The impact of ISGylation during *Mycobacterium tuberculosis* infection in mice

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20 **Abstract**

21 *Mycobacterium tuberculosis* infection results in 1.5 million deaths annually. Type I
22 interferon (IFN) signaling through its receptor IFNAR correlates with increased severity
23 of disease, although how this increases susceptibility to *M. tuberculosis* remains
24 uncertain. ISG15 is one of the most highly induced interferon stimulated genes (ISGs)
25 during *M. tuberculosis* infection. ISG15 functions by conjugation to target proteins
26 (ISGylation), by noncovalent association with intracellular proteins, and by release from
27 the cell. Recent studies indicated that ISG15 can function via conjugation-independent
28 mechanisms to suppress the type I IFN response. These data raised the question of
29 whether ISG15 may have diverse and sometimes opposing functions during *M.*
30 *tuberculosis* infection. To address this, we analyzed ISGylation during *M. tuberculosis*
31 infection and show that ISGylated proteins accumulate following infection in an IFNAR-
32 dependent manner. Type I IFN and ISG15 both play transient roles in promoting
33 bacterial replication. However, as the disease progresses, ISGylation deviates from the
34 overall effect of type I IFN and, ultimately, mice deficient in ISGylation are significantly
35 more susceptible than IFNAR mice. Our data demonstrate that ISGs can both protect
36 against and promote disease and are the first to report a role for ISGylation during *M.*
37 *tuberculosis* infection.

38 **Keywords:** mycobacteria; ISG15; interferon; pathogenesis; infection; immune response

1. Introduction

As one of the most successful pathogens in the world, *Mycobacterium tuberculosis* was reported to cause 9 million new cases of tuberculosis (TB) and 1.5 million TB related deaths in 2014 [1]. TB is a complex disease comprised of multiple stages that involve pathologies derived from both the pathogen and the host immune response to the infection. Primary infection with *M. tuberculosis* occurs when aerosolized bacteria are inhaled and phagocytosed by alveolar macrophages. The interaction of *M. tuberculosis* with recruited macrophages leads to a proinflammatory response and the recruitment of inflammatory cells to form a granuloma [2]. In an immunocompetent individual, T lymphocytes recruited to the granuloma secrete cytokines to activate a robust anti-mycobacterial defense that controls the primary infection by reducing bacterial numbers to uncultivable levels. However, due to the delayed accumulation of T cell responses within the lung, humans are unable to completely clear the *M. tuberculosis* infection, and instead develop a clinical state referred to as latency [3]. Approximately 15–20% of immunocompetent people who become tuberculin skin test positive after exposure to *M. tuberculosis* reactivate the infection after a variable period of latent infection. Reactivated infection leads to acute TB where the immune system is unable to control *M. tuberculosis* replication and the individual experiences the clinical symptoms of TB. The immune response to *M. tuberculosis* infection shapes much of the disease itself and a better understanding of the immune response to infection is imperative to develop new strategies to combat the TB epidemic.

An important immune response to infection is the production of interferons (IFNs). Type

II IFN (IFN- γ) is produced mainly by activated T lymphocytes and NK cells and is a potent activator of antimicrobial activities in macrophages [4]. It is well established that IFN- γ is critical for host resistance to intracellular bacterial infection, including *M. tuberculosis*, and deficiencies in IFN- γ signaling result in dramatic susceptibility to mycobacterial infections in both humans and mice [5–7]. Type I IFN (predominantly IFN- α and IFN- β) have been best studied during viral infection where they are critical for establishing an anti-viral immune response, and mice lacking type I IFN signaling are highly sensitive to viral infection [8]. In contrast to its role in viral infection, induction of type I IFN signaling has been shown to exacerbate intracellular bacterial infections including *Listeria monocytogenes* [9], *Francisella tularensis* [10], and *M. tuberculosis* [11–16]. Hypervirulent strains of *M. tuberculosis* induce higher levels of type I IFN than less virulent isolates [13,17] and leukocytes from patients with active, uncontrolled *M. tuberculosis* infection express higher levels of type I IFN-inducible genes than latently infected or uninfected people [18]. Understanding the roles of type I IFN signaling is essential for understanding TB disease pathology and outcomes.

Most reports of *M. tuberculosis* infection of mice lacking the type I IFN receptor, IFNAR, support a role for type I IFN signaling in exacerbating *M. tuberculosis* infection. C57BL/6 *IFNAR*^{-/-} mice infected with *M. tuberculosis* have 3 – 5 fold lower bacterial titers in the spleen [15], lung [16], and lymph node [16] during acute infection as compared to controls. Similarly, *IFNAR*^{-/-} mice in a 129 background showed increased control of both virulent laboratory strains and hypervirulent clinical strains of *M. tuberculosis*, resulting in 0.5 – 2 logs lower bacterial titers in the lungs [11,14] and increased survival [11,17]. Additionally, exogenously stimulating type I IFN signaling during *M. tuberculosis*

infection of mice leads to an increase in pulmonary burden and necrosis in lesions, coupled with decreased survival [12,17]. However, studies in mice from different genetic backgrounds have reported higher bacterial burden early during *M. tuberculosis* infection in the absence of IFNAR [19], suggesting some complexities in this system.

The mechanisms by which type I IFN impacts *M. tuberculosis* infection and whether it predominantly affects bacterial replication and/or inflammation is not well defined, but it presumably involves one or more of the interferon stimulated genes (ISGs) [20]. Type I IFN signaling through IFNAR activates transcription of over 100 ISGs and several of these have been shown to be upregulated during *M. tuberculosis* infection [21,22]. One of the most highly induced proteins by type I IFN [20] and during *M. tuberculosis* infection [21], is ISG15, a member of the ubiquitin family that can be conjugated to target proteins in a process termed ISGylation. Similar to ubiquitin, ISGylation requires the coordinated activities of three enzymes: the activating E1, the conjugating E2, and the ligating E3 enzymes [23]. UbE1L is the only known E1 enzyme for ISG15 and is necessary for most of the ISG15 conjugation in the cell [23]. As such, *UbE1L*^{-/-} mice lack almost all ISG15 conjugates but have higher than normal levels of free ISG15 [24]. Unlike ubiquitination, ISGylation does not target proteins to the proteasome, but instead affects protein activity or localization [23,25]. ISG15 can also function in a non-conjugated form by blocking intracellular protein-protein interactions [23,26,27] or by being released into the extracellular milieu [28]. Several immunomodulatory activities have been attributed to extracellular ISG15, including its ability to induce T cell-dependent NK cell proliferation, augment lymphokine activated killer activity, stimulate the production of IFN- γ , induce DC maturation, and function as a chemotactic factor for

neutrophils [23,29,30]. ISG15 does not contain a signal sequence and no cell surface receptor has been identified. Therefore, the mechanism of ISG15's cytokine-like effects is yet to be ascertained.

Bogunovic *et al.* recently reported that a group of patients with Mendelian susceptibility to mycobacterial diseases have mutations in *ISG15* that prevent its expression [31]. The authors show that ISG15 functions through an extracellular cytokine-like mechanism to control *M. tuberculosis* infection by synergizing with IL-12 to induce secretion of IFN- γ from T and NK cells. Consequently, the authors conclude that ISG15-dependent production of IFN- γ is diminished in ISG15^{-/-} patients, which leads to improper control of *Mycobacterium bovis* Bacille Calmette-Guérin (BCG) infection. In a follow-up report, Zhang *et al.* show that the absence of intracellular ISG15 in patients' cells also prevents the accumulation of USP18, a potent negative regulator of type I IFN [32]. For both the extracellular and intracellular activities of ISG15 presented by these studies, conjugation of ISG15 to its targets is proposed to not be required. These findings raised a number of questions, including what contribution conjugated, free, and released ISG15 have on the type I IFN response to *M. tuberculosis* infection and whether ISG15 always counteracts the type I IFN response during *M. tuberculosis* infection. We have investigated these questions by analyzing the production of ISG15 and its conjugates during *M. tuberculosis* infection and characterizing the outcome of *M. tuberculosis* infection in mice deficient in type I signaling (*IFNAR*^{-/-}), ISG15 (*ISG15*^{-/-}), or ISG15 conjugates (*UbE1L*^{-/-}).

2. Materials and methods

129 2.1. Bacteria and media

130 *Mycobacterium tuberculosis* Erdman was cultured at 37 °C in 7H9 (broth) or 7H10
131 (agar) (Difco) medium supplemented with 10% oleic acid/albumin/dextrose/catalase
132 (OADC), 0.5% glycerol, and 0.05% Tween 80 (broth).

133 2.2. Mice

134 Mice were bred and housed at Washington University School of Medicine in accordance
135 with all federal and university guidelines, under specific-pathogen-free conditions (8).
136 Wild-type C57BL/6J mice (Catalog no. 000664) were purchased from Jackson
137 laboratory (Bar Harbor, ME), bred and maintained in our facilities. *ISG15*^{-/-} mice
138 (provided by Dr. Klaus-Peter Knobeloch; University Clinic Freiburg, Germany) and
139 *Ube1L*^{-/-} mice (provided by Dr. Dong-Er Zhang; University of California San Diego
140 School of Medicine) were generated as previously described [33,34]. *Ube1L*^{-/-} and
141 *ISG15*^{-/-} mice were fully backcrossed (>99% identity) to C57BL/6; by congenic SNP
142 analysis through Taconic Laboratories (Hudson, NY). *IFNAR*^{-/-} mice were initially
143 obtained from M. Aguet at the Swiss Institute of Experimental Cancer Research
144 (Epalinges, Switzerland) and were fully backcrossed onto the C57BL/6 background.

145 2.3. Infection of mice with *M. tuberculosis*

146 Before infection, exponentially replicating *M. tuberculosis* bacteria were washed in PBS
147 + 0.05% Tween 80, and sonicated to disperse clumps. 7- to 10-week-old female mice
148 were exposed to 8×10^7 colony forming units (CFU) of *M. tuberculosis* in an Inhalation

Exposure System (Glas-Col), which delivers ~100 bacteria to the lung per animal. Bacterial burden was determined by plating serial dilutions of lung and spleen homogenates onto 7H10 agar plates. Plates were incubated at 37 °C in 5% CO₂ for 3 weeks prior to counting colonies. All procedures involving animals were conducted following the National Institute of Health guidelines for housing and care of laboratory animals and performed in accordance with institutional regulations after protocol review and approval by the Institutional Animal Care and Use Committee of The Washington University in St. Louis School of Medicine (protocol #20130156, Analysis of Mycobacterial Pathogenesis). Washington University is registered as a research facility with the United States Department of Agriculture and is fully accredited by the American Association of Accreditation of Laboratory Animal Care. The Animal Welfare Assurance is on file with OPRR-NIH. All animals used in these experiments were subjected to no or minimal discomfort. All mice were euthanized by CO₂ asphyxiation, which is approved by The Panel on Euthanasia of the American Veterinary Association.

2.4. Immunoblot analysis

Lungs and spleen homogenate were diluted with 2X Laemmli buffer and resolved by SDS-PAGE followed by transfer to PVDF membrane. Membranes were incubated with a polyclonal serum against ISG15 [35] or β -actin (Sigma-Aldrich).

2.5. Histology of infected organs

For histology, mouse lungs were fixed and stored in 10% buffered formalin until processing. Lungs were then dehydrated using ethanol and processed by The Elvie L.

170 Taylor Histology Core Facility at Washington University, where they were paraffin-
 171 embedded, sectioned, and stained with haemotoxylin-and-eosin. Slides were visualized
 172 using an Olympus BX51 light microscope (Olympus) equipped with a MicroPublisher 5.0
 173 digital camera (Q Imaging).

174 2.6. RNA extraction and quantification.

175 Following tissue disruption by bead-beating (MP Biosystems), RNA was extracted from
 176 *M. tuberculosis*-infected lungs and spleens using the RNeasy Kit according to
 177 manufacturer's guidelines (Qiagen 74106). cDNA was made with SuperScript III reverse
 178 transcriptase using oligo-dT primers (Life Technologies 18080-051). Quantitative real-
 179 time PCR (qRT-PCR) was performed using iTAQ SYBR Green (BioRad 172-5121).
 180 Transcript levels were normalized to actin and expressed as a fold change as compared
 181 to WT samples from that time point. Statistical differences were analyzed with one-way
 182 ANOVA followed by Tukey's multiple comparison test. *ifnb1* was detected with
 183 PrimePCR assay (BioRad 10025636), the remaining genes were detected using the
 184 following primers: *actin*- ACCTTCTACAATGAGCTGCG and
 185 CTGGATGGCTACGTACATGG; *ifng*- CCTAGCTCTGAGACAATGAACG and
 186 TTCCACATCTATGCCACTTGAG; *tnfa*- CTTCTGTCTACTGAACTTCGGG and
 187 CAGGCTTGTCACCTCGAATTTTG; *il12a* (p40)- ACTCCCCATTCCTACTTCTCC and
 188 CATTCCCGCCTTTGCATTG; *il12b* (p35)- ACAGATGACATGGTGAAGACG and
 189 TCGTTCTTGTGTAGTTCCAGTG; *ifit1*- AGAGTCAAGGCAGGTTTCTG and
 190 TGTGAAGTGACATCTCAGCTG; *ifi44*- CCCCTGCCATTTATTCTGTGT and
 191 CGGATGGTTTGATGTGATTGG

3. Results

3.1. Free and conjugated ISG15 accumulate during *M. tuberculosis* infection in a type I IFN-dependent manner.

Interfering with type I IFN signaling by deletion of IFNAR is associated with greater resistance to *M. tuberculosis* during chronic infection [14] and, therefore, we hypothesized that ISGs, including ISG15, would be expressed and function at this same time. To determine whether this was the case, we monitored the accumulation of ISGylated proteins in the lungs and spleens of C57BL/6 mice (wild-type, WT) before and after infection with aerosolized *M. tuberculosis*. As previously reported [36], there is a basal amount of free ISG15 (15 kDa) expressed in WT uninfected mouse lungs and spleens, but relatively low amounts of ISG15 conjugates (>20 kDa, multiple bands) (Fig. 1A and 1B). The levels of free ISG15 and ISG15-conjugates were increased in the lungs (Fig. 1A) and spleens (Fig. 1B) of WT mice at 77, 100, and 245 days post-infection, demonstrating that *M. tuberculosis* infection induces an accumulation of ISG15 and ISGylated proteins.

Uninfected mice lacking IFNAR had dramatically lower levels of free ISG15 and ISGylated proteins in their lungs and spleens as compared to WT mice (Fig. 1A and 1B). In addition, the levels of free ISG15 and ISGylated proteins remained lower in *IFNAR*^{-/-} mice following *M. tuberculosis* infection as compared to infected WT mice. These data show that ISG15 production and ISGylation in uninfected mice as well as during *M. tuberculosis* infection is largely type I IFN-dependent. Notably, there is a

detectable induction of free ISG15 and ISGylated proteins at 77 dpi in the IFNAR^{-/-} mice, suggesting that *M. tuberculosis* infection can induce ISG15 activity via a type I IFN-independent mechanism. As expected, ISG15^{-/-} mice have no detectable ISG15 in lungs and spleens before or after *M. tuberculosis* infection. Mice deficient in UbE1L accumulate free ISG15 in the lung and spleen throughout chronic *M. tuberculosis* infection but do not generate the majority of ISG15 conjugates, as expected given the role of UbE1L as the E1 ligase for ISG15 [24]. The identities of the protein species larger than free ISG15 in UbE1L^{-/-} lungs at some timepoints (Fig. 1A) are unknown, but may represent ISG15 conjugates that are formed independently of UbE1L

Taken together, these data demonstrate that ISG15 and ISGylation is upregulated in response to *M. tuberculosis* infection in a largely type I IFN dependent manner and this expression is sustained through 245 days post-infection, indicating a continual type I IFN response to the bacterial infection. UbE1L^{-/-} mice lack most ISGylated proteins, but produce similar and sometimes higher levels of free ISG15 in the lung and spleen. Together these data provide the first documentation that ISG15 and ISGylated proteins are induced by *M. tuberculosis* infection and sustained throughout chronic infection.

3.2. Loss of ISG15 expression and type I IFN signaling have transient effects on bacterial burden during *M. tuberculosis* infection in mice.

Type I IFN signaling has been reported to have both positive and negative effects on bacterial burdens during *M. tuberculosis* infection [11,14–16,19]. To determine the contribution of ISG15 in the type I IFN response to *M. tuberculosis* infection, bacterial

titers were monitored in mice lacking IFNAR or ISG15 during both acute and chronic infection. Bacterial titers in the lungs of infected *IFNAR*^{-/-} mice were significantly lower than in WT mice during acute infection (Fig. 2A, 71% lower at 7 days post infection). The bacterial burdens in the lungs of *IFNAR*^{-/-} mice approached WT levels as the infection progressed, starting at 21 days post infection, which corresponds with the accumulation of IFN-γ producing T cells in the lung [37]. These data indicate that type I IFN has a transient role in promoting higher bacterial titers in the lungs, which wanes over time in the mouse model of infection.

Loss of ISG15 (*ISG15*^{-/-} mice) resulted in significantly lower bacterial burden in lungs at 77 days post *M. tuberculosis* infection (Fig. 2A) indicating that the production of ISG15 by WT mice is detrimental at this timepoint. Similar to the trend observed in *IFNAR*^{-/-} mice, the bacterial burdens in the lungs of *ISG15*^{-/-} mice approached the levels observed in WT mice as the infection progressed. Thus, ISG15's role in promoting higher bacterial burdens in the lungs of infected mice is also transient despite the continued type I IFN-dependent accumulation of ISG15 throughout chronic infection (Fig. 1A).

Analysis of spleens from infected mice revealed that at 7 days post infection, *ISG15*^{-/-} mice had significantly lower (79% lower) bacterial burden in their spleens than WT mice, with two *ISG15*^{-/-} mice having bacterial burdens at this time point that were lower than the limit of detection of 10 CFU (Fig. 2B). At this timepoint, *IFNAR*^{-/-} mice also appear to have lower burden in the spleen (one mouse was below the limit of detection), however no significant difference was found at this timepoint when comparing *IFNAR*^{-/-} mice to

either WT or *ISG15*^{-/-} mice. At 77 days post infection, spleens from *IFNAR*^{-/-} mice had significantly less bacterial burden as compared to WT mice, indicating that IFNAR signaling in WT mice is detrimental at this timepoint. Interestingly, bacterial burdens in the spleens of *ISG15*^{-/-} mice were significantly higher than *IFNAR*^{-/-} mice at this time point (P value < 0.01 by Kruskal-Wallis with Dunn's Multiple Comparison test), and similar to WT mice. Therefore, at 77 days post *M. tuberculosis* infection in the spleen, the resistance to *M. tuberculosis* replication conferred by loss of the type I IFN response can not be attributed to loss of ISG15 induction, indicating other ISGs are likely playing a significant role in the response in this organ at this time point. By 100 days post infection, the bacterial titers in WT, *IFNAR*^{-/-}, and *ISG15*^{-/-} mice were no longer significantly different, illustrating a similar transient effect as observed in the lung where bacterial burdens wane as the disease progresses. To determine whether ISG15's role in promoting higher bacterial burdens requires its conjugation to target proteins, we monitored the bacterial titers in *M. tuberculosis* infected *UbE1L*^{-/-} mice, which are unable to produce most ISG15 conjugates, but retain WT levels of free ISG15 in lungs and spleens (Fig. 1A and 1B). The bacterial burdens in *M. tuberculosis* infected *UbE1L*^{-/-} mice displayed similar trends as observed in *ISG15*^{-/-} mice during both acute and chronic infection (Fig. 2), however, the bacterial titers were never significantly different than those observed in WT mice. Together, these data show that both ISG15 and type I IFN signaling can promote higher *M. tuberculosis* burden during infection, however these effects vary depending on both the organ and timepoint being analyzed.

3.3. ISGylation differs in its impact on mouse survival and cytokine responses following *M. tuberculosis* infection as compared to loss of type I IFN signaling.

Deletion of IFNAR in a 129 background results in improved survival following *M. tuberculosis* infection [11,14,17], however, whether this would be true in C57BL/6 mice remained to be tested. By monitoring survival in our infection experiments, we observed that the median survival time of *M. tuberculosis* infected *IFNAR*^{-/-} mice (C57BL/6 background) was 64 days later than the WT controls (Fig. 3A). In contrast, the median survival time of *M. tuberculosis* infected *ISG15*^{-/-} mice was 23 days earlier than WT mice (Fig. 3A and [31]) and 87 days earlier than *IFNAR*^{-/-} mice. However, the survival times of these mice were not significantly different from each other.

Previous reports using ISG15-deficient human cells attribute the role for ISG15 in protecting the host from mycobacterial infections to the free forms of ISG15, and not dependent on ISGylation [31,32]. To directly test if ISGylation was important for promoting survival of *M. tuberculosis* infected mice, we also monitored the survival of infected *Ube1L*^{-/-} mice as compared to WT, *IFNAR*^{-/-}, and *ISG15*^{-/-} mice. *Ube1L*^{-/-} mice succumbed to *M. tuberculosis* infection significantly more rapidly than *IFNAR*^{-/-} mice (Fig. 3A), with a median survival time of 120 days earlier than the *IFNAR*^{-/-} mice. These data demonstrate that even though the accumulation of ISGylated proteins during *M. tuberculosis* infection occurs mostly in a type I IFN dependent manner (Fig. 1), the role of ISGylation in promoting survival of infected mice diverges from the negative effect of the type I signaling pathway as a whole on the survival of mice infected with *M. tuberculosis*.

Although *IFNAR*^{-/-} mice survive significantly longer than *Ube1L*^{-/-} mice following *M. tuberculosis* infection, there is no difference in bacterial burden between any of the

strains tested at these later time points (Fig. 3B). This suggests that the altered survival is likely related to differences in pathology and host tissue damage as opposed to bacterial replication. To examine this possibility we performed histological analysis of the infected lung tissue at 100 and 275 days post-infection (Fig. 3B) and found that the lungs from all strains of mice contained numerous lesions at these late time points and there were no obvious differences in lesion structure. Similar results were observed in the spleens at 100 days post-infection. This does not exclude differences in the inflammatory response that are not discernable by this method and determining the differences in the responses of these mice that affect survival outcomes will require further investigation.

The decreased survival observed with *ISG15*^{-/-} mice during *M. tuberculosis* infection reproduces a similar finding by Bogunovic *et al.* [31]. Bogunovic *et al.* concluded that the importance of ISG15 in human cells to control mycobacterial infections was related to its ability to synergize with IL-12 to induce secretion of IFN- γ from human T and NK cells. In a follow-up report, Zhang *et al.* show that in human cells ISG15 can allow for the accumulation of USP18, a potent negative regulator of type I IFN. In the absence of ISG15, USP18 accumulation does not occur and type I IFN responses are enhanced [32]. Given these data, we investigated whether there were differences in type I and type II IFN signaling in the *ISG15*^{-/-} and WT mice by analyzing the transcript levels of *ifng*, *tnfa*, *il12a*, and *il12b* for type II IFN signaling and *ifnb1*, *ifit1*, and *ifi44* for type I IFN signaling in lungs and spleens at 77 days post-infection (Fig. 3C). *ifit1*, and *ifi44* were chosen because these transcripts were shown in the Zhang *et al.* report to be affected by ISG15 in human cells [32]. We found that *ifng*, *tnfa*, *il12a*, and *il12b* transcripts were

mostly unaffected by loss of ISG15, except for *il12a*, which was actually significantly higher in the lungs of *ISG15*^{-/-} mice compared to WT mice. This corroborates a previous report showing that loss of ISG15 in mice does not affect IFN- γ production and secretion [34]. Some type I IFN associated transcripts were significantly affected by loss of IFNAR in the spleen, but not the lung. *ifnb1*, *ifit1*, and *ifi44* transcript levels were lower in the spleens of *IFNAR*^{-/-} mice as compared to WT mice, with *ifnb1* and *ifi44* being statistically significantly lower, supporting their regulation by type I IFN. In *ISG15*^{-/-} mice, *ifnb1* and *ifi44* transcript levels were unchanged from WT mice, indicating that ISG15 did not regulate the expression of these transcripts. In contrast, *ifit1* transcripts were significantly more abundant in the spleens of *ISG15*^{-/-} mice compared to WT and *IFNAR*^{-/-} mice, demonstrating that this transcript is divergently regulated by ISG15 and type I IFN. The detection of effects of loss of type I IFN signaling or ISG15 on cytokine expression specifically in the spleen and not the lung may represent cell-type or organ-specific roles for these pathways during *M. tuberculosis* infection.

Previous reports in human cells show that it is the free, unconjugated form of ISG15 that affects IFN signaling[31,32]. To determine whether ISGylation was important for the effects of ISG15 on type I IFN regulated gene expression in mice during *M. tuberculosis* infection, we analyzed the same transcripts in the lungs and spleens of *Ube1L*^{-/-} mice. The trends for the transcript levels in mice deficient in ISGylation were similar to those deficient in ISG15 (Fig. 3C), indicating that the effect of ISG15 on *ifit1* is most likely due to its conjugated form. This correlates with our data that *ISG15*^{-/-} mice were not more susceptible to *M. tuberculosis* infection than *Ube1L*^{-/-} mice and supports that the intracellular and released forms of free ISG15 do not have a significant impact on the

response of mice to *M. tuberculosis* infection. Therefore, in both mice and humans, ISG15 negatively affects the expression of *ifit1* during *M. tuberculosis* infection, however the mechanisms by which ISG15 achieves this diverge where in humans the effect appears independent of ISGylation as opposed to ISGylation-dependent in mice.

4. DISCUSSION

Type I IFN signaling has been correlated to increased severity of tuberculosis disease in both humans and mice [11,13,16–18,38,39]. In mice, neutralization of type I IFN during *M. tuberculosis* infection leads to increased levels of IL-12, IL-1 α , and IL-1 β , which are important to develop the adaptive immune response that controls *M. tuberculosis* [17,40]. Exogenous stimulation of type I IFN signaling during *M. tuberculosis* infection of mice results in decreased levels of the protective cytokines TNF- α and IL-12 [12,13]. A recent study of IFN responses in human leprosy revealed a correlation between type I IFN signaling and progressive lepromatous lesions and showed that an IFN- γ -induced antimicrobial response was inhibited by IFN- β , which may explain the greater severity of disease [41]. Similarly, experiments with *L. monocytogenes* indicate that type I IFN induction results in reduced IFN γ -dependent gene expression and reduced expression of the IFN γ receptor by both infected and bystander macrophages [42]. Together these data suggest that signaling through IFNAR has an immunosuppressive effect during bacterial infection. However, *in vitro* *M. tuberculosis* infection of bone marrow derived macrophages has shown that type I IFN signaling is required for early production of inducible nitric oxide synthase [43], an important defense against *M. tuberculosis*. In addition, type I IFNs were found to have a beneficial effect against pulmonary TB in a

clinical trial [44]. Type I IFN signaling also serves a protective function in the absence of IFN- γ signaling [16]. These reports highlight the complex functions of type I IFN and the difficulty of defining a clear role for this cytokine during *M. tuberculosis* infection despite its importance in the immune response. Notably, IFN- α and IFN- β are also administered clinically to treat diseases such as viral infection, some cancers, and multiple sclerosis [45]. Given that these treatments can cause reactivation of latent *M. tuberculosis* [46,47], it is imperative to understand the downstream effects of these cytokines on *M. tuberculosis* infection and disease outcome.

The complicated and sometimes contradictory effects of type I IFN are likely due in part to the fact that signaling through IFNAR activates transcription of over 100 ISGs. Several of these ISGs have been shown to be upregulated during *M. tuberculosis* infection [21,22], however, the roles of individual ISGs during bacterial infections remain mostly unknown. In this report we investigate the role of two ISGs, ISG15 and Ube1L. Previous studies have detected ISG15 production following *M. bovis* BCG infection of cells in culture [31] and upon treatment of cultured cells with Gram-negative lipopolysaccharide [33]. However, this manuscript is the first to report the induction of ISG15 protein and ISGylation during *M. tuberculosis* infection *in vivo*. We have shown that this induction is sustained throughout chronic infection in the mouse model of infection and is largely type I IFN-dependent. However, at 77 dpi, some ISG15 and ISGylation is induced in IFNAR^{-/-} mice, which may relate to a recent report demonstrating that *Listeria* infection induces ISG15 expression and ISGylation in a type I IFN-independent manner [48]. During *M. tuberculosis* infection, type I IFN signaling and ISG15 make organ-dependent and time-dependent contributions to the control of

bacterial burden and cytokine expression. As the disease progresses, UbE1L deviates from the effects of type I IFN and, ultimately, ISGylation protects the mouse from succumbing to *M. tuberculosis* infection. In contrast, at these late stages of infection, type I IFN is detrimental to the survival of the mouse [11,17]. The absence of differences in bacterial burden between any of the strains tested positions the type 1 IFN response as more important for controlling inflammation than controlling bacterial titers. Importantly, in the absence of type I IFN signaling, a decrease in ISGylation is no longer detrimental, as evidenced by the improved survival of the *IFNAR*^{-/-} mice. This may reflect the importance of ISG15-mediated repression of the expression of certain ISGs that will otherwise not be induced in the *IFNAR*^{-/-} mice during *M. tuberculosis* infection, rendering ISG15 unnecessary.

A recent report showed that both *ISG15*^{-/-} and *UbE1L*^{-/-} mice exhibit increased susceptibility to lethality during Influenza A virus and Sendai virus infections without affecting viral replication [49]. During Influenza A virus and Sendai virus infections, loss of ISGylation did not significantly alter the recruitment of inflammatory cells to the lungs after infection. Rather, the modification of protein targets by ISG15 altered the ability of the respiratory epithelium to tolerate and recover from damage induced during infection. It is possible that a similar wound healing mechanism is involved in the susceptibility of *UbE1L*^{-/-} mice to *M. tuberculosis*. However, this will require further investigations to determine if this is the case.

A number of questions remain regarding the role of ISGylation during *M. tuberculosis* infection, including what the targets of ISGylation are during infection, whether they are

host- and/or pathogen-derived, and which target(s) function to allow increased bacterial titers versus better host survival. Both host and virus-derived targets have been described for ISG15 [23]. In addition, viruses have evolved mechanisms of inhibiting ISGylation [50], and it is possible that bacteria have also evolved mechanisms to modulate this system, however this concept has yet to be investigated. Future mechanistic studies will be necessary to evaluate these possibilities and to elucidate the complex role of ISG15 in an already complicated type I IFN response to bacterial infections. Our findings demonstrate that the type I IFN response to *M. tuberculosis* infection induces both protective and harmful effects for the host, and raises the question of whether the activities of certain ISGs are balanced differently in individual people to lead to different disease outcomes, possibly contributing to the variability in reactivation.

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579

580 **FIGURE LEGENDS**

581 **Figure 1. Expression of free ISG15 and ISG15-conjugates is induced in the lungs**
 582 **and spleens of *M. tuberculosis* infected mice and is dependent on type I IFN**
 583 **signaling. (A) Lung and (B) spleen homogenates from C57BL/6 WT (WT), *IFNAR*^{-/-}**

584 (IFNAR), *ISG15*^{-/-} (ISG15), and *UbE1L*^{-/-} (UbE1L) mice, analyzed for expression of free
 585 ISG15 (15 kDa) and ISGylated conjugates (>20 kDa, various bands) by immunoblot.
 586 Each lane represents an organ homogenate collected from an individual mouse either in
 587 the absence of infection (uninfected) or following aerosol infection with *M. tuberculosis*
 588 at various days post infection (dpi). Actin (lower panels) was detected as a loading
 589 control.

590 **Figure 2. The effects of mice lacking type I IFN signaling, ISG15, or ISGylation on**
 591 **bacterial burden during *M. tuberculosis* infection.** C57BL/6 WT (squares), *IFNAR*^{-/-}
 592 (circles), *ISG15*^{-/-} (triangles), and *UbE1L*^{-/-} (inverted triangles) mice were infected with
 593 aerosolized *M. tuberculosis* and the bacterial titers from (A) lungs and (B) spleens were
 594 determined at various time points of acute and chronic infection. The mean CFU/organ
 595 ± the SEM for at least 4 mice per strain from 2 separate infections is shown; each
 596 symbol represents an individual mouse. Statistical analysis was performed using
 597 Kruskal-Wallis with Dunn's Multiple Comparison test; * *P* value of <0.05, ** *P* value of
 598 <0.01, and *** *P* value of <0.001. n.s. is not statistically significant. Shown are the
 599 statistical analyses of each strain compared to WT.

600 **Figure 3. Effects of mice lacking type I IFN signaling, ISG15, or ISGylation on**
 601 **survival following *M. tuberculosis* infection.** (A) The survival of C57BL/6 WT
 602 (squares, n=14), *IFNAR*^{-/-} (circles, n=11), *ISG15*^{-/-} (triangles, n=15), and *UbE1L*^{-/-}
 603 (inverted triangles, n=14) mice infected with aerosolized *M. tuberculosis* was monitored.
 604 The median survival time of *IFNAR*^{-/-} mice was 65 days longer than C57BL/6 (WT) mice,
 605 while *ISG15*^{-/-} mice had a median survival time of 87 days earlier than *IFNAR*^{-/-} mice.

606 Mice lacking UbE1L displayed significantly increased susceptibility to *M. tuberculosis*
607 infection as compared to *IFNAR*^{-/-} mice, with a median survival time of 120 days earlier
608 than the *IFNAR*^{-/-} mice (** *P* value of <0.01, Mantel-Cox test). (B) Histology of lungs and
609 spleens stained with haemotoxylin-and-eosin (H&E) and visualized with a light
610 microscope. (C) Analysis of transcriptional responses in whole lung or spleen samples
611 taken at 77 days post-infection (dpi). Transcript levels were normalized to actin, and are
612 expressed as a fold change as compared to WT samples from that time point. Statistical
613 differences were analyzed with one-way ANOVA followed by Tukey's multiple
614 comparison test. *P* values: * *P* < 0.05, ** *P* < 0.01, *** *P* < 0.001. If no asterisks are
615 shown, the comparison was not significantly different.

616

Figure 1

Figure 2

A

B

Figure 3

A

B

C