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UFMylation Suppresses Type I IFN Signaling During *M. tuberculosis* Infection of
Human Macrophages

By

Nicholas Garelis

A dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in

Molecular and Cell Biology

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor Jeffery S. Cox, Chair

Professor Daniel A. Portnoy

Professor Sarah A. Stanley

Professor Russell E. Vance

Summer 2024

Abstract

UFMylation Suppresses Type I IFN Signaling During *M. tuberculosis* Infection of Human Macrophages

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Professor Jeffery S. Cox, Chair

Type I Interferons (Type I IFNs) are critical for host defense against a wide range of viral infections but can enhance the pathogenicity of several bacterial pathogens, including *Mycobacterium tuberculosis* (Mtb)¹⁻⁴. Given the significance of Type I IFN signaling in determining Mtb infection outcomes we sought to uncover new molecular mechanisms controlling Type I IFN activation during mycobacterial infection. We performed a genome-wide CRISPR interference screen in human macrophages to identify genes that regulate IFN- β , during infection with *M. marinum* (Mm). In addition to known regulators of Type I IFN, we identified UFL1, which encodes the E3 ligase for the ubiquitin-like protein UFM1, as a major regulator of IFN- β during mycobacterial infection. Depletion of other components of the UFMylation pathway, DDRGK1 and UFM1, also resulted in increased IFN- β , indicating that UFMylation activity is required for Type I IFN regulation. Deficiency in UFMylation resulted in increased production and secretion of IFN- β during macrophage infection with both Mm and Mtb compared to control cells. Additionally, silencing UFL1 resulted in increased expression of several interferon stimulated genes and other pro-inflammatory genes, including TNF and IL-6, at both the mRNA and protein level. Transcriptional profiling revealed a surprisingly broad increase in pro-inflammatory responses of UFL1-deficient cells during Mtb infection, particularly for interferon-stimulated genes. Our data reveals an unexpected role of the UFMylation pathway in suppressing Type I IFN, a pro-inflammatory immune pathway with detrimental effects on Mtb infection outcome.

Additionally, we investigated aspects of how Type I IFN is induced by Mtb – specifically the mechanistic basis for phagosome permeabilization by Mtb. Phagosome permeabilization correlates with the induction of Type I Interferon and is a critical step of bacterial intracellular pathogenesis. *M. tuberculosis* is hypothesized to permeabilize the host phagosome using a specialized secretion system, ESX-1⁵. It has not been possible to determine the specific factors directly mediating ESX-1-catalyzed permeabilization, as disruptions of single ESX-1 substrates are

reported to abrogate or impair secretion of total ESX-1 secretion -- a phenomenon termed "co-dependent secretion" -- however this rule may have some exceptions⁶⁻⁹. This study tests the hypothesis that the ESX-1 substrates EspE and EspF are required for phagosomal disruption and found that they were not absolutely required for phagosomal disruption in *M. marinum*. This study also finds that phthiocerol dimycocerosate, PDIM, a lipid present in the outer leaflet of the mycobacterial envelope appeared to be dispensable for surface-localization of EspE^{10,11}. This study also investigated the sequence elements sufficient for ESX-5-mediated secretion of fusion constructs but was inconclusive.

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"If you don't know where you are going, you'll end up someplace else." -Yogi Berra

Chapter 1: Introduction

***Mycobacterium tuberculosis* and Type I Interferons**

Mycobacterium tuberculosis (Mtb) is responsible for the most deaths globally by a single bacterial pathogen¹². The pathogenesis of Mtb is driven by both bacterial factors as well as hyper-activation of host immune responses, including Type I Interferon (Type I IFN) signaling^{2,3,13,14}. Type I IFNs, including IFN- β and several IFN- α s, comprise a family of immune signaling molecules with strong antiviral and pro-inflammatory effects. While Type I IFNs may be protective in the context of certain bacterial infections, they potently inhibit antibacterial host responses and exacerbate infection of many pathogenic bacteria, including *Mycobacterium tuberculosis*^{1–4,13–18}.

Type I IFN signaling leads to decreased resistance to tuberculosis (TB) in mice and is strongly correlated with promoting active Mtb disease in humans^{13,19}. Indeed, interferon signaling precedes transition to active Mtb disease in human patients and mutations in the Type I IFN receptor (IFNAR) that reduce Type I IFN signaling correlates with Mtb resistance¹⁷. Additionally, when hepatitis patients were treated with Type I IFN therapy, reactivation of Mtb occurred in some cases²⁰. The precise mechanism by which Type I IFNs inhibit Mtb control remains incompletely understood, although impairment of IL-1 signaling involving IL-1ra seems to be a major contributor to Mtb susceptibility, while inhibition of IFN- γ and TNF signaling may also contribute^{21–24}.

In vitro studies suggest that the cGAS-STING-IRF3 pathway, in which cGAS senses DNA in the host cytosol, is important for initiating IFN- β transcription during Mtb infection of macrophages, although Type I IFN production may also be influenced by other pathways and receptors^{5,23–26}. These pathways include TLR7/9 which sense nucleic acids, the RIG-I/MAVS pathway that recognizes RNA, the Nod receptors that recognize cell wall components, and TLR2 that, in the case of mycobacteria, recognizes lipids and glycoproteins from the mycobacterial cell envelope²⁴. These pathways converge on the synthesis and secretion of Type I IFNs which further amplify the response by signaling through the type I IFN receptor (IFNAR), thereby triggering additional production of Type I IFNs. While many regulators of these pathways have been discovered – and, in many cases, have been demonstrated to be important determinants for Mtb control – there are likely additional pathways that regulate production of Type I IFNs^{5,24,26}. Due to the consistent findings that Type I IFNs and regulators of Type I IFNs are important determinants of Mtb infection outcomes in both human and mouse studies, we sought to uncover additional factors that serve to regulate Type I IFNs.

UFMylation

UFMylation is a ubiquitin-like conjugation system which remains poorly understood but has been recently implicated in endoplasmic reticulum (ER) homeostasis and autophagy of the ER (ER-phagy)^{27,28}. Regulation of IFN- β by

UFMylation during bacterial infection has not been previously reported but ER function has been linked to immune responses via the unfolded protein response (UPR)²⁹. The UPR is activated by altered ER homeostasis and unfolded proteins, and is mediated through three main ER sensors, IRE1 α , ATF6, and PERK, which activate transcription factors XBP1, ATF6f, and ATF4, respectively. These transcription factors have a range of effects, including the upregulation of chaperones for proper protein folding and the promotion of degradation of misfolded proteins, but also modulation of immune responses³⁰.

ESX-1

M. tuberculosis (*Mtb*) is hypothesized to permeabilize the host phagosome via a Type VII specialized secretion system, termed ESX-1, although little is known about this system, or how it may damage the phagosomal membrane^{5,31,32}.

In *Mtb*, the secretion system itself is thought to be encoded by two loci, the ESX-1 locus, which includes at least 20 genes and spans approximately 25 kilobases, and the *espACD* locus, a second, distal locus of 3 genes⁷. In one simplified model of *Mtb* pathogenesis, aerosolized *Mtb* is inhaled into the lungs and subsequently phagocytosed by alveolar macrophage where *Mtb* remains surrounded by a phagosomal membrane – the final point evidenced by colocalization of *Mtb* with phagosomal markers and EM visualization of a bilayer surrounding intracellular *Mtb*^{33–35}. *Mtb* may then disrupt the phagosomal membrane, as suggested by several points of evidence, including: 1. Recruitment of host galectins to the phagosome, a common indicator of phagosomal membrane damage. 2. The FRET-based, CCF4-AM assay, which detects phagosome-cytosol flux by cleavage of a fluorescent dye loaded into the host cytosol by bacterial-derived beta-lactamases. 3. Activation of the host STING pathway, which senses DNA and cyclic dinucleotides in the host cytosol, and is stimulated by other pathogenic bacteria dependent on damage to the host phagosome^{5,36,37}. Importantly, these phenomena require a functional ESX-1, but very few mechanistic details are known³¹. ESX-1 was the first Type VII secretion system identified, but it is now recognized *Mtb* possesses four other paralogous systems: ESX-2, 3, 4, and 5, and, numerous homologous systems have since been discovered across actinobacteria and even highly divergent systems in firmicutes³⁸. Like ESX-1, these homologous systems remain poorly understood.

ESAT-6

An early model of ESX-1 phagosome permeabilization was that it was mediated by the ESX-1 substrate ESAT-6, a small protein of 95 amino residues encoded by *esxA*, which was thought to permeabilize host membranes like the phagosome^{32,39}. This was based on observations that included: 1. *Mm* deficient for *esxA* failed to escape host phagosomes³². 2. Recombinant ESAT-6 could disrupt liposomes *in vitro*³². The first point was determined to be misleading after it was reported that Δ *esxA* strains were deficient in all ESX-1 secretion⁴⁰. Similar findings were reported for other ESX-1 substrates: deletion of single ESX-1 substrates

abrogated or impaired all ESX-1 secretion^{6,40}. This phenomenon has been called “co-dependent” secretion, although some exceptions have been reported and the mechanism underlying this is not well understood – but it is commonly hypothesized substrates either form part of the secretion apparatus or play a role in regulating secretion – perhaps enforcing a specific order of secretion. Therefore, the finding that Δ esxA strains of Mm were deficient in phagosome escape can be explained by a deficiency in total ESX-1 secretion.

The second point, that recombinant ESAT-6 possessed pore-forming activity *in vitro*, has been brought into doubt after reports that ESAT-6 membranolytic activity could be from residual detergent from purification³¹. Therefore, with these subsequent findings, it is now uncertain how *Mtb* permeabilizes the phagosome; however, it is important to qualify that none of these reports definitively rule out ESAT-6 as being the pore-forming toxin of ESX-1, but simply cast doubt on the evidence that it is.

The mycobacterial envelope

Mycobacteria are particularly noted for their unusual cell envelope, which consists of a phospholipid plasma membrane, a peptidoglycan cell wall, and an external outer membrane (OM)⁴¹. While the plasma membrane and cell wall appear similar in composition to those of commonly studied bacteria, the mycobacterial OM is rather unique, and consists of an outer leaflet with a complex mixture of unique lipids, and an inner leaflet of extremely long fatty acids covalently linked to the cell wall via a glycan intermediate⁴¹.

PDIM

Phthiocerol dimycocerosate, PDIM, is present in the outer leaflet of the mycobacterial OM^{41,42}. Strains deficient for either PDIM biosynthesis, or its trafficking to the OM, are moderately, but not fully, impaired for the phagosomal perforation catalyzed by ESX-1^{43,44}. However, these strains are reported to have normal secretion of ESX-1 substrates, with perhaps even increased secretion into the culture filtrate⁴⁴.

Goals of this dissertation

Here we sought to contribute to the understanding of how Type I IFN is regulated during *Mtb* infection and to determine how the ESX-1 system of *Mtb* and Mm catalyzes phagosome perforation – a critical step in *Mtb* pathogenesis that is also required to induce Type I IFN^{5,45,46}. To study how Type I IFN is regulated, we used a genome-wide CRISPRi screen approach to identify novel regulators of IFN- β . This genome-wide CRISPRi screen approach relied heavily on an ISRE-IFN- β reporter, which was previously used in combination with a genome-wide screen to successfully identify an important transporter of cyclic dinucleotides into cells, and hence appeared a promising approach to identify other factors controlling Type I IFN⁴⁷. Understanding the regulation of Type I IFN during *Mtb* pathogenesis and identifying new regulatory mechanisms of Type I IFN is important due the significant

role of Type I IFNs in Mtb pathogenesis – a finding that has been demonstrated in both human and mouse studies^{1–4,13–18}. The second goal, of determining how the ESX-1 system of Mtb and Mm catalyzes phagosome perforation, is significant as phagosome perforation capability correlates with Mtb pathogenesis: Mtb strains deficient for ESX-1 are both highly attenuated and incapable of phagosome perforation^{36,46}. Despite this correlation between pathogenesis and phagosome perforation, many details remain unclear. We sought to determine how phagosome perforation contributes to Mtb pathogenesis and the mechanistic basis for phagosome perforation. To answer these questions, we use a variety of different genetic approaches, testing whether specific genes are necessary for phagosome perforation in Mm, and testing whether phagosome perforation could be disentangled from ESX-1 secretion activity – historically a confounding variable in the study of phagosome perforation by Mtb and Mm.

Chapter 2: UFMylation suppresses Type I IFN signaling during *M. tuberculosis* infection

Additional contributors: Luteijn RD, Raulet DH, Cox JS

The following is currently in preparation as a manuscript for submission to a journal

Chapter 2.1: Results

CRISPR interference screen for regulators of IFN- β during mycobacterial infection

To methodically uncover host genes that regulate IFN- β during mycobacterial infection, we conducted a genome-wide CRISPR interference (CRISPRi) screen using the THP-1 human monocyte reporter cell line, which can be differentiated into macrophages. As a model for Mtb infection, we used *Mycobacterium marinum* (Mm), which is reported to recapitulate key aspects of Mtb IFN-induction, including a dependence on ESX-1, a bacterial type VII secretion system, and the host factor, STING. These monocytes encode an ISRE-IFN- β reporter gene consisting of an IFN- β 1 promoter and multiple interferon-stimulated response element (ISRE) sequences that drive expression of tdTomato, as a readout for IFN- β -induction and can be monitored for expression using flow cytometry (Fig 1A). We tested different infection conditions with Mm and found that at a multiplicity of infection (MOI) of 5 after 24 hours, the IFN- β reporter was robustly induced (Fig 1B). To test whether this reporter could be used for a CRISPR screen, STING, a gene required for induction of IFN- β during mycobacterial infection was knocked down using a catalytically dead Cas9 fused to the transcriptional repression domain KRAB^{5,48}. Indeed, knockdown of STING lowered reporter induction to background levels. Additionally, consistent with previous studies of IFN- β and mycobacterial infection, wild-type Mm also induced a more potent response than ESX-1-deficient Mm (Fig 1B). Thus, this reporter cell line faithfully reproduced IFN- β induction during infection, allowing us to monitor individual cells by flow cytometry.

Next, we conducted a genome-wide CRISPR interference screen to identify new regulators of IFN- β during infection. We generated a library of THP-1 cells transduced with constructs containing dCas9-KRAB, the IFN- β reporter, and the Weissman genome-wide gRNA CRISPRi library⁴⁸. We infected this library with Mm at an MOI 5, and after 24 hours of infection, used fluorescence-activated cell sorting (FACS) to collect the highest and lowest quartiles of reporter-expressing cells (Fig 1C). Genomic DNA from these populations was harvested, and the region containing the gRNAs was amplified by PCR and deep-sequenced using an Illumina HiSeq 4000 to identify enriched gRNAs in each subpopulation. We ranked each gene for positive and negative enrichment using the robust rank algorithm (RRA) from the MAGeCK package⁴⁹. Non-targeting control gRNAs were evenly distributed between subpopulations, while targeting gRNAs showed biased distributions, consistent with a successful screen.

To identify pathways and protein complexes required for IFN- β regulation, we used MAGeCKFlute to perform gene set enrichment analysis (Fig 1E). The top enriched categories included a variety of signaling pathways directly related to Type I IFN signaling, although unexpected categories such as neuroblast polarity appeared (Fig 1E). Overall, the statistically strong enrichment of gene sets related to Type I IFN signaling validated the methodology of the screen.

The screen identified several known regulators of IFN- β as positive controls, such as STING, STAT1, IRF9, and IFNAR1, indicating that during the screen cells were activated by both intrinsic STING signaling and autocrine-paracrine IFN- β signaling (Fig 1D and 1F). IRF3, a transcription factor downstream of STING, was also among the top hits. Similar to a previous report using this library, only one gRNA for STING (TMEM173) showed enrichment in the high-IFN- β subpopulation, while other gRNAs targeting STING were not differentially enriched, likely due to low efficiency in knockdown of STING. In the hyper-responsive population, the top-ranked gene was USP18, an ISG15 deubiquitinase known to negatively regulate IFN- β . The high rankings of known positive and negative regulators of IFN- β further validated the methodology of the screen. Furthermore, we identified genes not previously associated with IFN- β regulation, including UFL1, a gene involved in UFMylation, a process not previously linked to IFN- β , but with emerging roles in ER homeostasis.

UFL1 suppresses transcription of IFN- β and IFIT1 during Mtb infection

To test the new putative regulators of IFN- β signaling during Mtb infection, we chose a set of eight genes identified in the screen for testing with individual gRNA targeting experiments. For each gene, we selected the top enriched gRNA and used lentiviral transduction to target these genes for knockdown in THP-1-dCas9 cells and, as positive controls, included known regulators, such as USP18. Six of the targets did not give rise to a phenotype, although we do not know the efficacy of the knockdown (Fig 2A). However, knockdown of UFL1 and NEDD8 resulted in an increase in IFN- β reporter activity, similar to the USP18 positive control, although the increase for NEDD8 was not statistically significant. Because NEDD8 has been reported to alter IFN- β , we focused on UFL1. UFL1 is an E3 protein ligase that catalyzes the attachment of a ubiquitin-like modifier UFM1 to target proteins (Fig 2A). UFL1 is an E3-like enzyme involved in UFMylation, the process of covalently linking UFM1, a ubiquitin homolog, to selected substrates. We also observed hyper-induction of the ISRE-IFN- β reporter in UFL1 cells treated with recombinant human IFN- β (Fig 2B). RT-qPCR on RNA harvested from control and UFL1-depleted cells verified that the knockdown was highly effective (97% depletion) (Fig 2C).

Next, we sought to directly test the reporter and screen findings with RT-qPCR and validate our findings with Mtb infection. UFL1-depleted THP-1 cells had more IFNB1 transcripts induced by Mtb infection, demonstrating an

approximately 2-fold increase relative to control cells at both 4 hpi with an MOI of 10, and 16 hpi with an MOI of 5, while the difference was less pronounced at MOI 20, 4 hpi (Fig 2D and 2E). Additionally, the lowest MOI tested, MOI 2.5, had minimal phenotype at 4 hpi but demonstrated a 2-fold increase at 16 hpi. We also observed no IFN- β induction for both genotypes in the uninfected condition. After noting higher levels of IFN- β at the transcript level in UFL1 KD macrophages, we hypothesized that these cells would also hyper-induce ISGs after Mtb infection. Indeed, UFL1 knockdown also increased infection-induced transcripts of the interferon-induced gene, IFIT1, at 4 hpi, MOI 10, by approximately 2-fold according to RT-qPCR analysis. Although the phenotypes for IFIT1 mostly mirrored IFN- β , IFIT1 differed from IFN- β in that UFL1 KD macrophages demonstrated elevated IFIT1 transcripts in the uninfected condition (Fig 2F). To begin to test whether the effect of UFL1 knockdown was specific to IFN- β , we measured mRNA levels of another pro-inflammatory cytokine, IL-6, and found that its levels were increased 4- to 6-fold in UFL1 knockdown cells (Fig 2G). Similar to transcripts for IFN- β , there was no induction of IL-6 in the uninfected conditions. For the three transcripts tested, encoding IFN- β , IFIT1, and IL-6, there were maximal differences in induction from Mtb infection at an MOI of 10 for 4 hpi and MOI 5 for 16 hpi. Taken together, these data confirm the results of the CRISPRi screen and demonstrate that UFL1 plays a significant role in negatively regulating pro-inflammatory innate immune signaling during Mtb infection.

These results indicate that UFL1 plays a significant role in innate immunity, specifically as a negative regulator and inhibitor of IFN- β production during Mtb infection, potentially through increased transcription, or potentially through other mechanisms such as changes to mRNA stability. Additionally, these individual KD studies validate the genes identified by the CRISPRi screen for their substantial involvement in IFN- β regulation.

UFMylation components DDRGK1 and UFM1 suppress IFN- β

UFMylation describes the sequential activation and conjugation of ubiquitin-like UFM1 to target substrates via an E1 (UBA5), E2 (UFC1), and E3 (UFL1) in a pathway analogous to ubiquitin conjugation²⁷. Also involved is the accessory factor DDRGK1, which is reported to function in recruiting UFL1 to the ER, where both DDRGK1 and UFL1 localize²⁷. Importantly, UFM1 and DDRGK1 were also identified in our screen, indicating that functioning of the entire UFMylation pathway is important for IFN- β regulation (Fig 3A). To comprehensively test if components of the UFMylation pathway in addition to UFL1 are required for IFN- β regulation, we designed CRISPRi constructs to knockdown UBA5, UFC1, UFL1 and DDRGK1.

Attempts to deplete UBA5 and UFC1 yielded no viable cells (data not shown), perhaps due to their involvement in other essential pathways, while knockdown of DDRGK1 and UFM1 led to enhanced ISRE-IFN- β -reporter signal with treatment of recombinant IFN- β , phenocopying UFL1 knockdown, suggesting that functional

UFM1-conjugation is important for IFN- β attenuation rather than UFL1 alone (Fig 3B). A number of substrates for UFMylation have been reported, but the best understood is RPL26, a ribosomal protein – UFL1 is thought to catalyze the ligation of UFM1 to Ribosomal Protein L26 (RPL26) as part of its role in clearing stalled ribosomes, er homeostasis, and er-phagy, however attempts to deplete RPL26 were also unsuccessful and yielded no viable cells, likely due to the essential role of RPL26 in ribosomal function (data not shown) although one guide against RPL26 was moderately enriched in our screen. Another recently reported substrate of UFL1 is CYB5R3, however, it was not substantially enriched in our screen (Fig 3A) and individual knockdown did not result in an appreciable increase in IFN- β (Fig 3B)⁵⁰.

Together, the results suggest a model whereby functional UFM1 conjugation is critical for IFN- β regulation rather than UFL1 regulating IFN- β independently of its role in UFMylation.

UFMylation suppresses pro-inflammatory cytokine secretion during Mtb infection

To test the role of UFL1 during cytokine secretion, we used a flow cytometry-based multiplex immunoassay to measure an array of cytokines whose production is activated during innate macrophage activation. In agreement with our mRNA results, we observed elevated IFN- β and IL-6 in UFL1-depleted macrophages, which were elevated approximately 3-fold and 30-fold respectively compared to control cells infected with Mtb (Fig 4). While the changes in IFN- β protein levels from UFL1-depleted cells correspond well to changes in transcript levels measured by RT-qPCR, the changes in IL-6 protein levels were considerably greater than the changes observed for il6 transcripts by RT-qPCR (Fig 4). This analysis also revealed that nearly all the other cytokines in this panel were significantly elevated in UFL1-KD macrophages infected with Mtb relative to control cells. TNF protein levels were approximately 10-fold higher relative to control cells infected with Mtb, along with IFNL1, IL12p70, IFN- α 2, IL10, and IL1beta, which were elevated in Mtb-infected UFL1-deficient cells relative to control cells to varying degrees (Fig 4).

The results indicate a surprisingly broad effect of UFL1 in innate immune inflammatory signaling, specifically as a suppressor of IFN- β , TNF, and IL-6 production.

Transcriptional profile of UFMylation-deficiency during Mtb infection

Our results show that the effects of UFMylation in inflammatory signaling extend beyond increased IFN- β transcription. To comprehensively determine how UFMylation alters the global macrophage transcriptional response to Mtb infection, we performed RNA-seq on mRNA samples harvested from Mtb-infected UFL1-knockdown and control cells at 4 hpi with Mtb with an MOI of 10, and from uninfected cells. Differential gene-expression analysis found that, during Mtb infection, 521 genes were significantly differentially upregulated with

UFL1-knockdown and 191 genes were significantly differentially downregulated (Fig 5A). Of the 521 genes differentially upregulated with UFL1-knockdown, 251 were induced specifically in response to Mtb infection.

Next, we moved to inspection of these differentially regulated genes (Fig 5B). We first found that one of the most significantly down-regulated genes was UFL1, which validated the RNA-seq and CRISPRi methodology (Fig 5A and 5B). Further analysis of the RNA-seq data at the gene-level confirmed our prior findings for IFN- β : There was approximately 2-fold increased transcripts for IFN- β from UFMylation-deficient macrophages during Mtb infection that closely matched out RT-qPCR results (Fig 2D and 5B). Also consistent with our findings from RT-qPCR, transcripts for IFIT1, and IL-6 were increased approximately 2-fold and 4- to 6-fold, respectively, with UFMylation-deficiency (Fig 2E-G and 5B). We then observed that many other inflammatory genes were also significantly elevated in Mtb-infected UFL1-deficient macrophages compared to Mtb-infected control cells, which included IL12B, MCP1, MCP2, and ACOD1, which were elevated 3- to 5-fold (Fig 5B). These included a variety of ISGs, such as GBP1, IFI44, IFT2, IFIT3, and CXCL11, which were also upregulated 3- to 5-fold with UFMylation-deficiency during Mtb infection (Fig 5B). Some of the other most-altered genes included ADORA2A, an adenosine receptor reported to suppress apoptosis by regulating TNF, which had 8-fold more transcripts in UFMylation-deficient, Mtb-infected macrophages, and IFNL1, which encodes a cytokine for Type III Interferon signaling, which was elevated 10-fold in UFMylation-deficient, Mtb-infected macrophages (Fig 5B)⁵¹.

We then performed pathway analysis of the differentially regulated genes from Mtb-infection (Fig 5C). The most statistically significant enriched pathways were 2'-5'-oligoadenylate synthetase activity, IL-10 signaling, interferon alpha/beta signaling, and chemokine signaling (Fig 5C). The IL-10 signaling set was attributed to upregulation of a broad list of inflammatory genes that included IL1A, IL6, IL12B, and TNF, while the interferon alpha/beta signaling set was attributed to a list that included IFNB1 along with several IFIT and GBP genes. The 2'-5'-oligoadenylate synthetase set was attributed to upregulation of the eponymous OAS1/2/3 genes, while chemokine signaling was attributed to upregulation of over a dozen inflammatory chemokines, such as CCL4, CCL5, and CCL19.

As past reports indicate that UFMylation is involved in ER homeostasis and ER stress, we also examined pathways associated with ER function. While not reaching the top most enriched pathways, several gene sets related to ER stress achieved statistical significance (FDR < 0.05). One of the ER stress-related genes driving these categories was PPP1R15A/GADD34, which functions to restore ribosomal activity after ER stress induces a block on general protein translation⁵². If UFMylation does impact ER homeostasis and deficiency causes ER stress, then upregulation of PPP1R15A/GADD34 may help explain the unimpaired secretion of cytokines we observed with UFMylation-deficient cells during Mtb infection.

All together, our transcriptional profile during Mtb infection finds that UFMylation-deficiency caused an upregulation of a wide-variety of inflammatory genes and pathways, including IL1A, TNF, a large number of ISGs, and a large number of inflammatory chemokines. This indicates UFMylation functions to broadly dampen inflammatory immune signaling during Mtb infection.

Transcriptional profile of UFMylation-deficiency in the absence of infection

Additionally, we examined the transcriptional profile of UFMylation-deficient cells versus control cells in the absence of infection. Differential gene-expression analysis found that 915 genes were significantly differentially upregulated with UFL1-knockdown and 745 genes were significantly differentially downregulated (Fig 5D). Manual inspection of these differentially regulated genes revealed upregulation of many ISGs, including GPB2, IFI30, OASL, IFIT1, and IRF7, although interferon-related pathways were not amongst the top most enriched pathways (Fig 5D and 5E). Pathway analysis revealed the most enriched pathways to be “vertebrate eye-specific patterning,” attributed to enrichment of the genes for CD11b and multiple serum complement components, and “transcription factor AP-1 complex” which regulates inflammatory responses and was attributed to the genes FOS, cJUN, JUNB, and JUND (Fig 5E). Overall, gene set enrichment from uninfected cells was several orders of magnitude statistically weaker than gene set enrichment from infected cells, and hence major transcriptional effects caused by UFMylation-deficiency appear to primarily manifest with an additional stimulus such as Mtb infection.

In conclusion, these findings indicate UFMylation functions to broadly dampen inflammatory immune signaling, with the most pronounced effects observed with Mtb infection.

Chapter 2.2: Discussion

While type I IFNs play a critical role in effective anti-viral immunity, substantial evidence from both humans and mouse studies indicates that type I IFNs can hinder the control of Mtb during infection^{2,3,13–18}. In this study, to learn more about underlying regulation of type I IFN production during Mtb infection, we performed a genome-wide CRISPRi screen in human THP-1 cells for genes that modulate production of type I IFNs during infection, using *M. marinum* as a model for Mtb. While we identified a number of genes known to be involved in the induction type I IFNs, such as STING and USP18, we found that the UFMylation pathway regulates production of IFN- β during Mtb infection^{5,14,27}.

As one of the most substantiated roles for UFMylation is the regulation of endoplasmic reticulum (ER) stress and ER-phagy, our findings suggest a possible interplay between ER stress and expression of type I IFN and ISGs during Mtb infection, with ER stress likely potentiating the type I IFN response elicited by Mtb^{28,53–55}. Mechanistically, UFMylation is reported to function in protein quality

control during ribosome co-translational translocation – ribosomes bound to the ER that stall during protein translocation trigger the conjugation of UFM1 to ribosomal protein RPL26 and ER protein RPN1, which may cause either the degradation of the stalled complex, by targeting it to lysosomes, or dissociation of the ribosome subunits from the ER translocon^{28,53–55}. Defects in UFMylation lead to ER stress – likely arising from the accumulation of misfolded protein-ribosome complexes stalled in the ER translocon – and increased Xbp1 mRNA splicing to the mature form – which occurs downstream of the IRE1 ER stress response pathway. Importantly, it has previously been reported that dendritic cells and macrophages synthesize greater quantities of IFN- β when treated with ER stressors, such as thapsigargin and tunicamycin, and TLR ligands in combination, compared with TLR ligands alone⁵⁶. This phenomenon was dependent on Xbp1, suggesting that Xbp1 activation may be involved in the signaling pathway that leads to enhanced IFN- β in UFMylation-deficient cells during Mtb infection. Indeed, we found that levels of IFN- β were decreased in cells deficient for both XBP1 and UFL1 compared to cells deficient solely for UFL1.

Due to evidence that type I IFNs can hinder the control of Mtb during infection, it is likely that deficiencies in UFMylation, which lead to increased levels of IFN- β , will promote Mtb disease in mice and humans. Although the precise mechanisms by which type I IFNs interfere with Mtb control remains unclear, recent literature suggests that the inhibition of IFN- γ signaling by type I IFNs may contribute to the loss of Mtb control as functional IFN- γ signaling is critical for Mtb resistance². Additionally, it has been previously found that UFMylation also plays a role in regulating IFN- γ responsiveness in macrophages – demonstrating that UFMylation-deficient cells have increased NO production in response to IFN- γ – which was shown to be dependent on Xbp1⁵⁷. Although IFN- γ and IFN- β signaling overlap to some degree, they produce distinct and even opposing effects in the context of a broad range of bacterial and viral infections. Because UFMylation may elevate both responsiveness to IFN- γ and production of IFN- β , it is possible that enhanced IFN- γ responsiveness may overcome and suppress the IFN- γ -inhibitory effects of type I IFNs, rendering these mice resistant to Mtb infection relative to control mice. If experimental findings confirm this hypothesis, it would provide further support for the hypothesis that type I IFNs have deleterious effects during Mtb infection due to their interference with IFN- γ signaling.

While type I IFNs are detrimental to Mtb control, type I IFNs are traditionally known for their antiviral properties. Hence, we hypothesized that elevated levels of IFN- β from UFMylation could be protective during viral infection and, indeed UFMylation is critical to hepatitis A virus (HAV) infection, where HAV translation depends on UFM1 conjugation⁵⁸. The mechanism behind this observation is not clear, but is consistent with our findings suggesting the enhanced production of type I IFNs arising from loss of UFMylation.

Tao et al. found that UFL1 promotes an antiviral immune response by regulating STING independently of UFMylation activity, by sequestering STING from ubiquitination by TRIM29⁵⁹. In contrast, they reported both murine macrophages and human epithelial cells showed decreased production of IFN- β during HSV-1 and vaccinia virus infection after siRNA-mediated knockdown of UFL1. These contrasting findings may reflect differences in cell type, differences arising from siRNA knockdowns compared to CRISPR, or possibly due to the action of pathogen virulence factors that may inhibit or promote steps of these different pathways.

UFMylation also has several reported links to tumor surveillance and the detection of DNA damage. One study found that UFMylation plays a role in maintaining the stability of the tumor suppressor protein p53 by antagonizing ubiquitination while another report concluded that UFMylation of histone H4 by UFL1 was an important step for activation of ATM, a kinase that functions in DNA damage response^{60,61}. While type I IFNs are known for their antiviral properties, they also have anti-neoplastic effects. Based on these reports and our findings, UFMylation may also act as a safeguard against tumorigenesis but, if lost, may potentiate type I IFN anti-tumor responses. While past studies found increased tumorigenesis in mice with UFMylation deficiency, this was carried out with tumor cell lines that appear to be insensitive to IFN- β due to secondary mutations associated with the tumor cell line^{60,62}. Hence, future studies may be carried out to determine whether UFMylation deficiency results in IFN- β -dependent tumor resistance.

Our work reveals a link between UFMylation and IFN- β during mycobacterial infection. Further investigation of the relationship between UFMylation and type I IFNs signaling during Mtb infections may determine the mechanism by which UFMylation affects these responses and whether they determine the outcome of Mtb infection in animals. Such studies will provide important insights into how type I IFN signaling impacts Mtb disease and reveal how we may be able to better treat Mtb by manipulating regulators of type I IFN signaling such as the UFMylation pathway.

Chapter 2.3: Figures

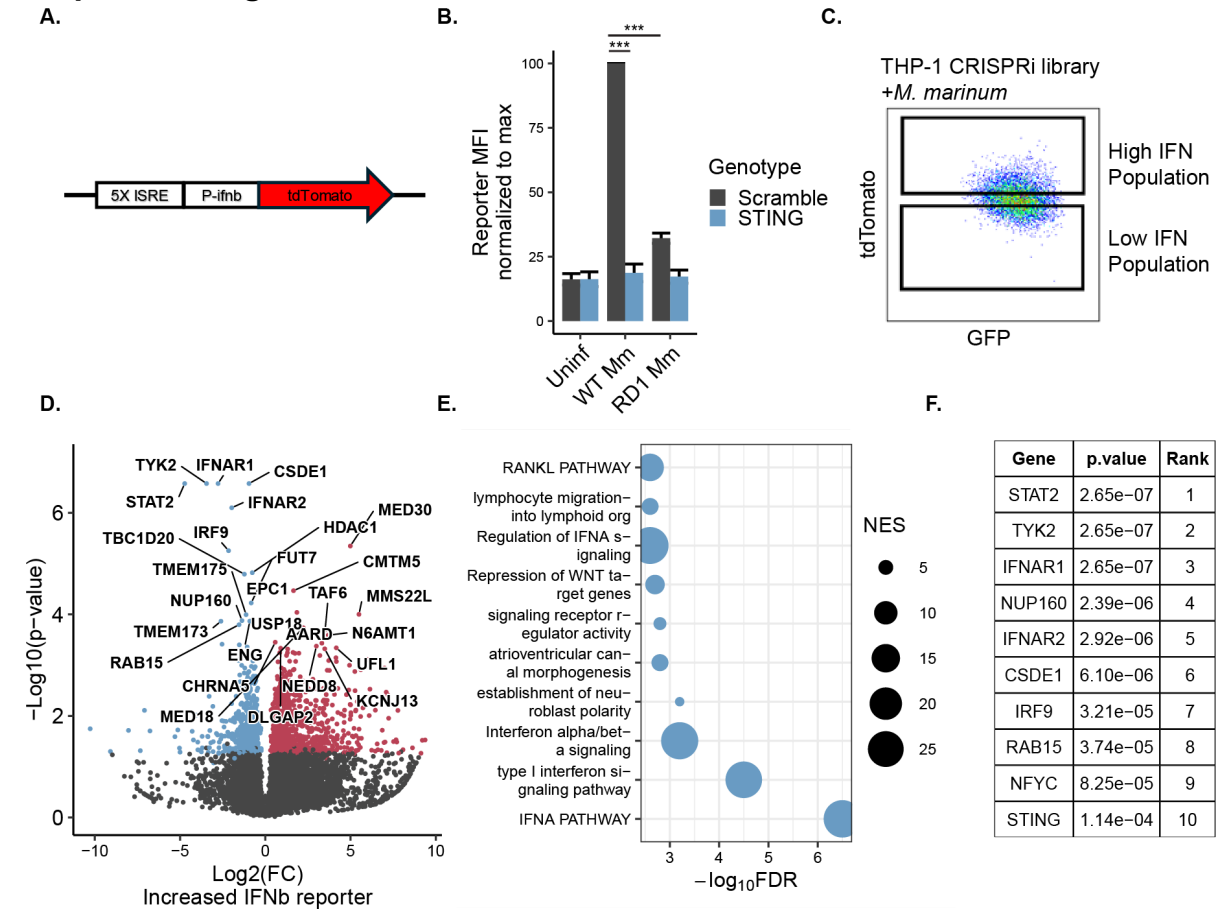


Fig 1. CRISPR interference screen for regulators of IFN- β during mycobacterial infection. (A) Design of the interferon genetic reporter with tdTomato expression driven by the IFN- β promoter with interferon-stimulatory response elements (ISREs). (B) Control (non-targeting) THP-1 cells and STING KD THP-1 cells were infected with *M. marinum* of the indicated genetic background. At 24 hpi, reporter expression was measured by flow cytometry. (C) A CRISPRi library of approximately 100,000 gRNA targeting approximately 20,000 genes was transduced into THP-1 reporter cells and infected with *M. marinum*. Cells were sorted into the top 33% and bottom 33% as measured by reporter expression for analysis. (D) Volcano plot showing results from the CRISPRi screen using p-values from the MAGECK analysis package. (E) Gene set enrichment analysis of CRISPRi results, testing for enrichment in the top 200 genes as ranked by Robust Rank Aggregation with MAGECK. (F) Top 10 genes enriched in the low-reporter population as ranked by Robust Rank Aggregation. Error bars represent SEM from 2 biological replicates. * $p < 0.05$., ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ by paired t-test.

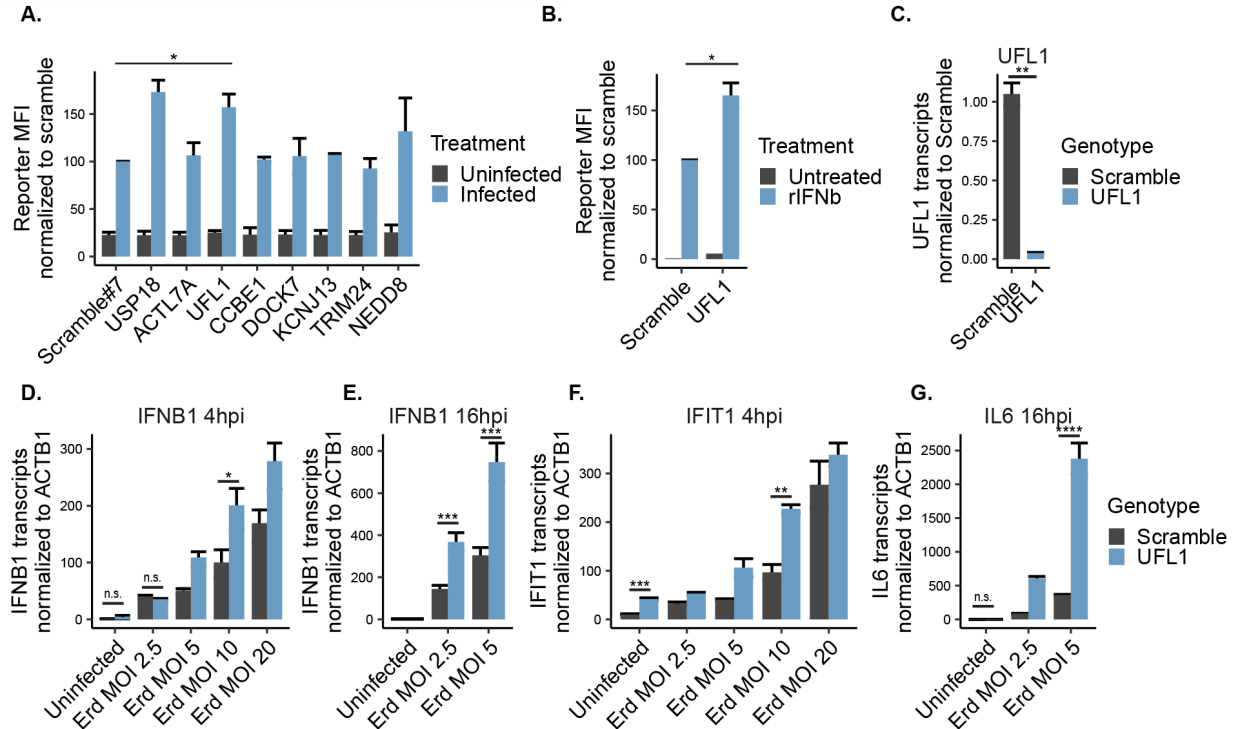


Fig 2. UFL1 suppresses transcription of IFN-β and IFIT1 during Mtb infection.

(A). THP-1 cells with gRNA against the gene indicated were infected with WT *M. marinum* MOI 5 or left untreated and reporter expression was measured by flow cytometry at 24 hpi. (B). THP-1 cells were transduced with either a gRNA for knockdown of UFL1 or with a non-targeting control gRNA, and then treated with 100 U/mL of human recombinant interferon beta, or left untreated. After 24 hours, reporter expression was measured by flow cytometry. (C). THP-1 cells with either a gRNA for knockdown of UFL1 or with a non-targeting control gRNA, and UFL1 transcripts were measured by RT-qPCR. (D-G). THP-1 cells with either gRNA for knockdown of UFL1 or a non-targeting control gRNA were either left untreated or infected with WT *M. tuberculosis* (Erdman) at the indicated MOI for 4 hr or 16 hr, and transcripts for IFN-β, IFIT1, and IL-6 were measured by RT-qPCR. Error bars represent SD from 2 technical replicates (A-C) and SD from 3 technical replicates (D-G). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ by unpaired t-test.

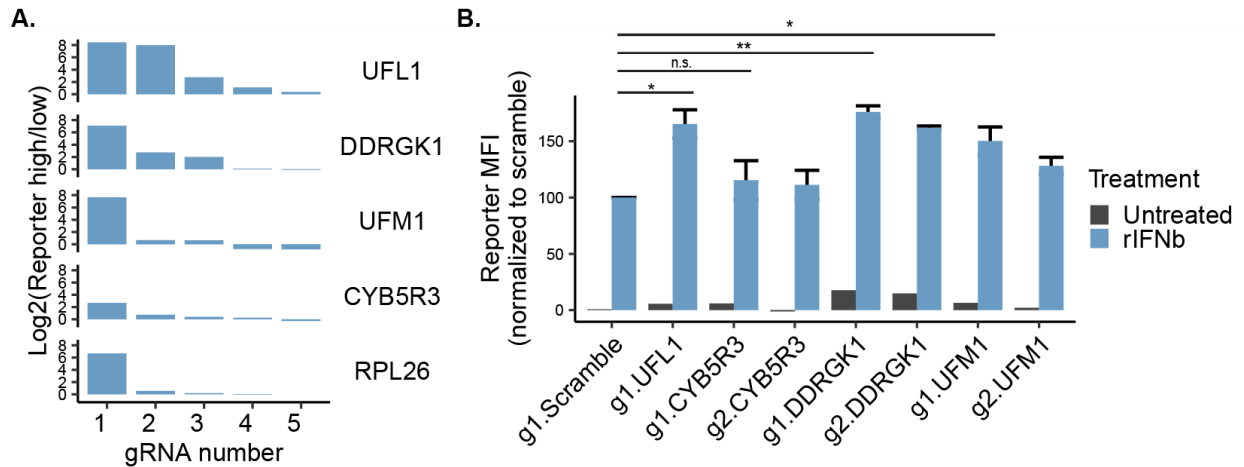


Fig 3. UFMylation components DDRGK1 and UFM1 suppress IFN- β . (A) Log2 fold enrichment of gRNA against the indicated gene from the genome CRISPRi screen. (B) THP-1 cells with gRNA against the gene indicated were treated with 100 U/mL of human recombinant interferon beta or left untreated. At 24 hpi, reporter expression was measured by flow cytometry. Error bars represent SD from 2 technical replicates. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 by unpaired t-test.

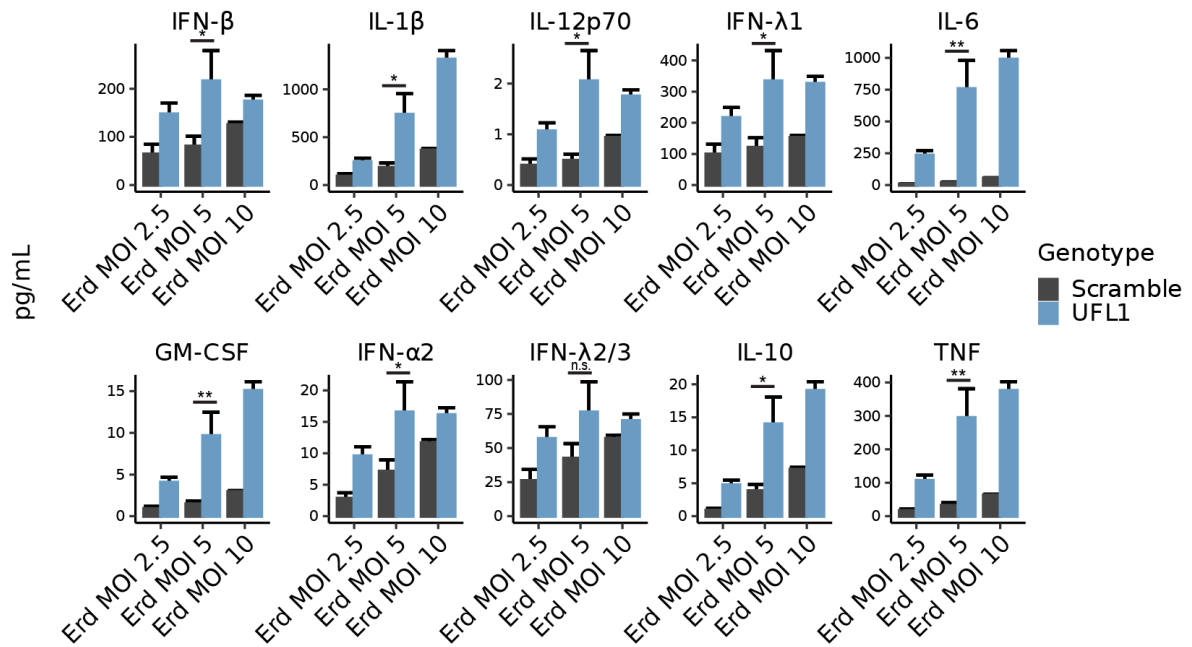
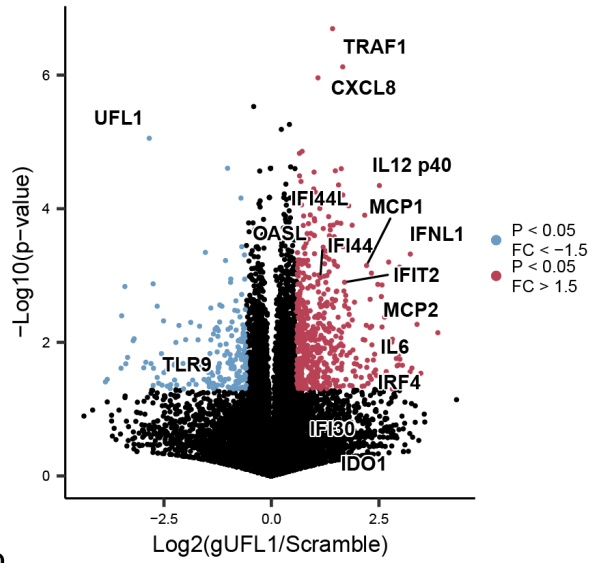
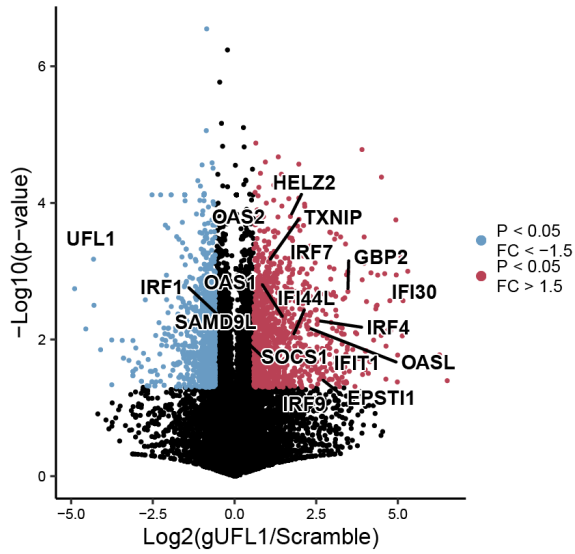


Fig 4. UFL1 suppresses IFN-β and other cytokines at the protein level during *Mtb* infection. THP-1 cells with either gRNA for knockdown of UFL1 or a non-targeting control gRNA were either left untreated or infected with WT *M. tuberculosis* (Erdman) at the indicated MOI for 24 hr. Supernatants were then harvested and protein levels were measured by cytometric bead array. Error bars represent SD from 3 technical replicates. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 by unpaired t-test.

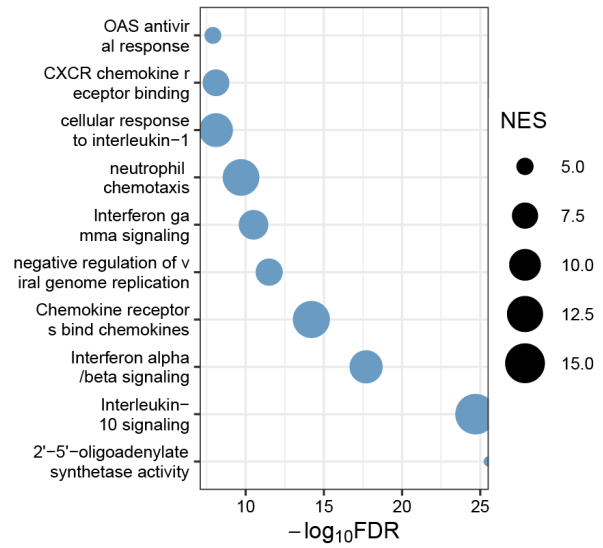
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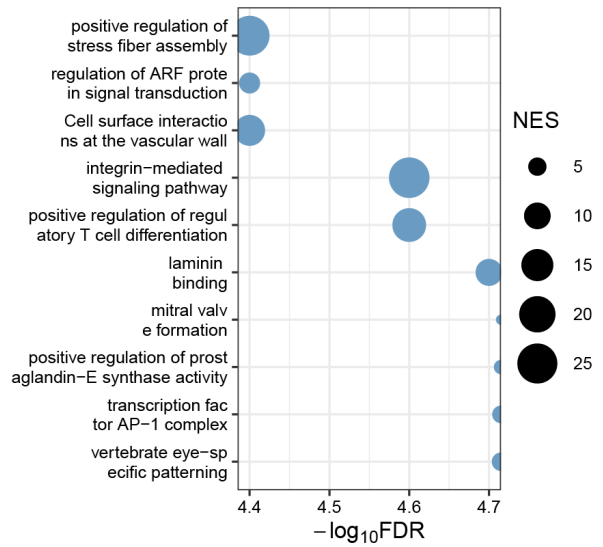
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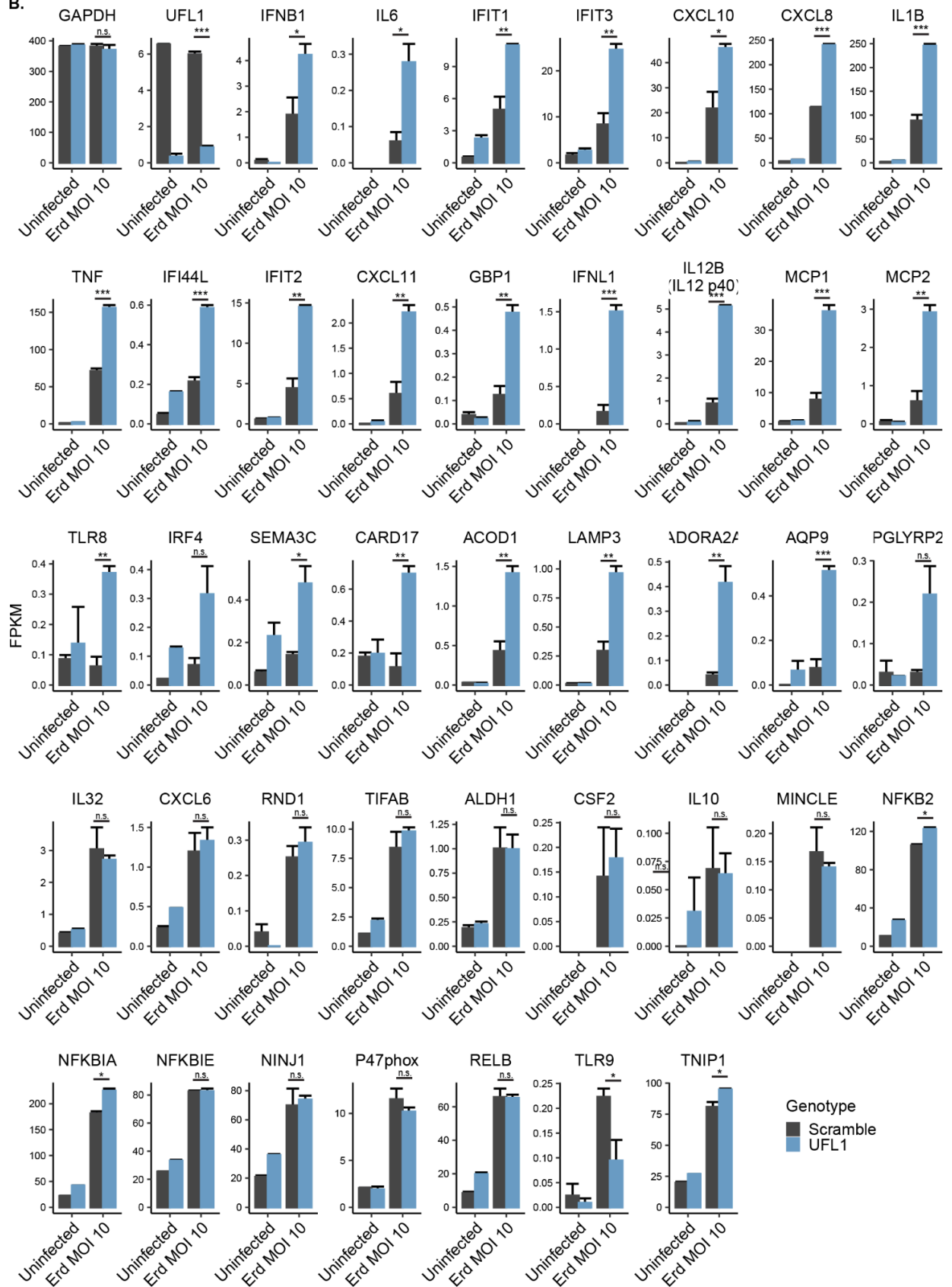


Fig 5. Transcriptional profile of UFL1 deficiency during Mtb infection. (A-B).

THP-1 cells with gRNA against UFL1 non-targeting control gRNA were infected with WT *M. tuberculosis* (Erdman) at the indicated MOI for 4 hr. RNA was harvested and analyzed by RNA-seq. Results presented by volcano plot (A) or individual plots (B). (C). Gene set enrichment analysis RNA-seq results, testing for enrichment in genes differentially regulated by UFL1-knockdown and specifically induced by infection. (D). Same as (A) but with uninfected cells. (E). Gene set enrichment analysis RNA-seq results, testing for enrichment in genes differentially regulated by UFL1-knockdown in the absence of infection. Error bars represent SEM from 3 biological replicates for infected conditions and SEM from 2 biological replicates for uninfected conditions. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ by unpaired t-test.

Chapter 2.4: Methods

RT-qPCR

For RNA analysis, cells were harvested in TRIzol at the specified time points and RNA was isolated using PureLink RNA Mini Kits (Invitrogen) and digested with TURBO DNase (Thermo Fisher). cDNA was synthesized with SuperScript III cDNA Synthesis Kit (Thermo Fisher) using 500 ng of RNA. Real-time quantitative PCR (RT-qPCR) was performed using iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories).

RNA-seq

>150 ng of DNase-treated total RNA, prepared as described for RT-qPCR, was submitted to Novogene for further processing, which included rRNA depletion and library preparation. Libraries were sequenced on an Illumina NovaSeq X Plus with 150 paired end reads, targeting at least 20M reads per sample. Raw reads were pre-processed using HTStream (<https://s4hts.github.io/HTStream/>)(version 1.3.3) to filter out adapter sequences, polyA tails, reads from PhiX, reads that were too short (<50 bp), read ends with low quality (<Q20), and reads from PCR duplication. Pre-processed reads were quantified using STAR aligner (version 2.7.9a)⁶³. The edgeR package was used to determine differentially expressed genes⁶⁴. Normalization was performed using trimmed mean of M-values.

Mycobacterial culture

M. tuberculosis (strain Erdman) and *M. marinum* (strain M) were used for macrophage infections. *M. tuberculosis* was grown to log phase in 7H9 liquid media (BD) supplemented with 10% Middlebrook OADC (Sigma), 0.5% glycerol, 0.05% Tween80 in roller bottles at 37°C. *M. marinum* was grown to log phase in 7H9 liquid media (BD) supplemented with 10% Middlebrook OADC (Sigma), 0.5% glycerol, 0.20% Tween80 in roller bottles at 30°C.

Macrophage infection

For infections with mycobacteria, macrophages were infected as previously described⁶⁵. Briefly, mycobacteria cultures were washed twice with PBS. Aggregates were removed by low-speed centrifugation at 60 xg for 5 min and gentle sonication, before being resuspended in cell media. Cell monolayers were overlaid with the bacterial suspension and centrifuged for 10 min at 1,000 xg for 'spinfection.'

Lentiviral transductions

CRISPR guide sequences targeting genes of interest were derived from the Weissman v2 guide library⁴⁸. Oligonucleotides for the chosen gRNAs were cloned into pLentiGuide-Puro (Addgene #52963) or lenti-gRNA hygro (Addgene #104991), and checked by Sanger sequencing. 293T cells were lipofected with pLentiGuide-Puro, psPAX2, and pMD2.G using Lipofectamine 3000 and OptiMem according to manufacturer's instructions to generate lentivirus for transduction. Target cells were overlaid with lentivirus and spinfected at 1000xg for 2 hr at 32°C in

media supplemented with 10 µg/ml protamine sulfate. Two days post-transduction, 4 µg/ml puromycin was added to THP-1 cells, and cells were selected in puromycin for 3 days.

Cell culture

THP-1 cell line was purchased from ATCC and cultured in RPMI (Gibco) supplemented with 10% FBS and 5 mM L-glutamine. Primary murine bone marrow-derived macrophages (BMMs) were isolated and cultured in BMM media (DMEM with 10% FBS, 2 mM L-glutamine, 10% MCSF, 11 mg/mL sodium pyruvate) as previously described^{48,65}.

CRISPRi Screen

The THP-1 CRISPRi library was generated as previously described⁴⁷. Briefly, the THP-1 reporter cell line was transduced with the human genome-wide CRISPRi v2 library containing approximately 100,000 gRNAs targeting approximately 20,000 genes. Cells were transduced and propagated to maintain at least 5x10⁷ transduced cells, representing 500X coverage of the gRNA library. The transduction efficiency was optimized for around 20% to minimize the chance of multiple integrations per cell. Two days after transduction, cells were cultured in the presence of puromycin for two days. Cells were infected with Mm as described in the infection. Approximately 24 hours post infected, cells were sorted on the basis of tdTomato expression using the BD Influx cell sorter and BD FACSAria Fusion cell sorter. The cells were sorted into two populations on the basis of tdTomato expression: The highest 33% of tdTomato expressing cells and lowest 33% of tdTomato expressing cells. Genomic DNA was isolated using NucleoSpin Blood kits (Macherey-Nagel) and digested for 18 hours with SbfI-HF (NEB). The gRNA cassette was isolated by gel electrophoresis with the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel), and the DNA was then amplified by PCR using primers with Illumina sequencing adapters. Indexed samples were pooled and sequenced on an Illumina HiSeq-4000. The screen was done for 3 independent biological replicates with a total coverage of >500X.

Screen read mapping

CRISPRi samples were mapped and analyzed using the MAGeCK package for R⁴⁹. MAGeCK robust rank algorithm (RRA) was used to score all the genes by negative or positive enrichment. Non-targeting gRNAs were used as a control in enrichment tests. For validation experiments, the gRNA sequence with the greatest magnitude log fold change was cloned into pLentiGuide and used to create single gene knockdowns.

Gene set enrichment

For gene set enrichment, the MAGeCKFlute package was used with the hyper-geometric test and the REACTOME, GO, and BIOCARTA gene sets⁶⁶. For the CRISPRi screen, genes with a rank of <200 were to test for enrichment, and for the

RNA-seq, genes with a fold change of at least 1.5 and a p-value <0.05 were used to test for enrichment unless otherwise indicated.

Cytokine analysis

For measurement of cytokines, supernatants were collected and filtered twice by 0.22 μ m filter at the specified time points and MOIs. Cytokines were measured in the supernatants using BioLegend LegendPlex Plate (Hu Anti-Virus Response 13-plex) and DuoSet ELISA kits (R&D systems) according to the manufacturer's instructions.

Flow cytometry

The tdTomato reporter cell was generated and used as previously described⁴⁷. Briefly, after approximately 24 hours of infection or stimulation, tdTomato expression was measured by flow cytometry using a BD LSR Fortessa.

Chapter 3: ESX secretion and phagosome permeabilization

Chapter 3.1 Results

Role of PDIM in surface localization

As numerous ESX substrates have been reported to be surface-localized proteins and ESX-1-mediated membrane damage is reported to require bacterial cell-to-host cell contact, we hypothesized it is the surface localization of ESX-1 substrates that is hindered in the absence of PDIM^{31,67,68}.

To test this model of PDIM function, EspE was localized in a PDIM-deficient *M. marinum* versus wild type *M. marinum*. To assay localization, the established method of genapol-X80 detergent extraction was used to collect the surface fraction from bacterial strains⁶⁹. A $\Delta mmpL7$ strain was used for PDIM-deficient *M. marinum* as PDIM is trafficked to the OM via the transporter MmpL7 and $\Delta mmpL7$ strains are reported to lack PDIM in the OM⁷⁰. To detect EspE in the surface fraction, a EspE-3xFlag construct was used in combination with immunoblotting with anti-Flag primary antibodies. By immunoblot, there were no discernable differences in the quantity of EspE in the surface fraction, although we cannot rule out small changes that could be detected by more sensitive methods (Fig 6).

M. marinum espE knockout

One report indicated that loss of putative ESX-1 chaperone, EspH, abrogated secretion of the ESX-1 substrates EspF and EspE, but had minimal effects on secretion of other ESX-1 substrates, suggesting that EspE and EspF are required for phagosomal perforation, but not ESX-1 secretion, which led us to hypothesize that EspE could be the pore-forming toxin of ESX-1⁶⁹.

To determine whether EspE catalyzes phagosomal perforation, we generated a $\Delta espEFG$ *M. marinum* strain to test for phagosome perforation. As a first test, the strain was shown to be defective in hemolysis and could only be rescued by complementation with all three genes: *espE* *espF* and *espG*, although the rescue was partial and statistics were not performed as the experiment was not repeated (Fig 7A). Next, to investigate the origin of the partial rescue of complementation, RT-qPCR was performed to test expression of the three genes and the efficacy of complementation. RT-qPCR indicated: 1. The antibiotic resistance cassette used to select for the deletion was driving transcription of a large portion of *espG* that hadn't been deleted and likely other downstream genes, perhaps causing the poor rescue from complementation with *espEFG*. 2. The *espF* complementation failed to restore transcription of *espF* in some constructs, which could likely be attributed to design of the complementation plasmid: the plasmid was designed according to reports that *espE* and *espF* did not exist in an operon and hence the plasmid did not include *espF*'s natural promoter from *espE*, although statistics for significance were not performed as the experiment was not repeated (Fig 7B)⁷¹.

To circumvent these issues, a clean $\Delta espEF$ *M. marinum* strain was generated and assayed for phagosomal perforation. To test for phagosomal perforation, we checked for induction of IFN- β by the IFN- β THP-1 reporter cells after infection with WT and $\Delta espEF$ *M. marinum*, as IFN- β correlates with ESX-1 activity and phagosomal disruption in both mycobacteria and other bacterial pathogens such as *L. monocytogenes*^{4,5}. According to the IFN- β reporter, the $\Delta espEF$ *M. marinum* strain had normal induction of IFN- β compared control strains, indicating *espEF* was not required for phagosomal perforation, although statistics for significance were not performed as the experiment was not repeated (Fig 7C). To confirm this finding from the reporter, IFNB1 transcriptional induction was assayed by RT-qPCR, which also showed the $\Delta espEF$ *M. marinum* strain had normal induction of IFN- β compared control strains, further indicating *espEF* was not required for phagosomal perforation, although statistics were not performed as the experiment was not repeated (Fig 7D). From this data, it cannot be ruled out that EspE and EspF function redundantly with other effectors to catalyze phagosome perforation.

PPE protein chimeras

To test whether *espE* functions in phagosomal disruption redundantly with other ESX-1 effectors, we sought to re-route EspE through ESX-5 and then evaluate whether this restored phagosomal disruption in an ESX-1-deficient strain. A previous publication identified a homologous region within the *esx-1* substrate PPE68 and the ESX-5 substrate PPE18 that, once swapped between the two proteins, re-routed PPE68 through ESX-5⁷². Structural alignments using PHYR2 identified a region of EspE which was structurally homologous to the region identified for rerouting PPE68 (Fig 8A)⁷³. As a first test, we swapped this identified region for EspE and the ESX-5 substrate PPE18 and, as a second test, we fused the predicted C-terminal domains of EspE to the predicted N-terminal domains of PPE18 (Fig 8B). We transformed these constructs into RD1 *M. marinum* and tested for phagosomal perforation. To test this, we used induction of IFN- β by the IFN- β reporter cells after infection with WT, RD1 transformed with empty plasmid, and RD1 transformed with these PPE18-EspE fusion constructs. According to the IFN- β reporter, the PPE18-EspE fusions *M. marinum* strain had minimal induction of IFN- β and phenocopied the RD1-empty-construct strains, indicating the *espE* fusions did not restore phagosomal perforation, although statistics for significance were not performed as the experiment was not repeated (Fig 8C). To assay secretion of these constructs into both the culture filtrate and onto the bacterial surface, the established methods of TCA-precipitation of culture filtrate, and genapol-X80 detergent extraction of bacterial pellets respectively were used. To detect secreted proteins, 3xFlag tags were used in combination with anti-Flag immunoblotting. By immunoblot, there was no discernable secretion of the fusion constructs (Fig 8D).

Next, we hypothesized that the EspE-fusion constructs might be secreted in RD1 strains due to missing cytosolic chaperones caused by the large (~10 kb) RD1 deletion. To test whether missing chaperones were the reason for the failure of the

EspE-fusion constructs to be secreted, we transformed the EspE-fusion constructs into a wild-type background and repeated the secretion assays. By immunoblot, there was no discernable secretion of the fusion constructs in a wild-type background (Fig 8E).

PE and PPE protein fusions

As the initial EspE-fusions were not secreted by ESX-5, we carried out further studies to try to identify the sequence elements sufficient for ESX-5-mediated secretion. To identify the sequence elements, we utilized C-terminal fusions to mCherry and also to nano-Luciferase (nLuc), which has previously been used to dissect secretion by Type III secretion systems (Fig 9A)⁷⁴. By immunoblot, there was no discernable secretion of the PPE-mCherry and PPE-nLuc fusions (Fig 9B). We hypothesized the C-terminal fusions could be interfering with secretion as there are several subfamilies of PPE proteins, each with conserved C-terminal motifs and potentially both N-terminal and C-terminal sequence elements may be required for secretion. We also hypothesized certain subfamilies of PE and PPE proteins may be more or less amenable to transport of heterologous fusion domains. Therefore, we tried internal nLuc fusions to an array of pe and ppe proteins from different subfamilies (Fig 9C). By immunoblot of culture filtrate and genapol-X80 extraction, there was no discernable secretion of the PE-nLuc and PPE-nLuc fusions (Fig 9D).

Heterologous expression of Listeriolysin O

Bacterial pathogens of genera such as *Shigella*, *Salmonella*, and *Yersinia* employ Type III secretion systems to deliver a range of effectors capable of not only pore formation, but also manipulation of host processes, such as vesicle trafficking, phagocytosis, and inflammatory responses⁷⁵. Past work has demonstrated that strains of Mtb and Mm deficient for ESX-1 are highly attenuated, however the mechanism by which ESX-1 contributes to pathogenesis is still not fully understood and the only known function of ESX-1 is membrane damage, particularly phagosomal membrane damage^{31,46}. It is possible that ESX-1 possesses additional functions in addition to pore-formation necessary for full virulence. To explore whether the sole pathogenic function of ESX-1 is pore-formation, we sought to generate ESX-1-deficient strains complemented with the pore-forming toxin, LLO, encoded by hly from *L. monocytogenes*, to restore pore-formation⁷⁶. Future findings on differences between wild-type and RD1::hly would help unravel whether ESX-1 has roles in pathogenesis other than pore-formation. Initial attempts to constitutively overexpress LLO did not result in induction of IFN- β by the IFN- β reporter cells, although statistics for significance were not performed as the experiment was not repeated (Fig 10A). Further tests revealed constitutively overexpressing LLO appeared to not be well tolerated: the only transformants recovered had mutations in the P-smyc promoter driving LLO expression (Fig 10B). Subsequent attempts used anhydrotetracycline (atc) inducible expression and a range of LLO expression levels which did not result in apparent toxicity. To assay for phagosomal damage, we tested for induction of IFN- β by the ISRE-IFN- β THP-1 reporter cells as a proxy for phagosomal disruption. According to the IFN- β reporter, the RD1::hly *M. marinum*

strain had minimal induction of IFN- β compared control strains, indicating the hly constructs were not causing phagosomal perforation, although statistics for significance were not performed as the experiment was not repeated (Fig 10C). To determine whether this was because of poor expression of LLO we immunoblotted for LLO in lysates after induction and observed robust bands matching the expected size of LLO (Fig 10D).

These initial constructs were designed without Sec or Tat signals based on previous reports that LLO without a Sec signal conveyed phagosomal damage in *E. coli*⁷⁷. Our initial expectation was that a Sec signal should result in LLO trapped in the mycobacterial periplasm and hence not facilitate secretion, however, it's possible that Sec or Tat signals would increase LLO release from mycobacteria. Initial attempts to fuse the Sec signal from the mycobacterial protein, Mpt-32, under inducible expression were inconclusive and will require future experiments to definitively evaluate.

Chapter 3.2 Discussion

The role of PDIM in *Mtb* pathogenesis and ESX-1 function remains unclear. While this study did not find a role for PDIM in surface localization of ESX substrates, it remains a distinct possibility that the interaction between ESX substrates and the surface of the bacteria is still altered in the absence of PDIM – perhaps the interaction is lessened to a degree not detected by immunoblot, the interaction of substrates other than EspE is altered, or the conformation is altered in such a manner that activity is impaired. Alternatively, there are reports of PDIM interacting with the macrophage phagosome membrane, and perhaps this facilitates the intercalation of the pore-forming toxin of the ESX-1 system – if such a toxin does exist.

In terms of investigating a potential pore-forming toxin of *Mtb*, the preliminary findings from this study suggest EspE and EspF are not the pore-forming toxin, but it cannot be ruled out that EspE and EspF function redundantly with other effectors to catalyze phagosome perforation, or that there is a divergence between ESX-1 of *Mtb* and of *Mm* – that is, it remains possible EspE is the pore-forming toxin of *Mtb* but not *Mm*. To unravel the mechanism of ESX-1-mediated phagosome pore-formation, it is likely that further studies of the sequence elements that control secretion would be helpful. This study suggests some of the simpler models of sequence elements for secretion are unlikely to be the case: the data suggests the N-terminal domain is sufficient for secretion of heterologous cargo.

Another significant question concerning ESX-1 that remains is how ESX-1 contributes to pathogenesis. It may be that ESX-1's sole contribution to pathogenesis is pore-formation, alternatively it may be that ESX-1 has roles yet undiscovered. In *M. smegmatis*, ESX-1 is reported to have roles in horizontal gene transfer and it could be ESX-1 in *Mtb* and *Mm* has additional roles in pathogenesis

or unrelated to pathogenesis. A key method to uncover this would be restoring phagosome permeabilization in ESX-1 deficient strain using an alternative pore-forming toxin, such as LLO. While this study did not appear successful in restoring phenotypes related to phagosome permeabilization in LLO, it is possible alternative constructs, perhaps involving different secretion signal fusions may be successful and allow ESX-1 activity to be disentangled from pore-formation. Alternatively, if the pore-forming toxin is identified, point mutants that interrupt membranolytic activity may help answer this question.

Chapter 3.3 Figures

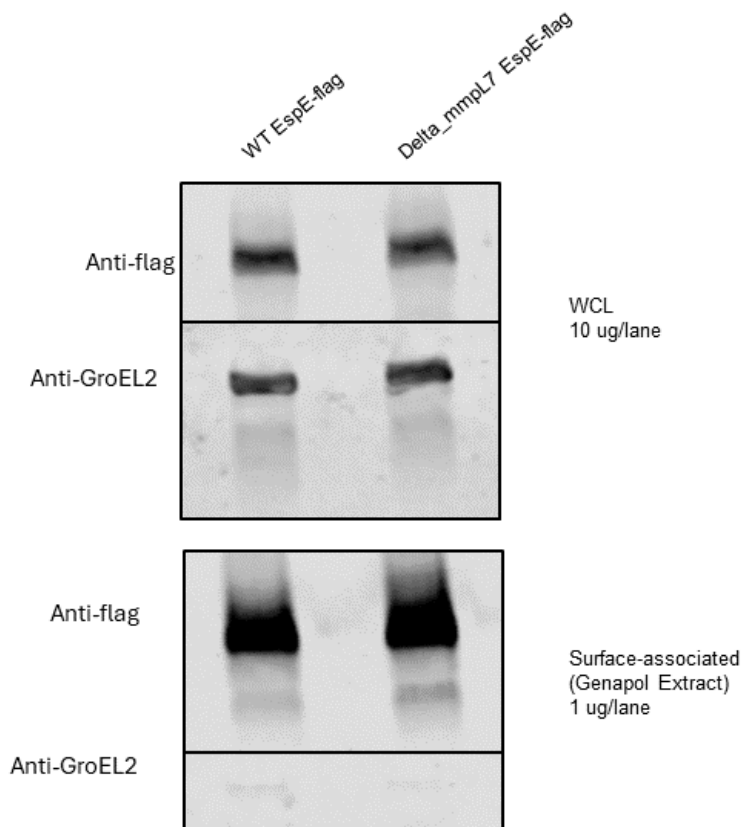


Fig 6: PDIM mutants are not defective for proper surface-localization of EspE.

M. marinum strains of either wild-type or $\Delta mmpL7$ were grown in 7H9 medium with OADC and 0.20% Tween 80 to mid-log phase. Bacteria were then washed twice and resuspended at an OD600 of 0.40 in 7H9 medium supplemented with 0.2% glycerol and 0.2% dextrose for 16 hours. Bacterial pellets were incubated with 0.5% Genapol X-080 in PBS for 30 minutes at room temperature with gentle rotation, centrifuged, and the supernatants were concentrated by trichloric acid (TCA), and the resulting protein pellets were washed with acetone and resuspended in LDS buffer with beta-mercaptoethanol. Proteins were visualized using antibodies against Flag and GroEL.

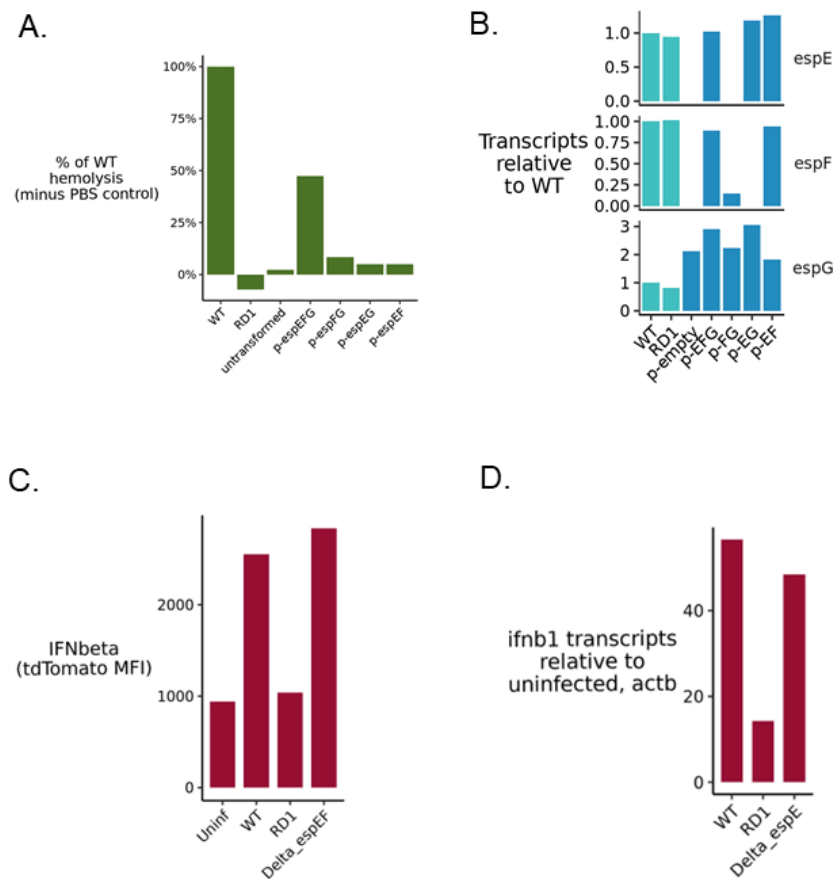


Fig 7: EspE is not required for IFN β induction in *M. marinum*. (A) *M. marinum* strains of the indicated genotypes were grown to mid-log phase, washed, and mixed with washed defibrinated sheep red blood cells were washed five times and diluted in PBS. After 2 hours at 33°C absorbance was measured at 405 nm. (B) *M. marinum* strains of the indicated genotypes were grown to mid-log phase, then RNA was harvested and analyzed by RT-qPCR. (C) THP-1 cells were infected with the indicated strain of *M. marinum*, MOI 5, or left untreated, and reporter expression was measured by flow cytometry at 24 hpi. (D) THP-1 cells were infected with WT *M. marinum* MOI 10 for 4 hr, and transcripts for IFN- β were measured by RT-qPCR. Statistics were not performed as the experiments were not repeated.

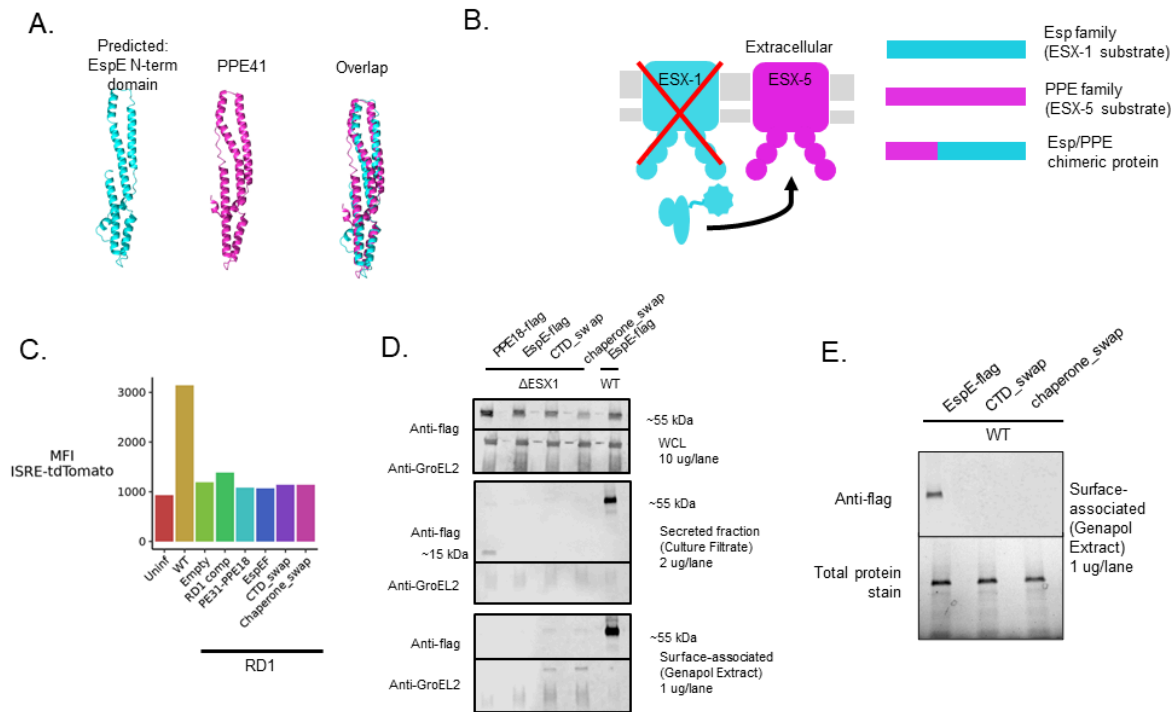


Fig 8: Attempts to re-route EspE through ESX-5 using EspE-PPE

fusions/chimeric constructs. (A). Crystal structures of the Mtb PPE protein, PPE1, compared to structural predictions of the N-terminal domain of EspE from PHYRE2. (B) Schematic showing the rerouting of PPE-EspE fusion from ESX-1 to ESX-5. (C) THP-1 cells were infected with the indicated strain of *M. marinum*, MOI 5, or left untreated, and reporter expression was measured by flow cytometry at 24 hpi. (D-E) *M. marinum* strains of the indication genotype were grown to mid-log phase, resuspended at an OD600 of 0.40 in 7H9 medium supplemented with 0.2% glycerol and 0.2% dextrose for 16 hours, and the resulting bacterial pellets were incubated with 0.5% Genapol X-080 in PBS for 30 minutes at room temperature with gentle rotation. Proteins were visualized using antibodies against Flag and GroEL. Statistics were not performed as the experiments were not repeated.

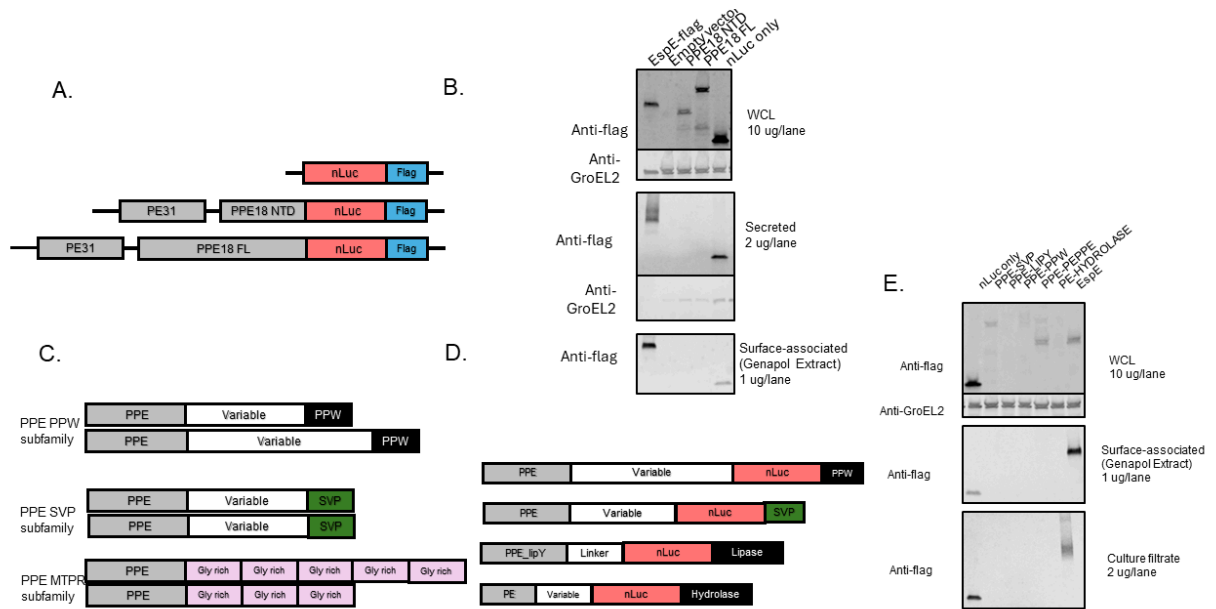


FIG 9: Testing signal sequences for ESX secretion using PPE C-terminal and internal fusions. (A) Schematic showing PPE proteins with C-terminal fusions to nano-Luciferase. (B). *M. marinum* strains of the indicated genotype were grown to mid-log phase, resuspended at an OD600 of 0.40 in 7H9 medium supplemented with 0.2% glycerol and 0.2% dextrose for 16 hours, and the resulting bacterial pellets were incubated with 0.5% Genapol X-080 in PBS for 30 minutes at room temperature with gentle rotation. Proteins were visualized using antibodies against Flag and GroEL. (C). Bioinformatically identified subfamilies of PPE proteins. (D). Schematic showing PPE proteins with internal fusions to nano-Luciferase. (E). Same as (B) with different strains.

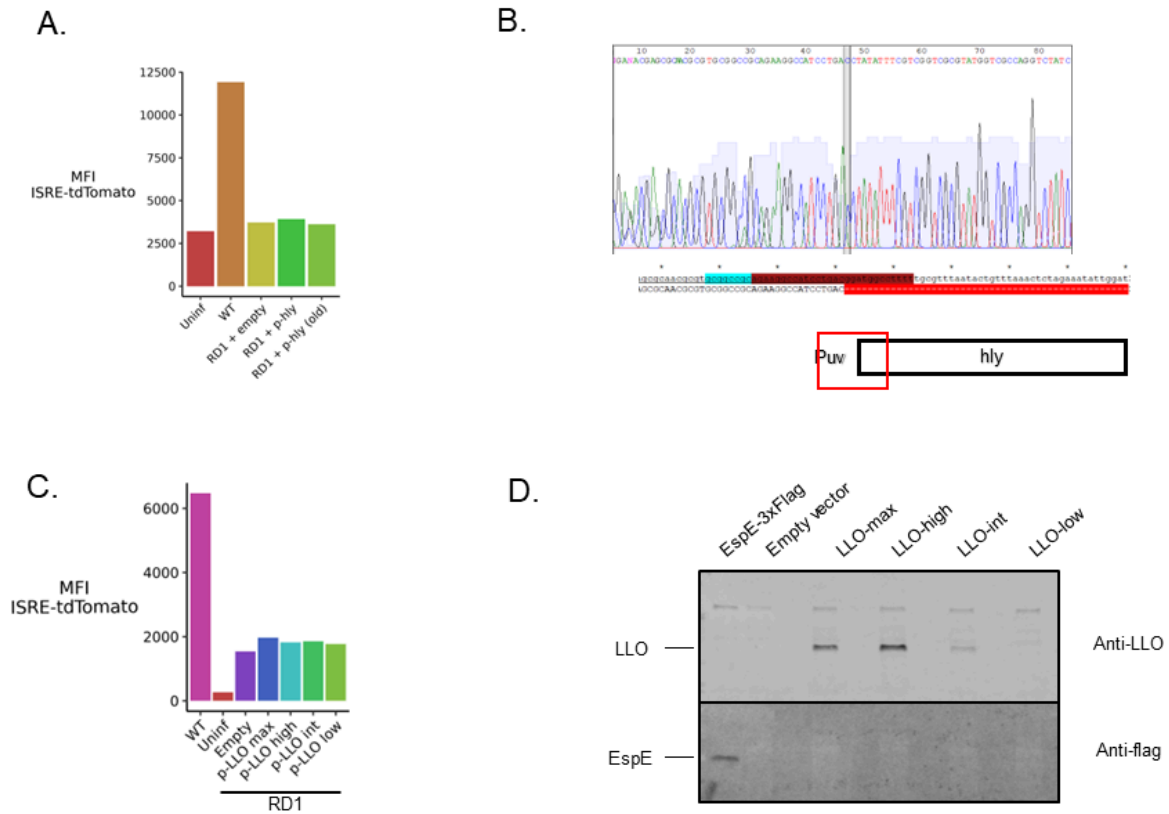


FIG 10: LLO does not restore IFN̳ induction in *M. marinum*. (A). THP-1 cells were infected with the indicated strain of *M. marinum*, MOI 5, or left untreated, and reporter expression was measured by flow cytometry at 24 hpi. (B). Sanger sequencing of constructs constitutively expressing hly. (C) THP-1 cells were infected with the indicated strain of *M. marinum*, MOI 5, or left untreated, and reporter expression was measured by flow cytometry at 24 hp. Strains of *M. marinum* were induced for expression by treatment with 100 ng/mL of anhydrotetracycline for approximately 16 hours. (D) *M. marinum* strains of the indication genotype were grown to mid-log phase, resuspended at an OD600 of 0.40 in 7H9 medium supplemented with 0.2% glycerol and 0.2% dextrose for 16 hours, and the resulting bacterial pellets were incubated with 0.5% Genapol X-080 in PBS for 30 minutes at room temperature with gentle rotation. Proteins were visualized using antibodies against LLO and Flag peptide. Statistics were not performed as the experiments were not repeated.

Chapter 3.4 Methods

Hemolysis assay

M. marinum strains were until mid-log phase. Bacteria were collected by centrifugation, washed once in PBS and diluted in PBS. Bacteria were quantified by absorbance measurement at OD600. Defibrinated sheep red blood cells were washed five times and diluted in PBS. Red blood cells and bacteria were mixed in a final volume of 100 μ l in a 96 well-plate, centrifuged for 5 minutes, and incubated at 33°C. After 2 hours, cells were resuspended, centrifuged, and 80 μ l of supernatant was transferred to a flat-bottom 96-wells plate and measured at 405 nm.

Secretion assays

M. marinum cultures were grown in 7H9 medium with OADC and 0.20% Tween 80 to mid-log phase. Bacteria were then washed twice and resuspended at an OD600 of 0.40 in 7H9 medium supplemented with 0.2% glycerol and 0.2% dextrose for 18 hours. For culture filtrate fractions, supernatants were filtered by 0.22 μ m filter, concentrated by trichloric acid (TCA), and the resulting protein pellets were washed with acetone and resuspended in LDS buffer with beta-mercaptoethanol. For Genapol extracts, bacterial pellets were incubated with 0.5% Genapol X-080 in PBS for 30 minutes at room temperature with gentle rotation, centrifuged, and the supernatants were concentrated by trichloric acid (TCA), and the resulting protein pellets were washed with acetone and resuspended in LDS buffer with beta-mercaptoethanol. For whole cell lysate, cells were lysed by bead beating for 5 minutes, clarified by centrifugation, concentrated by trichloric acid (TCA), and the resulting protein pellets were washed with acetone and resuspended in LDS buffer with beta-mercaptoethanol. Proteins were visualized using antibodies against Flag (Ms monoclonal ANTI-FLAG M2) and GroEL (Rb polyclonal, Assay Designs, SPS-875).

Mycobacterial Strain Construction

Mycobacterial deletion strains were created by homologous recombination using specialized transducing phage as previously described⁷⁸. Briefly, 5' and 3' regions upstream and downstream of the target gene were cloned flanking the loxP-zeoR-loxP cassette of plasmid pMSG360⁷⁹. The plasmid was then used to produce specialized transducing phage at high-titer phage using *M. smegmatis*, and this phage was transduced into target mycobacteria.

RT-qPCR

For RNA analysis, cells were harvested in TRIzol at the specified time points and RNA was isolated using PureLink RNA Mini Kits (Invitrogen) and digested with TURBO DNase (Thermo Fisher). cDNA was synthesized with SuperScript III cDNA Synthesis Kit (Thermo Fisher) using 500 ng of RNA. Real-time quantitative PCR (RT-qPCR) was performed using iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories).

Mycobacterial culture

M. tuberculosis (strain Erdman) and *M. marinum* (strain M) were used for macrophage infections. *M. tuberculosis* was grown to log phase in 7H9 liquid media (BD) supplemented with 10% Middlebrook OADC (Sigma), 0.5% glycerol, 0.05% Tween80 in roller bottles at 37°C. *M. marinum* was grown to log phase in 7H9 liquid media (BD) supplemented with 10% Middlebrook OADC (Sigma), 0.5% glycerol, 0.20% Tween80 in roller bottles at 30°C.

Macrophage infection

For infections with mycobacteria, macrophages were infected as previously described⁶⁵. Briefly, mycobacteria cultures were washed twice with PBS. Aggregates were removed by low-speed centrifugation at 60 x g for 5 min and gentle sonication, before being resuspended in cell media. Cell monolayers were overlaid with the bacterial suspension and centrifuged for 10 min at 1,000 x g for 'spinfection.'

Cell culture

THP-1 cell line was purchased from ATCC and cultured in RPMI (Gibco) supplemented with 10% FBS and 5 mM L-glutamine. Primary murine bone marrow-derived macrophages (BMMs) were isolated and cultured in BMM media (DMEM with 10% FBS, 2 mM L-glutamine, 10% MCSF, 11 mg/mL sodium pyruvate) as previously described^{48,65}.

Flow cytometry

The tdTomato reporter cell was generated and used as previously described⁴⁷. Briefly, after approximately 24 hours of infection or stimulation, tdTomato expression was measured by flow cytometry using a BD LSR Fortessa.

Chapter 4: Conclusions

Although the details of how ESX-1 permeabilizes the phagosome to induce Type I IFN remain opaque, we found previously unrecognized genes that regulate expression of IFN- β during mycobacterial infection. We found that induction of IFN- β is inhibited by UFL1, a ligase of the UFMylation pathway, indicating that UFL1 may have a significant function in the host immune response. We further demonstrated that the UFMylation pathway, rather than UFL1 alone plays this role as we identified similar roles of three key proteins in the UFMylation pathway: UFL1, DDRGK1, and UFM1.

Mechanistically, defects in UFMylation lead to ER stress potentially due to the accumulation of stalled ribosomes in the ER translocon⁸⁰. Additionally, dendritic cells and macrophages treated with ER stressors and TLR ligands in combination, rather than with TLR ligands alone, are reported to express greater levels of IFN- β ²⁹. These increased levels of IFN- β were dependent on Xbp1, leading us to hypothesize a model in which Xbp1 mediates enhanced IFN- β levels and that Xbp1 may be required for increased IFN- β in UFMylation-deficient cells during Mtb infection²⁹. Future should be done to determine whether increased levels of IFN- β are dependent on xbp1 such that levels of IFN- β are decreased in cells with XBP1-UFL1 double knockdown compared to cells with UFL1-only knockdown.

Evidence from mouse studies suggests that type I IFNs can increase susceptibility to Mtb during infection and hence it is possible that UFMylation-deficiency, which leads to more Type I IFNs, may worsen Mtb disease^{2,3,13-18}. The mechanism by which Type I IFNs counteract Mtb control remains unclear, but some literature suggests that Type I IFNs may inhibit IFN γ signaling, which could contribute to Mtb susceptibility as impaired IFN γ signaling significantly increases Mtb susceptibility^{23,81}. Because UFMylation appears to elevate levels of Type I IFNs and other inflammatory cytokines, such as IFN γ , it's possible that elevated IFN γ levels may compensate for the IFN γ -inhibitory effects of Type I IFNs. To test these hypotheses, future studies should be undertaken to experimentally determine whether UFMylation-deficient mice are susceptible or resistant to Mtb and whether levels of Type I IFNs and IFN γ are elevated as predicted. While UFMylation deficiency results in embryonic lethality in mice, conditional deletions, such as myeloid specific, are viable.

Due to the multitude of findings that Type I IFNs and regulators of Type I IFNs are important determinants of Mtb infection outcomes in human and mouse studies, there is a great potential for value in identifying the genetic determinants underlying regulation of Type I IFNs. This report provides new data on IFN- β regulation during mycobacterial infection, identifying a role for UFMylation in suppressing IFN- β during Mtb infection. Further studies on UFMylation's role in Type I IFN signaling during Mtb infections is necessary to understand how UFMylation influences Mtb infection outcomes. This would offer insights into the impact of Type I IFN signaling on Mtb

disease and potentially reveal new treatment strategies by targeting regulators of Type I IFN signaling, such as the UFMylation pathway.

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