; Matsuoka and Stainier, 2018)



**Figure 3.4:** Around 60 hours post infection, macrophages interacting with the extracellular bacteria at the site of infection begin to express *vegfaa*, as reported by the *vegfaa:eGFP<sup>pd260</sup>* transgene. In (A), a time series can be seen showing *vegfaa*-expressing macrophages and in (B), macrophages can be seen engaging with what appears to be extracellular bacteria that has accumulated over the course of the infection.

(Subsection 2.2.4). We demonstrated previously that cis-cyclopropanated trehalose 6-6'-dimycolate (TDM) is required for the induction of *vegfaa* and angiogenesis at the site of infection. Furthermore, we found that genetic blockade of Vegfaa signaling was sufficient to abolish angiogenesis during infection with wild-type mycobacteria (Walton et al., 2018). Taken together, these findings suggest that the failure to induce *vegfaa* is a primary contributor to the loss of angiogenesis in *pcaA*-deficient granulomas.

To study this phenomenon further, we began by examining the kinetics of *vegfaa* induction to identify the cellular source of Vegfaa during granuloma formation. To test whether macrophages were playing this role, we developed a macrophage-specific reporter using the previously described *acod1* promoter (also known as *irg1*), Tg(*irg1:tdTomato<sup>xt40</sup>*) (from here, *irg1:tdTomato*). *irg1* has been found to be expressed specifically in zebrafish macrophages and is upregulated during infection



**Figure 3.5:** At later time points, after *vegfaa* induction, blood vessels proliferate and begin to grow toward the site of infection in association with nearby macrophages. (A) shows this process occurring over the time period studied and culminating in a new vessel nearly spanning the intersomitic vasculature. (B) displays the accumulation of extracellular bacteria and macrophage killing over this time period.

(Sanderson et al., 2015; Kwon et al., 2022). We then crossed this line with the *vegfaa* reporter line TgBAC(*vegfaa:eGFP<sup>pd260</sup>*) (*vegfaa:eGFP* throughout) (Karra et al., 2018) and infected double transgenic *irg1:tdTomato; vegfaa:eGFP* progeny with *M. marinum* expressing eBFP2 (*Mm-eBFP2*) to simultaneously visualize bacteria, macrophage localization, and *vegfaa* production *in vivo* (Takaki et al., 2013).

We began imaging at a time point that preceded robust induction of *vegfaa:eGFP* but would allow us to capture the maximum time span of these events. We observed an increase in *vegfaa* reporter signal over time that appeared largely localized to macrophages (Figure 3.4A). We observed that bacteria initially grew primarily intracellularly within individual macrophages at 36 hours post infection but began to grow in characteristic extracellular cords by approximately 84 hours post infection with little to no intracellular containment at this site by 96 hours post infection (Figure 3.4B). The increase in extracellular growth coincided with the induction of eGFP signal in macrophages at 64 hours (Figure 3.4A), suggesting that, at low overall

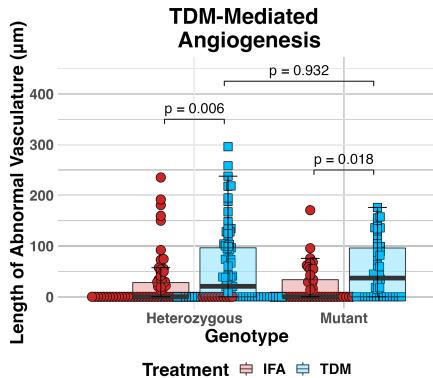
burden, intracellular detection is unable to induce *vegfaa* expression while extracellular engagement correlates with *vegfaa* expression during early stages of granuloma formation (Figure 3.4).

We next visualized the production of angiogenic vessels throughout infection in parallel to our characterization of *vegfaa* induction. Due to an inability to separate discrete emission wavelengths using two GFP reporter lines, we were unable to examine all four components (bacteria, *vegfaa* induction, macrophages, and vasculature) simultaneously. To relate this process directly to the angiogenesis observed in mycobacterial granulomas, we crossed the *irg1:tdTomato* macrophage reporter to the Tg(*kdrl:eGFP<sup>s843</sup>*) (from here, *kdrl:eGFP*) line, which labels vascular endothelium (*irg1:tdTomato; kdrl:eGFP*) (Jin et al., 2005). Under the same conditions and burden at which we infected the *vegfaa* and macrophage dual reporter line, we observed robust vascularization at approximately 96 hours post-infection, subsequent to initial granuloma formation and *vegfaa* induction (Figure 3.5A, B).

### 3.3.2 Genetic *card9* Deficiency Does Not Compromise

#### Angiogenesis During Mycobacterial Infection

Given these observations suggesting that macrophages engaging extracellular bacteria are an important source of *vegfaa* expression, we interrogated pattern recognition receptor (PRR) signaling pathways that had been implicated in host responses to TDM, a major external component of the mycobacterial cell envelope. We had previously found that *myd88* was dispensable for the induction of angiogenesis in response to TDM *in vivo* (Figure 3.6) (Bowdish et al., 2009; Walton et al., 2018). This suggested that the described TLR2-mediated responses that function downstream of TDM de-

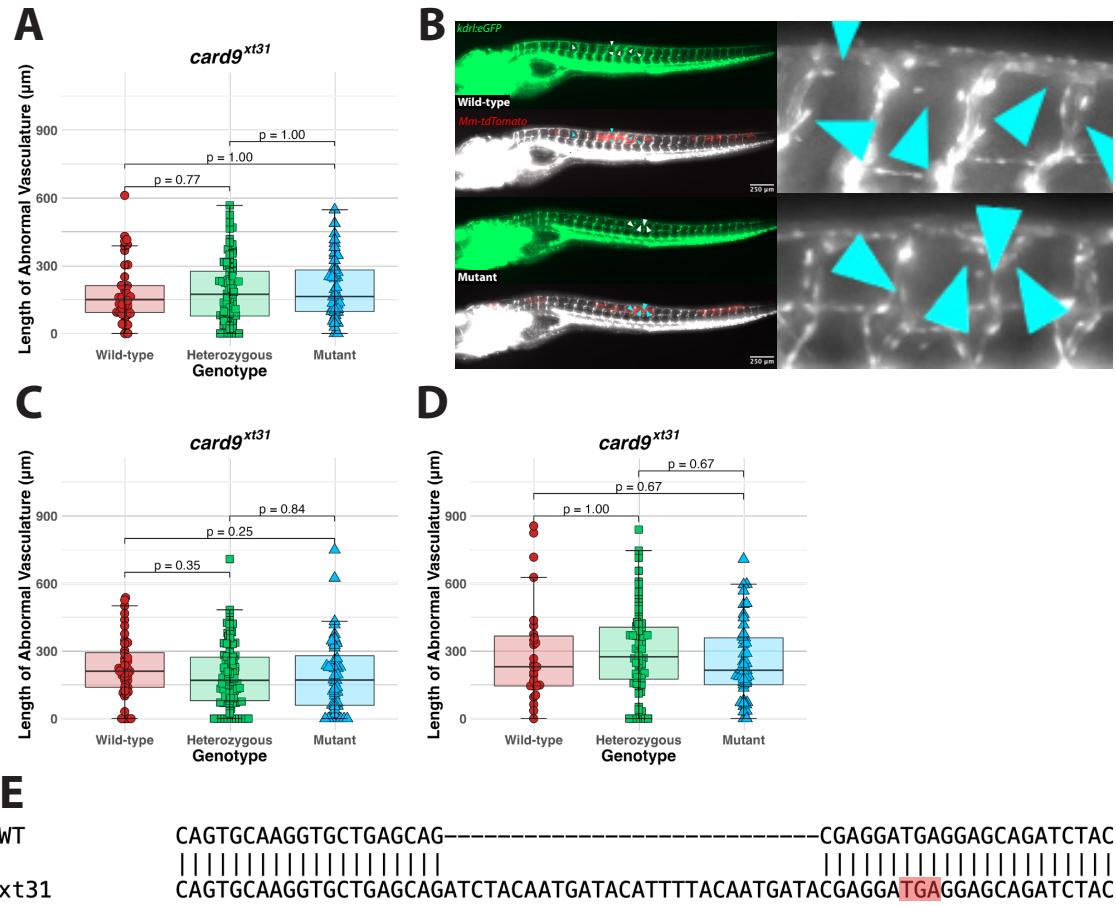


**Figure 3.6:** *myd88* is dispensable for angiogenesis during infection, suggesting a TLR- and IL-1R-independent signaling pathway is necessary for inducing angiogenesis downstream of TDM detection. Larval zebrafish were injected with IFA or TDM, imaged and quantitated, and then matched to genotype. Adapted from personal contributions to Walton et al. (2018).

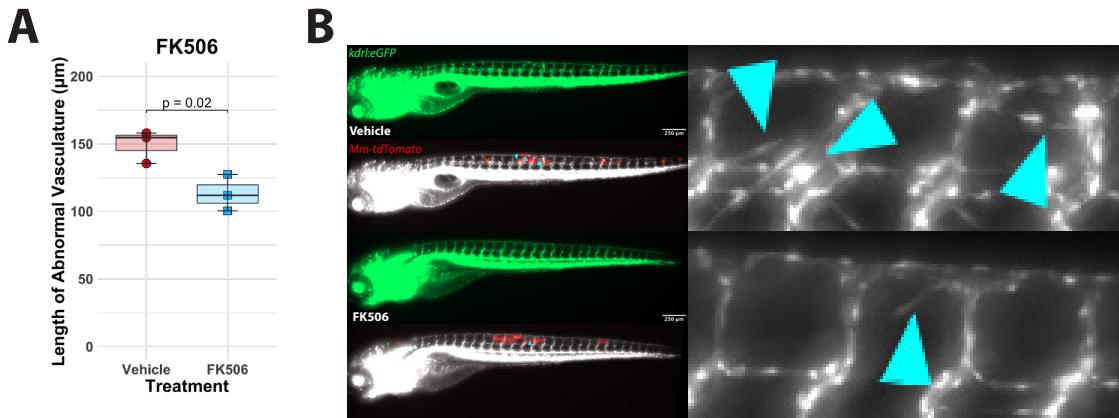
tection in some contexts were unlikely to be required for this process<sup>2</sup>. Rather, we found that the Fc $\gamma$ R homologs in zebrafish, *fcer1g* and *fcer1gl*, are required for the full angiogenic response to TDM (Walton et al., 2018), implicating C-type lectin receptors signaling in mediating this response (Richardson and Williams, 2014; Zhao et al., 2014).

As many of the downstream activities of C-type lectin receptors have been ascribed to the activation of CARD9-NF- $\kappa$ B signaling (Goodridge et al., 2009; Lobato-Pascual et al., 2013; Zhao et al., 2014; Williams, 2017; Deerhake et al., 2021), we assessed what role this pathway might play in angiogenesis during mycobacterial infection. We developed a *card9* knockout zebrafish line using CRISPR/Cas9 that carries a 28 bp insertion, resulting in an early stop after 59 amino acids (*card9<sup>xt31</sup>*) (Figure 3.7E). We then assayed these animals in the *kdr1:eGFP* transgenic background by incrossing *kdr1:eGFP*; *card9<sup>xt31/+</sup>* animals and infecting the resulting offspring with tdTomato-

<sup>2</sup>This could not, however, rule out any potential role for CD14-mediated signaling independent of TLR2. One issue is that the zebrafish do not have an identified CD14 homolog, but additional data clarifies things somewhat.



**Figure 3.7:** *card9*, the canonical downstream adaptor from C-type lectin signaling, is not required for angiogenesis during mycobacterial infection. (A), (C), and (D) show three replicates of this experiment from genotype-blind experiments. (B) shows a representative example of the effect seen, where no differences can be ascertained between genotypes. (E) displays the 28 base pair insertion generated, with the new early stop codon highlighted in red.

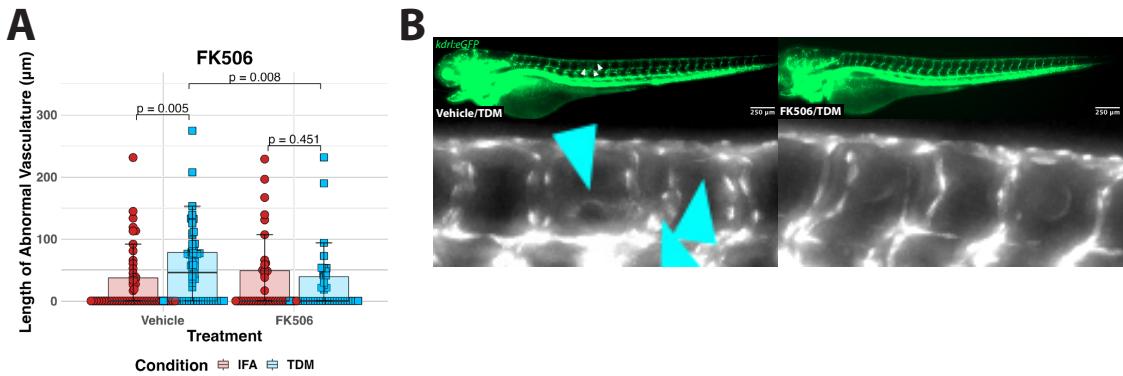


**Figure 3.8:** Treatment of infected larval zebrafish with the calcineurin-NFAT inhibitor FK506 at 125nM results in a reduction in infection-induced angiogenesis, suggesting a role for this pathway in inducing angiogenesis during infection.

fluorescent *M. marinum* (*Mm*-tdTomato) at 2 days post fertilization (Jin et al., 2005; Oehlers et al., 2015)(Figure 3.7A, B). We quantitated the resulting aberrant vasculature at 4 days post-infection under genotypic blinding and post hoc matched these measurements to genotype. To my surprise, there were no significant differences between the three genotypes across three independent replicates (Figure 3.7A, C, D), suggesting either redundancy between multiple established pathways or the existence of an alternative pathway downstream of TDM detection that was *fcer1g/fcer1gl*-dependent, but independent of both *myd88* and *card9*.

### 3.3.3 Pharmacological Inhibition of NFAT Activation Limits Angiogenesis in Response to Mycobacteria and TDM

Although many of the physiological consequences of C-type lectin receptor induction are often ascribed to CARD9-NF- $\kappa$ B signaling, this PRR class is also known to activate a distinct transcription factor family with known roles in immunity – the



**Figure 3.9:** Treatment of infected larval zebrafish with the calcineurin-NFAT inhibitor FK506 at 125nM results in a reduction in TDM-induced angiogenesis, suggesting a role for NFAT signaling in inducing TDM-dependent pro-angiogenic signaling.

nuclear factor of activated T cells, or NFAT (Goodridge, Simmons, and Underhill, 2007; Deerhake et al., 2021). This calcium-responsive transcription factor pathway is best described in its role regulating T cell biology, but there are numerous reports describing various roles for the members of this pathway in other cell types, including macrophages (see Section 1.4) (Symes, Gearan, and Fink, 1998; Jones, Conover, and Symes, 2000; Crabtree and Olson, 2002; Horsley and Pavlath, 2002; Elloumi et al., 2012). Given that there are four mammalian members of this pathway and six zebrafish homologs with potentially overlapping functions, we began with a pharmacological approach to globally inhibit NFAT signaling through all six zebrafish isoforms. Although this comes with caveats with specificity – as described in Subsubsection 1.4.2, NFATC1 has important roles in angiogenesis within the endothelium – it offers an opportunity to assess the general roles of this pathway as a first-pass approach.

We first infected 2 days post fertilization *kdrl:eGFP* larval zebrafish with *Mm*-tdTomato in the trunk and treated them with 125 nM FK506, a clinically utilized calcineurin inhibitor that blocks NFAT activation, for the duration of the experiment

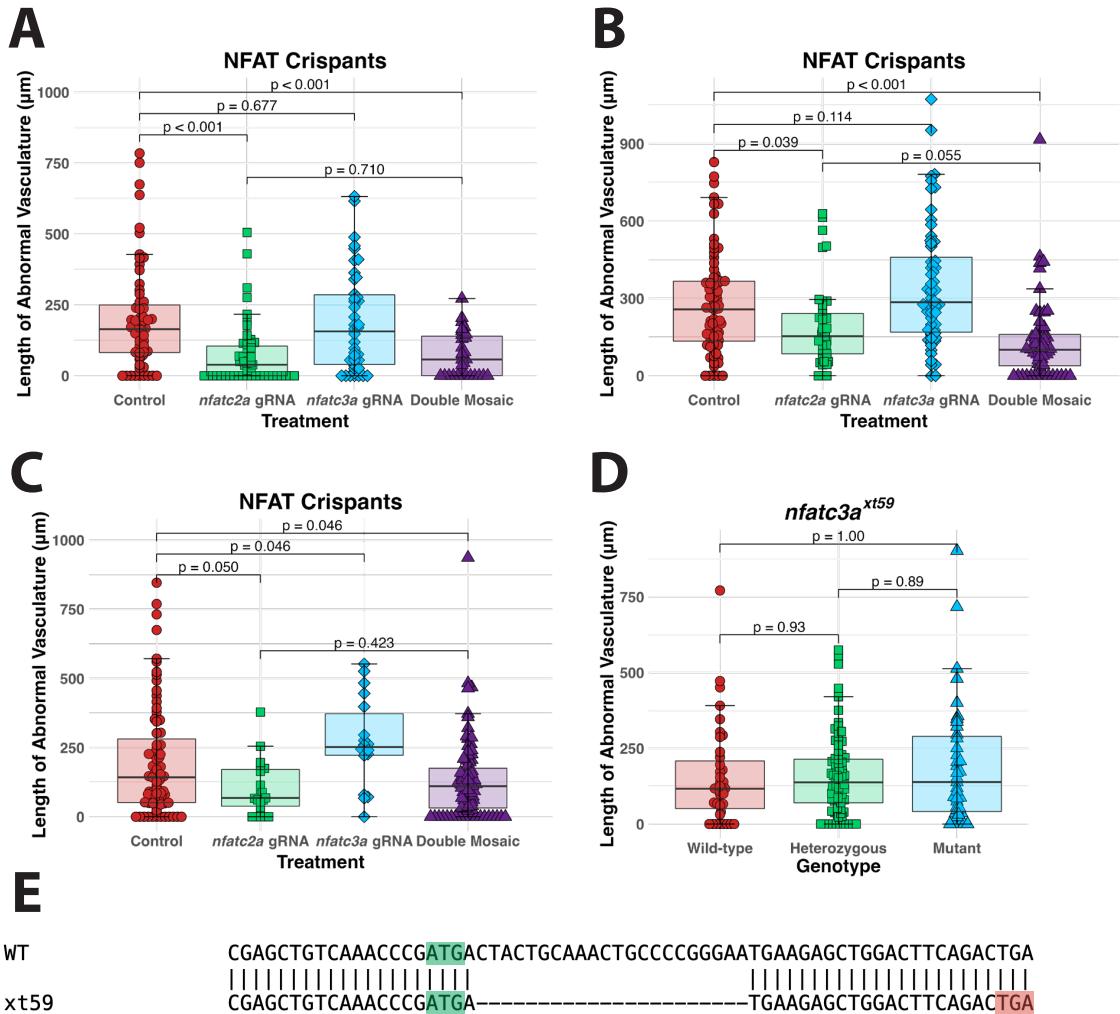
(Ellis, 1995) (Subsection 1.4.1). This minimal dose of FK506 was chosen due to developmental toxicities observed at higher doses in my hands and is in line with the dosage used by others (Kujawski et al., 2014). We imaged the fish at 4 days post infection and quantitated the degree of vasculature induced in the presence and absence of inhibitor under computational blinding. Even with at this low dose of FK506, we noted a small, but statistically significant reduction in the mean degree of neovascularization at this time point, consistent with a role for NFAT in controlling angiogenesis in response to *M. marinum* infection (Figure 3.8A, B) (Kujawski et al., 2014). To ask whether this effect was specific to recognition of TDM, we injected purified TDM or vehicle (incomplete Freund's adjuvant; IFA) alone into the trunks of 2 days post fertilization larvae. Treatment with FK506 resulted in a statistically significant reduction in the degree of angiogenesis induced at 2 days post injection (Figure 3.9A, B), suggesting that this pathway was specifically relevant to TDM-dependent angiogenesis.

### 3.3.4 The Isoform *nfatc2a* is Specifically Required for Angiogenesis During Infection

Combining our observations on the correspondence of granuloma formation and the induction of *vegfaa* with our data implicating the NFAT pathway, we sought to identify NFAT isoforms that were enriched in granuloma macrophage populations. Aside from investigations made into *nfatc1*, which is restricted to the endocardium, lymphatic vessels, and the notochord during much of zebrafish development (see Subsubsection 1.4.2) (Pestel et al., 2016; Shin et al., 2019; Bagwell et al., 2020), little is known of the expression patterns of these genes in zebrafish<sup>3</sup>, especially in

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<sup>3</sup>As mentioned previously in Subsubsection 1.4.2, the NFATC4 isoform is thought to not be expressed in the hematopoietic compartment, an observation backed up by some more recent data



**Figure 3.10:** Assays in mosaic, CRISPR/Cas9 RNP-injected larval zebrafish suggested that *nfatc3a* was dispensable for the induction of angiogenesis during infection. Panels (A), (B), (C) show the results of three replicates of these mosaic injections. (D) shows the results of the same assay in a stable knockout background of *nfatc3a*, confirming the preliminary results from the mosaic assay. (E) shows the nature of the mutation in *nfatc3a*<sup>xt59</sup>.

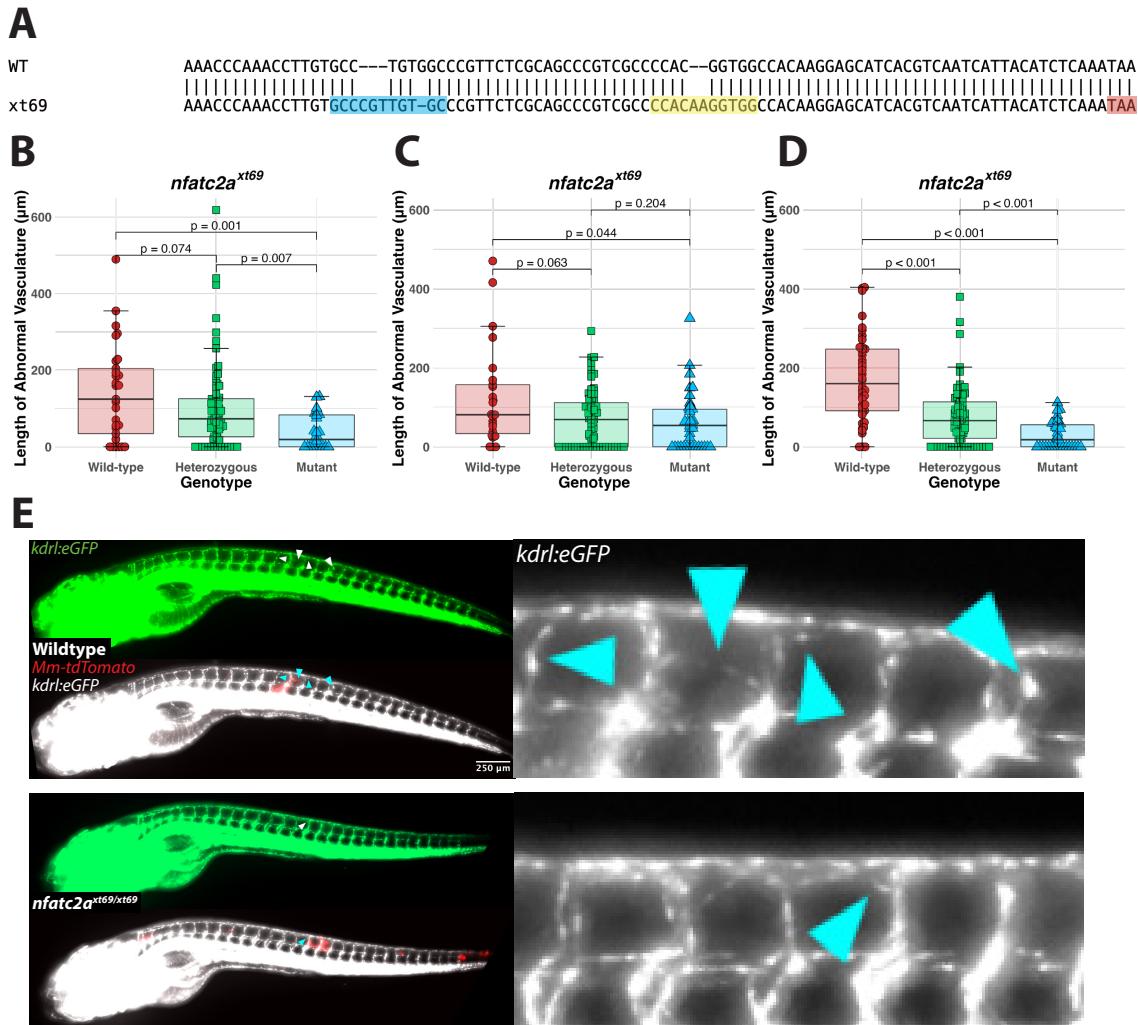
the context of infection. We first made use of published scRNA-seq datasets from mycobacterial granulomas in zebrafish and non-human primates for *nfat* transcripts that were robustly expressed in granuloma macrophages at the population level and identified both zebrafish *nfatc2a* and *nfatc3a* as plausible candidates (Cronan et al., 2021; Gideon et al., 2022).

To examine potential roles for *nfatc2a* and *nfatc3a* in granuloma-associated angiogenesis in vivo, we first screened F<sub>0</sub> CRISPR-injected mosaic knockouts (“crispants”) to rapidly evaluate these candidate genes (Subsection 2.5.1). Using this approach, similar to that used previously by other groups, we assessed the relative roles of these two isoforms individually and in tandem, measuring the angiogenic response to mycobacterial infection in the *kdr1:eGFP* background (Jao, Wente, and Chen, 2013; Hoshijima, Juryneac, and Grunwald, 2016; Wu et al., 2018; Hoshijima et al., 2019; Kroll et al., 2021). We found that *nfatc2a* inhibition resulted in a ~50-80% reduction in angiogenesis (Figure 3.10A-C). In contrast, *nfatc3a* had no effect on the length of ectopic blood vessels present (Figure 3.10A-C). The dual targeted double mosaics were statistically indistinguishable from the *nfatc2a* injected fish alone (Figure 3.10A-C). This allowed us to prospectively identify *nfatc2a* as an NFAT isoform required for full angiogenic response to mycobacteria while *nfatc3a*, despite expression in overlapping cell populations, appeared to be entirely dispensable for this process at this point in time (Figure 3.10A-C).

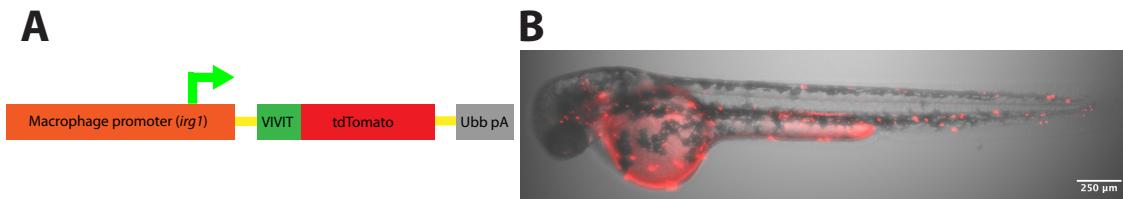
We then established stable, germline transmitting indel mutant alleles for both genes to validate our results from mosaic animals. Recapitulating our results in the F<sub>0</sub> generation, the *nfatc3a<sup>xt59</sup>* mutation carrying a 22 bp deletion (leading to an early stop codon at amino acid 9 in exon 1) had no effect on angiogenesis at 4

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as well (Peuker et al., 2022).



**Figure 3.11:** *nfatc2a* is required for the angiogenic response to infection. Use of the stable mutant line *nfatc2a<sup>xt69</sup>* demonstrated an important role for this pathway in inducing angiogenesis. (A) diagrams the mutation I established. (B), (C) and (D) show three replicates of the infection experiment, demonstrating robust and reproducible reduction in angiogenesis in both the heterozygous and mutant conditions relative to wild-type. (E) shows a representative example of the effect seen, with robust angiogenesis under wild-type conditions and a significant reduction with *nfatc2a<sup>xt69/xt69</sup>*.



**Figure 3.12:** Design of the *irg1*:*VIVIT-tdTomato* construct. (A) shows the overall layout of the construct, with *irg1* driving the expression of the VIVIT peptide conjugated to tdTomato. (B) shows a representative *irg1*:*VIVIT-tdTomato*<sup>xt38</sup> larva.

days post infection (Figure 3.10D, E). We then developed a knockout line of *nfatc2a* bearing a net 4 bp insertion leading to an early stop codon in the second exon (at amino acid 273, frameshifted after amino acid 247), prior to the DNA-binding domain (*nfatc2a*<sup>xt69</sup>) (Figure 3.11A). We repeated our angiogenesis assay using larvae from incrosses of *kdr1:eGFP*; *nfatc2a*<sup>xt69/+</sup> animals that produced expected Mendelian ratios of wild-type, heterozygous, and homozygous mutant offspring. Consistent with the results from mosaic animals, homozygous knockout of *nfatc2a* was sufficient to reduce the degree of angiogenesis present in larval zebrafish at 4 days post infection (Figure 3.11B-E). Importantly, given the known role of NFAT isoforms in T cell function, these defects emerged prior to the developmental emergence of functional T cells, which does not occur until approximately 6 days post fertilization as the thymus develops (Trede et al., 2004). However, whole animal knockouts could not address potential roles for cell types other than macrophages in mediating this process.

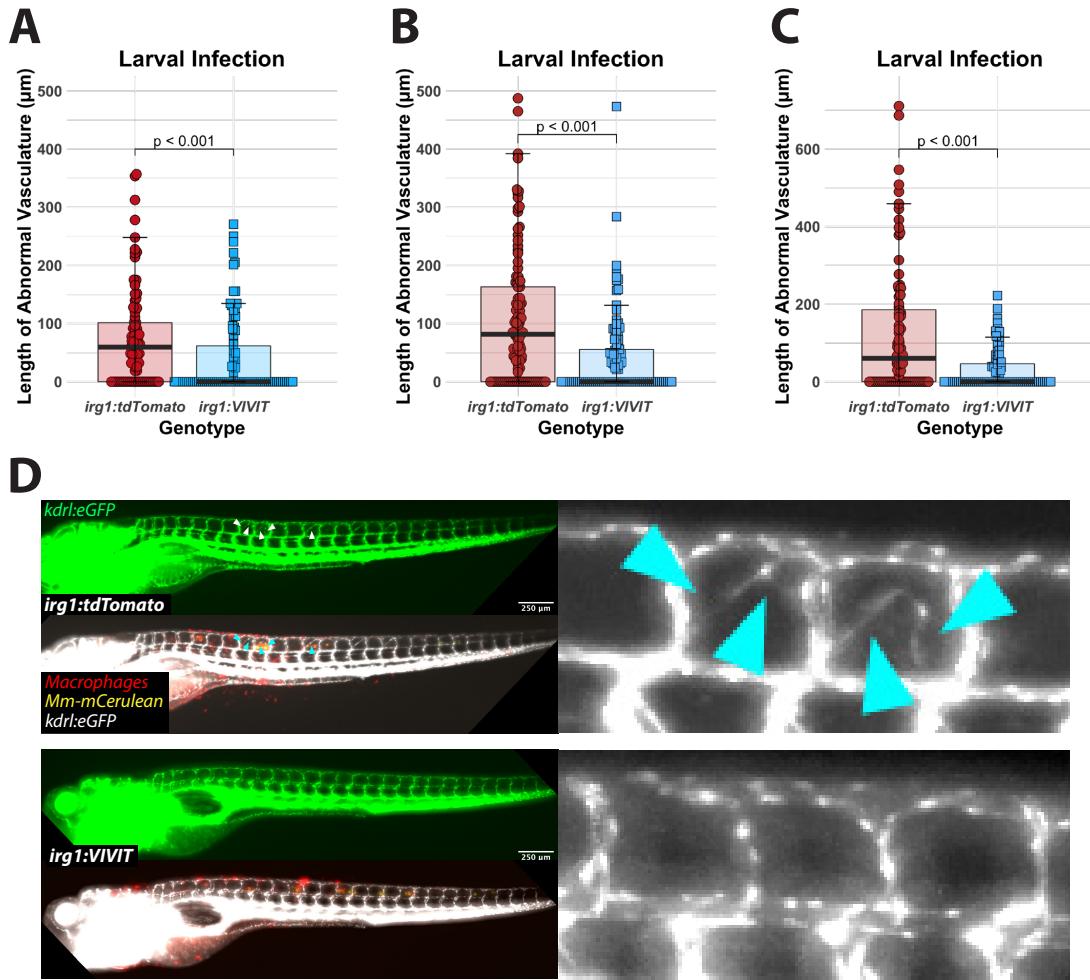
### 3.3.5 Macrophage-NFAT is Essential for Angiogenesis

#### Induction *in vivo*

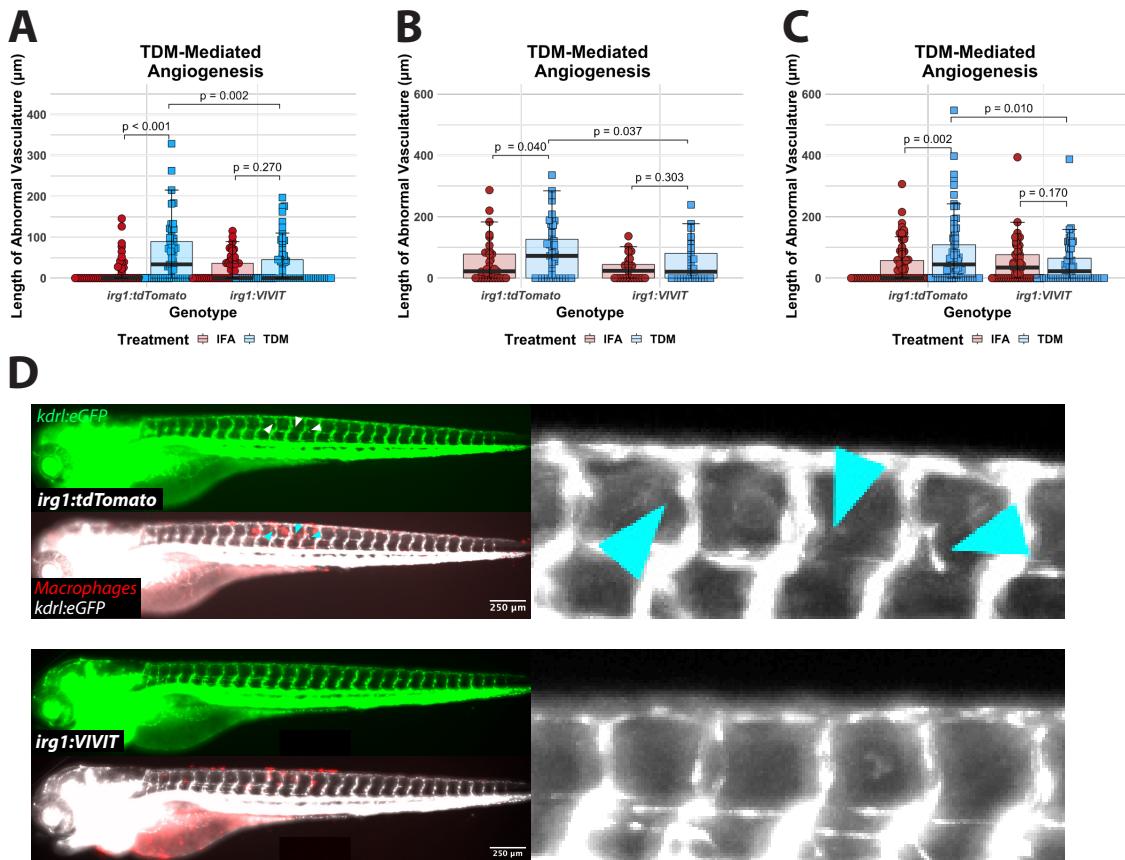
Given our observations on *vegfaa* induction in macrophages at the granuloma, we tested whether NFAT signaling was required specifically in macrophages for granuloma-associated angiogenesis. For *in vivo* inhibition of macrophage NFAT signal-

ing during infection, we applied an approach that takes advantage of the NFAT-inhibitory peptide VIVIT which competitively inhibits calcineurin-dependent activation of all the NFATc isoforms (Aramburu et al., 1999). This approach has been successfully used as an exogenous treatment in cell culture (Deerhake et al., 2021) and mice (Noguchi et al., 2004; Elloumi et al., 2012; Rojanathammanee et al., 2015), through ectopic overexpression in cell culture (McCullagh et al., 2004), and, more recently, in mice (Poli et al., 2022; Peuker et al., 2022). We developed a transgenic zebrafish line in which VIVIT is expressed specifically in macrophages, Tg(*irg1*:VIVIT-*tdTomato*<sup>xt38</sup>) (from here, simply *irg1*:VIVIT) (Figure 3.12A, B) (Sanderson et al., 2015). We assessed whether the macrophage-specific expression of VIVIT would be sufficient to reduce the degree of angiogenesis during infection in the trunk with wild-type *M. marinum* expressing mCerulean (*Mm-mCerulean*). We found that macrophage-specific VIVIT expression significantly reduced angiogenesis in response to infection (Figure 3.13A-D). This suggested a macrophage-specific role for NFAT signaling downstream of mycobacterial detection that was necessary to induce angiogenesis, presumably through the *nfatc2a* isoform.

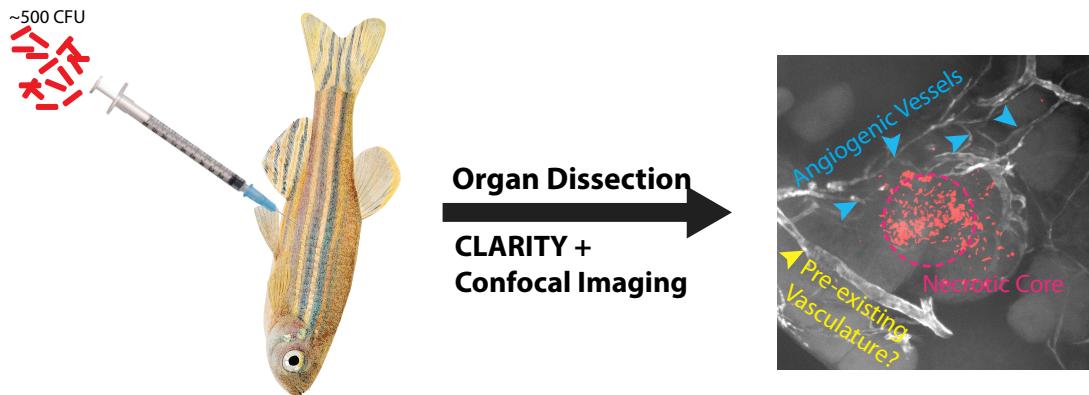
To ask more directly whether the decreased angiogenesis observed in the NFAT-deficient macrophages was via the TDM-mediated pathway, we used the TDM injection assay we had developed previously. We injected TDM or the IFA vehicle into the trunk of 2 days post fertilization larval zebrafish and measured the resulting angiogenesis at 2 days post injection (Walton et al., 2018). TDM was sufficient to induce angiogenesis *in vivo* and this effect was dependent upon functional NFAT signaling, with the degree of TDM-induced angiogenesis reduced to the level of the vehicle alone in *irg1*:VIVIT-*tdTomato* animals compared to *irg1*:*tdTomato* controls (Figure 3.14A-D).



**Figure 3.13:** Macrophage-specific VIVIT expression in larval zebrafish inhibits infection-induced angiogenesis. (A), (B), and (C) show three replicates of this infection experiment and a robust reduction in the amount of angiogenesis with expression of the VIVIT peptide compared to *irg1:tdTomato*-only controls. (D) shows representative images of the effect observed, with reduced angiogenesis seen in the VIVIT condition.



**Figure 3.14:** Macrophage-specific VIVIT expression reduces TDM-induced angiogenesis in the larval zebrafish. (A), (B), and (C) display three independent replicates of this experiment; (D) demonstrates the effect by representative images showing the reduction seen in the VIVIT-expressing condition.



**Figure 3.15:** Schematic of the infection and CLARITY-clearing approach used for adult infection experiments. Adult zebrafish (>12 weeks post fertilization) are infected interperitoneally with 500 CFU of fluorescent *M. marinum* and then organs are harvested and fixed approximately 14 days later, cleared with detergent, and then imaged.

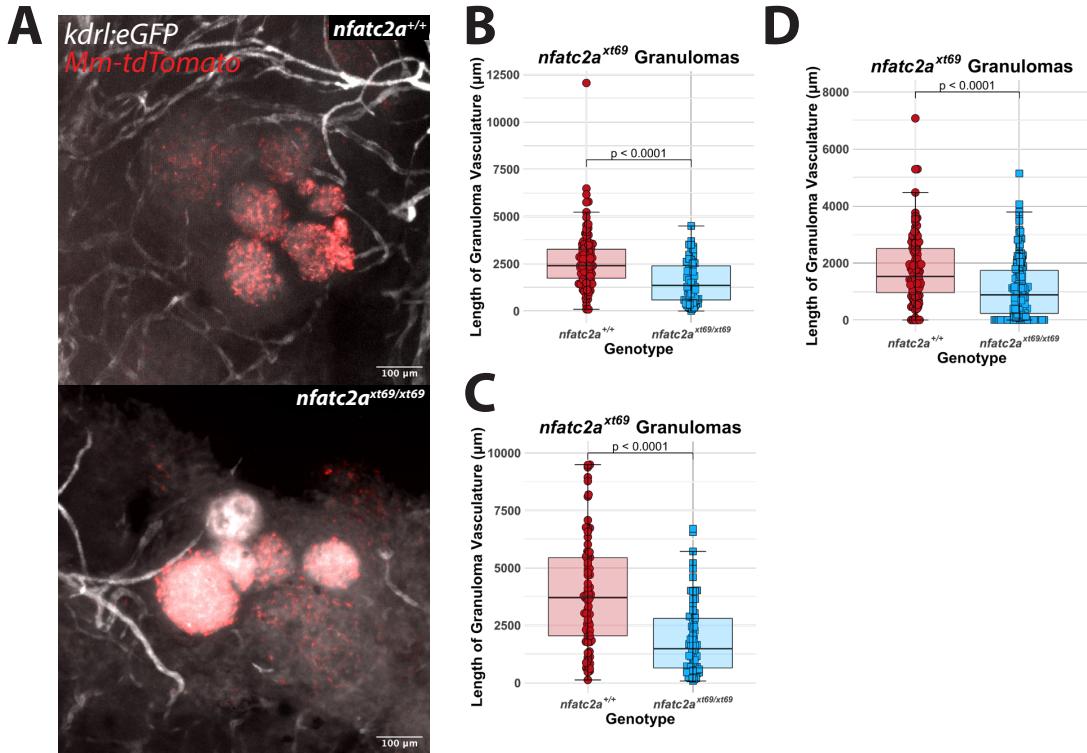
### 3.3.6 NFAT Activation is Essential for Angiogenesis in Adult Granulomas

Adult zebrafish are equipped with both innate and adaptive immunity and form mycobacterial granulomas that histologically mirror epithelioid human tuberculosis granulomas (Swaim et al., 2006), including induction of a surrounding vascular network (Cronan et al., 2015). To assess whether our findings in the larvae translated to a longer-term context in the presence of adaptive immunity, we infected adult *kdr1:eGFP; nfatc2a<sup>xt69/xt69</sup>* zebrafish and *kdr1:eGFP; nfatc2a<sup>+/+</sup>* siblings with *Mm*-tdTomato and examined their peritoneal organs at 18 days post infection after CLARITY-based clearing (Chung and Deisseroth, 2013; Cronan et al., 2015). Cleared organs were then imaged by spinning disk confocal microscopy (Figure 3.15). We measured the total vascular network surrounding the granulomas in a programmatically blinded fashion (Salter (2016) and Section 4.7) and found that *nfatc2a<sup>xt69/xt69</sup>* fish

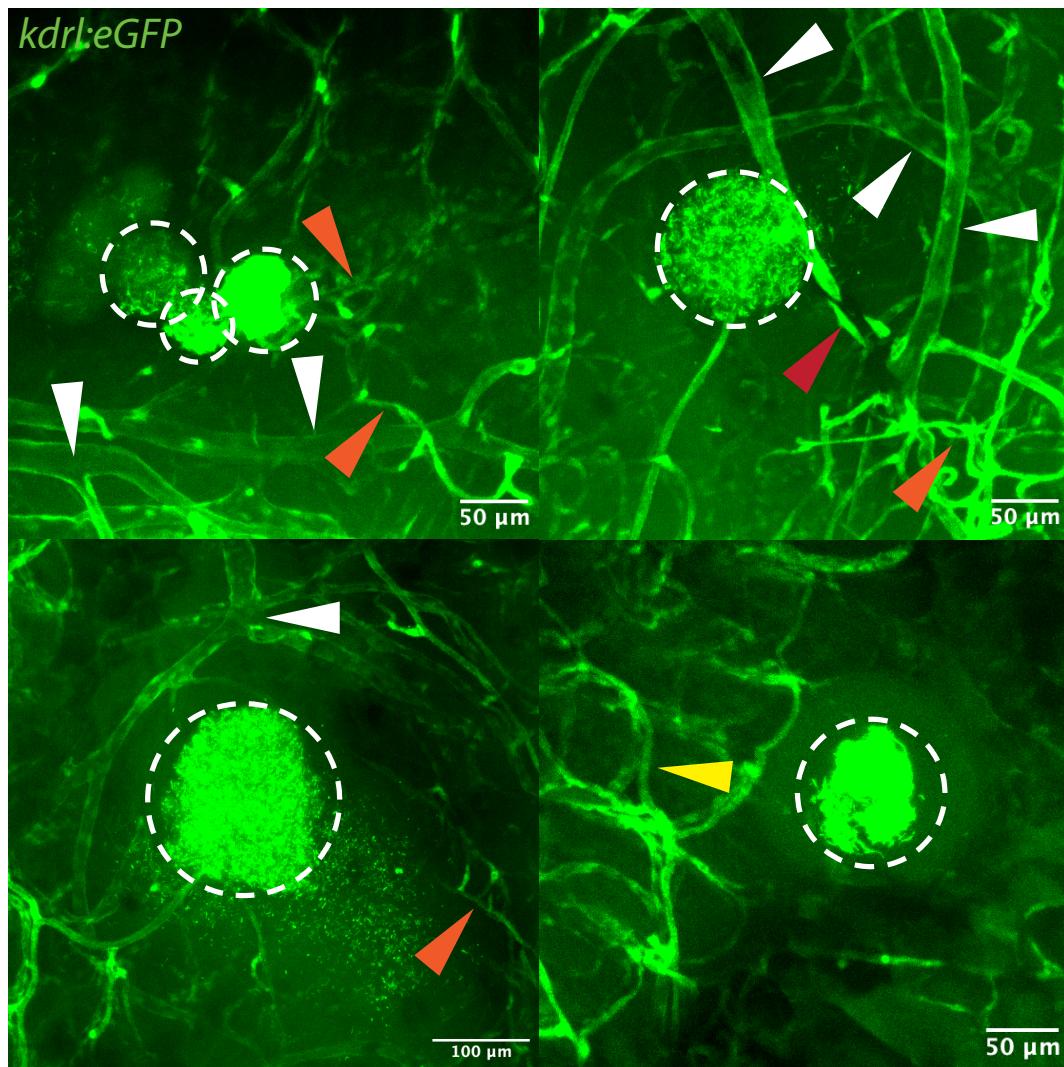
had a significant reduction (~50%) in the length of the vascular network compared to wild-type siblings, further validating this gene as important for the angiogenic response *in vivo* (Figure 3.16A-D). These putatively neovascular vessels tend to be highly branched and to be comprised of a limited number of cells with small or non-existent luminal volume, indicating that they are still in the sprouting stage of angiogenesis and suggesting a potential failure to mature over time, perhaps indicative of leakiness or other vascular defects, although further characterization would be required. We observed robust effects that are likely understated in our quantitation, as we could not make any formal distinction between thicker, existing vasculature present at baseline that happens to fall nearby the granuloma and the characteristic neovascularization more intimately associated with the granuloma and present in wild-type but reduced in *nfatc2a* mutants (Figure 3.17).

### **3.3.7 Macrophage-specific NFAT Inhibition in Mature Granulomas Reduces Angiogenesis**

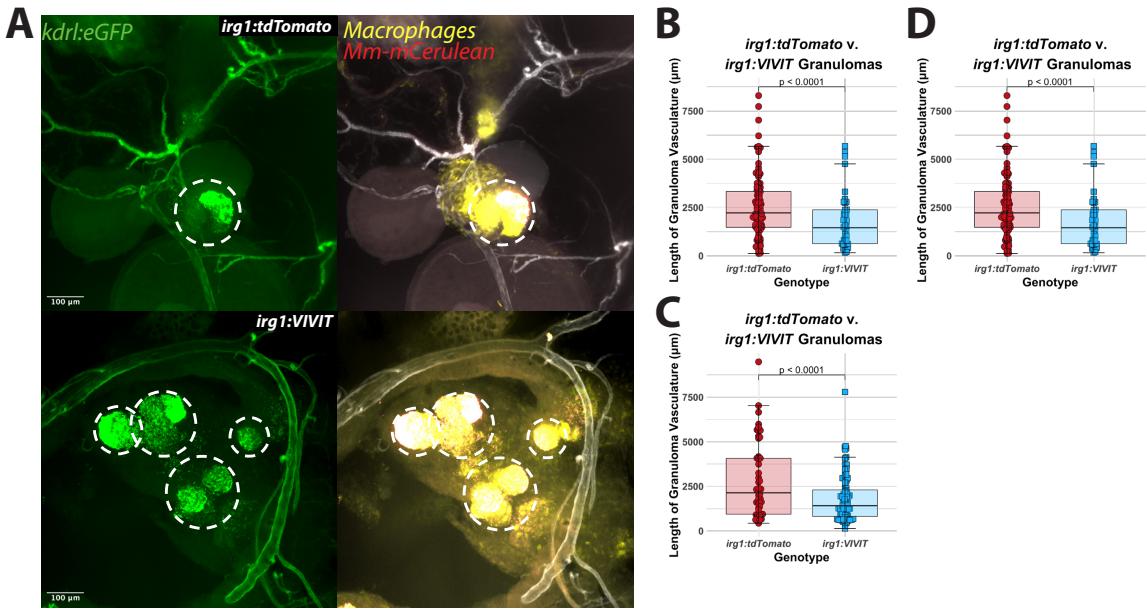
We next evaluated whether macrophage-specific NFAT inhibition had similar effects on vascularization in adult zebrafish. We infected adult *irg1:tdTomato; kdrl:eGFP* and *irg1:VIVIT-tdTomato; kdrl:eGFP* double transgenic zebrafish with *Mm-mCerulean* and examined visceral organs at 14 days post infection. We used confocal imaging to visualize individual CLARITY-cleared organs and measured the total length of granuloma-proximal vasculature under blinding as above (Salter, 2016). We found that the degree of vascularization was significantly reduced around granulomas from *irg1:VIVIT-tdTomato* fish as compared to *irg1:tdTomato* fish (Figure 3.18A-D). The extent of the vascular network in the *irg1:VIVIT-tdTomato* condition was notably restricted in most cases or solely comprised of more mature, luminal vessels, suggesting



**Figure 3.16:** *nfatc2a* is required for robust angiogenesis in established granulomas. (A) shows images comparing wild-type and *nfatc2a<sup>xt69/xt69</sup>* granulomas and a substantial reduction in the overall angiogenic effect can be seen. This is quantitated in (B), (C), and (D), with a 50% reduction in the total vasculature visible in the proximity of each granuloma.



**Figure 3.17:** Mycobacterial granulomas display significant heterogeneity in vascularization and many are in physical proximity to mature, likely pre-existing vessels. One of the drawbacks of the adult angiogenesis measurements is significant background seen in the proximity of these granulomas, as seen in this figure.



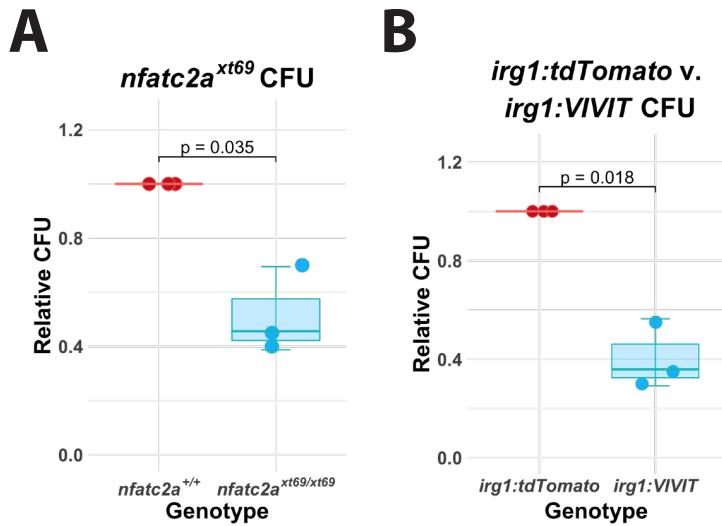
**Figure 3.18:** Macrophage-VIVIT expression inhibits granuloma angiogenesis in the adult zebrafish infection model. (A) shows images comparing *irg1:tdTomato* and *irg1:VIVIT-tdTomato* granulomas and a substantial reduction in the overall angiogenic effect can be seen. This is quantitated in (B), (C), and (D), with a 50% reduction in the total vasculature visible in the proximity of each granuloma.

a total failure to induce an angiogenic response (Figure 3.18A). These findings, consistent with our previous data from both larval zebrafish infections in the *irg1:VIVIT-tdTomato* background and in the *nfatc2a* mutant adult fish, point to a critical role for macrophage-specific NFAT activation in inducing the angiogenic response at mycobacterial granulomas. Furthermore, this establishes that NFAT function is broadly conserved from early larval infection through to the mature necrotic granulomas that characterize adult infection.

### 3.3.8 Inhibition of NFAT Signaling Results in Decreased Bacterial Burden

We had previously shown that inhibition of granuloma-associated vascularization is associated with decreased bacterial burden. Mycobacterial mutants unable to induce vascularization ( $\Delta pcaA$ ), and either genetic or pharmacological inhibition of VEGF or CXCR4 signaling all result in lower bacterial burden, presumably due to functions of the aberrant vasculature promoting bacterial growth and/or inhibiting bacterial killing (Rao et al., 2005; Glickman, Cox, and Jacobs, 2000; Oehlers et al., 2015; Walton et al., 2018) or through more direct pro-bacterial activities of VEGFA (Harding et al., 2019). To examine the effect on burden of inhibition of NFAT signaling, we performed colony forming unit (CFU) assays at timepoints after the induction of angiogenesis and granuloma maturation. We infected *nfatc2a<sup>+/+</sup>* and *nfatc2a<sup>xt69/xt69</sup>* adult zebrafish with *Mm-tdTomato* and plated them for CFU at 24 days post infection. We found that knockout of *nfatc2a* resulted in a ~50% decrease in median colony number compared to wild-type after extended infection (Figure 3.19A).

Finally, we evaluated the impact of macrophage-specific NFAT inhibition on whole organism bacterial burden. We infected adult zebrafish possessing either the *irg1:tdTomato* or *irg1:tdTomato* transgenes with *Mm-tdTomato* and then homogenized and plated these fish at 18 days post infection. We found that macrophage expression of the VIVIT peptide resulted in a median reduction of ~60% of the bacterial burden in these fish at this time point relatively soon after the formation of necrotic granulomas and robust induction of angiogenesis (Figure 3.19B).

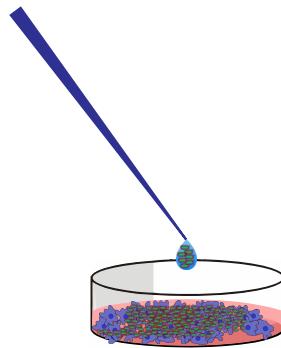


**Figure 3.19:** Inhibition of NFAT signaling results in a reduction in mycobacterial CFU at time points after granuloma formation. (A) shows a reduction in bacterial burden in *nfatc2a* mutant fish while (B) shows a similar reduction in burden in VIVIT-expressing fish.

### 3.3.9 Pharmacological Inhibition of NFAT in Human

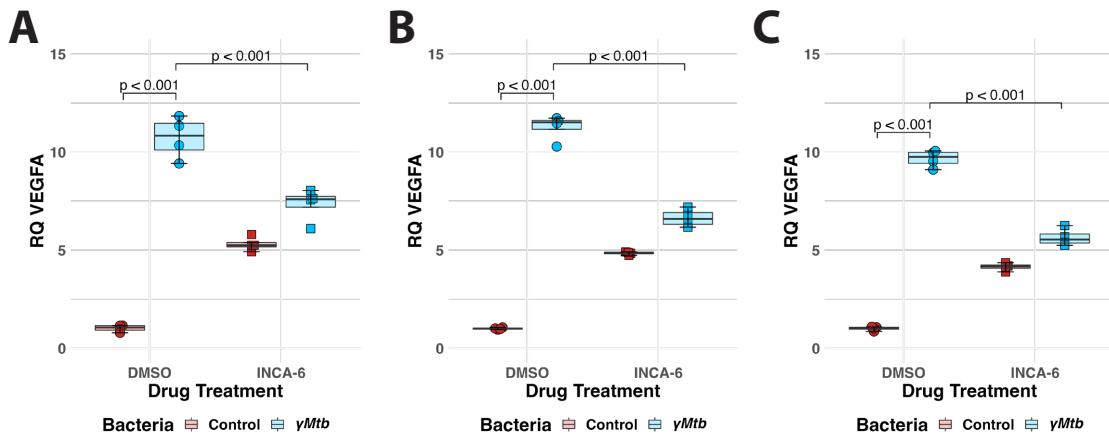
#### THP-1 Macrophages Limits VEGFA Induction by *Mycobacterium tuberculosis*

The zebrafish mycobacterial infection model shares important conserved features with *M. tuberculosis* infection of humans, host response and granuloma angiogenesis (Swaim et al., 2006; Datta et al., 2015; Oehlers et al., 2015; Cronan et al., 2021). In addition, important aspects of the response to cyclopropanated TDM appears to be largely maintained between zebrafish and humans (Walton et al., 2018; Rao et al., 2005). We next asked whether our findings discovered *in vivo* with the zebrafish-*M. marinum* model were conserved in human cells exposed to *M. tuberculosis*. We developed a cell culture model of macrophage-*M. tuberculosis* interactions using differentiated THP-1 monocytic cells exposed to  $\gamma$ -irradiated *Mycobacterium tuber-*

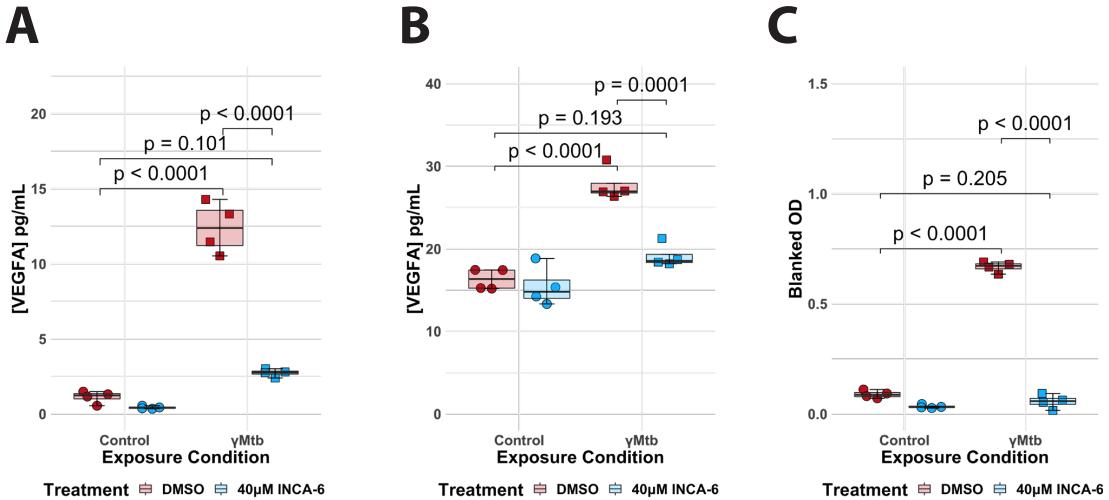


**Figure 3.20:** In our extracellular exposure model, clumps of gamma-irradiated *M. tuberculosis* is added to monolayers of THP-1 macrophages to stimulate them via primarily extracellular exposure rather than by phagocytosis.

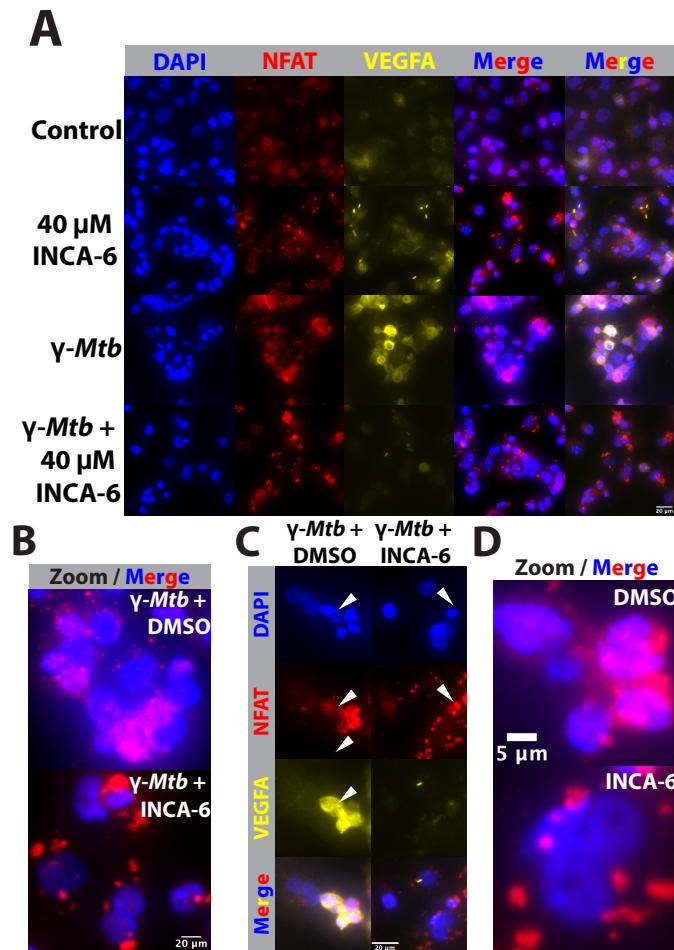
*culosis H37Rv ( $\gamma Mtb$ ), which produces the full spectrum of TDM species, presented to the cell in their native configuration (as compared to heat-killed *M. tuberculosis*, which disrupts cell envelope structure and organization) (Romero et al., 2014; Secanella-Fandos et al., 2014) (Figure 3.20) (Subsection 1.1.5). We found that exposure of differentiated THP-1 macrophages to  $\gamma Mtb$  was sufficient to induce VEGFA transcription as well as VEGFA secretion (Figure 3.21A-C, Figure 3.22A-C). To examine whether NFAT signaling is required for production and secretion of VEGFA we treated THP-1 macrophages with the small molecule inhibitor INCA-6, which specifically disrupts the interaction between the NFAT family members and their activating phosphatase, calcineurin (Roehrl, Wang, and Wagner, 2004; Roehrl et al., 2004). Strikingly, treatment of THP1 cells with INCA-6 during  $\gamma Mtb$  exposure significantly inhibited transcriptional induction of *VEGFA* (Figure 3.21A-C), as well as VEGFA secretion (Figure 3.22A-C). Immunofluorescence revealed robust translocation of NFAT (using an NFATC2 antibody) that was broadly correlated to VEGFA signal (Figure 3.23A-D, Figure 3.24A-C). Taken together these experiments suggest that human NFAT signaling is required for VEGF production in response to *M. tuberculosis* exposure.*



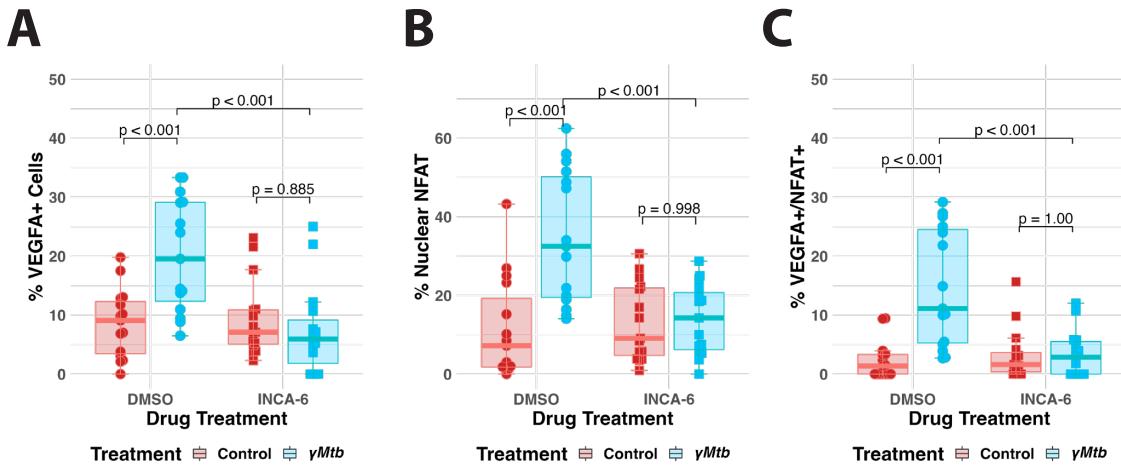
**Figure 3.21:** Treatment of THP-1 macrophages with gamma-irradiated *M. tuberculosis* results in the induction of *VEGFA* that is sensitive to inhibition by INCA-6, a calcineurin-NFAT inhibitor. Three independent replicates are shown in (A), (B), and (C).



**Figure 3.22:** Treatment of THP-1 macrophages with gamma-irradiated *M. tuberculosis* results in the secretion of VEGFA and is sensitive to inhibition by INCA-6, a calcineurin-NFAT inhibitor. Three independent replicates are shown in (A), (B), and (C). The standard curve for the replicate in C was generated improperly and was unable to be used, so raw blanked OD values are provided instead.



**Figure 3.23:** Immunofluorescence imaging of gamma-irradiated *M. tuberculosis* exposed THP-1 macrophages reveals robust NFAT protein translocation into the nucleus after exposure that corresponds to the induction of VEGFA. (A) shows robust VEGFA induction that can be inhibited by addition of INCA-6. (B) shows a magnified view of the NFAT nuclear translocation seen in (A). (C) shows further images and the correspondence between NFAT activation and VEGF production. (D) shows the reduction in nuclear localization from the images seen in (C).



**Figure 3.24:** Quantitation of various aspects of the response seen by immunofluorescence after gamma-irradiated *M. tuberculosis*-exposure and INCA-6 treatment. (A) shows the overall percentage of VEGFA+ cells seen in each condition, with a significant increase with  $\gamma Mtb$  exposure that is inhibited by INCA-6. (B) shows a parallel phenotype, where  $\gamma Mtb$  induces NFAT nuclear localization that is sensitive to INCA-6 addition. (C) shows the percentage of the total cells in the field of view that demonstrate both NFAT nuclear localization and VEGF expression.

### 3.3.10 Requirement of human NFATC2 for VEGFA induction

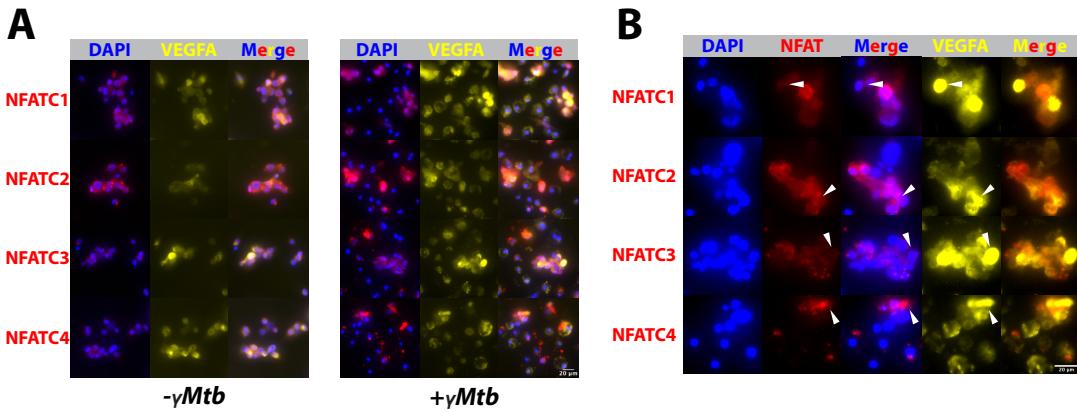
To identify functionally important NFAT human isoforms, we exposed THP-1 macrophages to  $\gamma Mtb$  and subsequently used the secretion inhibitor brefeldin A to lock VEGFA within secreting cells. Simultaneous staining for each of the four human NFATc proteins along with VEGFA allowed us to identify NFAT isoforms that underwent changes in expression and localization and correlate this with VEGFA production (Figure 3.25A). While THP-1 macrophages express all of the isoforms to varying degrees, the most intense co-staining with VEGF was found with NFATC2 (Figure 3.25B). Additionally, while each of the isoforms displayed structural and localization alterations (albeit not always nuclear) after  $\gamma Mtb$  exposure, only NFATC2 showed robust nuclear localization that appeared to correspond to VEGFA induction

in individual cells (Table 3.1). While some NFAT isoform translocation was observable with at least NFATC1 and, minorly, NFATC3, this generally had, at best, modest correspondence to the degree or presence of VEGFA production (Figure 3.26). While NFATC1 was correlated with VEGFA induction, the effect was much weaker than that seen with NFATC2. We quantified this effect by counting the number of VEGFA-expressing, NFAT nuclear localized cells and normalized to the number of VEGFA-expressing cells in total and found that NFATC2 most tightly corresponded to the induction of VEGFA in  $\gamma Mtb$ -exposed cells Figure 3.26. Given the strong correlation for NFATC2 with nuclear localization and VEGFA production after  $\gamma Mtb$  exposure, expression data from zebrafish and non-human primate granulomas, as well as the *in vivo* zebrafish results implicating macrophage *nfatc2a* in *vegfaa* production and angiogenesis, we focused on human NFATC2 as the key isoform.

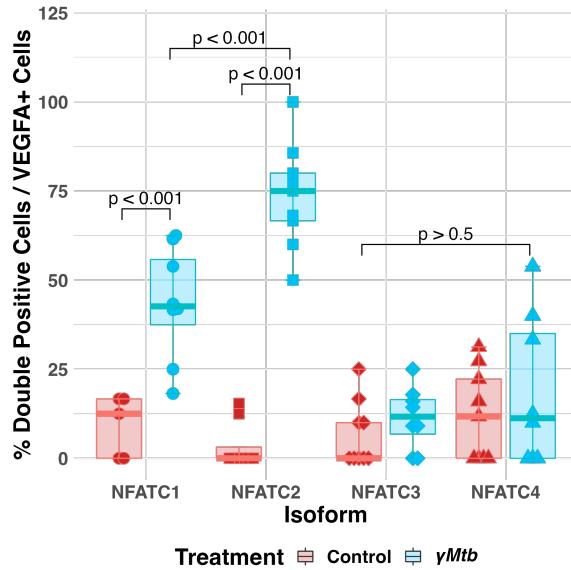
**Table 3.1:** Relative correspondence between the nuclear localization and VEGFA induction across all four NFAT isoforms. We observed nice correspondence between NFATC2 and VEGFA and a lesser association between NFATC1 and VEGFA while the other two isoforms displayed no notable relationship between their nuclear localization and VEGFA induction.

	$-\gamma Mtb$	$+\gamma Mtb$	<b>Relationship to VEGFA?</b>
NFATC1	+	++	Modest relationship between nuclear localization and VEGF expression.
NFATC2	++	+++	Nuclear localization generally corresponds to VEGF expression.
NFATC3	+	++	Weak relationship between nuclear localization and VEGF expression.
NFATC4	+/-	+	Expression increased but not obviously nuclear in most cells.

To test a functional role for human NFATC2 in macrophage induction of VEGFA



**Figure 3.25:** To identify particular NFAT isoforms that may be important for VEGFA production downstream of *M. tuberculosis* detection, immunofluorescence was performed against each of the isoforms. NFATC2 showed the most consistent induction of all of the isoforms. (A) shows alterations in the protein production and localization with and without *M. tuberculosis*. (B) displays a representative field where multiple VEGFA-expressing cells can be seen, but only nuclear localization of NFATC2 seems to correspond with this expression.

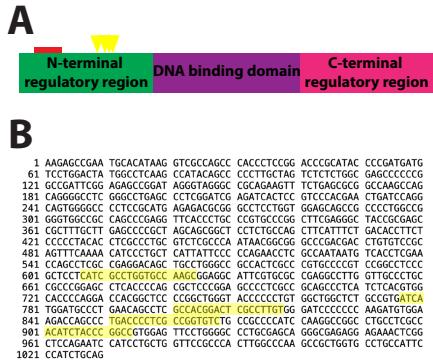


**Figure 3.26:** We quantitated the immunofluorescence images from THP-1 macrophages stained with antibodies against the four NFAT isoforms to assess the contribution of each protein to the VEGFA expression phenotype. Indeed, we found that NFATC2 most tightly corresponded to VEGFA induction by measuring double positive cells (those with both VEGFA expression and NFAT nuclear localization) and normalized to the total number of VEGFA positive cells.

**Table 3.2:** Nearest gene neighbors for the ST sgRNAs (all are intergenic).

sgRNA	Nearest Gene Neighbor (Chromosome)
hU6 ST	CNTN1 (12)
mU6 ST	MTDH (8)
7SK ST	OPN5 (6)
hH1 ST	ENSG00000249941 (5)

during  $\gamma Mtb$  exposure, we used a lentivirus-mediated CRISPR/Cas9 approach to introduce high-efficiency disruption of NFATC2. Using techniques inspired by the zebrafish and backported to cell culture, we simultaneously expressed four distinct guide RNAs targeting NFATC2 or safe-targeting controls, to maximize the percentage of puromycin-resistant cells possessing complete null mutations (Wu et al., 2018). We compared these cells to those transduced with lentiviruses expressing safe-targeting control sgRNAs. (Figure 3.27, Table 3.2, Subsection 2.8.5) (Kabadi et al., 2014; Sanjana, Shalem, and Zhang, 2014; Morgens et al., 2017; Kitamura and Kaminuma, 2021). Due to technical challenges associated with long-term culture of THP-1 cells and to address heterogeneity among cellular responses, we focused these assays on VEGFA induction in these cells by immunofluorescence after  $\gamma Mtb$  exposure. Because the N-terminal epitope recognized by our NFATC2 antibody was upstream of the targeted sites, we were unable to examine functional protein levels directly and simultaneously in the immunofluorescence images (Figure 3.29A). However, we found that transduced cells targeted by NFATC2 lentivirus generally failed to induce VEGFA while safe-targeting control lentivirus-transduced cells responded normally (Figure 3.28A-B), an effect that can be quantitated by percentage of VEGFA+ cells by multiple metrics (Figure 3.30A-C). These cells also demonstrated evidence of nonsense-mediated decay by NFATC2 transcript levels (Figure 3.29B). Thus, macrophage NFATC2-mediated induction of VEGFA downstream of mycobacterial TDM

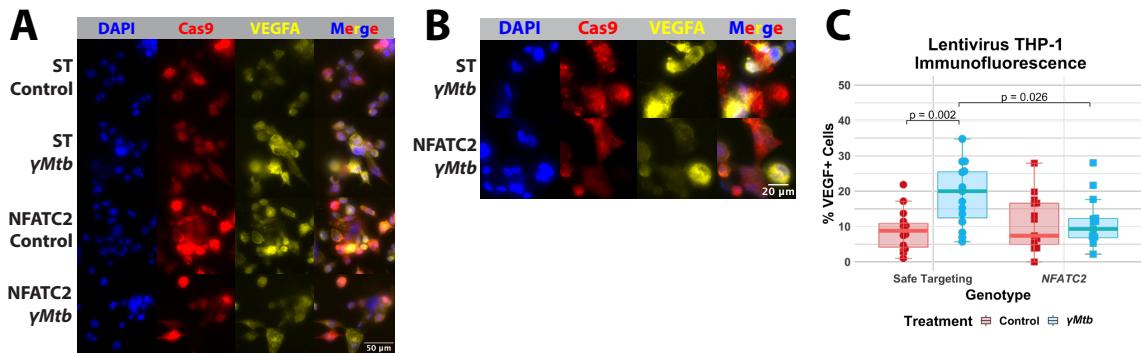


**Figure 3.27:** Based on our findings in THP-1 macrophages and the larval zebrafish, NFATC2 was selected for genetic targeting in the THP-1 macrophages. (A) displays the location of the four sgRNAs selected (in yellow arrows) and the location of the antibody epitope (red bar). (B) shows, on the genomic sequence itself, the location of the four sgRNAs.

exposure is conserved from zebrafish to human cells exposed to *M. tuberculosis*.

## 3.4 Discussion

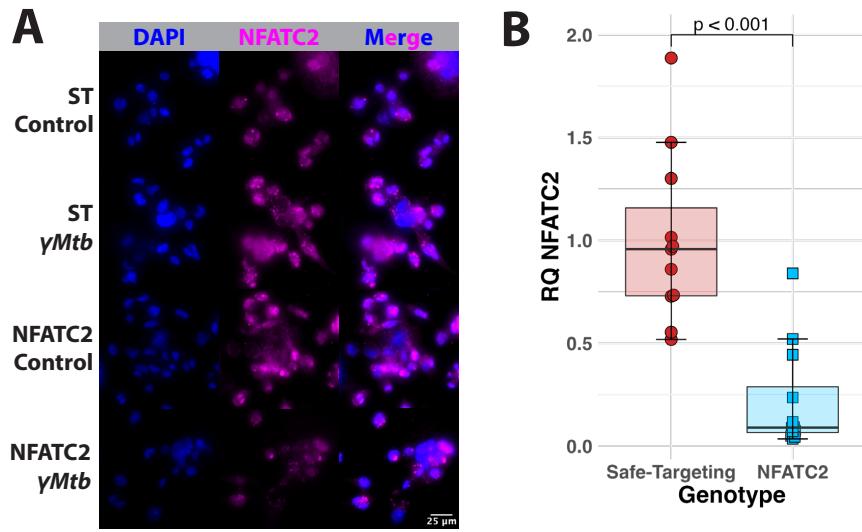
This work uncovers an unexpected and novel role for macrophage NFAT activation in immune responses to pathogenic mycobacteria and the maladaptive angiogenic responses that occur during infection. This activation of NFAT is driven through recognition of bacterial cis-cyclopropanated trehalose 6-6'-dimycolate,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



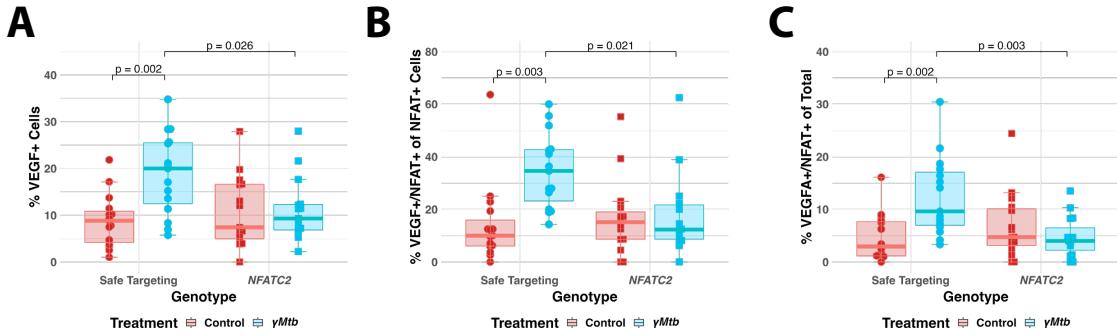
**Figure 3.28:** Lentivirus-mediated CRISPR/Cas9 targeting of NFATC2 results in reduced VEGFA expression after gamma-irradiated *M. tuberculosis* exposure. (A) shows that Cas9-expressing NFATC2-targeted cells have a reduction in VEGFA production while similar Cas9-expressing cells with safe targeting control sgRNAs robustly induce VEGFA. (B) shows this magnified. Note the higher-expressing NFATC2-targeted cell has minimal Cas9 expression, suggesting that these effects may depend on the efficiency of targeting and other factors. (C) shows quantitation of the percentage of VEGFA+ cells in a given field of view, with a statistically significant reduction in the NFATC2-targeted condition.

VEGFA induction is a prominent feature of tuberculosis in human disease as well as in a number of animal models, including non-human primates, rabbits, mice, and zebrafish (Datta et al., 2015; Oehlers et al., 2015; Polena et al., 2016; Harding et al., 2019; Cronan et al., 2021; Gideon et al., 2022). We found that VEGF was produced specifically within newly arrived macrophages at nascent granulomas. Macrophage populations are critical to VEGF induction, as macrophage-specific inhibition of NFAT signaling as well as deletion of *nfatc2a* result in reductions in granuloma-associated angiogenesis. Using a human cell culture model, we found that NFATC2 was similarly engaged in human cells as amongst all NFAT isoforms, only NFATC2 underwent robust nuclear translocation in response to *M. tuberculosis* stimulation. Correspondingly, pharmacological inhibition of NFAT signaling in human cell culture as well as genetic inhibition of *NFATC2* resulted in reduced VEGFA production.

Although animal models of tuberculosis generally report high VEGF levels, there



**Figure 3.29:** Validation of the lentivirus-mediated knockout approach. (A) shows aberrant localization of NFATC2, suggesting that the remaining protein has been functionally disrupted. Note that the antibody binding site is N-terminal to the sgRNA sites, a technical oversight in this design that made it difficult to quantify reductions in NFATC2 protein levels after targeting. (B) Nonsense-mediated mRNA decay in suspension THP-1 monocytes is reduced by NFATC2 targeting relative to safe targeting controls.



**Figure 3.30:** Blinded quantification of our lentivirus-transduced CRISPR/Cas9 THP-1 macrophages demonstrating a reduction in the degree of VEGFA expression that corresponds to reduced NFATC2 nuclear responsiveness to  $\gamma$ Mtb. (A) shows the total percentage of VEGFA+ cells out of the total cells present. (B) is the percentage of cells with both VEGFA expression and NFATC2 nuclear translocation out of the total number of cells with NFATC2 nuclear translocation. (C) shows the percentage of the total number of cells that have both VEGFA expression and NFATC2 nuclear translocation. Together these data implicate NFATC2 in mediating the VEGFA response to  $\gamma$ Mtb exposure.

are few studies that center on VEGFA induction in cell culture infection models. Through high-resolution time-lapses and reporter lines, we found that *vegfaa* induction generally does not occur until the formation of initial granulomas and is generally correlated with the appearance of extracellular bacteria that could be recognized by incoming, likely uninfected macrophages. This concentration-dependent effect on signaling may reflect key aspects of the disease itself, wherein large masses of extracellular bacteria accumulate in the necrotic core of the granuloma, potentially triggering relatively insensitive and/or chronic C-type lectin signaling in this context. Given what is already known about the need for chronic stimulus to produce effective NFATC2 activation, this would coherently fit with existing models and would offer a mechanism wherein other NFAT isoforms could play other roles earlier in infection but NFATC2 is specific to later stages at high intensity and duration of activation (Yissachar et al., 2013; Kar and Parekh, 2015).

Consistent with the recognition of extracellular bacteria, exposure of human macrophage-like cells to  $\gamma$ -irradiated *M. tuberculosis* rapidly induced NFATC2-dependent VEGF signaling in a dose dependent manner. Standard cell culture infection models generally eliminate extracellular bacteria using gentamicin treatment and media changes, and so it is possible that engagement of this pathway by extracellular bacteria or TDM stimulation is a key component of this response. A survey of the literature and a variety (Lee et al., 2019; Pisu et al., 2020; Hall et al., 2021; Looney et al., 2021; Pu et al., 2021) of RNA-seq datasets from macrophage-*M. tuberculosis* infection experiments reveal modest or nonexistent induction of VEGFA, further supporting the notion that extracellular exposure to *M. tuberculosis* may be an important element of the angiogenic response and may reflect some aspects of the macrophage-*M. tuberculosis* interface within granulomas (Orme, 2014).

As its name suggests, the NFAT pathway plays an indispensable role in normal T cell biology. Accordingly, whole animal knockouts of NFAT in standard mouse models of *M. tuberculosis* infection – where granuloma formation itself may be limited – may have obscured a role for myeloid-specific effects of NFAT signaling (Via et al., 2012). This study investigated a role for NFATC2 in control of *M. tuberculosis* infection and found that NFATC2 was required for effective bacterial control and production of TNF- $\alpha$  and IFN- $\gamma$  in CD4 $^{+}$  T cells despite no perturbation of the expression of these genes in dendritic cells, suggesting an adaptive immune response-specific defect in bacterial control. This may be of little surprise, given the extensive contacts that T cells have with mycobacteria within the lesions that develop in standard mouse models. Such a defect in T cell responses may be important for bacterial control when T cells are able to access the bacteria, but this condition is not reflective of the biology of the granuloma in other models or humans. It would be interesting to explore the phenotype of a *LysM-Cre; Nfatc2<sup>f/f</sup>* mouse infected with tuberculo-

sis to isolate the macrophage-dependent phenotypes. However, given our findings on macrophage NFAT contributions to angiogenesis, which is poorly modeled in the mouse, either entirely new roles for NFAT may be uncovered or it may have little or no effect on bacterial growth and disease. In any case, the present results offer some tension with these historical findings and it may take additional work to definitively identify any role for NFATC2 in the overall pathogenesis of tuberculosis infection.

The zebrafish model, by looking at early timepoints, uncovered a role both in angiogenesis and, presumably as a consequence, bacterial control. Wholesale, longer-term inactivation of NFAT, which also plays important roles in T cells, would potentially compromise important aspects of a productive adaptive immune response during mycobacterial infection. While genetically manipulable animal models allow for cell-specific separation, any host-based therapeutic approaches might require cell-specific macrophage delivery methods (Hu et al., 2019; Mukhtar et al., 2020; Colombo et al., 2022), NFATC2-specific targeting (Kitamura and Kaminuma, 2021), and/or contend with the adaptive immune response<sup>4</sup>, an important aspect of host resistance during mycobacterial infection.

While little else has been studied in the context of NFAT-mycobacterial interactions, a previous publication assessed the contributions calcineurin activation on the mycobactericidal activity of macrophages and found that mycobacteria manipulate an actin-binding protein called Coronin-1 to block phagosomal-lysosomal fusion and do so by activating calcineurin (Jayachandran et al., 2007). In the absence of Coronin-1 or after treatment with calcineurin inhibitors, lysosomes efficiently fused with the mycobacteria-containing phagosomes, mediating bacterial killing. This effect was

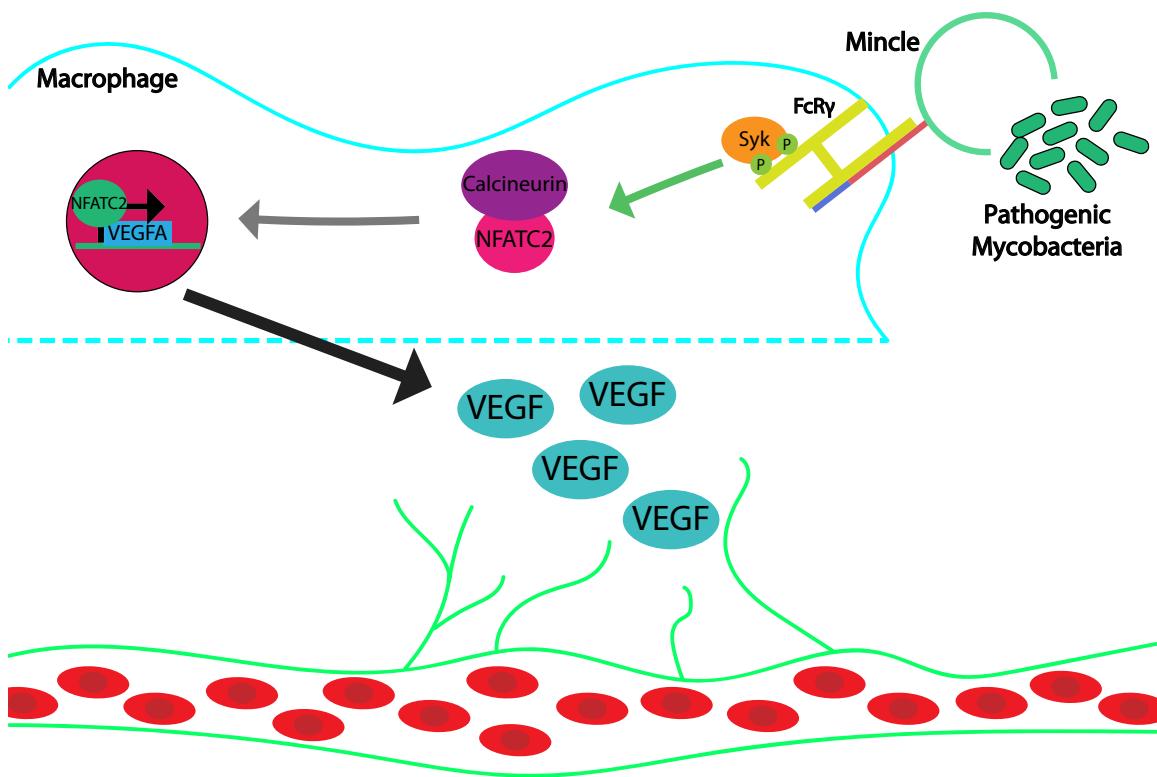
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<sup>4</sup>Although NFATC2 appears to be largely redundant with both NFATC1 and NFATC2 for many, but not all adaptive responses, it is still important to, as specifically as possible, target the desired pathway for minimal off-target side effects.

found to be independent of transcription, implicating calcineurin in somehow modulating the process of phagosomal-lysosomal fusion, potentially through some interaction with Coronin-1 and actin. However, a subsequent publication found that this process was independent of actin (Jayachandran et al., 2008), leaving it a yet-to-be resolved mystery how directly calcineurin-dependent activity blocks phagosomal-lysosomal fusion to facilitate mycobacterial survival and replication. If nothing else, this adds to the body of evidence suggesting that calcineurin/NFAT-based host directed therapies may have multiple modes of host-beneficial activity.

It remains unclear why NFATC2, but not any of the other isoforms, is specifically required in macrophages for the induction of VEGFA, given evidence that the others are present in resting macrophages (Figure 3.25). The functional distinctions between the isoforms have long been of basic interest, but relatively few specific differences between them have been identified beyond basal regulation to provide tissue-specificity and more recent findings describing layers of kinetic regulation with isoform-specific stimulation thresholds, nuclear retention, and more (Lyakh, Ghosh, and Rice, 1997; Rao, Luo, and Hogan, 1997; Kar et al., 2014; Kar and Parekh, 2015; Kar et al., 2016; Yissachar et al., 2013). Anecdotally, we observed substantially higher expression of NFATC2 by immunofluorescence after  $\gamma$ Mtb exposure even with INCA-6 treatment, suggesting that there may be interesting upstream regulatory mechanisms, potentially dependent on HIF-1 $\alpha$  or NF- $\kappa$ B. These novel levels of regulation offer opportunities for uncovering new features of the cell biology of NFAT.

NFAT is also likely to be involved in modulating the expression of other important pro-angiogenic chemokines, including CXCL12, the primary ligand for CXCR4. CXCR4 has been previously demonstrated to be critical for granuloma formation and angiogenesis (Torraca et al., 2017) and NFAT has been shown to be able to



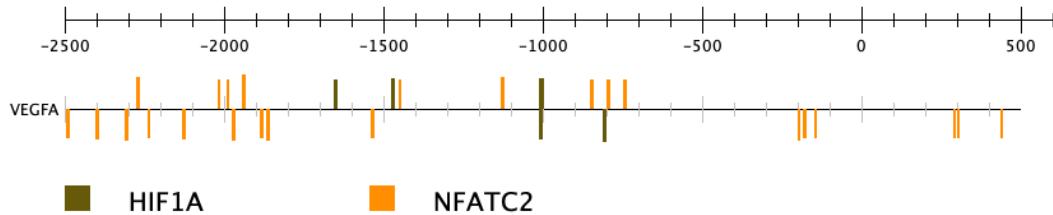
**Figure 3.31:** Upon completion of this work, we have identified a critical role for NFATC2 activation downstream of TDM detection in mediating the production of VEGFA by macrophages, which induces angiogenesis. This clarifies a major question in the field as to the underlying mechanisms of angiogenesis and opens new paths for investigation into the contributions of the NFAT signaling pathway to mycobacterial pathogenesis.

regulate CXCL12 expression, at least in osteoblasts (Sesler and Zayzafoon, 2013). It may be reasonable to think that NFATC2 is also mediating CXCL12 expression during infection and is acting as a more central regulator of angiogenic responses. This hypothesis warrants further study; in any case, the stimulus for and transcriptional mediators of the observed CXCR4-dependent granuloma angiogenesis should be identified to add to our understanding of how these processes occur.

Here, we identify the unique requirement for this single isoform in macrophages

to induce angiogenesis in response to mycobacterial infection (Figure 3.31). One hypothesis is that NFATC2 has binding partner(s) unique among NFAT isoforms required for its effect on the VEGFA promoter. Whether this is HIF-1 $\alpha$  (the canonical regulator of VEGFA) or one of the many previously described interacting partners is, as yet, unknown, but could be tested either *in vitro* or *in vivo* with genetic or chemical approaches (Subsection 5.2.3). However, higher order regulatory mechanisms that result in the production of VEGF in the absence of overt hypoxia have been understudied and this work proposes at least one potentially generalizable mechanism whereby NFATC2 activation results in VEGFA transcriptional upregulation, a process that can be inhibited with chemical and genetic intervention. Despite the widespread presence of putative NFAT binding motifs (5' -GGAAA-3' ) (Figure 3.32) in the proximal VEGFA promoter (Gearing et al., 2019), their influence on VEGFA transcription has been relatively unexplored as this specific effect is generally not seen in T cells or other cell types (Chang et al., 2004). This single publication from Chang et al. (2004) found that the -2400 site in the *VEGFA* promoter was specifically bound by NFATC proteins and acted to *repress* transcription. This is an intriguing hypothesis that might suggest that NFAT is able to repress *VEGFA* transcription at baseline but may act to induce it after stimulation. Such results would be consistent with the mild but reproducible increase in VEGFA we observe with INCA-6 treatment in our experiments in THP-1 cells (Figure 3.21).

As described in Section 1.4, NFAT is involved in the induction of a variety of cytokines including IFN- $\gamma$  and TNF- $\alpha$ , but whether NFAT-dependent transcriptional induction of these within macrophages has a role in the overall course of disease is unknown. This surely offers promise for the future as an expansion of the present work to incorporate a more complete view of the NFAT-dependent signals that influence mycobacterial disease progression.



**Figure 3.32:** NFATC2 and HIF-1 $\alpha$  putative binding sites in the VEGFA promoter as determined by Ciiider. There are several NFAT binding sites in the promoter, the function of which has largely yet to be determined.

A more comprehensive characterization of NFAT-dependent innate immune responses has begun in recent years (Deerhake et al., 2021; Peuker et al., 2022; Poli et al., 2022) (for a more detailed description, see Subsection 1.4.4), but this pathway has remained unstudied in the context of macrophage signaling during mycobacterial infection. Furthermore, this work draws a connection between the induction of calcium fluctuations – which can occur in response to many different developmental, homeostatic, and pathological stimuli, including to mycobacterial infection (Kusner and Barton, 2001; Jayachandran et al., 2007; Jayachandran et al., 2008; Matty et al., 2019; Malik, Denning, and Kusner, 2000) – to the angiogenic response to that stimulation. Our identification of NFAT regulation of VEGFA offers a novel approach to both pro- and anti-angiogenic intervention in various pathological contexts.

### 3.4.1 Limitations of the Study

The limitations of this study, like all studies, are innumerable. While I am proud of what I have accomplished in pursuit of this work, there are many opportunities for the future as well as further work to clarify the results we have found here. We have identified interesting macrophage biology mediating an important disease-relevant

phenotype during mycobacterial infection, but we do not see the same magnitude of bacterial burden change as with VEGF-inhibition alone (Oehlers et al., 2015), suggesting pleiotropy in the NFAT signaling pathway with inhibition resulting in both pro- and anti-bacterial effects. What these antimycobacterial aspects of NFAT signaling are is worth investigation to better understand the full corpus of possible consequences to NFAT inhibition strategies for the treatment of infectious disease and the potential risks for patients already taking calcineurin inhibitors. To do so, RNA-seq or similar should be employed to better delineate these alterations in gene expression profile. It would also be beneficial to work toward disentangling some of the other effects of NFAT inhibition by simultaneous treatment of the zebrafish with VEGFR2 inhibitors in the *irg1:VIVIT* background to directly test non-angiogenic features of NFAT in mycobacterial infection.

We have thus far been unable to identify the particular macrophage receptor responsible for detecting TDM in the zebrafish. While work in THP-1 macrophages may be able to more affirmatively link MINCLE or MCL to the activation of NFAT, identification of the zebrafish receptor would make a substantive contribution to model development as well as the evolution of C-type lectin receptors more broadly. Cell-type specific knockout of *nfatc2a* would also aid greatly in definitively linking this gene to VEGFA induction and angiogenesis, but the tools to do so are not yet operational; perhaps future studies can begin with such intent and focus more stringently on the role of this isoform strictly in macrophages.

Some technical limitations are also interfering with interpretation of some results. Generation of stable knockouts of NFATC2 in THP-1 macrophages would make for more robust and cleaner assays, although it has the limitation of odd clonal behavior and loses the heterogeneity that can be advantageous in dissecting some phenotypes.

The variable ploidy of these cells is also a challenge, so an alternative macrophage model or primary cells may be required to more effectively model some of these responses.

This macrophage-NFAT signaling pathway likely has important roles in other disease contexts that are not addressed here. Tuberculosis is the most common cause of granuloma formation, but it is far from the only cause, and any contributions of this pathway to granuloma biology in *Schistosoma*, *Cryptococcus*, or *Histoplasma* infections is not yet known. However, with the tools now available, these roles are likely to be easier to study in the future and new insights may be gleaned from such investigation. Angiogenesis is a common pathology in some autoimmune diseases, such as arthritis, and is prevalent among cancers; whether macrophage NFAT signaling might be mediating angiogenesis in these contexts is surely of future interest and may reveal novel aspects of the host-tumor interface.

We have validated important aspects of our observations in the zebrafish with a mammalian cell culture model, but subsequent studies may warrant further integration of mammalian models of tuberculosis infection where angiogenesis is present (rabbits, guinea pigs, macaques) or human patient samples to better understand certain aspects of the underlying biology and validate NFAT induction during tuberculosis infection and potential correspondence to VEGFA production.

# Chapter 4

## Novel Computational Approaches to Image Processing and Quantitation

High-resolution, high-throughput microscopy has opened possibilities for biological analysis that were inconceivable only a few short years ago, but the methods by which to analyze these data remain largely lacking. While heroic efforts have been made to use both standard thresholding methods as well as newer machine learning-powered methods to simplify and automate image processing, these approaches are often somewhat limited by the range of purposes for which they were designed and tested. While the open source nature of many of these approaches facilitates adaptation, this is both technically beyond the skillset of many scientists and impeded by, at best, variable quality documentation. Additionally, many of the tasks that experimenters seek to do are both relatively simple and highly repetitive but they are often unaware of the means by which to cut down on manual processing time in order to economize their time and energies. These scripts are a combination of automation scripts that will process particular image types into smaller and more informative images (compression of Z-stacks, time-series, etc.) and those meant for analysis of

image data (pixel intensity distribution across an image, bacterial burden within larval zebrafish). While none of these are of the caliber to open entirely new methods of analysis, I hope they are a catalyst for others in the zebrafish community at large to explore the potential for computational automation to save time and frustration in the process of analyzing often thousands of very large, data-rich images.

All of the scripts in their latest versions will be able to be found in perpetuity at <http://github.com/jaredbrewer/image-analysis>. A static version of these has been created at Zenodo (<https://doi.org/10.5281/zenodo.7036029>). Scripts at the end for RNA sequencing analysis are available at Zenodo (<https://doi.org/10.5281/zenodo.6981721>).

## 4.1 FIJI/ImageJ

ImageJ was first developed in embryo in the 1980s as a means of viewing scientific images on the Mac, but matured into the program we know today in the 1990s when it was ported to Java. The original ImageJ1 is still developed and maintained today and serves as the foundation of its functional successor, FIJI. Fiji Is Just ImageJ is essentially ImageJ2 bundled with a set of useful plugins that aid in visualizing and analyzing scientific imaging data. Preeminent among these bundled plugins is a library of programming languages that can be used to interface with the underlying Java-based APIs that make FIJI function. Among the options, Python is likely the most widely known and written programming language among biologists and this opens up a great deal of potential for object-oriented programming centered on the analysis of images.

The default language in ImageJ, the ImageJ1 Macro language (.ijm), is approximately based around Java but lacks defined sets of methods (for most purposes) and

objectification, has difficulty with interfacing with file systems, and exposes a great deal of underlying computer logic to the user. Take, for instance, the procedure for writing a *for* loop:

```
for (i = 0; i < 10; i++) {  
    print("Hello");  
}  
  
for i in range(1:10):  
    print("Hello")
```

The former example, written in the IJ1 macro language, exposes the user to looping over a set of indices from 0 to 10 and incrementing with each loop, uses curly braces to define the boundaries of the loop block, and requires each line to end with a semicolon. These are all standards features of many popular programming languages, but for FIJI, the key is to be as legible of a programming language as possible since the writers of most of these macros are *not* software engineers. Python, by contrast, increments for the user and will increment through an entire set of objects without needing to know how many objects are present. Blocks of code are delimited by spaces or tabs, so the relative location of the code on the line is informative as to what it relates to. Additionally, Python has well-studied and well-defined object types with specific associated methods that translate between all versions of Python. The benefits of the Python community only add to the value proposition of learning and using Python both generally and within ImageJ.

Not only this, but to loop through directories of files, the ImageJ macro language struggles even further, with little awareness of file structures or regular expressions whereas Python can trivially fetch lists of files by user-defined patterns from mixed directories and even search through subdirectories recursively.

## 4.2 Python

As alluded to, Python is a dynamically typed high-level programming language that is widely used for generating automation scripts of all varieties in addition to web server backend applications, data science, and even game creation (Rossum, 1995).

The advantages of Python are derived from the enormous community of developers who use Python and who continue to develop it as a language. In data science, the utility of pandas and numpy are unmatched and these alone make Python a language worth learning for any molecular biologist in the analysis of their quantitative data. Throughout this work, I have used Python both within the FIJI/ImageJ interpreter (which is based on Jython version 2.7.2) and via CPython 3.11, the standard distribution of Python, in the Terminal. While Jython is nominally different from standard Python, when written appropriately, it is simple enough to write version-universal code within the relatively narrow confines of what I have sought to accomplish thus far with it.

One of the key developments in the field of FIJI and Python is the release of PyImageJ, a complete API interface between CPython and ImageJ2, written in Python (Rueden et al., 2022). Going forward, it will be useful to reimagine some of these scripts and approaches to utilize the PyImageJ-defined API, as this is clearly the future of scripting in FIJI. Use of the image viewer in an *ad hoc* fashion is likely superior to an image-first approach for many tasks and PyImageJ cuts a lot of the unfortunate overhead from the graphical elements of FIJI. Unfortunately, such a translation is nontrivial as the command structure and imports have changed quite dramatically and in ways that are not as well documented as the base API. However, such an implementation allows FIJI/ImageJ to make use of pandas, numpy, and OpenCV

in Python, making for a more complete data science package and streamlining data collection and processing in a single language.

### 4.2.1 Napari

While FIJI/ImageJ have served as the key to the analyses presented both here and in Chapter 3, it is not the only image analysis software available. An alternative, written in pure Python, is napari, which is in the middle stages of alpha testing as of this writing (Sofroniew et al., 2022). While not yet feature complete, it is a lightweight alternative to FIJI and, with proper optimization, can likely outperform FIJI in the long run. By shedding dependence on Java, memory management and performance are able to be improved while the lowest-level image processing can be done in C invisible to the end user. At the present time, however, FIJI seems to be the most robust and widely accepted option and will likely remain so for the conceivable future. A bridging option, *napari-imagej* is in the works and promises to dramatically expand the napari feature set, but does not aid in napari independence from Java or older modalities.

## 4.3 R

As previously detailed (Section 2.10), R is a convenient and easy-to-learn object-oriented statistical programming language. This language has made a monumental contribution to data science and is used extensively throughout this work to analyze and visualize the resulting datasets. While less extensively used in this segment than Python, it is key to a lot of further analyses and is exhaustively used for all of the visualization seen in Chapter 3. Many of these same functions can be executed in Python, but I attempt to utilize the best tool for a given task at a given time and R

is often the best, most flexible way to work with arrays and data frames.

## 4.4 Maximum-Intensity Projection and Composite Image Generation

The fundamental premise of the ImageJ macro system is to simplify repetitive tasks to free up user time for higher forms of analysis and the default language (the ImageJ1 macro language) makes it easy to write procedural operations to be done on single images, but is difficult to scale to whole directories of images or accept various types of user input. I have thus developed a set of scripts that allow the user to rapidly generate maximum-intensity projections from sets of images and then generate composite images. This often condenses hundreds of megabytes of data into <30 MB, makes for more flexible image viewing and understanding, and allows the images to be opened in essentially any number (many hundreds to thousands) on any personal computer. These procedures are also internally memory managed, allowing them to run on most personal computers indefinitely to process the sometimes thousands or tens of thousands of images that can be generated over the course of an experiment with multiple wavelengths, Z stacks, XY positions, and times<sup>1</sup>. In our lab, we primarily use output files from epifluorescent Zeiss microscopes, which generate .czi files and from Metamorph connected to a custom spinning disk confocal system, which generates .tiff files.

The maximum intensity projection is an extremely common way of condensing multidimensional images into a single two-dimension representation by finding the

---

<sup>1</sup>If you have 4 channels across 48 stage positions, capturing every 30 minutes for 48 hours, you would have 18,432 individual images based on Metamorph's file saving structure; this can clearly get out of hand very quickly.

brightest pixel at every XY position and accepting it as the most “in focus” pixel. The success of this depends on images being properly exposed, but in most instances will generate a reasonably sharp image ready to be quantitated or presented. In any case, data spanning multiple Z stacks requires some degree of integration to be usefully presented to others or manual scanning through libraries of hundreds of images and these pipelines will facilitate that type of visualization at scale.

Source Code 4.1: This script allows the user to open as many files as their memory allotment will allow and then to Z project them one at a time with custom start and end positions. This ability often generates cleaner, sharper images by individually selecting the lowest and highest in-focus frames, but necessarily takes more time than a more automated approach.

```
from ij import IJ, ImagePlus
from ij.plugin import ZProjector
from ij.gui import NonBlockingGenericDialog
import os, sys, random
from os import path

gui = NonBlockingGenericDialog("MIP Ranges")

gui.addNumericField("First Slice: ", 0, 0)
gui.addNumericField("Last Slice: ", 0, 0)

gui.showDialog()

if gui.wasCanceled():
    IJ.error("Dialog cancelled!")
    exit()
# Everything below this ~should~ be in an else statement, but if it isn't
→ broke, don't fix it.

bot = gui.getNextNumber()
top = gui.getNextNumber()

imp = IJ.getImage()

if bot is 0 and top is 0:
    bot = 1
    top = int(imp.getImageStackSize())
if int(bot) > int(top):
```

```

IJ.error("Bottom is greater than top, invalid combination.")
exit()
if int(top) > int(imp.getImageStackSize()):
    IJ.error("Top is greater than the dimensions of the image - try again")
    exit()
if int(bot) < 1:
    IJ.error("Out of range - bottom number must be greater than or equal to
        ↵ 1")
    exit()

outputdir = str(imp.getOriginalFileInfo().directory)

proj = ZProjector.run(imp, "max", int(bot), int(top))
# This line is to show your MIP - if you want to see it, remove the #
# proj.show()

title = proj.getTitle()
out = path.join(outputdir, title)
IJ.saveAs(proj, "Tiff", out)

#Comment out these lines if you'd like to see your images before you
    ↵ close them.
imp.close()
proj.close()

rand = random.randint(1, 100)

if rand > 85:
    IJ.run("Collect Garbage", "")
else:
    pass

```

The use of Source Code 4.1 is to process a set of already opened images and generate maximum intensity projections from these and, optionally, save them back into the directory that they came from. This relatively simple set of GUI-guided processes allows the user to, in two clicks, accomplish a task that previously would have required a great deal of menuing to accomplish. The goal is narrow, but this execution is extremely useful when working through large numbers of images.

Source Code 4.2: A low overhead version of the manual maximum intensity projection script described above. Instead of opening all of the images first and then running the script, the script will processively open unanalyzed images one at a time and periodically garbage collect, allowing for entire directories to be processed at once on most reasonably modern computers.

```
from ij import IJ, ImagePlus
from ij.plugin import ZProjector
from ij.gui import NonBlockingGenericDialog, GenericDialog
import os, sys, random
from os import path

# This is a low-overhead version of the manMIPper that loads and displays
# images one at a time and only if needed.

gui = GenericDialog("File Directory: ")
gui.addDirectoryField("Z-Stacks Location", "~/Documents")
gui.addChoice("File Extension: ", [".czi", ".lif", ".lsm", ".tif",
    ".tiff", "Other"], ".tif")
gui.addStringField("Custom File Extension: ", "")
gui.showDialog()

basedir = str(gui.getNextString())

inputdir = basedir
outputdir = basedir + "MIPs/"
if not path.isdir(outputdir):
    os.makedirs(outputdir)

filenames = os.listdir(inputdir)

ext = str(gui.getNextChoice())
cusext = str(gui.getNextString())

extension = ""

if ext != "Other":
    extension = ext.lower()
elif ext == "Other":
    extension = cusext.lower()

rep_files = []

for files in filenames:
    if files.lower().endswith(extension) and "_thumb_" not in
        files.lower():
```

```

file = os.path.join(inputdir, files)
rep_files.append(file)

for f in rep_files:
    mips = [mip.lower() for mip in os.listdir(outputdir)]
    mip_title = str("MAX_" + os.path.basename(f)).lower()
    if mip_title.replace(extension, ".tif") not in mips:
        imp = IJ.openImage(f)
        imp.show()

gui = NonBlockingGenericDialog("MIP Ranges")
gui.addNumericField("First Slice: ", 0, 0)
gui.addNumericField("Last Slice: ", 0, 0)
gui.showDialog()

if gui.wasCanceled():
    IJ.error("Dialog cancelled!")
    exit()

bot = gui.getNextNumber()
top = gui.getNextNumber()

if bot is 0 and top is 0:
    bot = 1
    top = int(imp.getImageStackSize())
if int(bot) > int(top):
    IJ.error("Bottom is greater than top, invalid combination.")
    exit()
if int(top) > int(imp.getImageStackSize()):
    IJ.error("Top is greater than the dimensions of the image - try
    ↵ again")
    exit()
if int(bot) < 1:
    IJ.error("Out of range - bottom number must be greater than or
    ↵ equal to 1")
    exit()

proj = ZProjector.run(imp, "max", int(bot), int(top))
title = proj.getTitle()
out = path.join(outputdir, title)
IJ.saveAs(proj, "Tiff", out)
imp.close()
proj.close()
else:
    pass

```

```

rand = random.randint(1, 100)
if rand > 85:
    IJ.run("Collect Garbage", "")
else:
    pass

```

Source Code 4.2, while not always the correct choice depending on user preferences and system capabilities, is much less memory hungry than the original version above, but typically results in slightly slower overall operations due to the delay in opening images. If the images are on fast internal storage, then this is certain to be faster than Source Code 4.1, but when reading from external storage, the I/O limitations will likely make it faster to open all of the images prior to processing unless system memory is severely limited. Nevertheless, the nature of this makes it very generally useful on older systems.

Source Code 4.3: This script can be used in instances where the first and last stacks of a desired Z projection span the entire set of stacks provided. It will process an entire directory of images together and output the result into a subdirectory of the original.

```

from ij import IJ, ImagePlus
from ij.plugin import ZProjector
from ij.gui import GenericDialog
import os, sys, random
from os import path

gui = GenericDialog("File Directory: ")
gui.addDirectoryField("Z-Stacks Location", "~/Documents")
gui.addChoice("File Extension: ", [".czi", ".lif", ".lsm", ".tif",
    ".tiff", "Other"], ".tif")
gui.addStringField("Custom File Extension: ", "")
gui.showDialog()

basedir = str(gui.getNextString())

inputdir = basedir
outputdir = basedir + "MIPs/"
if not path.isdir(outputdir):
    os.makedirs(outputdir)

```

```

filenames = os.listdir(inputdir)

ext = str(gui.getNextChoice())
cusext = str(gui.getNextString())

extension = ""

if ext != "Other":
    extension = ext
elif ext == "Other":
    extension = cusext

rep_files = []

for files in filenames:
    if files.endswith(extension):
        file = os.path.join(inputdir, files)
        rep_files.append(file)

for f in rep_files:
    imp = IJ.openImage(f)
    if "MAX_" + imp.getTitle().rstrip(extension) + ".tif" not in
       os.listdir(outputdir):
        proj = ZProjector.run(imp, "max all")
        title = proj.getTitle()
        out = path.join(outputdir, title)
        IJ.saveAs(proj, "Tiff", out)
        imp.close()
        proj.close()
    rand = random.randint(1, 100)
    if rand > 85:
        IJ.run("Collect Garbage", "")
    else:
        pass

```

While the previous scripts have expected user input for each image, the skilled microscopist can select top and bottom slices that will suffice for generating Z projections during imaging itself. This means that the first and last slices of the projection are typically the first and last slices of the images *in toto*. This script takes a directory of images as an input and will perform total maximum intensity projections on all

of them and save in a new subdirectory. After observation, any that seem incorrect can then be processed with one of the preceding scripts, especially Source Code 4.1.

Source Code 4.4: An interface to functions allowing slices in a Z-stack to be kept or removed as desired through function calls. This can integrate into other workflows and be connected to the previous scripts through higher-order wrappers.

```
from ij import IJ, ImagePlus, ImageStack, WindowManager
from ij.gui import GenericDialog
from ij.measure import Calibration
from ij.plugin import Plugin
from ij.process import ImageProcessor
from os import path

# Defaults:
first = 1
last = 9999
inc = 2
imp = IJ.getImage()

def sliceKeeper(imp, first, last, inc):

    imp = IJ.openImage(imp)
    if not imp:
        IJ.noImage()
    stack = imp.getStack()
    if stack.getSize() is 1:
        IJ.error("Stack Required")
    title = imp.getTitle().split(".")[0]
    cal = imp.getCalibration()
    impdir = str(imp.getOriginalFileInfo().directory)

    def keepSlices(stack, first, last, inc):
        if last > stack.getSize():
            last = stack.getSize()
        newstack = ImageStack(stack.getWidth(), stack.getHeight())
        for slice in range(first, last + 1, inc):
            if slice > stack.getSize():
                break
            ip = stack.getProcessor(slice)
            newstack.addSlice(ip)
        imp = ImagePlus(title + "_kept", newstack)
        imp.setCalibration(cal)
        outputdir = path.join(impdir, title)
```

```

IJ.saveAs(im, "Tiff", outputdir)

keepSlices(stack, first, last, inc)

def sliceRemover(im, first, last, inc):

    im = IJ.openImage(im)
    if not im:
        IJ.noImage()
    stack = im.getStack()
    if stack.getSize() is 1:
        IJ.error("Stack Required")
    title = im.getTitle().split(".") [0]
    cal = im.getCalibration()
    immdir = str(im.getOriginalFileInfo().directory)

    def removeSlices(stack, first, last, inc):
        if last > stack.getSize():
            last = stack.getSize()
        newstack = stack.duplicate()
        for slice in range(first, last + 1, inc):
            if slice > stack.getSize():
                break
            newstack.deleteSlice(slice)
        im = ImagePlus(title + "_cut", newstack)
        im.setCalibration(cal)
        outputdir = path.join(immdir, title)
        IJ.saveAs(im, "Tiff", outputdir)

    removeSlices(stack, first, last, inc)

```

FIJI/ImageJ comes with a built-in option to keep and remove particular slices, but the native plugin does not readily fit into object-oriented programming pipelines like those used by Python and Java. Thus, I have adapted the underlying logic of these plugins to be wrapped in various other scripts through calls to the defined functions, *sliceKeeper* and *sliceRemover*. For instance, this allows calls to the maximum intensity projection plugins to be funneled into this plugin to simultaneously generate the kept slices as well as the maximum intensity projection of those kept slices for some

useful improvements to data integrity.

## 4.5 Surface Plot Analysis for Cellular Distribution of Labeled Proteins<sup>2</sup>

The distribution of a protein across the cell body can indicate changes in function of the protein and alterations in cell behavior, but analysis of such changes is not quite trivial. There are methods for drawing lines and plotting fluorescence intensity, but this fails to integrate the entire cell in three dimensions in to the analysis as it proves to be only one dimensional along that line. To analyze the distribution of ARPC2 in BLaER1 macrophages during *M. tuberculosis* infection, I developed a novel analysis pipeline that integrated the 3D Surface Plot plugin of ImageJ. This pipeline takes a raw image, isolates the desired channel, opens it in 3D Surface Plot for visualization, and then takes the user-generated output of 3D Surface Plot to find the maximum fluorescence along the longest axis of the cell. As this analysis is designed for macrophages, which can develop long spindles, this allows for quantitative proximal/distal differences in the localization of a protein to be determined.

Source Code 4.5: A script to isolate a single cell within a frame.

```
from ij import IJ, ImagePlus
from ij.io import DirectoryChooser, FileSaver
from ij.plugin import ChannelSplitter, ZProjector
from ij.plugin.frame import RoiManager
from ij.gui import GenericDialog
import os, sys
from os import path

gui = GenericDialog("File Directory: ")
gui.addDirectoryField("Cell Images", "~/Documents")
```

<sup>2</sup>Taken from personal contributions to Saelens et al. (2022).

```

gui.addChoice("File Extension: ", [".czi", ".lif", ".lsm", ".tif",
    ↵ ".tiff", "Other"], ".tif")
gui.addStringField("Custom File Extension: ", "")
gui.addNumericField("Reference Channel: ", 2, 0)
gui.addNumericField("Measurement Channel: ", 1, 0)
gui.showDialog()

basedir = str(gui.getNextString())

inputdir = basedir
outputdir = basedir + "/Splits/"
if not path.isdir(outputdir):
    os.makedirs(outputdir)

filenames = os.listdir(inputdir)

ext = str(gui.getNextChoice())
cusext = str(gui.getNextString())

extension = ""

if ext != "Other":
    extension = ext
elif ext == "Other":
    extension = cusext

rep_files = []

for files in filenames:
    if files.endswith(extension):
        if not files.startswith("."):
            file = os.path.join(inputdir, files)
            rep_files.append(file)

for f in rep_files:
    imp = IJ.openImage(f)
    proj = ZProjector.run(imp, "max all")
    imps = ChannelSplitter.split(proj)
    for i in imps:
        title = i.getTitle()
        out = path.join(outputdir, title)
        IJ.saveAs(i, "Tiff", out)
        ImagePlus.close(i)

###

```

```

splits = os.listdir(outputdir)

tifs = []

for files in splits:
    if files.endswith(".tif"):
        file = os.path.join(outputdir, files)
        tifs.append(file)

names = []

for tif in tifs:
    base = str(tif.split("-", 1)[1])
    names.append(base)

names = set(names)

rm = RoiManager.getRoiManager()

tifs.sort(reverse = True)

ref = str(int(gui.getNextNumber()))
meas = str(int(gui.getNextNumber()))

for name in names:
    for tif in tifs:
        if tif.startswith(outputdir + "C" + ref) and tif.endswith(name):
            a = IJ.openImage(tif)
            IJ.setAutoThreshold(a, "MinError dark")
            IJ.run(a, "Analyze Particles...", "size=100.00-Infinity clear
                include add")
            ra = rm.getRoisAsArray()
            rm.reset()
            ImagePlus.close(a)
        if tif.startswith(outputdir + "C" + meas) and tif.endswith(name):
            b = IJ.openImage(tif)
            title = b.getTitle().lstrip(outputdir + "C" + meas + "-")
            out = path.join(outputdir, title)
            b.show()
            for r in ra:
                rm.addRoi(r)
            rm.select(b, 0)
            IJ.run(b, "Clear Outside", "")
            rm.reset()

```

```

IJ.saveAs(b, "Tiff", out)

fin = GenericDialog("All Done!")
fin.showDialog()

```

The first step of this pipeline is to isolate the cell using some reference channel, which can also be the channel you are measuring if necessary, although I would recommend using some sort of pan-cytosolic or plasma membrane marker to more thoroughly mark the outline of the cell and to use that instead. The script presents a simple GUI and allows the user to select the needed parameters and will then process entire folders of images into isolated cells in a single channel. A missing feature is that it needs to be able to isolate each cell from images that contain more than one cell, to save the user time on isolating cells in their own separate files by hand. This could conceivably be done by integrating logic around coping ROI contents into new images, but that is a work-in-progress at this time.

The output is a folder of single-channel files ready to be input into 3D Surface Plot, which can be launched with the following:

```

from ij import IJ, ImagePlus

imp = IJ.getImage()

IJ.run(imp, "3D Surface Plot",
      '''plotType=1
colorType=3
drawAxes=0
drawLines=0
drawText=0
grid=256
drawLegend=0
smooth=8.5
backgroundColor=000000
windowHeight=600
windowWidth=720'''')

```

The image then needs to be rotated until it is along its longest axis in XY and then oriented precisely perpendicular in XZ (this can be done by turning the axes back on temporarily, although they must be turned off before the next step). This will give the longest two dimensional axis of the image and show a series of peaks and valleys corresponding to a smoothed-over rendition of the image. This can then be exported and, if desired, saved using:

```
from ij import IJ, ImagePlus
from os import path

outputdir = "" # Insert save directory as a string.
imp = IJ.getImage()
title = imp.getTitle()

out = path.join(outputdir, title)
IJ.saveAs(imp, "Tiff", out)
```

I recommend saving them for reference later, as the quantitation can occasionally be bugged by strange smoothing, etc. Any outliers will need to be inspected by hand and saving the intermediates can help a great deal with that. Additionally, saving allows further automation in the next step (although, as we will see, automating across all open images is also now possible and will be built into these options in the future).

Source Code 4.6: A script to automatically capture the signal at each point along an image and save it to a CSV file.

```
from ij import IJ, ImagePlus
from ij.gui import GenericDialog
from csv import writer
from os import path

gui = GenericDialog("Surface Plot Measure")
gui.addDirectoryField("File Location: ", "~/Documents")
gui.showDialog()
```

```

basedir = str(gui.getNextString())

inputdir = basedir
outputdir = basedir + "csvs/"
if not path.isdir(outputdir):
    os.makedirs(outputdir)

filenames = os.listdir(inputdir)

plot_files = []

for files in filenames:
    if files.startswith("Surface_Plot") and files.endswith(".tif"):
        file = os.path.join(inputdir, files)
        plot_files.append(file)

for f in plot_files:
    imp = IJ.openImage(f)
    title = imp.getTitle()

    IJ.run(imp, "8-bit", "")
    IJ.setRawThreshold(imp, 1, 255)
    IJ.run(imp, "Make Binary", "method=Default")

    width = imp.getWidth()
    height = imp.getHeight()

    pixel_width = []

    for num in range(0, width-1):
        pixel_width.append(num)

    pixel_height = []

    for num in range(0, height-1):
        pixel_height.append(num)

    max_coord = []

    for x_coord in pixel_width:
        for y_coord in pixel_height:
            if imp.getPixel(x_coord, y_coord)[0] == 255:
                max_coord.append([imp.getTitle(), x_coord, y_coord])

    csv_file = path.join(outputdir, title.split(".", 1)[0] + ".csv")

```

```

with open(csv_file, "a") as csv:
    writer = writer(csv)
    for coord in max_coord:
        writer.writerow(coord)

```

This script is key to capturing the points along the abstracted profile of the cell. It is relatively simple in that it just writes to a CSV each XY coordinate where there is a signal, which can then be condensed by finding the maximum point at each X position in R, which is the final step in the analysis.

Source Code 4.7: A script to conduct computational filename blinding from the command line written in Python.

```

# Jared Brewer
# Created: 21 July 2022
# Last Edited: 21 July 2022
# 3D Surface Profile Plot Analysis

library(ggplot2)
library(ggbeeswarm)
library(ggsignif)
library(dplyr)
library(DescTools)
library(stringr)

# Plug in the path were the .csv files are - make sure they are the only
# CSVs in the directory -
# unless you want to get more creative with some regex.

counts.dir <- ""
counts <- list.files(counts.dir, full.names = T)

cells <- data.frame()

# Our objective here is to combine all the individual CSV files into a
# single file, but to do a series of operations on it first.
# We read in the file, then aggregate into the minimum value
# (the data is inverted - need min, not max.)
# We then do some basic math operations to normalize the data across the
# length of the cell.

```

```

for (file in counts) {
  if (endsWith(file, ".csv")) {
    cells <- read.csv(file, header = F) |>
      aggregate(V3 ~ V1 + V2, data = _, min) |>
      mutate(pct_x = (V2 - min(V2))/(max(V2) - min(V2)),
             pct_y = (V3 - min(V3))/(max(V3) - min(V3))) |>
      mutate(x_pct_bin = ntile(pct_x, n=100),
             x_pct_corr = pct_x*100,
             y_pct_corr = -pct_y*100+100,
             treat = str_extract(V1, "WTCAFA|WTMAFA"),
             rep = as.numeric(str_extract(V1, "\\\d+$"))) |>
      rbind(cells)
  }
}

aucs <- data.frame()

# Now we want to measure the area under the curve for each replicate.
# We use nested for loops to read each treatment and each replicate for
# each treatment and filter.
# Then, we normalize across the total Y range, calculate the AUCs, and
# select only the columns we need.

for (t in unique(cells$treat)) {
  for (r in unique(cells$rep)) {
    aucs <- filter(cells, treat == t) |>
      filter(rep == r) |>
      mutate(y_pct_corr = y_pct_corr/sum(y_pct_corr)) |>
      mutate(per = AUC(x_pct_corr, y_pct_corr, from = min(0), to =
        max(25))/
        AUC(x_pct_corr, y_pct_corr, from = min(25), to =
        max(75)),
            mid = AUC(x_pct_corr, y_pct_corr, from = min(37.5), to =
        max(62.5))) |>
      select(per, mid, treat, rep) |>
      unique() |>
      na.omit() |>
      rbind(aucs)
  }
}

t.test(aucs$per ~ aucs$treat)

# Plot the AUCs - must add your own labels.

```

```

auc.plot <- ggplot(aucs, aes(x = treat, y = per)) +
  geom_beeswarm(aes(shape = treat, size = 3)) +
  geom_boxplot(aes(fill = treat), alpha = 0.25, outlier.shape = NA,
    ↵  fatten = 5) +
  stat_boxplot(geom='errorbar', width = 0.25) +
  xlab("Genotype") + ylab("AUC") +
  geom_signif(y_position = c(0.6), xmin = c(1), xmax = c(2), annotations
    ↵  = c("p = 0.002"), textsize = 5.5, color = "black") +
  scale_x_discrete(limits = c("", ""), labels = c("", "")) +
  scale_fill_manual(name = "", labels = c("", ""), values = c("black",
    ↵  "black")) + theme(legend.position = "none") +
  guides(color = "none") + theme_minimal() +
  theme(text = element_text(size = 20, face = "bold"), plot.title =
    ↵  element_text(hjust = 0.5)) +
  theme_prism() +
  theme(legend.position = "none")

```

This R script takes the CSV format output from FIJI/ImageJ and processes it by finding the maximum value along the curve and using that as the peak relative fluorescence intensity for that X position and then calculates the area under the curve across a defined interval. For instance, the middle 50% of the cell can be defined as "central" and the outer 25% can be defined is "distal" and these values normalized to one another and then compared across different experimental groups. It was found that BLAER1 cells infected with *M. tuberculosis* expressing ancestral *esxM* had less distal localization of ARPC2, suggesting that this bacterial effector was altering actin dynamics in these cells.

## 4.6 py-LaRoMe

The original purpose of LaRoMe (<https://github.com/BIOP/ijp-LaRoMe>) is to extract various features from images and is distributed as an ImageJ plugin. However, like many FIJI/ImageJ plugins, the design of LaRoMe is purely operational. In my attempts to utilize CellProfiler (Carpenter et al., 2006; Kamentsky et al., 2011; Mc-

Quin et al., 2018; Stirling et al., 2021) to automate capture of various details about the THP-1 macrophages from Subsection 3.3.9 and Subsection 3.3.10, I wished to incorporate LaRoMe into Python pipelines to increase my throughput of analysis. This, however proved challenging based on the original design of the plugins, as they did not accept an image as an argument and expected to use the currently active image window.

I therefore completely rewrote these plugins explicitly so that they would accept an image as the primary argument, which allows for easy integration into looping structures. In the process, the entire plugin was translated from Java to Python. It is hoped that in the future, these code bases can be reconciled so that the existing distribution of LaRoMe, managed by BIOP, can incorporate both the excellent GUI options already existing and allow for interfacing through scripts for high-throughput applications.

Source Code 4.8: A Python translation of the FIJI function “Label image to ROIs” from LaRoMe. This function allows the user to take images generated from CellProfiler and convert them into a set of regions of interest in the ROI Manager.

```
from ij import IJ, ImagePlus
from ij.gui import Wand, PolygonRoi, Roi
from ij.plugin import Duplicator
from ij.plugin.frame import RoiManager
from ij.process import ImageProcessor
import os

def labelsToROIs(imp):

    rm = RoiManager.getRoiManager()

    def L2R(imp):
        ip = imp.getProcessor()
        wand = Wand(ip)

        width = imp.getWidth()
        height = imp.getHeight()
```

```

pixel_width = []

for num in range(0, width - 1):
    pixel_width.append(num)

pixel_height = []

for num in range(0, height - 1):
    pixel_height.append(num)

ip.setColor(0)

for y_coord in pixel_height:
    for x_coord in pixel_width:
        if ip.getPixel(x_coord, y_coord) > 0:
            wand.autoOutline(x_coord, y_coord)
            if wand.npoints > 0:
                roi = PolygonRoi(wand.xpoints, wand.ypoints, wand.npoints,
                                  Roi.TRACED_ROI)
                roi.setPosition(imp.getCurrentSlice())
                ip.fill(roi)
                rm.addRoi(roi)
            rx = rm.getRoisAsArray()
            rm.reset()
            rois = []
            for r in rx:
                if r not in rois:
                    rois.append(r)
                    rm.addRoi(r)

            rm.runCommand(imp, "Show All")

copy_imp = imp.duplicate()
rm.reset()

dimensions = copy_imp.getDimensions()
nChannels = dimensions[2]
nSlices = dimensions[3]
nFrames = dimensions[4]

if (nChannels > 1 and nSlices > 1) or (nChannels > 1 and nFrames > 1)
    or (nSlices > 1 and nFrames > 1):
    print(imp.getTitle() + " is a hyperstack (multi c , z or t), please
          prepare a stack (single c, either z-stack or t-stack) from it.")

```

```

    pass
elif nChannels > 1 or nSlices > 1 or nFrames > 1:
    for stack in range(1, copy_imp.getImageStackSize() + 1):
        copy_imp.setPosition(stack)
        L2R(copy_imp)
else:
    L2R(copy_imp)

```

Source Code 4.9: A Python translation of the FIJI function “ROIs to label image” from LaRoMe. This allows the user to use a set of ROIs to regenerate a label image, useful for creating masks on existing images and comparing areas between different channels.

```

from ij import IJ, ImagePlus
from ij.gui import Wand, PolygonRoi, Roi
from ij.plugin import Duplicator, Concatenator
from ij.plugin.frame import RoiManager
from ij.process import ImageProcessor
import os, re

def ROIsToLabels(rois, imp_path = None):

    rm = RoiManager.getRoiManager()
    rm.reset()
    rm.runCommand("Open", rois)
    rc = rm.getRoisAsArray()

    if not imp_path:
        pxm = []
        pym = []
        lrs = []
        for r in rc:
            lr = list(r)
            for l in lr:
                lrs.append(str(l).lstrip("java.awt.Point"))
        for lr in lrs:
            px = re.findall("x=\d+", lr)
            for p in px:
                pxm.append(int(p.lstrip("x=")))
            py = re.findall("y=\d+", lr)
            for p in py:
                pym.append(int(p.lstrip("y=")))

```

```

imp_title = os.path.splitext(os.path.basename(rois))[0]
imp = IJ.createImage(imp_title, "8-bit black", max(pxm), max(pym), 1)
elif imp_path:
    imp = IJ.openImage(imp_path)

def R2L(imp):
    label_imp = imp.duplicate()
    IJ.run(label_imp, "Grays", "")
    IJ.run(label_imp, "16-bit", "")
    rc = rm.getRoisAsArray()

    stackN = label_imp.getImageStackSize()
    isStack = bool()
    if stackN > 1:
        isStack = True

    if int(len(rc)) in range(1, 255):
        IJ.run(label_imp, "8-bit", "")
    elif int(len(rc)) in range(256, 65535):
        IJ.run(label_imp, "16-bit", "")
    else:
        IJ.run(label_imp, "32-bit", "")

    for stack in range(1, stackN + 1):
        label_imp.getStack().getProcessor(stack).setValue(0)
        label_imp.getStack().getProcessor(stack).fill()

    for i in range(1, len(rc)):
        if isStack:
            label_imp.setPosition(rc[i].getPosition())
        label_imp.getProcessor().setValue(i + 1)
        label_imp.getProcessor().fill(rc[i])

    label_imp.setTitle("ROIsToLabels_" + imp.getTitle())
    imp_dir = os.path.dirname(rois)
    limp_title = label_imp.getTitle()
    IJ.saveAs(label_imp, "Tiff", os.path.join(imp_dir, limp_title))

    rm.reset()

dimensions = imp.getDimensions()
nChannels = dimensions[2]
nSlices = dimensions[3]
nFrames = dimensions[4]

```

```

if (nChannels > 1 and nSlices > 1) or (nChannels > 1 and nFrames > 1)
    ↵ or (nSlices > 1 and nFrames > 1):
        print(imp.getTitle() + " is a hyperstack (multi c , z or t), please
            ↵ prepare a stack (single c, either z-stack or t-stack) from it.")
        pass
elif nChannels > 1 or nSlices > 1 or nFrames > 1:
    for stack in range(1, imp.getImageStackSize() + 1):
        imp.setPosition(stack)
        R2L(imp)
else:
    R2L(imp)

```

Source Code 4.10: A Python translation of the FIJI function “ROIs to Measurement Image”. This combines the a defined set of ROIs (probably from labelsToROIs.py) and a raw image and generates an image that graphically represents measurements such as area or circularity.

```

from ij import IJ, ImagePlus
from ij.gui import Roi
from ij.plugin import Duplicator
from ij.plugin.frame import RoiManager
from ij.process import ImageProcessor, ImageStatistics
import math, os

def ROIsToMap(imp_path, rois, column_name):

    rm = RoiManager.getRoiManager()
    rm.reset()
    rm.runCommand("Open", rois)

    label = "Measure"
    choices = ["area", "angle", "anglevert", "ar", "circumference",
        ↵ "major", "minor", "mean",
        "median", "mode", "min", "max", "perimeter", "xcenterofmass",
        ↵ "ycenterofmass"]

    if str(column_name).lower() not in choices:
        print("Invalid column_name, try again")
        exit()

    def R2M(imp):

        imp2 = imp.duplicate()
        saveloc = str(imp.getOriginalFileInfo().directory)

```

```

title = imp.getTitle()

if imp.getBitDepth() == 32:
    IJ.run(imp2, "16-bit", "")

stackN = imp2.getImageStackSize()
isStack = bool()

if stackN > 1:
    isStack = True

for stack in range(1, stackN + 1):
    imp2.getStack().getProcessor(stack).setValue(0)
    imp2.getStack().getProcessor(stack).fill()

IJ.run(imp2, "32-bit", "")

rc = rm.getRoisAsArray()

filling_value = 0

for i in range(0, len(rc)):
    if isStack:
        imp.setPosition(rc[i].getPosition())
        imp2.setPosition(rc[i].getPosition())
        ip = imp.getProcessor()
        ip.setRoi(rc[i])

        ip2 = imp2.getProcessor()
        ip2.setRoi(rc[i])

        ip_stats = ip.getStatistics()

        if column_name.lower() == "area":
            filling_value = ip_stats.area
        elif column_name.lower() == "angle":
            filling_value = ip_stats.angle
        elif column_name.lower() == "anglevert":
            filling_value = ip_stats.angle - 90
        elif column_name.lower() == "ar":
            filling_value = ip_stats.major / ip_stats.minor
        elif column_name.lower() == "circumference":
            filling_value = 4 * math.pi * ip_stats.area /
                math.pow(rx[i].getLength(), 2)
        elif column_name.lower() == "major":

```

```

        filling_value = ip_stats.major
    elif column_name.lower() == "minor":
        filling_value = ip_stats.minor
    elif column_name.lower() == "mean":
        filling_value = ip_stats.mean
    elif column_name.lower() == "median":
        filling_value = ip_stats.median
    elif column_name.lower() == "mode":
        filling_value = ip_stats.mode
    elif column_name.lower() == "min":
        filling_value = ip_stats.min
    elif column_name.lower() == "max":
        filling_value = ip_stats.max
    elif column_name.lower() == "perimeter":
        filling_value = rc[i].getLength()
    # elif column_name.lower() == "pattern":
    #     roi_name = rc[i].getName()
    #     r = Pattern.compile(pattern)
    #     m = r.matcher(roi_name)
    #     if m.find():
    #         group = m.group(1)
    #     try:
    #         filling_value = Float.parseFloat(group)
    elif column_name.lower() == "xcenterofmass":
        filling_value = ip_stats.xCenterOfMass
    elif column_name.lower() == "ycenterofmass":
        filling_value = ip_stats.yCenterOfMass

    ip2.setValue(filling_value)
    ip2.fill(rc[i])
    imp2.setProcessor(ip2)

    imp2.setTitle(column_name.lower() + "_" + title)
    imp2.show()
    imp2.setRoi(0, 0, imp2.getWidth(), imp2.getHeight())
    IJ.saveAs(imp2, "Tiff", os.path.join(saveloc, imp2.getTitle()))

R2M(imp)

```

## 4.7 Experimental Blinding via a Single-Click Command Line Interface<sup>3</sup>

Source Code 4.11: A script to conduct computational filename blinding from the command line written in Python.

```
#!/usr/bin/python3

import os, csv, string, random, sys, argparse
from os import path

def blindrename(folder, r = False):

    # Root = bad for so many reasons.
    if os.geteuid() == 0:
        exit(f"For safety reasons, you must NOT be root when running
            ↵ renamer.py. Please become a non-root user, make sure that user
            ↵ has permissions to write to all files in {folder}, and try
            ↵ again.")

    # Needs a folder, if none is provided, exit.
    if not folder:
        exit("You must supply a folder name. I will randomly rename all files
            ↵ in that folder, and create a key file in .CSV format in that
            ↵ folder.")

    # The folder needs to already exist.
    if not path.isdir(folder):
        exit(f"{folder} is not a valid, existing folder. Try again - typo
            ↵ maybe?")

    # And it presumably needs to have some number of non-hidden files in
    ↵ it. Seems harmless if it is empty, but not expected behavior.

    contents = os.listdir(folder)
    if len(contents) == 0:
        exit("Looks like that folder is empty!")

    # Avoid double renaming.
    if path.exists(os.path.join(folder, "keyfile.csv")):
```

---

<sup>3</sup>Implementation from Brewer et al. (2022b), original conception from Salter (2016).

```

    exit("Keyfile already exists. Have you already randomized that
         ↵   folder?")

 csvfile = open(path.join(folder, "keyfile.csv"), "w")
 writer = csv.writer(csvfile)
 writer.writerow(["original", "file.path"])

 chars = str(string.ascii_letters + string.digits)

 print("Renaming: ")

for dirpath, subdirs, files in os.walk(folder):
    subdirs[:] = [d for d in subdirs if not d.startswith(".")]
    files = [f for f in files if not f.startswith(".")]
    if r:
        old_names = [path.join(dirpath, f) for f in files]
    else:
        old_names = [path.join(folder, f) for f in files if dirpath is
                     ↵   folder]
    for old_name in old_names:
        if path.isfile(old_name):
            if path.basename(old_name) == "keyfile.csv":
                pass
            else:
                base = path.basename(old_name).rsplit(os.extsep, 1)
                # This ensures that there is a 1 index for files with no
                ↵   extension
                base.append("")
                cloaked_name = "".join(random.choices(chars, k = 5))
                if base[1]:
                    new_name = path.join(dirpath, cloaked_name + "." + base[1])
                if not base[1]:
                    new_name = path.join(dirpath, cloaked_name)
                print(old_name, " to ", new_name)
                writer.writerow([old_name, new_name])
                os.rename(old_name, new_name)

    print("Finished!")

def unblind(folder):

    if os.geteuid() == 0:

```

```

exit(f"For safety reasons, you must NOT be root when running
    ↵ renamer.py. Please become a non-root user, make sure that user
    ↵ has permissions to write to all files in {folder}, and try
    ↵ again.")

# Needs a folder, if none is provided, die
if not folder:
    exit("You must supply a folder name. I will randomly rename all files
        ↵ in that folder, and create a key file in .CSV format in that
        ↵ folder.")

# The folder needs to already exist.
if not path.isdir(folder):
    exit(f"{folder} is not a valid, existing folder. Try again - typo
        ↵ maybe?")

# And it presumably needs to have some number of non-hidden files in
    ↵ it. Seems harmless if it is empty, but not expected behavior.

contents = os.listdir(folder)
if ".DS_Store" in contents:
    contents.remove(".DS_Store")
if len(contents) == 0:
    exit("Looks like that folder is empty!")

# Needs a keyfile to function.
if not path.exists(os.path.join(folder, "keyfile.csv")):
    exit("No keyfile?")

 csvfile = open(path.join(folder, "keyfile.csv"), "r")
 reader = csv.reader(csvfile)

print("Renaming: ")

for file in reader:
    masked = file[1]
    original = file[0]
    if path.exists(masked):
        print(masked + " to " + original)
        os.rename(masked, original)

print("Finished!")

if __name__ == '__main__':

```

```

parser = argparse.ArgumentParser(description='Blind rename/unblind a
    ↵ folder of files')
parser.add_argument("function", nargs = 1, action = "store", help =
    ↵ "Which command would you like to run? [blindrename] or [unblind]")
parser.add_argument("folder", nargs = 1, action = "store", help =
    ↵ "Provide the folder you wish to rename. Drag and drop is usually
    ↵ fine.")
parser.add_argument("--r", default = False, required = False, action =
    ↵ "store_true", help = "Do you want to rename files in the
    ↵ subdirectories as well? If so, provide [--r]")
args = parser.parse_args()
print(args)
if "blindrename" in args.function:
    blindrename(args.folder[0], args.r)
if "unblind" in args.function:
    unblind(args.folder[0])

```

In the process of data collection (especially image acquisition), the experimenter will typically assign each file a logical name indicative of what it contains. However, these file names are also a critical breakdown in analytical blinding and ways to avoid this are essential to prevent the introduction of excess experimenter bias into the process of analysis. This problem is clearly widespread, but solutions are difficult to come by. Individuals can have labmates or others to rename folders or files to obfuscate their contents, but this has the danger of the human element – it would not be that difficult to lose track of any alterations and make subsequent quantitation worthless. Thus, a computationally robust method is required.

Salter (2016) saw this issue and elegantly addressed it through a Perl script (Wall, Christiansen, and Orwant, 2000). The author generated a script that would allow you to provide a single argument – a folder – and have the contents renamed and output a keyfile. This implementation was excellent and used throughout my work, but as time passed, I found it lacking in two major areas: recursion and the ability to undo the renaming. I thus generated a Python implementation that was able

to (optionally) recursively move through subdirectories, would provide the exact file path as the original name of the file (useful for feeding into R or FIJI), and came with the ability to provide a folder and keyfile and name all the files back to the original name. In my opinion, these features make for a more complete blinding solution and will hopefully be adopted more widely by users looking to reduce their experimental bias.

This script can be used by first making it executable:

```
chmod +x ./renamer.py
```

And then calling it by:

```
./renamer.py [function] [file directory to rename] [--r]
```

Where the “–r” argument is either left absent (for no recursion) or is provided “–r” for folder recursion through all non-hidden subdirectories. False is the default for safety. This is a reasonable user-friendly option via the command line is should make experimental blinding much easier. The function is either “blindrename” or “unblind” and the directory can be provided as a naked string (that is, no “” required).

## 4.8 User-Friendly Analysis of RNA Sequencing Data using Kallisto/Sleuth in a Python Environment<sup>4</sup>

While an old technology today, the analysis of RNA sequencing data still unfairly remains a challenge for the technologically naïve researcher. To ameliorate part of this problem, in the course of the work in Saelens et al. (2022), I developed a set

---

<sup>4</sup>Taken and expanded from personal contributions to Saelens et al. (2022).

of pipelines for the analysis of RNAseq data using a combination of Kallisto and Sleuth, a pair of analysis and visualization applications that utilize pseudoalignment to calculate read counts and then display them in a Shiny application via R (Pimentel et al., 2017).

Conducting this portion of the work required acquainting myself with a number of commonly used computational tools, including cmake and a deeper knowledge of Python and how that can translate into generating a broadly useful cross-platform tool to analyze complex sequencing data. Doing so also required interfacing with FTP and other networking functions and navigating server directories to fetch reference cDNA from Ensembl.

While these were originally implemented as two parallel scripts, one for bacteria and the other for eukaryotes, they have since been consolidated into a single all-purpose script that requests different input based on what is available. The issue with bacterial analysis is that Ensembl has discontinued generating bioMarts for bacterial genomes and has adopted an unpredictable folder structure for fetching these files via FTP. Thus, the user will have to provide the reference transcriptome of their bacterial strain of choice, which can generally be acquired from species-specific databases (Mycobrowser being the notable one here) or from the set of available strains on NCBI or Ensembl. Nonetheless, this should offer a guided experience in the command line to the analysis of RNA sequencing data and provide output that can then be analyzed using either DESeq2 or Sleuth.

Source Code 4.12: A guided command line application for the analysis of bulk RNA-seq data using Kallisto.

```
#!/usr/bin/env python3

# macOS ONLY
```

```

# People need to have installed Anaconda for this to work. Presumably
# that can be done easily enough. Alt: execute another script first.

import re, os, ftplib, subprocess, glob, sys, shutil, platform

# This gives the script some self awareness. It finds itself and changes
# the working directory to that path (temporarily).
# This is important for executing the brew_installer.sh script.
script_path = os.path.dirname(os.path.realpath(__file__))
os.chdir(script_path)

# Welcome to the era of Apple Silicon.

if "macOS" in platform.platform():
    # This will check to see if several important system programs are
    # installed in sequential order.
    # If they are not, then it executes a script to install them, may
    # require user password.
    if not shutil.which('xcode-select'):
        subprocess.run(['./brew_installer.sh'])
    if not shutil.which('brew'):
        subprocess.run(['./brew_installer.sh'])
    else:
        subprocess.run(["brew", "install", "hdf5"])
    if "arm64" in platform.platform():
        kallisto = "./kallisto_as"
    elif "x86_64" in platform.platform():
        kallisto = "./kallisto_intel"
    elif "Linux" in platform.platform():
        kallisto = "./kallisto_linux"
        subprocess.run(["sudo", "apt-get", "install", "libhdf5-dev"])

from termcolor import colored, cprint
import Bio

fastq_dir = input("Enter the directory of your FASTQ files (drag and drop
# is fine): ")
fastq_dir = fastq_dir.strip()
try:
    os.chdir(fastq_dir)
except FileNotFoundError:
    text = colored("Looks like that directory does not exist - restart the
# script and try dragging and dropping the folder directly into
# Terminal.", "red")
    print(text)

```

```

    sys.exit(1)
except PermissionError:
    print(text)
    sys.exit(1)

phylum = input(colored('Is your organism an [animal], [plant], [fungus],
    ↵ [protist], or [metazoan]? Input one of the options within the
    ↵ brackets: ', 'cyan'))
phylum = phylum.lower()
organism_name = input(colored("Input the 'Genus species' for your
    ↵ reference organism: ", 'magenta'))
org_split = organism_name.lower().split()
org_dir = "_" .join(org_split)

# This is where the user-friendliness comes in: use Ensembl's highly
    ↵ regular patterning (for eukaryotes) to fetch the needed file for
    ↵ supported organisms.

# Vertebrates/Model Animals:
if 'ani' in phylum:
    try:
        with ftplib.FTP('ftp.ensembl.org') as ftp:
            ftp.login('anonymous')
            ftp.cwd('/pub/current_fasta/{}/cdna/'.format(org_dir))
            for filename in ftp.nlst(pattern):
                fhandle = open(filename, 'wb')
                ftp.retrbinary('RETR ' + filename, fhandle.write)
                fhandle.close()
    except ftplib.error_perm:
        text = colored("It appears that your organism is not supported. Try
            ↵ running again with a closely related species or check
            ↵ spelling.", 'red')
        print(text)
        sys.exit(1)

# Fungi:
if 'fu' in phylum:
    try:
        with ftplib.FTP('ftp.ensemblgenomes.org') as ftp:
            ftp.login('anonymous')
            ftp.cwd('/pub/current/fungi/fasta/{}/cdna/'.format(org_dir))
            for filename in ftp.nlst(pattern):
                fhandle = open(filename, 'wb')
                ftp.retrbinary('RETR ' + filename, fhandle.write)
                fhandle.close()

```

```

except ftplib.error_perm:
    text = colored("It appears that your organism is not supported. Try
    ↵ running again with a closely related species or check
    ↵ spelling.", 'red')
    print(text)
    sys.exit(1)

# Plants:
if 'pl' in phylum:
    try:
        with ftplib.FTP('ftp.ensemblgenomes.org') as ftp:
            ftp.login('anonymous')
            ftp.cwd('/pub/current/plants/fasta/{}/cdna/'.format(org_dir))
            for filename in ftp.nlst(pattern):
                fhandle = open(filename, 'wb')
                ftp.retrbinary('RETR ' + filename, fhandle.write)
                fhandle.close()
    except ftplib.error_perm:
        text = colored("It appears that your organism is not supported. Try
        ↵ running again with a closely related species or check
        ↵ spelling.", 'red')
        print(text)
        sys.exit(1)

# Protists:
if 'pr' in phylum:
    try:
        with ftplib.FTP('ftp.ensemblgenomes.org') as ftp:
            ftp.login('anonymous')
            ftp.cwd('/pub/current/protists/fasta/{}/cdna/'.format(org_dir))
            for filename in ftp.nlst(pattern):
                fhandle = open(filename, 'wb')
                ftp.retrbinary('RETR ' + filename, fhandle.write)
                fhandle.close()
    except ftplib.error_perm:
        text = colored("It appears that your organism is not supported. Try
        ↵ running again with a closely related species or check
        ↵ spelling.", 'red')
        print(text)
        sys.exit(1)

# Metazoans:
if 'met' in phylum:
    try:
        with ftplib.FTP('ftp.ensemblgenomes.org') as ftp:

```

```

        ftp.login('anonymous')
        ftp.cwd('/pub/current/metazoa/fasta/{}/cdna/'.format(org_dir))
        for filename in ftp.nlst(pattern):
            fhandle = open(filename, 'wb')
            ftp.retrbinary('RETR ' + filename, fhandle.write)
            fhandle.close()
    except ftplib.error_perm:
        text = colored("It appears that your organism is not supported. Try
                       ↵ running again with a closely related species or check
                       ↵ spelling.", 'red')
        print(text)
        sys.exit(1)

# Error Handling:
valid_responses = ('ani', 'fu', 'pl')
if not any(s in phylum for s in valid_responses):
    text = colored("Looks like you've entered an improper phylum. Try
                   ↵ again!", "red")
    print(text)
    sys.exit(1)

# Looks like everything works up to here now.

# Define some potentially useful variables that I can plug into
# subprocess.
kallisto = './kallisto'
index = 'index'
quant = 'quant'

# Fetch the index file (name independent, it just needs to end in the
# pattern defined above)
# All Ensembl cDNA files should have that precise formatting.

pattern = '*.cdna.all.fa.gz'

ref_cdna = glob.glob(pattern)
ref_cdna = ref_cdna[0]

exts = ('*.fastq.gz', '*.fq.gz', "*.fastq", "*fq")

threads = int(os.cpu_count())

# This checks whether you already have an index and, if so, whether you
# want to build a new one.

```

```

index_checker = os.path.isfile('kallisto_index')

if index_checker:
    indexed = input(colored("Would you like to build a fresh transcriptome
    ↪ index? This is optional [Y/N] ", 'green'))
    if indexed == "Y":
        if "bac" in phylum:
            ref_cdna = input(colored("Provide a path to the reference cDNA for
            ↪ your organism: ", "green")).rstrip()
            subprocess.call([kallisto, index, '-i', 'kallisto_index', ref_cdna])
        else:
            pass
    else:
        if "bac" in phylum:
            ref_cdna = input(colored("Provide a path to the reference cDNA for
            ↪ your organism: ", "green")).rstrip()
            subprocess.call([kallisto, index, '-i', 'kallisto_index', ref_cdna])

while True:
    valid = ('pe', 'se')
    read_type = input(colored("Are your reads paired-end [PE] or
    ↪ single-end [SE]? Paired-end reads have two files for each
    ↪ condition - one with '_1' and one with '_2' [PE/SE] ", "yellow"))
    if read_type.lower() not in valid:
        text = colored("Looks like you had a typo in the last prompt!",
        ↪ "red")
        print(text)
        continue
    else:
        break

# This script runs for PE reads - it can estimate fragment length from
    ↪ this and doesn't need you to provide the information.

cnt = 0

if 'PE' in read_type.upper():
    fastqs = glob.glob("./*_1.f*q*")
    try:
        for forward in fastqs:
            reverse = forward.replace("_1.f", "_2.f")
            matcher = re.search("_1.f.*q*", forward).group(0)
            dir = forward.replace(matcher, "")
            subprocess.run([kallisto, quant,
            '-i', 'kallisto_index',

```

```

        '-o', dir + '_quant',
        '--bias',
        '-b', '200',
        '-t', threads,
        forward, reverse])
    cnt += 1
    print(cnt)
except:
    text = colored("Looks like something went wrong.", "red")
    print(text)
    sys.exit(1)
else:
    pass

# This needs a little bit more user-engagement - average fragment length
↳ and the SD are required, but can be substituted by guesses if it is
↳ not known (it rarely is).

cnt = 0

if 'SE' in read_type.upper():
    fastqs = glob.glob("./.*.f*q*")
    frag_len = 150
    standard_dev = 10
    frags = input("If known, input estimated average fragment length (from
      ↳ FastQC). If not known, hit enter: ")
    if not frags:
        frags = frag_len
    sd = input("If known, input the standard deviation of the fragment
      ↳ length (from FastQC). If not known, hit enter: ")
    if not sd:
        sd = standard_dev
    try:
        for read in fastqs:
            matcher = re.search(".f.*q*", read).group(0)
            dir = read.replace(matcher, "")
            subprocess.run([kallisto, quant,
                           '-i', 'kallisto_index',
                           '-o', dir + '_quant',
                           '--bias',
                           '-b', '200',
                           '-t', threads,
                           '--single',
                           '-l', frags,
                           '-s', sd,

```

```

        read])
        cnt += 1
        print(cnt)
    except:
        text = colored("Looks like something went wrong.", "red")
        print(text)
        sys.exit(1)
else:
    pass

input(colored("All finished! You are ready to proceed with further
← analysis and visualization in RStudio.", 'green', 'on_white'))

```

This script automates many of the most confusing steps for end users by providing an FTP backend to, whenever possible, fetch the needed files aside from the .FASTQ files from the sequencer. Additionally, the use of Kallisto over more traditional alignment methods like STAR or Bowtie allows this to be trivially run on any modern computer without requiring the user to interface with a computing cluster, which is a relatively steep learning curve for many. Kallisto has other advantages as it is much faster and also more accurate than other alignment methods thanks to its pseudoalignment strategy versus more traditional, computationally intensive find-and-match approaches. Kallisto is also robust to unprocessed .FASTQ files, allowing the user to, in many cases, skip adapter trimming and other preprocessing steps. While there is still a place for STAR and HISAT alignments for *de novo* transcript discovery, in most instances, Kallisto will suffice for the task.

Source Code 4.13: Pipeline for the visualization of Kallisto-aligned RNA seq data using Sleuth. This version supports both eukaryotes and bacteria, albeit through two distinct methods of gathering gene lists.

```

# Sleuth Analysis of Kallisto Output
# Created: 29 September 2017
# Last Edited: 5 August 2022
# Jared Brewer

```

```

# You have to provide exactly two things:
# The directory where you did everything and set the working directory
# to that location using setwd()
# A sample table formatted with two columns: sample and condition and
# named sample_table.txt
# After running, you'll get an interactive window where you can play with
# your data and look for effects.
# There is merit in breaking out comparisons if you have a bunch of
# conditions...

setwd("~/esxM")

if (!require("BiocManager", quietly = TRUE))
  install.packages("BiocManager")
BiocManager::install(version = "3.15")

BiocManager::install(c("rhdf5", "biomaRt"))

install.packages("devtools")
devtools::install_github("pachterlab/sleuth")

library(sleuth)

library(tidyverse)
library(biomaRt)
library(data.table)
library(pheatmap)
library(tidyr)

# This analysis was optimized for animals - biomaRt access to bacterial
# genomes has been discontinued and is a mess.
mart <- read_tsv("./gene_list.txt")
t2g <- as.data.frame(mart>Name)
names(t2g)[1] <- "target_id"
t2g.2 <- t2g[!duplicated(t2g$target_id),]
t2g.2 <- as.data.frame(t2g.2)
names(t2g.2)[1] <- "target_id"
t2g.2$gene_id <- t2g.2$target_id

# Eukaryotes only:
mart <- useMart(biomart = "ENSEMBL_MART_ENSEMBL", dataset =
  "hsapiens_gene_ensembl")
t2g <- getBM(attributes = c("ensembl_transcript_id_version",
  "ensembl_gene_id", "external_gene_name"), mart = mart)

```

```

t2g <- rename(t2g, target_id = ensembl_transcript_id_version, ens_gene =
  ↵  ensembl_gene_id, ext_gene = external_gene_name)
# 

files.kal <- list.files(pattern = "./*_quant")
files.dirs <- sapply(files.kal, function(id) file.path(".", id))
sample.table <- read.table("./sample_table.txt", header = T)
sample.table <- dplyr::mutate(sample.table, path = files.dirs)

so <- sleuth_prep(sample.table,
  full_model = ~condition,
  target_mapping = t2g.2,
  extra_bootstrap_summary = T,
  aggregation_column = "target_id")
so <- sleuth_fit(so, ~condition, "full")
so <- sleuth_fit(so, ~1, 'reduced')
so <- sleuth_lrt(so, 'reduced', 'full')
# so <- sleuth_wt(so, which_beta = 'condition', which_model = 'full')

sleuth_table <- sleuth_results(so, 'reduced:full', 'lrt', show_all =
  ↵  FALSE)
sleuth_significant <- dplyr::filter(sleuth_table, pval <= 0.05)

sleuth_live(so)

de <- subset(sleuth_table, pval < 0.05)
paths <- enrichPathway(gene = trimws(de$entrez), pvalueCutoff = 0.2,
  ↵  readable = T, organism = "Mycobacterium_tuberculosis_h37rv")

tpm.mat <- sleuth_to_matrix(so, which_df = "obs_raw", which_units =
  ↵  "tpm")
tpm <- as.data.frame(tpm.mat)

# Read straight from Sleuth matrix:
setDT(tpm, keep.rownames = "genes")
esx <- tpm |> filter(endsWith(genes, "_5")) |> column_to_rownames(var =
  ↵  "genes")
esx <- as.matrix(esx)
write.csv(x = tpm.mat, file = "esx_5_tpm.csv")
pheatmap(esx, show_colnames = F, annotation_legend = T, filename =
  ↵  "rnaseq_heatmap_070622.png", width = 6, height = 2)

# Read from saved .csv file (this is the method used for the actual
  ↵  figure):
esx <- read.csv("./esx_5_tpm.csv", header = T)

```

```
row.names(esx) <- esx$target_id  
esx <- esx |> filter(endsWith(target_id, "_5"))  
esx <- esx[-c(1:2)]  
esx <- as.matrix(esx)  
pheatmap(esx, show_colnames = F, annotation_legend = T, filename =  
  "rnaseq_heatmap_070622.png", width = 6, height = 2)
```

Due to policy changes by Ensembl, retrieving pre-packaged marts for bacterial genomes is now impossible on the grounds that the number of bacterial strains has outgrown Ensembl's ability to provide these marts. Despite the difficulties this creates for finding reference gene lists for even the most common bacterial species (including even *Escherichia coli*), this can be subverted by converting gene lists available from species-specific repositories into a mart-like object that can then be utilized for the downstream steps. No user wants to look at a list of Ensembl IDs after quantitation, and so provision of common gene names is an important component of any analysis. This analysis pipeline was generated specifically for *Mycobacterium tuberculosis*, but could easily be modified to support almost any species.

The logic for fetching and processing bioMarts remains in the script, for use with eukaryotic species. As many of the later steps converge, the user can choose to run particular sets of lines based on their organism. Future versions might wrap some GUI elements or starting booleans to only execute the appropriate commands when needed, but in my experience, it is possible to get a new user working with this script with minimal overall effort.

## 4.9 Bacterial Burden Analysis by Fluorescence Intensity in a Semi-Automated Manner with a User-Friendly Graphical Interface

One of the major routine tasks in the field of zebrafish-*M. marinum* host-pathogen interactions is the quantitation of the total bacterial burden per larva. While it has been well established that the integrated fluorescence intensity of the image corresponds well to the colony forming units of bacteria present, the larval zebrafish has particular challenges. Many of these are attributable to autofluorescence from the yolk and pigment cells or the physical background of the imaging surface, but it is important to avoid catching these in the quantitation as these can vary greatly from fish to fish and are difficult to subtract *post hoc*. However, through clever approaches to image pre-processing it is possible to eliminate these sources of misquantitation and streamline analysis to minimize user intervention.

Source Code 4.14: This graphical user interface allows for automatic background subtraction from images of *M. marinum*-infected larval zebrafish and then quantitation of the remaining signal above a manually set threshold that captures as much of the true signal as possible.

```
from ij import IJ, ImagePlus
from ij.measure import ResultsTable, Measurements
from ij.plugin.frame import RoiManager
from ij.process import AutoThresholder
from ij.plugin import ZProjector
from ij.plugin.filter import ParticleAnalyzer
from ij.gui import GenericDialog, NonBlockingGenericDialog
import os, random, csv, sys, re
from os import path
```

```

def burden(directory, chan, min_threshold, ext, screen_threshold = "Otsu
    ↵ dark", proj_save = False, proj_show = False, imp_show = False,
    ↵ fish_channel = None, outline_threshold = "Triangle dark", brightfield
    ↵ = False, subset = 0, man_psize = 100000):

# directory is a string with path to the files you wish to analyze.
# chan is an integer of the channel # with the bacteria.
# min_threshold is an integer of the minimum threshold value (empirically
    ↵ determined by checking a few images)
# ext is the file extension (" .czi", ".lsm", ".tif" are some examples) as
    ↵ a string
# screen_threshold is the threshold algorithm to find the yolk as a
    ↵ string. I found that "Otsu dark" works well, but others may be better
    ↵ in other circumstances.
# proj_save is a boolean that determines whether or not to save the MIPs
    ↵ as you go.
# proj_show is a boolean to determine whether to show the MIPs as they
    ↵ are processed. A bit memory intensive, but valuable for sanity
    ↵ checking.
# imp_show is a boolean for whether or not to show the source image (not
    ↵ recommended except with small datasets)
# fish_channel is an integer for the channel containing something we
    ↵ could use as the basis for an outline of the fish.
# outline_threshold is a string with the thresholding algorithm to find
    ↵ the whole fish (you want something generous, but probably not
    ↵ MinError or similar).
# brightfield is a boolean telling whether or not the selected
    ↵ fish_channel is the brightfield channel. This can be useful and very
    ↵ convenient, but requires some additional processing to be useful.
# subset is to run the function over a subset of the total number of
    ↵ images. Provide an integer value less than the total number of images
    ↵ in the directory. If subset is selected, the projections will
    ↵ automatically show.
# man_psize is a manual particle size value for the "Analyze
    ↵ Particles..." function. Default has worked well for me, but can be
    ↵ changed if needed.

filenames = os.walk(str(directory))
bfiles = []

if ext.startswith("."):
    # Rigid binding of extension per se.
    ext = ext.replace(".", "")

```

```

# This logic is a little over-complex for the task, but essentially I
# want to pull out the extension and then use it to match the end of
# the line. Why do it in two steps? It makes the code more flexible
# for ".tif" vs ".TIFF" and could allow future use of the ext in
# downstream applications if needed.
for dirpath, subdir, files in filenames:
    for file in files:
        mext = re.search("\." + ext + "\S*$", file, re.IGNORECASE)
        if mext:
            if file.endswith(mext.group()):
                bfiles.append(path.join(dirpath, file))

# Make sure all our measurements are set properly.
IJ.run("Set Measurements...",
"area mean min limit display redirect=None decimal=3")

rm = RoiManager.getRoiManager()
rt = ResultsTable.getResultsTable()
data = []

if subset > len(bfiles):
    wrong_subset = NonBlockingGenericDialog("Subset Error")
    wrong_subset.addMessage("The subset is greater than the number of
    files - running on whole directory.")
    wrong_subset.showDialog()
    subset = 0
if subset:
    bfiles = bfiles[0:int(subset)]
    proj_show = True
else:
    subset = 0

valid_thresholds = [str(i + " dark") for i in
    AutoThresholder.getMethods()]
valid_thresholds.append("Try all")
valid_thresholds.append("None")

if screen_threshold == "Try all" or outline_threshold == "Try all":
    if screen_threshold == "Try all":
        for f in bfiles:
            imp = IJ.openImage(f)
            proj = ZProjector.run(imp, "max all")
            proj.setC(int(chan))
            IJ.run(proj, "Auto Threshold", "method=[Try all] dark")
    if outline_threshold == "Try all":

```

```

for f in bfiles:
    imp = IJ.openImage(f)
    proj = ZProjector.run(imp, "max all")
    proj.setC(int(fish_channel))
    if brightfield:
        IJ.run(proj, "Find Edges", "slice");
    IJ.run(proj, "Auto Threshold", "method=[Try all] dark")
else:
    for i, f in enumerate(bfiles):
        imp = IJ.openImage(f)
        if imp_show:
            imp.show()
        if imp.getDimensions()[3] > 1:
            proj = ZProjector.run(imp, "max all")
        else:
            proj = imp.duplicate()
        if proj_show:
            proj.show()
        proj.setC(int(chan))
    if screen_threshold not in valid_thresholds:
        screen_threshold = "Otsu dark"

    if screen_threshold != "None":
        IJ.setAutoThreshold(proj, str(screen_threshold))

    IJ.run(proj, "Analyze Particles...",
           "size="+str(man_psize)+"-Infinity clear include add slice")

    if rm.getCount() > 1: # This is the dumbest source of a
                           # downstream error I have ever encountered. rm.getCount is an
                           # attribute and rm.getCount() is a method = two different
                           # outcomes, but no evaluation error.
        rm.runCommand(proj, "Select All")
        rm.runCommand(proj, "Combine")
        rm.runCommand(proj, "Delete")
        rm.runCommand(proj, "Add")

    rm.select(proj, 0)

    IJ.run(proj, "Clear", "slice")
    IJ.run(proj, "Select None", "")
    IJ.run(proj, "Remove Overlay", "")
    rm.reset()

```

```

# Build some logic for using the outline of the fish if provided.
# Brightfield images probably need even more work, but this will
# *probably* work for fluorescent channels.
if fish_channel:
    proj.setC(int(fish_channel))
    if brightfield:
        IJ.run(proj, "Find Edges", "slice");
    if outline_threshold not in valid_thresholds:
        outline_threshold = "Triangle dark"
    IJ.setAutoThreshold(proj, outline_threshold)
    IJ.run(proj, "Analyze Particles...",,
          "size="+str(man_psize)+"-Infinity clear include add slice")
    proj.setC(int(chan))
    ra = rm.getRoisAsArray()
    for r in ra:
        rm.addRoi(r)
    if rm.getCount() > 1:
        rm.runCommand(proj, "Select All")
        rm.runCommand(proj, "Combine")
        rm.runCommand(proj, "Delete")
        rm.runCommand(proj, "Add")

    rm.select(proj, 0)

else:
    IJ.run(proj, "Select All", "")

# We want this to work for any bit depth image, but due to
# limitations of the underlying plugin, may only work for 16-bit
# and lower images. Something weird could happen with 24-bit RGB
# images, but I can't stop people from doing weird things.

if proj.getBitDepth() == 24: # This should only be color images -
# seems a little risky.
    IJ.run(proj, "8-bit", "")
elif proj.getBitDepth() > 16: # The thresholds don't work for
# 32-bit images and FIJI doesn't really understand 10 or 12 bit
# images.
    IJ.run(proj, "16-bit", "")

IJ.setRawThreshold(proj, int(min_threshold),
                   2**int(proj.getBitDepth()) - 1)
IJ.run(proj, "Measure", "")
row = rt.getRowAsString().split("\t")

```

```

row.insert(0, path.join(imp.getOriginalFileInfo().directory,
    ↵ imp.getOriginalFileInfo().fileName))
if i == 0:
    headings = rt.getColumnHeadings().split("\t")
    headings.insert(0, "file_path")
data.append(row)

if not imp_show:
    imp.close()
if not proj_show:
    proj.close()

if proj_save:
    out = path.join(directory, proj.getTitle())
    IJ.saveAs(proj, "Tiff", out)

rand = random.randint(1, 100)
if rand > 85:
    IJ.run("Collect Garbage", "")
else:
    pass

# Write out the results to a CSV file for further processing
↳ (probably in R, but also fine for other options.)

with open(path.join(directory, "burden.csv"), "w") as csvfile:
    writer = csv.writer(csvfile)
    writer.writerow(headings)
    for datum in data:
        writer.writerow(datum)

gui = GenericDialog("Finished")
gui.addMessage("All Done! Output is located in " + directory)
gui.showDialog()

def burden_gui():

    values = [str(x + " dark") for x in AutoThreshold.getMethods()]
    values.append("Try all")
    values.append("None")

# Build the GUI elements.
gui = NonBlockingGenericDialog("Bacterial Burden Measurement")
gui.addDirectoryField("Image Folder: ", " ~/Documents")

```

```

gui.addChoice("File Extension: ", [".czi", ".lif", ".lsm", ".tif",
    ↵ "Other"], ".tif")
gui.addStringField("Custom File Extension: ", ".jpeg")
gui.addNumericField("Bacterial Fluorescence Channel #: ", 1)
gui.addNumericField("Minimum Threshold: ", 200)
gui.addMessage("'Try all' requires a subset to be selected - the fewer
    ↵ the better. Default = 5")
gui.addChoice("Background Removal Threshold: ", values, "Otsu dark")
gui.addCheckbox("Save MIPs?", False)
gui.addCheckbox("Show MIPs?", False)
gui.addCheckbox("Show Images?", False)
gui.addCheckbox("Use Fish Outline?", False)
gui.addToSameRow()
gui.addNumericField("Channel # for Fish Outline: ", 2)
gui.addChoice("Fish Threshold: ", values, "Triangle dark")
gui.addCheckbox("Brightfield Image?", False)
gui.addCheckbox("Only do a subset?", False)
gui.addToSameRow()
gui.addNumericField("If yes, how many images to try: ", 0)
gui.addNumericField("Manual Particle Size: ", 100000)
gui.showDialog()
if gui.wasCanceled():
    canceled = GenericDialog("Canceled")
    canceled.showDialog()
    sys.exit(0)

# Parse all of the GUI elements into variables that can be plugged into
    ↵ the burden function.
directory = str(gui.getNextString())
ext = str(gui.getNextChoice())
if ext == "Other":
    ext = str(gui.getNextString()).lower()
else:
    dump = gui.getNextString()

chan = int(gui.getNextNumber())
min_threshold = int(gui.getNextNumber())
screen_threshold = str(gui.getNextChoice())
proj_save = gui.getNextBoolean()
proj_show = gui.getNextBoolean()
imp_show = gui.getNextBoolean()
do_fish = gui.getNextBoolean()
if do_fish:
    fish_channel = int(gui.getNextNumber())
    outline_threshold = str(gui.getNextChoice())

```

```

brightfield = gui.getNextBoolean()
else:
    dump = gui.getNextNumber()
    dump = gui.getNextChoice()
    dump = gui.getNextBoolean()
    fish_channel = None
    outline_threshold = "Triangle dark"
    brightfield = False
subsetter = gui.getNextBoolean()
if subsetter:
    subset = int(gui.getNextNumber())
else:
    dump = gui.getNextNumber() # Another extraordinarily stupid source of
    ↵ error - every entry /must/ be accessed or you're going to
    ↵ frameshift - was feeding 0 into man_psize when subset = False and
    ↵ getting a correspondingly bad result (all points included).
    subset = 0
if screen_threshold == "Try all":
    subsetter = True
    if not subset:
        subset = 5
if outline_threshold == "Try all":
    subsetter = True
    if not subset:
        subset = 5
man_psize = int(gui.getNextNumber())

burden(directory = directory,
        chan = chan,
        min_threshold = min_threshold,
        ext = ext,
        screen_threshold = screen_threshold,
        proj_save = proj_save,
        proj_show = proj_show,
        imp_show = imp_show,
        fish_channel = fish_channel,
        outline_threshold = outline_threshold,
        brightfield = brightfield,
        subset = subset,
        man_psize = man_psize)

burden_gui()

```

This graphical user interface facilitates automatic processing of arbitrary numbers

of images at once by allowing users to select various parameters to test for appropriateness in their particular experiment. The underlying logic will automatically create Z projections if applicable and then use those for subsequent analysis. Users are encouraged to select a subset of images to start and the computational thresholds are used to capture any objects over a certain size for background removal, which will typically only capture the yolk and any background fluorescence. This approach also allows for more generous manual thresholds to be selected for quantitation, an issue that often arises in manual approaches to fluorescence quantitation due to the need to accommodate this autofluorescence.

It is my hope that this application, after further beta testing and refinement can supplant these manual approaches and replace them with something that free researcher time to conduct more experiments rather than spend many hours drawing circles around zebrafish in order to measure the bacterial burden of these fish. Additionally, this could in principle be adapted to measuring other aspects of zebrafish biology, from macrophage clustering to transcriptional reporter signals. The elegance of this approach is that it utilizes open-source and well-defined mechanisms for measuring signal over noise through the implementation of the automatic thresholds and wraps a set of utility functions within an interface that avoids the need for the end user to write burdensome macros to accomplish the same goal. This approach, after a minutes-long period of optimizing is able to measure the burden of thousands of larvae in mere minutes. The output can then be spot-checked for accuracy, as in a small subset of the larvae parts of the background may not be perfectly removed and these can then be reprocessed either manually or with altered parameters.

# Chapter 5

## Conclusions and Future Directions

At the conclusion of the present work, many new questions have been generated while others remain unanswered. This work has accomplished two primary goals: defining the intracellular signaling pathway within macrophages that is responsible for inducing angiogenesis during mycobacterial infection (the NFAT pathway) – a long-standing question in the field of tuberculosis pathogenesis – and setting the stage for future work to simplify and automate common procedures commonly used in the analysis of imaging data relevant to both zebrafish and tissue culture research. Some of these lingering questions will be addressed in the coming weeks and months while others will stretch over the course of many years or decades as we delve into deeper and deeper understandings of the fundamental processes governing the nature of the angiogenic response to tuberculosis infection and how and when this can be a fruitful target for therapeutic intervention.

This leaves a set of important questions, pertinent to model development, deeper understanding of the biology of NFAT within (granuloma) macrophages, the intersections between this pathway and other, known pathways involved in angiogenic responses, and the future of imaging analysis in the context of ever-growing compu-

tational power.

- What is the TDM receptor in zebrafish and do they have an as-yet unannotated MINCLE homolog?
- How or why is NFATC2 special and is it sufficient to induce VEGF?
- How does the NFAT pathway alter other aspects of macrophage behavior potentially relevant to tuberculosis biology and does this pathway intersect with HIF-1 $\alpha$  signaling?
- Aside from TDM, do mycobacteria have other mechanisms for manipulating the host angiogenic response and, if so, what are they and how does that enhance our overall understanding of this process?
- Are our findings on the nature of NFATC2 in inducing tuberculous angiogenesis relevant to other disease contexts where VEGF signaling plays an important role?
- Could NFAT offer a meaningful mechanism for inhibiting angiogenesis in the context of disease as a host-directed therapy?

These questions, among many others, are the subject of this concluding chapter; it is hoped that a comprehensive presentation of these questions will stimulate future generations to pursue answers and that these will further inform our understanding of the pathogenesis of tuberculosis toward the goal of eradicating this disease.

## 5.1 The Zebrafish MINCLE

As discussed in Subsection 1.3.5, data from human cell culture and mice has implicated MCL and MINCLE as the primary C-type lectin receptors for TDM, which

induces a variety of downstream responses, seemingly including the upregulation of VEGF and downstream angiogenesis (see Chapter 3). However, the precise identity of the homolog of MCL or MINCLE in the zebrafish remains unknown. These two proteins arose from a tandem duplication and inversion at an unknown point in evolutionary history, although the two are ubiquitous across reptiles, birds, and mammals (Miyake et al., 2013; Richardson and Williams, 2014). Given the strong, bidirectional selective pressure on both host and pathogen to modulate host PRR activity, these divergences are expected even between closely related species (Rambaruth et al., 2015). This diversification is especially notable among CLRs: mice have no fewer than eight putative DC-SIGN homologs and a great deal of work was done to narrow down the functional ones in order to model human disease (Garcia-Vallejo and Kooyk, 2013); on the other hand, the bovine homolog of MINCLE was readily identifiable but had diverged in non-critical domains from the human MINCLE (Feinberg et al., 2016; Furukawa et al., 2013; Feinberg et al., 2013). Indeed, the murine Mincle is only 67% identical to the human MINCLE, despite an overlapping set of known ligands (Matsumoto et al., 1999). These aspects of structural diversity add unique complexities to the identification of any putative functional homolog in the fish, which may have substantially diverged from the ancestral protein as well as the mammalian versions. Despite these challenges, such identification would both substantially advance the zebrafish-*M. marinum* model and deepen our understanding of shared mechanisms of detection and response to C-type lectin receptor ligands.

Despite these challenges, there is an abundance of evidence that zebrafish possess an as-yet unidentified MINCLE (and/or MCL) homolog including, but not limited to: a long evolutionary history alongside pathogenic mycobacteria, the clear, *myd88*-independent inflammatory response to purified TDM, and the *in vivo* attenuation of mutants lacking fully mature TDM. Zebrafish have long been speculated to have

functional homologs of other C-type lectin receptors despite difficulty in their identification (Petit et al., 2019). MINCLE has not been previously linked to angiogenesis, but the identification of this receptor in zebrafish would allow us to better understand the relevant pathway in humans; indeed, the role of MINCLE during infection is unclear given that some groups have demonstrated that it contributes to bacterial control while others have seen no effect (Behler et al., 2012; Behler et al., 2015; Heitmann et al., 2013; Lee et al., 2012). This has translational implications for modulating the activity of the human MINCLE to enrich for host-beneficial responses and also basic science implications in revealing the diversification of a receptor that maintains the ability to detect a common ligand.

Unlike MINCLE, the basal receptor MCL has clear roles in mediating protection against mycobacteria (Wilson et al., 2015). This might imply that the basal recognition capacities of MCL and potentially later downstream events that depend on MCL/MINCLE interactions are more important than the contributions of MINCLE alone. What is needed is a full locus-deletion mouse that removes both MCL and MINCLE to enable studies on the function of TDM detection *per se in vivo*. While the development of a mouse model to accomplish this would be an excellent contribution, we may be able to fill in some gaps using the zebrafish if we are able to identify the correct homolog in the zebrafish genome.

Although large amino acid segments of CLRs are able to undergo radical changes in primary sequence with few deleterious effects, there are several domains that have been identified as absolutely essential for binding to TDM. A specific set of criteria for this selection are listed in Table 5.1 (Alenton et al., 2017; Feinberg et al., 2016; Feinberg et al., 2013; Bird et al., 2018; Furukawa et al., 2013; Zelensky and Gready, 2005). Based on these criteria, we have identified three putative homologs with

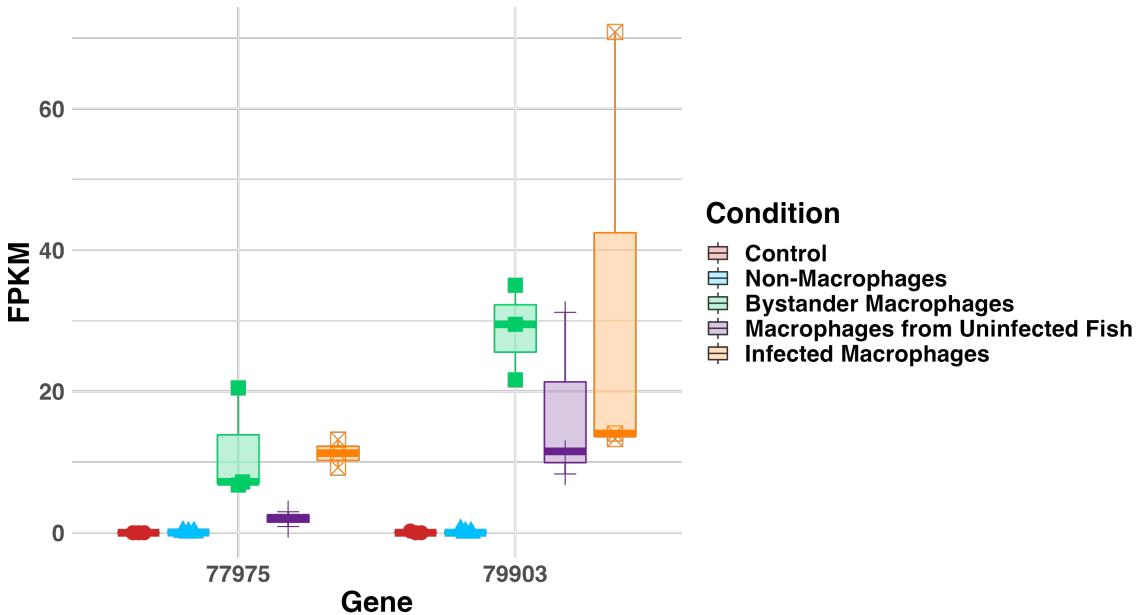
>50% amino acid similarity to the human CLEC4E in the carbohydrate binding domain (Table 5.1) and have identified transmitting nonsense mutations in each of them (Table 5.2). As further evidence, two of these homologs (77975 and 79903) are organized in tandem, mirroring the genomic organization of MINCLE and MCL in mammals.

**Table 5.1:** Criteria used to select putative zebrafish homologs of the human MINCLE.

Criteria	Rationale
Possesses a gEPNn motif	Of the two major carbohydrate recognition domain motifs, the EPN motif is known to bind glucose-derived sugars while QPD motifs are known to bind galactose-derived sugars. As trehalose is a di-glucose and MINCLE and MCL both possess this EPN motif, this is an important first-pass selection criterion.
Lacks an intracellular ITAM motif	In humans, both MINCLE and MCL use Fc $\gamma$ R to signal as they lack their own ITAM motif. While not an essential quality to detect and respond to TDM, this would strengthen the similarities between the two; we have also published data implicating Fc $\gamma$ R in the zebrafish, which argues in favor of this shared layer of similarity as well.
Induced by infection	Using existing RNA-seq datasets, expression of these genes under inflammatory stimulus is an important indicator that they may be acting similarly to MINCLE, which is an inducible gene responsive to various inflammatory stimuli.
Transmembrane helix	These surface receptors use a single-pass transmembrane helix to remain bound to the plasma membrane and transduce signals.
Hydrophobic amino acids in the CRD	One of the defining biochemical features of MINCLE is a small hydrophobic pocket that appears to be useful for binding to the mycolate tails of TDM; the presence of such a pocket would be evocative of further similarity to MINCLE.

Multiple approaches can be taken to identify the capacity of these proteins to bind TDM and generate meaningful biological responses. Going forward, I propose to take a biochemistry-first approach to this question as it enables greater flexibility in responding to new data and starts with a foundation of known interactions. Thus, I will utilize established methods of TDM blotting (Jegouzo et al., 2014) and wash recombinant carbohydrate recognition domain-streptavidin fusion protein lysate across them and then detect these interactions using standard biotin-horseradish peroxidase detection. This will provide a quantifiable readout for both presence/absence of an interaction but also the strength of the interaction. Should none of these proteins efficiently bind TDM, it is relatively trivial to generate new chimeric proteins and test a range of others present in the zebrafish genome. This data can then be used to go back into the zebrafish to assess the *in vivo* consequences of this interaction and also allow for more flexibility in approach – rather than seeking the receptor, we can explore phenotypes that may be altered in this context across both angiogenic responses and more general immune responses.

In the zebrafish itself, I would actually propose to take a few steps backward and return to a mosaic-based screening approach. While the long-term goal should be to generate a full locus-spanning deletion of 77975 and 79903, it may be worthwhile to use the zebrafish as a screening platform to determine the potential contributions of these two genes separately and together in the angiogenesis phenotype and CRISPR/Cas9 ribonucleoprotein injections allow for such rapid screening and planning for whether it makes sense to invest the time and energy in generating a full deletion mutant. Given the unlikelihood of being able to ever generate a crossover event to combine the two existing mutations, some alternative approach is clearly going to be required and this seems like a great place to start in pursuit of the zebrafish TDM receptor(s). These two genes make for especially compelling candidates as they



**Figure 5.1:** Comparison of the expression profiles of two of the putative MINCLE homologs, ENSDARG00000077975 and ENSDARG00000079903 based on existing RNA-seq datasets in the Tobin lab. Both of these genes are generally restricted to macrophages and are induced by the presence of mycobacterial infection, an effect exceptionally notable for 77975. This provides some evidence that these genes are regulated by mycobacterial infection and may be playing some role in this context.

are macrophage-specific and are induced by infection, even in bystander macrophages (Figure 5.1).

Additionally, it may be of some use to study the specific human MINCLE-mycobacteria interactions in the context of a whole immune system. Thus, going forward, it would be logical for future researchers to develop transgenic zebrafish that express human versions of MINCLE and MCL in macrophages and, perhaps, neutrophils, to assess the contributions of the human protein to conserved responses. This may also help to clarify some of the conflicting data in the literature around the role of MINCLE by using an overexpression model to capture the effect of excess MINCLE signaling. In the long term, gene replacement of the native MINCLE-like homolog

**Table 5.2:** Putative zebrafish MINCLE homologs with key details about their native structure and mutants that have been generated thus far.

Gene ID	Length (a.a.)	CRD	Similarity	Mutation	Site (a.a.)
56379	263	kEPNn	50.7%	::13	43
79903	263	gEPNn	53.3%	::13	207
77975	170	gEPNn	60.3%	Δ8	108

with the human MINCLE would allow for a more authentically humanized model of macrophage biology within the zebrafish. Use of this as a complementing strategy if we can identify the zebrafish MINCLE would also serve as a more comprehensive humanized model to study the MINCLE-specific contributions to angiogenesis and bacterial control.

## 5.2 Integration of Hypoxia Signaling

The literature is replete with descriptions of HIF-1 $\alpha$  regulation of VEGFA production and signaling; the logical means by which to alleviate local hypoxia is through the recruitment of vasculature carrying oxygenated blood. This allows angiogenesis to occur when necessary and for the vasculature to remain quiescent under homeostatic conditions, where intravital oxygen concentrations are maintained at a high level. However, in areas of pathogen invasion, tumor growth, or tissue damage, the local oxygen concentration can fall, triggering the activation of the HIF-1 $\alpha$  signaling pathway. HIF-1 $\alpha$  is an oxygen-sensing protein that is expressed and rapidly degraded under normoxic conditions but stabilized under hypoxia. Under standard oxygen concentrations, two classes of regulatory proteins mediate prolylhydroxylation and proteosomal degradation of HIF-1 $\alpha$  in a process dependent on molecular oxygen.

Alternatively, HIF-1 $\alpha$  can be induced through transcriptional alterations in the

homeostatic stoichiometry of HIF-1 $\alpha$  itself and the two families of regulatory proteins, PHD and FIH. This allow HIF-1 $\alpha$  to be induced under normoxic conditions, the presumed whole-body state of the zebrafish larva. This normoxia activation has been found to be important for myeloid immune responses, including those downstream of MINCLE activation, and may play an important role especially in the early signaling events of mycobacterial infection (Nishi et al., 2008; Schatz et al., 2016; Schoenen et al., 2014; Thompson et al., 2017).

In the environment of the granuloma, both the host and pathogen must adapt to reduced oxygen tension. Mycobacteria can temporarily revert into a non-replicating state known as persistence, but this is not a viable strategy for long-term evolutionary success (Ehrt, Schnappinger, and Rhee, 2018; Stewart, Robertson, and Young, 2003; Manabe and Bishai, 2000; Pandey and Sasseeti, 2008; Bentrup and Russell, 2001). However, during persistence, the bacteria are extremely difficult to kill as most antitubercular drugs are only effective on replicating bacteria (Veatch and Kaushal, 2018). Even during active growth, the bacteria and associated host cells must alter their metabolism to accommodate for reduced oxygen availability, with important consequences for host immunity (Harper et al., 2012; Tsai et al., 2006; Prosser et al., 2017; Rustad et al., 2009; Galagan et al., 2013). Despite our superficial knowledge about the importance and contributions of hypoxia in the lifestyle of *M. tuberculosis*, we do not have a clear mechanism to genetically manipulate these responses or to differentiate the roles of hypoxia *per se* from the activity of HIF-1 $\alpha$  signaling. Thus, going forward, new tools are going to be required to study not only the contributions of HIF-1 $\alpha$  in mycobacterial infections, but even more importantly, how those contributions intersect with the role of NFAT in inducing VEGFA production and angiogenesis in this environment.

### 5.2.1 HIF for HIF's Sake

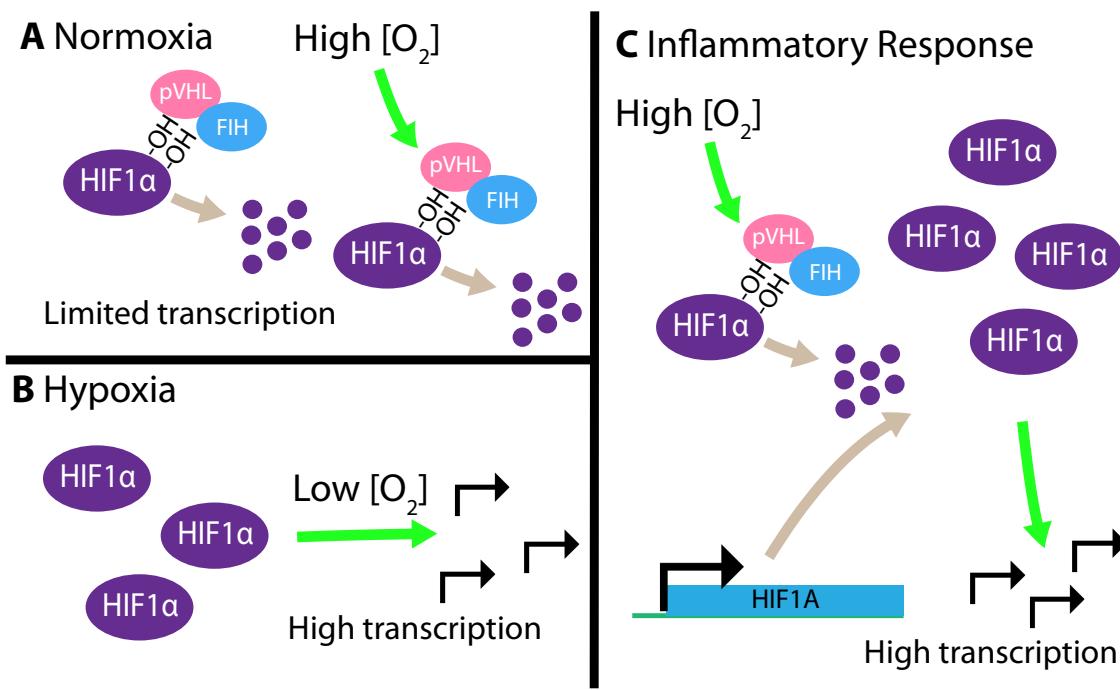
Several groups, the most notable of which being Philip Elks's lab, have studied the contributions of HIF-1 $\alpha$  signaling to the immune response to mycobacterial infections<sup>1</sup>, but there remain several needs as yet unaddressed. The Elks lab has utilized both dominant-negative and dominant-active versions of the zebrafish *hif1ab*<sup>2</sup> to modulate the activity of this pathway, particularly in neutrophils, and have found that activation of HIF-1 $\alpha$  prolongs inflammation and improve mycobacterial clearance, suggesting that at early time points, HIF- $\alpha$  plays a protective role in infection (Elks et al., 2011; Elks et al., 2013; Hammond, Lewis, and Elks, 2020). It was also found that HIF-1 $\alpha$  is important for the induction of TNF- $\alpha$ , which is a critical protective factor during infection (Lewis and Elks, 2019; Flynn et al., 1995).

HIF-1 $\alpha$  mediates both priming and responding to diverse insults. Hypoxia is able to induce the expression of *tlr2* and *tlr6*, which are important for broad responses to various infections, including *M. tuberculosis* (Kuhlicke et al., 2007). However, once induced by the pathogen itself, HIF-1 $\alpha$  is a critical inducer of *il1b* and IL-1 $\beta$  which, as we have seen is an important dimension of the host defense against mycobacterial infections (Ogryzko et al., 2019). Indeed, HIF is able to be activated indirectly by C-type lectin signaling, suggesting it may play a role in major antifungal and mycobacterial responses (Elder et al., 2019; Friedrich et al., 2017). As might be expected, genetic stabilization of HIF fosters more robust protective responses against multiple pathogens, making this a potential target for agonism as a means of fostering

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<sup>1</sup>There are also described roles for HIF-2 $\alpha$  in regulating certain aspects of neutrophil biology during mycobacterial infection, but this is beyond the scope of the presently proposed work (Thompson et al., 2014; Elks et al., 2015).

<sup>2</sup>As is all too common, but rarely quite so penetrant, zebrafish have two copies of all of the HIF- $\alpha$  subunits, making for an expansive coding space for these genes in the zebrafish genome and each copy appears to have some distinct effects, making for both opportunity and frustration (Elks et al., 2015).



**Figure 5.2:** Schematic demonstrating the different states of HIF-1 $\alpha$ . (A) shows the standard normoxic state of HIF-1 $\alpha$ , where it is rapidly degraded by oxygen-dependent enzymes; (B) these enzymes are inhibited in hypoxia, leading to HIF-1 $\alpha$ -dependent transcription. (C) Immunological stimuli can result in HIF-1 $\alpha$  transcription, leading to functional activation even in the absence of overt hypoxia.

immune defense (Schild et al., 2020). This extends to macrophages specifically, which can exert improved killing of *M. tuberculosis* after HIF-1 $\alpha$  activation (Li et al., 2021). Different physiological conditions within the host can impair HIF activation and HIF-dependent responses, including high blood glucose, adding a potential dimension to the immunosuppression experienced by many diabetics (Teran et al., 2022). On the other hand, glucose influx is important for initial glycolysis initiation, so there is likely to be some degree of Goldilocks effect where the right amount of glucose can facilitate glycolytic metabolism but too much overwhelms the system and too little starves the cells (Stunault et al., 2018).

This work nicely complements work from Didier Stainier's lab, which used a combination of *hif1aa* and *hif1ab* mutant zebrafish and new macrophage-specific transgenic tools to manipulate the HIF-1 $\alpha$  signaling pathway and found that this pathway, specifically in macrophages, was critical for mediating developmental angiogenesis. Unlike the behaviors seen in neutrophils, specific expression of even a wild-type *hif1ab* in macrophages was toxic to the cells and rendered them impotent (Gerri et al., 2017). This toxicity is likely due to sequestering of important binding partners, but may be due to metabolic changes that leave the cells unable to produce sufficient ATP, albeit by two divergent mechanisms – wild-type *hif1ab* may produce a strict reliance on glycolysis while dominant-negative *hif1ab* may force oxidative phosphorylation at rates exceeding the ability to produce pyruvate. This evokes an important function of this pathway in these cells that current tools remain unable to address.

Other studies have implicated HIF-1 $\alpha$  signaling in inhibiting macrophage necrosis in mycobacterial granulomas, indicating that this signaling pathway may exert some protective effects in particular contexts, although this study focused on *M. avium* granulomas, which different in some respects from *M. tuberculosis* granulomas

(Cardoso et al., 2015).

To further the study of the HIF pathway in the context of mycobacterial infection, a set of new tools should be made: one is a set of reporter constructs to better identify both hypoxia and transcriptional induction of *hif1ab* in macrophages and the other is a conditional approach to the expression of dominant negative and dominant active versions of HIF-1 $\alpha$  in macrophages, potentially enabling new cell-autonomous understanding of the role of this pathway in mycobacterial pathogenesis and angiogenesis.

HIF-1 $\alpha$  signaling is also implicated in various aspects of tumor biology and, as mentioned in Subsection 1.5.1, is important in mediating the angiogenic response in cancer. It also plays important functions in other aspects of cancer biology, including modulating tumor pH and the extracellular matrix (Dayan et al., 2008). These studies within tumors have also revealed interesting layers of transcriptional regulation within the HIF-1 $\alpha$  regulon and the functional differences between FIH and PHD, where PHD proteins are very strictly dependent on environmental oxygen while FIH has a much higher affinity for oxygen and can operate even at relative hypoxia. Given the different domains targeted by these proteins, HIF-1 $\alpha$  can exert biphasic effects at varying oxygen tension, with certain genes upregulated at modest hypoxia and additional genes induced under severe hypoxia (Dayan et al., 2008). Such gradients of response may be critical to understanding this pathway during mycobacterial infection, where the timing of these changes in relative oxygen concentration may reflect distinct stages of disease.

Previous work in our lab using *in situ* hybridization for *phd3* mRNA revealed the upregulation of this gene surrounding the mycobacteria, indicating hypoxia (Oehlers et al., 2015). The Elks lab generated a transgenic line using a bacterial artificial chromosome containing the promoter for *phd3* that expresses GFP (Santhakumar et al.,

2012), which serves as a useful spatial and temporal readout for HIF-1 $\alpha$  transcription factor activity across different tissues. However, this tool is unable to distinguish between normoxic and hypoxic activation or different cell types, so new tools are required to better address these questions.

During conditions of hypoxia, HIFs are degraded through hydroxylation in the oxygen dependent degradation domain (ODD) that contains two proline residues that are hydroxylated by PHD proteins, leading to proteasomal degradation. This ODD has been shown to be both necessary and sufficient to direct oxygen-dependent degradation, so it seems reasonable to use a macrophage-specific promoter to drive expression of ODD linked to a fluorescent protein as a reporter for granuloma hypoxia. This would be stabilized at low oxygen concentration while being constitutively degraded under normoxia. This would allow for a clearer report of the degree of present hypoxia and complement existing tools, including hypoxyprobe (Cousins et al., 2016; Huang et al., 1998).

In parallel, a reporter is needed to provide a readout of normoxic activation of HIF-1 $\alpha$ , which is predominantly thought to be regulated at the transcriptional level. Therefore, either ectopic expression or direct protein fusion strategies would be appropriate to the study of this pathway. If some promoter could be identified that responded comparably to the native *hif1ab* promoter or a CRISPR-mediated knockin could be generated, this would be a useful reporter of transcription-level induction of HIF-1 $\alpha$ , a process likely relevant to inflammatory responses during infection. This, in tandem with the previously mentioned ODD transgenes would allow for a more thorough dissection of the relative contributions of hypoxia *per se* and the activity of HIF-1 $\alpha$ .

As previous transgenic attempts have failed, the expression of dn-hif1ab and da-

*hif1ab* in macrophages will require new approaches. It seems that misregulation of HIF-1 $\alpha$  results in some sort of developmental toxicity in the macrophage, so it is critical that HIF is only modulated in the time and place where it is most relevant. Thus, using established estradiol-responsive constructs, a set if fish should be made expressing dn-*hif1ab* and da-*hif1ab* covalently linked to ER50 degradation domains, which sequester proteins in the cytosol and target them for degradation except when in the presence of tamoxifen (Miyazaki et al., 2012). This would allow for the creation of HIF-modulating transgenes within macrophages that would be expected to have reduced toxicity and allow for time-targeted modulation of this pathway. Like the previous tools, this seems especially relevant in the context of mycobacterial granulomas from adult zebrafish, which are known to be hypoxic and more closely resemble human granulomas. Alternatively, and perhaps even more elegantly, dominant-negative *hif1ab* could be created endogenously, through knock-in of a Cre-flippable trap cassette into intron 7 or 8; if placed in the “off” orientation, this would allow for normal HIF-1 $\alpha$  activity while induction of Cre<sup>ERT2</sup> would then allow for macrophage-specific activation of a dominant-negative version of the protein through introduction of a splice acceptor-stop codon-polyadenylation sequence. Even further creative approaches could enable tissue-specific induction of dominant-active HIF-1 $\alpha$  through a similar approach by knocking in the remaining cDNA with the hydroxylated proline residues mutates and placing this, too, under control of an invertible *loxP* pair. These are likely to reveal not only new aspects of HIF modulation of angiogenesis, but broader impacts on the overall response to infection, especially given the central role of HIF-1 $\alpha$  in altering macrophage metabolism.

### 5.2.2 Macrophage Metabolism in Immunity

Upon activation, macrophages undergo a metabolic switch that corresponds, in part, to their longevity and function (Kolliniati et al., 2022). While immediate-responding<sup>3</sup> macrophages begin to utilize aerobic glycolysis for metabolism as a way to rapidly (albeit inefficiently) generate energy, longer-term macrophages utilize oxidative phosphorylation to optimize energy consumption over a long course of engagement (Shi et al., 2019; Kiran et al., 2016; Viola et al., 2019; Langston, Shibata, and Horng, 2017; Taylor and Scholz, 2022). Thus, while macrophages may initially use bursts of glycolysis to fuel rapid, oxidizing responses, this is unsustainable over the long term and these macrophages must either transition to the use of oxidative phosphorylation, disperse, or perish (Odegaard and Chawla, 2011; Howard and Khader, 2020). These processes are thought to be predominantly regulated by a combination of HIF-1 $\alpha$ , AKT, and mTOR, although this does not exclude the possibility of other factors influencing these changes (Covarrubias, Aksoylar, and Horng, 2015). The reason for such interest in macrophage metabolism is many fold: there is comparatively little known about how these processes influence the biology of the granuloma itself, it intimately involves HIF-1 $\alpha$  signaling (a classic mediator of angiogenesis), and is clearly an important dimension of a variety of protective immune responses and we currently lack a model that integrates what is most likely the most important second messenger of eukaryotes (divalent calcium) with macrophage metabolism, which may allow for integration of the NFAT signaling pathway into these broader alterations in macrophage biology.

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<sup>3</sup>In the literature, these are so-called “M1” macrophages and while useful to acknowledge the inflammatory and activated state of these cells, it is not clear to me nor does it seem likely that macrophages strictly use only one metabolic modus at a time, although they are relatively depleted of mitochondria (Biswas and Mantovani, 2012).

This burst of glycolysis also generates important intermediates that can be used for additional cellular processes which might have otherwise been consumed by the citric acid cycle (Viola et al., 2019; Kelly and O'Neill, 2015). These initial responses are regulated in part by the activation of HIF-1 $\alpha$ . One of the earliest observations on this point evaluated the consequences of loss of function of HIF-1 $\alpha$  on the ATP concentrations within myeloid cells and found dramatically reduced levels during inflammation (Cramer and Johnson, 2003). Wound sites and other sites of insult tend to have reduced oxygen concentrations, which drives this metabolic shift as well as the previously described induction of VEGFA to alleviate this hypoxia, which can act to modulate both endothelial and further macrophage responses.

The earliest events of the glycolytic switch in macrophages is triggered by the inducible nitric oxide synthase, which generates NO that inhibits mitochondrial respiration. The net effect is a reduction in oxidative phosphorylation capacity by the cell and a need for the utilization of an alternative mode of metabolism, which is supplied by aerobic glycolysis. Subsequently, HIF-1 $\alpha$  activation by succinate and  $\alpha$ -ketoglutarate drives the transcription of glucose transporters and lactate dehydrogenase to block pyruvate utilization by the mitochondria (Russell, Huang, and VanderVen, 2019; Galvan-Pena and O'Neill, 2014). This increase in glucose import has been shown to be important during leprosy, an effect that may be more general (Medeiros et al., 2016; Montoya-Rosales et al., 2016; Vance et al., 2019). HIF-1 $\alpha$ , as we have seen, also induces the expression of cytokines and chemokines, making it an integrated part of the overall response to detection of PAMPs, the model of which is LPS (see Subsection 1.3.2). As a major mediator of inflammatory responses, this glycolytic phenotype allows for rapid, energetically expensive response to immediate threats but is unsustainable over the long-term, leading to a net shift back to oxidative phosphorylation.

In order to switch back to oxidative phosphorylation, HIF-1 $\alpha$  must be downregulated and alternate pathways induced; rather than nitric oxide synthase consuming cellular arginine to make nitric oxide<sup>4</sup> and citrulline, arginase needs to be induced to produce urea and ornithine (Palmieri et al., 2020; Qualls and Murray, 2016). Indeed, arginine is at the very center of tuberculosis immunometabolism and its byproducts – through nitric oxide and itaconate – determine important aspects of host immunity. However, it is now known that LPS can paradoxically induce arginase, which would argue that our current knowledge of the factors that contribute to these different states (if they are to be states as such) is lacking (El Kasmi et al., 2008). A further metabolite in this process, itaconate, has a range of important immunomodulatory and directly antimicrobial properties (He et al., 2021). Additional amino acid biosynthesis pathways also seem to contribute to the process of granuloma formation, but the precise roles of these are somewhat unclear. One of the issues with the study of mutants in discrete genes in these pathways is that much of this has been done *in vitro* under the standard binary LPS or IL-4/IL-13 polarization conditions or in the mouse, where more comprehensive profiling is possible but the knockout of *Arg1* or *Nos2* might not fundamentally change the cellular identity of these populations although it may shift them on the M1-M2 dichotomy by the formal definitions. Nevertheless, the relative contributions of HIF-1 $\alpha$  to the metabolic state of the macrophages depends on the interplay of formal hypoxia as well.

In cancer, tumor-associated macrophages undergo similar shifts in metabolism and many of these are driven by HIF-1 $\alpha$ . Inhibition of glycolysis in tumor-associated macrophages resulted in a reduction in tumor burden through a resulting increase in anti-tumor activity through what was described as increased M1-like phenotypes

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<sup>4</sup>This production of nitric oxide is both an important antimicrobial function and is classically used to define M1 macrophages via detection of inducible nitric oxide synthase.

(Mehla and Singh, 2019; Rabold et al., 2017). While glycolysis is, of course, required for many different metabolic pathways, it may be the case that in the absence of the ability to undergo glycolysis, other pathways are able to compensate within M1 macrophages to exercise these phenotypes while the increased metabolic demands of M2 macrophages result in them polarizing into more inflammatory states. Most of the macrophages in the tumor microenvironment engage the TCA cycle for energy production, partially as a product of their long periods of stimulation and need for a more sustainable supply of energy that does not produce as many reactive oxygen species (Yu et al., 2020). The net effect is that blockade of HIF-1 $\alpha$  in cancer cells results in diminished tumor growth and may be a useful target to metabolically reprogram tumors and tumor-associated macrophages (Hong, Lee, and Kim, 2004).

The specific utilization of lipids as energy sources might impact the outcomes of mycobacterial infection, although this is not presently well understood. Mycobacteria depend on cholesterol and other host lipids for a carbon source and for entry into macrophages, so manipulations of the ability for macrophages to uptake, but not then catabolize, host lipids as an energy source may play a role in the growth of the bacteria (Gatfield and Pieters, 2000). Inflammatory macrophages are known to induce lipogenesis to enhance phagocytosis, a process likely to benefit mycobacterial growth (Yan and Horng, 2020). On the other hand, fatty acid oxidation is more associated with anti-inflammatory types of macrophages, implying that these cell types may be more inclined toward the import of exogenous lipids (Yan and Horng, 2020); if mycobacteria can prevent the macrophages from catabolizing these lipids, then that would improve bacterial growth (Nazarova et al., 2019). As we now know that granuloma macrophages express PPAR- $\gamma$ , a more thorough dissection of potential bacterial secreted effectors that alter macrophage metabolism may be targets for altering the polarization state of these macrophages to facilitate improve antibacterial

responses (Phan, Goldrath, and Glass, 2017; Gideon et al., 2022; Cronan et al., 2021; Chawla et al., 2001; Wilson, Mayr, and Weichhart, 2019).

Mycobacteria benefit, in part, from phagocytosis by macrophages undergoing glycolysis because this process also results in fatty acid biosynthesis, an important component of the bacterial energetic diet; these bacteria can also make use of glycolytic intermediates as a carbon source, suggesting that uptake by nominally inflammatory macrophages primarily undergoing glycolysis may in some ways benefit the bacteria (Escoll and Buchrieser, 2019; Mehrotra et al., 2014; Shi et al., 2019). *M. tuberculosis*, through the use of TNT, depletes the macrophage of NAD<sup>+</sup>, which triggers metabolic adaptation in the macrophage to increase the supply of NAD<sup>+</sup> and transition back into a more oxidative phosphorylation status (Howard and Khader, 2020). The products of glycolysis in macrophages include known antimycobacterial compounds like nitric oxide and prostaglandins, making this a delicate balance ultimately determined by the bacterial ability to manipulate the macrophage into a state that allows it to replicate while minimizing risks from the potentially antibacterial responses (Osada-Oka et al., 2019). However, these metabolic changes also induce the production of IL-1 $\beta$ , which is known to be protective against mycobacteria (Corcoran and O'Neill, 2016; Ogryzko et al., 2019; Fremond et al., 2007; Shi et al., 2019). However, the macrophages have developed strategies to accumulate lipids for their own utility while preventing bacterial access, predominantly through sequestration in lipid droplets (Laval, Chaumont, and Demangel, 2021). Limitation of macrophage access to cholesterol through a variety of mechanisms has the potential to modulate nutritional immunity against the bacteria (Babunovic et al., 2022; Pandey and Sasse蒂, 2008; Yang et al., 2014d), but a more comprehensive evaluation of the overall metabolic drivers in macrophages during immune responses is still very much a work in progress.

Metabolic changes mediated by HIF-1 $\alpha$  are thought to underly what is known as “trained immunity.” The induction of aerobic glycolysis within macrophages primes them to respond more vigorously to future insults, an effect that appears to be able to last weeks or months (Cheng et al., 2014). As HIF-1 $\alpha$  is a known mediator of IL-1 $\beta$  induction, it appears that increased transcription but not increased processing of IL-1 $\beta$  is one of the mechanisms by which this process is able to work (Arts et al., 2018). Trained immunity may even serve as a transgenerational mechanism of protection against insults (Katzmarski et al., 2021), although this is controversial (Kaufmann et al., 2022; Katzmarski et al., 2022). The synthesis of the effects on metabolism with the effects on macrophage biology and interactions with surrounding stroma in mediating some of the effects of trained immunity still require a great deal of investigation, although this is certainly yet another realm where major families of immunologically relevant transcription factors surely play some role.

Under sustained immunological stimulation in hypoxia, such as that found in a tuberculous granuloma, metabolic reprogramming toward oxidative phosphorylation still occurs in macrophages (Wilson, Mayr, and Weichhart, 2019; Mehrotra et al., 2014). The conflict between the production of VEGF, the M2-like identity of many granuloma macrophages that express VEGF, and the downregulation of HIF-1 $\alpha$  in M2 macrophages raises important questions about the factors that were presumed to be regulating VEGF production prior to the present work, which presents the alternative model of a C-type lectin receptor-NFAT transduction pathway able to induce VEGF. These effects appear to be mediated by inappropriate responses within the macrophage that downregulate glycolysis; upregulation of glycolysis in these cells can result in vascular normalization through cellular starvation of the vasculature (Wenes et al., 2016). It may be that, under this model, that the metabolic status of the macrophages is unimportant for VEGF production, although this would be, to

some degree, in contradiction with other parts of the literature. A further mystery is why VEGFA is so frequently considered an M2 cytokine when its transcription was thought to be dependent on HIF-1 $\alpha$ , a gene induced under M1 conditions. A more thorough integration with the findings of this work with the general metabolic status of granuloma macrophages that produce VEGF normally will be important for resolving some of these quandaries on the contribution of HIF-1 $\alpha$  to granuloma macrophage metabolism, antibacterial responses, and angiogenesis.

The metabolite profile of granulomas also comprise an interesting aspect of this work. While not directly connected to the metabolism of the host *per se*, this is a reflection of the complex interaction between host and pathogen to generate a milieu of metabolic byproducts that may play important roles in immunity. Profiling has been done in guinea pig granulomas and has found signatures suggesting a mixed polarization state with the presence of lactate as well as enrichment of aspartate and glutamate and glutathione, suggesting simultaneous hypoxia and oxidative stress indicative of multiple different stimuli altering the metabolic state of the macrophages over time (Somashekar et al., 2011).

### 5.2.3 HIF-NFAT Interactions

A tempting hypothesis is that HIF-1 $\alpha$  and NFATC2 are regulating one another to induce the expression of VEGFA. The model most consistent with the existing literature is that NFATC2 is upregulating the transcriptional expression of HIF-1 $\alpha$  under circumstances where HIF-1 $\alpha$  is already being stabilized; these interactions could also be reciprocal, where HIF-1 $\alpha$  activation induces the expression of further NFATC2, consistent with our previous observations (Section 3.4). This would feed-forward the induction of VEGFA and may be essential for regulating these responses in normoxic

environments or very early in infection.

A piece of standalone support for this model is a study conducted in mast cells that demonstrated a calcineurin-NFAT dependent upregulation of HIF-1 $\alpha$  at the transcriptional and protein levels. Treatment of these mast cells with ionomycin, which increases cytosolic calcium concentrations, caused these effects in a way that was able to be inhibited by tacrolimus treatment (Walczak-Drzewiecka et al., 2008). Additionally, this effect was exacerbated by culture under 1% O<sub>2</sub>, a description that corresponds well to the behavior of NFATC3 in the myocardium, where hypoxia increased expression of endothelin-1 in a way that is sensitive to superoxide and the activity of NFATC4, which can also be activated by hypoxia (Frutos et al., 2011; Ramiro-Diaz et al., 2014; Moreno et al., 2015). Additional studies found that hypoxia activated NFATC3 to promote pulmonary smooth muscle proliferation in a way that might promote pulmonary hypertension (Hou et al., 2013). Hypoxia can also increase proliferation of human fibroblasts through NFATC2 independently of HIF-1 $\alpha$  but dependent on HIF-2 $\alpha$  (Senavirathna et al., 2018). None of these studies but the last investigated a mechanism to integrate this activation of NFAT under hypoxia with the roles of HIF-1 $\alpha$  despite these phenotypes having been previously described to correspond with HIF-1 $\alpha$  activity (Cui et al., 2021; Qi et al., 2017; Li et al., 2014; Thackaberry et al., 2002; Sonanez-Organis et al., 2016). Even the Senavirathna et al. only investigated this effect within a relatively limited scope (proliferation) and not in respect to the diversity of phenotypes that are stimulated by hypoxia.

To study these interactions, it seems that combinatorial chemical inhibition strategies could be utilized, although HIF-1 $\alpha$  inhibitors are relatively sparse in number and understudied (Viziteu et al., 2016). However, this would only scratch the surface in pursuit of epistatic interactions between these proteins and would neglect the poten-

tial for protein-protein interactions in mediating part of the effect. A structure based approach targeting known interacting domains on HIF-1 $\alpha$  and its DNA binding domain may reveal important transactivation role for HIF-1 $\alpha$  as well as resolve some questions about the direct versus indirect nature of NFAT regulation of hypoxia responses. Much of this work could be done in a heterologous model (HEK-293T cells, for instance) to more efficiently assess these types of binary interactions. Larger scale screening approaches by two-hybrid would be another potential avenue for such a project to go as the interacting partners of various domains of NFATC2 remain only partially resolved. Proximity ligation approaches, which will be discussed moreso later on, offer another possibility for dissecting the HIF-1 $\alpha$  interactome. Unfortunately, the state of reagents to work with toward these ends in the zebrafish are somewhat limited and additional groundwork would be required to study some of these processes in an *in vivo* context, although this seems like an excellent direction for a future Ph.D. project.

### 5.3 Cotranscriptional Interplay

To further dissect the contributions of other transcription factors to the NFAT-dependent angiogenesis response, we can make use of our THP-1 macrophage platform to study the role of NFAT interacting partners in the overall response to *M. tuberculosis* exposure. While wild-type NFATC2 is able to interact with a panoply of other transcription factors through both C- and N-terminal domains, the domains important for particular binary interactions have been teased out over time, largely by Anjana Rao's lab. Thus, to simultaneously test the sufficiency of NFATC2 in inducing transcriptional responses, the importance of AP-1 transcription factor binding, and the ability for NFATC2 itself to bind DNA, I have developed a set of expression plasmids

that drive expression of a range of different modified NFATC2 proteins (Table 5.3).

**Table 5.3:** Lentiviral expression constructs to assess the role of NFATC2 domains for induction of VEGFA signaling.

Plasmid	Utility
pLEX:mPapaya	Empty expression vector driving expression of only the conjugated fluorescent protein, for background comparison.
pLEX:CA-NFAT1	Expression of a constitutively active NFATC2 that drives transcriptional responses independent of calcineurin.
pLEX:CA-NFAT1-ΔDBD	Expression of a constitutively nuclear NFATC2 that is unable to bind DNA, to assess the contributions of NFAT binding on the induction of VEGFA.
pLEX:CA-NFAT1-ΔRIT	Expression of a constitutively active NFATC2 unable to interact with AP-1 transcription factors, to determine the contribution of this family to the VEGFA response.
pLEX:CA-NFAT1-ΔDBD-ΔRIT	Expression of a constitutively nuclear DNA binding domain mutant also unable to interact with AP-1 transcription factors.

These plasmids will enable a genetics-first dissection of the activity of NFATC2 and its potential sufficiency to induce transcription of VEGFA. While, as noted in Section 3.4 and seen in Figure 3.32, the VEGFA promoter has a number of putative NFAT binding sites, the activity of these remain unknown. Using these new genetic tools in the defined environment of THP-1 macrophages it should be possible to dissect the function of these sites. Distant future approaches may utilize deeper genetic probing to identify particular sites of especial importance, as has been done in Chang et al. (2004). While that publication found one site that was bound by NFAT in the myocardium, the particular binding site of importance may vary by cell

type and circumstance.

It is also possible that non-AP-1 transcription factors are important for NFAT activity and this is a trickier proposition to address, but could be studied through either immunoprecipitation-mass spectrometry or biotin tagging approaches via BioID (Roux et al., 2012). The advantage of the former is the identification of more stable interactions and could be done in tandem with DNase-seq, ChIP-seq, or ATAC-seq to identify promoter occupancy of this isoform. The latter may be a technically less challenging approach, however, and may provide a useful readout of the total set of interactions experienced by NFATC2 over the course of  $\gamma$ Mtb exposure. The former can also be done in genetically unmodified cells while the latter would require some (minimal) additional cloning and generation of yet another lentivirus for the purpose of tagging NFATC2 with one of the several biotin ligases now available (Cho et al., 2020). Both seem useful and complementary and should be considered going forward to more comprehensively understand the NFAT interactome during mycobacterial infections. I hypothesize that there are as-yet unobserved protein-protein interactions between NFATC2 and HIF-1 $\alpha$  that would potentially shed light on the transcriptional regulation of VEGFA in diverse contexts including in cancer and autoimmunity.

### 5.3.1 NFAT:AP-1 Interactions

The most exhaustively studied and first identified transcriptional partner for NFAT is the AP-1 transcription factor superfamily, which includes proteins in the JUN, FOS, ATF, and MAF families (Boise et al., 1993). While some of these may strike the reader as being critical regulators of cell growth and tumorigenesis, they also play critical roles in other aspects of normal biology including in immune responses

(Macian, Lopez-Rodriguez, and Rao, 2001; Eferl and Wagner, 2003). AP-1 binding sites are often adjacent to NFAT binding sites and these two transcription factors<sup>5</sup> may cooperate to drive the transcription of VEGFA. These cooperative interactions may be close in proximity or over a long distance and may be direct protein:protein interactions or more indirect. Regardless, given the imminent importance placed on NFAT:AP-1 interactions, this seems to be an excellent place to start on a targeted list of potential interacting partners.

A few approaches can be taken. The first, utilizing the previously described pLEX:CA-NFAT-ΔRIT constructs, would give an indication about the importance of direct interactions with NFATC2, as these mutate the critical residues for complexing with AP-1 proteins. Additionally, there are a number of relatively broad inhibitors against AP-1 that could be used to test whether inhibition of AP-1 alone alters the VEGFA transcriptional response to mycobacteria *in vitro* or the overall angiogenic response *in vivo* (Makino et al., 2017; Huang et al., 1997). It has been previously demonstrated that AP-1 can activate VEGFA transcription as well as VEGFD, suggesting that this pathway may have as-yet unappreciated roles in inducing VEGFA during immune responses and may act in concert with NFAT to execute these functions<sup>6</sup> (Shih and Claffey, 2001; Debinski et al., 2001; Wang et al., 2016; Josko and Mazurek, 2004; Guo et al., 2022).

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<sup>5</sup>To avoid providing unnecessary amounts of detail, I will refer to AP-1 as a monolithic “transcription factor” much of the time. While each of the proteins in this family serve different functions, the subtleties are perhaps beyond the scope of this prospective overview. The guiding question of this section -- is an AP-1 transcription factor cooperating with NFAT to drive VEGFA transcription – is sufficiently broad that future experimentation would almost certainly eventually seek to identify, at the gene level, which is responsible.

<sup>6</sup>More classical data suggests that HIF-1α-dependent VEGF upregulation is independent of AP-1, although this data may be limited by the scope of the time and the particular experimental conditions. Or hypoxia-induced VEGFA may be independent of AP-1, but pathogen-induced VEGFA is dependent on it – these all remain to be explored (Finkenzeller, Technau, and Marme, 1995).

### 5.3.2 NFAT:NF $\kappa$ B

As has been discussed previously (see Section 1.4), the interplay between NFAT and NF- $\kappa$ B is widely discussed but the precise biological consequences of these two transcription factor families in inducing VEGFA remain unknown. It has previously been shown that NF- $\kappa$ B is able to induce the transcription of VEGFA in various contexts (Xie et al., 2010; Greenberger et al., 2010; Lukiw et al., 2003) and is important for VEGFA expression in macrophages (Kiriakidis et al., 2003). Lukiw et al. (2003) even uncovered important interactions between HIF-1 $\alpha$  and NF- $\kappa$ B in the induction of VEGFA during hypoxia, suggesting the potential for there to be multiple layers of signaling required to induce VEGFA during particular context and which may vary based on the stimulus.

The overarching theme in studies of NFAT-NF- $\kappa$ B interactions is that they can either be antagonistic or cooperative and *a priori* prediction of which is likely to occur has been elusive (Khalaf, Jass, and Olsson, 2013; Fisher et al., 2006). Foundational studies in this field found cooperative roles for NF- $\kappa$ B and NFAT in regulating the expression of IFN- $\gamma$  (Sica et al., 1997) and that they were differentially induced by alterations in cellular calcium (Dolmetsch et al., 1997). Direct dimerization was first suggested with NFAT5, which established a model for the study of these interactions in the NFATc isoforms (Lopez-Rodriguez et al., 2001). These proteins also directly interact to synergistically promote cardiac hypertrophy, roles consistent with those described for NFATC2, NFATC3, and NFATC4 (Subsubsection 1.4.2, Subsubsection 1.4.2, and Subsubsection 1.4.2) (Liu et al., 2012). And as we have seen in the context of osteoclast biology, NF- $\kappa$ B can bind to the promoter of NFATC1<sup>7</sup> to

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<sup>7</sup>The relevance to other NFAT proteins remains unknown, but at least NFATC2 has putative NF- $\kappa$ B binding sites in the proximal promoter.

drive increased expression to allow for osteoclast differentiation (Asagiri et al., 2005). However, activation of the specific NF- $\kappa$ B member RELB has been shown to inhibit NFATC1 expression, so there may be important regulatory layers based on the particular NF- $\kappa$ B family member engaged – a critical consideration that is difficult to account for with present knowledge (Zhao et al., 2015). And in the context of TLR agonism, NFATC3 nuclear occupancy was important for NF- $\kappa$ B responses but was not induced by TLRs and could either enhance or inhibit NF- $\kappa$ B responses (Minematsu et al., 2011; Conboy et al., 1999). In the endothelium, the balance of angiogenesis is determined by the balance of competitive promoter occupancy between NF- $\kappa$ B and NFAT through the expression of FASL and cFLIP, respectively (Aurora et al., 2010). NF- $\kappa$ B-dependent expression of FASL drives endothelial apoptosis while NFAT-dependent expression of cFLIP promotes cellular survival, despite both proteins binding both promoters. This pairwise antagonism between these two transcription factors offers an intriguing model that should be further explored in other contexts. Yet other regulatory factors may be involved in regulating these roles, but this serves as an interesting potential regulatory mechanism worthy of investigation in the context of macrophage VEGFA signaling given the composite evidence of NF- $\kappa$ B importance for macrophage expression of VEGFA and the known layers of regulation surrounding NFAT and NF- $\kappa$ B signaling and the data presented here on the role of NFAT in mediating this process during mycobacterial infection.

One of the interesting observations that came out of the work studying the NFATC2-dependent transcriptional responses to *M. tuberculosis* was the observation that  $\gamma$ *Mtb*-exposed THP-1 macrophages seemed to have greater production of NFATC2 protein by immunofluorescence, an unusual potential regulatory mechanism only rarely described in historical literature (Asagiri et al., 2005; Aramburu et al., 1995) and then only for NFATC1. The concept that there may be some sort of up-

stream priming event that stimulates NFATC2 production and/or downregulation of NFATC2 protein degradation offers the potential for a new layer of regulation of NFAT activity. Further experimentation will need to be done to confirm this and I would propose RNA-seq as an excellent first-attempt to categorize some of the macrophage-specific responses to extracellular exposure to *M. tuberculosis*.

## 5.4 New Genetics Tools for the Study of NFAT Signaling

One of the dominant tools in the field for the study of intracellular calcium flux is the use of GCaMP, a modified green fluorescent protein that fluoresces in response to calcium binding (Nakai, Ohkura, and Imoto, 2001). Since its initial development, many interactions have developed allowing for ever-finer detection of various aspects of cellular calcium concentrations and localization. These tools have been used in the fish to detect and manipulate cellular behavior in both fixed and freely moving fish (Beerman et al., 2015; Kim et al., 2017). These tools have clear promise in better understanding the biology of NFAT activation, but likely need tethering to either the channels or proteins themselves or a specific cellular compartment to increase spatial resolution; a whole-cell approach is no longer sufficient for the proper understanding of NFAT activity in this context and finer resolution would greatly aid in the identification of future mechanisms. Given findings from Kar and Parekh (2015) on the importance of nuclear calcium in regulating the activity of at least NFATC3, it would be beneficial to have new tools to monitor these alterations in real time.

An initial approach would take advantage of some of the new technologies available in the zebrafish (reviewed in Subsection 2.5.2) by generating an endogenously

GCaMP-tagged *nfatc2a* in the zebrafish to monitor the timing of calcium flashes and how those correspond to the activation state of the protein, able to be visualized by intercompartmental trafficking. By using high temporal and spatial resolution imaging offered by LightSheet (Reynaud et al., 2008), it would be possible to monitor on the seconds resolution the activation status of Nfatc2a within macrophages in response to mycobacterial infection. This finely tuned reporting behavior would reveal key details about the timing and kinetics of NFAT activation during infection and how that activation corresponds to the induction of *vegfaa* and angiogenesis.

In the long-term, it is likely to be worth exploring the possibility of generating a floxed *nfatc2a* allele to allow study of this protein in a particular cellular context. While the use of VIVIT allows for gross examination of NFAT-dependent phenotypes, it would be beneficial to be able to study individual isoforms' contributions to given phenotypes. This is technically already possible, but is rather laborious. Should newer approaches to the generation of knock-in zebrafish lines emerge, this should be a foremost consideration in the continuation of this work.

Such a tool either on its own or by contrast with the *irg1:VIVIT* line should be analyzed by RNA-seq during mycobacterial infection to capture a comprehensive profile of the transcriptional changes resulting from inhibition of NFAT within macrophages. Bulk RNA-seq approaches are likely appropriate for this task and could be sourced from either macrophages in the granuloma from adult zebrafish or from macrophages in the early stages of granuloma development in the larva. This would provide an essential comprehensive profile of the NFAT-dependent responses in these cells *in vivo* likely to also be a useful resource for the community at large in dissecting critical pathways for defense against mycobacteria and designing therapeutic options that properly balance the desire to inhibit deleterious responses while enhancing protective

ones.

It will also be interesting to start teasing out non-macrophage cellular contributions to angiogenesis. While we have already seen that depletion of macrophages reduces the angiogenic phenotype in response to mycobacterial infection (Oehlers et al., 2015), it is not known if other cells types may be able to compensate at other stages of infection. I have developed a *lyz:VIVIT-tdTomato* transgenic line that would allow for the functional dissection of these roles within neutrophils, in which NFAT may be playing distinct roles during infection, as has been described in other contexts (Herbst et al., 2015; Vymazal et al., 2021; Vega et al., 2007). This would offer a new horizon of NFAT contributions during infection that could serve as an entire new Ph.D. project.

To more directly study the contributions of macrophage alone, however, we could apply the *irf8<sup>st95</sup>* fish line, which lacks macrophages due to loss of a transcription factor essential for macrophage development, to study these processes in the larva and the adult (Shiau et al., 2015; Xu et al., 2012; Tamura et al., 2005). Ambitious approaches would incorporate reconstitution of the macrophage population in an otherwise wild-type *irf8<sup>st95/st95</sup>* with *nfatc2a<sup>-/-</sup>* macrophages to more precisely identify the requirement for these cells in angiogenesis. A slightly simpler approach would adopt the strategy used by Cronan et al. (2021) to conduct whole kidney marrow transplantation into *myb* mutant larvae, reconstituting the entire hematopoietic system with *nfatc2a* mutant cells. Any of these approaches should allow for deeper study of macrophage NFATC2 biology in the zebrafish model.

## 5.5 Mycobacterial Interactions with Other Aspects of Vascular Biology

The focus of the present work has been on a rather coarse readout of vascular biology: angiogenesis or not. However, as was discussed briefly in Section 1.5, the biology of the endothelium is far more complex than just sprouting angiogenesis and the interaction of the granuloma with the vascular is more complex as well. This makes it imperative to develop a more nuanced understanding of how these other factors may contribute to the pathology of the disease and how NFAT signaling may modulate these factors as well.

A radical approach that may be of some utility in addressing some of these more challenging questions would require the development of a new model wherein *ex vivo* cultured granulomas are co-cultured with endothelial cell lines to start addressing some of the contributions of different genetic factors to the ability of the granuloma to alter endothelial cell biology more directly. While it is known that human umbilical vascular endothelial cells (HUVECs) can respond to zebrafish Vegfaa, it may be worthwhile to explore a more native model. Endothelial cell lines from other fish species have been established and it could be of great utility to explore the possibility of establishing something similar for the zebrafish (Pham et al., 2017; Luque et al., 2014). A genetically tractable system for generating these lines would allow for both granuloma-derived signals and endothelial responses to be measured in a more tightly controlled environment. Given many of these objectives involve more cell biology than simply the growth of vessels, the ability to observe them in greater isolation may prove necessary if these are to be effectively addressed.

### 5.5.1 Vascular Permeability

In addition to the overall growth of blood vessels, VEGFA has important roles in maintaining the integrity of blood vessels both through weakening integrin-mediated connections between neighboring cells and through further signaling to the neighboring mural cells, including pericytes, to modulate the ability of vascular luminal contents to access neighboring tissue in the direction of the source of VEGFA (Park-Windhol and D'Amore, 2016; Claesson-Welsh, 2015). However, VEGFA is not the only chemokine capable of modulating the integrity of the endothelium; another, related pathway known as the angiopoietin system, is also known to play a major role in maintaining the structure of the endothelium and preventing (or encouraging) vascular leakage which may be critical for certain developmental events as well as effective immune responses.

A previous study from the Tobin lab found that angiopoietin signaling was a critical mediator of vascular permeability during mycobacterial infection, a finding consistent with that of tumors, where ANG2 signaling induces alterations in the biology of the endothelium through the Tyrosine kinase with Ig and EGF homology domain 2 (TIE2) (Oehlers et al., 2017; Duran et al., 2021; Goel, Wong, and Jain, 2012; Thurston and Daly, 2012). Inhibition of angiopoietin signaling resulted in a reduction in bacterial burden and improved vessel normalization, likely in part by blocking the function of VEGFA signaling. However, more recent findings have noted that granuloma macrophages do not seem to be a major source of angiopoietins<sup>8</sup>, which creates a bit of a conundrum: if we know that these genes are expressed during infection and play a role in the overall course of disease, where are they coming from

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<sup>8</sup>This is at least true of the 14 days post infection time point at which the samples were taken and similar findings can be found from Gideon et al. (2022) at four and ten weeks post infection.

(Cronan et al., 2021)?

Angiopoietin signaling is a complex affair, with multiple ligands and receptors, but the focus is centered on the bifunctional ligand, angiopoietin-2<sup>9</sup> (ANGPT2 or ANG2) and the receptor TIE2<sup>10</sup>. While at homeostasis and at some dosages ANG2 can activate TIE2 to promote vascular stability, in the context of inflammation, it becomes antagonistic through combinatorial effects with various cytokines, and induces vascular permeability (Augustin et al., 2009). ANG1 is the standard agonist of this pathway and the activity of ANG1 maintains vascular integrity and prevents the expression of inflammatory integrins (Augustin et al., 2009). This leakiness induced by ANG2 results in a deregulated vascular environment that promotes tumor and, seemingly, bacterial growth; normalization with antibodies induces vascular regression and exerts anti-tumor effects. This also implicates ANG2 as an important mediator of angiogenic processes in concert with VEGF. It is known that endothelial cells can produce their own ANG2 to signal in an autocrine and paracrine manner as part of normal vascular biology. Additionally, stromal cells in nearby tissues can respond to a cytokine environment and induce the expression of ANG2 (Huang et al., 2010; Hato, Tabata, and Oike, 2008; Thurston and Daly, 2012). This provides neighboring cells and the endothelium itself an important role in regulating endothelial biology and may be at play in the context of the granuloma.

If nearby fibroblasts, pericytes, or the endothelium itself are producing ANG2, as would be suggested by Gideon et al. (2022) and paralleling tumor biology, then we

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<sup>9</sup>Angiopoietin-1 is the main ligand during development and homeostasis, where knockout of ANG1 recapitulates the phenotypes of TIE2 knockout, but during pathology, alterations in ANG2 levels are the primary focus (Akwii and Mikellis, 2021). There are also a range of angiopoietin-like proteins that serve diverse functions that may be of interest but are target for far future investigation in this direction (Hato, Tabata, and Oike, 2008).

<sup>10</sup>TIE1 primarily plays a complementary role with TIE2 as a heterodimer – this is a bit beyond the present topic but for further reading, Savant et al. (2015) and La Porta et al. (2018) have excellent studies on how TIE1 contributes to angiogenesis in development and cancer.

are missing out on crucial aspects of stromal-granuloma interactions in the dataset from Cronan et al. (2021). What gene networks might be regulating these processes and are macrophages a major source of a key inducing cytokine(s) (Augustin et al., 2009)? These are difficult questions to address with existing tools and the gene duplication of the zebrafish presents fresh difficulties. Others in the lab have developed CRISPR-Cas9 mutations in zebrafish *angpt2a*, *angpt2b*, and *tie2*, which may be useful in dissecting some of these effects. Studies using these lines and, potentially, the development of novel *angpt2* transgenic reporters or application of broader microdissection approaches and further scRNA-seq would help to narrow on the cellular identity of ANG2-producing cells in the granuloma and how modulation of those cells could offer an alternative pathway toward vascular normalization in the granuloma either independent of or in concert with VEGF-targeting therapies. As we have seen with bevacizumab, it will likely be necessary to develop a multi-pronged approach to vascular targeting and a more thorough study of this pathway during mycobacterial infection would constitute a major contribution in that direction (Huang et al., 2010; Saharinen, Eklund, and Alitalo, 2017). An unlikely, but plausible, hypothesis would be that NFAT in macrophages is regulating the production of a cytokine that is stimulating ANG2 production by fibroblasts, pericytes, or the endothelium. Such an intercellular signaling network would be a fascinating new aspect of granuloma angiogenesis biology worth further exploration. Despite the great deal that is known about the roles of ANG2-TIE2 signaling during cancer, it is not clear if there are discrete stimuli that are able to upregulate ANG2 expression or secretion by the endothelium and identification of such may serve as a further upstream mechanism of host-directed intervention in various diseases (Huang et al., 2010). Interestingly, the angiopoietin-TIE2 system is also functions in and is important for lymphatic development, a topic that will be returned to in Section 5.6 (Eklund, Kangas, and

Saharinen, 2017).

### 5.5.2 Pericytes

Pericytes are critical stromal cells that support various aspects of vascular cell biology. These cells are derived from myeloid and mesenchymal precursors and express a number of different pattern recognition receptors, making them important in regulating vascular inflammation and, potentially vascular responses to infections (Yamazaki and Mukouyama, 2018; Stark et al., 2013). Despite these important functions in maintaining the vascular barrier, limiting vascular permeability, regulating angiogenesis, and more, essentially nothing is known about their contributions to tuberculosis (Bergers and Song, 2005). In addition, there are interesting subpopulations, especially within the brain, that have macrophage-like functions while remaining affiliated with the vascular endothelium; these cells likely conduct interesting immune processes about which we know essentially nothing (Venero Galanternik et al., 2017; Balabanov et al., 1996). Slightly more is known about their function in cancer, which may establish a model for our understanding in infectious disease.

Within tumors, pericyte coverage of vessels is highly variable and almost universally perturbed and pericytes are thought to play a bimodal role where high pericyte coverage fosters tumor growth while low pericyte coverage facilitates metastasis (Ribeiro and Okamoto, 2015; Saharinen, Eklund, and Alitalo, 2017). Additionally, low pericyte coverage of blood vessels is associated with increased ANG2 expression, suggesting that pericytes-endothelial contacts or paracrine signaling may be important for limiting vascular permeability in addition to any physical role these cells may have in prevent vascular leakage. Because pericytes do not depend on VEGFA signaling for their maintenance, they are often left behind in the aftermath of anti-

angiogenic therapy, where they are able to serve as a path for vascular regrowth after withdrawal of therapy (Keskin et al., 2015; Ribeiro and Okamoto, 2015). This suggests that a combination of anti-angiogenic therapy and pericyte-targeting therapy might be able to achieve more complete and sustained vascular regression.

The targeting of pericytes also offers another approach to understanding the function of vascular leakiness in influencing granuloma biology as others have demonstrated that pericytes are critical mediators of vascular integrity and that abolition of these cells can actually improve peri-tumor vasculature and restrict tumor growth (Keskin et al., 2015). Pericytes and the endothelium also communicate through a variety of immunological pathways, including inflammasome activation and IL-1 $\beta$  signaling, making their interactions able to be interfered with by true inflammatory signals during disease (Kozma et al., 2021).

Other types of mural cells also exist that serve to support the vasculature and these may be of interest to study as well. For instance, Bower et al. (2017a) found that lymphatic vessels that run along blood vessels regulate their elaboration during vascular development, suggesting that crosstalk between these two critical circulatory systems may be important for the overall progression of angiogenesis in disease as well. These, in addition to these interesting macrophage-like pericytes demonstrate that there is much left unknown about these cells and a more comprehensive spatial and transcriptional profiling of them in various disease states is needed to fully understand how the mural cells enveloping the vasculature respond to various stimuli (Yamazaki and Mukouyama, 2018; Stark et al., 2013; Kozma et al., 2021; Balabanov et al., 1996).

Luckily, there already exist tools within the zebrafish that can allow for preliminary dissection of some of the roles of pericytes during mycobacterial infection. The

TgBAC(*pdgfrb:mCitrine*<sup>s1010</sup>) zebrafish line labels pericytes throughout development and may allow us to visualize some aspects of their biology using CLARITY techniques in the adult zebrafish granuloma as described in Section 2.6 (Vanhollebeke et al., 2015). Alterations in pericyte localization, vascular engagement, or even interactions with and within the granuloma itself may suggest an important role for these cells. Additionally, little is known about the contributions of the signaling molecules that these cells utilize for their normal biology and how those may influence disease. Use of genetic mutations in *pdgfrb* (the major signaling molecule to induce pericyte differentiation) or tissue-targeted ablation approaches (*pdgfrb:nitroreductase*<sup>11</sup> or similar) may offer a look at the impact these cells have on the overall development of tuberculosis disease and their contributions to angiogenesis. Of all the as-yet unstudied stromal cells likely to influence the progression of disease, these strike me as the most interesting population given their heterogeneity and known functions in cancer.

### 5.5.3 Extracellular Matrix

Although this dissertation has not particularly addressed the biochemistry of the focal chemokine – VEGFA – it is an intriguing protein with multiple layers of regulation, including the existence of multiple splice variants. The two major ones VEGFA<sup>121</sup> and VEGFA<sup>165</sup> have distinct capacities for binding to the extracellular matrix (ECM), with VEGFA<sup>165</sup> binding to heparin while VEGFA<sup>121</sup> freely diffuses through ECM (Fearnley et al., 2016; Shibuya, 2011). These differences are important as they may determine different aspects of the overall angiogenic response and may offer an additional mechanism for subversion by the bacteria through engagement with the extracellular matrix.

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<sup>11</sup>Nitroreductase is a bacterial enzyme that reduces metronidazole into a toxic by-product that kills cells and can be used to ablate specific cell populations, although this clearly has the potential to induce some immune responses to all of the dead cells (Sharrock et al., 2021).

## Tetranectin

An interesting happenstance in my early graduate school career was a few months spent on the study of the zebrafish gene *clec3ba*, which is one of the two zebrafish homologs of the human *CLEC3B*, which encodes for the gene tetranectin. Tetranectin is a plasminogen<sup>12</sup>-binding protein produced by macrophages with very few described roles (Nielsen, Clemmensen, and Kharazmi, 1993). As implied by the gene name, tetranectin is a C-type lectin which we had naïvely thought might be a candidate for the MINCLE homolog based on the expression pattern by RNA-seq. This protein is able to block the binding of plasmin to the extracellular matrix, which will reduce the rate of fibrin degradation (Mogues et al., 2004). Because so little is known about the biology of tetranectin aside from its structure (it exists as a trimer), some basic details about its role in plasminogen binding, and its potential as a biomarker for various cancers, this could offer a really novel opportunity to study something unexpected in the pathogenesis and angiogenic response to mycobacterial infection (Holtet et al., 1997; Nielsen et al., 1997; Graversen et al., 1998). One of the only modern papers I could find on this gene found that expression of tetranectin is protective during sepsis but that excessive endocytosis of tetranectin by macrophages resulted in increased pyroptosis and exacerbation of inflammation (Chen et al., 2020). This suggests that tetranectin can exert anti-inflammatory functions during this disease and could be a promising target to agonize during sepsis.

In my work on this gene, I found that disruption of *clec3ba* by CRISPR/Cas9 reduced the amount of angiogenesis in response to TDM injection in the larval zebrafish

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<sup>12</sup>Plasminogen is an essential component of the fibrin-degradation pathway that clears fibrin clots. The balance of fibrin deposition and plasmin degradation determines the hemodynamics of clot formation. Fibrinogen is one of the many targets of aspirin that allows it to effectively thin the blood to prevent heart attacks (Bjornsson, Schneider, and Berger, 1989; Keragala and Medcalf, 2021).

in a manner nearing statistical significance. While we realized that we were barking up the incorrect metaphorical tree in pursuit of the zebrafish MINCLE, these results are suggestive evidence that this gene may be playing a role in mediating angiogenesis, potentially in a broader sense but perhaps restricted to TDM or mycobacteria. In the search for non-VEGF mechanisms to inhibit angiogenesis, more active pursuit of these sorts of observations may provide new drug targets to either block or enhance angiogenesis.

## Plasminogen

Given tetranectin's role in binding plasminogen, it begs the question of whether plasminogen itself may have some important role in mediating pathological angiogenesis. Previous studies had found that plasminogen knockout mice had reduced angiogenic responses to VEGF, implicating plasmin degradation of fibrin in facilitating vessel growth (Oh, Hoover-Plow, and Plow, 2003). However, loss of plasminogen results in fibrosis as there is no longer an effective mediator to degrade these fibrin deposits. Given that mycobacteria bind and activate plasminogen using alpha-enolase, it seems logical to expect that this protein serves pro-bacterial functions (Monroy et al., 2000). Indeed, interaction with plasminogen inhibits phagocytosis, but this does not really address the role of this pathway in the granuloma (Echeverria-Valencia et al., 2019). To address this, it will be useful to generate knockout zebrafish able to model loss of function in plasminogen and fibrinogen. The role of fibrin and fibrin degradation in the granuloma is generally poorly understood so uncovering new roles of these hemodynamic processes are likely to reveal novel mediators of angiogenesis and host defense against mycobacteria.

The target of plasminogen, fibrin, also plays an important role in mediating the cellular effects of TDM. TDM bound to fibrinogen inhibits cellular necrosis and pro-

motes the formulation of the granuloma whereas in the absence of fibrinogen, extensive cellular necrosis is able to occur (Sakamoto et al., 2010). This would suggest a suppressive or immunomodulatory effect of fibrin on TDM; in the absence of plasmin to degrade fibrin, these effects may be magnified and angiogenesis further reduced. If that is the case, then local blockade of plasmin in the granuloma could be an interesting experimental approach to altering the granuloma microenvironment to be more hostile to bacterial growth. Some of these interactions have begun to be studied in recent years with a study from Horte et al. (2019) demonstrating that fibrinogen mutant zebrafish had improved bacterial control due to mycobacterial induction of coagulation, a process known to be induced by TDM (Retzinger et al., 1982; Retzinger, 1987; Donnachie, Fedotova, and Hwang, 2016). It is possible that, depending on the degree of fibrin deposition, various effects can be exerted where not enough may be neutral or neutral-positive while exacerbation may offer benefits as well (Venkatasubramanian et al., 2016). These interactions, especially those between platelets and macrophages, warrant further study and it will be interesting to dissect plasmin/fibrin interactions as aspects of granuloma formation, the granuloma microenvironment, and angiogenesis (Feng et al., 2014; Lugo-Villarino and Neyrolles, 2014).

#### 5.5.4 Other Angiogenic Stimuli

Vascular biology is vastly complex and there are many potentially influential angiogenic stimuli. As has already been discussed, Torraca et al. (2017) found a critical role for CXCR4 signaling in inducing angiogenesis in response to mycobacterial infection, but determining the signaling underlying CXCL12 expression from macrophages would certainly be of some interest. If NFAT is mediating the production of CXCL12, then this may offer an opportunity to intervene in a broadly pro-angiogenic pathway

to exert host benefit by multiple mechanisms against which the pathogen may have little recourse.

Another critical pro-angiogenic signaling pathway is driven by the expression of TGF- $\beta$ . Transforming growth factor beta is produced by macrophages and other immune cells, but also exerts autocrine functions that facilitate the upregulation of angiogenic signals, including VEGFA, as well as directly influencing angiogenesis by priming endothelial cells (Jeon et al., 2007; Ferrari et al., 2009; Goumans, Liu, and Dijke, 2009). Given the known roles for NFAT signaling in mediating the downstream responses to TGF- $\beta$ , it is of interest to explore the role of this somewhat mysterious cytokine in regulating macrophage-endothelial crosstalk and potentially important roles upstream of VEGFA in priming endothelial cells for proliferation (Cobbs and Gooch, 2007). Engoglin is a part of the TGF- $\beta$  receptor complex and is a transmembrane protein that is induced in endothelial cells during inflammation to mediate the responses of the endothelium to TGF- $\beta$  and other related cytokines and is thought to mediate certain aspects of blood vessel diameter and maturation after VEGFA-mediated angiogenesis (Liu et al., 2014; Sugden et al., 2017). This pathway thus makes a compelling case as being potentially dysfunctional during granuloma angiogenesis and may be an additional target that can facilitate vascular perfusion and normalization to improve drug delivery.

Endothelins are short peptides that regulate the vasculature that have previously been shown to play a role in tuberculosis. *M. tuberculosis* secretes an enzyme that liberates endothelin from the pro-peptide to allow for active signaling and this enhances bacterial growth (Correa et al., 2014). While this was originally proposed to be mediated by macrophage recognition of active endothelin, an alternative hypothesis is that this peptide is being activated by *M. tuberculosis* to modulate aspects

of vascular biology, either through endothelin's roles in vasoconstriction or promoting endothelial survival. Such functions are comparatively understudied relative to VEGFA and may play important roles in mycobacterial subversion of the endothelial system independently or in synthesis with TDM.

A final point is the potential role for novel modes of cellular differentiation to play a role in modulating host angiogenesis. Studies in development and cancer have identified an mesenchymal to endothelial transitions that allow for the direct incorporation of mesenchymal stem cells and macrophages into the structure of the endothelium itself whereby these cells adopt defining characteristics of the endothelium, including expression of vascular endothelial cadherin (Ubil et al., 2014; Wu et al., 2007; Zhang et al., 2008a). The contribution of the diverse types of macrophages within the granuloma, including those expressing both VEGFR2 and TIE2, to the vasculature is largely unknown, but the incorporation of mycobacterially subverted macrophages into the vasculature may be a further mechanism of coopting this system for the benefit of the invading bacteria (Cortie et al., 2014; Hall et al., 2012). Many further studies would be required to address this, but longer-term fate mapping of *flk1<sup>+</sup>*, *irg1<sup>+</sup>* cells may open some possibilities for evaluating this process.

## 5.6 Lymphangiogenesis

This project has focused strictly on the proliferation and growth of vascular endothelial cells during mycobacterial infections, a process known as (hem)angiogenesis. However, a parallel process exists concerning the lesser studied lymphatic vascular system and this is known as lymphangiogenesis. The evolutionary segregation of blood and lymph circulation has led to distinct but very closely related mechanisms of regulation during development and pathology, which suggests a potential role for

this pathway during tuberculosis infection as well. While this process is less extensively studied, it is a critical component of the vascular development of vertebrate organisms and is essential for proper fluid homeostasis and immune system function, as lymphatic vessels are the route along which antigen presenting cells “drain” into the lymph nodes to prime the adaptive immune system. Failures of lymphangiogenesis result in lymphoedema and general problems with fluid balance (Makinen et al., 2001). In the context of disease, the lymphatic system is essential for proper immune behavior, but during chronic conditions like cancer or tuberculosis, lymphangiogenesis can be subverted by the insult for their own benefit. In cancer, lymphangiogenesis is utilized to provide additional routes of metastasis away from the primary tumor; the role in tuberculosis is comparatively less well studied (Huang et al., 2010; Augustin et al., 2009; Duong, Koopman, and Francois, 2012; Stacker et al., 2014).

Lymphangiogenesis is primarily mediated through an analogous set of ligand-receptor pairs to that seen in hemangiogenesis. VEGFC and VEGFD<sup>13</sup> are the major chemokines that bind VEGFR3 on the lymphatic endothelium to stimulate lymphangiogenesis (Alitalo, Tammela, and Petrova, 2005; Kuchler et al., 2006; Haiko et al., 2008), a process which depends on functional ERK signaling, much like hemangiogenesis (Shin et al., 2017; Shin et al., 2016). During development, a portion of the cardinal vein differentiates into lymphatic progenitors (Yaniv et al., 2006; Nicenboim et al., 2015) and begins to elaborate into a full lymphatic system by approximately 3 days post fertilization in the larval zebrafish; these budding vessels are guided by arterial cues, which allows much of this system to parallel the blood endothelial system (Jung et al., 2017; Bussmann et al., 2010). This process ultimately leads to a complete lymphatic system able to drain excess fluid and remove insults from within

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<sup>13</sup>At least VEGFD acts non-redundantly and is also able to stimulate hemangiogenesis in the zebrafish and, given the findings presented in Subsection 1.5.1, both of these likely to play roles in other angiogenic processes (Bower et al., 2017b).

tissues, although zebrafish are not known to possess lymph nodes as mammals do and the primary sites of antigen presentation lie elsewhere (Lessen et al., 2017; Onder et al., 2017).

During cancer growth, the tumor itself and the tumor associated macrophages that surround it both contribute to lymphangiogenesis, which provides a means of dissemination for the tumor to distal body sites. Lymphangiogenesis and VEGFC/D can facilitate metastasis in the tumor although it is not clear whether this mechanism operates solely through induction of lymphangiogenesis or if VEGFC/D can act directly on the tumor itself, analogously to VEGFA autocrine activity (Stacker et al., 2014). The primary site of initial metastasis – to the lymph node – is a symptom of the lymphatic involvement in this disease. Of course, it was conceptually possible that the lymphatic endothelium was a bystander, but it is clear that the lymphatic system actively grows and remodels in response to tumor signals. It has since been demonstrated that VEGFC overexpression by cancer cells in the tumor drives increases lymphangiogenesis and early lymph node metastasis, implicating active manipulation on the part of the tumor in this process (Duong, Koopman, and Francois, 2012). Several different tumors also overexpress the lymphangiogenic effector CCBE1, which aids in processing VEGFC into its active form to potentiate lymphatic growth (Hogan et al., 2009; Le Guen et al., 2014; Li et al., 2018a; Song et al., 2020; Zhao et al., 2018).

Tumor-associated macrophages have been linked to this process through tumor-directed activation of the inflammasome that drives IL-1 $\beta$  processing to increase lymphangiogenesis (Weichand et al., 2017). This interesting mechanism provides the tumor multiple means of inducing a full spectrum of different signals that facilitate its spread. Macrophages more broadly also play key roles in the induction of in-

inflammatory lymphangiogenesis, also partially mediated by IL-1 $\beta$  as well the induced expression of VEGFC and VEGFD during insult. Some sort of systematic promoter comparison, either computationally or *in vivo*, would help to decipher the regulatory mechanisms for each isoform. After tissue damage, it is critical to direct the growth of new lymphatic vessels toward the injury in order to facilitate drainage and immune access, but excessive lymphatic inflammation is harmful. An additional mechanism that macrophages can use to induce lymphangiogenesis is through IL-33 production, which induces a complex intracellular cascade that include nitric oxide signaling to drive inflammation (Han et al., 2017). Similarly, TNF- $\alpha$  has been shown to induce lymphangiogenesis by stimulating production of VEGFD<sup>14</sup> (Hong et al., 2016). Conveniently, the zebrafish is able to be utilized to model many of these processes in inflammatory lymphangiogenesis, allowing us to adapt it to the study of these processes in mycobacterial infection (Okuda et al., 2015).

To date, there have been two major sets of studies on the role of the lymphatic system in mycobacterial infection, which is a relative indictment on the attention that has been paid to this potentially significant contributor to disease. The first, from Harding et al. (2015), examined this pathway through chemical inhibition to block VEGFR3 activity. This study found that reduction of lymphangiogenesis resulted in decreased proliferation of *M. tuberculosis*-specific T cells, suggesting that lymphangiogenesis blockade reduced access of antigen-presenting cells to the lymph nodes to stimulate T cell proliferation. However, this was done in the context of BCG infection, so the effects on bacterial burden were unable to be ascertained. Future studies on this pathway during infection with virulent *M. tuberculosis* seems to be in order as well as a more thorough examination of the consequences of this on disease

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<sup>14</sup>Could it be through NFAT? Perhaps and if so that would add another putatively positive aspect to targeting this pathway.

progression over time. While blocking lymphangiogenesis may alter the ability of the immune system to generate a robust adaptive response, it may also alter cellular dynamics earlier in infection in host-beneficial ways, but these things are currently unknown. A synthesis of the larval and adult models of zebrafish infection seem to be promising methods for assessing some of these impacts.

The second studies interrogated the function of lymphatic endothelium as a replicative niche for *M. tuberculosis* (Lerner et al., 2016; Lerner et al., 2020). Although tuberculosis is classically a pulmonary infection and the pulmonary niche is the only effective route of transmission, the most common extrapulmonary presentation of tuberculosis is in the lymph nodes, a disease known from antiquity as scrofula. The infection of the lymph nodes implied that the bacteria had a productive replicative niche in the lymph nodes, which could have simply been macrophages, but this study found that mycobacteria could directly infect the lymphatic endothelium (Lerner et al., 2016). Indeed, it appears that lymphatic endothelial cells are a relatively permissive host cell type, as the bacteria will cord within these cells as a mechanism of host immune evasion (Lerner et al., 2020). This highlights a binary role for lymphangiogenesis during infection, where it may be important for priming certain adaptive immune responses, but may also offer a convenient replicative niche; the net effect remains in need of study and the zebrafish may offer an ideal model in which to do so.

There are an abundance of tools in the zebrafish to study lymphangiogenic processes. The *lyve1* transgenic lines allow for visualization of lymphatic vessels throughout life, where they appear at approximately 3 days post fertilization (Okuda et al., 2012). This allows both larval and adult zebrafish to be used to study these processes, unlocking the full spectrum of disease. Additionally, a number of genetic tools exist

to allow for study of this process. Notably, the *hsp70l:sFlt4<sup>bns82</sup>* line is able to potently inhibit the generation of lymphatics through overexpression of a soluble decoy VEGFR3 after heat shock (Matsuoka et al., 2016). Together, these lines can offer both a means of visually assessing the state of the lymphatic endothelium as well as block lymphangiogenesis at various time points. Pioneering studies should evaluate the degree of lymphangiogenesis induced by *M. marinum* infection and, thorough genetic inhibition, study the role of this at both early and late stages of infection in the larva and the adult. It is my hypothesis that, especially in the adult, there is extensive lymphatic involvement that contributes to infection and, like previous studies, may provide an extragranulomatous replicative niche during earlier stages of infection (Lerner et al., 2016). The ability to study the contributions of lymphatics within a model that presents with necrotic, epithelioid granulomas offers the opportunity to also evaluate them as a potential route of dissemination complementary to hematogenous dissemination.

There are several proposed strategies to block lymphangiogenesis in these diverse contexts. One is, of course, through pharmacological inhibition of VEGFR3 signaling in a manner analogous to that of bevacizumab, but this may be subject to subversion through compensatory VEGFR2 signaling. Another is to block aspects of the lymphatics that are required for growth, such as integrin  $\alpha 5$  (Dietrich et al., 2007) or fatty acid oxidation (Wong et al., 2017). The future of this remains uncertain, although these drugs may act as excellent adjuvants to existing antihemangiogenic therapies.

## 5.7 Generalizability

One of the major questions is whether or not the findings I have shown in Chapter 3 are able to be generalized to other disease contexts. The most notable possibility is in cancer, where tumor-associated macrophages are well known to produce VEGFA to induce tumor angiogenesis (Subsubsection 1.5.4). Are these tumor associated macrophages detecting some tumor-derived ligand to induce these responses or is the production of VEGFA a product of a complex set of secreted signals that create the proper microenvironment? It may be that such a distinction is one without an underlying need for discrimination as the key lies in whether these signals – whatever they are – are inducing NFAT nuclear translocation. To my knowledge, no study has been done to look at the localization of NFAT in tumor associated macrophages from either cancer models or patient biopsies and this would make for one avenue to begin exploring potential contributions of this pathway to cancer progression.

The zebrafish and the tools I have made would also provide an excellent means to beginning to study these phenomena. For instance, the zebrafish *mitfa:BRAF<sup>V600E</sup>* model could be used to induce melanomas in the zebrafish and then further histological profiling can be done to further examine the effects on tumor mass, vascularization, and other macrophage responses to the tumor in the *irg1:VIVIT* background. Complementary use of similar mouse models with *LysM-Cre*; *Nfatec<sup>fl/fl</sup>* or *ROSA26:lox-STOP-lox-VIVIT* lines already developed may offer further insight on this process in a mammalian context. The precise biology of tumor associated macrophages, while extensively described, is not particularly well understood and genetic approaches like these may offer further insight into some master regulators of tumor associated macrophage responses and present opportunities for reprogramming

toward more effective tumor clearance.

Other infectious diseases are known to induce the formation of granulomas or manipulate the vasculature in various ways. A great many diseases induce the formation of new blood vessels for their own purposes while other actively suppress the host angiogenic response (Osherov and Ben-Ami, 2016). For instance, *Cryptococcus neoformans* is a well-known granulomatous infection, where it can either conceal itself within quiescent granulomas within the lung or in active, fulminant granulomas within the brain. Infection begins within macrophages where the yeast has to adapt to a new, hostile environment and subvert these responses for its own benefit, a process which has been described in some detail over the course of scientific investigation (Coelho, Bocca, and Casadevall, 2014). However, little is known or described about the associations between cryptococcal granulomas and the vasculature, with the subject left unmentioned even in the most recent review on the subject (Ristow and Davis, 2021). More generally, the biology of the cryptococcal granuloma remains understudied and contributions of the surrounding stromal cells largely unknown and with few known host determinants despite many studies having been conducted on the contributions of C-type lectin receptors to host defense (Campuzano et al., 2017). However, C-type lectin signaling seems to have a mostly neutral effect on *Cryptococcus* infection despite CARD9 playing a critical role in host defense (Nakamura et al., 2007; Campuzano et al., 2017; Walsh et al., 2017). Whether VEGFA and angiogenesis play a role in this context would certainly be interesting and, given the facility with which larval zebrafish can be infected with *Cryptococcus*, this may be a convenient platform to begin studying some of these interactions, as well as the macrophage-specific consequences of NFAT inhibition, as NFAT is thought to play important roles in protection against various fungal diseases (Tenor et al., 2015; Davis et al., 2016; Greenblatt et al., 2010; Goodridge, Simmons, and Underhill, 2007;

Bojarczuk et al., 2016). The ability to adapt an existing model in the zebrafish to capture elements of this disease offers a compelling means of studying the contributions of NFAT signaling and angiogenesis in the innate responses to this prevalent disease of the immunocompromised (Lin and Heitman, 2006). Indeed, given that the existing antifungal drug itraconazole has known anti-angiogenic activity, it may be of interest to attempt to repurpose this drug as an anti-angiogenic therapeutic option in other contexts (Nacev et al., 2011).

*Histoplasma* is a further fungal infection that is known to induce granuloma formation and for which there seems to be additional descriptions of the vascular involvement of this infection. Similar to other infections, enhanced stabilization of HIF-1 $\alpha$  in this context resulted in improved bacterial killing (Friedrich et al., 2017; Friedrich et al., 2019). Despite these potentially beneficial roles for known pro-angiogenic pathways in the innate defense against *Histoplasma*, this does not resolve any role for the angiogenesis itself in this process. However, during later stages of granulomatous infection, extensive vasculitis in the periphery of these foci has been noted by histological examination (Mukhopadhyay and Katzenstein, 2010; Kauffman, 2007; Mann, Fogarty, and Kincaid, 2000). Interestingly, itraconazole is the primary therapy used for histoplasmosis and the anti-angiogenic contributions of this therapy to the overall course of disease would certainly be of some interest in further clarifying the mechanisms of action (Kauffman, 2007). Models exist in the mouse for the study of this infection and have been well-described, but further work to characterize the host and pathogen factors underlying the granulomatous response in this disease seems to be in order (Heninger et al., 2006). Not only would this potentially reveal alternative treatment options, but would highlight further aspects of potentially generalizable granuloma biology.

Schistosomiasis is a classical model of what are called fibroid granulomas and this disease of the urogenital tract is known to induce VEGF-dependent angiogenesis that benefits the growth of the parasitic eggs (Shariati et al., 2011; Pereira et al., 2013; Figueiredo et al., 2015; Chabon et al., 2014; Loeffler et al., 2002). However, the precise underlying mechanisms of this angiogenesis induction are unknown and appears to be mediated by specific macrophage-mediated detection of parasite ligands, but these have not been identified (Loeffler et al., 2002). Identification of the underlying mechanism of VEGF-induction by *Schistosoma* has implication in not only improving disease outcomes, but managing the hypertensive pathology of schistosomiasis, which is linked to parasite-induced VEGF production (Chabon et al., 2014). The potential that this pathway is mediated by NFAT should be further explored, which is now possible in the zebrafish and might offer a compelling model for identifying both parasite ligands and host pathways in this disease (Takaki et al., 2021).

The role of angiogenesis in mold infections is the inverse, where *Aspergillus* is thought to inhibit the host VEGFA response in order to maintain itself within a vascular niche (Kontoyiannis, 2010). While the host responds by producing abundant VEGFA<sup>15</sup>, the fungus produces gliotoxin to destroy newly formed vessels and may also produce factors that suppress HIF-1 $\alpha$  (Ben-Ami, 2013). It thus becomes a compelling option that there may be non-targeted pathways by the fungus that could be repurposed to induce angiogenesis; NFAT activators may serve such a purpose by driving VEGFA expression, which has been suggested to be a potential host-directed therapeutic target in aspergillosis.

Rheumatoid arthritis is an autoimmune condition characterized by aberrant inflammation in the joints, which is often mediated by macrophages and T cells and for

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<sup>15</sup>Could this be downstream of C-type lectin receptors?

which anti-TNF- $\alpha$  therapy has proven remarkably effective (Feldmann, 2002). The macrophage and vascular involvement suggests a potential role for some intra-macrophage signaling pathway that induces the production of VEGFA, but the identity of this pathway is unknown, although assumed to be through the HIF-1 $\alpha$  (MacDonald et al., 2018). As anti-angiogenesis therapy has been shown in animal models to improve symptoms, this may offer another alternative to treatment for arthritis where other treatments have been unsuccessful (Paleolog, 2002; Elshabrawy et al., 2015). Indeed, treatment of patients with rheumatoid arthritis with tacrolimus ameliorates symptoms and has been shown to aid in treatment of otherwise non-responsive arthritis (Dutta and Ahmad, 2011). This mechanism could be through activity of tacrolimus on T cells, but may play equally important roles in inhibiting macrophage TNF- $\alpha$  and VEGF production to improve symptoms. Alternatively, parts of the angiogenic response in arthritis are known to be mediated by aberrant TLR activation, which drives the production of ANG2 and, although we do not yet know if NFAT signalling mediates angiopoietin expression, this could be another potential mechanism of action here (Saber et al., 2011).

It may be the case that activation of C-type lectin receptors more broadly is able to induce the production of VEGFA, which would tie this pathway into a vast array of diseases given the vast variety of ligands able to agonize this pathway. Alternatively, it may be the case that pathogen-specific subversion of these receptors induces different relative proportions of the downstream pathways; if this were the case, the underlying mechanisms would be extremely revealing as to the nature of C-type lectin signaling and selective downstream pathway induction.

## 5.8 A Pressing Need for More Objective Approaches to Image Analysis

While microscopy serves as the basis of much experimentation and is ubiquitous in the zebrafish field, these sorts of data remain extremely difficult to analyze in an objective manner. While blinding approaches (Section 4.7) can help, what is truly needed is the development of truly more objective analytical approaches to glean quantitation from images. These features should, often, be discrete features that can be differentiated from the background but in practice this is not always the case. As often as not, human interpretation of these images is required to differentiate apparent from true effects, a problem that is magnified by heterogeneity within the samples. As we saw throughout Chapter 4 the manual aspects of image processing and analysis can be creatively subverted with existing tools, but to end-to-end analyze images and extract various parameters would both ease the labor of the microscopist but would likely unlock new levels of analysis that are currently impractical or impossible.

The answer, so it seems, lies in intelligent application of machine learning algorithms. While these approaches are functionally in their infancy, it is already possible to attempt to train a model to analyze diverse aspects of images and classify them into distinct categories; it is more difficult to use those models to do things like precisely measure subtle changes. In the future, these will be important improvements to pursue with the goal of making scientific image analysis more reproducible, robust, and informative.

### 5.8.1 Approaches to Measuring Angiogenesis

Many have attempted and few have succeeded at creating approaches that are able to automate the measurement of angiogenesis in the zebrafish. While there seem to be a number of FIJI plugins that can measure blood vessel growth *in vitro*, the *in vivo* context presents some unique challenges even within such a developmentally defined model. Although there had been earlier utilities (Heath, Bicknell, and Simms, 2017), probably the first earnest attempt (from only this year) focused on developmental angiogenesis in the brain of the larval zebrafish and was able to develop a machine-learning methodology to monitor quantitative features of the zebrafish cranial vasculature over time – an unquestionably impressive feat (Kugler et al., 2022). However, this is somewhat narrowly focused on its intended vascular context and I had substantial difficulty in translating this approach for use in intersegmental vasculature. While tailored approaches for each discrete problem is how we have gotten into this situation of being unable to utilize other's tools for our purpose, it may be that either the approach taken by Kugler et al. needs to be accommodated somewhat to other regions of vasculature or bespoke tools will have to be made for each possible application.

To do so, I would propose a few key steps. First, it is important to develop a landmarked model of angiogenesis over the course of development that is able to provide a robust reference with acceptable built-in tolerances. While developmental angiogenesis in the zebrafish is highly stereotyped, there is unquestionably a degree of fluctuation from fish to fish in the exact trajectory. Once such a model has been established, the model must be able to align new images to this reference and then detect any differences between the spectrum of possible references and the image presented to capture measurements about the aberrations – total length and volume,

intersegmental location, correspondence to bacterial burden (if present), etc. It may be that this can be accomplished with traditional thresholding-based approaches, but training a machine learning model in PyTorch or TensorFlow and integrating with the new PyImageJ may be more feasible at scale and more generally applicable. This is a project worth substantial effort if the use of the larval zebrafish intersomitic vasculature is to be used in future endeavors to measure vascular perturbations and would likely advance this model in and of itself by enabling more consistent measurements across individuals and labs.

### 5.8.2 High-Throughput Quantitation of Cell Features

Unlike the zebrafish, which is a complex multicellular organism, extracting useful information from cells in tissue culture *should* be comparatively easier, but as I have experienced, this is not always the case. Subtle variations in fluorescence intensity and patterning and algorithmic preference for within-image automated thresholding versus pan-image thresholding makes it difficult to accurately threshold cellular features at low magnification, which allows for more complete capture of fields of cells versus the biased selection of individual cells. While it is relatively easy to segment cells at high magnification with the use of proper markers, none of my attempts at automated quantitation of the images for the THP-1 experiments came to fruition. My desire was an objective capture of the nuclear localization of NFAT in these cells and both a binary call of VEGFA expression and some ability to, in a granular way, measure the amount of VEGFA being produced per cell out of approximately 100 cells per field of view. CellProfiler (Carpenter et al., 2006; Kamentsky et al., 2011; McQuin et al., 2018; Stirling et al., 2021) did an acceptable job at segregating nuclei and capturing large punctae of NFAT, but struggled to measure the diffuse staining patterns seen with VEGFA and would often omit cells that, by eye, I called as

positive no matter the thresholding pattern used. Additionally, it was challenging to develop overlapping sets of measurements that allowed me to isolate individual cells with nuclear NFAT and VEGFA expression – a cytoplasmic marker likely would have helped, as would have sparser plating of the cells themselves. However, these workarounds in many ways defeat the aspirations of high-content imaging. While I have no delusion about the imaging done for this work being “high content,” as it becomes possible to automate the collection of terabytes of imaging data, it must be similarly possible to analyze these gigapixels of images in an efficient manner while also extracting the maximum amount of biologically relevant data from them.

While I do not think that machine learning is the answer to all that ails, it does seem likely to me that a properly trained model could allow for precisely these sorts of analyses. By developing a model that demonstrates the correspondence between particular cellular features, it should be reasonable to allow the model to intelligently make judgements based on the proximity and intensity of nearby pixels of relevance. Again, I suspect that one of the sources of issue in my previous attempts was the lack of a pan-cytosolic or plasma membrane marker able to effectively delineate neighboring cells. Further study on the factors that best facilitate automated image analyses in cell culture is evidently needed.

## 5.9 NFAT Targeting as a Host-Directed Therapy?

The elephant in the room at the conclusion of this work is whether or not pharmacological targeting of NFAT could be a viable option to use as a host-directed therapeutic against mycobacterial infections, including tuberculosis. While we do not yet know, based on our preliminary experiments restricted to targeting either the individual isoform *NFATC2* or NFAT signaling in macrophages, it may be a promis-

ing avenue for clinical study once more specific approaches have been developed to the targeting of this pathway. As mentioned in Section 3.4, there are a number of ways to overcome the traditional limitations of calcineurin inhibition either through the development of more targeted inhibitors (like cell-permeable VIVIT or INCA-6), cell-specific targeting with liposomes or AAVs, isoform-specific targeting to minimize the scope of the inhibition, location-specific delivery via nebulization, or some combination of all of the above. At present, it would require more exhaustive study on the disease outcomes of broad NFAT inhibition in humans to begin to develop a model for targeting this pathway during disease.

It has been, to various degrees, established that tacrolimus treatment is able to inhibit angiogenesis, although we are now able to propose a model where, at least in some instances, part of this angiogenesis inhibition is mediated by a failure of macrophages to produce VEGFA after inhibition of NFAT (Shen et al., 2022; Turgut et al., 2011). However, the synthesis of various factors that contribute to tacrolimus's ability to prevent angiogenesis may prove to be a strength. By targeting macrophages and the endothelium simultaneously, it may be possible to circumvent cellular resistance mechanisms within the granuloma that may attempt to induce further VEGFA to alleviate hypoxia. A more thorough study on the role of NFAT in the endothelium would certainly clarify some of these opportunities.

One of the major questions is whether NFAT targeting improves the efficacy of existing antimycobacterial therapies based on time to clearance. This too can be modeled reasonably effectively in the zebrafish model and is an approach that could have important ramifications for this as a host-directed therapy. If NFAT inhibition synergizes with existing therapies to accelerate bacterial killing, then that would certainly present a compelling argument for such a strategy; conversely, if NFAT

inhibition reduces bacterial burden but hampers drug delivery, such would be the death knell for this approach. Even in the latter instance, this should stimulate further study to understand what endothelial features make for the most effective antimicrobial delivery and seek pathways that facilitate those specific alterations.

As an additional and perhaps more readily translated possibility, topical tacrolimus is already widely used clinically to treat atopic dermatitis and may be useful as an adjunctive therapy to treat either non-tuberculous mycobacterial infections in humans, including *M. marinum*, *M. ulcerans*, and *M. abscessus* or leprosy. The potential utility of existing topical treatment to improve drug delivery to these largely topical infections is a compelling possibility that could likely be tested quickly in pre-clinical models of the former list of pathogens. Indeed, confirmation of the utility of such an approach in these other diseases may encourage further study on their utility in the context of *M. tuberculosis*.

## 5.10 Closing Remarks

The work presented here resolves a critical question in the world of tuberculosis pathogenesis and opens new doors in the fields of macrophage biology and angiogenesis by uncovering an unexpected and surprising role for the nuclear factor of activated T cells transcription factor family in mediating vascular endothelial growth factor production by macrophages and resulting angiogenesis during mycobacterial infection. Such a work has necessarily synthesized aspects of diverse disciplines within the biological sciences to address the standing question of what transcriptional regulation may be influencing the outgrowth of blood vessels during tuberculosis. While there is undoubtedly much more to learn about various factoring influencing the activation of the angiogenic response, I believe this opens new doors to the study of the NFAT

signaling pathway in the broader pathogenesis of a variety of mycobacterial infections after years of neglect. While other transcription factors have been deeply characterized in the innate immune response to infection, this critical player has comparatively languished. With this work adding to the body of studies newly emphasizing the critical importance of this pathway in various conditions, further studies are likely to reveal previously unknown levels of import in infection, cancer, autoimmunity, and more.

The conduct of this work has required creativity and perseverance through a years-long process of personal and scientific growth. It has been an intensely human process of experimentation and discovery, success and failure, open-mindedness and a willingness to follow the data where it leads. Science succeeds whenever it is moving us, individually and collectively, toward a more complete understanding of the nuance and chaos of world around us.

# Appendix A

## Reagents Used

**Table A.1:** List of Antibodies Used

Reagent or Resource	Source	Identifier
polyclonal goat anti-human VEGFA antibody	R&D Systems	#AF-293
monoclonal mouse anti-Cas9 antibody	Cell Signaling	#7A9-3A3
Normal Goat IgG Control	R&D Systems	#AB-108-C
rabbit anti-human NFATC1 serum (against NH <sub>2</sub> -CVSPKTTDPEEGFPRGLGA, residues 210 to 227)	Lyakh, Ghosh, and Rice, 1997; Symes, Gearan, and Fink, 1998	#801
rabbit anti-human NFATC2 serum (against NH <sub>2</sub> -CSPPSGPAYPDDVLDYGLK, residues 53 to 70)	Lyakh, Ghosh, and Rice, 1997; Symes, Gearan, and Fink, 1998	#1777
rabbit anti-human NFATC3 serum (against NH <sub>2</sub> -DLQINDPEREFLERPSRDHL, residues 130 to 149)	Lyakh, Ghosh, and Rice, 1997; Symes, Gearan, and Fink, 1998	#1689
rabbit anti-human NFATC4 serum (against NH <sub>2</sub> -GRDLSGFPAPPGEPPA, residues 886 to 902)	Lyakh, Ghosh, and Rice, 1997; Symes, Gearan, and Fink, 1998	#889

rabbit anti-human NFATC4 serum (against NH <sub>2</sub> -CDSKVVFIERGPDGKLQWEE, residues 614 to 632)	Lyakh, Ghosh, and Rice, 1997; Symes, Gearan, and Fink, 1998	#890
rabbit anti-human pan-NFAT serum (against NH <sub>2</sub> -SDIELRKGETDIGRKNTRC)	Lyakh, Ghosh, and Rice, 1997; Symes, Gearan, and Fink, 1998	#796
donkey anti-goat IgG Alexa Fluor 647	ThermoFisher	#A-21447
donkey anti-goat IgG Alexa Fluor 555	ThermoFisher	#A-21432
donkey anti-rabbit IgG Alexa Fluor 647	ThermoFisher	#A-31573
donkey anti-rabbit IgG Alexa Fluor 555	ThermoFisher	#A-31572
donkey anti-mouse IgG Alexa Fluor 555	ThermoFisher	#A-31570
donkey anti-mouse IgG Alexa Fluor 488	ThermoFisher	#A-21202

**Table A.2:** Bacterial Strains

Reagent or Resource	Source	Identifier
<i>Mycobacterium marinum</i> M	ATCC	#BAA-535
<i>Mycobacterium marinum</i> M / pMSP12:mCerulean	Oehlers et al., 2015	N/A
<i>Mycobacterium marinum</i> M / pMSP12:tdTomato	Cambier	2014/A
Gamma-irradiated <i>Mycobacterium tuberculosis</i> H37Rv	BEI	#NR-49098
NEB 5-alpha Competent <i>Escherichia coli</i> (High Efficiency)	NEB	#C2987H
NEB 10-beta Competent <i>Escherichia coli</i> (High Efficiency)	NEB	#C3019H
NEB Stable Competent <i>Escherichia coli</i> (High Efficiency)	NEB	#C3040H

**Table A.3:** Chemicals

Reagent or Resource	Source	Identifier
Trizol	Ambion	#15596026

MicroAmp Fast Optical 96-Well Reaction Plate with Barcode, 0.1 mL	Applied Biosystems	#4346906
Spawning Tanks	Aquaneering	#ZHCT100
Baking soda (sodium bicarbonate)	Arm & Hammer	#426292
Insulin Syringes	BD	#08290-3284-38
Tuberculin Syringe (27G)	BD	#309623
SDS, 20%(w/v) solution, 1L	Bio-Basic	#SD8119
40% acrylamide	Bio-Rad	#1610140
2% bis-acrylamide	Bio-Rad	#1610142
Artemia	Brine Shrimp Direct	#BSEP6LB
Polymyxin B sulfate	Cayman Chemical	#14157
INCA-6	Cayman Chemical Roehrl et al., 2004	#21812
T-75 Flasks	CellStart	#658170
Molecular Biology Grade Water	Corning	#46000CI
10x PBS	Corning	#46013CM
7H10	Difco	#262710
7H9	Difco	#271310
Chloroform	EMD Millipore	#CX1055
16% Methanol-free Paraformaldehyde	EMS	#15710
Triton X-100	Fisher Scientific	#BP151
Dimethyl sulfoxide (DMSO)	Fisher Scientific	#BP231
Mineral oil	Fisher Scientific	#BP2629
Tween-80	Fisher Scientific	#BP337
Sodium chloride	Fisher Scientific	#S271
1x PBS	Gibco	#10010-023
Sodium pyruvate	Gibco	#11360
Amphotericin B	Gibco	#15290-026
HEPES	Gibco	#15630
Instant Ocean Sea Salt	Instant Ocean	#SS15-10
Hygromycin B solution	Invitrogen	#10687010
4-well Cell Culture Slides	MatTek	#CCS-4
35 mm Dish, No. 1.5 Coverslip, 14 mm Glass Diameter, Uncoated	MatTek	#P35G-1.5-14-C
Tris (base)	Millipore	#648311
Millex-SV 5.0 µm	Millipore	#SLSV025LS
T4 DNA Ligase	NEB	#M0202S
Taq 5x Master Mix	NEB	#M0285L
LongAmp Taq	NEB	#M0323L

rSAP		NEB	#M0371L
Q5 High-Fidelity DNA Polymerase		NEB	#M0491L
Q5 High-Fidelity 2X Master Mix		NEB	#M0492L
Deoxynucleotide (dNTPs) Solution Mix		NEB	#N0447L
XbaI		NEB	#R0145L
DpnI		NEB	#R0176L
XmaI		NEB	#R0180L
PflMI		NEB	#R0509L
MwoI		NEB	#R0573L
FseI		NEB	#R0588L
NotI		NEB	#R3189L
RNA Cleanup Kit (50 µg)		NEB	#T2040L
6.5mm ceramic beads		Omni	#19-682
Petri dishes for embryonic zebrafish		Sarstedt	#83-3902-500
FK506 (tacrolimus)	Selleck Chemicals		#S5003
Methanol	Sigma-Aldrich		#179337
Ammonium chloride	Sigma-Aldrich		#254134
24:1 chloroform:isoamyl alcohol	Sigma-Aldrich		#25666
Sodium azide	Sigma-Aldrich		#71290
Boric acid	Sigma-Aldrich		#B0394
Sodium phosphate monobasic monohydrate	Sigma-Aldrich		#D2158
Fetal bovine serum	Sigma-Aldrich		#F2442
Incomplete Freund's adjuvant (IFA)	Sigma-Aldrich		#F5506
Glycerol	Sigma-Aldrich		#G7757
Glucose solution	Sigma-Aldrich		#G8769
OADC	Sigma-Aldrich		#M0678
Phorbol-12-myristate-13-acetate (PMA)	Sigma-Aldrich		#P148
Tween-20	Sigma-Aldrich		#P1754
1-phenyl-2-thiourea	Sigma-Aldrich		#P7629
RPMI-1640	Sigma-Aldrich		#R8758
trehalose 6-6'-dimycolate (TDM) from M. bovis	Sigma-Aldrich		#T3034
Tyloxapol	Sigma-Aldrich		#T8761
100x Tris-EDTA (TE)	Sigma-Aldrich		#T9285
Polybrene	Sigma-Aldrich		#TR-1003-G
BeadBug homogenizer tubes with 2.8mm stainless steel beads	Sigma-Aldrich		#Z763829-50EA
Dry fish food	Skretting		#GEMMA Micro 500

DAPI Fluoromount-G	SouthernBiotech	#0100-20
Tricaine-S (MS-222)	Syndel	#ANADA 200-226
Brefeldin A Solution (1000X)	ThermoFisher	#00-4506-51
BP Clonase II	ThermoFisher	#11789020
LR Clonase II Plus	ThermoFisher	#12538120
FastDigest Esp3I (IIIs class)	ThermoFisher	#FD0454
Calcium chloride	VWR	#BDH9224
Potassium chloride	VWR	#BDH9258
Sodium phosphate dibasic heptahydrate	VWR	#BDH9296
2,2'-Azobis[2-(2-imidazolin-2-yl)propane]dihydrochloride	Wako Chemicals	#VA-044
Magnesium chloride	WardÅ s Scientific	#470301

**Table A.4:** Commercial Assays

Reagent or Resource	Source	Identifier
MeltDoctor HRM Master Mix	Applied Biosystems	#4415450
Luna Universal qPCR Master Mix	NEB	#M3003X
Human VEGF DuoSet ELISA	R&D Systems	#DY293B-05
LunaScript RT SuperMix Kit	NEB	#E3010L
HiScribe T7 High Yield RNA Synthesis Kit	NEB	#E2040S

**Table A.5:** Cell Lines

Reagent or Resource	Source	Identifier
THP-1 monocytic cells	ATCC	#TIB-202
HEK-293T	ATCC	#CRL-2316

**Table A.6:** Model Organisms and Strains

Reagent or Resource	Source	Identifier
<i>Danio rerio</i> strain *AB	ZIRC	#ZDB-GENO-960809-7

Tg( <i>irg1:tdTomato</i> <sup>xt40</sup> )	This work	N/A
Tg( <i>irg1:VIVIT-tdTomato</i> <sup>xt38</sup> )	This work	N/A
Tg( <i>kdrl:eGFP</i> <sup>s843</sup> )	Jin et al., 2005	N/A
TgBAC( <i>vegfaa:eGFP</i> <sup>pd260</sup> )	Karra et al., 2018	N/A
<i>nfatc2a</i> <sup>xt69</sup>	This work	N/A
<i>nfatc3a</i> <sup>xt59</sup>	This work	N/A
<i>card9</i> <sup>xt31</sup>	This work	N/A

**Table A.7:** Recombinant DNA and Plasmids

Reagent or Resource	Source	Identifier
p5E <i>irg1</i>	Addgene Sanderson et al., 2015	#188698
pME VIVIT NS	This work; Addgene	#188699
p3E <i>tdTomato</i>	Addgene Walton et al., 2015	#188700
pDEST tol2 Ubb pA	Addgene Walton et al., 2015	#188701
pME <i>tdTomato</i>	Addgene Oehlers et al., 2015	#135202
p3e Ubb pA	Addgene Walton et al., 2015	#188702
pTol2 <i>irg1:VIVIT-tdTomato</i>	This work	
pTol2 <i>irg1:tdTomato</i>	This work	

pLV hUbC-Cas9-P2A-Puro_- BsmBI-sgRNA	This work, derived from Kabadi et al., 2014; Sanjana, Shalem, and Zhang, 2014; Addgene	#188703
pLV hUbC-Cas9-P2A-Puro sgRNA α NFATC2	This work, Addgene	#188704
pLV hUbC-Cas9-P2A-Puro sgRNA α Safe Targeting Loci	This work, Addgene	#188705
phU6 NFATC2	This work, Addgene	#188708
pmU6 NFATC2	This work, Addgene	#188709
p7SK NFATC2	This work, Addgene	#188710
phH1 NFATC2	This work, Addgene	#188711
phU6 ST	This work, Addgene	#188712
pmU6 ST	This work, Addgene	#188713
p7SK ST	This work, Addgene	#188714
phH1 ST	This work, Addgene	#188715
psPAX2	Addgene	#12260
pMD2.G	Addgene	#12259
sfGFP-C1	Pedelacq et al., 2006, Addgene	#54579

**Table A.8:** Software

Application	Source/Citation
R, 4.2.1	(R Core Team, 2022)

RStudio, 2022.06 "Spotted Wakerobin"	(RStudio Team, 2022)
FIJI/ImageJ2, 2.5.0	(Schindelin et al., 2012; Rueden et al., 2017)
ImageJ, 1.53s	(Schneider, Rasband, and Eliceiri, 2012)
Python/Jython, 2.7.18	(Rossum, 1995)
ggplot2, 3.3.5	(Wickham, 2009; Wickham, 2016; Wickham et al., 2022a)
dplyr, 1.0.9	(Wickham et al., 2022b)
gghighlight, 0.3.3	(Yutani, 2022)
ggbeeswarm, 0.6.1	(Clarke and Sherrill-Mix, 2017)
ggsignif, 0.6.3	(Ahlmann-Eltze and Patil, 2021)
blindrename.pl, 1.0	(Salter, 2016)
scales, 1.2.0	(Wickham and Seidel, 2022)
extrafont, 0.18	(Chang, 2022)
reshape, 0.8.9	(Wickham, 2022)
RColorBrewer, 1.1-3	(Neuwirth, 2022)
FSA, 0.9.3	(Ogle et al., 2022)
HRM Software, 3.0.2	ThermoFisher

**Table A.9:** Equipment

Equipment	Source	Identifier
MP Bio FastPrep 24 Classic (Bead Mill)	MP Bio	#116004500
Applied Biosystems 7500 Fast Real-Time PCR System	ThermoFisher	#4351106
Nikon Stereomicroscope	Nikon	#SMZ745
Nikon High Intensity Illuminator	Nikon	#NI-150
Eppendorf Femtojet 4x	Eppendorf	#5253000025
Precision Plant Growth Chamber, 504 L	ThermoFisher	#PR505755L
Zeiss AxioObserver Z1	Zeiss	N/A
X-Cite 120Q	Excelitas	#12-63000
Cryostat	Leica	#CM1860

**Table A.10:** Oligonucleotides

Description	Sequence (5' to 3')
VIVIT sense oligo	GCCATCATGGCAGGACCACACCCGGT GATTGTTATCACTGGACCACATGAGG AG
VIVIT anti-sense oligo	CTCCTCATGTGGTCCAGTGATAACAA TCACCGGGTGTGGTCCTGCCATGATG GC
VIVIT attB1 primer F	GGGGACAAGTTGTACAAAAAAGCAG GCTGCCATCATGGCAGGACC
VIVIT attB2 primer R	GGGGACCACTTGTACAAGAAAGCTG GGTACTCCTCATGTGGTCCAGTG
irg1 promoter cloning primer F	CCCTATAGTGAGTCGTATTAC
irg1 promoter cloning primer R	TCCCTTTAGTGAGGGTTAAT
nfatc2a gRNA 1	TAATACGACTCACTATAGGGCTGCGA GAACGGGCCACGTTTAGAGCTAGAA
nfatc2a gRNA 2	TAATACGACTCACTATAGGCAGCCCG TCGCCCCACGGGTTTAGAGCTAGAA
nfatc3a gRNA 1	TAATACGACTCACTATAGGCAGTT GCAGTAGTCATGTTTAGAGCTAGAA
nfatc2a PfMI sequencing/genotyping primer F (+ BGH Reverse)	TAGAAGGCACAGTCGAGGCTCGAGGC TTTCTGGAGACCTCTGTCC
nfatc2a PfMI sequencing/genotyping primer R (+ GAG Reverse)	TGACACACATTCCACAGGGCTCTAG AGGTTGCCCTCATATCCTGC
nfatc2a MwoI genotyping primer F	CCTCTATGCAAACGCACCTACG
nfatc2a MwoI genotyping primer R	GTGATGCTCCTGTGGCAC
nfatc3a sequencing primer F (+ M13F-40)	GTTTCCCAGTCACGACCAGAACGGTC GAGCAGTTGG
nfatc3a sequencing primer R	AACGTGTTCGCCTTGC
nfatc3a HRMA primer F	AAAGAGTCGGTGTACATAGACGGG
nfatc3a HRMA primer R	CGAAGATCAGTCTGAAGTCCAGC
card9 sequencing primer F (+ M13F-40)	GTTTCCCAGTCACGACCAGAACGGTC CTCATCAAGACC
card9 sequencing primer R	CTTCAGATTGTCTTCAGAACTCTTAC C
card9 HRMA primer F	CCTTATCTGAGACAGTGCAAGGTGC

card9 HRMA primer R	TTACCAACTTGC GGCGTCTG
VIVIT genotyping primer F	ATT CAGAGCTCGCACAGG
VIVIT genotyping primer R	ATCTCGAACTCGTGGCC
human VEGFA qPCR primer F	GAGGAGGGCAGAACATCACG
human VEGFA qPCR primer R	ACAGGATGGCTGAAGATGTACTCG
human TNF qPCR primer F	GAGGCCAAGCCCTGGTATG
human TNF qPCR primer R	CGGGCCGATTGATCTCAGC
human GAPDH qPCR primer F	CTGGGCTACACTGAGCACC
human GAPDH qPCR primer R	AAGTGGTCGTTGAGGGCAATG
hU6 ST sgRNA Sequence mU6 ST sgRNA Sequence 7SK ST sgRNA Sequence hH1 ST sgRNA Sequence	GATGGTGACAGTTGTCGA GCTAA GTACTCTAACAGG GTGGATAACTTCCTGAGT GTGCAGTTCTCCGGGTTG
hU6 NFATC2 sgRNA Sequence mU6 NFATC2 sgRNA Sequence 7SK NFATC2 sgRNA Sequence hH1 NFATC2 sgRNA Sequence	GACACCGGCGAGGGGTCA GCTTGGCACCAAGGCGATG GCCACGGACTCGCCTTGT GGCCGGGTAGATGTGGCG
Common Tail Oligo	AAAAGCACCGACTCGGTGCCACTTT TCAAGTTGATAACGGACTAGCCTTAT TTTAAC TTGCTATTCTAGCTCTAAA AC

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# Biography

Jared was born in the mountains of Appalachia in Barbourville, KY in 1994. After graduating valedictorian from Barbourville High School, he enrolled at Transylvania University in Lexington, KY where he received a Bachelor of Arts degree in Biology and Political Science, *summa cum laude* in May 2016. In the fall of 2016, he began his Ph.D. at Duke University in the Molecular Genetics and Microbiology department, having been awarded a James B. Duke Fellowship. He then joined the lab of David Tobin in the summer of 2017. He attended the Cold Spring Harbor course “Programming for Biologists” in the fall of 2017, where he learned Python. In his time at Duke, he has contributed to a number of publications, including “Cyclopropane Modification of Trehalose Dimycolate Drives Granuloma Angiogenesis and Mycobacterial Growth through Vegf Signaling” by Walton et al. (2018), “A non-canonical type 2 immune response coordinates tuberculous granuloma formation and epithelialization” by Cronan and Hughes et al. 2021, “An Ancestral Mycobacterial Effector Promotes Dissemination of Infection” by Saelens and Sweeney et al. 2022 and “Macrophage NFATC2 Drives Angiogenic Signaling During Mycobacterial Infection”, his primary thesis publication. He was awarded a Ruth L. Kirschstein National Research Service Award F31 fellowship from the National Heart, Lung, and Blood Institute to assist in the completion of his thesis work.