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Toll-like receptor deficient mice reveal how innate immune signaling influences *Salmonella* virulence strategies

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Summary

Pathogens utilize features of the host response as cues to regulate virulence gene expression. *Salmonella enterica* serovar Typhimurium (ST) sense Toll-like receptor (TLR)-dependent signals to induce *Salmonella* Pathogenicity Island 2 (SPI2), a locus required for intracellular replication. To examine pathogenicity in the absence of such cues, we evaluated ST virulence in mice lacking all TLR function (*Tlr2*$^{-/-}$×*Tlr4*$^{-/-}$×*Unc93b1*$^{3d/3d}$). When delivered systemically to TLR-deficient mice, ST do not require SPI2 and maintain virulence by replicating extracellularly. In contrast, SPI2 mutant ST are highly attenuated after oral infection of the same mice, revealing a role for SPI2 in the earliest stages of infection, even when intracellular replication is not required. This early requirement for SPI2 is abolished in *MyD88*$^{-/-}$ x*TRIF*$^{-/-}$ mice lacking both TLR- and other MyD88-dependent signaling pathways, a potential consequence of compromised intestinal permeability. These results demonstrate how pathogens use plasticity in virulence strategies to respond to different host immune environments.

Introduction

Receptors of the innate immune system evolved to detect highly conserved microbial features called pathogen associated molecular patterns (PAMPs) (Janeway, 1989, Medzhitov, 2007). This strategy enables recognition of diverse microbial species with a fairly limited set of receptors. The Toll-like receptors (TLRs) localize to the plasma membrane and endosomes and bind diverse microbial ligands, including lipopolysaccharide (LPS), bacterial lipoproteins, flagellin, and nucleic acids. Receptor activation leads to recruitment of adaptor proteins MyD88 and/or TRIF, and subsequent signal transduction contributes to the initiation of both innate and adaptive immunity (Kawai and Akira, 2010).

TLRs control a number of antimicrobial effector mechanisms that contribute to the earliest aspects of the host response to infection. Many of these effector mechanisms are initiated by expression of cell adhesion molecules, cytokines and chemokines, and antimicrobial peptides (AMPs) (Newton and Dixit, 2012). TLRs also induce non-transcriptional effects like accelerated phagosome maturation, involving rapid acidification and production of

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reactive oxygen and nitrogen species (Blander and Medzhitov, 2004, Blander and Medzhitov, 2006, Arpaia et al., 2011). These activities are critical for the destruction and clearance of engulfed or invading microbes. Thus, TLRs regulate multiple aspects of the immediate host response to infection, which explains why mice lacking TLR function succumb to pathogen challenge (Kawai and Akira, 2010).

Pathogens employ virulence mechanisms that enable survival despite recognition by the innate immune system. For example, some bacteria can survive and replicate inside host cells either by escaping from the phagocytic vacuole or by altering its antimicrobial properties (Casadevall, 2008). In this manner, intracellular pathogens avoid being killed in the phagosome while creating a replicative niche shielded from extracellular antimicrobial responses. Whatever the precise strategy, it is generally true that expression of virulence genes must be tightly regulated (Mekalanos, 1992). In addition to being energetically costly, some of these factors are sensed by innate immune receptors, so unnecessary expression can be detrimental (Vance et al., 2009, Miao and Warren, 2010). Thus, pathogens utilize environmental cues to precisely coordinate virulence gene expression. Defining these cues, as well as pathogen behavior in their absence, is an important step toward understanding the pathogenesis of a given microbe.

These concepts of virulence gene regulation certainly apply to the enteric pathogen *Salmonella enterica* serovar Typhimurium (ST). ST encodes two distinct type three secretion systems (TTSSs) within large genomic regions termed *Salmonella* Pathogenicity Island (SPI) 1 and SPI2 (Srikanth et al., 2011). The SPI2 locus, encoding TTSS-2 and associated effector proteins, is expressed in the phagosome and functions to transform this characteristically antimicrobial compartment into a replicative niche (Figueira and Holden, 2012). Notably, ST requires TTSS-2 for systemic dissemination and virulence (Fields et al., 1986, Leung and Finlay, 1991). Recent data indicate that SPI2 gene products are also expressed in the gut and may facilitate intracellular transit from the apical to the basolateral side of intestinal epithelial cells (IECs) (Muller et al., 2012, Brown et al., 2005). While progress has been made in characterizing the molecular triggers responsible for phagosomal SPI2 induction (Arpaia et al., 2011, Deiwick et al., 1999, Cirillo et al., 1998), the physiological cues responsible for SPI2 expression in the gut are less well defined.

While the 13 described TLRs bind a variety of distinct PAMPs, most of these receptors converge upon the signaling adaptor MyD88 (Kawai and Akira, 2010). To reveal phenotypes masked by redundancy, the collective role of TLRs has been largely studied using mice lacking MyD88. However, MyD88 also mediates signaling from interleukin (IL)-1, IL-18, and IL-33 receptors as well as other non-TLR/IL-1R family receptors (Sun and Ding, 2006, O'neill, 2008, He et al., 2010), complicating interpretation of phenotypes in these mice. Furthermore, MyD88-deficient mice have reduced intestinal AMPs and IgA, factors critical to maintain gut homeostasis (Vaishnava et al., 2011, Frantz et al., 2012). To overcome potential caveats associated with MyD88-deficient mice, we have intercrossed mice lacking TLR genes or function to investigate how immune activation by TLRs influences pathogen virulence strategies and the regulation of virulence gene expression.

Our previous work showed that ST use TLR-dependent phagosomal acidification as a cue to express SPI2 genes and replicate inside bone marrow-derived macrophages (BMMs). Thus, despite possessing reduced TLR function, mice lacking TLR2, TLR4, and TLR9 ($Tlr2^{-/-}\times Tlr4^{-/-}\times Tlr9^{-/-}$ or TLR2×4×9) were *less* susceptible to ST infection compared to mice lacking only TLR2 and TLR4 ($Tlr2^{-/-}\times Tlr4^{-/-}$ or TLR2×4) because of a defect in intracellular ST replication (Arpaia et al., 2011). While this work established the connection between TLR signaling and ST virulence, we could not address why a significant proportion of TLR2×4×9 mice still succumb to oral infection. There are at least two possible

explanations for this partial phenotype: either residual TLR signaling supports intracellular replication *in vivo* (that is not detectable in BMMs *in vitro*), or ST maintain pathogenicity by an alternative virulence strategy that does not require TTSS-2 for replication. To distinguish between these two possibilities, we compared ST virulence and the requirement for SPI2 in a panel of mice that included animals that specifically lack TLR signaling. We found that 'TLR-deficient' mice were quite susceptible to systemic infection, and ST maintain virulence in these mice because a lack of inflammation permits extracellular replication that does not require SPI2 proteins. Additionally, the comparison between TLR-deficient and mice lacking MyD88 revealed an unexpected requirement for SPI2 for productive oral infection by ST.

Results

TLR-mediated responses to ST are absent in TLR-deficient BMMs

To determine if ST can maintain virulence in a TLR-deficient environment, we intercrossed TLR2×4 mice with *Unc93b1*$^{3d/3d}$ (3d) mice to generate *Tlr2*$^{-/-}$×*Tlr4*$^{-/-}$×*Unc93b1*$^{3d/3d}$ mice (TLR2×4×3d or 'TLR-deficient'). Since TLR1 and TLR6 operate as heterodimers with TLR2, the function of these receptors is disrupted in *Tlr2*$^{-/-}$ mice (Ozinsky et al., 2000). 3d mice have an inactivating mutation in *Unc93b1*, a gene encoding a trafficking chaperone required for proper localization of many TLRs (Kim et al., 2008). Our group and others have shown that 3d mice exhibit a specific defect in signaling from TLRs -3, -7, -8, -9, -11, -12, and -13 (Lee and Barton). Of note, while 3d mice were originally reported to have a defect in antigen presentation, subsequent studies have been unable to confirm this observation (Deguine et al., 2013, Tabeta et al., 2006). Moreover, we unexpectedly found that Unc93b1 is also required for TLR5 trafficking, as TLR5 failed to acquire endoglycosidaseH (EndoH)-resistant glycosylation in immortalized BMMs (iBMMs) derived from 3d mice (Figure S1A). Additionally, TLR5-expressing 3d iBMMs did not respond to flagellin stimulation (Figure S1B). Thus, while we cannot completely rule out the possibility of TLR signaling independent of Unc93b1, TLR-deficient mice should lack all known TLR function while retaining MyD88 function downstream of other receptors.

We compared the responsiveness of BMMs from our various strains after stimulation with TLR ligands or infection with ST. While wild type (WT), TLR2×4, and TLR2×4×9 BMMs maintained responsiveness to the expected TLR ligands, TLR2×4×3d BMMs and BMMs lacking MyD88 and TRIF (*MyD88*$^{-/-}$×*TRIF*$^{-/-}$, MYxTR) did not respond to any of the ligands tested (Figures 1A and 1B). Additionally, while WT BMMs robustly produced both nitrite and TNF-α in response to ST, the residual response observed in TLR2×4×9 cells was eliminated in TLR2×4×3d BMMs, suggesting that TLR-mediated responses to ST are abrogated in TLR2×4×3d mice (Figures 1A and 1B). To assess murine responses directly, we measured serum proinflammatory cytokines in WT and TLR2×4×3d mice infected with ST by the intraperitoneal (IP) route. While WT mice exhibited increases in serum cytokines, TLR2×4×3d animals did not produce any measurable CCL2 or TNF-α at early time points after ST infection (Figure S1C).

To ensure that MyD88 remained functional in TLR2×4×3d mice, we measured responsiveness to IL-18 and IL-1. After IP injection of IL-1α recruitment of neutrophils (NEs) and inflammatory monocytes (IMs) to the peritoneal cavity was comparable in WT and TLR2×4×3d mice (Figures 1C and S1D). Moreover, NK1.1^{+} cells from WT and TLR2×4×3d mice produced similar levels of IFN-γ in response to stimulation with IL-18 and IL-12 (Figure 1D). As expected, MYxTR mice failed to respond to both cytokines (Figures 1C, 1D, and S1D). These results suggest that TLR2×4×3d mice lack TLR responses to ST yet retain MyD88-dependent signaling downstream of other receptors.

To determine if ST can replicate inside TLR2×4×3d BMMs, we used immunofluorescence microscopy (IF) to compare the number of ST per cell in TLR2×4 and TLR2×4×3d BMMs at 1 and 24 hours-post infection (hpi). In contrast to TLR2×4 BMMs that were previously shown to support intracellular ST replication (Arpaia et al., 2011), the number of ST per cell did not significantly increase in TLR2×4×3d BMMs (Figure 1E). Collectively, these results suggest that TLR2×4×3d mice represent a suitable model to study pathogen virulence in the absence of TLR signaling.

ST are highly virulent in TLR2×4×3d mice

We hypothesized that ST maintains virulence in TLR2×4×9 mice either by exploiting residual TLR signaling for intracellular replication or by using an alternative virulence mechanism altogether. If residual signaling is exclusively responsible for the observed virulence in TLR2×4×9 animals, then TLR2×4×3d mice should be more resistant to infection than TLR2×4×9 mice. In contrast to this prediction, TLR2×4×3d mice were significantly more susceptible to oral infection than TLR2×4×9 mice (Figure 2A). TLR2×4×3d animals also had significantly higher bacterial burdens in their gut tissues and more incidences of dissemination to the spleen and liver when compared to TLR2×4×9 mice (Figure 2B). These results suggest that ST may utilize an alternative, perhaps TTSS-2-independent, replication mechanism in TLR2×4×3d mice.

ST maintain virulence in TLR2×4×3d mice by TTSS-2 independent replication

To determine if ST require TTSS-2 for virulence in TLR-deficient mice, we compared the ability of wild type (wt) and SPI2-deficient (spi2-) ST to replicate in these animals. Since oral infection has colonization bottlenecks that can increase variability, we used the IP route for greater control over the dose and synchrony of infection. First, we established that TLR2×4×3d mice infected by IP injection were, similar to oral gavage, highly susceptible to wt ST (Figure 3A). Next, we injected a panel of mice with either wt or spi2- ST and measured spleen and liver colonization at 1 day post-infection (dpi). Surprisingly, we detected no difference between wt and spi2- ST colonization in any murine genotype, although both strains replicated to higher CFUs in mice with less TLR function (Figure 3B). Our inability to detect a difference between wt and spi2- ST in WT mice is at odds with the well-documented role for SPI2 during systemic infection (Fields et al., 1986, Leung and Finlay, 1991). One likely explanation is that replication in WT mice is minimal at 1 dpi, so the requirement for SPI2 for virulence may not be as apparent.

To compare more fairly whether SPI2 expression is advantageous in each mouse strain, we measured colonization after the bacteria had undergone many rounds of replication and also used a competitive infection to directly measure any difference between wt and spi2- ST. Under these conditions, wt ST had a significant advantage in WT and TLR2×4 mice that support TTSS-2 dependent intracellular replication (Figures 3C and S2). In contrast, wt and spi2- ST replicated equally well in TLR2×4×3d and MYxTR mice, indicating that SPI2-encoded genes are dispensable for virulence in the absence of TLR signaling. Interestingly, while SPI2 gene expression was advantageous in the majority of TLR2×4×9 mice, spi2- ST replicated similarly to wt ST in a few animals (Figures 3C and S2). These findings imply that both SPI2 TTSS-dependent and -independent replication strategies can occur in TLR2×4×9 mice and may contribute to the split phenotypes observed here and in previous studies (Arpaia et al., 2011). To determine definitively if SPI2 gene products are required for virulence in each background, we monitored survival after systemic infection with spi2- ST. In contrast to WT, TLR2×4, and TLR2×4×9 mice, TLR2×4×3d and MYxTR animals were highly susceptible to spi2- ST (Figure 3D). This result confirms that TTSS-2 is indeed dispensable for virulence in mice lacking all TLR signaling. In addition, while some

replication can occur independently of TTSS-2 in TLR2×4×9 mice, expression of the SPI2 locus is required for full virulence in the presence of residual TLR signaling.

Inflammatory responses to ST are impaired in TLR2×4×3d mice

It is conceivable that TLR-deficient mice permit TTSS-2-independent replication because impaired innate immune responses render the extracellular space a 'safe' replicative niche. To characterize inflammatory responses in the various backgrounds, we infected mice with wt ST and measured splenic cellular infiltration. Both TLR2×4×3d and MYxTR mice did not exhibit appreciable increases of NEs or IMs (Figure 4). Differences in bacterial colonization could not account for the lack of inflammation seen in TLR2×4×3d and MYxTR mice, as the CFUs were comparable in all genotypes (Figure S3). These data show that early inflammatory responses are impaired in TLR2×4×3d mice, potentially permitting unchecked replication in the extracellular space.

TTSS-2 independent replication can occur extracellularly in TLR2×4×3d mice

To determine if TTSS-2 independent replication can occur in the extracellular space, we used gentamicin, a bactericidal antibiotic that cannot readily cross eukaryotic cell membranes. 24 h after systemic infection with wt ST, cohorts of TLR2×4 and TLR2×4×3d mice received an intravenous dose of gentamicin and were sacrificed 6 h later. If ST replicate extracellularly in TLR2×4×3d mice, then gentamicin-treated TLR2×4×3d animals would yield significantly less CFUs than untreated TLR2×4×3d mice. Conversely, since ST replicate inside TLR2×4 cells (Figure 1E and (Arpaia et al., 2011)), gentamicin treatment should not affect colonization of TLR2×4 mice. While organs harvested from all TLR2×4 mice were colonized similarly, TLR2×4×3d mice treated with gentamicin displayed significant decreases in colonization (79% in the spleen and 91% in the liver) (Figure 5A). This result indicates that the vast majority of ST present in TLR2×4×3d mice were extracellular. To ensure that these results were due to differences in bacterial localization and not a consequence of TLR2×4×3d mice having higher burdens, we infected a set of TLR2×4 mice and waited 4 d before gentamicin treatment. Despite the fact that CFUs were roughly 3 logs higher, treated and untreated TLR2×4 mice again displayed similar bacterial burdens indicating that the majority of the ST present in TLR2×4 mice are intracellular (Figure 5B).

TTSS-2 is required during oral infection of TLR2×4×3d mice

The data generated using systemic infections support a model where, in the absence of TLR signaling, ST do not require the SPI2 TTSS for virulence and can replicate extracellularly. We next examined if the same mechanisms explained ST virulence in TLR2×4×3d mice during the natural, oral route of infection (Figure 2A). In direct contrast to the results found with systemic administration, spi2- ST were largely avirulent in orally-infected TLR-deficient mice (Figures 3D, 6A, and Table S1). To confirm that this unexpected result was not strain-specific, we assessed the virulence of additional ST strains with mutations in SPI2 TTSS structural components. TLR2×4×3d mice were also resistant to oral infection with SseB and SseC mutant ST strains (Figures 6B, 6C, and Table S1). These results suggest that the SPI2 TTSS is required for full virulence in the context of oral infection, even in absence of TLR signaling.

The experiments described thus far were all performed using mice that express a functional Nramp1 protein (*Nramp1*$^{R/R}$), which enhances resistance to ST. To determine if the lack of virulence of spi2- ST in TLR-deficient mice required Nramp1, we tested spi2- ST virulence in TLR2×4×3d mice expressing non-functional Nramp1 (*Nramp1*$^{S/S}$). Similar to TLR2×4×3d *Nramp1*$^{R/R}$ mice (Figures 6A, 6B, 6C), TLR2×4×3d mice on the *Nramp1*$^{S/S}$ background were also largely resistant to oral infection with spi2- ST (Figure 6D and Table

S1). Thus, SPI2 is required for productive oral infection, even in mice lacking all TLR function and functional Nramp1.

The fact that spi2- ST were generally avirulent when delivered orally to TLR2×4×3d mice suggested that SPI2 serves a function other than facilitating intracellular replication during the systemic phase of infection. It was recently reported that TTSS-2 may be required for ST to transit through IECs to access the underlying gut lamina propria (Muller et al., 2012). To establish if the block in virulence is at the level intestinal colonization, we compared wt and spi2- ST bacterial burdens in different anatomical compartments of orally infected mice. In contrast to wt ST, spi2- ST could not consistently be cultured from the small intestinal tissue, Peyer's Patches, MLN, spleens, or livers of WT or TLR2×4×3d mice (Figure 6E). In contrast, wt ST were able to access each of these tissues in both WT and TLR2×4×3d mice and showed increases in CFU from 3 to 5 dpi. wt ST also replicated to significantly higher numbers in TLR2×4×3d mice, likely through unchecked extracellular replication after crossing the epithelium. These results suggest that the block in spi2- virulence occurs very early in this murine typhoid model, perhaps before bacteria cross the epithelial layer or at least shortly thereafter. Moreover, the fact that wt ST are still virulent when delivered orally to TLR2×4×3d mice indicates that SPI2 expression for the purposes of intestinal colonization does not require TLR-dependent cues.

MYxTR mice are susceptible to oral infection with spi2- ST

We next considered whether the requirement for SPI2 during oral infection could be bypassed with a further reduction of immune function. Strikingly, in contrast to TLR-deficient animals, MYxTR mice were significantly more susceptible to oral infection with SPI2-encoded TTSS ST mutants (Figures 6A, 6B, 6C, and Table S1). Based on these results, we hypothesized that SPI2 gene products are necessary for intestinal breach and/or colonization and that MYxTR mice are susceptible to spi2- ST because of compromised intestinal barriers and/or immune function. To determine if barrier function is impaired in MYxTR mice, we compared the susceptibilities of WT and MYxTR mice to oral infection with wt ST, spi2- ST, and a non-invasive ST strain deficient in both TTSS-1 and TTSS-2 function (spi1-/2- ST). TTSS-1 drives non-phagocytic IECs to engulf ST, facilitating the first step in conventional intestinal invasion (Phoebe Lostroh and Lee, 2001). The mutant strains were equally virulent in MYxTR animals, confirming that these mice are unable to contain non-invasive ST (Figure 7A). spi2- ST were also avirulent in WT mice on the *Nramp1*$^{S/S}$ background, indicating that the requirement for the SPI2 TTSS for virulence after oral infection is not only observed in TLR-deficient mice (Figure 7A).

To directly measure intestinal barrier permeability in MTxTR mice, we orally gavaged mice with FITC-dextran and measured translocation into the sera by tracking FITC fluorescence. As expected, FITC-dextran did not cross the barrier efficiently in WT mice, while mice treated with dextran sodium sulfate salts (DSS) to intentionally disrupt the intestinal barrier had increased levels of serum FITC (Figure 7B). Neither TLR2×4×3d nor MYxTR mice showed statistically significant increases in serum FITC when compared to WT mice; however, a subset of MYxTR mice did display increased FITC-dextran translocation relative to WT and TLR2×4×3d mice. While this trend toward increased barrier permeability does correlate with the susceptibility to non-invasive ST in a subset of MYxTR mice, we cannot conclude from these assays that gross barrier defects in MYxTR mice are responsible for the increased susceptibility. Nevertheless, our combined analyses suggest that, at some level, the intestinal barrier is less functional in MYxTR mice than in TLR2×4×3d mice.

Another explanation for MYxTR mice being susceptible to non-invasive ST could be the inability to respond to inflammasome-dependent cytokines. In other words, spi2- ST may access the submucosa in all mouse genotypes, but mice with intact TLR and/or IL-1/IL-18

signaling can clear the infection due to enhanced inflammatory cell infiltration. To test this idea, we orally infected *IL-1R*$^{-/-}$ (lacking responsiveness to IL-1α and IL-1β) and *Caspase1*$^{-/-}$*/11*$^{-/-}$ (lacking functional IL-1β and IL-18) mice with spi2- ST. Both strains were completely resistant to infection with spi2- ST (data not shown). Therefore, in the context of functional TLRs, IL-1 and/or IL-18 are not required for clearance of spi2- ST. However, in an environment lacking TLR signaling, inflammasome-dependent cytokines could trigger inflammatory responses responsible for host resistance. Therefore, we tested if NE infiltration contributed to host defense in TLR2×4×3d mice. Injecting mice with α-Gr1 antibodies before and after oral infection with ST resulted in complete ablation of NEs for the first 5 days of infection (Figure S4A and S4B). As a positive control for functional NE depletion *in vivo*, we treated cohorts of WT mice as shown in Figure S4A and monitored survival after oral infection with fully virulent wt ST. As seen previously, neutropenic WT mice were significantly more susceptible to oral ST infection (Figure 7C) (Vassiloyanakopoulos et al., 1998, Seiler et al., 2000). In contrast, the same α -Gr1 treatment did not significantly increase the susceptibility of TLR2×4×3d mice orally infected with spi2- ST, indicating that differences in NE recruitment are not responsible for the differential susceptibility of TLR-deficient and MYxTR mice (Figure 7D). Rather, the absence of signaling pathways dependent on MyD88, likely functioning downstream of TLRs as well as other receptors, and TRIF compromise the host barrier and permit intestinal colonization by ST in the absence of essential virulence factors.

Discussion

ST is a versatile pathogen that has acquired genes and regulatory mechanisms that facilitate virulence in a number of biological niches. This resourcefulness is also reflected by the variety of host species within which ST can cause disease as well as the ability of ST to survive in non-host ecological environments. Here we used ST in a murine typhoid mouse model to monitor pathogen virulence and the outcome of infection in the absence of TLR signaling. Our results reveal that ST have the ability to maintain pathogenicity in the absence of customary environmental cues. We also demonstrated that ST evolved to induce the SPI2 virulence locus using both TLR-dependent and TLR-independent biological triggers. Lastly, comparing the susceptibilities of TLR- and MyD88-deficient mice to spi2- ST revealed an absolute requirement for TTSS-2 for virulence after oral infection.

Plasticity of Pathogen Virulence Strategies

To examine the nature of ST pathogenesis in TLR2×4×9 animals, we determined the requirement for SPI2 in these mice and also generated TLR2×4×3d animals to address the issue of residual TLR signaling. We found that wt ST had a competitive advantage over spi2- ST in the majority of TLR2×4×9 mice after IP infection. Thus, despite the inability to replicate in TLR2×4×9 BMMs *in vitro* (Arpaia et al., 2011), ST can use TTSS-2 to replicate in TLR2×4×9 cells *in vivo*. As TTSS-2 was not required for replication in TLR2×4×3d mice, we hypothesized that ST assumed an alternative replication strategy in these animals. Indeed, a lack of inflammatory cell infiltration appears to render the extracellular space a viable replicative niche in TLR2×4×3d mice. In addition to demonstrating the flexibility of virulence strategies used by a facultative pathogen, this work implies that the TLR-dependent inflammation may be a selective driver for the evolution and maintenance of an intracellular replication strategy.

We propose that the replicative niche used by ST is dictated by innate immune signaling, and that the relationship between ST pathogenesis and host innate immune function can be represented in a gradient model (Figure S5A). While WT, TLR2×4, and TLR2×4×9 mice generate inflammatory responses that render the extracellular space an unviable replicative

niche, these genotypes support intracellular replication *in vivo*. Despite the fact that a similar virulence strategy is used in these three genotypes, the resistance of each strain differs dramatically. WT mice are most resistant, attributable to a TLR4-mediated cell death pathway that exposes ST to extracellular antimicrobial responses (Broz et al., 2012, Arpaia et al., 2011). When compared to TLR2×4 mice, the enhanced resistance of TLR2×4×9 mice suggests that, while still intracellular, the replicative niche in TLR2×4×9 mice is perhaps less optimal (Arpaia et al., 2011). On the other end of the spectrum, TLR2×4×3d and MYxTR mice do not permit efficient intracellular replication; nevertheless, impaired inflammatory responses - including a lack of reactive oxygen and nitrogen species (Grant et al., 2012, Vazquez-Torres et al., 2000) - allow for replication independent of the SPI2 TTSS that can occur extracellularly. Thus, the level of TLR signaling can dictate the replicative niche most suitable for ST, and TLR2×4×9 mice appear to represent a “tipping point”, as intermediate or split phenotypes likely represent the fact that neither replication strategy is optimal for ST in this background.

TLR-dependent and TLR-independent Cues Induce SPI2 Genes

Considering that spi2- ST were highly virulent when delivered systemically to TLR2×4×3d mice, it was initially difficult to reconcile why SPI2 was required for oral infection of these same mice, especially considering that SPI2 has been classically characterized as critical for systemic virulence. However, it has been demonstrated that SPI2 genes are expressed as quickly as 15 minutes after intestinal delivery in a ligated loop model (Brown et al., 2005), and recent work using intravital microscopy has shown that after apical entry, spi2- ST were less able to transit to the basolateral side of IECs (Muller et al., 2012). These data suggest an updated model of intestinal invasion whereby ST require SPI2 gene products to transcytose the polarized monolayer and access the underlying lamina propria (Figure S5B). Here, we used TLR-deficient mice to demonstrate that TTSS-2 is required for productive oral infection and, in contrast to the phagosome, SPI2 induction in the intestine is independent of TLR signaling.

The anatomical discrepancy in TLR dependence for SPI2 induction could be due to muted TLR signals in the intestine, one of the many mechanisms to maintain homeostasis with PAMP-expressing gut commensals (Abreu et al., 2001, Chassin et al., 2010). In a climate of downregulated TLR function, ST may have evolved to recognize other, TLR-independent signals for intestinal SPI2 induction. While independent of TLRs, it is formally possible that another signaling pathway may induce environment cues similar to those found in a mature phagosome such that the same downstream molecular inputs and sensors used for phagosomal SPI2 induction also function in the intestine. Also of interest is the nature of SPI2 function in these two physiologically distinct niches. That is, are the same SPI2 gene products used for intracellular replication also required for intestinal colonization, or are different subsets of SPI2 genes required for these seemingly distinct functions? Lastly, the exact location of SPI2 induction in the gut is unclear. An intestinal invasion model that requires SPI1 for IEC invasion and SPI2 for transcytosis comes with the assumption that the timing of expression of each locus is regulated. Additionally, there are no known conditions that simultaneously induce both SPIs *in vitro*, suggesting reciprocal regulation (Deiwick et al., 1999). More research is needed to address the environmental cues, expression profile, and spatiotemporal nature of SPI2 induction in the intestine.

TLR2×4×3d and MYxTR Mice Reveal the Requirement for SPI2 for Intestinal Invasion

An important aspect of this study is the differential susceptibilities of TLR-deficient and MYxTR mice to oral infection with spi2- ST. One hypothesis for the enhanced susceptibility of MYxTR mice may relate to reports of compromised intestinal barriers in these animals. Gut commensals are controlled largely through secretion of mucins, AMPs, and IgA into the

intestinal lumen (Brown et al., 2013), and mice lacking MyD88 in their IECs display decreases in these proteins (Vaishnava et al., 2011, Frantz et al., 2012). Such deficiencies result in global changes in bacterial populations and breakdown of intestinal barrier integrity, and as a result MyD88-deficient mice experience increased microbial translocation and susceptibility to experimentally induced colitis (Rakoff-Nahoum et al., 2004, Slack et al., 2009). While we cannot completely rule out that TLR2×4×3d mice have residual TLR function, the distinction between TLR-deficient and MYxTR animals shown here emphasizes the benefit of using TLR2×4×3d mice to evaluate the specific role of TLR signaling.

The fact that TLR-deficient mice were resistant to oral infection with spi2- ST revealed a role for SPI2 gene products during ST pathogenesis: intestinal breach and/or colonization. In contrast to this conclusion, previous work utilizing SPI2 mutant ST has produced several instances where SPI2 mutant strains have been cultured from the MLN and systemic tissues after oral infection (Coombes et al., 2005, Coburn et al., 2005, Hapfelmeier et al., 2005, Fierer et al., 2012). However, these studies were conducted using colitis models that involve pretreating mice with antibiotics prior to ST infection. It is well established that antibiotic-treated mice are dysbiotic; correspondingly, dybiosis compromises the intestinal barrier by allowing certain bacterial communities to outgrow, access the intestinal wall, and damage IECs (Kamada et al., 2013, Stecher et al., 2013). Therefore, similar to MYxTR mice, mice pretreated with antibiotics could allow non-invasive bacteria to breach the intestine without the need for elaborate virulence strategies. In support of this notion, SPI1 is also not required for intestinal invasion in animals pre-treated with antibiotics (Coombes et al., 2005, Hapfelmeier et al., 2005). For this reason, we believe the importance of SPI2 during oral infection has been previously underappreciated. Indeed, there are examples of typhoid-based studies that do not pre-treat animals with antibiotics and show a drastic defect in invasiveness by strains lacking SPI2 (Cirillo et al., 1998, Buckner et al., 2011, Shea et al., 1996). Here, by contrasting TLR-deficient and MYxTR mice, we were able to unequivocally demonstrate that the SPI2 virulence locus is required for intestinal infection.

Experimental Procedures

More details regarding reagents and experiments can be found in the Supplemental Experimental Procedures.

Bacterial Strains and Culture

Streptomycin-resistant (STR^R) *Salmonella enterica* serovar Typhimurium strain SL1344 was obtained from S. Falkow (Stanford University). SL1344 mutant strains *ssaV::kan PrpsMgfp*, *sseB::kan*, *sseC::kan*, and *ssaV::kan orgA::tet PrpsM-gfp* were obtained from D. Monack (Stanford University).

Mice and *in vivo* Infections

All mice were bred and maintained in specific pathogen-free conditions, and all experiments were conducted according to protocols approved by University of California Animal Care and Use Committee. Experiments were conducted on 6 to 12 weeks age- and sex-matched mice. Unless noted otherwise, all of the studies described here use mice with a functional Nramp1 protein ($Nramp1^{R/R}$) (Arpaia et al., 2011). See Supplementary Experimental Procedures for descriptions of strains and backcrossing analyses.

For oral infections, mice were fasted overnight. The following morning, mice were given indicated concentrations of ST in 0.1 ml PBS by OG using 22g feeding needles (Braintree Scientific). For systemic infections, mice were given the indicated amounts of ST in 0.1 ml

PBS by IP injection using 27g needles (BD Biosciences). For survival, infected mice were monitored daily. Animals displaying physical signs of distress or significant body weight loss (≥15% original weight) were euthanized by CO_2 asphyxiation followed by cervical dislocation. For *in vivo* gentamicin protection experiments, infected mice received a retro-orbital intravenous injection of 5 μg/g body weight gentamicin (Gibco). For α-Gr1 NE depletion experiments, mice were mock treated or given 250 μg anti-Gr1 (clone RB6-8C5, a kind gift from D. Portnoy, UC Berkeley) in 0.1 ml PBS by IP injection on days -1, 2, and 5.

CFU analyses

Organs from euthanized mice were harvested at the indicated time points and kept on ice. Organs were homogenized in PBS using a Polytron PT2100 homogenizer (Kinematica) and plated using the Autoplate Spiral Plating System (Advanced Instruments).

Flow Cytometry

Red blood cells were removed from single cell suspensions using ACK Lysing Buffer (Gibco). Cells were then washed, filtered through Nytex nylon mesh (Fisher Scientific), counted, and stained for flow cytometry. Live singlet events were interrogated using LSRII or Fortessa flow cytometers (BD Biosciences) and analysis was performed using FlowJo 6 Software (Tree Star).

Measurement of cytokine responses

To determine IL-1 responsiveness, mIL-1α (2.5 ng/mouse, eBioscience) was delivered in 0.1 ml PBS by IP injection. Peritoneal cells were processed and stained for flow cytometry. To determine IFN-γ production by NK cells, spleens were made into a single cell suspension and cultured overnight with mIL-12p70 (2 ng/ml, eBioscience) and/or mIL-18 (10 ng/ml, R&D). The next morning, 1× GolgiPlug (BD Biosciences) was added for 4 h. Non-adherent cells were removed and processed for ICS.

BMM Experiments

BMM were differentiated from bone marrow using macrophage colony-stimulating factor as previously described (Arpaia et al., 2011). EndoH assays on iMacs were performed as previously described (Lee et al., 2013). For all ST treatments, RP-10 without Penicillin/Streptomycin (RP-10 no P/S) was used. ST infected cells were subjected to centrifugation (5 min, 750 rpm) to facilitate uniform phagocytosis, and after 30 min incubation, were washed and given media containing 10 or 25 μg/ml gentamicin. For ICS, 1×10^6 BMMs or 2×10^5 iMacs were treated with TLR ligands or ST. After 30 min, cells received 1× GolgiPlug and were incubated an additional 4–5 h before processing for ICS. For analysis of nitric oxide production, 1×10^5 mIFN-γ activated (100 U/ml, R&D Systems) BMMs were treated with TLR ligands or ST. Supernatants were analyzed for nitrite content using the Griess Reagent System (Promega) 30h later. IF experiments were performed as previously described (Arpaia et al., 2011). Enumeration was performed in a blinded fashion, and bacteria from 10 random non-overlapping Z-stacked images were counted (at least 75 BMMs per genotype per time point).

Measurement of Splenic Cellular Recruitment

Spleens harvested from euthanized mice were minced with scissors in RP-2.5 (2.5% FBS) containing collagenase VIII (1 mg/ml, Sigma) and DNAse (10 μg/ml, Sigma). Minced tissues were incubated at 37°C for 45 min with gentle agitation. After straining digested tissues, single cell suspensions made were processed and stained for flow cytometry.

Measurement of Barrier Disruption

Mice were pre-bled to determine background signal following by OG with 10 mg FITC-dextran (4kDa, Sigma) in 0.2 ml PBS. Total serum was isolated 4 h later by cardiac puncture. Serum was diluted 1:5 in PBS and fluorescence was measured with excitation and emission wavelengths of 485 and 538, respectively, using a SpectraMax M2 microplate reader (Molecular Devices). Fluorescence values were quantified using a standard curve. As a positive control, WT mice were given 3% dextran sulfate sodium salt (36–50 kDa, MP Bio) *ad libitum* in their drinking water for 7 d prior to FITC-dextran gavage.

Statistical Analysis

Where applicable, the indicated statistical analyses were performed using Prism Software v5 (Graph Pad). All survival comparisons were made using the log-rank (Mantel-Cox) test and all t-tests were two-tailed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- *Tlr2*$^{-/-}$×*Tlr4*$^{-/-}$×*Unc93b1*$^{3d/3d}$ (TLR-deficient) mice lack all TLR signaling
- SPI2 mutant *Salmonella* are virulent when given systemically to TLR-deficient mice
- *Salmonella* can replicate extracellularly in TLR-deficient mice
- SPI2 is required for productive oral infection of TLR-deficient mice

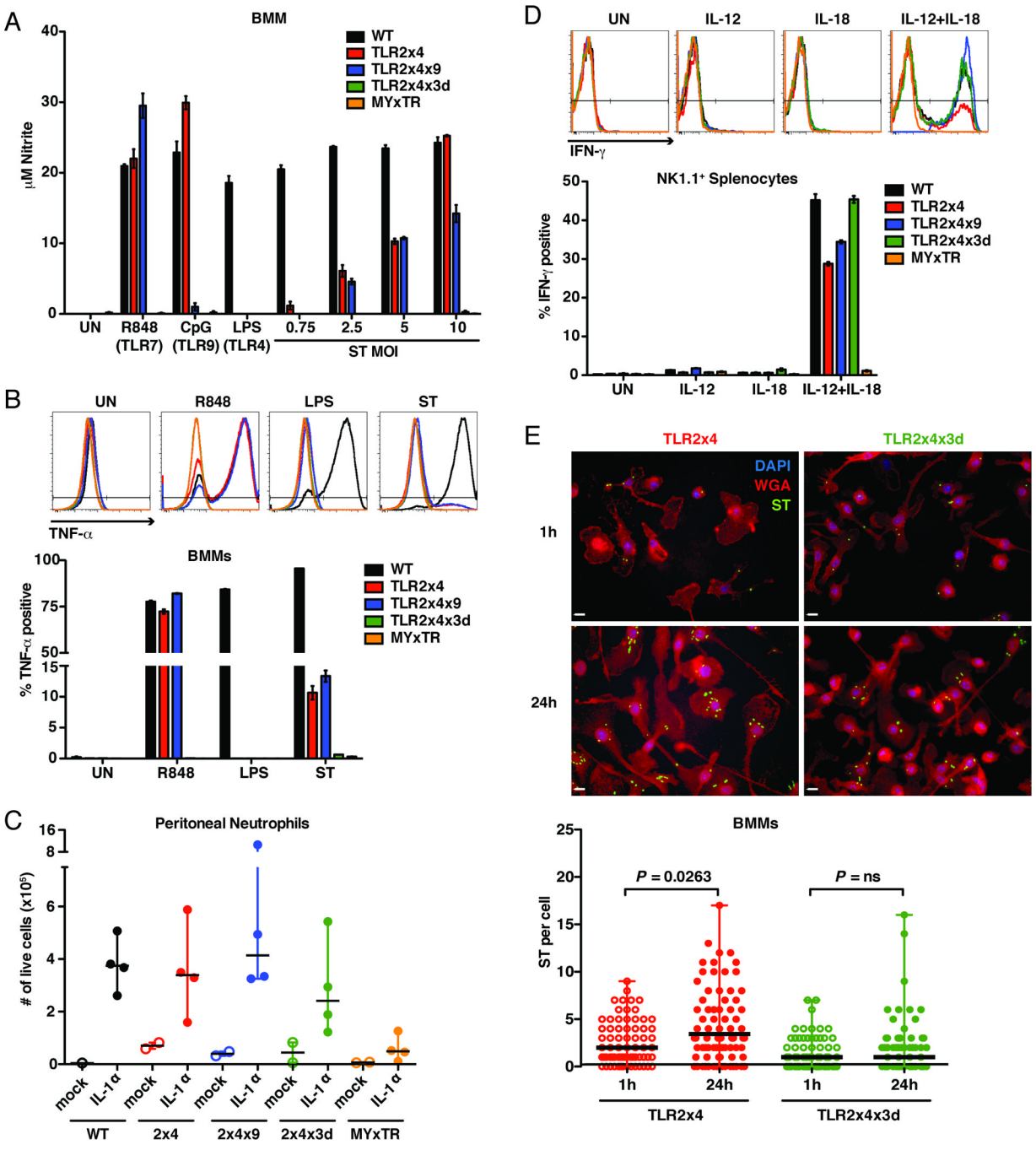

Figure 1. TLR2×4×3d BMMs do not respond to ST infection and do not support efficient intracellular replication

(A) Prior to stimulation, BMMs of the indicated genotypes were treated overnight with mouse interferon (mIFN)-γ.BMMs were then left untreated (UN) or treated with TLR7 ligand R848, TLR9 ligand CpG oligonucleotide (CpG), TLR4 ligand LPS, or infected with an increasing multiplicity of infection (MOI) of wt ST. Nitrite (a byproduct of nitric oxide) production was measured 24 hours (h) later by Griess assay. Data are representative of six independent experiments and are shown as mean ± standard error of the mean (SEM). **(B)** BMMs of the indicated genotypes were left untreated (UN) or treated with R848, LPS, or with ST (MOI=5). TNF-α production was measured 4.5 h later by intracellular cytokine

staining (ICS) and flow cytometry. Top: Representative histograms. Bottom: Quantified data representative of three independent experiments and shown as mean ± SEM. **(C)** Mice of the indicated genotypes were given IP injections of phosphate-buffered saline (PBS, mock) or PBS containing mIL-1α. 24 h after injection, peritoneal lavage fluid was collected and analyzed by flow cytometry. Live singlet $CD11b^+$ events were interrogated for Ly6C and Ly6G positivity. Shown are total numbers of NEs ($CD11b^+Ly6C^+Ly6G^+$). Data are representative of three independent experiments and shown as median ± range. **(D)** Total splenocytes from indicated mice were isolated and cultured overnight with no treatment (UN), mIL-12 alone, mIL-18 alone, or a combination of mIL-12 and mIL-18. IFN-γ production by $NK1.1^+$ cells was measured by ICS and flow cytometry. Top: Representative histograms. Bottom: Quantified data representative of three independent experiments and shown as mean ± SEM. **(E)** BMMs of the indicated genotypes were infected with ST (MOI=5), treated with gentamicin to kill extracellular bacteria, and processed for IF at 1 and 24 hpi. Top: Representative micrographs, Bar=10 μ m. Bottom: Quantified data representative of two independent experiments and shown as median ± range. Significance was determined by the Mann-Whitney test. WGA, wheat germ agglutinin; ns, not significant. See also Figure S1.

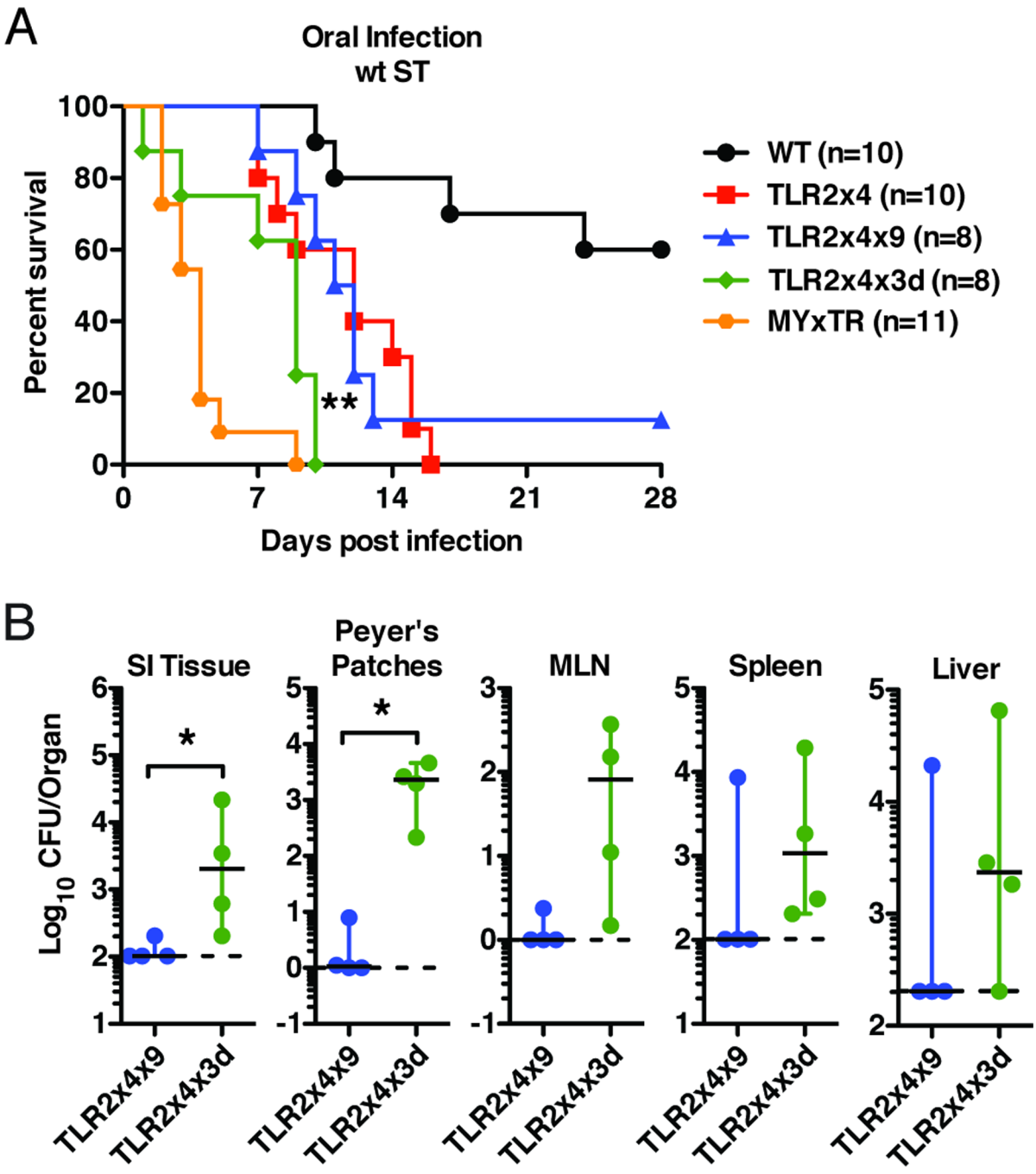

Figure 2. TLR2×4×3d mice are more susceptible to oral ST infection than TLR2×4×9 mice

(A) Mice of the indicated genotypes were infected by oral gavage (OG) with 1×10^8 colony-forming units (CFU) of wild type (wt) ST and monitored for survival. When comparing TLR2×4×9 and TLR2×4×3d mice, $^{**}P = 0.0088$. Data are representative of four independent experiments. **(B)** Mice of the indicated genotypes were infected by OG with 1×10^9 CFUs of ST and sacrificed at 3 dpi for organ harvest and enumeration of bacterial burdens. Significance ($^{*}P<0.05$) was determined by the Mann-Whitney test. Data are representative of three independent experiments and shown as median ± range. Dotted lines indicate the limit of detection (LOD). SI, small intestine; MLN, mesenteric lymph nodes.

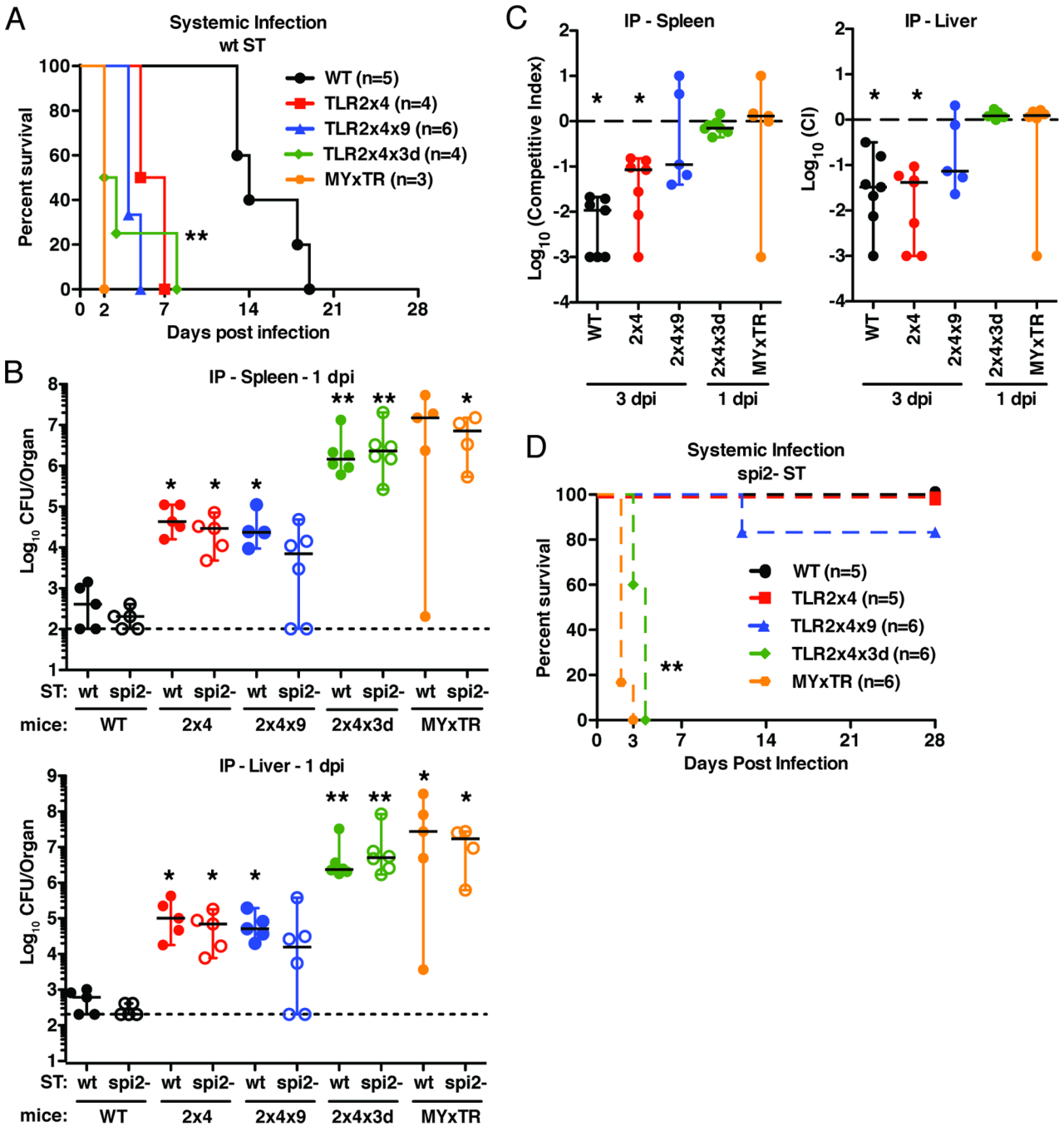

Figure 3. The SPI2 TTSS is not required for replication or virulence in TLR2×4×3d mice

(A) Mice of the indicated genotypes were infected by IP injection with 5×10^2 CFU of wt ST and monitored for survival. When comparing WT and TLR2×4×3d mice, $^{**}P = 0.0025$. Data are representative of two independent experiments. **(B)** Mice of the indicated genotypes were infected by IP injection with 5×10^2 CFU of wt ST (wt) or *ssaV::kan* ST (spi2-) and sacrificed at 1 dpi for enumeration of bacterial burdens in their spleens and livers. Significance ($^{*}P < 0.05$, $^{**}P<0.01$) was determined by the Mann-Whitney test. *P* values above wt ST samples were compared to wt ST CFUs in WT mice while *P* values above spi2- ST samples were compared to spi2- ST CFUs in WT mice. Data are

representative of two independent experiments and are shown as median ± range. Dotted lines indicate the LOD. **(C)** Mice of the indicated genotypes were infected by IP injection with a 1:1 mixture wt and spi2- ST (5×10^2 total CFU) and sacrificed at the indicated time points for enumeration of bacterial burdens. Data are expressed as the $\log_{10}$ of the competitive index (CI) for the spleens and livers. The CI is calculated by dividing the output ratio (spi2- CFU/wt CFU) to the corresponding input ratio. Values >0 indicate that spi2- ST outcompeted wt ST, values <0 indicate that wt ST outcompeted spi2- ST, and a CI=0 (dashed lines) indicates that the strains had equal fitness. When spi2- ST were undetectable, the Log_{10}(CI) was arbitrarily set at -3 and when wt ST were undetectable, the Log_{10}(CI) was arbitrarily set at 1. Significance (*$P < 0.05$) was determined using the Wilcoxon matched-pairs signed rank test on raw CFU values. Data are representative of four independent experiments and are shown as median ± range. **(D)** Mice of the indicated genotypes were infected by IP injection with 5×10^2 CFU of spi2- ST and monitored for survival. When comparing WT and TLR2×4×3d mice, **$P = 0.0031$. Data are representative of two independent experiments. See also Figure S2.

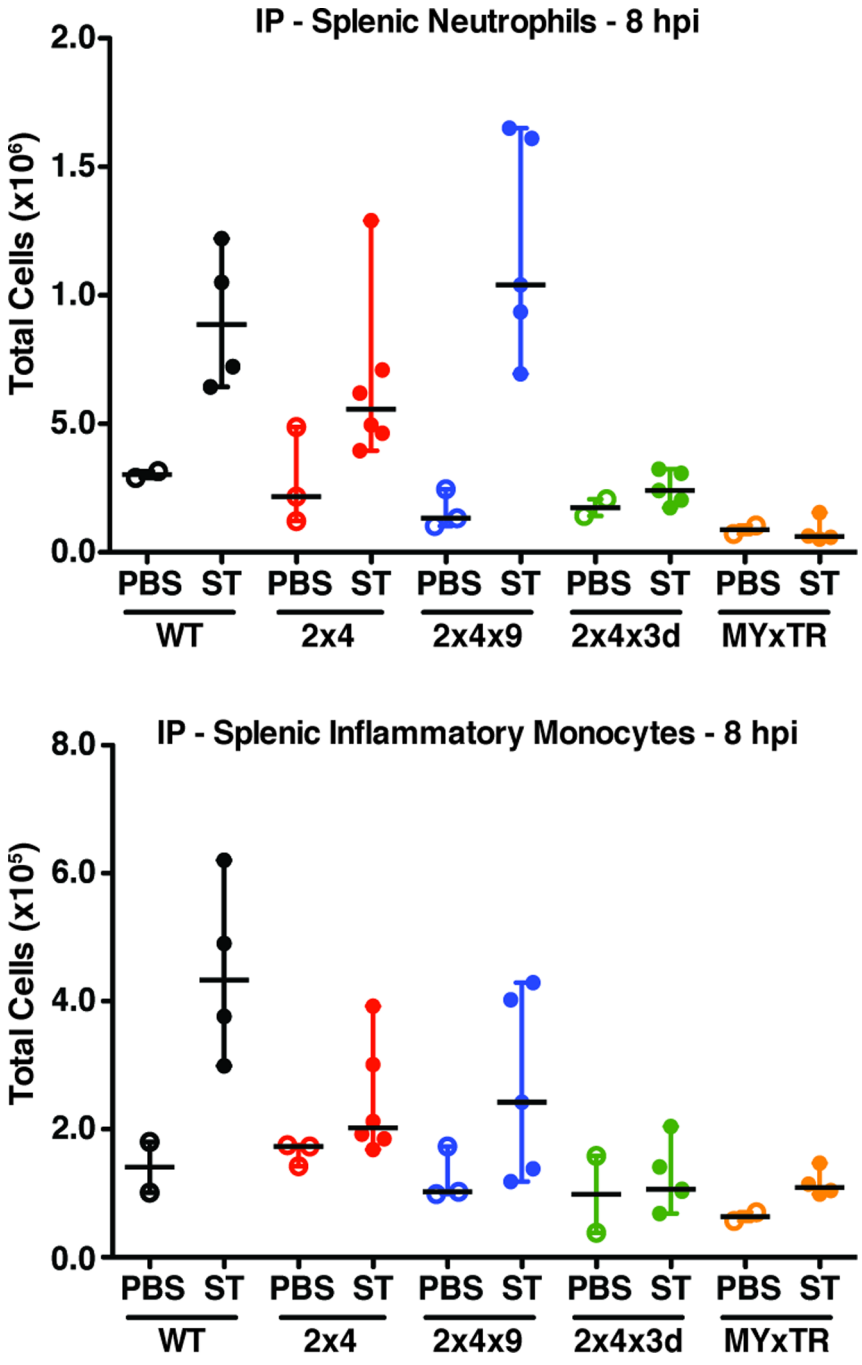

Figure 4. TLR2×4×3d have less inflammation in response to ST at 8 hpi

Mice of the indicated genotypes were infected by IP injection with PBS or 5×10^2 CFU wt ST and sacrificed at 8 hpi. Harvested spleens were dissociated, processed for flow cytometry, and analyzed for NE and IM recruitment as shown in Figure S1C. Data are representative of three independent experiments and are shown as median ± range. See also Figure S3.

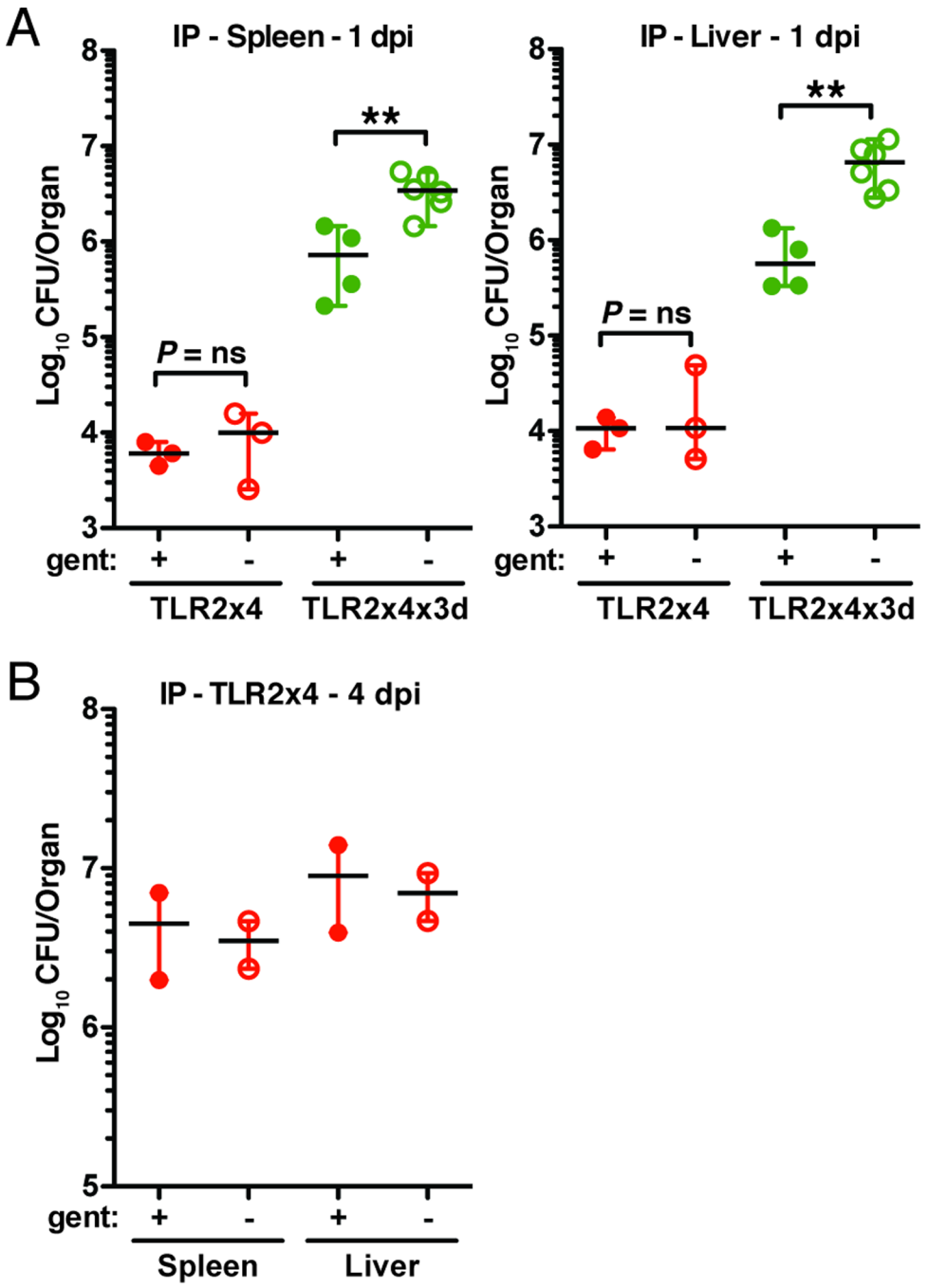

Figure 5. TLR2×4×3d mice support extracellular ST replication

(A) Mice of the indicated genotypes were infected by IP injection with 5×10^2 CFU of wt ST and 24h after infection received an intravenous injection of gentamicin (gent, 5 μ g/g body weight). Mice were sacrificed 6h post gentamicin treatment and bacterial burdens in their spleens and livers were measured. Data are representative of at least three independent experiments and are shown as median ± range. Significance (**$P < 0.05$) was determined by the Mann-Whitney test. **(B)** Mice were manipulated as in **(A)** with the exception of allowing the infection to progress for 4 d before gentamicin treatment. Data are representative of two independent experiments and shown as median ± range.

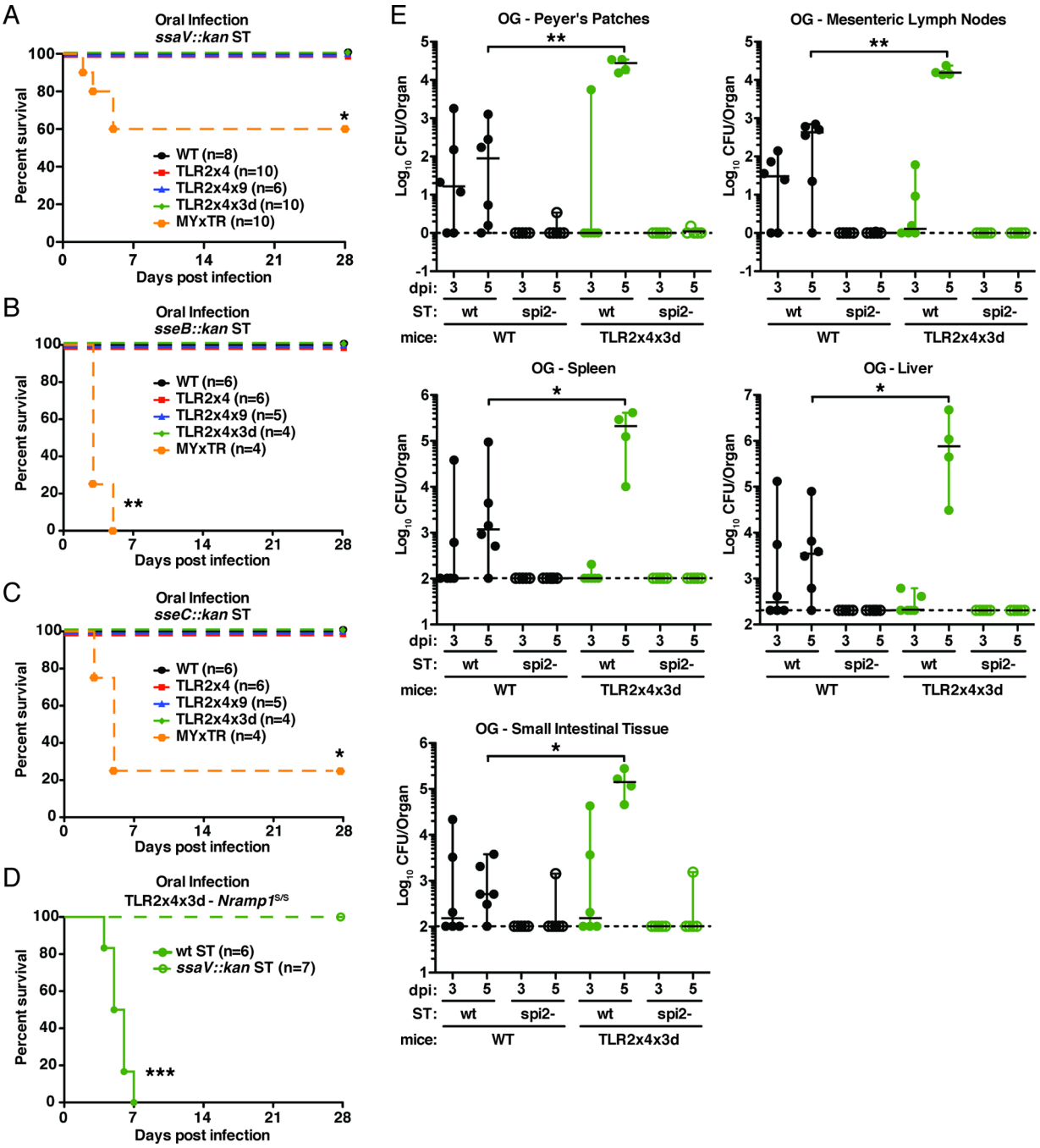

Figure 6. SPI2 is required for virulence in a murine typhoid model

(A–C) Mice of the indicated genotypes were infected by OG with 1×10^9 CFU of either **(A)** *ssaV::kan* ST, **(B)** *sseB::kan* ST, or **(C)** *sseC::kan* ST and monitored for survival. When comparing TLR2×4×3d and MYxTR mice in **(A)** $*P = 0.0295$, in **(B)** $**P = 0.0058$, and in **(C)** $*P = 0.0455$. Data are representative of two independent experiments. **(D)** TLR2×4×3d mice expressing a nonfunctional Nramp1 ($Nramp1^{S/S}$) were infected by OG with 1×10^9 CFU of wt ST or *ssaV::kan* ST and monitored for survival. $***P = 0.0002$. Data are representative of two independent experiments. **(E)** WT and TLR2×4×3d mice were infected by OG with 1×10^9 CFU of wt ST or spi2- ST (*ssaV::kan*) and were sacrificed at 3 or 5 dpi

for enumeration of bacterial burdens in the indicated tissues. Significance (*$P < 0.05$, **$P<0.01$) was determined by the Mann-Whitney test. Data are representative of at least three independent experiments and are shown as median ± range. See also Table S1.

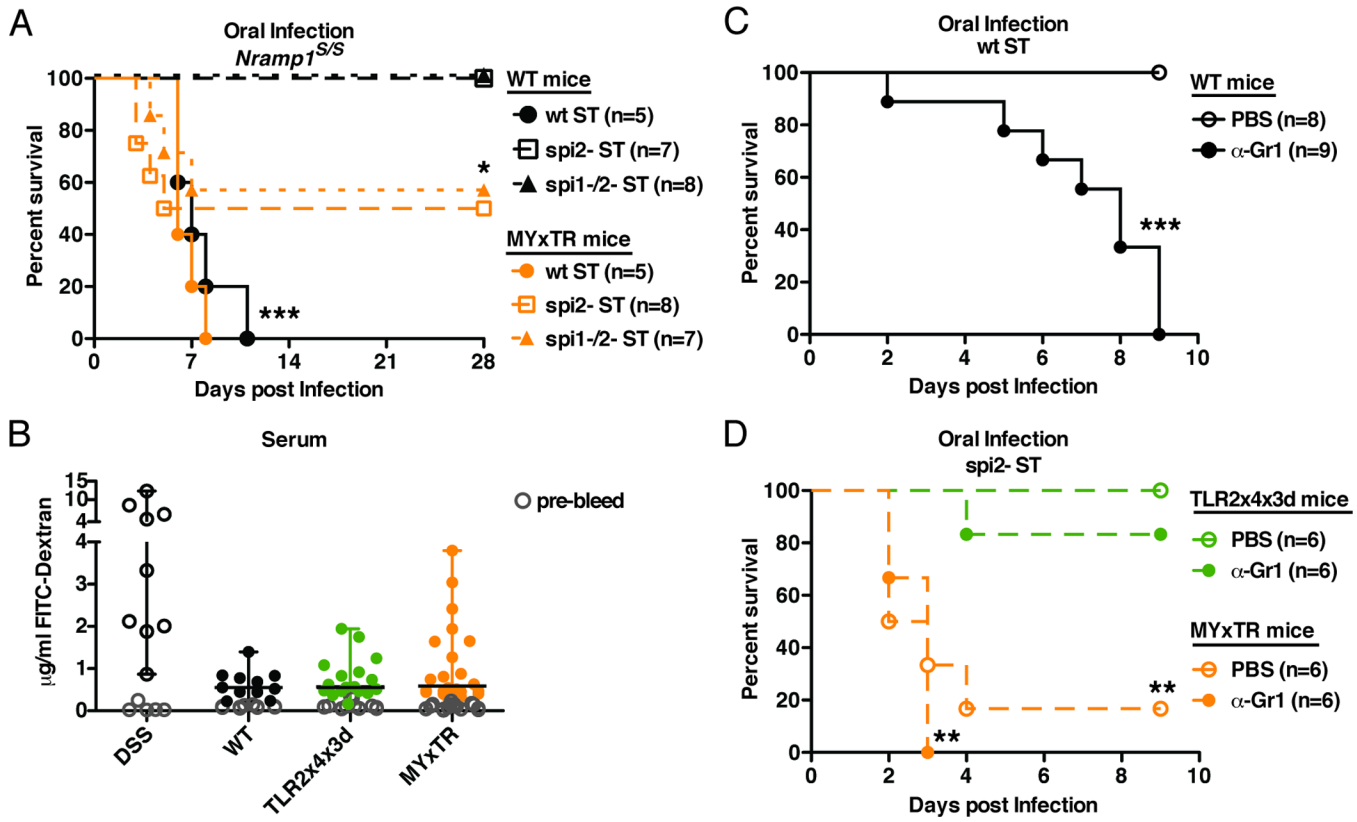

Figure 7. MYxTR mice are susceptible to oral infection with mutant ST strains

(A) WT and MYxTR mice on the *Nramp1*$^{S/S}$ background were infected by OG with 1×10^{9} CFU of wt St, spi2- ST (*ssaV::kan*), or spi1-/2- ST (*orgA::tet ssaV::kan*) and monitored for survival. When comparing WT and MYxTR mice infected with spi2- ST, **P* = 0.0354. When comparing WT and MYxTR mice infected with spi1-/2- ST, **P* = 0.0452. When comparing WT mice infected with wt or spi2- ST, ****P* = 0.0003. Data are representative of three independent experiments using either *Nramp1*$^{R/R}$ or *Nramp1*$^{S/S}$ mice. **(B)** Mice of the indicated genotypes were pre-bled (gray open circles) and given 10 mg FITC-Dextran by OG. Mice were terminally bled 4h later and the amount of FITC-dextran present in the serum was measured. Data are the collective results from three independent experiments using both *Nramp1*$^{R/R}$ and *Nramp1*$^{S/S}$ mice, which within each genotype yielded indistinguishable results. DSS, WT *Nramp1*$^{R/R}$ mice treated with DSS. **(C and D)** Mice of the indicated genotypes receiving IP injections of either PBS or α -Gr1 antibodies were infected by OG with 1×10^{9} CFU of **(C)** wt ST or **(D)** spi2- (*ssaV::kan*) ST and monitored for survival. In **(C)**, ****P* = 0.0002. In **(D)**, when comparing PBS treated cohorts, ***P* = 0.0039; when comparing α -Gr1-treated cohorts, ***P* = 0.0012; when comparing TLR2×4×3d or MYxTR cohorts, *P* = ns. Data are representative of two independent experiments. See also Figure S4.