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

Shauna M. Dauphinee[1,2], **Etienne Richer**[3], **Megan M. Eva**[1,2], **Fiona McIntosh**[4], **Marilène Paquet**[5], **David Dangoor**[6], **Christoph Burkart**[6], **Dong-Er Zhang**[7], **Samantha Gruenheid**[8], **Philippe Gros**[1,2,9], **Marcel Behr**[10], and **Danielle Malo**[1,2,10]

[1]Department of Human Genetics, McGill University, Montreal, QC, Canada

[2]Complex Traits Group, McGill University, Montreal, QC, Canada

[3]Department of Biology, McGill University, Montreal, QC, Canada

[4]Montreal General Hospital, McGill University, Montreal, Quebec, Canada

[5]Département de pathologie et de microbiologie, Faculté de Médecine Vétérinaire, Université de Montréal, Saint-Hyacinthe, Québec

[6]Moores Cancer Center, University of California San Diego, La Jolla, CA, USA

[7]Department of Pathology, University of California San Diego, La Jolla, CA, USA

[8]Department of Microbiology and Immunology, McGill University, Montreal, QC, Canada

[9]Department of Biochemistry, McGill University, Montreal, QC, Canada

[10]Department of Medicine, McGill University, Montreal, QC, Canada

Abstract

Host genetics plays a key role in susceptibility to S*almonella* 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 (*Usp18*^{Ity9}), which confers increased susceptibility to *Salmonella* Typhimurium. USP18 functions to regulate type I IFN signaling and as a protease to remove ISG15 from substrate proteins. *Usp18*^{Ity9} mice are susceptible to infection with *Salmonella* Typhimurium and have increased expression and function of ISG15, but *Usp18*^{Ity9} mice lacking *Isg15* do not show improved survival with *Salmonella* challenge. Type I IFN signaling is increased in *Usp18*^{Ity9} 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 IL-1β and IL-17. Furthermore, *Usp18*^{Ity9} mice are more susceptible to infection with *Mycobacterium tuberculosis*, have increased

Corresponding author: Danielle Malo, McGill University, 3649 Sir William Osler Promenade, Montreal, QC, H3G 0B1, Canada, danielle.malo@mcgill.ca, Tel: 514-398-3907, Fax: 514-398-2603.

CONFLICT OF INTEREST
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bacterial load in 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 *Usp18*^{Ity9} mice to *Salmonella* infection and that hyperactivation of signaling leads to increased IL-10, deregulation of autophagic markers and increased proinflammatory cytokine production.

Keywords

USP18; innate immunity; Salmonella; mycobacteria; type I IFN; ISGylation; autophagy

INTRODUCTION

Salmonella enterica are facultative, intracellular, gram-negative enterobacteria that cause a range of enteric diseases in mammalian hosts. The human restricted serovars *Salmonella enterica* Typhi and Paratyphi are the causal agents of Typhoid fever, affecting more than 27 million people worldwide and resulting in greater than 200 000 deaths each year through contaminated food and drinking water (1). In contrast, infection with the *Salmonella 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*-nitrosurea (ENU) mutagenesis screen. Our ENU screen has previously identified a loss of function mutation within the gene ubiquitin specific peptidase 18 (*Usp18*^{Ity9}), 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 IFN signaling through 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 *Usp18*^{Ity9} 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 (6, 7)). 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 cells (9) 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).

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 (12)). Upon entry into the cell, *Salmonella* reside within specialized vesicles called *Salmonella* containing vacuoles (SCV). Damage to the SCV 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) (14–17). 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 play an important role in either upregulating (IFNγ, TNFα, IL-1β) or inhibiting (IL-4, IL13, 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*^{Ity9} mice lies within the IFNAR2-binding region of USP18 (3, 4), as well as the importance of type I IFN signaling in bacterial infection, we sought to determine if the IFNAR regulatory function of USP18 is compromised in *Usp18*^{Ity9} mice and whether hyperactivation of type I IFN signaling contributes to the pathogenesis of infection. In this study, we show that *Usp18*^{Ity9} 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^{Ity9} 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 JAK/STAT activation (4, 5, 19). To address the relative importance of these two functions in *Usp18*^{Ity9} mice, we first transferred the *Usp18*^{Ity9} allele from the mixed genetic background of C57BL/6 × 129S1 × DBA/2J (3) to homogenous 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*^{Ity9} 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*^{Ity9} 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*^{Ity9} 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 to mice on a DBA/2J or a mixed genetic background (Fig. 1A, left and right panel respectively, and (3)). Consistent with our earlier findings in original mixed-strain background mice, 129S1.Cg-*Usp18*^{Ity9} and D2.Cg-*Usp18*^{Ity9} mice had significantly increased bacterial load in the spleen and liver post-infection compared with wild-type mice (Fig. 1B, left and right panel respectively). In addition, 129S1.Cg-*Usp18*^{Ity9} and D2.Cg-*Usp18*^{Ity9} mice had elevated levels of serum IL-6 (Fig. 1C, left and right panel respectively), indicative of a systemic pro-inflammatory response. However, in contrast to *Usp18*^{Ity9} 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*^{Ity9} and D2.Cg-*Usp18*^{Ity9} mice compared to wild-type controls (Fig. 1D, left and right panel respectively). Moreover, the transient decrease in IFN-γ production observed in mixed background mutant mice was not seen at days 1 and 5 post-infection in 129S1.Cg-*Usp18*^{Ity9} mice compared to 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 pro-inflammatory 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*^{Ity9} mice.

Loss of ISG15 had no impact on early susceptibility of Usp18^{Ity9} mice to *Salmonella* infection although it mediated delayed susceptibility in wild-type mice

To determine the contribution of ISG15 to the *Usp18*^{Ity9} phenotype, we examined ISG15 expression and function *in vivo* and *in vitro*. 129S1.Cg-*Usp18*^{Ity9}mice have increased spleen *Isg15* mRNA expression both prior to infection and following *Salmonella* challenge (Fig. 2A) and circulating ISG15 post-infection (Fig. 2B). Such increased expression is likely due to the enhanced type I IFN signaling in *Usp18*^{Ity9} mice since *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 BMDM from 129S1 wild-type and 129S1.Cg-*Usp18*^{Ity9} 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 LPS (Fig. 2C) compared to wild-type BMDM, suggesting that the loss of functional USP18 in *Usp18*^{Ity9} mutant mice contributes to a decrease in deISGylation. To determine if the *Usp18*^{Ity9} mutation affected the enzymatic function of human USP18, we

have used USP18 mutant 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^{L365F} transfected cells. As observed in *Ity9* mutant mice, there was a reduction in deISGylation in HEK293T cells transfected with USP18^{L365F} compared to wild-type USP18. Levels of ISGylation in USP18^{L365F} transfected cells were comparable to those detected in the absence of USP18 (Fig. 2D).

To examine the impact of increased ISG15 and ISGylation *in vivo*, we generated *Usp18/Isg15* double-deficient mice by intercrossing *Usp18^{Ity9}* mice and *Isg15* knockout mice. We showed that the *Isg15* genotypes (+/+, +/− or −/−) had no impact on survival to infection in *Usp18^{Ity9}* mice (Fig. 2E). Correspondingly, there was no difference in spleen and liver bacterial load 4 days post-infection between *Usp18^{Ity9}Isg15$^{+/+}$* and *Usp18^{Ity9}Isg15$^{-/-}$* mice (Fig. 2F). Interestingly, *Usp18$^{+/+}$* mice lacking *Isg15* showed a slight decrease in survival later during infection (Fig. 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 post-infection (Fig 2G). These results demonstrate that increased *Isg15* and ISGylation are not primarily responsible for the susceptibility phenotype observed in *Usp18^{Ity9}* mice, although loss of *Isg15* does mediate susceptibility and increased bacterial load later during S*almonella* infection in wild-type *Usp18* mice.

Blocking type I IFN receptor signaling improves survival of Usp18^{Ity9} 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 JAK and the type I IFN receptor (4). The mutation in *Usp18^{Ity9}* mice lies within the IFNAR2-binding region of USP18 suggesting that it may interfere with its regulatory function. We have previously shown that *Usp18^{Ity9}* mice on a mixed background have increased levels of *Ifnb* transcript and increased STAT1 phosphorylation downstream of the receptor (3). Similarly, 129S1.Cg-*Usp18^{Ity9}* mice showed increased basal *Ifnb* mRNA expression (Fig. 3A) and increased STAT1 activation following *Salmonella* infection (Fig. 3B). Increased STAT1 activation was also observed in HeLa cells transfected with human USP18^{L365F} following stimulation with IFNα (Fig. 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^{Ity9}* mice, we treated mice with the IFNα/β receptor 1 (IFNAR1)-specific MAR1-5A3 monoclonal antibody one day prior to infection with *S.* Typhimurium. This antibody has been shown to potently inhibit type I IFN receptor signaling in mouse models of infection (22). *Usp18^{Ity9}* mice that were pretreated with the MAR1-5A3 antibody showed improved survival following *Salmonella* infection compared to mice that received an isotype control (Fig. 3D). The MAR1-5A3 treated mice also had significantly reduced bacterial loads in the spleen and liver (Fig. 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^{lty9} 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, (23), we examined whether the increase in type I IFN signaling in *Usp18^{lty9}* mice affected IL-10 production. At 8 days post-infection, 129S1.Cg-*Usp18^{lty9}* mice had elevated *Il10* transcript in the spleen (Fig. 4A) and an increase in circulating IL-10 (Fig. 4B). Consistent with the finding that STAT3 is activated downstream of the IL-10 receptor (24), we also observed that infected *Usp18^{lty9}* mice had elevated levels of phosphorylated STAT3 in the spleen (Fig. 4C).

Several studies have demonstrated a role for autophagy in innate immunity to *Salmonella* infection (14, 15). Since IL-10 is an inhibitor of autophagy (25), we next asked whether the increase in IL-10 in *Usp18^{lty9}* mice affected the expression of autophagy markers *in vivo*. We collected lysates from spleen tissues of *Usp18^{lty9}*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 (26). Indeed, *Usp18^{lty9}*mice showed increased accumulation of p62 following infection compared to wild-type controls (Fig. 4D), and this was not a consequence of increased p62 transcript (Fig. 4G). To further study the impact of *Usp18^{lty9}* 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 phosphatidylethanolamine modification to facilitate association with *Salmonella* during infection (27). 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^{lty9}* mice in comparison to wild-type mice before and during *Salmonella* infection, although to a greater extent following bacterial infection (Fig. 4E). During *Salmonella* infection, an additional autophagy receptor, optineurin (OPTN), is recruited to ubiquitylated bacteria in the cytosol and, following phosphorylation by TBK1, binds LC3 to bring *Salmonella* to the autophagosome (16). Therefore, we assessed the levels of OPTN in *Usp18^{lty9}* mice to further define the impact of the mutation on autophagy during *Salmonella* infection. We found that *Usp18^{lty9}* mice had decreased levels of *Optn* transcript and OPTN protein after infection (Fig. 4F and H).

Generation of ROS by NADPH oxidase is important for the induction of autophagy, LC3 recruitment to phagosomes and restriction of intracellular replication of *Salmonella* Typhimurium (28). Consistent with our finding that *Usp18^{lty9}* mice have deregulated autophagy marker expression following *Salmonella* infection *in vivo*, we also observed that *Usp18^{lty9}* macrophages have decreased ROS production after exposure to heat-killed *Salmonella* (Fig. 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^{lty9}* mutant mice, we investigated whether inhibition of Type I IFN in *Usp18^{lty9}* mice would affect IL-10 levels in circulation. We found that the increase in IL-10 observed in *Salmonella*-infected mice given the IgG control was

diminished in mice treated with the type I IFN neutralizing antibody (Fig. 4J). Moreover, inhibition of type I IFN in mutant mice was also sufficient to restore levels of OPTN following *Salmonella* infection (Fig. 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 Th17 response

IL-1β is a pro-inflammatory cytokine important for innate immunity to *Salmonella* infection, but in excess, can result in endotoxemia (29). Autophagy regulates pro-IL-1β production and an accumulation of cellular p62 due to deficient autophagy can promote activation of NF-κB and subsequently induce pro-IL-1β (30). 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 post-infection (Fig. 5A–B). In addition, explanted splenocytes from *Salmonella* Typhimurium-infected mice produced more IL-1β compared to control mice (Fig. 5C). Since IL-1β release can lead to an increase in IL-23 secretion, (31) and together, potently induce the secretion of IL-17 by Th17 cells (32), 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 (Fig. 5D–E) and increased production of IL-17 from CD4^{+} T cells (Fig. 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 rodentii*) and mycobacteria (*Mycobacterium bovis* BCG and *Mycobacterium 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 to both wild-type littermates and DBA/2J mice following aerosol infection with the highly virulent *M. tuberculosis* H37Rv strain (Fig. 6A). Decreased survival correlated with significantly higher bacterial burden in both the lung and spleen of *M. tuberculosis* infected *Usp18*^{Ity9} mice (Fig. 6B). At necropsy 6 weeks post-infection, *Usp18*^{Ity9} mutant mice showed large infected foci with extensive necrotic centre and increased lymphohistiocytic inflammatory cell infiltration in the lung compared to littermate control mice with a greater percentage of the lung affected by inflammation (Supplemental Fig. 1, left and middle panels). Moreover, Zeihl-Neelsen staining of acid-fast bacilli revealed significantly more *Mycobacterium tuberculosis* bacteria in the lungs of mutant mice compared to wild-type controls (Supplemental Fig. 1, right panels). As observed during the course of *Salmonella* infection, *Usp18*^{Ity9}mice showed elevated levels of circulating ISG15 and several cytokines including IFNγ, TNFα, and IL-10 (Fig. 6D). In addition, *Usp18*^{Ity9}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 (Fig. 6E). Together, these observations suggest that *Usp18*^{Ity9} 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*^{Ity9} during *Salmonella* infection. Our *in vitro* and *in vivo* data are consistent with those from *Usp18* knockout mice where LPS-stimulated macrophages have increased ISGylation compared to wild-type mice (33). The robust expression of ISG15 and ISGylation in response to *Salmonella* infection due to the loss of USP18 activity does not appear to play a major role in susceptibility to infection of *Usp18* mutant mice. Loss of *Isg15* in *Usp18*^{Ity9} 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*^{Ity9} 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 IFNγ and provide protective immunity to infection (34–36). Although the expression and secretion of IFN-γ in *Usp18*^{Ity9} mutant mice was not affected by the 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 *Mycobacterium tuberculosis* infection has been also reported (35). 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 *Usp18*^{Ity9} 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, 11). 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 (37). 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 (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-1β and IL-6 These cytokines most likely act in synergy with other cytokines (IFNγ and TNF) that were also upregulated during infection, to cause septic shock, tissue damage, and death. In parallel, we showed that *Usp18*^{Ity9} 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 (38). 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 ROS production in LPS-stimulated macrophages and neutrophils (39, 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-1β is increased at both the transcript and protein levels in the spleen of infected mice, which contrasts with the observation that IFN-β is able to limit pro-IL-1β availability and IL-1β maturation (23) and that elevated type I IFN inhibits *M. tuberculosis*-induced IL-1β mRNA expression in macrophages (41). Moreover, type I IFN was shown to inhibit production of IL-1β from myeloid cells *in vivo* (42), resulting in a loss of IL-1β-mediated control of bacterial burden (43). This discrepancy may be attributed to an increase in p62 post-infection in *Usp18*^{Ity9} mice since accumulation of p62 and subsequent activation of NF-κB has been shown to increase IL-1β (30). We do observe an increase in IL-23 and IL-17 in the spleen *Usp18*^{Ity9} mutant mice which is consistent with reports of an IL-1β-dependent increase in IL-23 resulting in enhanced IL-17 secretion from T cells (31, 32). USP18 was recently shown to play an important role in adaptive immunity by controlling Th17 cell differentiation (44). USP18 knock out T cells were shown to be deficient in Th17 differentiation, which contrasts with our observation that *Usp18*^{Ity9} mutant mice had elevated levels of *Il17* transcript and increased production of IL-17 from CD4^{+} T cells during *Salmonella* infection. This apparent discrepancy could be explained by the fact that the *Usp18*^{Ity9} mutation does not affect the peptidase domain of USP18 and Liu *et al.* reported that the enzymatic activity of USP18 was important for controlling Th17 differentiation. The exact mechanism underlying increased IL-1β and IL-17 in *Usp18*^{Ity9} mutant mice is not fully understood, however, we suggest that inhibition of autophagy by type I IFN signaling may be one of the mechanisms. This hypothesis is based on the reported observations that inhibition of autophagy promotes inflammasome activity and increases IL-1β production (45) and that IL-17 is increased in *M. tuberculosis* infected mice deficient in autophagy (46) and a prolonged Th17 response contributes to the pathogenesis of the infection (47). Autophagic degradation of intracellular bacteria is an important host defense mechanism during infection. Recently, invading *Salmonella* were shown to induce a transient amino acid starvation as a result of membrane damage leading to the induction of autophagy and host protection (48). Yet, *Salmonella* effector molecules including SseL, a deubiquitinase, and SopD2, a regulator of SCV integrity, contribute to the inhibition of autophagy during infection (49, 50), suggesting that bacterial evasion of the host autophagic response is key to bacterial replication. Low levels of autophagy lead to accumulation of p62 and decreased LC3II (51). Thus, the increased p62 and decreased LC3II observed in *Usp18*^{Ity9} mice *in vivo* raises the possibility that autophagy may be impaired in *Usp18*^{Ity9} mutant mice although a more comprehensive evaluation of autophagy *in vitro* will be necessary to draw a final conclusion.

The role of type I IFN in the regulation of mycobacteria infections is highlighted by recent studies showing that IFNAR$^{-/-}$ mice are more resistant to *M. tuberculosis* infection and that patients with active tuberculosis have a characteristic IFN-inducible gene signature (52, 53). An increase in the type I IFN response was also reported in lepromatous leprosy skin lesions when compared to self-healing tuberculoid lesions caused by *Mycobacterium leprae in vivo* (54). We show in the current paper that *Usp18*^{Ity9} mice are more susceptible to *Mycobacterium tuberculosis* infection and have increased bacterial load in lung and spleen, elevated inflammatory cytokine production and more severe lung pathology. As shown during *Salmonella* infection and in contrast with previous studies (35), the increases in circulating ISG15 and IFNγ do not appear to be protective during infection in *Usp18*^{Ity9}

mice. This finding demonstrates that USP18 plays a broad role during infection with intracellular bacteria, additional studies will provide further insight into the mechanism of susceptibility in *Usp18*^{Ity9} mice during mycobacterial disease.

In summary, this work describes the biological significance of type I IFN signaling in the survival of *Usp18*^{Ity9} mice following *Salmonella* infection and outlines a model of susceptibility resulting from increase in proinflammatory cytokine production as a consequence of hyperactivation of type I IFN signaling, and increased IL-10 production, leading to deregulated expression of IL-1β and autophagy markers that results in increased bacterial burden and septic shock in *Salmonella*-infected *Usp18*^{Ity9} mice. Our studies reveal that a mutation in human USP18 corresponding to the *Usp18*^{Ity9} 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 #5797).

Mice

The *Usp18*^{Ity9} mutation was originally identified on a mixed C57BL/6 × 129S1/SvImJ × 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), resulting in mice that are >99.8% 129S1 or DBA/2J. *Isg15* knockout (B6.129P2-*Isg15*^{tm1Kpk}/J; Jackson Laboratories) were crossed with *Usp18*$^{+/+}$ or *Usp18*^{Ity9} mice to generate F1 mice that were heterozygous for *Isg15* and *Usp18*^{Ity9} or the wild-type allele. These mice were intercrossed to generate mice that were homozygous for *Usp18*^{Ity9} 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 7–12 weeks of age were infected intravenously with *Salmonella* Typhimurium strain Keller, as described by us previously (3). Mice were infected in the caudal vein and monitored twice daily for survival. Alternatively, spleens and livers were collected, homogenized in saline and CFUs were determined by plating of serial dilutions on trypticase soy agar plates.

ELISA

Serum was obtained from the blood of infected mice and cytokines were assayed by ELISA (eBioscience) according to the manufacturers directions.

RNA extraction and quantitative RT-PCR

Total RNA was isolated from mouse tissue using the TRIzol reagent (Invitrogen Life Technologies). 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) on a StepOnePlus apparatus (Applied Biosystems). The Ct values for the genes of interest were normalized to the housekeeping gene TATA-binding protein (TBP). The relative expression of the gene was calculated as $2^{-\Delta\Delta Ct}$.

Bone marrow-derived macrophages

Femurs were collected from 8–12 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 RBCs lysed for 5 min using a commercial RBC Lysis Buffer (Sigma-Aldrich). Cells were resuspended in complete medium (RPMI supplemented with 10% heat-inactivated FBS, 2 mM glutamine, 100 μg/mL Penstrep and 30% L929 conditioned medium as a source of murine CSF. L929 supernatant was replenished every 2 days prior to cell counting and plating.

Immunoblotting

Protein lysates were prepared using the CellLytic-M reagent (Sigma-Aldrich) according to the manufacturers directions. Proteins were quantified by the Bradford method (Bio-Rad) and Western blotting was carried out using 25–50 μg of protein resolved by SDS-PAGE, transferred to PVDF membrane and immunoblotted as indicated. β-actin was used to assess protein loading.

Plasmids and transfection

The wild type USP18 expression constructs were described previously (55). $USP18^{L365F}$ mutant constructs were generated by site directed mutagenesis. ISG15 isopeptidase activity and type I IFN inhibitor activity of wild type and mutant USP18 were examined using an ex vivo ISGylation system in 293T cells and in HeLa cells, respectively, as described previously (55, 56).

MAR1-5A3 treatment

Mice were pretreated with 2 mg of either MAR1-5A3 or mouse IgG_1 isotype control antibody (Leinco Technologies, Inc.) 1 day prior to infection with 1000 CFUs of *Salmonella* Typhimurium.

Flow cytometry

Spleens were harvested from 8–12 week old mice, macerated and passaged through a 70 μM cell strainer. RBC lysis was performed using 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 (10×10^6 cells) using CytoFix/CytoPerm (BD Biosciences). Briefly, cells were stimulated *in vitro* (4 h) with PMA (50 ng/mL; Sigma-Aldrich) and Ionomycin (500 ng/mL; Sigma-Aldrich) and intracellular transport was

inhibited using GolgiStop (BD Biosciences). Cells were stained with antibodies to CD4, CD3, B220 and IL-17 and gating was performed to include $CD3^+CD4^+B220^-IL\text{-}17^+$ cells. All samples were analysed using a FACSCanto (BD Biosciences) with FlowJo software (Tree Star).

ROS measurements

BMDM were plated in non-tissue-culture treated six-well dishes and infected with heat-killed Salmonella (MOI = 50, 6 h). Complete DMEM was aspirated, cells were washed with PBS and incubated with CM-H_2DCFDA (10 μM, 30 min; Invitrogen) in serum-free medium. Cells were washed with warm PBS, then removed from the well using cold PBS containing 10 mM EDTA, pelleted at 1200 r.p.m., resuspended in cold PBS containing 1% FBS and analysed using a FACSCanto (BD Biosciences) with FlowJo software (Tree Star). Mean fluorescence intensity values were calculated as fold change over uninfected cells.

In vivo Mycobacterium tuberculosis infections

Mycobacterium tuberculosis H37Rv was grown at 37°C in Middlebrook 7H9 medium (Difco Laboratories) containing 0.05% Tween-20 (Sigma-Aldrich) and 10% albumin-dextrase-catalase (ADC) supplement (Becton Dickson and Co.). Bacteria were delivered by aerosol using an inhalation exposure system (In-Tox Products) and infectious dose was confirmed by enumeration of bacteria within the lungs of control mice at 24 hours post-infection. Mice were euthanized at 6 weeks post-infection, organs were homogenized in PBS and bacterial burden was determined by serial dilution on Middlebrook 7H10 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 prior to immunohistochemical analysis or stored in RNA later (Ambion). 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 analysed using a two-tailed Student's *t*-test using the GraphPad Prism statistical program. P values of less than 0.05 were considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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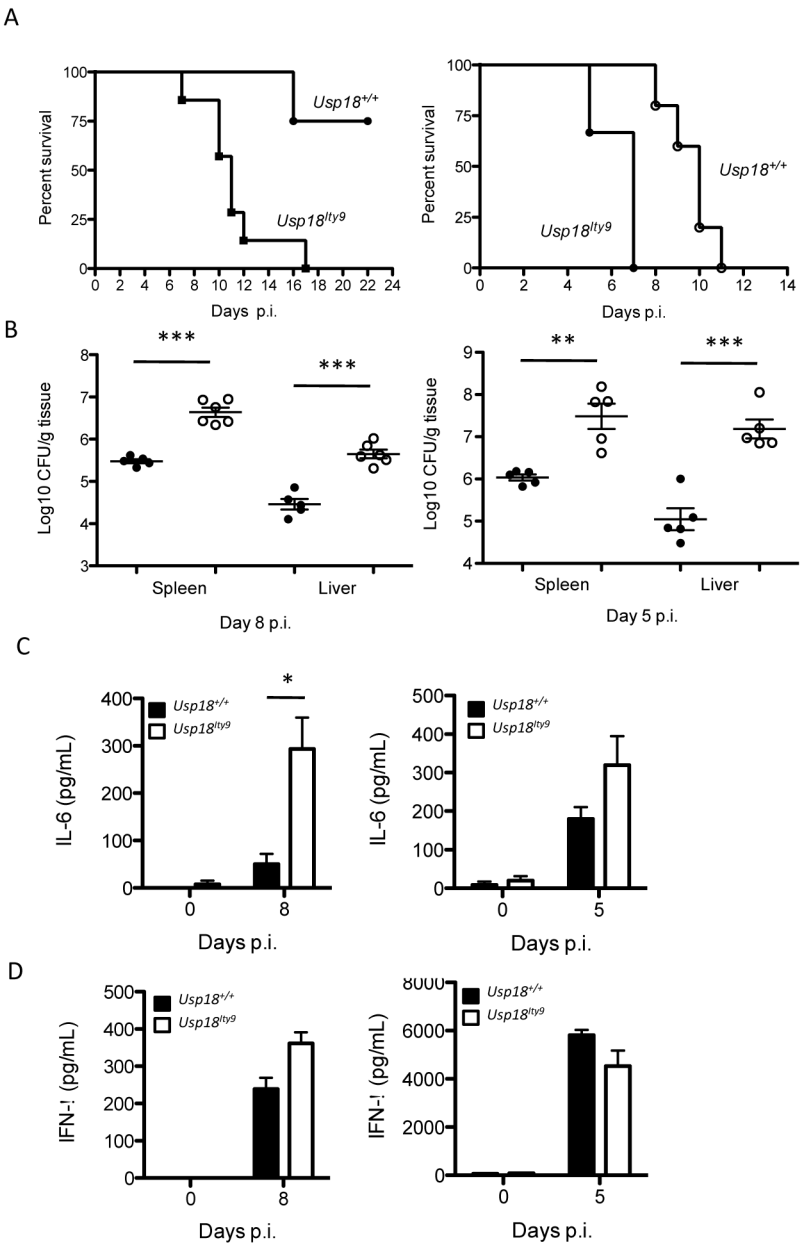

Figure 1. Genetic background affects the allelic expression of 129S1.Cg-*Usp18*^{Ity9} and D2.Cg-*Usp18*^{Ity9}

Mice were infected intravenously with 1.5 X 10^4 CFUs *Salmonella* Typhimurium isolate Keller and (A) survival was monitored for 24 days (129S1.Cg-*Usp18*^{Ity9}, left panel; Log-rank (Mantel-Cox) p <0.001) or 14 days (D2.Cg-*Usp18*^{Ity9}, right panel; Log-rank (Mantel-Cox) p <0.007); *Usp18*$^{+/+}$ (n=4), *Usp18*^{Ity9} (n=7), (B) bacterial load was measured in the spleen and liver at 8 days post-infection (p.i.) (129S1.Cg-*Usp18*^{Ity9}, left panel; *** p < 0.0001) or 5 days p.i. (D2.Cg-*Usp18*^{Ity9}, right panel; ** p = 0.0015 and *** p = 0.0002) (*Usp18*$^{+/+}$, dark circles and *Usp18*^{Ity9}, open circles), and (C–D) cytokines were measured at 8 days p.i. (129S1.Cg-*Usp18*^{Ity9}, left panel) or 5 days p.i. (D2.Cg-*Usp18*^{Ity9}, right panel) in the serum by ELISA in *Usp18*$^{+/+}$ (black, n=5), *Usp18*^{Ity9} (white, n=5); * p = 0.01.

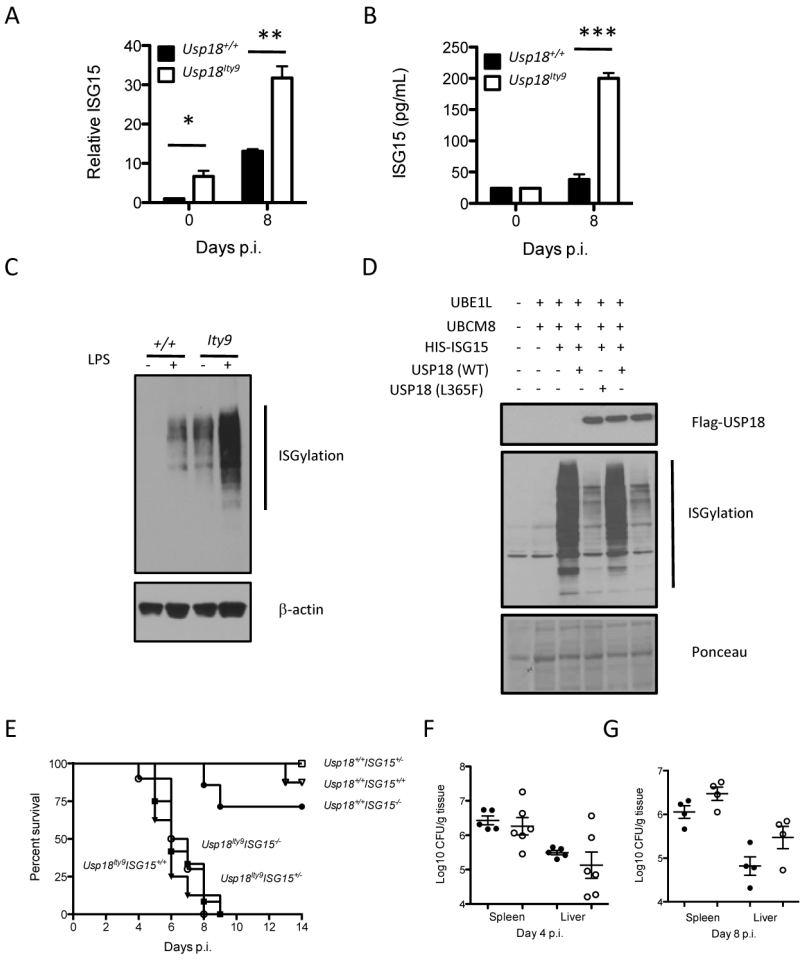

Figure 2. The susceptibility of 129S1.Cg-*Usp18*^{Ity9} mice to *Salmonella* infection is not due to increase ISG15 expression and function

(A–B) *Usp18*$^{+/+}$ (black, n=3) and 129S1.Cg-*Usp18*^{Ity9} (white, n=3) mice were infected intravenously with 10^3 CFUs *Salmonella* Typhimurium for 8 days and (A) RNA was extracted from spleen for qRT-PCR (*p = 0.017, **p = 0.0035) and (B) ISG15 was measured in the serum by ELISA (*** p < 0.0001) (C) Western blot analysis of bone marrow derived macrophages stimulated with 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) Survival of mice infected with *Salmonella* Typhimurium for 14 days; *Usp18*^{Ity9}*Isg15*$^{+/+}$ (n=8), *Usp18*^{Ity9}*Isg15*$^{+/-}$(n=12), *Usp18*^{Ity9}*Isg15*$^{-/-}$ (n=10), *Usp18*$^{+/+}$*Isg15*$^{+/+}$ (n=8), *Usp18*$^{+/+}$*Isg15*$^{+/-}$ (n=8), *Usp18*$^{+/+}$*Isg15*$^{-/-}$ (n=7); Log-rank (Mantel-Cox) p <0.0001 and (E–F) bacterial load in the spleen and liver was measured at (F) 4 days p.i. in *Usp18*^{Ity9} mice (left panel, *Usp18*^{Ity9}*Isg15*$^{+/+}$, dark circles and *Usp18*^{Ity9}*Isg15*$^{-/-}$) and (G) 8 days p.i. in *Usp18*$^{+/+}$ (right panel, *Usp18*$^{+/+}$*Isg15*$^{+/+}$, dark circles and *Usp18*^{Ity9}*Isg15*$^{-/-}$, open circles).

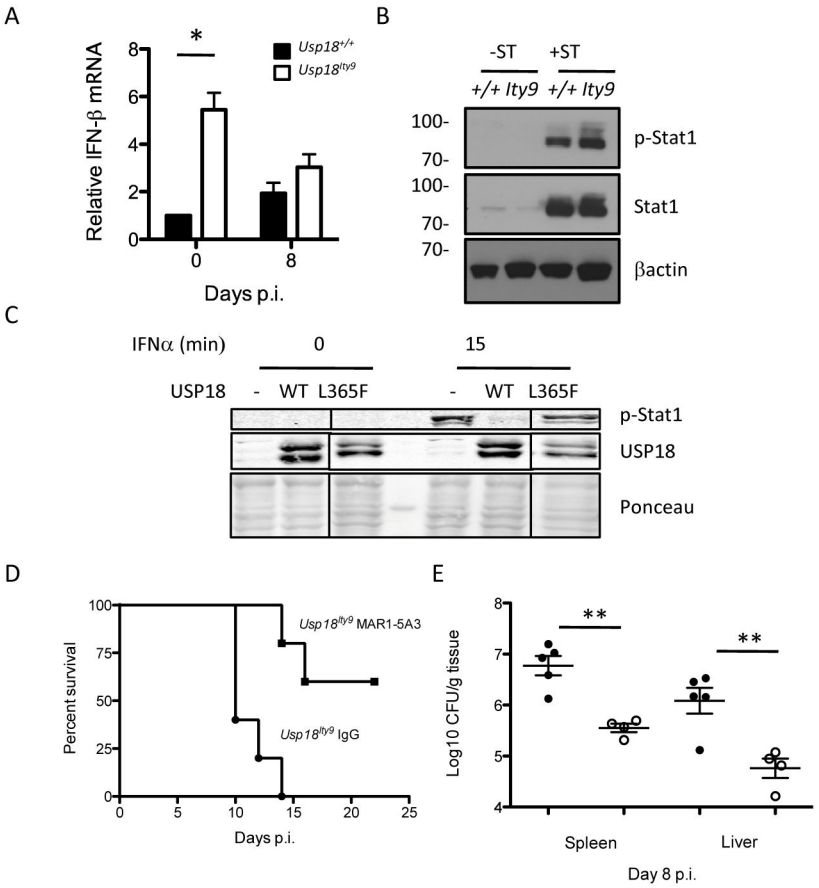

Figure 3. Blocking type I IFN signaling partially rescues the susceptibility of 129S1.Cg-*Usp18*^{Ity9} mice to *Salmonella* infection

(A–B) *Usp18*$^{+/+}$ (black, n=3) and 129S1.Cg-*Usp18*^{Ity9} (white, n=3) mice were infected intravenously with 1.5 X 10^4 CFUs *Salmonella* Typhimurium for 8 days and (A) RNA was extracted from spleen for qRT-PCR (*p = 0.015) (B) Western blot analysis was performed on spleen tissue lysates; n=3 mice/genotype (C) Hela cells were transduced with retrovirus for expressing the wild type and mutant USP18 as indicated, stimulated with IFNα (3000 U/mL, 15 min) and immunoblotting was performed. Total protein staining with Ponceau S was used to confirm equal loading. (D–E) *Usp18*^{Ity9} mice were given MAR1-5A3 or isotype control 1 day prior to infection with *Salmonella* Typhimurium and (D) survival was monitored for 22 days p.i.; n=5 mice/treatment; Log-rank (Mantel-Cox) p <0.005 and (E) bacterial load was measured in the spleen and liver in at 8 days p.i. (IgG, dark circles and MAR1-5A3, open circles; **p = 0.001 and 0.005 for spleen and liver, respectively).

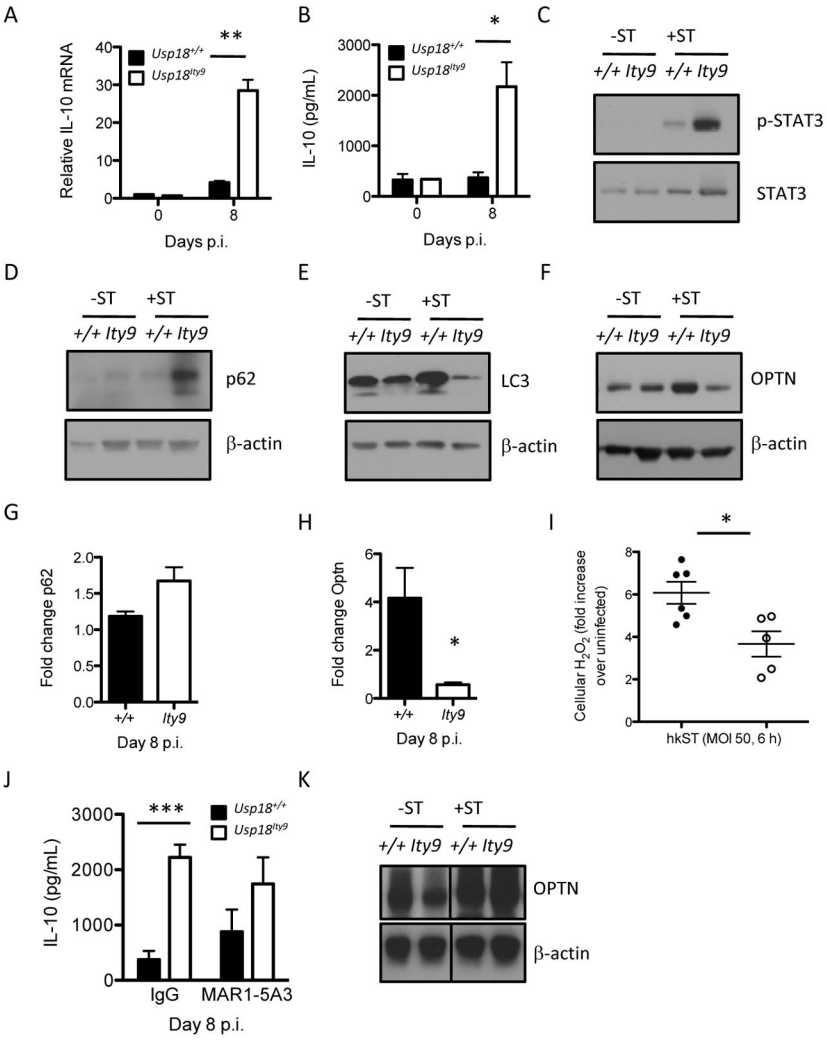

Figure 4. 129S1.Cg-*Usp18*^{lty9} have increased IL-10 and deregulated expression of autophagy markers

Mice were infected intravenously with *Salmonella* Typhimurium for 8 days and (A) RNA was extracted from spleen for qRT-PCR; (**p = 0.001), (B) IL-10 was measured in the serum by ELISA (*p = 0.019), and (C–F) Western blot analysis was performed on spleen tissue lysates using antibodies for STAT3 or p-STAT3 (C), p62 (D), OPTN (E) and LC3 (F). (G–H) RNA was extracted from spleen for qRT-PCR (*p = 0.05); (I) BMDM were infected with heat-killed *Salmonella* (MOI = 50), stained with CM-H_2DCFDA and analyzed by FACS. Data is represented as mean fluorescence intensity fold changes (*p = 0.01); (J) IL-10 was measured in the serum of MAR1-5A3 or IgG control treated mice at 8 days p.i. by ELISA (***p = 0.0005); (K) Western blot 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.

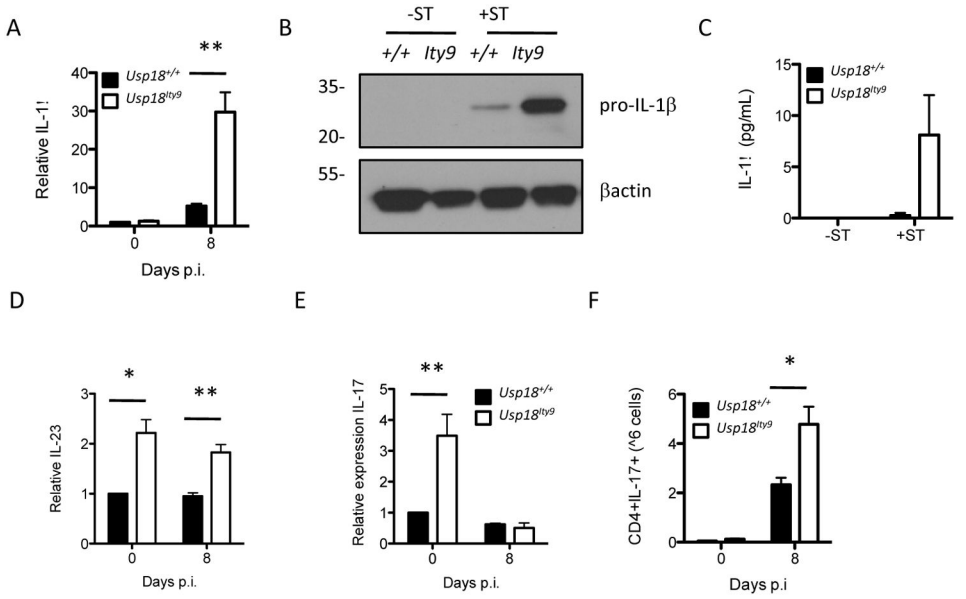

Figure 5. 129S1.Cg-*Usp18*^{Ity9} have increased IL-1β and an elevated Th17 response

Mice were infected intravenously with 1.5 X 10^4 CFUs *Salmonella* Typhimurium for 8 days and (A) RNA was extracted from spleen for qRT-PCR (** p = 0.009), (B) Western blot analysis was performed on spleen tissue lysate using an antibody for IL-1β, (C) Splenocytes were harvested and cultured for 24 h prior to measuring IL-1β in the supernatant by ELISA, (D–E) RNA was extracted from 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 2 experiments.

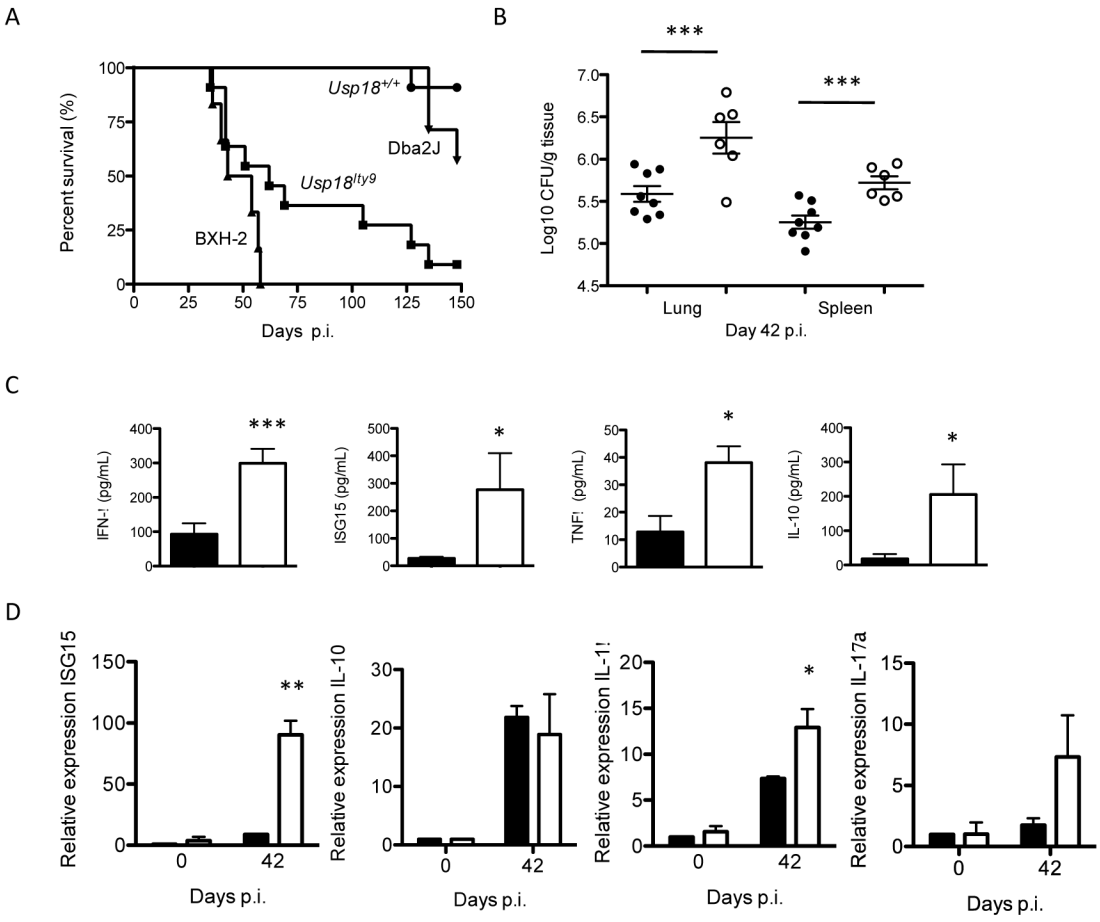

Figure 6. *Usp18*^{Ity9} are more susceptible to *M. tuberculosis* infection

Mice were infected by aerosol with 100 CFUs of H37Rv and (A) survival was monitored for 150 days p.i. ($p < 0.001$), n=11 mice/genotype from 2 experiments (B) bacterial load was measured in the lung and spleen (*Usp18*$^{+/+}$, dark circles and *Usp18*^{Ity9}, open circles) at 42 days p.i. (***p = 0.005 and 0.001 for lung and spleen, respectively), (C) cytokines were measured in the serum by ELISA at day 42 p.i. n=6–8 mice/genotype and (D) RNA was extracted from lung for qRT-PCR n=3 mice/genotype (*p = 0.05, **p = 0.002).