Contribution of increased ISG15, ISGylation and deregulated type I IFN signaling in Usp18 mutant mice during the course of bacterial infections

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Host genetics has a key role in susceptibility to Salmonella Typhimurium infection. We previously used N-ethyl-N-nitrosourea (ENU) mutagenesis to identify a loss-of-function mutation within the gene ubiquitin-specific peptidase 18 (Usp1889), which confers increased susceptibility to Salmonella Typhimurium. Usp18 functions to regulate type I interferon (IFN) signaling and as a protease to remove ISG15 from substrate proteins. Usp1889 mice are susceptible to infection with Salmonella Typhimurium and have increased expression and function of ISG15, but Usp1889 mice lacking Isg15 do not show improved survival with Salmonella challenge. Type I IFN signaling is increased in Usp1889 mice and inhibition of type I IFN signaling is associated with improved survival in mutant mice. Hyperactivation of type I IFN signaling leads to increased IL-10, deregulated expression of autophagy markers and elevated interleukin (IL)-1ß and IL-17. Furthermore, Usp1889 mice are more susceptible to infection with Mycobacterium tuberculosis, have increased bacterial load in the lung and spleen, elevated inflammatory cytokines and more severe lung pathology. These findings demonstrate that regulation of type I IFN signaling is the predominant mechanism affecting the susceptibility of Usp1889 mice to Salmonella infection and that hyperactivation of signaling leads to increased IL-10, deregulation of autophagic markers and increased proinflammatory cytokine production.

INTRODUCTION
Salmonella enterica are facultative, intracellular, Gram-negative enterobacteria that cause a range of enteric diseases in mammalian hosts. The human-restricted serovars S. enterica Typhi and Paratyphi are the causal agents of Typhoid fever, affecting > 27 million people worldwide and resulting in >200 000 deaths each year through contaminated food and drinking water.1 In contrast, infection with the S. enterica serovar Typhimurium results in a self-limiting gastroenteritis in humans but, in mice, is an established model of fatal systemic disease, whereby infection leads to dissemination of the bacteria to the spleen and liver and activation of both innate and adaptive immune responses. Typhoid fever remains an important global health issue due to geographic spread as a result of foreign travel to areas of endemicity, including Africa and Asia.

The outcome and severity of infection with Salmonella is dependent on several parameters, including microbial virulence factors, environment, immune status and host genetics. Numerous quantitative trait loci influencing microbial pathogenicity have been discovered from the inherent differential susceptibility of inbred mouse strains.2 However, to identify additional gene candidates important during Salmonella infection, we have used a large-scale N-ethyl-N-nitrosourea mutagenesis screen. Our N-ethyl-N-nitrosourea screen has previously identified a loss-of-function mutation within the gene ubiquitin-specific peptidase 18 (Usp1889), which confers increased susceptibility to Salmonella Typhimurium.3 We have previously shown that the decreased survival in mice that carry a point mutation in Usp18 results from increased bacterial load in the spleen and liver, an increased inflammatory response and increased type I interferon (IFN) signaling through signal transducer and activator of transcription factor 1 (STAT1) activation.3 USP18 functions both to regulate the type I IFN signaling pathway and, independently, as a protease to remove ISG15 adducts from substrate proteins.4,5 However, the contribution of hyperactivation of type I IFN signaling and the host ISGylation pathway to the susceptibility of Usp1889 mice has not been fully characterized.

Although the role of type I IFN in the host response to viral infection is well established, its role during bacterial infection is more controversial with activities that are both favorable and detrimental for the host (reviewed in Trinchieri6 and Decker et al7). In addition to providing a protective role during infection, the production of type I IFN is also associated with suppression of the innate immune response through mechanisms that include decreasing the antibacterial production or function of IFN-γ,8 promoting the generation of IL-10-producing regulatory T cells9 and decreasing the recruitment of leukocytes to the site of infection.10 Thus, an increase in type I IFN signaling can lead to increased susceptibility of the host to bacterial infection.3,11

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Autophagy, a cellular degradation pathway, has emerged as a key component of the innate immune system through recognition and elimination of intracellular bacteria (reviewed in Deretic12). Upon entry into the cell, *Salmonella* reside within specialized vesicles called *Salmonella*-containing vacuoles. Damage to the *Salmonella*-containing vacuole membrane results in escape of the *Salmonella* to the cytosol and accumulation of polyubiquitinated proteins on the surface of the bacteria.13 Autophagy has been shown to limit the growth of *Salmonella* Typhimurium through association between ubiquitinated proteins and autophagic cargo receptors, including p62 (SQSTM1), NDP52 and optineurin (OPTN).4,11 Recognition of the bacteria by these receptors facilitates interaction with the autophagy-related gene 8/microtubule-associated protein 1 light chain 3 (ATG8/LC3) family of proteins at the phagophore, resulting in lysosomal fusion and degradation of the bacteria. Autophagy is regulated by both host and pathogen-derived immune signals. Several cytokines have been reported to have an important role in either upregulating (IFN-γ, tumor necrosis factor α (TNFα), IL-1β) or inhibiting (IL-4, IL-13, IL-10) autophagy.18 However, deregulation of autophagy as a consequence of increased cytokine production during *Salmonella* infection has not been described.

Given that USP18 suppresses type I IFN signaling and that the mutation in *Usp18*+/− mice lies within the IFNAR2-binding region of USP18,13,14 as well as the importance of type I IFN signaling in bacterial infection, we sought to determine whether the IFNAR regulatory function of USP18 is compromised in *Usp18*+/− mice and whether hyperactivation of type I IFN signaling contributes to the pathogenesis of infection. In this study, we show that *Usp18*+/− mice succumb to *Salmonella* infection due to an inability to regulate type I IFN signaling, which in turn results in increased IL-10 production and deregulation of autophagy cargo receptors. Furthermore, this influences the production of IL-1β and the amount of IL-17 produced from CD4+ T cells. These data demonstrate for the first time a link between type I IFN and autophagy in *Salmonella*-infected mice.

RESULTS

The genetic background significantly affects the expressivity of the *Usp18*+/− mutation

USP18 has two known functions, including the deconjugation of the ubiquitin-like modifier protein ISG15 from target proteins and the inhibition of type I IFN-induced Janus-activated kinase/STAT activation.15,19 To address the relative importance of these two functions in *Usp18*+/− mice, we first transfected the *Usp18*+/− allele from the mixed genetic background of C57BL/6 × 129S1 × DBA/2J to homozygous 129S1, DBA/2J or C57BL/6J inbred strains. This was particularly important to minimize the confounding effects of the mixed background on the expression of the *Usp18*+/− phenotype during infection and to have the mutation on the appropriate background for further evaluation of susceptibility to other important human pathogens. The genetic background significantly affected viability. *Usp18*+/− mutant mice were found in the expected frequencies in 129S1 and DBA/2J, whereas homozygous animals showed perinatal lethality in the C57BL/6J genetic background (data not shown). These findings are consistent with perinatal lethality observed in the C57BL/6J background for *Usp18*+/− mice.20

The transfer of the *Usp18*+/− mutation to 129S1 or DBA/2J background resulted in decreased survival to *Salmonella* Typhimurium infection although the susceptibility was delayed in the 129S1 background compared with mice on a DBA/2J or a mixed genetic background (Figure 1a, left and right panel, respectively, and Richer et al.). Consistent with our earlier findings in original mixed-strain background mice, 129S1.Cg-*Usp18*+/− and D2.Cg-*Usp18*+/− mice had significantly increased bacterial load in the spleen and liver postinfection compared with wild-type mice (Figure 1b, left and right panels, respectively). In addition, 129S1.Cg-*Usp18*+/− and D2.Cg-*Usp18*+/− mice had elevated levels of serum IL-6 (Figure 1c, left and right panels, respectively), indicative of a systemic proinflammatory response. However, in contrast to *Usp18*+/− mice on a mixed background, which showed a transient decrease in IFN-γ production following *Salmonella* infection, serum IFN-γ levels were not significantly different in 129S1.Cg-*Usp18*+/− and D2.Cg-*Usp18*+/− mice compared with wild-type controls (Figure 1d, left and right panels, respectively). Moreover, the transient decrease in IFN-γ production observed in mixed background mutant mice was not seen at days 1 and 5 postinfection in 129S1.Cg-*Usp18*+/− mice compared with control (data not shown).

Taken together, these results demonstrate that the increased susceptibility of *Ity9* mutant mice is 100% penetrant when the mutation is transferred to a homogenous 129S1 or DBA/2J genetic background, albeit with variable expressivity that could be explained by the inherent degree of susceptibility of the background strains, the DBA/2J presenting an intermediate phenotype and the 129S1 being highly resistant to infection.21 The susceptibility is paralleled by an increase in bacterial load at systemic sites of infection and an increase in proinflammatory cytokines, collectively contributing to the clinical phenotype. The difference in the IFN-γ response in the 129S1 and DBA/2J congenic mice suggests that strain-specific modifier genes, most likely contributed from the C57BL/6J background, may be involved in the regulation of IFN-γ levels in *Usp18*+/− mice.

Loss of ISG15 had no impact on early susceptibility of *Usp18*+/− mice to *Salmonella* infection although it mediated delayed susceptibility in wild-type mice

To determine the contribution of ISG15 to the *Usp18*+/− phenotype, we examined ISG15 expression and function in vivo and in vitro. 129S1.Cg-*Usp18*+/− mice have increased spleen *Isg15* mRNA expression both before infection and following *Salmonella* challenge (Figure 2a) and circulating ISG15 postinfection (Figure 2b). Such increased expression is likely due to the enhanced type I IFN signaling in *Usp18*+/− mice as ISG15 is a well-known IFN-inducible gene. To determine whether the increase in *Isg15* mRNA expression correlated to an increase in ISG15 conjugation to other proteins, we isolated bone marrow-derived macrophages (BMDM) from 129S1 wild-type and 129S1.Cg-*Usp18*+/− mutant mice and examined protein ISGylation. We found that ISG15 conjugation was significantly increased in mutant cells, under basal conditions and in cells stimulated with lipopolysaccharide (Figure 2c) compared with wild-type BMDM, suggesting that the loss of functional USP18 in *Usp18*+/− mutant mice contributes to a decrease in delISGylation. To determine whether the *Usp18*+/− mutation affected the enzymatic function of human USP18, we have used *Usp18* mutant protein constructed with the corresponding mutation in the human sequence (L365F) to transfect HEK293T cells. The levels of *Usp18* protein expression were similar in wild-type and *Usp18*+/− transfected cells. As observed in mutant mice, there was a reduction in delISGylation in HEK293T cells transfected with USP1A1365F compared with wild-type USP18. Levels of ISGylation in *Usp1A1365F*-transfected cells were comparable to those detected in the absence of USP18 (Figure 2d).

To examine the impact of increased ISG15 and ISGylation in vivo, we generated *Usp18*/+*Isg15* double-deficient mice by intercrossing *Usp18*+/− mice and *Isg15* knockout mice. We showed that the *Isg15* genotypes (+/+, +/+ or –/− or –/−) had no impact on survival to infection in *Usp18*+/− mice (Figure 2e). Correspondingly, there was no difference in the spleen and liver bacterial load 4 days postinfection between *Usp18*+/−/− and *Usp18*+/−*Isg15*/− mice (Figure 2f). Interestingly, *Usp18*+/− mice lacking *Isg15* showed a slight decrease in survival later during
infection (Figure 2e), and this was paralleled by an increase in bacterial load of 2.6-fold in the spleen and 4.5-fold in the liver at 8 days postinfection (Figure 2g). These results demonstrate that increased Isg15 and ISGylation are not primarily responsible for the susceptibility phenotype observed in Usp18Ity9 mice, although loss of Isg15 does mediate susceptibility and increased bacterial load later during Salmonella infection in wild-type Usp18 mice.

Blocking type I IFN receptor signaling improves survival of Usp18Ity9 mice to infection with Salmonella

Independent of its isopeptidase activity, USP18 is a negative regulator of type I IFN signaling through binding to the IFNAR2 receptor and blocking interaction between Janus-activated kinase and the type I IFN receptor. The mutation in Usp18Ity9 mice lies within the IFNAR2-binding region of USP18, suggesting that it may

Figure 1. Genetic background affects the allelic expression of 129S1.Cg-Usp18Ity9 and D2.Cg-Usp18Ity9. Mice were infected intravenously with 1.5 × 10⁶ colony-forming units (CFUs) Salmonella Typhimurium isolate Keller and (a) survival was monitored for 24 days (129S1.Cg-Usp18Ity9, left panel; Log-rank (Mantel–Cox) \( P < 0.001 \)) or 14 days (D2.Cg-Usp18Ity9, right panel; Log-rank (Mantel–Cox) \( P < 0.007 \); Usp18Ity9/(n = 4), Usp18Ity9/(n = 7), (b) bacterial load was measured in the spleen and liver at 8 days postinfection (p.i.) (129S1.Cg-Usp18Ity9, left panel; ***\( P < 0.0001 \)) or 5 days p.i. (D2.Cg-Usp18Ity9, right panel; **\( P = 0.0015 \) and ***\( P = 0.0002 \) (Usp18Ity9, dark circles; and Usp18Ity9, open circles) and (c and d) cytokines were measured at 8 days p.i. (129S1.Cg-Usp18Ity9, left panel) or 5 days p.i. (D2.Cg-Usp18Ity9, right panel) in the serum by enzyme-linked immunosorbent assay in Usp18Ity9/(black, n = 5) and Usp18Ity9/(white, n = 5); *\( P = 0.01 \). IFN, interferon; IL, interleukin.
Figure 2. The susceptibility of 129S1.Cg-Usp18<sup>+/−</sup> mice to Salmonella infection is not due to increase ISG15 expression and function. (a, b) Usp<sup>18<sup>+/−</sup></sup> (black, n = 3) and 129S1.Cg-Usp<sup>18<sup>+/−</sup></sup> (white, n = 3) mice were infected intravenously with 1.5 × 10<sup>7</sup> colony-forming units (CFUs) Salmonella Typhimurium for 8 days and (a) RNA was extracted from the spleen for quantitative reverse transcriptase–PCR (P = 0.017, **P = 0.0035) and (b) ISG15 was measured in the serum by enzyme-linked immunosorbent assay (**P < 0.0001). (c) Western blotting analysis of bone marrow-derived macrophages stimulated with lipopolysaccharide (LPS) for 18 h. (d) HEK293T cells were transfected as indicated, and immunoblotting was performed. Total protein staining with Ponceau S was used to confirm equal loading. (e, f) Survival of mice infected with Salmonella Typhimurium for 14 days; Usp<sup>18<sup>+/−</sup></sup>Isg15<sup>+/−</sup> (n = 8), Usp<sup>18<sup>+/−</sup></sup>Isg15<sup>+/−</sup> (n = 12), Usp<sup>18<sup>+/−</sup></sup>Isg15<sup>−/−</sup> (n = 10), Usp<sup>18<sup>+/−</sup></sup>Isg15<sup>−/−</sup> (n = 8), Usp<sup>18<sup>+/−</sup></sup>Isg15<sup>−/−</sup> (n = 8); Log-rank (Mantel–Cox) P < 0.0001 and (e, f) bacterial load in the spleen and liver was measured at (f) 4 days postinfection (p.i.) in Usp<sup>18<sup>+/−</sup></sup> mice (left panel, Usp<sup>18<sup>+/−</sup></sup>Isg15<sup>+/−</sup>, dark circles; and Usp<sup>18<sup>+/−</sup></sup>Isg15<sup>−/−</sup>, open circles) and (g) 8 days p.i. in Usp<sup>18<sup>+/−</sup></sup> mice (right panel, Usp<sup>18<sup>+/−</sup></sup>Isg15<sup>+/−</sup>, dark circles; and Usp<sup>18<sup>+/−</sup></sup>Isg15<sup>−/−</sup>, open circles).
interfere with its regulatory function. We have previously shown that Usp18<sup>ly9</sup> mice on a mixed background have increased levels of Ifnb transcript and increased STAT1 phosphorylation downstream of the receptor. Similarly, 129S1.Cg-Usp18<sup>ly9</sup> mice showed increased basal Ifnb mRNA expression (Figure 3a) and increased STAT1 activation following Salmonella infection (Figure 3b). Increased STAT1 activation was also observed in HeLa cells transfected with human USP18<sup>L365F</sup> following stimulation with IFN-α (Figure 3c). Together, these results indicate that in addition to enzyme inactivation this single amino-acid mutation of USP18 leads to loss of the inhibitory function of USP18 in type I IFN signaling.

To determine the impact of type I IFN signaling on the survival of 129S1.Cg-Usp18<sup>ly9</sup> mice, we treated mice with the IFN-α/β receptor 1 (IFNAR1)-specific MAR1-5A3 monoclonal antibody 1 day before infection with <i>S. Typhimurium</i>. This antibody has been shown to potently inhibit type I IFN receptor signaling in mouse models of infection. Usp18<sup>ly9</sup> mice that were pretreated with the MAR1-5A3 antibody showed improved survival following Salmonella infection compared with mice that received an isotype control (Figure 3d). The MAR1-5A3 treated mice also had significantly reduced bacterial loads in the spleen and liver (Figure 3e). Taken together, these results suggest that loss of the regulation of the type I IFN signaling pathway that is normally imparted by USP18 contributes to the increase in susceptibility of mutant mice to Salmonella infection.

Usp18<sup>ly9</sup> mice have increased IL-10 and deregulation of the levels of autophagy substrates

Given that type I IFN has been shown to induce IL-10 in a STAT1-dependent manner, we examined whether the increase in type I IFN signaling in Usp18<sup>ly9</sup> mice affected IL-10 production. At 8 days postinfection, 129S1.Cg-Usp18<sup>ly9</sup> mice had elevated Il10 transcript in the spleen (Figure 4a) and an increase in circulating IL-10 (Figure 4b). Consistent with the finding that STAT3 is activated downstream of the IL-10 receptor, we also observed that infected Usp18<sup>ly9</sup> mice had elevated levels of phosphorylated STAT3 in the spleen (Figure 4c).

Several studies have demonstrated a role for autophagy in innate immunity to Salmonella infection. As IL-10 is an inhibitor of autophagy, we next asked whether the increase in...
IL-10 in Usp18<sup>−/+</sup> mice affected the expression of autophagy markers. We collected lysates from spleen tissues of Usp18<sup>−/+</sup> mice and wild-type controls and assessed the levels of the autophagy cargo receptor, p62 (SQSTM1), which has been shown to accumulate in vivo in conditions where autophagy is repressed. Indeed, Usp18<sup>−/+</sup> mice showed increased accumulation of p62 following infection compared with wild-type controls (Figure 4d), and this was not a consequence of increased p62 transcript (Figure 4g). To further study the impact of Usp18<sup>−/+</sup> on autophagic markers, we measured LC3 conversion in the spleen of wild-type and mutant mice during infection. LC3 is a ubiquitin-like protein that undergoes...

**Figure 4.** 129S1.Cg-Usp18<sup>−/+</sup> have increased interleukin (IL)-10 and deregulated expression of autophagy markers. Mice were infected intravenously with Salmonella Typhimurium for 8 days and (a) RNA was extracted from the spleen for quantitative reverse transcriptase–PCR (qRT-PCR; **P** = 0.001), (b) IL-10 was measured in the serum by enzyme-linked immunosorbent assay (ELISA; *P* = 0.019), and (c–f) western blotting analysis was performed on spleen tissue lysates using antibodies for signal transducer and activator of transcription factor 3 (STAT3) or p-STAT3 (c), p62 (d), optineurin (OPTN) (e) and light chain 3 (LC3) (f). (g, h) RNA was extracted from the spleen for qRT-PCR (*P* = 0.05); (i) bone marrow-derived macrophages were infected with heat-killed Salmonella (MOI = 50), stained with CM-H<sub>2</sub>DCFDA and analyzed by fluorescence-activated cell sorter. Data are represented as mean fluorescence intensity fold changes (*P* = 0.01); (j) IL-10 was measured in the serum of MAR1-5A3 or immunoglobulin G (IgG) control-treated mice at 8 days postinfection (p.i.) by ELISA (**P** = 0.0005); (k) Western blotting analysis of OPTN was performed on spleen tissue lysates from MAR1-5A3 or IgG control-treated mice at 8 days p.i. β-Actin was used as a loading control.

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phosphatidylethanolamine modification to facilitate association with *Salmonella* during infection. The conversion of LC3 (LC3I) to its lipidated form (LC3II) is correlated with the formation of autophagosomes. We found that the intensity of the LC3I and LC3II bands was decreased relative to β-actin in the *Usp18*Ity9 mice in comparison to wild-type mice before and during *Salmonella* infection, although to a greater extent following bacterial infection (Figure 4e). During *Salmonella* infection, an additional autophagy marker, OPTN, is recruited to ubiquitinated bacteria in the cytosol and, following phosphorylation by TBK1, binds LC3 to bring *Salmonella* to the autophagosome. Therefore, we assessed the levels of OPTN in *Usp18*Ity9 mice to further define the impact of the mutation on autophagy during *Salmonella* infection. We found that *Usp18*Ity9 mice had decreased levels of *Optn* transcript and OPTN protein after infection (Figures 4f and h).

Generation of reactive oxygen species (ROS) by NADPH oxidase is important for the induction of autophagy, LC3 recruitment to phagosomes and restriction of intracellular replication of *Salmonella* Typhimurium. Consistent with our finding that *Usp18*Ity9 mice have deregulated autophagy marker expression following *Salmonella* infection in vivo, we also observed that *Usp18*Ity9 macrophages have decreased ROS production after exposure to heat-killed *Salmonella* (Figure 4i). Together, these data suggest that the diminished levels of ROS and OPTN result in a failure of autophagy to proceed, thus leading to an accumulation of p62 and lack of LC3 conversion.

To further demonstrate that type I IFN is important in the control of IL-10 and autophagy during *Salmonella* infection in *Usp18*Ity9 mutant mice, we investigated whether inhibition of type I IFN in *Usp18*Ity9 mice would affect IL-10 levels in circulation. We found that the increase in IL-10 observed in *Salmonella*-infected mice given the immunoglobulin G control was diminished in mice treated with the type I IFN-neutralizing antibody (Figure 4j). Moreover, inhibition of type I IFN in mutant mice was also sufficient to restore the levels of OPTN following *Salmonella* infection (Figure 4k). Together, these data support our hypothesis that IL-10 and autophagic marker levels are affected by the levels of type I IFN in *Usp18*Ity9 mice.

*Usp18*Ity9 mice have increased IL-1β and an elevated T helper type 17 (Th17) response

IL-1β is a proinflammatory cytokine important for innate immunity to *Salmonella* infection but, in excess, can result in endotoxemia. Autophagy regulates pro-IL-1β production and an accumulation of cellular p62 due to deficient autophagy can promote activation of nuclear factor-κB and subsequently induce pro-IL-1β. Therefore, we evaluated the impact of the *Usp18*Ity9 mutation on IL-1β expression. We found that *Il1b* transcript and pro-IL-1β protein levels were increased in *Usp18*Ity9 at 8 days postinfection (Figures 5a and b). In addition, explanted splenocytes from *Salmonella* Typhimurium-infected mice produced more IL-1β compared with control mice (Figure 5c). As IL-1β release can lead to an increase in IL-23 secretion and, together, potently induce the secretion of IL-17 by Th17 cells, we next investigated the levels of these cytokines in *Usp18*Ity9 mice after *Salmonella* infection. We found that mutant mice had elevated levels of IL-23 and IL-17 transcript (Figures 5d and e) and increased production of IL-17 from CD4+ T cells (Figure 5f) showing that high IL-1β levels in *Usp18*Ity9 mutant mice induced a Th17 response.

*Usp18*Ity9 mice are susceptible to *Mycobacterium tuberculosis* infection

To further investigate the role of *Usp18* during bacterial infection, we tested whether the *Ity9* mutation would impact the susceptibility to another Gram-negative bacteria (*Citrobacter rodentium*) and mycobacteria (*Mycobacterium bovis* BCG and *M. tuberculosis*). *Usp18*Ity9 were not susceptible to *C. rodentium* as measured by survival analysis (data not shown) and bacterial shedding (data not shown) and to *M. bovis* BCG (data not shown). In contrast, we found that *Usp18*Ity9 mice showed significantly increased susceptibility to infection compared with both wild-type littermates and DBA/2J mice following aerosol infection with the highly virulent

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**Figure 5.** 129S1.Cg-**Usp18**Ity9 have increased interleukin (IL-1)β and an elevated T helper type 17 response. Mice were infected intravenously with 1.5 × 10⁴ colony-forming units (CFUs) *Salmonella* Typhimurium for 8 days and (a) RNA was extracted from the spleen for quantitative reverse transcriptase–PCR (qRT-PCR; **P = 0.009), (b) western blotting analysis was performed on spleen tissue lysate using an antibody for IL-1β, (c) splenocytes were harvested and cultured for 24 h before measuring IL-1β in the supernatant by enzyme-linked immunosorbent assay, (d, e) RNA was extracted from the spleen for qRT-PCR of (d) IL-23 (**P = 0.02, **P = 0.006) and (e) IL-17 (**P = 0.001), (f) flow cytometry of intracellular IL-17 in CD4+ T cells from the spleen (**P = 0.016). n = 3–5 mice/genotype from two experiments.
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M. tuberculosis H37Rv strain (Figure 6a). Decreased survival correlated with significantly higher bacterial burden in both the lung and spleen of M. tuberculosis-infected Usp18<sup>zy9</sup> mice (Figure 6b). At necropsy 6 weeks postinfection, Usp18<sup>zy9</sup> mutant mice showed large infected foci with extensive necrotic centre and increased lymphohistiocytic inflammatory cell infiltration in the lung compared with littermate control mice with a greater percentage of the lung affected by inflammation (Supplementary Figure S1, left and middle panels). Moreover, Ziehl–Neelsen staining of acid-fast bacilli revealed significantly more M. tuberculosis bacteria in the lungs of mutant mice compared with wild-type controls (Supplementary Figure S1, right panels). As observed during the course of Salmonella infection, Usp18<sup>zy9</sup> mice showed elevated levels of circulating ISG15 and several cytokines, including IFN-γ, TNFα and IL-10 (Figure 6d). In addition, Usp18<sup>zy9</sup> mice also showed increased lung mRNA expression of Il17, Il1b and Isg15, with no difference in the mRNA expression of Il10 between wild-type and mutant mice (Figure 6e). Together, these observations suggest that Usp18<sup>zy9</sup> mutant mice are more susceptible to mycobacterial infection as a result of increased bacterial load and excessive inflammatory response.

**DISCUSSION**

In the current paper, we examined the contribution of the deISGylating and type I IFN regulatory functions of Usp18<sup>zy9</sup> during Salmonella infection. Our in vitro and in vivo data are consistent with those from Usp18 knockout mice where lipopolysaccharide-stimulated macrophages have increased ISGylation compared with wild-type mice. The robust expression of ISG15 and ISGylation in response to Salmonella infection due to the loss of Usp18 activity does not appear to have a major role in susceptibility to infection of Usp18 mutant mice. Loss of Isg15 in Usp18<sup>zy9</sup> mutant mice does not impact on the in vivo susceptibility, suggesting that deregulation of the ISGylation pathway is not the predominant mechanism of susceptibility in Usp18<sup>zy9</sup> mice. The secretion of ISG15 from various immune cells suggests that in addition to its role as a ubiquitin-like molecule, ISG15 acts as a cytokine that synergizes with IL-12 to increase IFN-γ and provide protective immunity to infection. Although the expression and secretion of IFN-γ in Usp18<sup>zy9</sup> mutant mice was not affected by high levels of ISG15, it is possible that the loss of ISG15 in its secreted form in the double Isg15 and Usp18-deficient mice may explain, in part, the inability to improve survival to infection. Indeed, Isg15-deficient mice carrying a wild-type allele at Usp18 showed increased susceptibility later during infection. Late susceptibility of Isg15-deficient mice to M. tuberculosis infection has been also reported. Identification of ISG15 substrate proteins, including those involved in adaptive immune responses, will provide insight on the mechanism of susceptibility later during bacterial infection.
On the other hand, our results indicate that Usp18Ity9 mice treated with a neutralizing antibody to the type I IFN receptor have improved survival and decreased bacterial load after Salmonella challenge. We have previously reported that signaling through the type I IFN pathway is deleterious to the host during Salmonella infection.\(^3,1\) In addition, IFNAR \(-/-\) mice are more resistant to infection with Salmonella Typhimurium as a result of increased macrophage necroptosis thereby permitting evasion of the host response.\(^3,7\) Type I IFNs have been shown to be detrimental to the host during bacterial infection through a number of mechanisms, including chemokine production, leukocyte recruitment, T-cell responses and host cell apoptosis, among others (reviewed in Trinchieri\(^6\)) suggesting that the mechanisms underlying the action of type I IFN are complex.

In Usp18 mutant mice, the proinflammatory immune response to infection becomes amplified and dysregulated as shown by excessive production of the cytokines IL-1\(\beta\) and IL-6. These cytokines most likely act in synergy with other cytokines (IFN-\(\gamma\) and TNF) that were also upregulated during infection, to cause septic shock, tissue damage and death. In parallel, we showed that Usp18Ity9 mice have elevated systemic IL-10. IL-10 is an anti-inflammatory cytokine that prevents damage to the host. IL-10 works in opposition to IL-6, which also signals through STAT3.\(^3,8\) The activation of IL-10 signaling does not appear to repress the expression of proinflammatory genes in Usp18 mutant mice although decreased ROS production in vitro was observed, which is consistent with studies showing that IL-10 inhibits macrophage ROS production in lipopolysaccharide-stimulated macrophages and neutrophils.\(^3,9,40\) The attenuated oxidative burst activity in Usp18 mutant mice may well explain the higher bacterial load observed in the spleens and livers of these animals.

In our model of infection, IL-1\(\beta\) is increased at both the transcript and protein levels in the spleen of infected mice, which contrasts with the observation that IFN-\(\gamma\) is able to limit pro-IL-1\(\beta\) availability and IL-1\(\beta\) maturation\(^5,2\) and that elevated type I IFN inhibits M. tuberculosis-induced IL-1\(\beta\) mRNA expression in macrophages.\(^3,7\) Moreover, type I IFN was shown to inhibit production of IL-1\(\beta\) from myeloid cells in vivo,\(^2\) resulting in a loss of IL-1\(\beta\)-mediated control of bacterial burden.\(^3,4\) This discrepancy may be attributed to an increase in p62 post-infection in Usp18Ity9 mice as accumulation of p62 and subsequent activation of nuclear factor-xB has been shown to increase IL-1\(\beta\).\(^3,30\) We do observe an increase in IL-23 and IL-17 in the spleen Usp18Ity9 mutant mice, which is consistent with reports of an IL-1\(\beta\)-dependent increase in IL-23 resulting in enhanced IL-10 production, leading to deregulated expression of IL-1\(\beta\) and autophagy markers that results in increased bacterial burden and septic shock in Salmonella-infected Usp18Ity9 mice. Our studies reveal that a mutation in human USP18 corresponding to the Usp18Ity9 mutation also affects the enzymatic and regulatory functions of USP18, suggesting that the findings presented here may be relevant to the function of human USP18 during infection.

**MATERIALS AND METHODS**

**Ethics statement**

All animal experiments were performed under guidelines specified by the Canadian Council on Animal Care. The animal use protocol was approved by the McGill University Animal Care Committee (protocol no.5797).

**Mice**

The Usp18Ity9 mutation was originally identified on a mixed C57BL/6 \(\times\) BALB/c background (99.8% 129S1 or DBA/2J genetic background (37.5%, 37.5%, 25%, respectively). Backcrossing from the original mixed background was accomplished by nine generations of inbreeding to the 129S1 or DBA/2J strains (Jackson Laboratories, Bar Harbor, ME, USA), resulting in mice that are >99.8% 129S1 or DBA/2J. Isg15 knockout (B6.129P2-Isg15tm1Kpk/J; Jackson Laboratories) were crossed with Usp18Ity9 or Usp18Ity9 mice to generate F1 mice that were heterozygous for Isg15 and Usp18Ity9 or the wild-type allele. These mice were intercrossed to generate mice that were homozygous for Usp18Ity9 or the wild-type allele and selected for wild-type Slc11a1. Mice were bred at the Goodman Cancer Research Centre Animal Facility.

**In vivo Salmonella infections**

Mice between 7 and 12 weeks of age were infected intravenously with Salmonella Typhimurium strain Keller, as described by us previously.\(^2\) Mice were infected in the caudal vein and monitored twice daily for survival. Alternatively, spleens and livers were collected, homogenized in saline and
colony-forming units were determined by plating of serial dilutions on trypticase soy agar plates.

Enzyme-linked immunosorbent assay (ELISA)

Serum was obtained from the blood of infected mice, and cytokines were assayed by ELISA (eBioscience, San Diego, CA, USA) according to the manufacturer’s directions.

RNA extraction and quantitative reverse transcriptase–PCR

Total RNA was isolated from mouse tissue using the TRIzol reagent (Invitrogen Life Technologies, Burlington, ON, Canada). First-strand cDNA was generated using MMLV-RT (Invitrogen) and random oligonucleotides as primers. Quantitative PCR was performed in duplicate for each transcript using SYBR green qPCR master mix (Applied Biosystems, Burlington, ON, Canada). Cells were resuspended in complete medium (RPMI supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 100 U/ml penstrep and 30% L929-conditioned medium as a source of murine colony-stimulating factor). L929 supernatant was replenished every 2 days before cell counting and plating.

Immunoblotting

Protein lysates were prepared using the Celllytic-M reagent (Sigma-Aldrich) according to the manufacturer’s directions. Proteins were quantified by the Bradford method (Bio-Rad, Mississauga, ON, Canada), according to the manufacturer’s directions. Total RNA was isolated from mouse tissue using the TRIzol reagent (Invitrogen Life Technologies, Burlington, ON, Canada) on a StepOnePlus apparatus (Applied Biosystems). The Ct values for the genes of interest were normalized to the housekeeping gene TATA-binding protein. The relative expression of the gene was calculated as $2^{-\Delta\Delta Ct}$.

BMDM

Femurs were collected from 8- to 12-week-old mice, and bone marrow was extracted by flushing the femurs with RPMI using a 25-G needle. A single-cell suspension was obtained by passage through a 25-G needle and red blood cells (RBCs) lysed for 5 min using a commercial RBC Lysis Buffer (Sigma-Aldrich, St Louis, MO, USA). Cells were resuspended in complete medium (RPMI supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine and 10% albumin-dextrase-catalase (ADC) supplement (Sigma-Aldrich) and 10% ADC (Sigma-Aldrich) and 10% ADC (Sigma-Aldrich), respectively, as described previously.

Flow cytometry

Spleens were harvested from 8- to 12-week-old mice, macerated and passed through a 70-μm cell strainer. RBC lysis was performed using the ACK lysing buffer, and cells were enumerated and plated for ELISA or for flow cytometry. Intracellular staining of IL-17 (eBioscience) was performed on prepared splenocytes (10 x 10^6 cells) using CytoFix/CytoPerm (BD Biosciences, Mississauga, ON, Canada). Briefly, cells were stimulated in vitro (4 h) with phorbol myristate acetate (50 ng/ml) and ionomycin (500 ng/ml), and intracellular transport was inhibited using GolgiStop (BD Biosciences). Cells were stained with antibodies to CD4, CD3, B220 and FITC-conjugated mouse anti-IL-17 antibody (BD Biosciences, Mississauga, ON, Canada). Cells were then fixed with 4% paraformaldehyde, permeabilized with 0.1% saponin and stained with antibodies to CD4, CD3 and B220. Data were analyzed using FACSDiva image analysis software (Becton Dickinson) or using the FlowJo software (Tree Star). Mean fluorescence intensity values were calculated as fold change over uninfected cells.

**In vivo M. tuberculosis infections**

*In vivo* M. tuberculosis H37Rv was grown at 37°C in Middlebrook 7H9 medium (Difco Laboratories, Mississauga, ON, Canada) containing 0.05% Tween-20 (Sigma-Aldrich) and 10% albumin-dextrase-catalase (ADC) supplement (Becton Dickson and Co., Mississauga, ON, Canada). Bacteria were delivered by aerosol using an inhalation exposure system (In-Tox Products, Moriarty, NM, USA), and infectious dose was confirmed by enumeration of bacteria within the lungs of control mice at 24 h postinfection. Mice were euthanized at 6 weeks postinfection, organs were homogenized in PBS and bacterial burden was determined by serial dilution on Middlebrook 7H110 agar (Difco Laboratories) plates supplemented with OADC enrichment (Becton Dickson and Co.) and BacTac Panta Plus (Becton Dickson and Co.). Serum was collected for ELISA, and tissues were either fixed in buffered formalin before immunohistochemical analysis or stored in RNA later (Ambion, Burlington, ON, Canada). For histology, representative slides were assessed by a pathologist and scored for degree of inflammation.

Statistical analyses

Results are expressed as means ± s.e.m. Data were analyzed using a two-tailed Student’s t-test using the GraphPad Prism statistical program (GraphPad Software, La Jolla, CA, USA). P-values of <0.05 were considered significant.

**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

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