TRIM14 IS A KEY REGULATOR OF THE TYPE I INTERFERON RESPONSE DURING MYCOBACTERIUM TUBERCULOSIS INFECTION

A Dissertation

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ABSTRACT

TRIM (tripartite motif) family proteins are distinguished as important players in the innate immune response to infection. While many TRIMs have been implicated in antiviral responses, their roles in regulating bacterial pathogenesis remain poorly defined. TRIM14 has been heavily implicated in anti-viral innate immune signaling through various protein-protein interactions. However, TRIM14's role during bacterial infection remains unclear. Here, I demonstrate that TRIM14 is a crucial negative regulator of type I interferon and interferon stimulated gene (ISG) expression during infection with *Mycobacterium tuberculosis*, a potent activator of cytosolic DNA sensing pathways. My data shows that TRIM14 directly interacts with the DNA sensing kinase TBK1 and that loss of TRIM14 leads to dramatic hyper-induction of IFNβ and ISGs in response to cytosolic nucleic acid agonists, including M. tuberculosis. Consistent with this phenotype, I report that loss of TRIM14 promotes phosphorylation of STAT3 at S754, leading to downregulation of negative regulators of ISG expression including SOCS3. Furthermore, in investigating ways in which TRIM14 could be regulated we discovered an isoform of TRIM14 that is upregulated upon *Mycobacterium tuberculosis* infection. In addition, TRIM14 is differentially ubiquitinated and phosphorylated upon bacterial infection. I propose here that TRIM14 has several layers of regulation in order to manipulate the host innate immune response against different pathogens. Ultimately, TRIM14 activity may prove to be a good therapeutic target as limiting its activity could promote clearance of *Mycobacterium tuberculosis* infection.

DEDICATION

I would like to dedicate this dissertation to my loving and supportive family, thank you for never giving up on me. To my daughter Delilah, never give up on your dreams, you can do all things through Christ who strengthens you.

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NOMENCLATURE

IFN- Interferon

ISG- Interferon Stimulated Gene

TRIM- Tripartite Motif

Mtb- *Mycobacterium tuberculosis*

BCG- Bacillus-Calmette Guerin

MDR- multi-drug resistance

cGAS- Cyclic GMP-AMP synthase

cGAMP- Cyclic guanosine monophosphate–adenosine monophosphate

STING- Stimulator of interferon genes

TBK1-Tank binding Kinase

IRF- interferon-regulatory factor

ISRE-Ifn stimulated response element

GAS- Gamma interferon activation site

CRISPR- Clustered Regularly Interspaced Short Palindromic Repeats

mtDNA- Mitochondrial DNA

IFNAR- interferon-α/β receptor

JAK1- Janus kinase

TYK2- Tyrosine kinase 2

ISGF3- Interferon-stimulated gene factor 3

BMDM- bone marrow derived macrophage

IL- Interleukin

TNF- Tumor necrosis factor

LC3- light chain 3

ATG5- Autophagy related 5

RING- really interesting new gene

TRIM- tripartite motif

PAMP- pathogen associated molecular pattern

MAVS- mitochondrial antiviral signaling protein

MDA5- melanoma differentiation associated gene 5

RIG-I- retinoic acid-inducible gene I

SOCS- Suppressor of Cytokine Signaling

STAT- Signal Transducer and Activator of Transcription

NO- Nitric oxide

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CHAPTER I

INTRODUCTION

The human pathogen *Mycobacterium tuberculosis* (Mtb) causes a devasting lung infection and is currently the number one infectious killer in the world, infecting over one-fourth of the world's population, with the highest prevalence of disease in regions of Africa and South East Asia (Figure 1)[1]. Developing nations account for 95% of active Tb cases and deaths [1]. These nations remain the most burdened countries due to lack of health care and appropriate treatment of disease. Misuse of anti-Tb antibiotics has also attributed to the rise of multi-drug resistant tuberculosis (MDR) in which only 56% of patients are successfully treated, assuming appropriate care is available [1]. Spread of disease is further propagated by environmental factors such as living and working conditions that lack proper air circulation. In addition, many individuals in these nations have an increased the risk of infection due to malnutrition, impaired immune system, or drug and alcohol abuse. Rates of HIV are also higher in these regions which makes an individual 19 times more likely to develop active Tb disease [2, 3]. Taken together, all of these things facilitate the spread of infection and make it increasingly difficult to eliminate disease even though prevention and treatment exist.

Unlike many other intracellular pathogens, Mtb is an ancient infection and has exclusively caused human disease for thousands of years [4]. Anecdotal evidence suggest Tb was found in ancient Egyptian mummies dating back to 2400BC [5, 6] however the first written documentation of Tb dates back 3300 years ago in India and

2300 years ago in China [7, 8]. Tuberculosis spreads when a person with active disease coughs, sneezes, or otherwise spreads aerosolized droplets containing the bacterium and these droplets are inhaled by an uninfected individual (Figure 2). These droplets carry Mtb into the depths of the lung where it meets the bodies first line of defense against pathogens, the alveolar macrophage. Macrophages recognize pathogen associated molecular patterns of Mtb through several different pattern recognition receptors such as TLR2, TLR4, TLR9, and NOD2 [9-11]. Despite the method of recognition, macrophages phagocytize Mtb and sequester the bacilli into phagosomes with the intent to kill, destroy, and alert surrounding cells of the infection. However, Mtb has used its long-standing relationship with its host to uniquely evolve several mechanisms that allow it to manipulate the macrophage in order to replicate and establish its niche. This evolution allows Mtb to remain latent within the host for decades and leads to difficulties in treatment and prevention of disease. Individuals with latent Tb infection, which accounts to around 1.7 billion people, have a 5-10% chance of developing active Tb and further spreading the infection [1]. Treatment of these individuals is crucial in eliminating and preventing spread of disease. Current treatment for infection is 6-9 months of antibiotic cocktails including isoniazid and rifampicin [1]. This long course of antibiotics requires appropriate dosage, quality drugs, and suitable length of treatment depending on age and health status. Strict adherence to this treatment is difficult and oftentimes inaccessible, which has given rise to several multidrug resistant (MDR) strains of Mtb [12]. These MDR strains are becoming increasingly more prevalent in the population faster than new antibiotics can be produced [12]. In addition, the only

vaccine approved and available, Bacillus-Calmette Guerin (BCG), is only effective in infants against Tb meningitis and miliary disease and provides no protection for adolescents or adults against pulmonary Tuberculosis [13, 14]. As antibiotic treatment and prevention methods for Mtb infection are becoming outdated and ineffective, it is crucial to better understand the interface between *Mycobacterium tuberculosis* and the host immune system in order to develop novel therapeutics. Specifically, development of host-directed therapies and better vaccines that focus on enhancing the immune system's fighting capabilities [15].

Mtb and the innate immune response

Bacteria generally have essential secretion systems that allow for the export of proteins across the cell membrane. In addition to these, bacteria with complex cell envelopes often have specialized secretion systems that allow for the delivery of proteins outside of the bacterium or even into the host cell. Often times these specialized secretion systems enhance virulence through the export of various virulence factors and effector proteins that augment the bacteria's ability to survive in the host.

Mycobacterium tuberculosis has five of these secretion systems, ESX-1 – ESX-5, all of which are classified as type VII secretion systems. Two of these, ESX-1 and ESX-3 have been shown to be essential for virulence and growth respectively [16]. The virulence associated secretion system, ESX-1 was the first to be identified and is the best characterized, numerous studies have shown mutations in the ESX-1 locus lead to attenuated phenotypes in both mice and macrophages [17-22]. In addition, there is strong evidence that demonstrates ESX-1 secretion is required for Mtb to access the host

cytosol likely by permeabilization the phagosome membrane[23, 24]. The exact secreted substrate(s) that are responsible for this remain to be identified. However, ESAT-6 has been identified as an ESX-1 substrate that can alter membrane integrity through inserting into/ interacting with the phagosome membrane [25-27].

Disruption of the phagosome leads to the activation of several immune defense pathways such as autophagy, DNA sensing, NOD sensing, and the inflammasome. Selective autophagy is an antimicrobial pathway that promotes the destruction of intracellular pathogens via various adapters that detect and target bacteria to the autolysosome [28]. There are several DNA sensors that can bind and detect DNA in the cytosol such as DAI, IFI16, DDX41, and AIM2-like receptors. However, cGAS has been implicated as the chief contributor to the activation of the pathway and ultimately results in expression of type I IFN and interferon stimulated genes (ISG)s. In addition, the host DNA sensors IFI16, IFI204, AIM2, as well as the NOD-like receptors NLRP1, NLRP3 and NLRP4 can induce inflammasome activation [29, 30]. The inflammasome is a multiprotein complex that promotes the maturation of proinflammatory cytokines IL-1β and IL-18 and induces cell death[31]. Taken together, it is clear disruption of the phagosome is an important stimulus of the innate immune response.

Mtb disruption of the phagosome membrane activates the innate immune response

Once Mtb is phagocytosed by the host macrophage, the ESX-1 secretion system secretes virulence associated proteins that help it subvert the macrophages fighting capabilities [21, 32-35]. As previously mentioned, some of these proteins such as ESAT-6 destabilize the phagosome membrane, consequently allowing Mtb proteins and dsDNA

access to the host cytosol, which activates the host's DNA-sensing pathway [23, 33-38]. The exact mechanism of how and why Mtb DNA is released in the phagosome is still poorly understood. However, the requirement for phagosome permeabilization in activating DNA-sensing has been shown to be ESX-1 dependent. One study actually measured this permeabilization event by using a fluorescent β-lactamase assay which showed phagosome integrity was disrupted in an ESX-1 dependent manner [24]. In addition, macrophages infected with an ESX-1 mutant strain of Mtb expressing an auto-activated form of LLO from *L. monocytogenes* (pore forming toxin) restored the mutant's ability to activate the DNA sensing pathway. This was seen by both the induction of IFNβ and ISGs and by an increase of targeting of GFP-LC3 to these ESX-1 +LLO containing autophagosomes[23, 24]. Furthermore, WT vs ESX-1 mutant infection of macrophages show ESX-1 is necessary for IRF3 translocation to the nucleus as well as induction of IFNβ and ISGs transcripts [24].

cGAS is critical for DNA sensing response to Mtb infection

The host cytosolic DNA sensor, cyclic GMP-AMP synthase (cGAS) was discovered to be the essential enzyme that binds DNA and activates the DNA sensing pathway by three separate studies. First, cGAS was identified through a purification study of fractionated cell lysates which had cGAMP synthesizing activity and further confirmed by overexpression studies which activated IRF3 and induced IFNβ transcription [39]. In addition, siRNA knockdown and knockout of cGAS in L929 cells inhibited IFNβ induction in response to DNA stimulation or viral infection with HIV, SeV, and HSV1 [39]. Finally, a large screen on antiviral activity of ISGs identified

cGAS as an ISG that broadly inhibits viral replication and genetic ablation of cGAS in mice corresponded to a failure to induce the IFN response and control viral infection [40]. A later study also showed the requirement for cGAS to induce the IFN response specifically during Mtb infection when cGAS KO BMDMs failed to induce IFNβ or ISGs in response to Mtb infection [36]. In addition, this same study also used a CHIP-like assay and discovered cGAS specifically binds to Mtb dsDNA in an ESX-1 dependent manner[36]. Collectively, the results show cGAS is an essential enzyme that binds DNA and activates the DNA sensing response.

Once cGAS recognizes Mtb dsDNA, it synthesizes the secondary messenger cGAMP which binds to and activates the stimulator of interferon genes (STING) which in turn activates TANK binding Kinase 1 (TBK1) (Figure 3). STING was actually discovered to be essential to inducing the DNA sensing response to Mtb infection prior to the discovery of cGAS. Manzanillo et al. infected STING and NOD1/2 KO BMDMs with wildtype Mtb and discovered that the adaptor STING but not NOD1/2 (as previously thought) was required for the induction of IFNβ and ISG transcripts in response to Mtb infection [24]. In addition, STING KO BMDMs failed to activate IRF3 translocation in response to Mtb infection[24]. As seen in figure 3, TBK1 kinase is a branching point in the innate immune response because activation of TBK1 leads to two opposing outcomes; the activation of the type I IFN response and selective autophagy [36, 41-43].

In addition to just bacterial DNA contributing to the type I IFN response, several studies suggest that host DNA also contributes to the induction of this response during

later time points of macrophage infection. First, a study infected BMDMs with several different strains of Mtb and compared the induction of IFNβ, mitochondrial stress, and mitochondrial DNA present in the cytosol[44]. They found that certain strains induced more mitochondrial stress which correlated to an increase in mitochondrial DNA in the cytosol and an increase in IFNβ induction[44]. Most recently, a separate study found that Mtb infection of macrophages manipulates the integrity of host membranes which causes release of host mtDNA and nuclear DNA into the cytosol and contributes to the induction of the IFN response[45]. Further studies need to be done to better understand the dynamics of the response in a macrophage. However, it seems initially bacterial DNA activates the response and later host mtDNA/nuclear DNA further contribute.

Interferons

Interferons are a group within the class II family of cytokines that consists of 6 interleukins which are closely related to IL-10 [46]. Interferons are released by the host in response to danger signals such as pathogens or even cancer cells. They are further classified into type I, type II, and type III based on their receptors, the cell type that makes them, and the cell type they stimulate. Type I IFNs mainly consist of IFNα and IFNβ and signal through the IFNAR receptor complex[47]. Type I IFNs have 17 additional subtypes, 13 of which are IFNα [48]. IFNγ is the only type II IFN and it signals through the IFNγ receptor composed of IFN-γR1 and IFN-γR2 subunits[46]. IFN-γ is secreted by T cells and NK cells and has been shown to induce expression of MHC molecules (class I and class II) and activate macrophage killing via nitric oxide production and the formation of granulomas[49, 50]. Type III IFNs are the most recently

discovered group of IFNs and are the most similar to IL-10 and signal through the IL28 receptor, this group consists of IFNλ1, IFNλ2, IFNλ3, and IFNλ4[51]. This receptor is only expressed in cells of epithelial origin as well as NKs and DCs and the current consensus is that type III IFNs play a unique role at mucosal surfaces in response to pathogens[52].

Type I IFNs are produced by several cell types including B cells, T cells, natural Killer cells, Dendritic cells, macrophages, and endothelial cells. They also have several functions as they can stimulate MHC class I expression, initiate production of CD8+ T cells, promote killing by NK cells, and induce expression of ISGs [53]. Type I IFNs are expressed in response to various innate immune stimuli that trigger a cascade of proteins to become activated and turn on the transcription factors IRF3 or IRF7. Once expressed, these IFNs can signal through the IFNAR receptor and activate STAT proteins to turn on the expression of hundreds of ISGs. Type I and type III IFNs activate the expression of genes that are considered antiviral while type II IFNs are considered antibacterial. However, this paradigm is shifting as more and more studies are uncovering the importance of ISGs in controlling bacterial infection. For example, GBPs are a group of ISGs turned on during the type I IFN response and they have been associated with protection against numerous intracellular pathogens [54].

Type I IFN response

TBK1 is a crucial kinase in the activation of the type I IFN response, as BMDMs lacking TBK1 also failed to induce IFIT1 and IFNβ transcription or IRF3 nuclear translocation in response to *M. tuberculosis* infection[24]. In order to induce the type I

IFN response, activated TBK1 phosphorylates the transcription factor IRF3, which then forms a dimer and translocates into the nucleus where it turns on transcription of IFNβ [55-57].

IFNβ is then released from the cell and detected through the IFNα receptor (IFNAR), which is composed of IFNAR1 and IFNAR2 subunits on the cell surface [58]. Once the receptor binds IFNβ, both subunits on the cell surface form a dimer that is endocytosed in order to activate their associated tyrosine kinases Janus kinase 1 (JAK1) and tyrosine kinase 2 (TYK2) [59, 60].

Once activated these two kinases recruit and phosphorylate the dimer of signal transducer and activator of transcription 1 (STAT1) and STAT2. The STAT1/STAT2 dimer then assembles with IRF9 to form the IFN-stimulated gene factor 3 (ISGF3) complex [58, 59, 61]. Finally, this ISGF3 complex translocates into the nucleus and binds IFN stimulated response elements (ISRE) to induce expression of hundreds of IFN stimulated genes (ISG) such as ISG15, IRF7, and iFIT1 (Figure 4) [59, 61, 62]. Transcription of these ISGs is specific to the type I IFN response, as typically STAT proteins initiate transcription at IFNγ activates sequence (GAS) elements [63]. Ultimately transcription of this response results in a positive feedback loop through the IFNAR receptor that further amplifies the Type I IFN response [64].

Unlike the type I IFN response to virus, which is critical to clearance of many viral infections, this response has been shown to be detrimental to the host during Mtb infection. Specifically, when mice are pretreated with type I IFN prior to Mtb infection, they succumb to the infection significantly sooner [65]. Whereas removing IRF3 using

CRISPR significantly decreased type I IFN and mice were resistant to Mtb infection [23]. More importantly, human studies have shown Tuberculosis patients with active disease have an elevated type I IFN signature and there is a correlation between HIV patients and Mtb susceptibility [66, 67]. An additional human study found a SNP in the IFNAR receptor gene, which leads to less IFN signaling, has been shown to be protective against Mtb infection [68].

Several studies suggest type I IFNs promote Mtb pathogenesis by inhibiting the protective IFNγ response, driving IL-10 production, and by defining the early myeloid cell population the responds to Mtb infection. IFNγ is crucial for the hosts antimicrobial response to Mtb infection as it induces protective cytokines such as IL-12, TNFα, and IL-1 [69-71]. IFNβ expression in macrophages infected with Mtb was shown to induce IL-10 production which is known to inhibit the Th1 innate immune response to Mtb infection, as well as block IFNγ antimicrobial pathways and protective cytokine expression [72-75]. Mice lacking the IFNAR receptor were protected from Mtb infection through altered migration of inflammatory monocytes and neutrophils and early depletion of neutrophils rescued the TB-susceptible mice to levels equivalent to the IFNAR KO mouse [76]. An additional study found that IFNα expression during Mtb infection preferentially induces monocyte differentiation of macrophages over dendritic cell differentiation which is an essential cell for the adaptive T cell response [77]. IL-1 has also been shown to be an important regulator of IFN and an anti-bacterial cytokine as it can induce eicosanoids that limit IFN production and promote bacterial control [78]. In addition, mice deficient in IL-1R or its adaptor MyD88 are extremely susceptible to

Mtb infection and [79, 80]. Finally, a more recent study has discovered Il1rn which encodes IL-1ra is induced in mice by IFN signaling during Mtb infection which ultimately binds to IL-1R1 blocks IL-1 signaling despite the high protein levels of IL-1 present in these mice [81]. Taken together, these studies demonstrate that high levels of IFN are associated with poor disease prognosis and enhanced Mtb pathogenesis.

Selective autophagy

As well as its involvement in the type I IFN response, TBK1 is also a crucial player in selective autophagy (Figure 3). TBK1 co-localizes with autophagy components LC3, NDP52, and p62 at the Mtb containing autophagosome and is required for the targeting of Mtb to the autophagosome [23, 82]. Selective autophagy has been shown to play a critical role in the control and destruction of Mtb infection [83-85]. Mice deficient in ATG5, a fundamental component in autophagy, have higher bacterial burdens in the lungs and spleen and are overall more susceptible to Mtb infection [23]. Several members of the small RNA family, mi-R17, promote Mtb replication in macrophages by inhibiting autophagy through various mechanisms [86-88]. Furthermore, isoniazid and pyrazinamide, two of the drugs used for Mtb treatment have been shown to function through activating autophagy and phagosome maturation [89]. Finally, autophagy has also been shown to play a critical role in bone marrow derived macrophages (BMDM) ability to clear Mtb infection [84, 89, 90].

Since activation of this DNA sensing pathway leads to two opposing outcomes during Mtb infection, there is a critical need to identify unique components and regulators of each response. Characterization of these will allow for development of novel

therapeutics that could activate selective autophagy to increase Mtb destruction while inhibiting the detrimental type I IFN response. We believe we have found one of these unique regulators, TRIM14 which our data has identified and shown is unique to the regulation of the type I IFN response.

TRIM family proteins

Tripartite motif family proteins (TRIM) are characterized based on the presence of three domains really interesting new gene (RING) domain, one or two B Boxes, the coiled-coil region, and the presence of a variable C-terminal region (Figure 5) [91-93]). The RING domain is an E3 ligase which gives TRIMs the ability to catalyze the final transfer of ubiquitin from an E2 ubiquitin conjugating enzyme to a protein substrate [94-96]. This can be linked through several different ubiquitin lysine residues and can be a single ubiquitin modification or several that form polyubiquitin chains [95, 97]. There are two types of B-boxes, B-box1 and 2 which are both different zinc-binding motifs [92, 98]. The function of these B-boxes is not well understood but it has been shown that they are necessary for efficient substrate binding for several TRIMs but not entirely required for the E3 ligase to function [99]. These B- box domains are also structurally similar to the RING domain which might also give them a role in the regulation of the RING domain itself. B-box2 is important for interactions with proteins and in formation of homo-multimers as seen [92, 98, 100]. The coiled-coil motif of TRIM proteins is known to be involved in homo- and hetero-multimerization of TRIMS and in promoting the formation of high molecular weight complexes with other proteins [101-103]. The C terminal region divides the TRIM family up further into subgroups, with the

PRY/SPRY domain being the most commonly found C-terminus (Figure 5) [93, 103]. The PRY/SPRY domain is generally recognized as a protein binding domain and can facilitate many different interactions depending on the TRIM protein [104].

TRIMs regulation of the innate immune response

TRIM family proteins have been established as important regulators of the innate immune response, with the majority of these regulators being characterized in the context of viral infection [100, 105-107]. Specifically, TRIM5α has been shown to block viral infection through an interaction with the viral capsid which results in premature virion disassembly and release of stimulatory PAMPS leading to antiviral gene expression [108-110]. It has also been shown to target human immunodeficiency virus type 1 (HIV-1) for autophagic degradation in Langerhans cells [111]. TRIM25 and TRIM4 have both been reported to activate RIG-I through K63-linked ubiquitin chains which induce the recruitment of MAVS to trigger downstream antiviral gene expression [112, 113]. TRIM65 similarly, promotes K63-linked ubiquitination of MDA5 which helps activate IRF3 and turns on IFN signaling and TRIM65 KO mice were more susceptible to encephalomyocarditis virus (EMCV) [114]. In contrast, TRIM40 has been shown to have an inhibitory role on antiviral signaling, specifically through K27- and K48-linked polyubiquitination of RIG-I and MDA5 resulting in TRIM40 deficient mice having enhanced IFNβ production after Vesicular Stromatis virus infection (VSV) [115].

TRIMs regulation of the type I IFN response

Furthermore, several TRIMs have been implicated in the regulation of IFNAR signaling and specifically the type I IFN response, both of which are crucial in

controlling inflammatory responses. TRIM6 was found to synthesize K48-linked ubiquitin chains onto IKKε which are required for its activation and downstream phosphorylation of STAT1 [107]. TRIM8 positively regulates the IFN response by protecting phosphorylated IRF7 from degradation which allows IRF7 to initiate transcription of ISGs [116]. TRIM8 also interacts with SOCS1 to promote its degradation and inhibit SOCS1-mediated downregulation of IFNγ signaling [61]. TRIM11 interacts with TBK1 through its coiled coil domain which inhibits IRF3 phosphorylation and downstream IFNβ production [117]. TRIM13 has been suggested to regulate both RIG-I and MDA5 through unknown mechanisms, as knockdown of TRIM13 led to significantly increased levels of IFNβ [118]. TRIM24 negatively regulates IFN production in conjunction with Retinoic acid receptor alpha by binding the STAT1 promoter to suppress its transcription [119]. TRIM26 is an interesting TRIM and requires further studies to interrogate its exact role in the response. One study showed overexpression of TRIM26 led to degradation of nuclear IRF3 through polyubiquitination ultimately resulting in downregulation of IFNβ production [120]. However, the exact opposite was found when TRIM26 knockdown resulted in less IRF3 activation and IFNβ induction [121]. In addition, TRIM26 recruited TBK1 through its associated with NEMO which further suggest TRIM26 in fact positively regulates IFNβ production [121]. TRIM27 targets TBK1 for degradation via k48-linked ubiquitin residues at lysine residue 251 and 372 [122]. TRIM28 associates with STAT1 in a manner that inhibits STAT1-mediated IRF1 gene expression [123] and has been shown to negatively regulate IFN-I production by promoting IRF7 SUMOylation

[124]. TRIM38 downregulates the TLR3-mediated type I IFN response by sending TRIF for proteasomal degradation via K48-linked polyubiquitination [125]. TRIMs are also negative regulators of the response in other species. For example, TRIM30α found in mice, negatively regulates TLR-mediated NF-κB activation by targeting TAB2 and TAB3 for degradation. In addition, TRIM44 and TRIM8 were found to negatively regulate the IFN response during RNA viral infection in fish [116, 126].

Despite the critical role of TRIMs in regulating viral infection and the innate immune response, relatively little is known about how they influence the outcome of bacterial infection. Almost certainly because these nucleic acid sensing pathways were thought to be exclusively used for viral detection and destruction. However, recent work has shown these pathways are also activated during intracellular bacterial infection of pathogens such as *M. tuberculosis*, *Legionella pnuemophila*, *Listeria monocytogenes*, *Francisella novicida*, and *Chlamydia trachomatis* [28]. A few papers have begun to touch on the topic of TRIMs effect during bacterial infection. A gene expression profiling study in human patients found that TRIM expression could be possible biomarkers of Tuberculosis infection [127]. While *Salmonella* Typhimurium effector protein SopA has been shown to target TRIM56 and TRIM65 in order to stimulate innate immune signaling through RIG-I and MDA5 [128, 129]. Finally, TRIM14 was shown to be anti-bacterial during *Listeria monocytogenes* infection through an unknown mechanism [130].

Regulation of TRIMs through splicing

Many of the canonical TRIM family proteins generate diversity by encoding several alternatively spliced isoforms (Figure 6) [92, 103]. Often times these encoded isoforms result in complete deletions of domains important for protein-protein interactions, for example the PRY/SPRY domain. As a whole, the roles of these isoforms are poorly characterized and very few have been experimentally investigated. Interestingly, in this small group of isoforms, a truncated form that regulates its own full-length canonical TRIM or other TRIMs is always present [98, 99, 131, 132]. TRIM5 is the best-characterized TRIM encoding these splice variants and it encodes shortened variants that both positively and negatively regulate full length TRIM5's functions [131]. Intriguingly, TRIM19 encodes splice variants that result in changes in the C-terminus that allow it to alter its localization in the cell and ultimately positively regulate IFNγ signaling [96, 132]. It is very plausible that this level of regulation is conserved among the TRIM family and all of these isoforms are used to regulate their own protein functions as well as the innate immune response in general.

TRIM14

TRIM14 is one of the few TRIMs that lacks the E3-ligase RING domain, it encodes the coiled-coil, B-box, and C-terminal PRY/SPRY domains [92] (Figure 7). The lack of this RING domain likely means it doesn't have the ability to catalyze ubiquitination of proteins. However, it has been shown to induce ubiquitination of influenza A NP protein [133].

TRIM14 was first observed to be highly expressed in HIV infected human lymphomas and Simian Immunodeficiency virus (SIV) infected monkey lymphomas [134, 135]. More recently it was discovered that TRIM14 expression is induced specifically by STAT1 binding to its promoter which now classifies it as an ISG [136].

Consistent with the rest of the family, TRIM14 has subsequently been heavily implicated in antiviral innate immunity and nucleic acid sensing pathways. TRIM14 directly influences replication of several RNA viruses specifically through its c-terminal PRY/Spry domain. TRIM14 inhibits the formation of the Smc-HBx–DDB1 complex important for hepatitis b viral replication by interacting with the viral HBX protein [136]. During hepatitis C infection, TRIM14 restricts viral replication by promoting k-48 linked ubiquitination and degradation of NS5A [137]. TRIM14 also induces K48-linked ubiquitination and proteasomal degradation of the influenza a nucleoprotein NP thus restricting influenza A viral replication [133].

TRIM14 is equally important in initiation of the type I IFN response through several different mechanisms. Overexpression of TRIM14 in 293T cells expressing cGAS and STING had significantly higher induction of IFNβ protein levels as compared to control cells [138].This phenotype was further investigated and it was discovered TRIM14 promotes cGAS stability by recruiting USP14 to remove K48-linked ubiquitin from cGAS which thereby prevents its autophagic degradation and facilitating the IFN response [138]. Further demonstrating the importance of TRIM14s contribution to cGAS stability, TRIM14 KO BMDMs had lower IFNβ protein levels 24 hours post VSV or HSV-1 infection [138]. As well as lowered IFNβ and ISG transcript expression 0-24

hours post VSV or HSV-1 infection which ultimately conferred with enhanced viral replication in both BMDMs and MEFs [138]. TRIM14 also localizes to the mitochondria and acts as an adaptor for retinoic acid-inducible gene-I (RIG-I)–like receptors (RLRs) and mitochondrial antiviral signaling platform protein (MAVS) where it recruits and facilitates interactions that activate the IFN and NF-kB pathways [139-143]. Specifically, during RNA viral infection, TRIM14 is a mitochondrial adaptor associated with MAVS that recruits the NF-κB essential modulator (NEMO) to stimulate antiviral signaling through IRF3 and NF-κB [139]. TRIM14 is also crucial to the assembly of the WHIP-TRIM14-PPP6C complex which is required for RIG-I mediated signaling through the MAVS signalosome [141, 142]. Finally, TRIM14 was just discovered as a novel positive regulator of noncanonical NF-κB signaling through its interaction with USP14 [143].

Modification of TRIM14

TRIM14 has several annotated spliced isoforms that can result in TRIM14 transcripts being targeted to non-sense mediated decay or resulting in a truncated version of the protein. One such spliced variant, TRIM14 202 encodes Exons 1-5 but it is missing exon 6 and subsequently all of the PRY/SPRY domain (Figure 7). In addition to splicing, TRIM14 is also modified by two types of ubiquitination that regulate its interactions. RNF125 is a known E3 ligase of TRIM14 that catalyzes K48-linked polyubiquitination and targets the protein for proteasomal degradation [140]. During SeV and VSV infection, TRIM14 undergoes K63-linked ubiquitination of K365 which is essential for its association with NEMO [139]. However, the E3 ligase responsible has

yet to be discovered. Finally, Bennet Penn and our own observations from the Jeff Cox lab shows that TRIM14 can be differentially ubiquitinated and phosphorylated during different bacterial infections (Figure 7) [144].

Regulation of the type I IFN response

Prolonged activation or dysregulation of the type I IFN response is detrimental to the host as it can lead to chronic inflammation and pathogenesis of autoimmune diseases dubbed "interferonopathies" which include Systemic lupus erythematosus, Sjögren's syndrome, Myositis, and Rheumatoid arthritis among others [145-148]. Under normal conditions, this pathway is tightly regulated to maintain immune homeostasis. Negative regulation occurs directly through protein-protein interactions or indirectly through post translational modifications (PTMs) such as ubiquitination and phosphorylation which can change the function of the protein or target it for degradation. These PTMs can also be removed as a means of regulating the protein by rendering it inactive. There are numerous negative regulators of the type I IFN response that can essentially target every level of the response. Pattern recognition receptors, their downstream kinases, and transcription factors are among the main proteins to be targeted as they are crucial to the initiation of the response. Two ISGs, RNF 135 and RNF 216, are E3 ligases that induce the proteasomal degradation of RIG-I, MDA5, and MAVS or TLR3, TLR4, and TLR 9 respectively [149, 150]. RIG-I is also inactivated through removal of K63-linked polyubiquitin chains by several deubiquitinating enzymes including USP3, USP15, USP21, and USP25 [151-154]. MAVS, which is just downstream of RIG-I during the RNA sensing cascade, is targeted for proteasomal degradation through K-48 linked

ubiquitination by several E3 ligases; MARCH5, RNF5, AIP4, and SMURF2 [155-157]. TBK1, which is also a critical kinase for the IFN response (among other things) has its activity modulated through phosphorylation, ubiquitination, and complex formation. To summarize, PPM1B, SHIP1, glucocorticoids, and GSK3β modulate TBK1 activity by targeting its phosphorylation [158-161]. SOCS3 mediates K48-linked ubiquitination of TBK1 which promotes its degradation [162]. SIKE, MIP-T3 and ISG56 complex use competitive inhibition to suppress TBK1 complex formation [163].

The IFNAR receptor and the second arm of the type I IFN response (JAK/STAT signaling) is also tightly regulated. Not only does the expression of ISGs upregulate the response, a negative feedback loop exists in which ISGs also limit the duration of the IFN response. Among these are ubiquitin specific peptidase (USP18) and the suppressor of cytokine signaling (SOCS) proteins, which act at the level of the IFNAR receptor as well as STAT proteins which function at the level of transcriptional regulation. USP18 disrupts the association of JAK1 and IFNAR receptor by directly binding the intracellular portion of IFNAR subunit 2, which effectively blocks IFN signaling [164]. STAT2 facilitates this interaction between USP18 and IFNAR2 by serving as an adaptor that enhances binding [165]. SOCS1 directly binds JAK1 in a way that prevents its phosphorylation and subsequent activation [166]. Together, SOCS1 and SOCS3 degrade IRF7 to mediate IRF7 transcription of ISGs [167]. SOCS3 also contains a kinase inhibitory region (KIR) which allows it to suppress JAK by inhibiting its kinase activity [168]. Moreover, SOCS3 is a feedback inhibitor of STAT3 in which it blocks phosphorylation of STAT3 by rendering JAK inactive [169]. Interestingly, STAT3 is in

fact a transcription factor of SOCS3 [170] and a negative regulator of the type I IFN response itself, as described in the next section [171, 172]. Furthermore, STAT3 and SOCS3 fine tune the specific type of immune response required by modulating each other's activity through various crosstalk mechanisms [173-175]. In addition to its interaction with USP18, STAT2 negatively regulates the IFN response through constitutive phosphorylation that prevents ISGF3 formation/DNA binding activities [176, 177].

STAT3 regulation of type I IFN response

In response to IFNAR activation through IFNα/β, STAT3 directly suppresses the IFN response by inhibiting formation of functional STAT1 homodimers, preventing ISGF3 from binding DNA, and limiting expression of ISGF3 components [166, 171, 178]. In the absence of STAT3, there is greater type I IFN production, STAT activation, and ISG gene expression which correlates to enhanced antiviral activity in macrophages whereas overexpression of STAT3 results in the exact opposite [172]. STAT3 also indirectly attenuates the IFN response in conjunction with phospholipid scramblase 2 (PLSCR2) and microRNAs (miRs) [87, 179]. More specifically, STAT3 is required for PLSCR2 to suppress recruitment of ISGF3 to ISRE elements [179]. STAT3 stimulates expression of miR221 and 222 which ultimately functions to stabilize STAT3 and increase STAT3 levels and downregulate expression of STAT1 and STAT2 [87] .

STAT3 and SOCS3 during Mtb infection

STAT3 and SOCS3 are activated downstream of numerous receptors so it is difficult to tease out the exact mechanisms through which Mtb manipulates and how

they influence the innate immune response (Figure 8). However, they have been shown to be critical in the outcome of Mtb infection. As mentioned previously, STAT3 is a transcription factor of SOCS3 and there is crosstalk between these two factors that helps regulate the innate immune response [170, 173-175]. Mtb has been shown to induce early activation of STAT3 and SOCS3 which can control the expression of protective innate immune cytokines such as IL-12 and TNF-α as well as suppress Nitric oxide induction in macrophages [180, 181]. In addition, SOCS3 -/- BMDMs are better at controlling Mtb replication likely due to higher levels of nitric oxide (NO). Low SOCS3 expression is also associated with latent TB infection which suggests it plays a protective role against the pathogen.

CHAPTER II

TRIM14 IS A KEY REGULATOR OF TYPE I IFN RESPONSE DURING MYCOBACTERIUM TUBERCULOSIS INFECTION

Overview

Tripartite motif family proteins (TRIMs) are well-characterized regulators of type I interferon (IFN) expression following cytosolic nucleic acid sensing. While many TRIMs are known to regulate innate immunity to viruses, their contribution to innate immune signaling and gene expression during bacterial infection remains largely unknown. Because *Mycobacterium tuberculosis* is a potent activator of cGAS-dependent cytosolic DNA sensing, we set out to investigate a role for TRIM proteins in regulating macrophage responses to *M. tuberculosis*. Here we demonstrate that TRIM14, a non-canonical TRIM that lacks an E3 ligase RING domain, is a critical negative regulator of the type I IFN response in macrophages. We show TRIM14 physically interacts with both cGAS and TBK1 and that macrophages lacking TRIM14 dramatically hyper induce interferon stimulated gene (ISG) expression following cytosolic nucleic acid agonist transfection, IFN-β treatment, and *M. tuberculosis* infection. Consistent with a defect in resolution of the type I IFN response, *Trim14* knockout (KO) macrophages have more phospho-Ser754 STAT3 relative to phospho-727 and fail to upregulate the STAT3 target *Socs3* (Suppressor of Cytokine Signaling 3), which is required to turn off IFNAR signaling. These data support a model whereby TRIM14 acts as a scaffold between TBK1 and STAT3 to promote phosphorylation of STAT3 at Ser727 and promote negative regulation of ISG expression. Remarkably, because *Trim14* KO macrophages hyper

induce antimicrobials like *Inos2*, they are significantly better than control cells at limiting *M. tuberculosis* replication. Collectively, these data reveal a previously unappreciated role for TRIM14 in resolving type I IFN responses and controlling *M. tuberculosis* infection.

Introduction

Mycobacterium tuberculosis, arguably the world's most successful pathogen, elicits a carefully orchestrated immune response that allows bacteria to survive and replicate in humans for decades. Infection of macrophages with *M. tuberculosis* sets off a number of pathogen sensing cascades, most notably those downstream of TLR2, which senses mycobacterial lipomannan [182, 183] and cGAS, which senses bacterial DNA in the host cytosol [36]. cGAS-dependent DNA sensing during *M. tuberculosis* infection elicits two distinct and somewhat paradoxical responses: targeting of a population of bacilli for destruction in lysosomes via ubiquitin-mediated selective autophagy, and activation of a type I interferon (IFN) gene expression program, which is inadequate at controlling bacterial pathogenesis *in vivo*. Because both selective autophagy and type I IFN have been repeatedly shown in animal and human studies to be hugely important in dictating *M. tuberculosis* infection outcomes [5-7], there is a critical need to elucidate the molecular mechanisms that drive their activation.

Many members of the TRIM family of proteins have emerged as important regulators of a variety of innate immune responses [6-8]. Defined on the basis of their tripartite domain architecture, TRIMs generally encode a RING domain with E3 ligase activity, a B-box that is a zinc-binding domain with a RING-like fold [9], and a coil-coiled domain that mediates dimer/multimerization and protein-protein interactions (10). In

addition to these domains, TRIMs encode a highly variable C-terminal domain. Since the initial discovery of TRIM5 as a potent HIV restriction factor (11), a variety of TRIMs have been shown to play critical roles in antiviral innate immunity through polyubiquitination of key molecules in DNA and RNA sensing cascades, including MDA5 by TRIM13, TRIM40, and TRIM65 (14-17), RIG-I by TRIM25 and TRIM40 (12, 13), and TBK1 by TRIM11 and TRIM23 (14). We are just beginning to appreciate the complex and dynamic network of regulatory factors, including TRIMs, that cells employ to up and downregulate innate immune signaling and gene expression (15).

Recent work has shown that cytosolic nucleic acid sensing pathways are also engaged during infection with a variety of intracellular bacterial pathogens, including *M. tuberculosis*, *Legionella pnuemophila*, *Listeria monocytogenes*, *Francisella novicida*, and *Chlamydia trachomatis* (16). Some of these pathogens, like *M. tuberculosis and C. trachomatis*, have been shown to activate cGAS via bacterial dsDNA (3, 17), while others like *L. monocytogenes* directly activate STING by secreting cyclic di-AMP (18). It is becoming increasingly clear that activation of the cytosolic nucleic acid sensing pathways provides some benefit to intracellular bacterial pathogens, and thus the ability to engage with and manipulate regulatory molecules like TRIM proteins is likely a conserved bacterial adaptation. *Salmonella* Typhimurium has been shown to secrete SopA, an effector molecule which targets TRIM56 and TRIM65 to stimulate innate immune signaling through RIG-I and MDA5 (19, 20). Likewise, TRIM8 has been shown to regulate inflammatory gene expression downstream of TLR3 and TLR4 during *Salmonella* Typhimurium-induced septic shock (21). In addition, ablation of TRIM72 in

alveolar macrophages enhances phagocytosis and clearance of *Psuedomonas aeruginosa* (22).

Realizing the huge potential for TRIM proteins in tipping the balance between pro- and anti-bacterial innate immune outcomes, we decided to study TRIMs during *M. tuberculosis* infection, specifically a non-canonical TRIM family member: TRIM14. Like most TRIMs, TRIM14 encodes a coiled-coil, a B-box, and a C-terminal PRY/SPRY domain, but curiously it lacks the E3 ligase RING domain, likely rendering it unable to catalyze ubiquitination of proteins. Consistent with it being a major player in antiviral innate immunity, TRIM14 has been shown to directly influence replication of several RNA viruses including influenza A via interaction with the viral NP protein (23), hepatitis B via interaction with HBx (24), and hepatitis C via interaction with NS5A (25). In the context of RNA sensing, TRIM14 has been shown to localize to mitochondria and interact with the antiviral signaling adapter MAVS (26). More recently, TRIM14 has been shown to promote cGAS stability by recruiting the deubiquitinase USP14 and preventing autophagosome targeting of cGAS (27).

Here, we demonstrate that TRIM14 is a crucial negative regulator of *Ifnb* and ISG expression during macrophage infection with *M. tuberculosis*. TRIM14 is recruited to the *M. tuberculosis* phagosome and can directly interact with both cGAS and the DNA sensing kinase TBK1. Deletion of *Trim14* leads to dramatic hyper induction of *Ifnb* and ISGs in response to several cytosolic nucleic acid agonists, including *M. tuberculosis*. In *Trim14* KO macrophages we observe preferential phosphorylation of the transcription factor STAT3 at Ser754 and lack of association of STAT3 with the chromatin loci of target genes

like Socs3, a crucial negative regulator of interferon α/β receptor (IFNAR) signaling. These data argue that TRIM14 acts as a negative regulator of cytosolic DNA sensing through bringing TBK1 and STAT3 together to promote phosphorylation of STAT3 at Ser727. Surprisingly, *Trim14* KO macrophages were remarkably efficient at limiting *M. tuberculosis* replication by virtue of overexpressing inducible nitric oxide synthase. Collectively, this work suggests that TRIM14 is a critical regulatory node of type I IFN induction and resolution in macrophages and highlights a previously unappreciated role for TRIM14 in anti-*M. tuberculosis* innate immunity.

Results

TRIM14 is a player in M. tuberculosis *infection of macrophages*

Having previously described a crucial role for the ESX-1 virulence associated secretion system in eliciting cGAS-dependent cytosolic DNA sensing and type I IFN expression during *M. tuberculosis* infection, we first set out to better define gene expression differences between a wild-type and a ΔESX-1 strain. Briefly, we infected bone marrow derived macrophages (BMDMs) with wild type *M. tuberculosis* (Erdman strain) and the Tn5370::Rv3877/EccD1 mutant (ΔESX-1) (28), which lacks a functional ESX-1 secretion system and has previously been shown to be defective in eliciting cGAS-dependent responses (29, 30). We performed RNA-seq at an established key innate immune time point of 4h and an average $\log_2$ fold-change of 4 biological replicates is depicted ($p < 0.05$). Consistent with previous microarray and RNA-seq data (30, 31), we observed dramatic induction of pro-inflammatory cytokines (*Il6*, *Il1b*) and antimicrobial molecules like *Nos2* in macrophages infected with both wild-type and ΔESX-1 *M.*

tuberculosis (Fig. 9A), alongside downregulation of several protein-coding genes (*Epha2*, *Gpr34*, *Rtn4rl1*), and noncoding RNAs (Gm13391, Gm15564, Gm24270) (Fig. 9B). To identify genes/pathways whose induction requires ESX-1 secretion, we performed Ingenuity Pathway Analysis (Qiagen) and identified "Interferon signaling" and "Activation of IRF by Cytosolic PRRs" as the major pathways enriched for ESX-1 dependent genes (Fig. 9C), in agreement with earlier data demonstrating a requirement for ESX-1 phagosome permeabilization for activation of type I IFN expression downstream of cGAS-dependent cytosolic DNA sensing (30). We next used RT-qPCR to measure expression of a number of important innate immune transcripts, both in BMDMs to validate our RNA-seq results and in RAW 264.7 murine macrophage-like cells (Fig. 9D and S1C), to justify our use of these genetically tractable cells moving forward. In analyzing lists of ESX-1-dependent upregulated genes, we noticed that several belonged to the TRIM family, consistent with TRIMs being ISGs (Fig. 9E) (32). Because so little is known about how TRIM proteins regulate anti-bacterial immunity, we set out to better understand how TRIMs influence cGAS-dependent innate immune outcomes during *M. tuberculosis* infection.

Based on its recent characterization as a regulator of cGAS stability (27), we elected to investigate a role for TRIM14 during *M. tuberculosis* infection. RT-qPCR analysis confirmed that *Trim14* expression was upregulated after *M. tuberculosis* infection of RAW 264.7 cells (Fig. 9F). Transfection of RAW 264.7 cells with dsDNA (ISD) (33) recapitulated this effect (Fig. 9F), suggesting that *Trim14* upregulation during *M. tuberculosis* infection occurs downstream of cytosolic DNA sensing. To further implicate

TRIM14 in *M. tuberculosis* infection of macrophages, we next asked whether TRIM14 protein associated with the *M. tuberculosis* phagosome. Using immunofluorescence microscopy and an antibody against endogenous TRIM14, we detected TRIM14 at about 30% of *M. tuberculosis* phagosomes, reminiscent of the number of phagosomes we have previously shown to be positive for ubiquitin (Ub) and LC3, two markers of selective autophagy (Fig. 9G) (34). Together, these data begin to suggest that TRIM14 is a player in the macrophage response to *M. tuberculosis*.

TRIM14 interacts with components of the DNA sensing pathway

Based on its recruitment to the *M. tuberculosis* phagosome, we hypothesized that TRIM14 may interact with one or more components of the cytosolic DNA sensing pathway (e.g. cGAS, TBK1) that we have previously observed to co-localize with *M. tuberculosis* (Fig. 10A and (3)). We transfected epitope-tagged versions of mouse TRIM14 (3xFLAG-TRIM14) and major components of the DNA sensing pathway (mouse HA-cGAS, HA-STING, and HA-TBK1) into murine embryonic fibroblasts (MEFs). Following 24 hours of expression, cells were fixed, and co-immunostained. Consistent with a previous report (24), we observed that TRIM14 co-localized with cGAS (Fig. 10B), while no co-localization between 3xFLAG-TRIM14 and HA-STING was seen. Intriguingly, we also detected substantial overlap between 3xFLAG-TRIM14 and HA-TBK1, suggesting that TRIM14 may interact with more than one component of the cytosolic DNA sensing pathway.

To further characterize this previously unappreciated association between TRIM14 and TBK1, we co-expressed mouse 3xFLAG-TRIM14 with mouse HA-cGAS,

HA-STING, HA-TBK1, and HA-IRF3 in HEK 293T cells, immunopurified each of the DNA sensing pathway components, and probed for interaction with TRIM14 by western blot. Consistent with our immunofluorescence microscopy data, we found that TRIM14 co-immunoprecipitated with both cGAS and TBK1 but not STING or IRF3 (Fig. 10C).

Next, to determine whether these biochemical associations interactions were direct, we performed surface plasmon resonance (SPR) experiments. Briefly, truncated versions of mouse TRIM14 (residues 247-440), human cGAS (residues 157-522), and mouse and human TBK1 (residues 11-657) were expressed using a baculoviral system. A portion of mouse IRF3 (residues 184-419) served as the negative control (Fig. 10D). Each of these protein truncations had previously been shown to be stably expressed at high levels and remain soluble when generated in insect cells (35-37). Equilibrium binding studies measured a binding affinity of 24.3 μM for binding between mTRIM14 and mTBK1 (Fig. 10E) and a slightly lower affinity of 42.6 μM for mouse TRIM14 and human TBK1 (Fig. 10F). We also measured a binding affinity of 25.8 μM between human cGAS and mouse TRIM14 (Fig. 17C). No binding was measured between mTRIM14 and mIRF3. Combined, these *in vivo* and *in vitro* biochemical data argue strongly for a direct, previously unreported interaction between TRIM14 and TBK1.

Loss of TRIM14 leads to type I IFN and ISG hyper induction during* M. tuberculosis *infection

To investigate the contribution of TRIM14 to cytosolic DNA sensing outcomes during *M. tuberculosis* infection, we first tested how knockdown of *Trim14* affects *Ifnb* gene expression. *Trim14* knockdown (KD) macrophages were generated by transducing

RAW 264.7 cells with lentiviral shRNA constructs designed to target the 3'UTR of *Trim14* or a control scramble shRNA (SCR). RT-qPCR analysis confirmed ~50% and 70% knockdown of *Trim14* using two different shRNA constructs (KD1 and KD2 respectively) (Fig. 11A). *Trim14* KD and control RAW 264.7 cells were either infected with *M. tuberculosis* or transfected with ISD to directly engage cGAS and *Ifnb* transcripts were measured after 4 hours. In both experiments, we observed lower levels of *Ifnb* transcript induction in *Trim14* KD cell lines compared to the SCR control (Fig. 11B and 11C), supporting a role for TRIM14 in the DNA sensing pathway. Since residual levels of TRIM14 protein in knockdown cell lines could potentially complicate interpretation of phenotypes, we decided to generate *Trim14* knockouts (KO) using CRISPR-Cas9. Briefly, *Trim14*-specific guide RNAs (gRNAs) were designed to target *Trim14* exon 1 and a GFP-specific gRNA was designed as a negative control. Two clones with distinct frameshift mutations that each introduced a stop codon early in exon 1 were identified and chosen for subsequent experimentation (Fig. 11D). Knockout of *Trim14* was confirmed by western blot using an antibody against the endogenous protein and by anti-TRIM14 immunofluorescence of control and *Trim14* KO cells (Fig. 11E).

In order to test how genetic ablation of *Trim14* affects cytosolic DNA sensing, *Trim14* KO and control macrophages were infected with wild type *M. tuberculosis* and RNA was collected over a 24h time-course of infection. Surprisingly, while we again measured lower *Ifnb* expression at 4h post-infection, we observed a dramatic hyper induction of *Ifnb* in the absence of TRIM14 at later infection time points (Fig. 11F). To determine the contribution of cytosolic DNA sensing to this phenotype, we transfected

Trim14 KO and control RAW 264.7 cells with ISD and again found significantly higher induction of *Ifnb* in the absence of TRIM14 at 8 hours and 24 hours post-transfection (Fig. 11G). To verify that the transcript changes we observed translated to differences in protein levels, we used Interferon Stimulated Response Element (ISRE) luciferase reporter cells to analyze IFN-β protein secretion in supernatants from cells 8- and 24-hours post-*M. tuberculosis* infection. Using relative light units as a proxy for IFN-β, we again observed hyper induction of IFN-β in the absence of TRIM14 (Fig. 11H). These data suggest that the major phenotype associated with *Trim14* ablation in RAW 264.7 macrophages is loss of negative regulation following cytosolic DNA sensing.

Having observed higher *Ifnb* transcript and protein levels in *Trim14* KO RAW 264.7 cells, we predicted that these cells would also hyper induce ISGs following treatment with innate immune agonists that stimulate IRF3 signaling downstream of cGAS or STAT signaling downstream of the Interferon α/β receptor (IFNAR). RT-qPCR analysis of RNA recovered over a time-course of either *M. tuberculosis* infection or ISD transfection showed hyper induction of *Ifit1*, *Isg15*, and *Irf7* (Fig. 12A and 12B). Likewise, high levels of *Ifit1*, *Isg15*, and *Irf7* were observed when cells were transfected with 1μg poly (I:C), a potent agonist of RNA sensing via RIG-I (Fig. 12C) or treated with recombinant IFNβ directly (Fig. 12D). Collectively, these results demonstrate that TRIM14 is needed for induction of *Ifnb*/ISGs immediately following innate immune sensing as well as for subsequent resolution of the response, arguing for a model whereby TRIM14 regulates cytosolic DNA sensing at two different nodes in the pathway.

TRIM14 regulates STAT3 activation through TBK1

Since we detected *in vivo* and *in vitro* interactions between TRIM14 and TBK1, we hypothesized that TRIM14-dependent misregulation of TBK1 activity could drive hyper induction of *Ifnb* and ISGs. TBK1 is a prolific innate immune serine/threonine kinase with many known targets(38, 39). One such target, STAT3 (Signal transducer and activator of transcription 3) has been repeatedly implicated in negatively regulating type I IFN responses (40, 41). Therefore, we set out to determine whether the presence of TRIM14 and its interaction with TBK1 was required to control STAT3 activity in macrophages.

Previous studies have demonstrated that TBK1 can directly phosphorylate STAT3 at Ser727 and Ser754 upon cytosolic DNA sensing (45) (Fig. 13A). Phosphorylation of STAT3 at Ser754 inhibits STAT3's ability to interact with target genes, while phosphorylation of STAT3 at Ser727 promotes STAT3 activity and transcription of STAT3 targets (42). To determine whether TRIM14 influences STAT3 phosphorylation, we transfected control and *Trim14* KO cells with ISD to activate TBK1 and analyzed STAT3 phosphorylation at Ser727 and Ser754 by immunoblot over a time-course. We observed substantially more phospho-Ser754 STAT3 and significantly less phospho-Ser727 STAT3 in the absence of TRIM14 (Fig. 13B), while loss of TRIM14 had no effect on JAK tyrosine kinase phosphorylation of STAT1 at Y701. These data suggest a role for TRIM14 in influencing TBK1's preference to phosphorylate particular serine residues in the transactivation domain of STAT3. To further implicate TRIM14 in mediating STAT3 activation by TBK1, we performed cellular fractionation experiments and measured the

amount of STAT3 in the nucleus following ISD transfection in *Trim14* KO and control cells by immunoblot. As expected, we observed significantly less STAT3 accumulation in the nuclei of *Trim14* KO cells (Fig. 13C).

We next predicted that TRIM14 can control TBK1's ability to phosphorylate STAT3 by interacting with both factors and bringing them together in a conformation that promotes phosphorylation at STAT3 S727 while inhibiting phosphorylation at S754.

We reasoned that since loss of TRIM14 caused hyper induction of ISGs via a TBK1/STAT3 dependent mechanism, then loss of either TBK1 or STAT3 would phenocopy loss of TRIM14. Indeed, previous studies have shown that *Stat3* KO MEFs and BMDMS hyper induce ISGs following viral infection (40). To test whether loss of TBK1 could also lead to ISG hyper induction, we harvested BMDMs from *Tbk1*$^{-/-}$/*Tnfr*$^{-/-}$ mice (44, 45) and treated them with recombinant IFNβ to directly engage with IFNAR and bypass the need for TBK1 to phosphorylate IRF3 and promote *Ifnb* expression (46, 47). Remarkably, we measured dramatic hyper induction of ISGs in *Tbk1*$^{-/-}$/*Tnfr*$^{-/-}$ BMDMs over a six-hour time course of IFNβ treatment (Fig. 13F). This result argues that TBK1 plays a crucial, yet mostly unappreciated, role in diminishing the type I IFN response downstream of IFNAR signaling and supports a model whereby TRIM14 downregulates type I IFN gene expression via TBK1-dependent phosphorylation of STAT3.

TRIM14 is required for STAT3-dependent transcription of Socs3, a negative regulator of the type I IFN response

Because an uncontrolled type I IFN response is deleterious to the host, cells have evolved multiple mechanisms to dampen type I IFN gene expression following pathogen

sensing or IFNAR activation. The hyper induction of *Ifnb* and ISGs we measure in *Trim14* KO macrophages is consistent with a loss of negative regulation; therefore, we hypothesized that expression of one or more negative regulators would be lower in *Trim14* KO cells. Inhibition of JAK1-STAT signaling is a well-characterized mechanism through which type I IFN signaling is downregulated (48). SOCS (Suppressor of cytokine signaling) family proteins are ISGs that dampen type I IFN responses by interfering with JAK1 kinase activity and limiting STAT signaling (41). USP18 (Ubiquitin Specific Peptidase 18) has similarly been shown to inhibit type I IFN expression by blocking interaction between JAK1 and the IFNAR2 subunit (49). To test whether *Trim14* KO cells were defective in expressing negative regulators of type I IFN, we measured *Socs3*, *Socs1*, and *Usp18* transcripts in control and *Trim14* KO RAW 264.7 cells infected with *M. tuberculosis* or transfected with ISD. Similar to all ISGs we examined in these studies, *Socs1* and *Usp18* were hyper induced in *Trim14* KO macrophages. However, we observed a specific defect in *Socs3* induction, suggesting that one or more *Socs3* transcription factors were impacted by loss of TRIM14 (Figure 14A and 14B).

Previous reports have shown that *Socs3* is a major target gene of STAT3 (50, 51). Having measured increased phosphorylation at the "inhibitory" Ser754 residue of STAT3 in *Trim14* KO macrophages, we hypothesized that lack of *Socs3* induction could be due to the inability of phospho-Ser754 STAT3 to bind at the *Socs3* promoter. To test this, we transfected control and *Trim14* KO cells with ISD and performed chromatin immunoprecipitation (ChIP) at 0h, 1h, and 6h following treatment using antibody directed against total STAT3 protein. Consistent with low *Socs3* transcription, we detected

significantly less recruitment of STAT3 to the *Socs3* genomic locus at both 1h and 6h after ISD transfection (Fig. 14D). We also detected lower STAT3 recruitment to other non-ISG target genes, including *Bcl3* and *Cxcl9* (Fig. 14D and 21), suggesting that loss of TRIM14 broadly impacts STAT3's ability to translocate to the nucleus and/or associate with DNA. From these data, we conclude that defective nuclear translocation of STAT3 and subsequent lack of *Socs3* transcriptional activation result in ISG hyper induction in the absence of TRIM14.

Loss of TRIM14 impacts the ability of macrophages to control infection

Having demonstrated an important role for TRIM14 in regulating the type I IFN response, we set out to investigate how loss of TRIM14 impacts cell-autonomous innate immune responses to viral and intracellular bacterial infection. To test how loss of TRIM14 impacts survival and replication of *M. tuberculosis*, we infected control and *Trim14* KO macrophages with M. *tuberculosis* expressing the *luxBCADE* operon from *Vibrio harveyi* (52) and bacterial replication was quantified as a measure of luminescence over a 72 hour time course (52, 53). Remarkably, we observed a dramatic inhibition of *M. tuberculosis* replication in *Trim14* KO macrophages (Figure 15A). Importantly, this lack of *M. tuberculosis* replication did not correspond to loss of cells, as infected monolayers remained completely intact at the 72h time point (Fig. 22). We also observed a significant inhibition of *M. tuberculosis* replication in *Trim14* KO RAW264.7 cells by enumerating colony forming units (CFUs) (Fig. 15B). To begin to identify the molecular mechanisms driving *M. tuberculosis* restriction in *Trim14* KO macrophages, we measured expression of several genes whose proteins have been purported to have direct bactericidal activity

against *M. tuberculosis* (54).(55, 56). RT-qPCR revealed hyper induction of inducible nitric oxide (*Inos2*) and guanylate binding proteins 1 and 5 (*Gbp1* and *Gbp5*) in *M. tuberculosis*-infected *Trim14* KO macrophages (Fig. 15C). We predict that the overabundance of one or more of these factors contributes to enhanced control of *M. tuberculosis* replication in the absence of TRIM14.

Because another report demonstrated that loss of TRIM14 leads to hyper replication of VSV, an enveloped RNA virus, we also infected TRIM14 KO and control RAW264.7 cells with VSV and followed viral replication and ISG expression by RT-qPCR over a 12 hour time-course. Although uptake of virus, as inferred by viral genome measurements at the 1h time point was very similar between the two genotypes, we observed a dramatic hyper replication of VSV in *Trim14* KO macrophages (Fig. 22B) concomitant with significantly higher *Ifnb* and ISG expression (Figure 22D). Although hyper induction of ISGs is seemingly at odds with hyper replication of VSV, we do consistently observe low basal levels of *Ifnb* and ISGs that in resting macrophages lacking TRIM14 (likely due to cGAS instability (27)), which may give the virus enough time to "take off" before TRIM14-dependent resolution of type I IFN responses kicks in.

Discussion

To prevent chronic inflammation and damage to host cells and tissues, potent innate immune responses like type I IFN induction require tight temporal control. Here, we demonstrate a previously unappreciated role for TRIM14 in resolving *Ifnb* and ISG expression following a variety of cytosolic innate immune stimuli. By providing evidence that TRIM14 can directly interact with both cGAS and TBK1, our work uncovers a

complex mechanism through which TRIM14 can both up and downregulate type I IFN responses in macrophages. Notably, we report that loss of TRIM14 has significant consequences on cell autonomous control of both bacterial and viral replication, with dramatic restriction of *M. tuberculosis* replication and uncontrolled replication of VSV observed in *Trim14* KO macrophages (Fig. 15A-B and 22). These results reveal a crucial role for TRIM14 in regulating macrophage innate immunity and point to TRIMs as potential targets for host-directed therapies designed to enhance a macrophage's antimicrobial repertoire.

Our data support a model whereby TRIM14 acts as a scaffold between TBK1 and STAT3, promoting TBK1-dependent phosphorylation of STAT3 at Ser727 and transcriptional activation of negative regulators of JAK/STAT signaling like SOCS3 (Figure 15D). There is mounting evidence that a complex network of post-translational modifications regulates STAT3's ability to dimerize, translocate to the nucleus, and/or bind DNA (41). In addition to inhibitory and activating STAT3 phosphorylation at Ser754 and Ser727 (42), respectively, several other modifications are known to control STAT3 activity, including acetylation at lysine 685 and phosphorylation of tyrosine 705, both of which increase the protein's ability to bind DNA and translocate to the nucleus (57, 58). We propose that in the context of DNA sensing, TBK1-dependent phosphorylation of STAT3 acts as a control point for ramping up or down the STAT3 transcriptional regulon and the presence of TRIM14 can tip this balance. It is curious that these two modifications (Ser727 and Ser754) have dramatic opposing effects on STAT3 activity, as both residues reside in the transactivation domain in close proximity. Structural studies will be needed

to shed light on how modulation of TBK1/STAT3 interactions by TRIM14 promote Ser727 phosphorylation over Ser754 phosphorylation. It is possible that the presence of TRIM14 makes one site more accessible either directly through interactions with STAT3 or by modulating interactions with other binding partners that influence availability of one serine over the others.

The apparent reliance of *Socs3* on STAT3 for its activation in our RAW 264.7 cells is also perplexing. In addition to being expressed by STAT3, depending on the cell type and context, *Socs3* can be transcribed by STAT1 and its promoter also contains AP-1, Sp3, and NFκB binding elements(59-61). The fact that these remaining transcription factors do not compensate for loss of STAT3 nuclear translocation in Trim14 KO macrophages (Fig. 13B) hints at the potential for crosstalk between STAT3 and other transcription factors, consistent with previous reports (62-64). The extent to which the entire STAT3-transcriptional regulon is impacted by loss of TRIM14 also remains unclear. Furthermore, following STAT3 expression of SOCS3, SOCS3 can actually downregulate STAT3 via a negative feedback loop (65, 66); future experiments will need to determine precisely how this loop is broken in *Trim14* KO macrophages. As STAT3 and SOCS3 are hugely important not only for controlling inflammatory responses during infection but also for regulating embryogenesis, cancer metastasis, and apoptosis, there is a critical need to understanding how TRIM14 can regulate their activation (67, 68).

Another recent study also shows a requirement for TRIM14 in VSV replication but reported that *TRIM14* KO macrophages had lower ISG expression compared to wild-type (27). The authors ascribed these phenotypes to TRIM14's role in promoting cGAS

stabilization and provide evidence that loss of TRIM14 allows for cGAS degradation via the E3 ligase USP14 that targets cGAS to p62-dependent selective autophagy. We also observed lower *Ifnb* in response to *M. tuberculosis* and ISD transfection for our earliest measurements (4 hours for *M. tuberculosis* infection; 2, 4, and 6 hours for ISD transfection) (Fig. 11B, F, and G), but the phenotype of *Trim14* KO macrophages shifts to hyper induction at later time points. It is not entirely clear what accounts for the discrepancies in our data, although notably, our analysis focuses almost exclusively on early time points following viral infection or innate immune activation (1-12 hours) as opposed to the 12-24 hours Chen *et al.* focused on during which cell death resulting from high viral titers may complicate measurement and interpretation of transcript abundance. Taking the conclusions from both studies into account, it seems likely that TRIM14 plays a dual role in type I IFN regulation whereby it interacts with cGAS to promote type I IFN expression and with TBK1/STAT3 to dampen it (Fig. 15D). Future work will need to investigate the precise spatiotemporal distribution of cGAS/TRIM14- and TBK1/TRIM14/STAT3-containing complexes over the course of type I IFN induction and resolution. It will also be important to investigate how and when TRIM14 itself is post-translationally modified. Recent work from Jia *et al*., provides evidence for RNF125-mediated polyubiquitination and proteasomal degradation of a mitochondrially-associated population of TRIM14 during viral infection (69). This post-translational modification and others could be critical for controlling whether TRIM14 influences type I IFN responses at the level of cGAS or TBK1/STAT3 or both.

Our finding that *Trim14* KO macrophages are better at controlling *M. tuberculosis* replication is quite surprising. As *M. tuberculosis* replicates very slowly (~24 hour doubling time), we propose that unlike VSV, whose replication can be influenced by low resting ISGs in *Trim14* KO cells, *M. tuberculosis* replication is restricted by hyper induction of ISGs that dominate after 4 hours of infection. It is unlikely that TRIM14's contribution to cGAS stability accounts *M. tuberculosis* restriction in *Trim14* KO macrophages, as our previous work showed that knocking out cGAS actually renders macrophages more permissive to *M. tuberculosis* infection, likely through loss of selective autophagy downstream of cytosolic DNA sensing (3). Consistent with these data and our model, another group has reported that siRNA knockdown of STAT3 in human macrophages enhances nitric oxide synthesis and restricts *M. tuberculosis* replication (70), although it is possible that TRIM14 also contributes to *M. tuberculosis* restriction through more direct mechanisms as well. Curiously, in the context of *Listeria monocytogenes* infection of STAT1-deficient fibroblasts, overexpression of TRIM14 was protective, suggesting TRIM14 may have ISG-independent antibacterial functions as well (71). Future experiments designed to investigate what proteins TRIM14 interacts with on *M. tuberculosis* phagosome and how loss of TRIM14 impacts maturation of the autophagosome will provide important insights into how TRIM14 controls *M. tuberculosis* replication and shed light on how we may be able to manipulate TRIM14 as a tuberculosis therapeutic.

CHAPTER III

TRIM14 REGULATION

Overview

TRIM14 influences nucleic acid sensing pathways and the innate immune response in a number of ways. In short, it contains a c-terminal PRY/SPRY domain that allows it to participate in several different protein-protein interactions. These interactions allow TRIM14 to indirectly induce the immune response at various levels. For example, TRIM14 stabilizes the nucleic acid sensor cGAS which results in the upregulation of the type I IFN response [138]. Prolonged activation of the immune response is detrimental to the host as it leads to chronic inflammation and autoinflammatory diseases such as lupus and psoriasis. Therefore, it is tightly controlled through the presence of negative feedback inhibition and other negative regulators of the response. Since TRIM14 is such an instrumental protein in the induction of this response, it is likely that layers of regulation also exist on TRIM14 itself. As previously stated, TRIM14 is differentially phosphorylated and ubiquitinated upon bacterial infection and encodes spliced isoforms[144]. Here, I investigate the possible regulation of TRIM14 through these post translational modifications and the presence of several different isoforms.

Modification of TRIM14

TRIM14 isoform

TRIM14 has several annotated mRNA coding sequences that encode for various spliced isoforms, most of which are predicted to be targeted to nonsense mediated decay and result in no protein. In order to investigate these possible isoforms in the context of

Mtb infection, we infected BMDMS with wild type Mtb and sent RNA for sequencing. RNA sequencing analysis revealed the abundance of each TRIM14 isoform that is alternatively spliced at rest and during infection. TRIM14 is primarily spliced into three different isoforms and the abundance of these changes upon Mtb infection (Figure 22). Interestingly, only two of these isoforms, TRIM14 and TRIM14 202, exist as proteins in the cell. The third major isoform that was found, TRIM14 205, results in a processed transcript but no protein. TRIM14 205 could possibly be a mechanism of regulating the amount of TRIM14 protein that exists in the cell, however this remains to be investigated. TRIM14 202 encodes a spliced isoform that is missing exon 6 and results in a truncated protein that is completely missing the c-terminal PRY/SPRY domain (Figure 7). The RNA sequencing results indicated that the abundance of TRIM14 202 goes up during Mtb infection at 4 hours. Next, I wanted to look at the dynamics of TRIM14 vs TRIM14 202 expression over a time course of Mtb infection. I did this by infecting RAW 264.7 macrophages with Mtb and collected RNA to be used for RT-qPCR analysis. In addition, I designed qPCR primers specific to TRIM14 exon 1 (total TRIM14), TRIM14 exon 6 (full length TRIM14) and TRIM14 202. I found that TRIM14 expression is induced overall, as expected (Figure 23). Interestingly, full length TRIM14 expression levels off at 8 and 24 hours post infection, while TRIM14 202 continues to increase and surpasses full length TRIM14 expression by 24 hours post infection (Figure 23). Taking these results together, I began to speculate that TRIM14 splicing is induced during Mtb infection to produce more TRIM14 202 that can ultimately restrict full length TRIM14. In addition to Mtb infection, I also wanted to explore if this phenotype occurred during DNA stimulation

or if it was specific to infection. I stimulated RAW 264.7 cells with ISD, collected RNA, and analyzed it by RT-qPCR as previously described. I found that TRIM14 202 expression is not significantly induced and expression is almost exclusively full length TRIM14 during DNA stimulation (Figure 24). This result was intriguing as normally DNA stimulation is a good proxy for Mtb infection and transcript expression is similar [36]. In order to determine the function of TRIM14 202 and get a better idea of what could be going on during Mtb infect, I took a step back and examined protein-protein interactions of TRIM14 and TRIM14 202.

Several TRIMs have been shown to be self-associated in the form of dimers or oligomerize to form higher order complexes with themselves or other TRIMs. This association has often been facilitated through the coiled coil domain [92, 109]. Although TRIM14 202 lacks the PRY/SPRY domain which is thought to facilitate most of the protein-protein interactions of TRIM14, it is very plausible that other interactions could be taking place through the B-box or coiled coil domains as these are the same for both proteins. I wanted to interrogate the idea that TRIM14 could interact with itself and possibly its isoform TRIM14 202 which could ultimately modulate how it functions in the cell. I cloned N-terminal tagged versions of both full length TRIM14 and TRIM14 202. First, I co-expressed HA TRIM14 with 3xFlag TRIM14 in 293T cells and performed flag immunoprecipitation (IP) and used western blot analysis to probe for both tagged versions of TRIM14. My negative control was 3xFlag GFP, as it should not be in a complex with anything in the cell. I found that TRIM14 can immunoprecipitate with itself (Figure 25). In addition, I repeated this experiment but instead co-expressed 2xStrep TRIM14 with

3xFlag TRIM14 202. Western blot analysis shows that TRIM14 202 also immunoprecipitated with TRIM14 (Figure 26). These results indicate that TRIM14 can be in a complex with itself and/or the isoform TRIM14 202. In light of these findings, I also sought to test whether TRIM14 202 protein interacts with TBK1. This would further confirm previous SPR experiments in which the PRY/SPRY domain of TRIM14 was the only piece required for interaction with TBK1. I cloned N-terminal tagged 2xStrep TBK1 and co-expressed it with either full length 3xFlag TRIM14 or 3xFlag TRIM14 202. As previously described, I performed Strep immunoprecipitation (IP) to pull down TBK1 and used western blot analysis to probe for anti-Strep and anti-Flag (Figure 27). As seen by Figure 27, TRIM14 is in a complex with TBK1 but TRIM14 202 is not. I am confident in the requirement of the PRY/SPRY domain for the interaction of TRIM14 and TBK1. However, I was intrigued by the thought that TRIM14 might have a preference to bind TRIM14 202 over TBK1. In order to test this, I repeated this co-immunoprecipitation experiment except this time I added in increasing amounts of TRIM14 202 to see if this might change the outcome of the interaction. I found that TRIM14 interacted with TBK1 no matter the amount of TRIM14 202 that was also present in the cell (Figure 28).

In addition to what complexes these isoforms might be forming, I also wanted to investigate if TRIM14 202 localizes differently in the cell than full length TRIM14. I performed immunofluorescence by overexpressing 3xFlag TRIM14 or 3xFlag TRIM14 202 in MEF cells and stained for the Flag tag. The localization patterns of TRIM14 and TRIM14 202 are quite different. TRIM14 appears as distinct puncta around one side of the nucleus in the cell, while TRIM14 202 appears scattered in smaller puncta throughout

the cytoplasm (Figure 29). In addition, I co-expressed 3xFlag TRIM14 202 with 2xStrep TRIM14 to investigate if co-localization occurs. Somewhat to my surprise, I found TRIM14 and TRIM14 202 do not co-localize together even though they able to form a complex together as seen by the IPs (Figure 26 & 30).

It is difficult to say what role each of these isoforms might be playing in the cell when they always exist together. In order to further interrogated the possible role of each isoform independently of each other, I stably expressed 3xFlag TRIM14 or 3xFLag TRIM14 202 in my *Trim14* KO macrophages I had previously made, described in chapter 2 (Figure 11). My main interest is how these isoforms might be involved in the context of the type I IFN response. Therefore, I plan to stimulate these stably expressing cells with ISD or IFNβ over a time course and used RT-qPCR of relevant transcripts as my read out of the response. In addition, I also plan to infect these cells with Mtb and see if there is a difference in response when different isoforms are present.

TRIM14 PTM

TRIM14 is not only alternatively spliced, it is also post-translationally modified through addition of ubiquitin or phosphoryl groups on different amino acid residues in response to bacterial infection (Figure 7). To determine if any of these residues are essential for TRIM14 function, we used site-directed mutagenesis to change each amino acid residue that is modified specifically during bacterial infection. Each of the Lysine residues where changed to an Arginine so it is no longer able to be ubiquitinated at that site. The phosphorylation site of TRIM14 at Serine 105 was mutated two different ways either from Serine to an Alanine to inhibit phosphorylation or Serine to Aspartate to mimic

phosphorylation. I stably expressed each of these mutants in RAW 264.7 cells and stimulated 3 of these ubiquitin mutants (K151R, K244R, and K262R) with ISD to look at how they respond in the context of the DNA sensing response. My control was RAW 264.7 cells stably overexpressing non-mutated TRIM14. I specifically chose these three to work with first because each of these Lysines is within a different domain of TRIM14. I speculated that each of these Lysines could be doing something different, and therefore all three might not be essential and I would see different response in at least one of them. I used RT-qPCR of *Ifnb* as my read out and found that overexpression of TRIM14 alone caused massive induction *Ifnb* (Figure 32). In comparison, all the TRIM14 mutants had significantly less *Ifnb* induction than wildtype TRIM14, suggesting each lysine is individually important to TRIM14s function (Figure 32). This was surprising to me, as I expected some redundancy in the system that would allow for function of TRIM14 with a single mutation. However, these were Lysine were chosen due to their ubiquitination during bacterial infection, so it would make sense that these sites would be essential during the immune response. In addition, I also treated this group with IFNβ to specifically test the IFNAR side of the response. Similarly, I found there was less induction of ISGS *Irf7* and *ifit1* in response to IFNβ as compared to wildtype (Figure 33). In the future I would like to infect the rest of the Trim14 mutant stable cell lines with Mtb and compare how they respond using RT-qPCR of type I IFN transcripts. I also want to test whether mutation of these residues affects TRIM14s ability to interact with TBK1. I would do this by using co-immunoprecipitation experiments in which I expressed 2xStrep TBK1 with each 3xFlag TRIM14 mutant. Overall, these results begin to suggest that TRIM14 is regulated

through splicing and post translational modifications as a way to regulate its role in the cell.

CHAPTER IV

MATERIAL AND METHODS

Cell Culture

RAW 264.7 macrophages, HEK293T, MEF, and LentiX cells were cultured at 37°C with 5% CO_2. Cell culture medium was comprised of High glucose, sodium pyruvate, Dulbecco's modified Eagle medium (Thermo Fisher) with 10% FBS (Sigma Aldrich) 0.5% HEPES (Thermo Fisher).

Co-immunoprecipitations

1.8 x 10^6 HEK293T cells in a 10cm plate were transfected with 1-10 µg of pDEST 3xFlag TRIM14, pDEST HA cGAS, pDEST HA STING, pDEST HA TBK1, pDEST HA IRF3, pDEST Flag STAT3, or pDEST HA TRIM14 using PolyJet In Vitro DNA Transfection Reagent. Cells were harvested in PBS+0.5M EDTA 24 hours post-transfection and pellets were lysed on ice with lysis buffer (50 mM Tris HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.075% NP-40) containing protease inhibitor (Pierce A32955). Strep-tactin superflow plus beads (Qiagen) were washed using buffer containing 5% 1M Tris at pH 7.4, 3% NaCl, and 0.2% 0.5M EDTA. 1000 µl of the cleared lysate was added to the beads and inverted for 2 hr at 4°C. Beads were then washed 4 times with wash buffer (50 mM Tris HCl pH 7.4 150 mM NaCl 0.5M EDTA, 0.05% NP-40) and eluted using 1x Biotin. Whole cell lysate inputs and elutions were boiled in 4x SDS loading buffer with 10% 2-mercapethanol. Proteins were run on SDS-PAGE gels (Bio-Rad) and then transferred to nitrocellulose membrane (GE Healthcare). Membranes were blocked in TBS (Odyssey Blocking buffer Li-COR) for 1 hour and incubated with primary antibody

overnight at 4°C. LI-COR secondary was used (IR Dye CW 680 goat anti-rabbit, IR Dye CW 680 goat anti-rat 680, IR Dye CW800 goat anti-mouse (LI-COR)) and developed with the Odyssey Fc by LI-COR. Immunoprecipitation experiment were also performed as stated above but with Pierce Anti-HA agarose (Thermo 26181). Beads were eluted three times at room temperature for 15 min each using Influenza Hemagglutinin (HA) peptide (Sigma Aldrich I2149).

Western Blot analysis

Protein samples were run on Any kD Mini-PROTEAN TGX precast protein gels (BioRad) and transferred to 0.45 μm nitrocellulose membranes (GE Healthcare). Membranes were incubated in the primary antibody of interest overnight and washed three times with TBS-Tween 20. Membranes were then incubated in secondary antibody for 1 hour and imaged using LI-COR Odyssey FC Imaging System. Primary antibodies used in this study: mouse monoclonal α-FLAG M2 antibody (Sigma-Aldrich, F3165), α-HA high affinity rat monoclonal antibody (Roche; 3F10), α-strep (Genscript A00626), α-phospho-Stat3 (Ser727) (Cell Signaling #9134), α-phospho-Stat3 (Ser754) (Cell Signaling #98543), α-Stat3 (124H6) Mouse mAb (Cell Signaling #9139), α-phospho-Stat1 (Tyr701) (58D6) Rabbit mAb #9167, α-TRIM14 G-15 (Santa Cruz sc79761), α-TRIM14 (Aviva ARP34737), and α-mouse monoclonal Beta-Actin (Abcam, #6276). Secondary antibodies used in this study: IR Dye CW 680 goat anti-rabbit, IR Dye CW 680 goat anti-rat 680, IR Dye CW800 goat anti-mouse (LI-COR), Alexfluor-488 anti-rabbit, Alexfluor-597 anti-rat, and Alexafluo-647 anti-mouse secondary antibodies for immunofluorescence (LI-COR).

Construction of sgRNA/Cas9 LentiCRISPR and viral transduction

Guide RNAs targeting the first exon of Trim14 were designed using the Broad online tool (https://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design). The top five hits were used to design five guide RNA constructs that were cloned into Lenti CRISPR vector (Puromycin) at the BsmB1 site. Constructs were sequenced and verified. Plasmids were then transfected into Lenti-X cells with PAX2 and VSVG packing plasmids. Virus was collected 24 and 48 hours post-transfection and stored at -80°C. RAW264.7 cells stably expressing Cas9 were then transduced with virus and selected using puromycin for 72 hours. Cells were then clonally selected using serial dilutions and clones were selected from wells calculated to contain a single cell. To verify the knockout, genomic DNA was collected from cells and the first exon of TRIM14 was amplified using PCR. This reaction was sent for sequencing to verify the mutation.

Generation of shRNA-expressing stable cell lines

To generate knockdown RAW 264.7 macrophages, plasmids of scramble non-targeting shRNA constructs and TRIM14 shRNA constructs targeted towards the 3' UTR of TRIM14 were transfected into Lenti-X cells with PAX2 and VSVG packing plasmids. Virus was collected 24 and 48 hours post-transfection and stored at -80°C. RAW264.7 cells were then transduced with virus and selected using hygromycin at 10mg/ml (Invitrogen) to select for cells containing the shRNA plasmid.

Macrophage stimulation

RAW 264.7, CRISPR/Cas9 RAW 264.7, or shRNA RAW 264.7 macrophages were plated on 12-well tissue-culture treated plates at a density of 3 $\times 10^5$ cells/well and allowed to grow overnight. Cells were then transfected with 1 μg/ml ISD or 1 μg/ml poly(I:C) with lipofectamine or treated with 200units recombinant mouse IFNB (pbl assay science Cat#12400-1).

***M. tuberculosis* Infection**

Low passaged lab stocks of each Mtb strain (Erdman strain WT, Erdman *luxBCADE*, or Erdman m-Cherry) were thawed for each experiment to ensure virulence was preserved. *M. tuberculosis* was cultured in roller bottles at 37°C in Middlebrook 7H9 broth (BD Biosciences) supplemented with 10% OADC, 0.5% glycerol, and 0.1% Tween-80. All work with Mtb was performed under Biosafety Level 3 (BSL3) containment using procedures approved by the Texas A&M University Institutional Biosafety Committee. To prepare the inoculum, bacteria grown to log phase (OD 0.6-0.8) were spun at low speed (500g) to remove clumps and then pelleted and washed with PBS twice. Resuspended bacteria were briefly sonicated and spun at low speed once again to further remove clumps. The bacteria were diluted in DMEM + 10% horse serum and added to cells at an MOI of 10 for RNA and cytokine analysis and MOI of 1 for microscopy studies. Cells were spun with bacteria for 10 min at 1000 x g to synchronize infection, washed twice with PBS, and then incubated in fresh media. RAW 264.7 or CRISPR/Cas9 RAW 264.7 macrophages were plated on 12-well tissue-culture treated plates at a density of 3×10^5 cells/well and

allowed to grow overnight. Where applicable, RNA was harvested from infected cells using 0.5 ml Trizol reagent at each time point.

***M. tuberculosis* survival/replication**

RAW 264.7 or CRISPR/Cas9 RAW 264.7 macrophages were plated on 12-well tissue-culture treated plates at a density of 2.5 x10^5 cells per well. Luminescence was read for Mtb *luxBCADE* by lysing in 400ul 0.5% Triton-X and splitting into two wells of a 96 well white plate and using the luminescence feature of the INFINITE 200 PRO by TECAN at 0, 24, 48, and 72 hours post infection.

RNA isolation and qPCR analysis

In order to analyze transcripts, cells were harvested in Trizol at the specified time points and RNA was isolated using Direct-zol RNA Miniprep kits (Zymo Research) with 1 hour DNase treatment. cDNA was synthesized with iScript cDNA Synthesis Kit (Bio-Rad). cDNA was diluted to 1:20 for each sample. A pool of cDNA from each treated or infected sample was used to make a 1:10 standard curve with each standard sample diluted 1:5 to produce a linear curve. RT-qPCR was performed using Power-Up SYBR Green Master Mix (Thermo Fisher) using a Quant Studio Flex 6 (Applied Biosystems). Samples were run in triplicate wells in a 96-well or 384 well plate. Averages of the raw values were normalized to average values for the same sample with the control gene, *beta-actin*. To analyze fold induction, the average of the treated sample was divided by the untreated control sample, which was set at 1.

Immunofluorescence Microscopy

Glass coverslips were incubated in 100µl poly-lysine at 37°C for 30 minutes. MEF cells were plated at a density of $2x10^4$ on glass coverslips in 24-well plates and left to grow overnight. Cells were then transfected with 250ng of the desired plasmid(s) using PolyJet. The next day cells were treated with 1µg ISD as described above. At the designated time points, cells were washed with PBS (Thermo Fisher) and then fixed in 4% paraformaldehyde for 10 minutes. Fixed cells were washed three times in PBS and permeabilized by incubating them in PBS containing 5% non-fat milk and 0.05% saponin (Calbiochem). Coverslips were placed in primary antibody for 1 hour then washed 3x in PBS and placed in secondary antibody. These were washed twice in PBS and twice in deionized water, followed by mounting onto a glass slide using ProLong Diamond antifade mountant (Invitrogen). Images were acquired on a Nikon A1-Confocal Microscope. DAPI nuclear staining (Thermo Fisher).

Co-localization experiments with *M. tuberculosis*

RAW 264.7 macrophages were plated on glass coverslips at a density of 3 $\times 10^5$ cells/well in 24-well plates. Cells were infected with m-Cherry *M. tuberculosis* at an MOI of 1 and fixed and stained as above at the designated time points. Colocalization of α-TRIM14 G-15 (Santa Cruz sc79761), with *M. tuberculosis* was visualized directly by fluorescence microscopy. A series of images were captured and analyzed by counting the number of bacteria that colocalized with the corresponding marker. At least one hundred events were analyzed per coverslip and each condition was performed with triplicate coverslips.

Protein Expression and Purification

The cDNA encoding mouse TRIM14 (residues 247 to 440), mouse IRF-3 (residues 184-419) were cloned into a modified pET28(a) vector containing an N-terminal Avi-His6-SUMO tag. Sequences of the plasmids were confirmed by DNA sequencing. The BL21 (DE3) cells were co-transformed with the pET28(a) plasmids coding for the target proteins and the pBirAcm plasmid coding for BirA and induced with 0.4 mM IPTG in the presence of 5 μg ml−1 biotin and cultured at 16 °C overnight. The Biotin-labelled-Avi-His6-SUMO proteins were purified using a nickel-NTA column followed by gel-filtration chromatography using a HiLoad 16/60 Superdex 75 column (GE Healthcare). Mouse and human TBK1 (residues 1 to 657) were cloned into the pAcGHLTc baculovirus transfer vector. The plasmid was transfected together with Baculo-Gold bright linearized baculovirus DNA (BD Biosciences) into sf9 insect cells to generate recombinant baculovirus. The original recombinant viruses were amplified for at least two rounds before the large-scale protein expression. The insect cells at a density of 2.5×10^6 cells/ml were infected by TBK1 recombinant baculovirus and cultured at 27°C and harvested 72 hours post infection. The cells were lysed in the buffer containing 150 mM NaCl, 0.2 M Tris-HCl, 1% NP-40, 1 mM PMSF at pH 8.0. The target protein in the supernatant was purified using nickel affinity chromatography followed by size-exclusion chromatography.

SPR Binding Study

The binding studies between mouse TRIM14 and TBK1 were performed using a Biacore X100 SPR instrument (GE Healthcare). Biotin-labeled SUMO-fusion TRIM14

was coupled on the sensor chip SA (GE Healthcare). Dilution series of TBK1 or IRF-3 (1.25, 2.5, 5, 10, 20 μM) in 1× HBS-EP+ buffer (GE Healthcare) were injected over the sensor chip at a flow rate of 30 μL/min. The single-cycle kinetic/affinity protocol was used in all binding studies. All measurements were duplicated under the same conditions. The equilibrium K_d was determined by fitting the data to a steady-state 1:1 binding model using Biacore X100 Evaluation software version 2.0 (GE Healthcare).

Chromatin Immunoprecipitation

Chromatin Immunoprecipitation (ChIP) was adapted from Abcam's protocol. Briefly, one confluent 15 cm dish of CRISPR/Cas9 RAW 264.7 macrophages were crosslinked in formaldehyde to a final concentration of 0.75% and rotated for 10 minutes. Glycine was added to stop the cross linking by shaking for 5 minutes at a concentration of 125 mM. Cells were rinsed with PBS twice and then scraped into 5 mL PBS and centrifuged at 1,000g for 5 min at 4C. Cellular pellets were resuspended in ChIP lysis buffer (50 mM HEPES-KOH pH7.5, 140 mM NaCl, 1 mM EDTA pH8, 1% Triton X-100, 0.1% Sodium Deoxycholate, 0.1% SDS Protease Inhibitors) (750 μL per 1×10^7 cells) and incubated for 10 min on ice. Cellular lysates were sonicated for 40 minutes (30sec ON, 30sec OFF) on high in a Bioruptor UCD-200 (Diagenode). After sonication, cellular debris was pelleted by centrifugation for 10 min, 4°C, 8,000 × g. Input samples were taken at this step and stored at −80°C until decrosslinking. Approximately 25 μg of DNA diluted to 1:10 with RIPA buffer was used for overnight immunoprecipitation. Each ChIP had one sample for the specific antibody and one sample for Protein G beads only which were pre-blocked for 1 hr with single

stranded herring sperm DNA (75 ng/μL) and BSA (0.1 μg/μL). The respective primary antibody was added to all samples except the beads-only sample at a concentration of 5 ug and rotated at 4°C overnight. Beads were washed 3x in with a final wash in high salt (500mM NaCl). DNA was eluted with elution buffer and rotated for 15 min at 30C. Centrifuge for 1 min at 2,000 × g and transfer the supernatant into a fresh tube. Supernatant was incubated in NaCl, RNase A (10 mg/mL) and proteinase K (20 mg/mL) and incubated at 65°C for 1 h. DNA was purified using phenol:chloroform extraction. DNA levels were measure by RT-qPCR. Primers were designed by tiling each respective gene every 500 base pairs that were inputted into NCBI primer design.

mRNA sequencing

RNA was sequenced from 4 biological replicates for each condition; Uninfected BMDMs, ESX-1-infected BMDMs, and M. tuberculosis-infected BMDMs. Raw reads were processed with expHTS (Street et al. 2015) to trim low-quality sequences and adapter contamination, and to remove PCR duplicates. Trimmed reads for each sample were aligned to the GRCm38 GENCODE primary genome assembly using STAR v.2.5.2b aligner (Dobin et al. 2013), and the GENCODE v.M10 annotation (gtf file). Each of the 4 replicates were merged into a single BAM file for further analysis. Prior to analysis, genes with expression less than 2 counts per million reads were filtered, leaving 11,808 genes. Differential gene expression was conducted using a single factor ANOVA model in the limma-voom Bioconductor pipeline. Log2 fold change values with a p-value <0.05 are represented in heatmaps where uninfected samples were the denominator and ESX-1 or

M.tuberculosis-infected samples were the numerator in their respective datasets. Heatmaps were generated with GraphPad Prism Software.

VSV infection

RAW 264.7 cells were seeded in 12-well plates at $8x10^5$ 16h before infection. Cells were infected with VSV-GFP virus at multiplicity of infection (MOI) of 1 in serum-free DMEM (HyClone SH30022.01). After 1h of incubation with media containing virus, supernatant was removed, and fresh DMEM plus 10% FBS was added to each well. At indicated times post infection, cells were harvested with Trizol and prepared for RNA isolation.

Statistics

Statistical analysis of data was performed using GraphPad Prism software (GraphPad). Two-tailed unpaired Student's t tests were used for statistical analyses, and unless otherwise noted, all results are representative of at least three independent biological experiments and are reported as the mean ± SD (n = 3 per group).

CHAPTER V

CONCLUSIONS

Taken together, my work has shown TRIM14 is an important regulator of the innate immune response to various stimuli, but more importantly the human pathogen *Mycobacterium tuberculosis*. In addition, I have also begun to explore regulation of TRIM14 itself which will be important to better understanding the dynamics of its interactions with several nodes of the innate immune response.

In Chapter 2, I discovered TRIM14 is a negative regulator of the type I IFN response during Mtb infection, as removal of TRIM14 resulted in sustained hyper induction of the type I IFN response. I proposed a model in which TRIM14 acts as a scaffold between TBK1 and STAT3 which ultimately promotes TBK1-dependent phosphorylation of STAT3 at Ser727 and transcriptional activation of negative regulators of JAK/STAT signaling like SOCS3. Although my data supports this model, the connections and mechanisms for how this occurs remain to be resolved. How exactly TRIM14 is modulating phosphorylation of STAT3 is a black box. I have not yet shown that TBK1, TRIM14, and STAT3 are all three in a complex together and this might be more of a transient event than a longstanding relationship. In order to interrogate this further it would be helpful to do immunoprecipitation assays with all three proteins. In addition, using a kinase dead TBK1 would give insight as to whether this interaction is also dependent on TBK1 activation. Furthermore, I have observed the interaction between STAT3 and TRIM14 specifically in macrophages is dependent on DNA

stimulation. Further exploration of the dynamics of this relationship under different innate immune agonists could be helpful as well.

There are several outstanding possibilities for how TRIM14 could be facilitating the phosphorylation of STAT3. TRIM14 could recruit STAT3 to TBK1 or vis versa. If TRIM14 is simply important in the recruitment of these proteins to each other, it could do so through modulating the protein-protein interactions of STAT3 and/or TBK1. It is also possible that the interaction of TRIM14 and STAT3 blocks Serine 754 and therefore promotes TBK1 phosphorylation at Serine 727 simply because it is more accessible. Structural studies of STAT3 and TRIM14 in a complex together would be helpful in resolving this question.

The fourth player in the TRIM14-STAT3 axis that is of notable importance during Mtb infection is SOCS3. My data strongly suggests *Socs3* expression during Mtb infection is dependent on STAT3 activation/phosphorylation. However, the lack of compensation in my TRIM14 cells is curious as other STAT proteins, such as STAT1 have also been shown to induce *Socs3* expression [184]. I would like to further explore the effect TRIM14 has on STAT3's entire transcriptional regulon as well as explore why STAT1 is not able to compensate for the loss of STAT3. A possible reason for lack of compensation is due to the important of STAT3's interactions and cross-talk with other transcription factors. For example, STAT3 and NFκB cooperate with each other in order to promote the development and progression of cancer [185]. In addition, STAT3 interacts with STAT1 in order to modulate and regulate inflammation [178]. One possibility is lower expression of these STAT3 genes in the absence of TRIM14 is due to

the lack of this important cross-talk. STAT3 also forms a heterodimer with STAT1 which prevents STAT1-dependent gene expression [178]. It is also possible that the high levels of pSTAT3 S754 in the absence of TRIM14 still form this dimer which is preventing STAT1 gene expression. As discussed in the introduction, *Socs3* expression is a negative feedback loop which regulates STAT3. How TRIM14 contributes to this regulation also remains to be interrogated. In addition to STAT3 and SOCS3 playing roles in controlling inflammatory responses during infection, they also plays several other roles in regulating embryogenesis, cancer metastasis, and apoptosis [175, 185]. Understanding how TRIM14 can regulate STAT3 activation will also be important to better understand these areas of research and develop possible treatments. Interestingly, TRIM14 also plays a role in malignant processes of several cancers such as breast and lung cancer [126, 186]. I hypothesize that the connection between STAT3, SOCS3, and TRIM14 goes beyond regulation of the immune response to infection and is important to general cell homeostasis and human health. It is quite possible that TRIM14 could be a good target for host-directed therapy against a number of diseases beyond Tuberculosis, including several types of cancer and autoinflammatory diseases.

Furthermore, I found removal of TRIM14 restricts Mtb replication in a macrophage. This was quite shocking as higher type I IFN and ISGS have historically conferred to worse infection outcomes. I further interrogated the mechanism for this replication defect and found that in addition to higher levels of ISGs, TRIM14 KO macrophages also have significantly more *Nos2* during Mtb infection. Nos2 is a key inflammatory agent that catalyzes the generation of nitric oxide which has ultimately been

shown to be important for the control of Mtb replication in macrophages as well as in vivo [187-189]. I provided strong evidence that this clearance phenotype was in fact through the uncontrolled expression of *Nos2* through my use of a Nos2 inhibitor during Mtb infection, which effectively restored Mtb replication and growth back to wildtype levels. Intriguingly, the Nos2 inhibitor had no effect on Mtb replication in wild type macrophages as seen previous studies in human macrophages [190, 191]. Mtb clearly has evolved ways to survive in the presence of Nos2 production which begs the question, is the amount of Nos2 the crucial factor in determining Mtb survival or is there more going on that is contributing the inhibition/control of Mtb replication. It will also be interesting to see if this phenotype holds up in an *in vivo* mouse infection model. I would expect that a TRIM14 KO mouse infected with Mtb would have higher levels of inflammation overall. I am curious to see if this would correlate to more or less control of Mtb infection due to our surprising phenotype of Mtb restriction in TRIM14 KO macrophages. Other studies have consistently shown higher inflammation leads to worse infection outcomes *in vivo* [23, 65, 66]. However, it seems there is more to the story than just inflammation being an important factor in prognosis. I speculate that Mtb prefers to induce inflammation through use of its effectors in an orchestrated manner that allows it to fully manipulate the timing of events that occur upon infection, as timing is critical. Mtb has been shown to induce early activation of STAT3 and SOCS3 which can control the innate immune response as well as suppress Nitric oxide induction [180, 181]. In addition, the timing and duration of IL-1 expression is extremely important in the context of Mtb infection as well. Several studies have shown expression of IL-1 early in infection is protective and IL-1 KO mice

are extremely susceptible to Mtb infection [78, 81, 192, 193]. However, prolonged IL-1 expression leads to increased neutrophil infiltration to the site of infection and exacerbates disease, as Mtb grows well inside neutrophils [194, 195]. IL-1 is also inhibited by the presence of IFNβ and NO [192]. I believe my work has just begun to scratch the surface of the complexities involved in the restriction of Mtb inside a macrophage. Further studies are most certainly needed to determine its impact on in vivo infection In order to further test the impact of inflammation on disease, I would take various autoimmune disease mouse models and infect them with wild type and ESX-1 mutant Mtb. This could give us a better idea of if pre-existing inflammation is better or worse at controlling Mtb infection. In addition, this could be informative on how the type of inflammation alters infection outcomes.

In addition to Nos2 production being elevated in my TRIM14 KO macrophages, I also observed significantly higher levels of guanylate-binding proteins (GBP)s. These have notoriously been associated with protection against intracellular pathogens and could be playing an additional role during Mtb infection [54]. I would like to investigate if there are GBPs on the Mtb containing phagosome during infection of a macrophage as they have been shown to coat other intracellular bacteria such as *Mycobacterium bovis* and *Shigella flexneri* [196, 197] . If this is the case, I would also follow up and test if there are more/less GBPs present upon removal of TRIM14 from the system, given the current data I would predict to see an increase. Despite GBPs ability to specifically coat or bind to the Mtb containing phagosome, they could certainly still be playing a crucial role in the control of Mtb replication through other mechanisms. GBPs also have been

proposed to contribute to bacterial control through vacuolar lysis, microbial escape to the cytosol, and activation of the inflammasome [54]. The direct connection of TRIM14 to these as well as the contribution of GBPs to the control of mtb replication is an obvious next step for further exploration.

As discussed in the introduction, selective autophagy is critical to control of Mtb replication in macrophages and TBK1 is a key kinase involved in this response in addition to just DNA sensing. It is quite plausible that TRIM14 is interacting with TBK1 in a way that upregulates the type I IFN response instead of autophagy. Removal of TRIM14 in a macrophage could enhance selective autophagy and be another explanation for the restriction of Mtb replication. I would like to test this by looking for the presence of TRIM14 at the Mtb-containing phagosome as well as those that are positive for autophagy markers such as LC3, ubiquitin, and autophagy specific adaptors. In addition, I would like to see if there is a difference in the targeting of Mtb to autophagosomes in the absence of TRIM14.

My work as well as another publication has established the interaction between TRIM14 and cGAS. However there is much about this relationship that remains to be interrogated. The authors propose a model in which TRIM14 inhibits USP14 ubiquitination of cGAS and therefore it no longer gets targeted for downstream degradation through p62-depedent selective autophagy. Removal of TRIM14 from their system found lower levels of *ifnb* transcript and IFNβ protein in response to viral infection from 12-24 hours post infection. I also observed lower *ifnb* in my TRIM14 KO macrophages at 4 hours post Mtb infection and 2,4,6 hours post DNA stimulation.

However, I see hyper-induction of *ifnb* and ISG transcripts at later time points. Interestingly, we both observed removal of TRIM14 leads to hyper replication of virus. Taking both my work and this study into account, I hypothesize that TRIM14 is interacting at two different nodes in the regulation of the type I IFN response. First, it helps promote the response through its interaction with cGAS and then later it is important for the repression of the response through its interaction with TBK1 and STAT3. Further studies on the dynamics of both of these TRIM14 complexes will be helpful in determining how TRIM14 is seemingly playing two opposing roles.

In addition to TRIM14s role(s) during Mtb infection, I believe a crucial avenue to explore is the regulation of TRIM14 itself. As discussed in Chapter 3, TRIM14 has a splice variant and several PTMs that occur during Mtb infection. I found that TRIM14 can interact with itself as well as its splice variant. It is possible that TRIM14 forms multimeric complexes with itself which preferentially facilitate certain interactions with binding partners over others. I also discovered several TRIM14 ubiquitin mutants fail to induce the IFN response as compared to overexpression of wild type TRIM14. Further investigation of why the ubiquitin modification at these sites is important could provide further evidence as to the mechanism of TRIM14s regulation on the immune response. In addition to my own work, Jia *et al*. provides evidence that RNF125 targets TRIM14 for proteasomal degradation via polyubiquitination during viral infection (69). Overall, my data and others have only begun to scratch the surface of TRIM14 regulation. Nonetheless, I believe understanding the significance of this level of regulation on

TRIM14 itself will help provide clues into the many roles TRIM14 has in controlling the innate immune response at the level of cGAS or TBK1/STAT3 or both.

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APPENDIX A

FIGURES

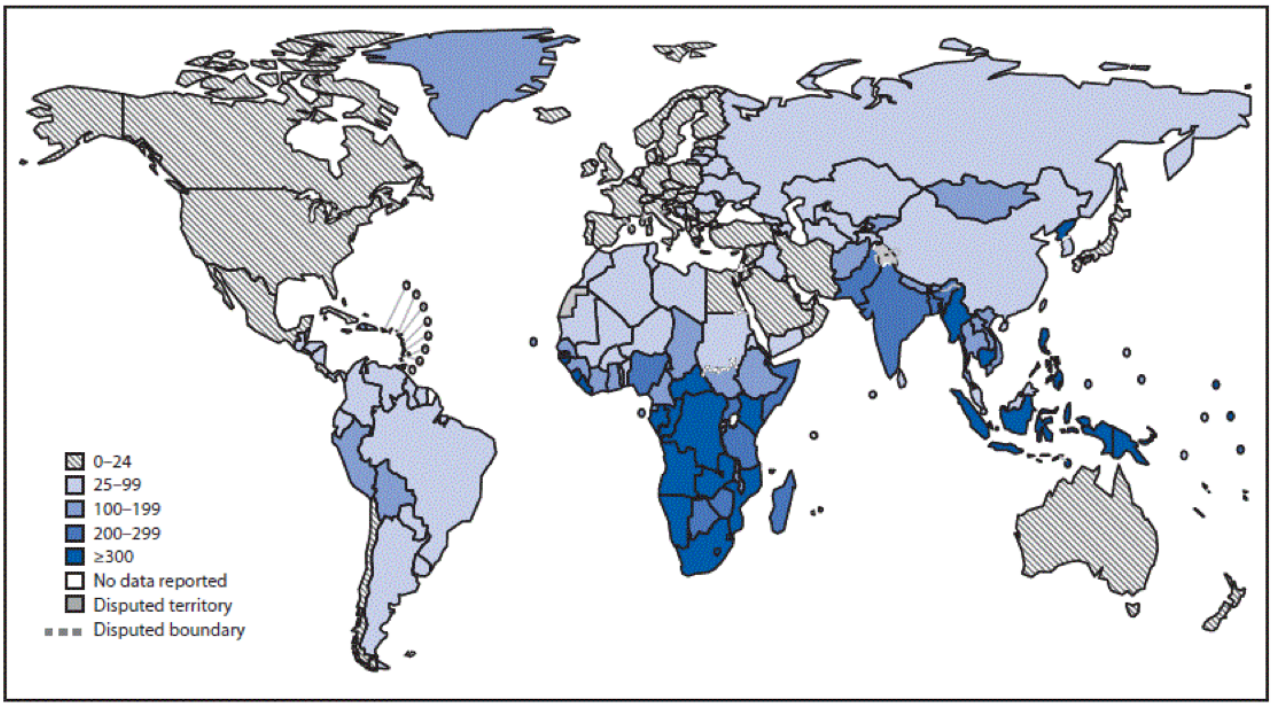


Figure 1 Worldwide incidence of Tuberculosis 2017

Annual cases of Tuberculosis per 100,000 for each population, showing regions Africa and South East Asia account for approximately 70% of global Tb infection. Figure reprinted from the World Health Organization (1).

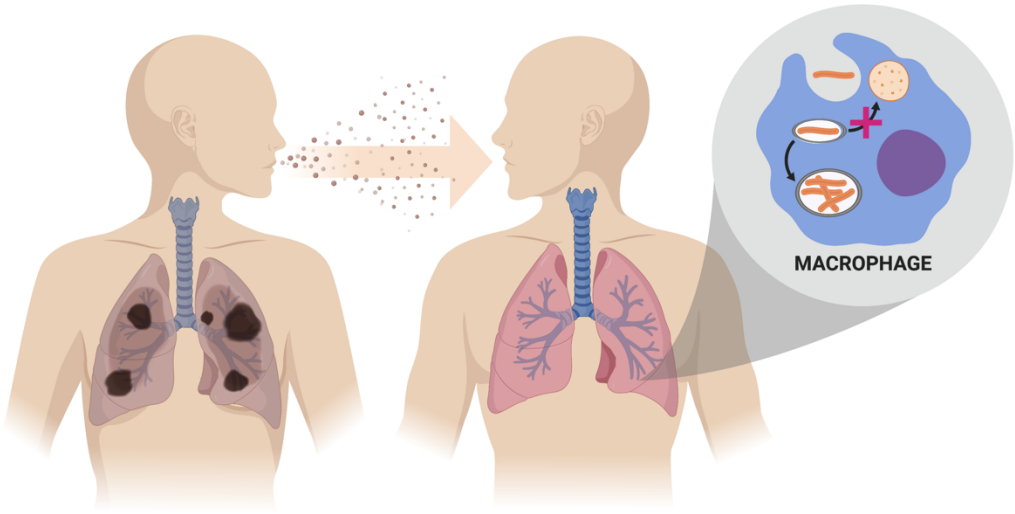


Figure 2 Tuberculosis infection cycle and spread

Tuberculosis is spread from person to person via aerosolized droplets from an individual with an active Tuberculosis infection. When an uninfected individual inhales these bacteria containing droplets, *Mycobacterium tuberculosis* gains access to lungs and is phagocytosed by the macrophage. Here it can subvert the immune system and establish a niche within the macrophage that allow it to remain dormant for decades. Figure provided by the Dr. Samantha Bell.

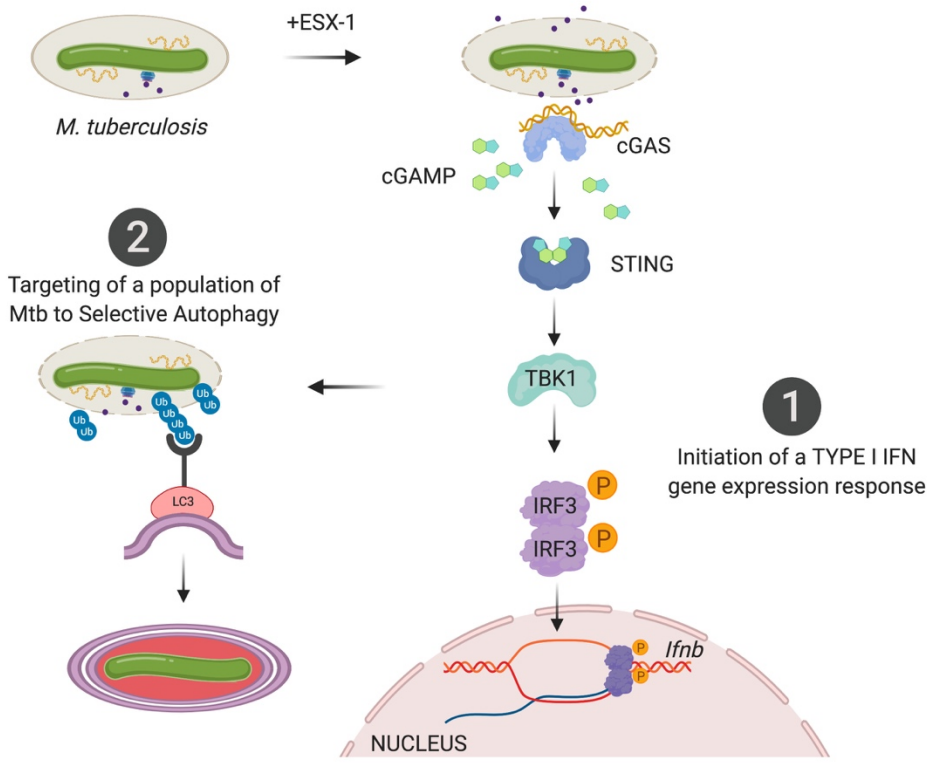


Figure 3 DNA sensing and Autophagy during Mtb infection

Once contained inside the macrophage, *Mycobacterium tuberculosis* uses is ESX-1 secretion system to secrete proteins that permeabilize the bacterium containing phagosome. Host cytosolic DNA sensor cGAS detects Mtb DNA and initiates the DNA sensing cascade leading to TBK1 activation. TBK1 is involved in two outcomes 1) initiation of the Type I IFN response and 2) targeting Mtb for destruction via selective autophagy.

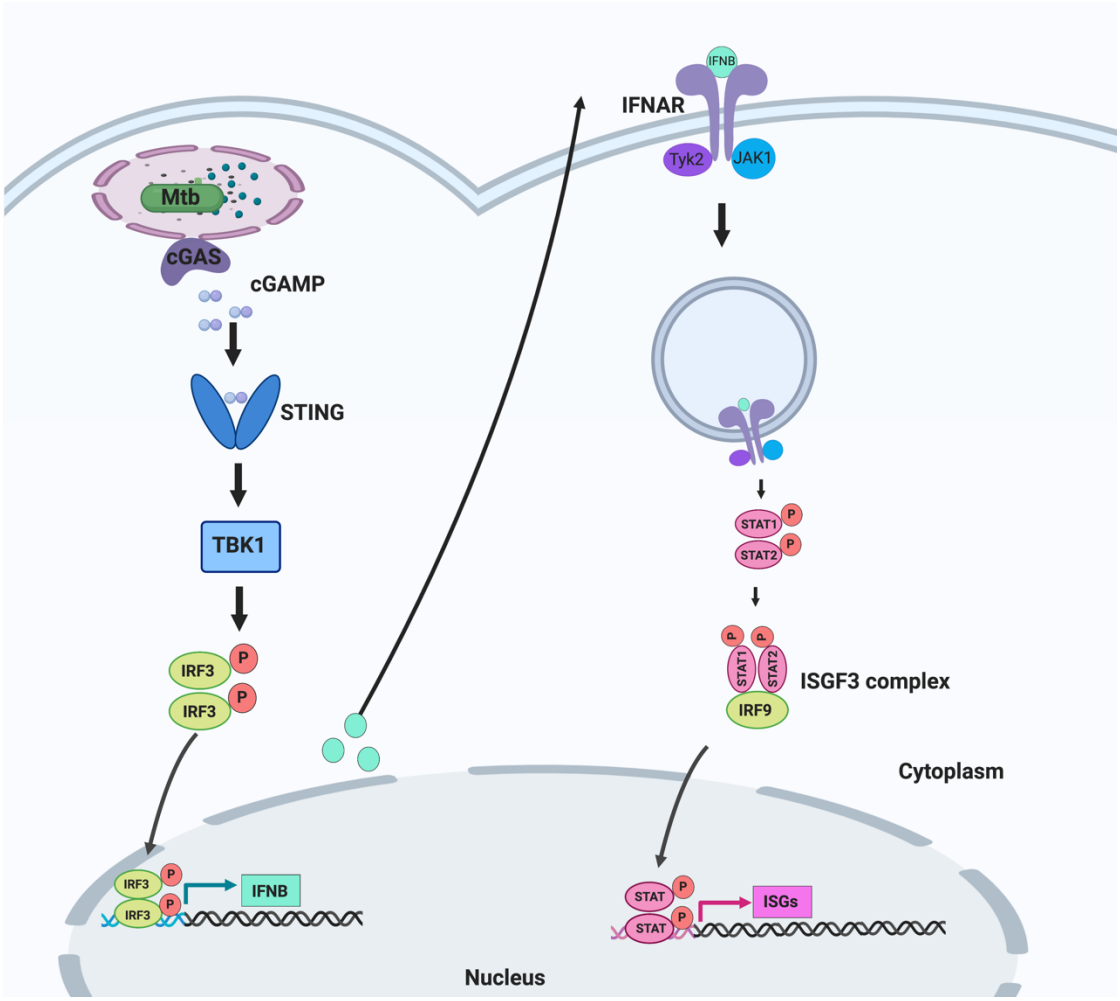


Figure 4 Type I IFN Response

Once contained inside the macrophage, *Mycobacterium tuberculosis* uses is ESX-1 secretion system to secrete proteins that permeabilize the bacterium containing phagosome. Host cytosolic DNA sensor cGAS detects Mtb DNA and initiates the DNA sensing cascade leading to TBK1 activation. TBK1 phosphorylates IRF3 which then forms a dimer and translocates into the nucleus where it initiates transcription of IFNβ. IFNβ. is released from the cell and detected by the IFNAR receptor on the cell membrane. This leads to the activation of associated kinases JAK1 and Tyk2. JAK1 and Tyk2 phosphorylate the STAT1/STAT2 dimer, which then forms the ISGF3 complex

along with IRF9. The ISGF3 complex translocates into the nucleus and binds to ISRE elements to turn on transcription of 100s of ISGs.

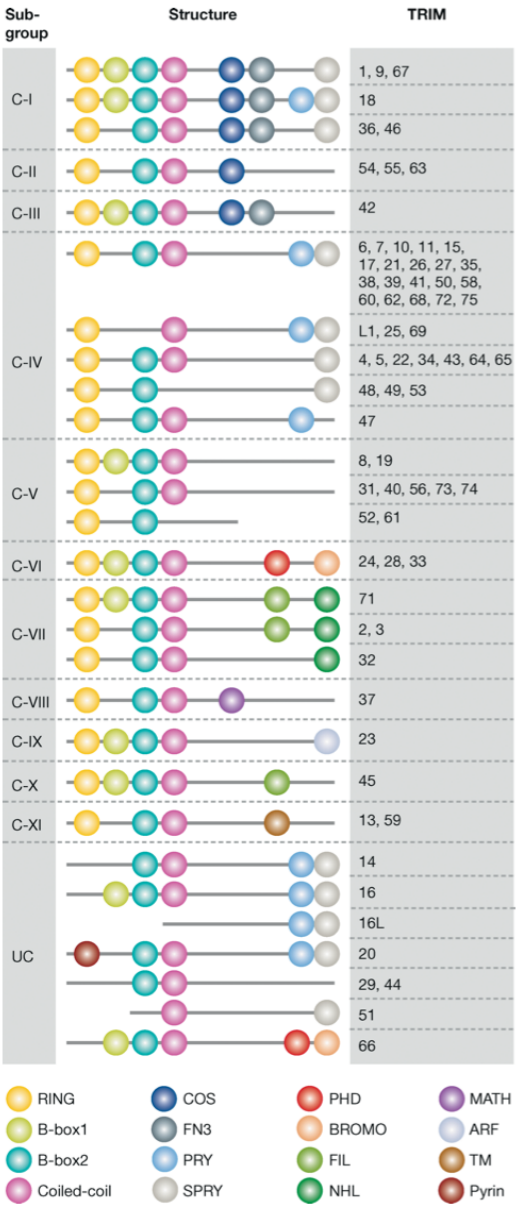


Figure 5 TRIM Family proteins

TRIM family proteins are characterized by having three domains; the RING, B Box, and Coiled coil with a variable C-terminus. They are grouped further into subfamilies based on their C-terminus. TRIM14 and a few other trims remain unclassified due to the lack of a RING domain. Figure reprinted from Kawai *et al*. [104]

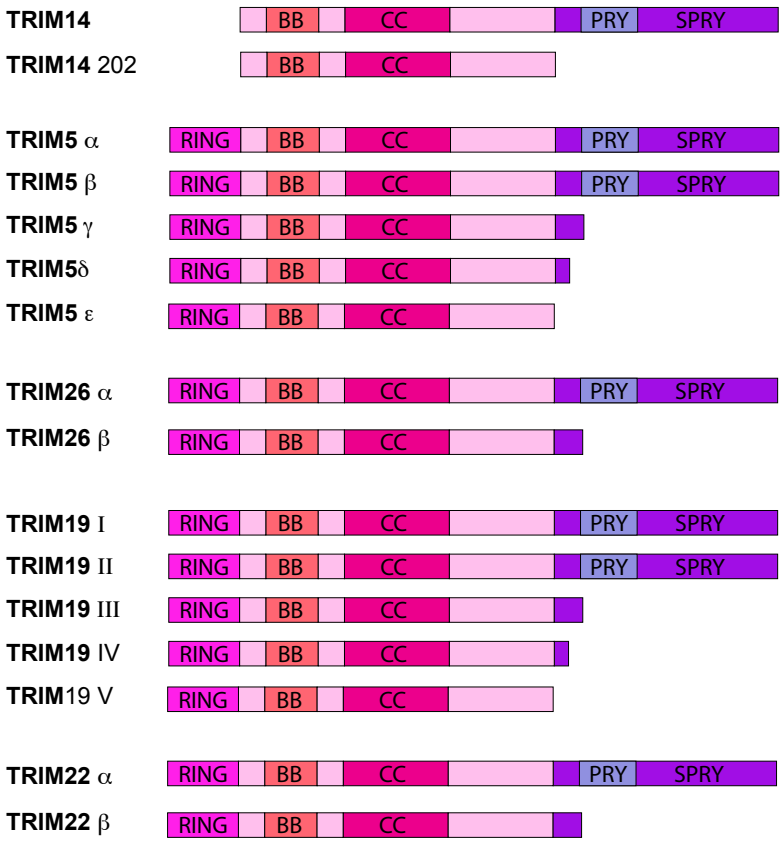


Figure 6 TRIM Spliced Isoforms

TRIM family proteins are known to encode alternatively spliced isoforms. Often times these encoded isoforms result in complete deletions of domains important for protein-protein interactions, for example the PRY/SPRY domain seen in purple. TRIM5 and TRIM19 have the best characterized isoforms in which the truncated isoforms regulate the canonical protein.

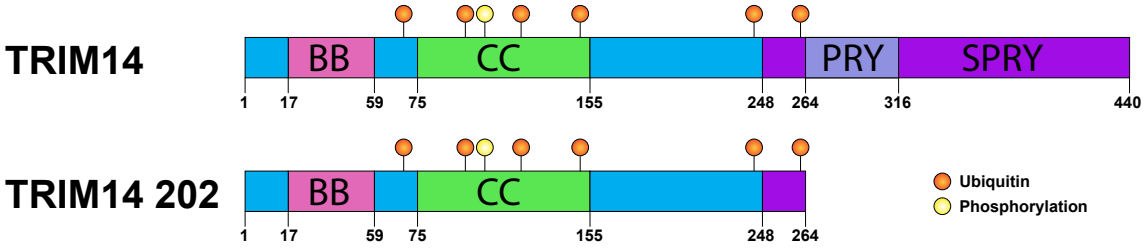


Figure 7 TRIM14 Modification

TRIM14 has two isoforms that encode proteins, canonical TRIM14 which has all 6 exons and TRIM14 202 which is missing Exon 6 and subsequently the c-terminal PRY/SPRY domain. TRIM14 is also differentially ubiquitinated and phosphorylated upon bacterial infection as noted by the orange and yellow circles respectively.

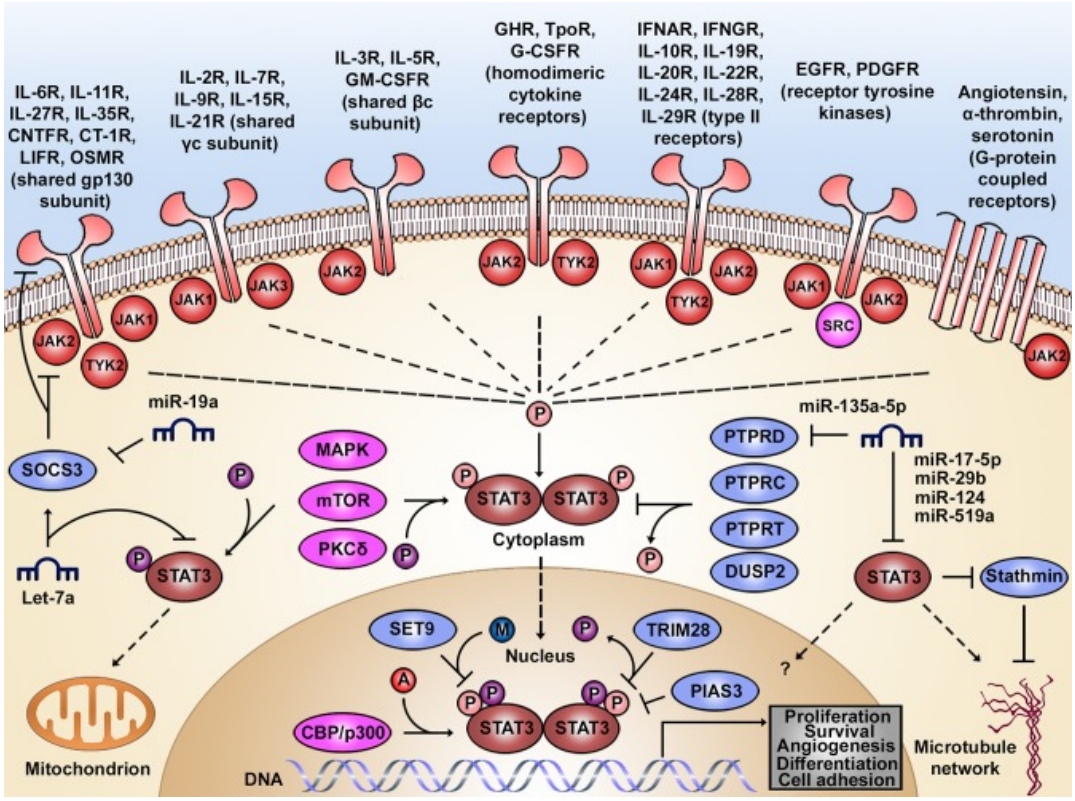


Figure 8 STAT3 activation

STAT3 is activated downstream of numerous receptors including the IFNAR receptor. There is tight control of STAT3 activation as is involved in regulation of innate immunity and plays several other roles in regulating embryogenesis, cancer metastasis, and apoptosis. Figure reprinted from Roca Suarez et al. [198]

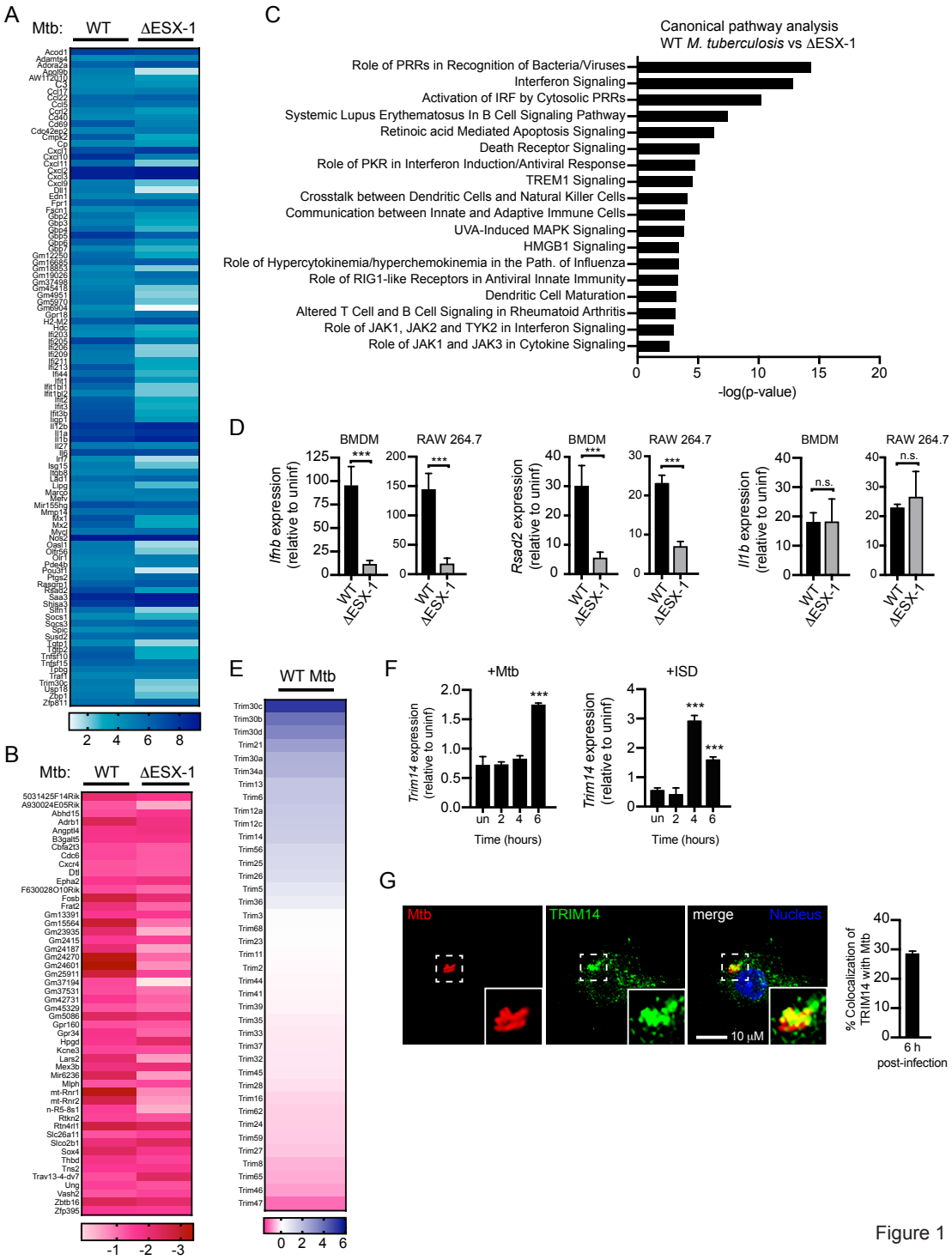


Figure 1

Figure 9 - TRIMs are players in the innate immune response to M. tuberculosis

(A,B) H eatmap of significant ($p < 0.05$) ge ne expression differences (log2 fold-change over uninfected) in BMDMs infected with WT vs ΔESX-1 M. tuberculosis (Mtb) Genes upregulated are displayed in blue. Genes downregulated are displayed in red. (C) IPA software analysis of cellular pathways enriched for differentially expressed genes in BMDMs infected with WT vs ΔESX-1M. tuberculosis. (D)RT -qPCR of transcripts in BMDMs & RAW 264.7 cells infected with WT M. tuberculosis(E) H eatmap of significant ($p < 0.05$) ge ne expression differences (log 2fold-change) i n TRIM family genes in BMDMS infected with WT vs ΔESX-1 M. tuberculosis (F)RT -qPCR of fold-change in Trim14 transcripts in BMDMs stimulated with ISD or infected with WT M. tuberculosis. G) RA W 264.7 cells infected with mCherry M. tuberculosis for 6 hours and immunostained for TRIM14. Statistical significance was determined using two-tailed students' t test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

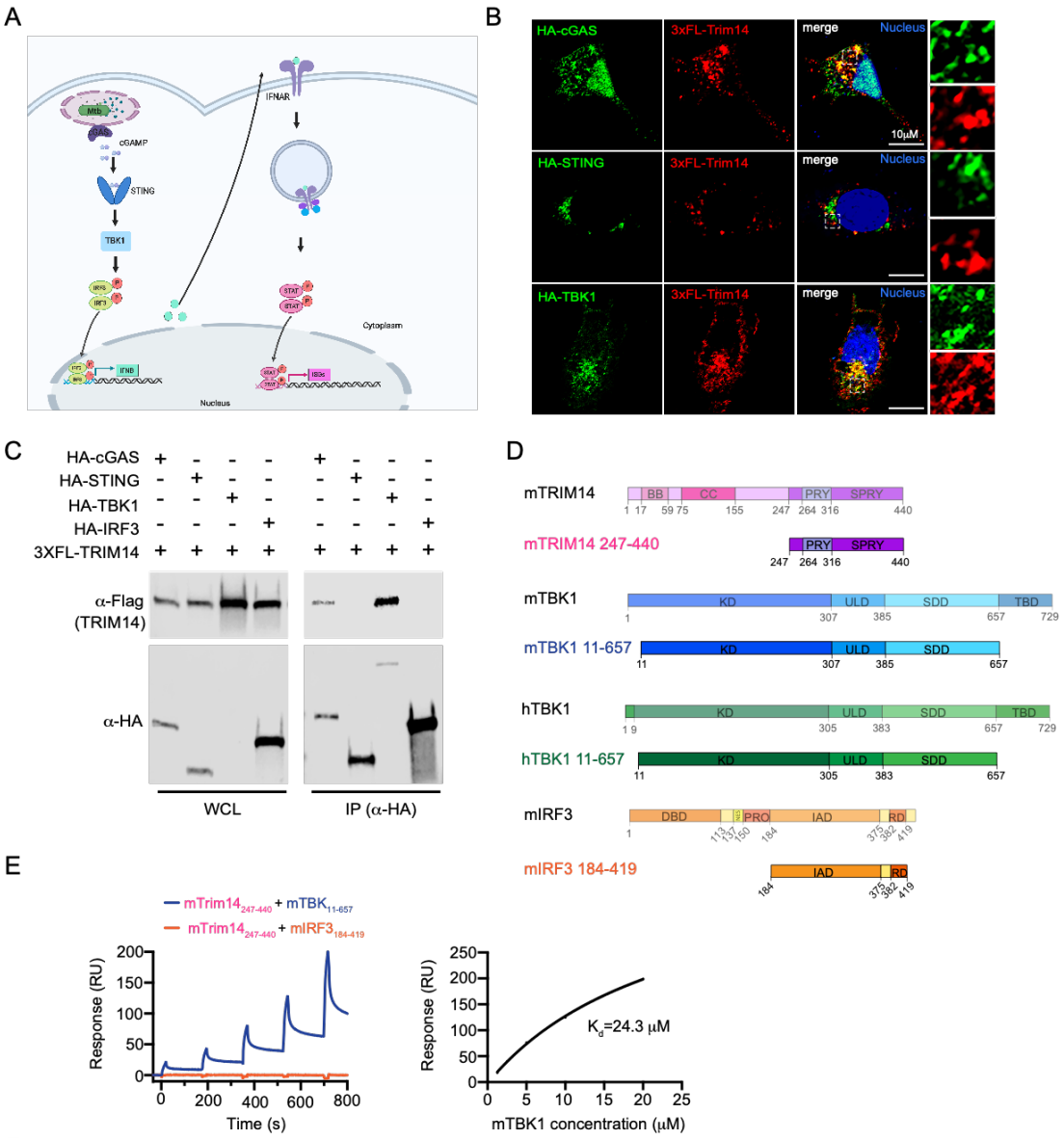
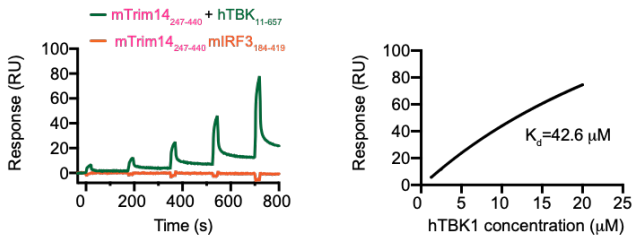


Figure 10 - TRIM14 interacts with both cGAS and TBK1 in the DNA sensing pathway

Figure 10 Continued

F



(A) Model of DNA sensing pathway during *M. tuberculosis* infection (B) Immunofluorescence microscopy of MEFs expressing 3xFLAG-TRIM14 with HA-cGAS , HA-STING , or HA-TBK1 co-stained with α-HA and α-Flag antibodies (C) Western blot analysis of co-immunoprecipitation of 3xFLAG-TRIM14 co-expressed with HA-cGAS , HA-STING , HA-TBK1, or HA-IRF3 in HEK 293T cells. Blot is representative of >3 independent biological replicates. (D) Diagram of mTRIM14, mTBK1, hTBK1, mIRF3 gene domains and truncations used in surface plasmon resonance (SPR) studies. (E) Equilibrium binding study of mTRIM14 and mTBK1 by SPR. mIRF3 was used as a negative control. Dissociation constant (K_d= 24.3μM) was derived by fitting of the equilibrium binding data to a one-site binding model. (F) As in (E) but with mTRIM14 and hTBK1. Dissociation constant (K_d= 42.6μM) was derived by fitting of the equilibrium binding data to a one-site binding model.

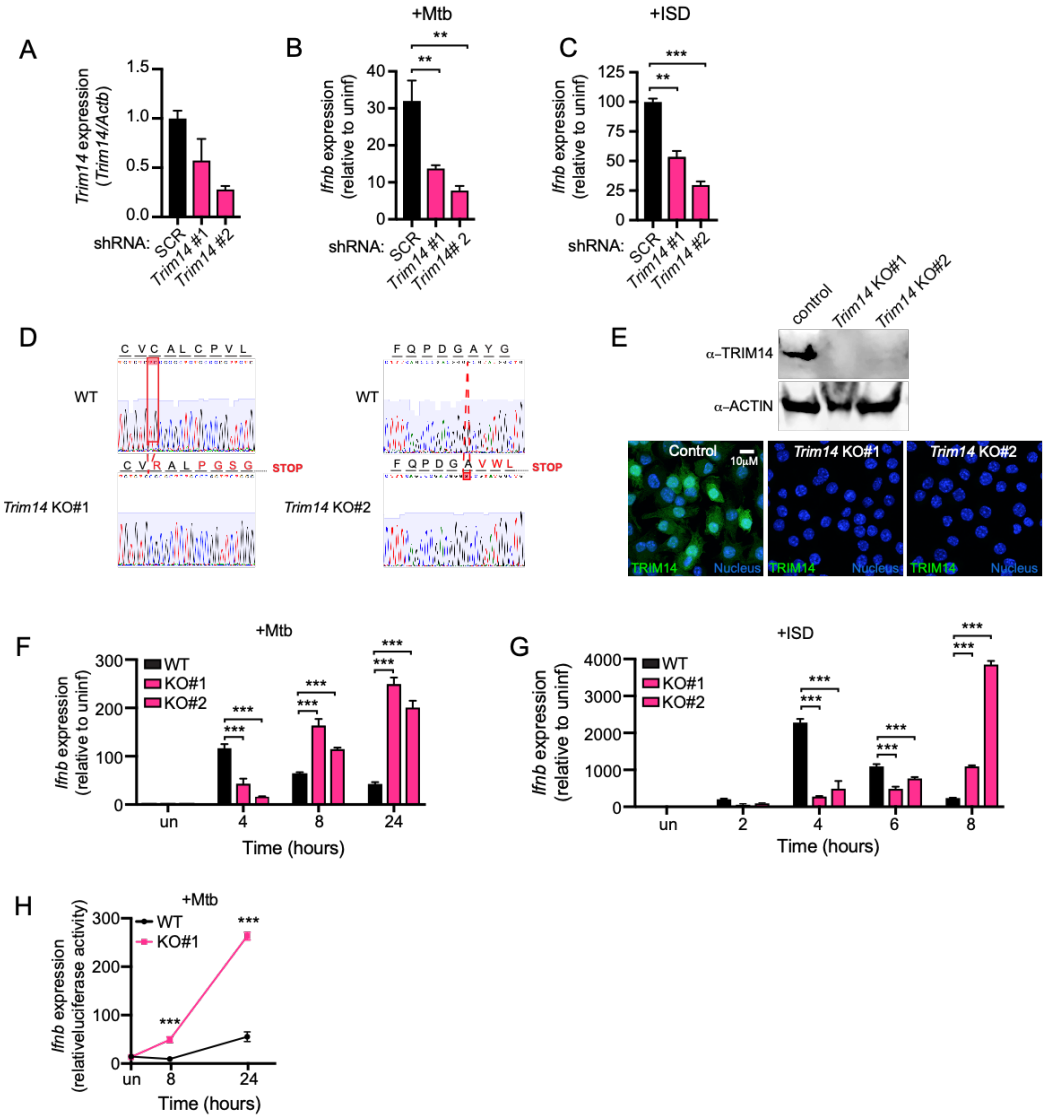


Figure 11 (3)- Loss of Trim14 leads to hyperinduction of *Ifnb* in response to *M. tuberculosis* and cytosolic DNA

(A) RT-qPCR of *Trim14* transcript in RAW 264.7 macrophages stably expressing shRNA to either SCR control or *Trim14*. (B) RT-qPCR of *Ifnb* transcript in RAW 264.7 macrophages stably expressing shRNA to either SCR control or *Trim14* infected with *M. tuberculosis* at 4h post infection. (C) RT-qPCR of *Ifnb* transcript in RAW 264.7

macrophages stably expressing shRNA to either SCR control or TRIM14 transfected with 1μg ISD at 4h post infection. (D) Sequencing chromatogram depicting mutations in *Trim14* gRNA CRISPR/Cas9 RAW 264.7 macrophages compared to GFP gRNA control (WT). (E) Western blot analysis and immunofluorescence microscopy of *Trim14* in WT vs *Trim14* KO RAW 264.7 macrophages using an anti-TRIM14 antibody. (F) RT-qPCR of *Ifnb* transcripts in WT and *Trim14* KO RAW 264.7 macrophages infected with *M. tuberculosis* at specified times after infection. (G) RT-qPCR of *Ifnb* transcripts in WT and *Trim14* KO RAW 264.7 macrophages treated with ISD at specified times after treatment. (H) ISRE reporter cells expressing luciferase with relative light units measured as a readout for IFNβ protein. Statistical significance was determined using two-tailed students' t test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

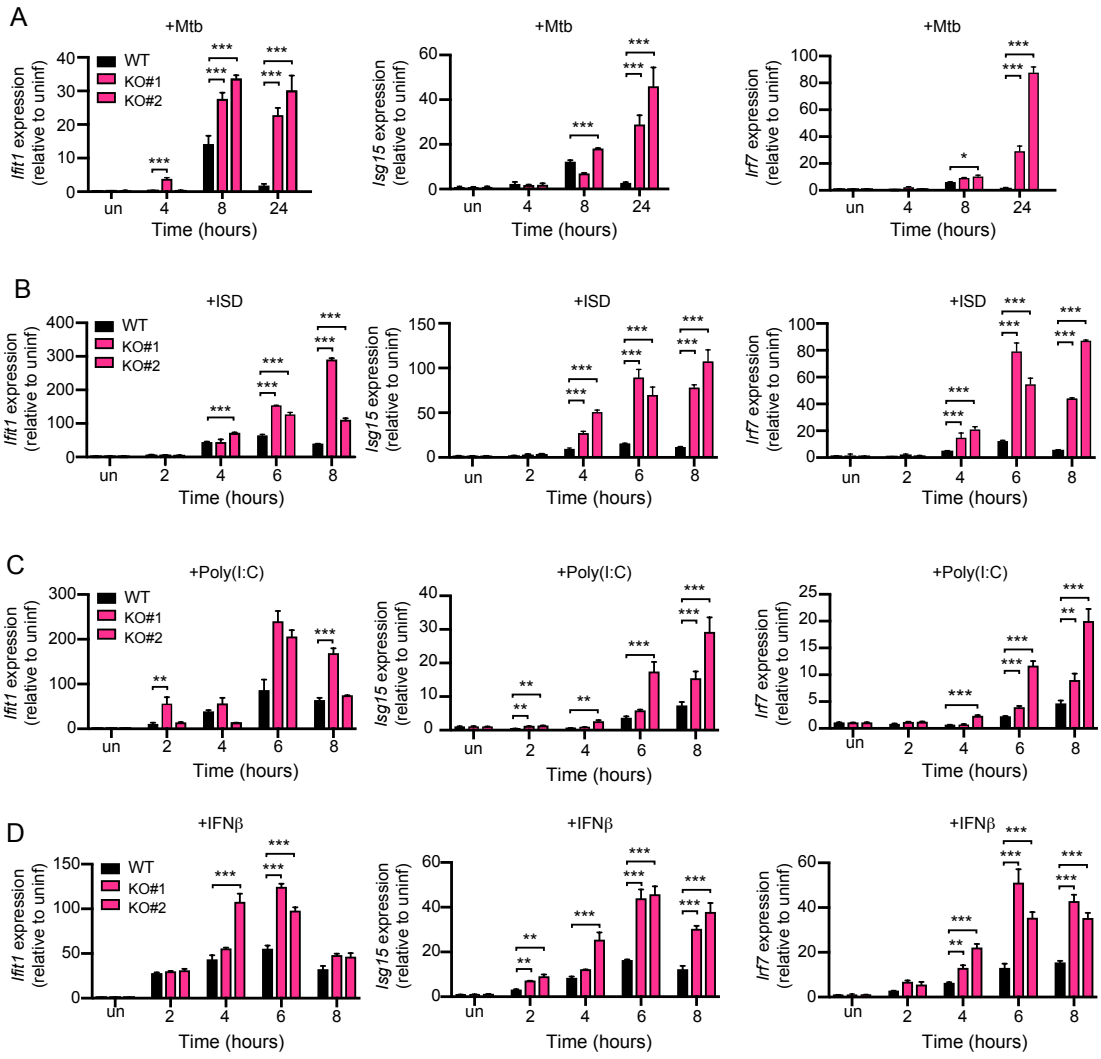


Figure 12 (4)- Loss of TRIM14 leads to hyperinduction of ISGs in response to multiple innate immune agonists

(A) RT-qPCR of *Ifit*, *Isg15*, *Irf7* transcripts in WT and *Trim14* KO RAW 264.7 macrophages infected with *M. tuberculosis* at specified times after infection. (B) RT-qPCR of *Ifit*, *Isg15*, *Irf7* transcripts in WT and *Trim14* KO RAW 264.7 macrophages transfected with 1μg ISD. (C) As in (B) but with poly(I:C) transfection. (D) As in (B) but

with IFNβ treatment (200 IU). Statistical significance was determined using two-tailed students' t test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

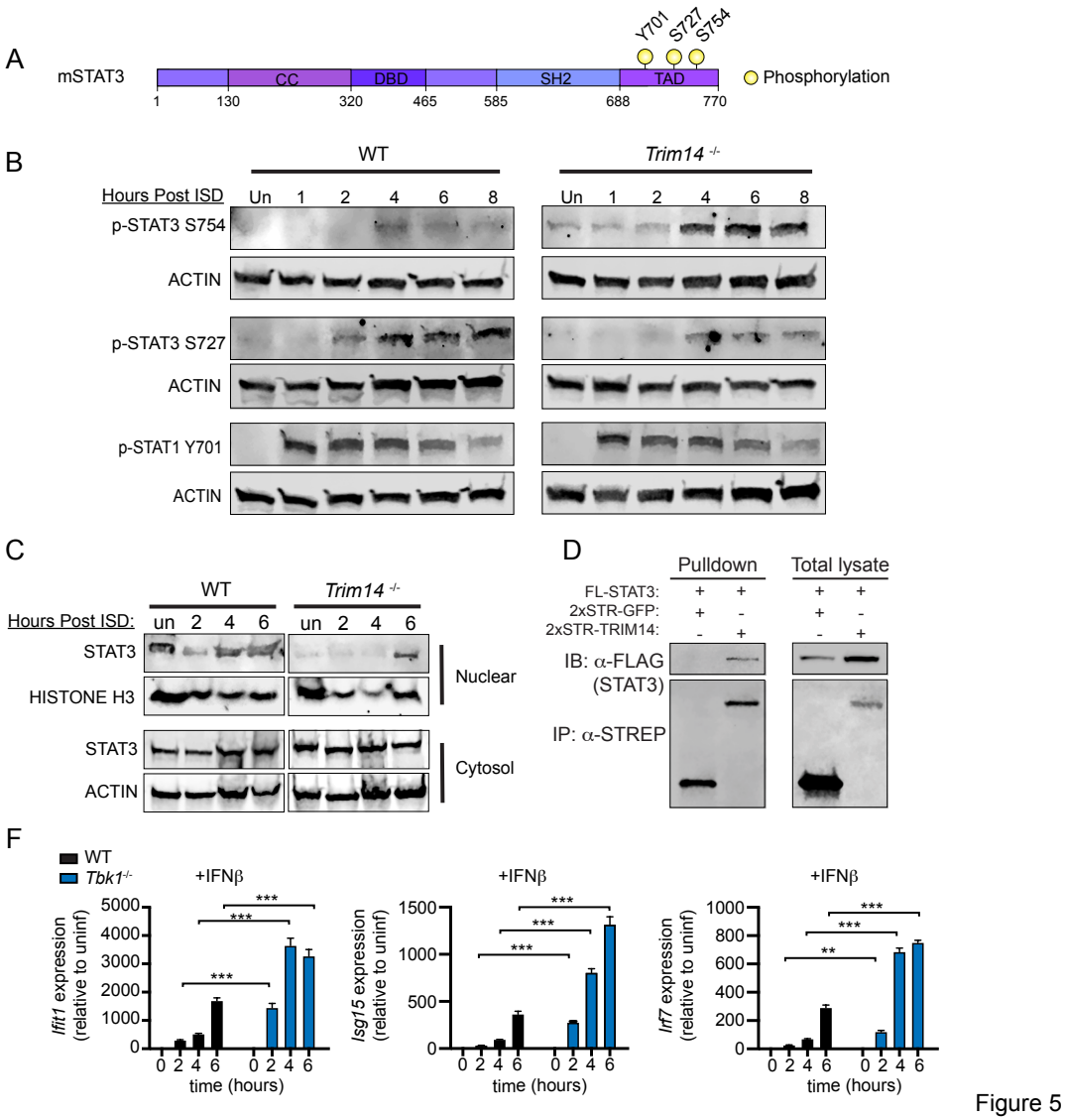


Figure 5

Figure 13 (5)- Loss of TRIM14 promotes inhibitory phosphorylation of STAT3 at Ser754

(A) Cartoon depiction of phosphorylation sites of STAT3. (B) Immunoblot of phospho-STAT3 Ser754, phospho-STAT3 Ser727, and phospho-STAT1 Y701 in WT and *Trim14* KO RAW 264.7 macrophages at 1, 2, 4, 6, 8h post-ISD transfection. ACTIN is shown as a loading control. (C) Cellular fractionation showing nuclear STAT3 in WT and

Trim14 KO RAW 264.7 macrophages treated with ISD at specified times after treatment. Histone 3 shown as loading/nuclear control (D) Co-immunoprecipitation and western blot analysis of HEK 293T cells co-transfected with FLAG-STAT3 and HA-TRIM14. (E) As in F, with FLAG-STAT3 and HA-TBK1 (F) RT-qPCR of *Ifit*, *Isg15*, *Irf7* transcripts in WT and *Tbk1* KO BMDMs treated with IFNβ. Western blots are representative of >3 independent biological replicates. Statistical significance was determined using two-tailed students' t test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

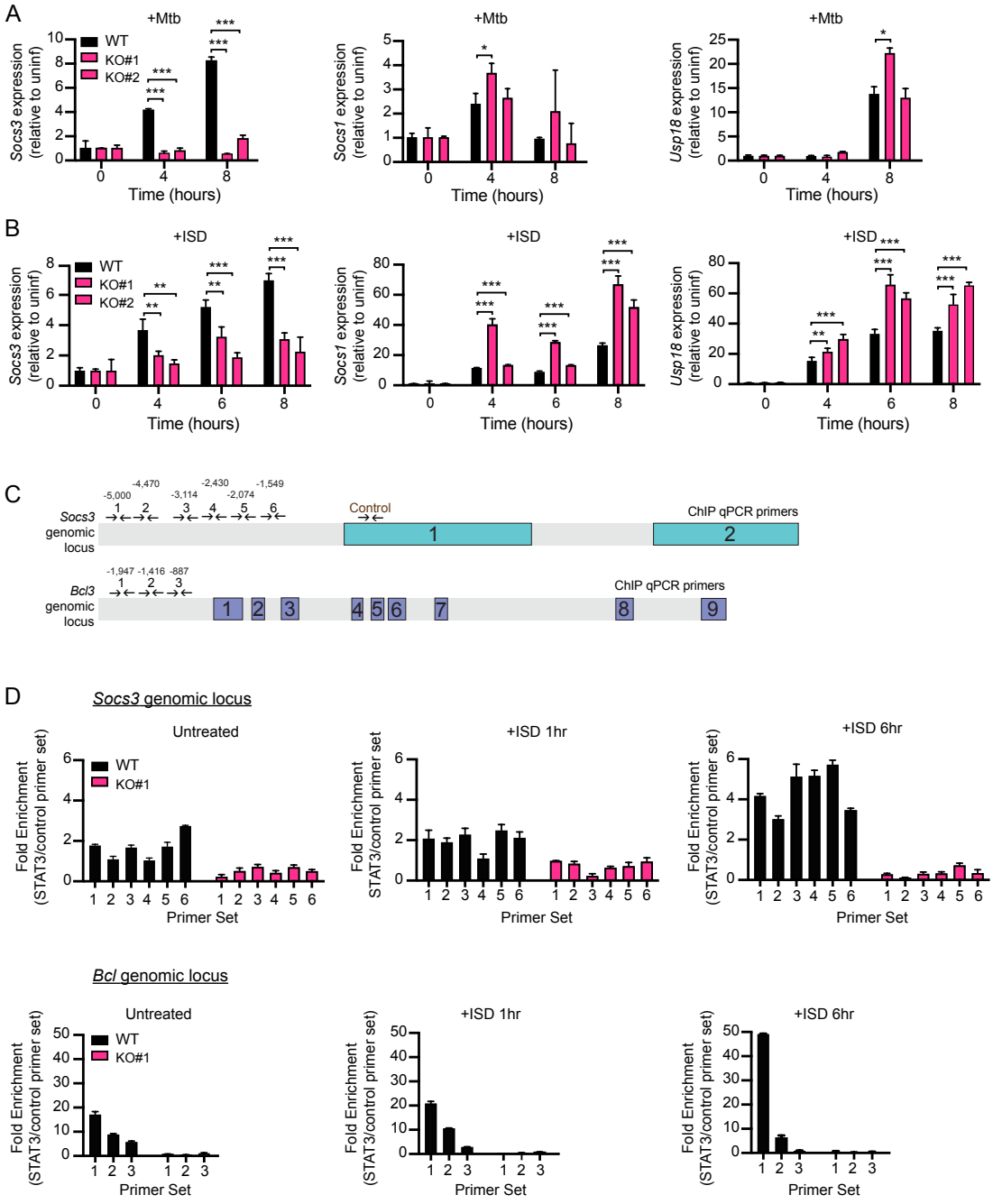
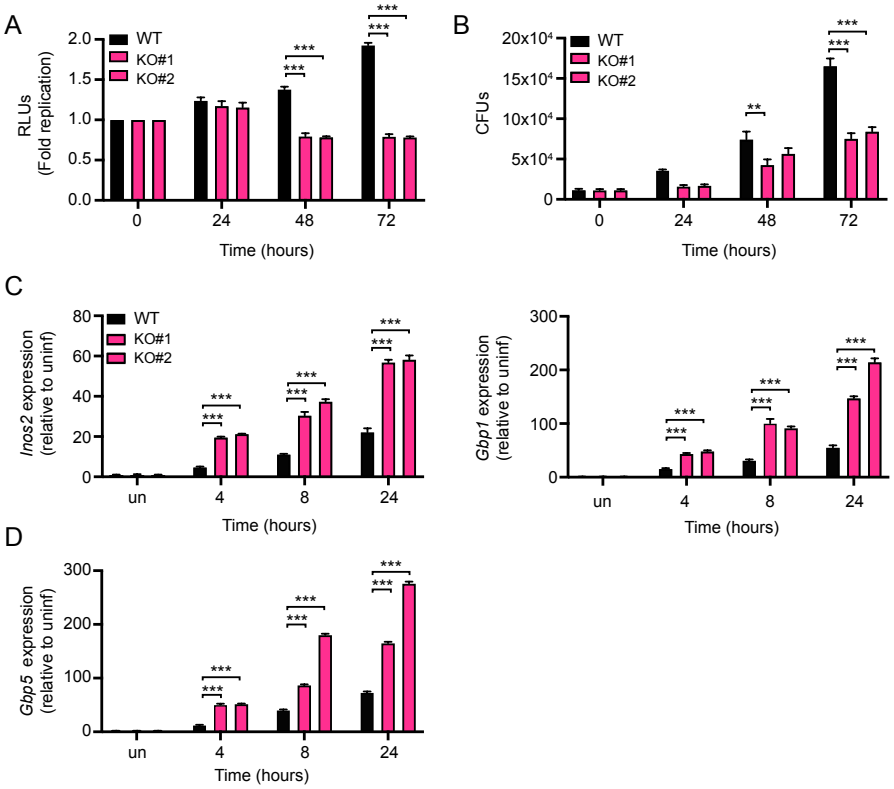


Figure 6

Figure 14 (6)- *Trim14* KO macrophages fail to induce expression of the negative regulator of the type I IFN response, Socs3

(A) RT-qPCR of *Socs3*, *Socs1*, and *Usp18* transcripts in WT and *Trim14* KO RAW 264.7 macrophages infected with *M. tuberculosis* at specified times after treatment. (B) RT-qPCR of *Socs3*, *Socs1*, and *Usp18* transcripts in WT and *Trim14* KO RAW 264.7 macrophages treated with ISD at specified times after treatment. (C) qPCR primers designed to tile *Socs3* and *Bcl3* genes for chromatin immunoprecipitation experiments (D) ChIP-qPCR of 3xFLAG-STAT3 associated genomic DNA from the *Socs3* and *Bcl3* loci in WT and *Trim14* KO RAW 264.7 macrophages transfected with 1μg ISD. Statistical significance was determined using two-tailed students' t-test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.



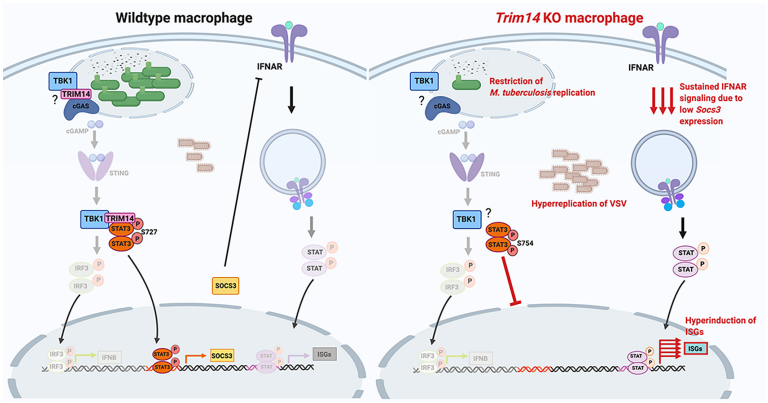


Figure 15 (7)- *Trim14* KO macrophages restrict *M. tuberculosis* replication but fail to control VSV replication

(A) Fold replication of Mtb *luxBCADE* in WT and *Trim14* KO RAW 264.7 macrophages. (B) CFUs in WT and *Trim14* KO RAW 264.7 macrophages infected with Mtb at 0h, 24h, 48h and 72h post-infection. (C) RT-qPCR of *iNos2, Gbp1*, and *Gbp5*

transcript levels in Mtb infected WT and *Trim14* KO RAW 264.7 macrophages. (D) Proposed model of TRIM14's dual roles in regulating cytosolic DNA sensing. TRIM14/cGAS interaction is required to inhibit proteasomal degradation of cGAS and TRIM14/TBK1 interaction is required to promote TBK1-dependent phosphorylation of STAT3 at Ser727 and activate transcription of negative regulators of the type I IFN response like *Socs3*.

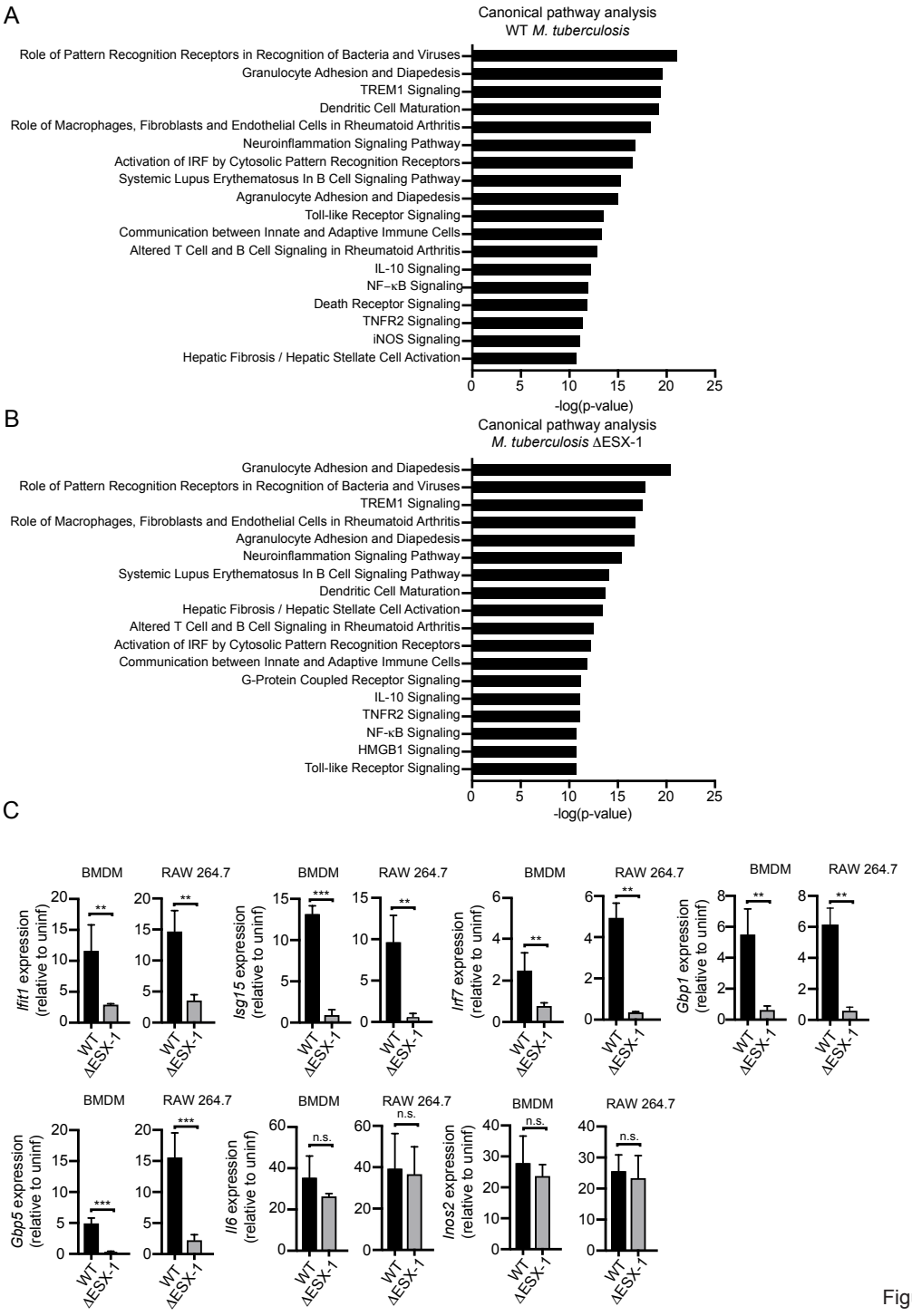


Figure S1

Figure 16 (S1)- RAW 264.7 and BMDMs induce a similar response to Mtb infection

(A) IPA software analysis of cellular pathways enriched for differentially expressed genes in BMDMs infected with WT *M. tuberculosis***.** (B) IPA software analysis of cellular pathways enriched for differentially expressed genes in BMDMs infected with ΔESX-1 *M. tuberculosis*. (C) RT-qPCR of transcripts in BMDMs & RAW 264.7 cells infected with WT *M. tuberculosis.*

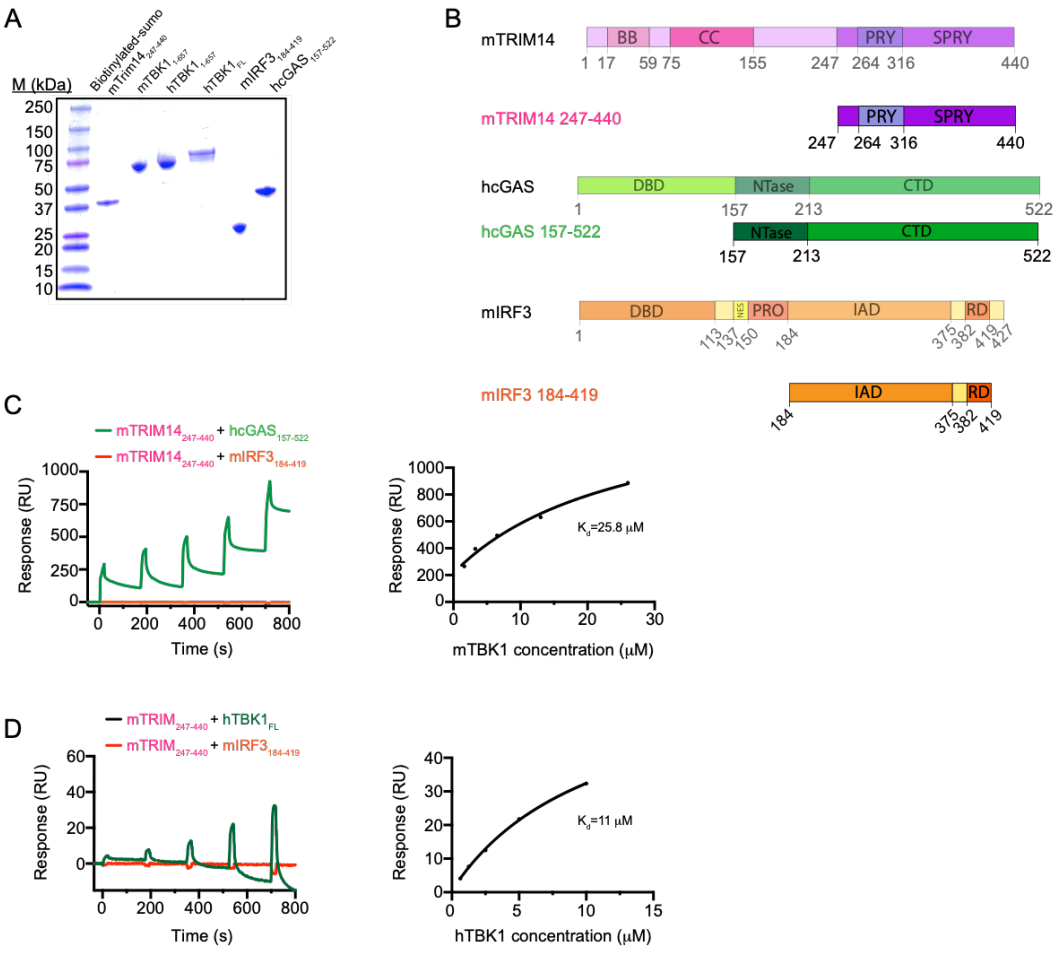


Figure 17 (S2)- TRIM14 directly binds cGAS

(A) Protein expression of mTRIM14, mTBK1, hTBK1, mIRF3, and hcGAS. (B) Diagram of mTRIM14, mIRF3, and hcGAS gene domains and truncations used in SPR studies. (C) Equilibrium binding study of mTRIM14 and hcGAS by surface plasmon resonance (SPR). mIRF3 was used as a negative control. Dissociation constant (K_d= 25.8 μM) was derived by fitting of the equilibrium binding data to a one-site binding model. Equilibrium binding study of mTRIM14 and hTBK1 by surface plasmon resonance (SPR).

mIRF3 was used as a negative control. Dissociation constant (K_d= 11 μM) was derived by fitting of the equilibrium binding data to a one-site binding model.

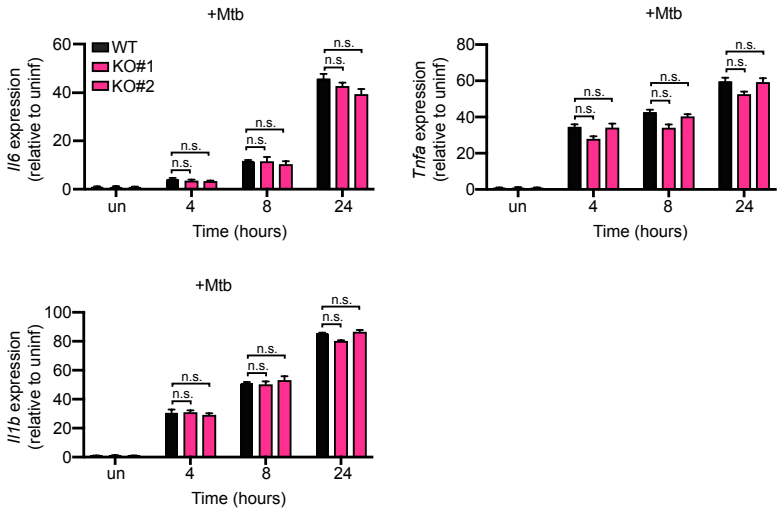


Figure 18 (S4)- TRIM14 KO respond similar to wild-type after LPS treatment

(A) RT-qPCR of *Ifnb, Ifit*, *Isg15*, *Irf7* transcripts in WT and *Trim14* KO RAW 264.7 macrophages transfected with 100ng/ml LPS

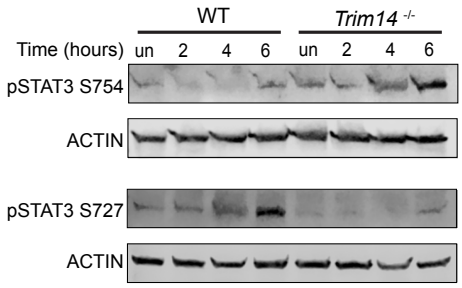


Figure 19 (S5)- TRIM14 KO has more pSTAT3 S754 in response to IFNβ

Immunoblot of phospho-STAT3 Ser754, phospho-STAT3 Ser727, and phospho-STAT1 Y701 in WT and *Trim14* KO RAW 264.7 macrophages at 1, 2, 4, 6, 8h post recombinant IFNβ treatment. ACTIN is shown as a loading control.

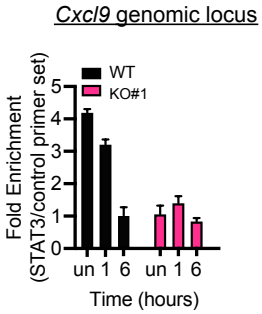


Figure 20 (S6)- STAT3 fails to bind Cxcl9 locus in TRIM14KO

ChIP-qPCR of 3xFLAG-STAT3 associated genomic DNA from the CXCL9 locus in WT and *Trim14* KO RAW 264.7 macrophages transfected with 1 μg ISD.

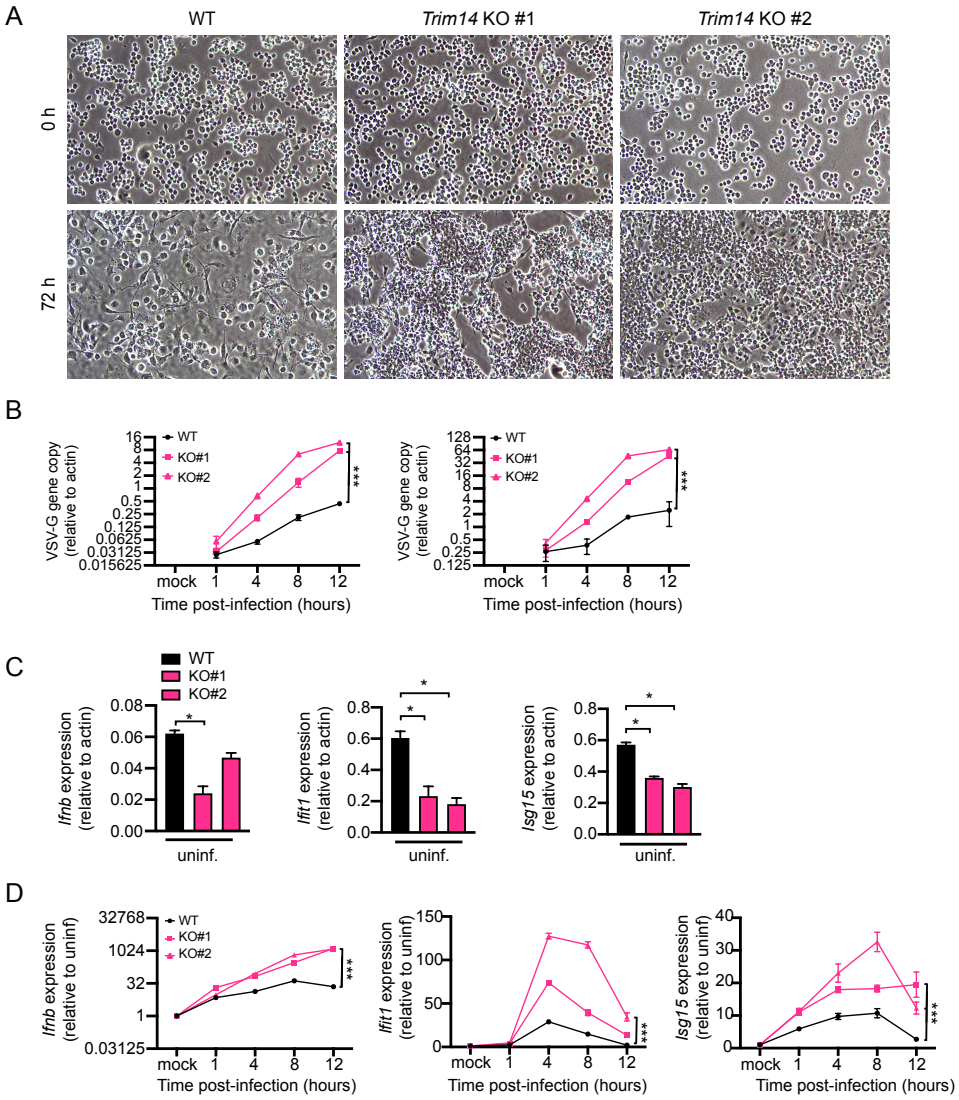


Figure 21 (S7) VSV hyper-replicates in TRIM14 KO

(A) Phase contrast images showing monolayers of WT and *Trim14* KO RAW 264.7 macrophages infected with Mtb *luxBCADE*. (B) Viral replication in WT and *Trim14* KO RAW 264.7 macrophages infected with VSV (MOI=1.0 or Mock infected) at 1h, 4h, 8h and 12h post-infection. (C) RT-qPCR of transcript levels in WT and Trim14 KO RAW 264.7 macrophages (D) RT-qPCR of transcript levels in WT and Trim14 KO RAW 264.7

macrophages infected with VSV (MOI=1.0 or Mock infected) at 1h, 4h, 8h and 12h post-infection.

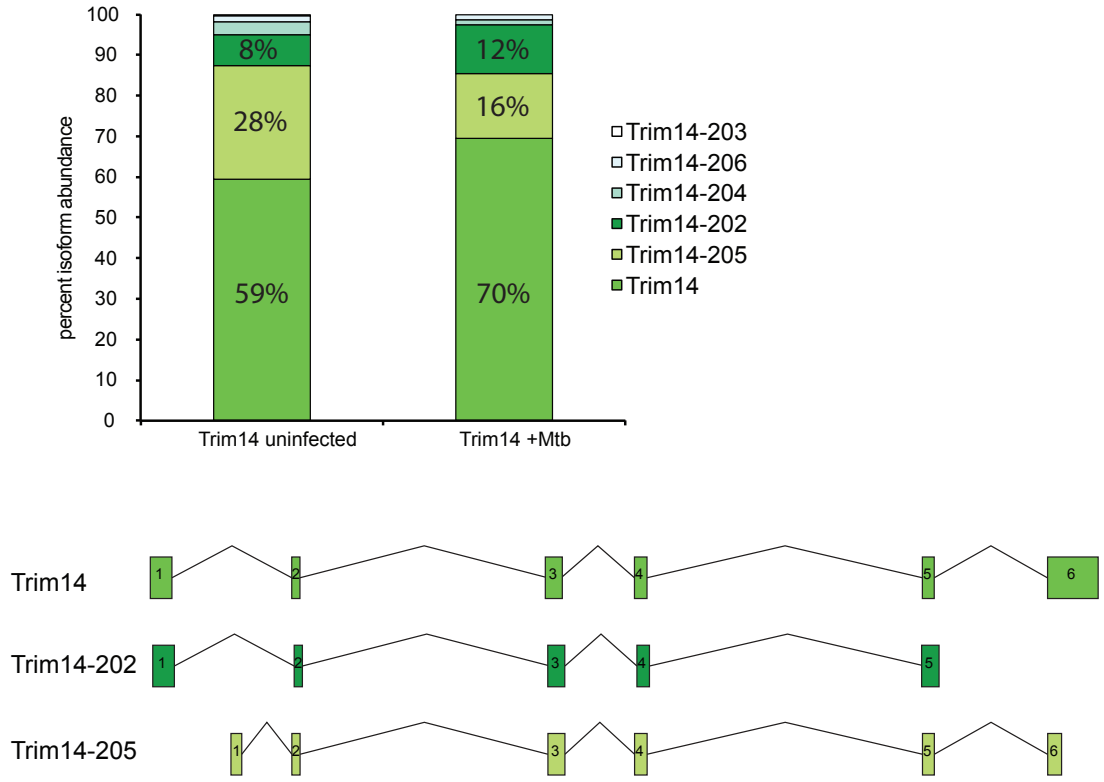


Figure 22 TRIM14 spliced isoforms during Mtb infection

RNA sequencing analysis in BMDMs at rest or infected with Mtb showing relative abundance of TRIM14 isoforms transcripts.

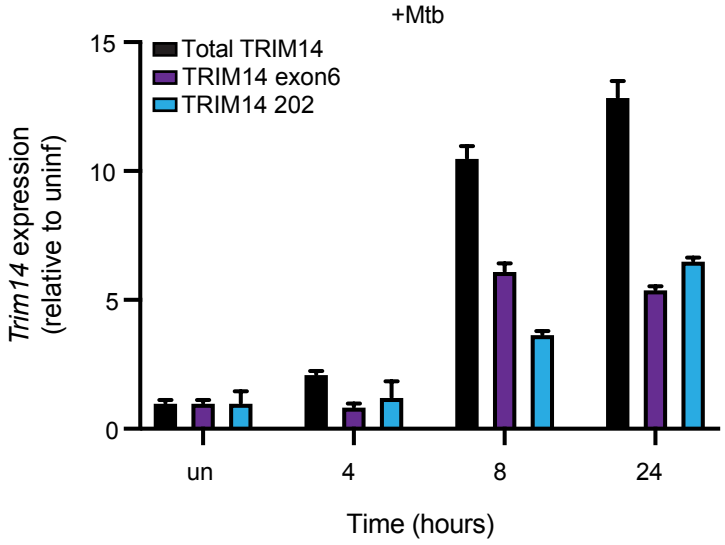


Figure 23 TRIM14 spliced isoform transcripts during Mtb infection

RT-qPCR of fold change in TRIM14 and TRIM14 202 transcripts in RAW 264.7 cells stimulated infected with Mtb.

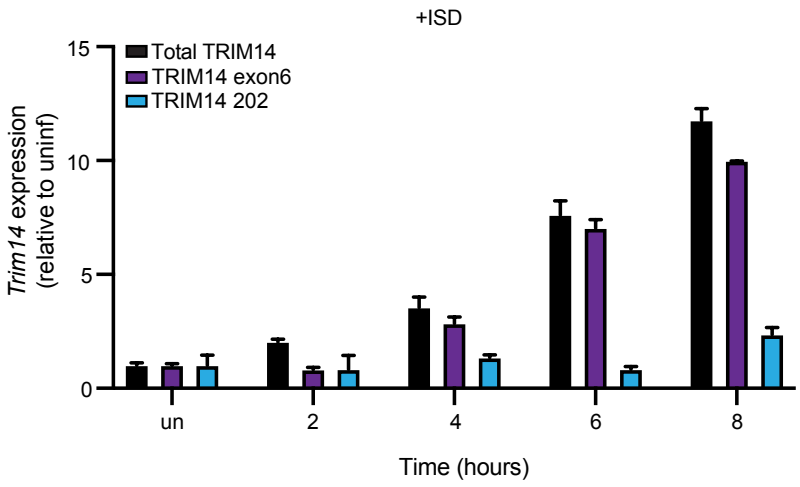


Figure 24 TRIM14 spliced isoform transcripts during DNA transfection

RT-qPCR of fold change in TRIM14 and TRIM14 202 transcripts in RAW 264.7 cells stimulated with ISD.

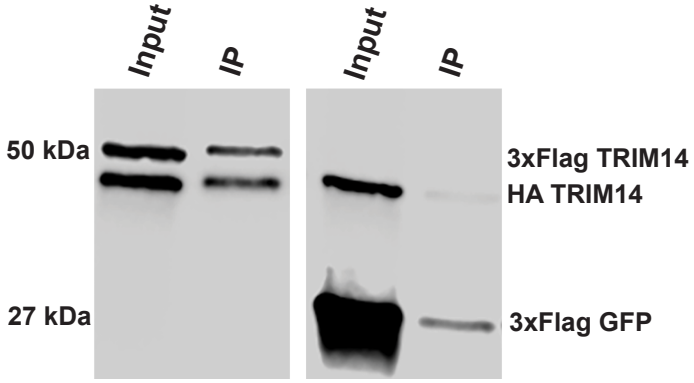


Figure 25 TRIM14 can form a complex with itself

Western blot analysis of Flag co-immunoprecipitation experiment in which 3xFLAG-TRIM14 was co-expressed with HA-TRIM14 in HEK 293T cells and anti-Flag resin was used to pull down 3xFlag TRIM14 or negative control 3xFlag GFP. Showing TRIM14 is in a complex with itself.

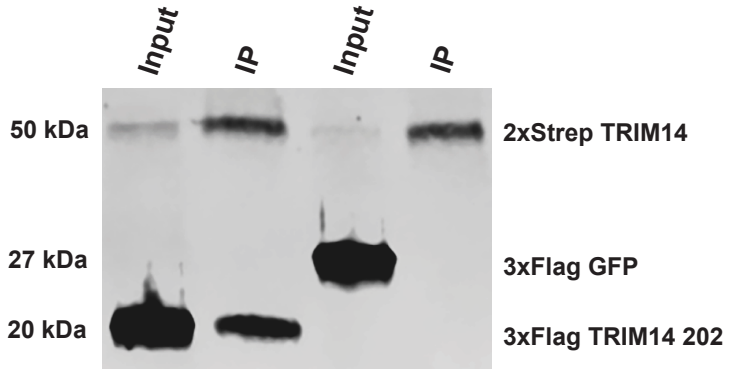


Figure 26 TRIM14 can form a complex with isoform 202

Western blot analysis of co-immunoprecipitation of 2xStrep-TRIM14 co-expressed with 3xFlag TRIM14 202 in HEK 293T cells. Showing TRIM14 202 in a complex with TRIM14, 3x Flag GFP as the negative control.

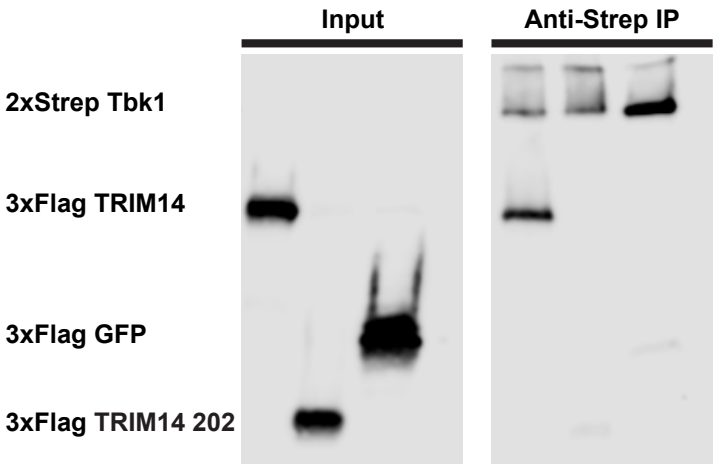


Figure 27 TRIM14 in complex with TBK1

Western blot analysis of co-immunoprecipitation of 2xStrep TBK1 co-expressed with either 3xFlag TRIM14 or 3xFlag TRIM14 202 in HEK 293T cells. Showing TRIM14 immunoprecipitated with TBK1 specifically through its PRY/SPRY domain.

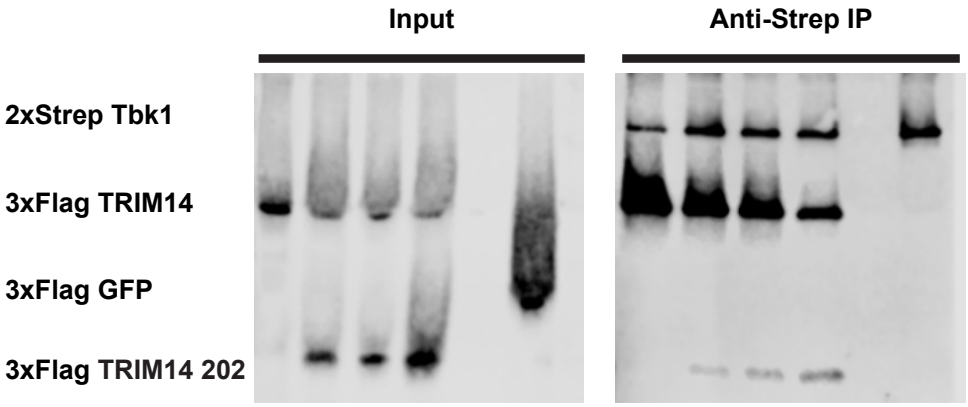


Figure 28 TRIM14 202 does not affect TRIM14-TBK1 interaction

Western blot analysis of co-immunoprecipitation of 2xStrep TBK1 co-expressed with 3xFlag TRIM14 and increasing amounts of 3xFlag TRIM14 202 in HEK 293T cells. Showing TRIM14 202 does not affect TRIM14s interaction with TBK1.

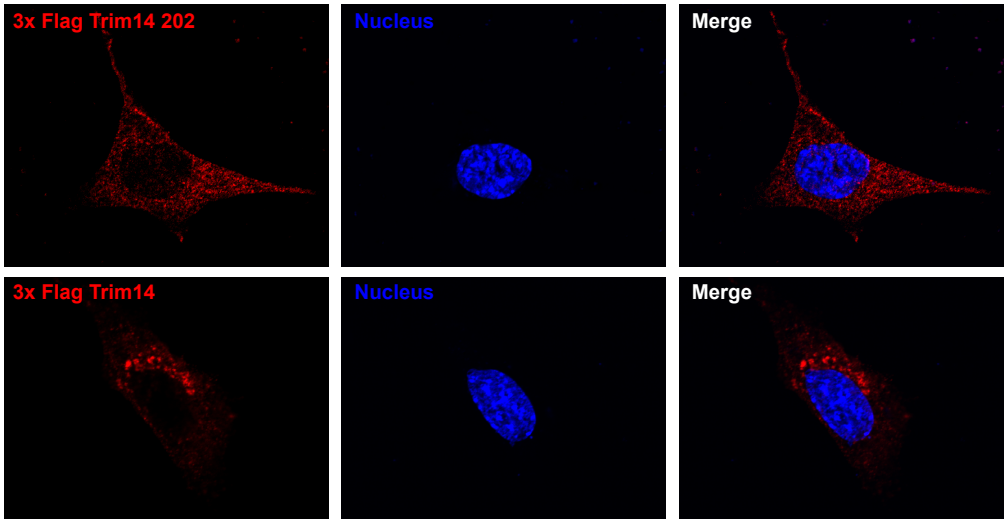


Figure 29 TRIM14 and TRIM14 202 localization

Immunofluorescence showing 2xStrep TRIM14 and 3xFlag TRIM14 202 co-expressed in MEF cells. TRIM14 and TRIM14 202 localize differently in the cell.

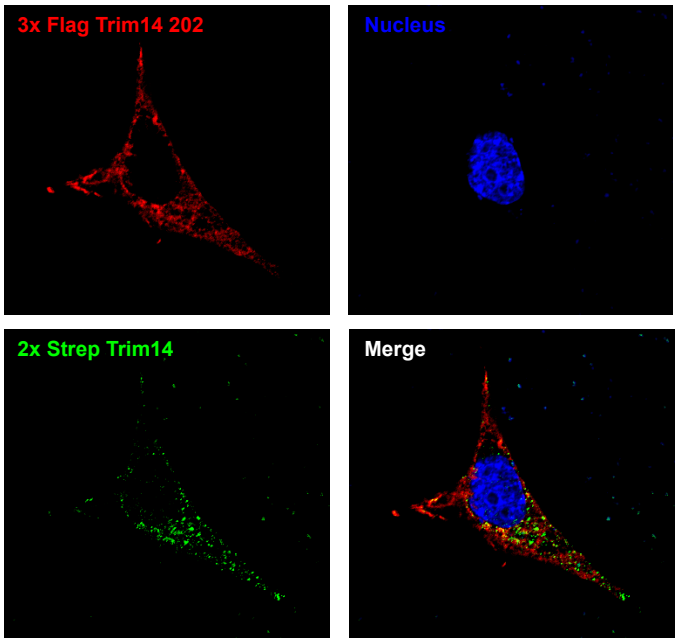


Figure 30 TRIM14 and TRIM14 202 localization

Immunofluorescence showing 2xStrep TRIM14 and 3xFlag TRIM14 202 co-expressed in MEF cells. TRIM14 and TRIM14 202 localize to different parts of the cell in the presence of one another.

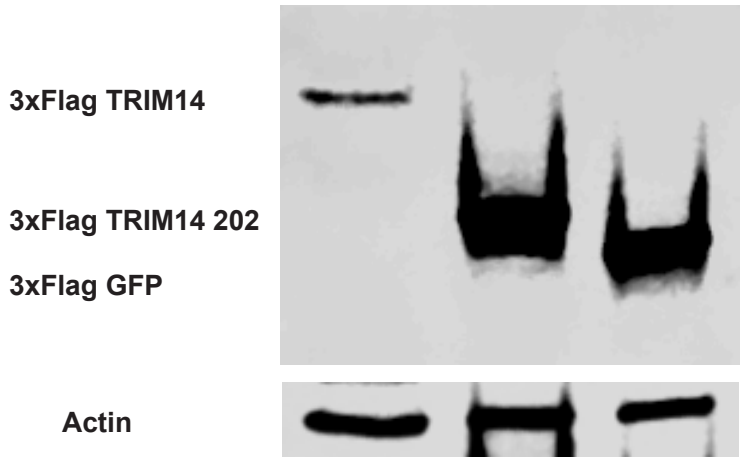


Figure 31 Trim14 KO Macrophages expressing TRIM14 and TRIM14 202

Western blot analysis of Trim14 KO RAW264.7 cells stably expressing either 3xFlag TRIM14, 3xFlag TRIM14 202, or 3xFlag GFP.

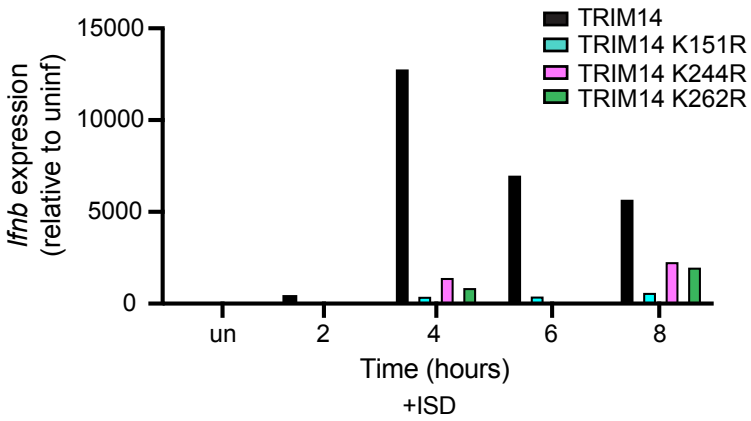


Figure 32 RAW 264.7 Macrophages expressing TRIM14 Ub Mutants

RT-qPCR of *ifnb* in RAW 264.7 cells stably expressing TRIM14 lysine mutants, stimulated with ISD.

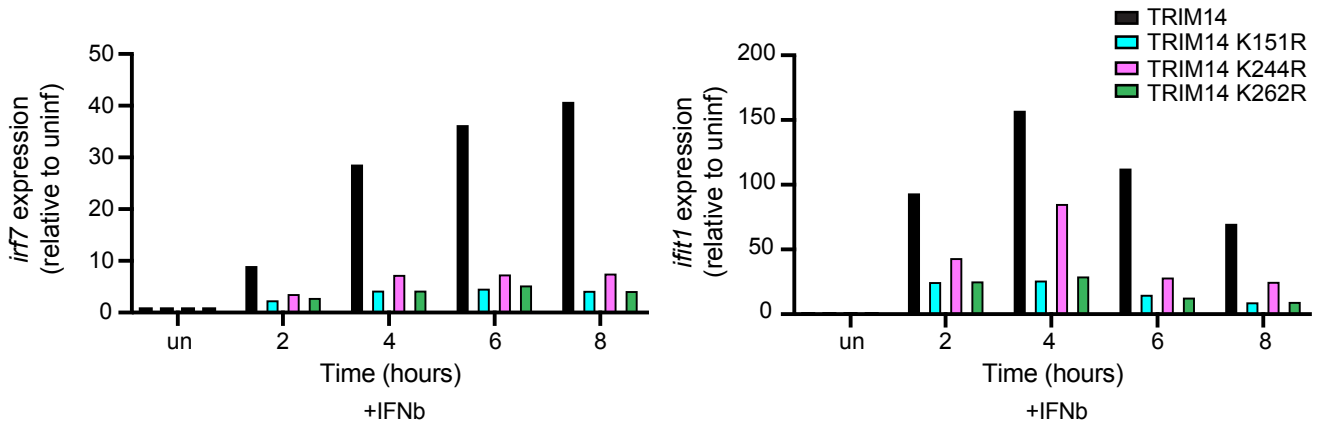


Figure 33 RAW 264.7 Macrophages expressing TRIM14 Ub Mutants

RT-qPCR of *irf7* and *ifit1* in RAW 264.7 cells stably expressing TRIM14 lysine mutants, stimulated with IFNβ.