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Immunological responses against *Salmonella enterica* serovar Typhimurium Braun lipoprotein and lipid A mutant strains in Swiss-Webster mice: Potential use as live-attenuated vaccines

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Abstract

We generated and characterized *Salmonella enterica* serovar Typhimurium mutants that were deleted for the genes encoding Braun lipoprotein (*lpp*) alone or in conjunction with the *msbB* gene, which codes for an enzyme required for the acylation of the lipid A moiety of lipopolysaccharide. Two copies of the *lpp* gene, designated as *lppA* and *lppB*, exist on the chromosome of *S. Typhimurium*. These mutants were highly attenuated in a mouse infection model and induced minimal histopathological changes in mouse organs compared to those seen in infection with wild-type (WT) *S. Typhimurium*. The *lppB/msbB* and the *lppAB/msbB* mutants were maximally attenuated, and hence further examined in this study for their ability to induce humoral and cellular immune responses. Importantly, infection of out-bred Swiss-Webster mice with the mutant *S. Typhimurium* generated superior T helper cell type 2 (Th2) responses compared to WT *S. Typhimurium*, as determined by measuring IgG subclasses and cytokines. WT *S. Typhimurium* induced higher levels of IgG2a in sera of infected mice, while the *lppB/msbB* and *lppAB/msbB* mutants mounted higher levels of IgG1 as determined by an enzyme-linked immunosorbent assay. Mice immunized with *lppB/msbB* and *lppAB/msbB* mutants rapidly cleared WT *S. Typhimurium* upon subsequent rechallenge, and naïve mice passively immunized with sera from animals infected with *S. Typhimurium* mutants were protected against subsequent challenge with WT *S. Typhimurium*. Splenic T cells produced higher levels of interferon-gamma following *ex vivo* exposure to WT *S. Typhimurium*, while splenic T cells infected with the above-mentioned two mutants evoked higher levels of interleukin-6. Further, mice infected with *lppB/msbB* and *lppAB/msbB* mutants showed much higher levels of splenic T cell activation as measured by CD44⁺ expression on CD4⁺ T cells by flow cytometry and by incorporation of ³H-thymidine compared to mice that were infected with WT *S. Typhimurium*. We expect the *lppB/msbB* and *lppAB/msbB* mutants to be excellent live-attenuated vaccine candidates, because they induced minimal inflammatory responses and evoked stronger and specific antibody and cellular immune responses.

Keywords

Salmonella Typhimurium; vaccine; immune responses; mouse model

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1. Introduction

Salmonella enterica, which belongs to the *Enterobacteriaceae* family, contains 2300 serogroups based on the structure of O-antigen of lipopolysaccharide (LPS). Although most of these serogroups are of animal origin, *S. Typhi* is strictly a human pathogen [1]. Diseases associated with *Salmonella* infections include self-limiting gastroenteritis and septicemia, and humans could be asymptomatic carriers of this pathogen for several years with the organism residing in hepatocytes and the gall bladder [2]. Although infections with salmonellae are of concern in both developing and developed countries, typhoid fever is highly prevalent in developing countries with an annual global incidence of approximately 16 million cases and 600,000 deaths [3]. Among different serogroups of *Salmonella*, *S. enterica* serovar Typhimurium is most commonly associated with human infections after consuming contaminated food and water. The organism specifically invades M cells and is then taken up by macrophages before being released into the blood stream to infect other organs [4,5].

Braun (murein) lipoprotein (Lpp) represents one of the most abundant components present in the outer membrane of bacteria belonging to the family *Enterobacteriaceae* [6,7]. Maturation of Lpp requires modification of the lipid moiety, which is catalyzed by enzymes, specifically glycerol transferase, *O*-acyl transferase, signal peptidase II, and *N*-acyl transferase [8]. Two functional copies of the *lpp* gene (designated as *lppA* and *lppB*) exist on the chromosome of *S. Typhimurium* 14028 located in tandem and separated by 82 bp [9]. Deletion of both copies of the *lpp* gene results in a *S. Typhimurium* mutant that is minimally invasive to epithelial cells, non-motile, and severely impaired in its ability to induce cytotoxicity in murine macrophages (RAW 264.7 cells) and T84 human colonic epithelial cells, possibly due to the reduced production of proinflammatory cytokines and chemokines (e.g., tumor necrosis factor- α [TNF- α] and interleukin [IL]-8) [9]. The *lpp* (*lppAB*) double knockout (DKO) mutant was avirulent in mice following oral and intraperitoneal (i.p.) challenges. Mice immunized with the *lppAB* mutant were protected from death when rechallenged with a lethal dose of wild-type (WT) *S. Typhimurium* [9].

All Gram-negative bacteria possess LPS with lipid A representing the biological active domain and containing fatty acids believed to contribute to the low-permeability barrier of the outer membrane of Gram-negative bacteria [10]. As is the case with Lpp, lipid modification of LPS by the addition of fatty acids is catalyzed by enzymes encoded by the genes *msbB* (multicopy suppressor of *htrB*), *htrB* (high temperature requirement), and *pagP* (PhoP-activated gene) that attach myristic, lauric, and palmitic acids, respectively, to lipid A [11,12].

Deletion of the *msbB* gene reduces toxicity associated with LPS by preventing the addition of a terminal myristyl group to the lipid A domain [13]. As a result of a reduced production of proinflammatory cytokines and nitric oxide synthase, the *msbB* mutant of *S. Typhimurium* evokes less mortality and tissue damage in mice compared to that seen with WT *S. Typhimurium* [14,15]. Therefore, *msbB* single knockout (SKO) mutants show a reduced septic shock response and hence increase the safety of these *S. Typhimurium* mutants for potential use as live-attenuated vaccines in humans [15].

WT *S. Typhimurium* releases LPS during both *in vitro* and *in vivo* growth [16]. LPS release is significantly enhanced during lysis of *S. Typhimurium* following exposure to antibiotics or human serum and this enhanced LPS release causes septic shock [17]. Likewise, Lpp is also a critical bacterial component in the induction and pathogenesis of septic shock. It induces the production of TNF- α and IL-6 in mouse and human macrophages *ex vivo* [5] and leads to lethal shock as a result of the production of these cytokines in both LPS-responsive and non-responsive mice [18–20]. More importantly, Lpp synergizes with LPS to induce production of

proinflammatory cytokines in mice, because Lpp binds to the toll-like receptor (TLR)-2, whereas LPS binds to TLR-4 and CD14 to activate host cells [20–22].

Therefore, we predicted that *lpp* mutants of *S. Typhimurium* with or without the deletion of the *msbB* gene would be excellent live-attenuated vaccine candidates. We reported that such *lpp* mutants (e.g., *lppA* and *lppB* SKO, *lppAB*, *lppA/msbB* and *lppB/msbB* DKO, and *lppAB/msbB* triple knockout [TKO]) are highly attenuated in *ex vivo* and *in vivo* models of *S. Typhimurium* infections [23]. In the present study, we investigated the immunological responses of *lpp/msbB* mutants and demonstrated that these *S. Typhimurium* mutants induced significantly higher IL-6 and minimal interferon-gamma (IFN- γ) from mouse splenic T cells and evoked superior T cell activation compared to WT *S. Typhimurium*. Further, *lppB/msbB* and *lppAB/msbB* mutant-infected mice showed significantly higher levels of IgG1, and mice immunized with the *S. Typhimurium* mutants rapidly cleared a subsequent infection with WT *S. Typhimurium*. Finally, passive immunization of naïve mice with sera from animals infected with *S. Typhimurium* mutants protected recipient mice against infection with WT *S. Typhimurium*.

2. Results

2.1. Mortality in Swiss-Webster mice after infection

Both Lpp and LPS of *S. Typhimurium* contribute to septic shock and mortality in a mouse model of salmonellosis [9,19,24]. Similar to in-bred C57BL/6 mice used in the salmonellosis model, out-bred Swiss-Webster mice are also sensitive to infection by *S. Typhimurium* [22]. We first compared mortality in mice infected with WT *S. Typhimurium* or the various mutant *S. Typhimurium* strains. Each experimental group contained ten mice, and animals were observed over a period of 30 days following infection *via* the intraperitoneal (i.p.) route. At a dose of 1×10^3 cfu, 60% of mice infected with WT *S. Typhimurium* died within 4 to 8 days. In contrast, animals infected with the same dose of *msbB*, *lppB*, *lppAB*, *lppB/msbB*, or *lppAB/msbB* mutants did not die (Fig. 1a). At a higher dose of 1×10^4 cfu, mice infected with the *msbB*, *lppB/msbB*, or *lppAB/msbB* mutants showed 100% survival rates (Fig. 1b). In contrast, all of the mice infected with 1×10^4 cfu of WT *S. Typhimurium* died within 4–5 days. The survival rate of mice was 90% and 70%, respectively, when animals were infected with 1×10^4 cfu of *lppAB* or *lppB* mutant strains (Fig. 1b). Since these latter mutants did not provide 100% survival to animals, these strains were not considered sufficiently attenuated to serve as vaccine candidates.

An even higher infectious dose (1×10^6 cfu) led to death in WT *S. Typhimurium*-infected mice within three days (Fig. 1c). At this dose, all of the mice infected with the *lppB* or *lppAB* mutant of *S. Typhimurium* also died, but death was somewhat delayed compared to WT *S. Typhimurium*-infected mice (three to four vs. two to three days). In contrast, 30% of the mice infected with 1×10^6 cfu of the *msbB* mutant survived, and importantly, 100% survival was observed in mice that were infected with either the *lppB/msbB* or the *lppAB/msbB* mutant (Fig. 1c). The *lppB/msbB* and *lppAB/msbB* mutants of *S. Typhimurium* were highly avirulent; 100% of the mice infected with even 1×10^7 cfu survived (Fig. 1d). At this dose, WT *S. Typhimurium*-infected mice died within three days.

2.2. Protection provided by mutants against subsequent challenge with WT *S. Typhimurium*

To determine whether initial infection with mutant *S. Typhimurium* induced protective immunity, we rechallenged mice that survived infection by 1×10^4 cfu of any of the mutant *S. Typhimurium* strains (Fig. 1b) with WT *S. Typhimurium*. For the rechallenge, 1×10^5 cfu of WT *S. Typhimurium* were injected 30 days after the first infection with mutant *S. Typhimurium*. Age-matched mice initially injected with phosphate-buffered saline (PBS)

served as a control group. All of the control mice injected with 1×10^5 cfu of WT *S. Typhimurium* died, whereas mice that survived infection with a mutant *S. Typhimurium* strain were protected (80 to 100% survival following rechallenge with WT *S. Typhimurium*) (Fig. 2a).

We also determined the protective effect of a higher dose of *lppB/msbB* and *lppAB/msbB* mutants of *S. Typhimurium* (1×10^6 cfu) in a mouse model. Thirty days after the initial infection, mice were rechallenged with WT *S. Typhimurium*. All of the mice initially infected with 1×10^6 cfu of *lppB/msbB* or *lppAB/msbB* mutant *S. Typhimurium* survived a rechallenge with 1×10^6 cfu of WT *S. Typhimurium*, while 60% (for *lppAB/msbB*) or 80% (for *lppB/msbB*) protection was achieved in mice rechallenged with 1×10^7 cfu of WT *S. Typhimurium* (Fig. 2b). We chose to initially infect mice with the mutants at a higher dose (i.e., 1×10^6 cfu) as all of the mice infected with 1×10^4 cfu of the *lppB/msbB* or *lppAB/msbB* mutant of *S. Typhimurium* died when rechallenged with 1×10^6 cfu of WT *S. Typhimurium* (data not shown). For the studies presented in Fig. 2b, we specifically used mutants *lppAB/msbB* and *lppB/msbB* as they were highly attenuated and did not kill mice even at a dose as high as 1×10^7 cfu (Fig. 1d).

2.3. Splenic bacterial load after infection

In early time points, we expected to find a reduced bacterial load in the spleens of mice infected with *lpp* mutants of *S. Typhimurium*, because our previous studies showed that deletion of the *lpp* genes affected invasiveness of *S. Typhimurium* into host cells [9]. To determine whether WT and mutant strains of *S. Typhimurium* differed in their ability to grow in infected hosts, we measured bacterial loads in the spleens of mice infected with 1×10^3 cfu of the various *S. Typhimurium* strains. At various times after infection, spleens were harvested from three mice per group. The spleens were homogenized, and bacteria were enumerated in serial log dilutions. The number of WT *S. Typhimurium* increased dramatically, reaching a maximal level of more than 1×10^6 cfu seven days after infection (Fig. 3a). The spleens of mice infected with mutant *S. Typhimurium* varied in their maximal bacterial loads, but the decline in splenic bacterial numbers was more rapid in mice infected with mutant *S. Typhimurium* than in mice infected with WT *S. Typhimurium*. Importantly, *lppAB/msbB* mutant *S. Typhimurium* did not survive in host cells, and no bacteria were detectable two days after infection. The second *S. Typhimurium* mutant strain that was highly protective in rechallenge experiments, the *lppB/msbB* mutant (Fig. 2b), reached maximal splenic numbers by day five and was completely eliminated from infected spleens by day seven (Fig. 3a).

To determine whether immunization with mutant *S. Typhimurium* prevented or retarded growth of WT *S. Typhimurium* upon rechallenge, we infected mice initially immunized with 1×10^3 cfu of either the *lppB/msbB* or the *lppAB/msbB* mutant, with 1×10^3 cfu of WT *S. Typhimurium*, 30 days after the first infection. Unimmunized mice (injected with PBS alone) were used as a control and were challenged with WT *S. Typhimurium* at the same dose. Seven days after rechallenge, we encountered very low bacterial numbers in the spleens of immunized mice, and we could not detect any bacterial growth 14 days after rechallenge (Fig. 3b). In contrast, we observed normal bacterial growth in the spleens of unimmunized control mice over a period of 14 days (Figs. 3a and b). To further evaluate the protective effect of immunization with 1×10^3 cfu of either the *lppB/msbB* or the *lppAB/msbB* mutant, we rechallenged immunized mice with 1×10^6 cfu of WT *S. Typhimurium*. As we observed at the lower dose of rechallenge, no surviving WT *S. Typhimurium* bacteria could be detected after 14 days in the spleens (data not shown). Our data indicated that WT *S. Typhimurium* was either rapidly cleared or killed in immunized mice.

2.4. Serum IgG levels in mice infected with WT or mutant strains of *S. Typhimurium*

In order to be an effective vaccine strain, the attenuated *S. Typhimurium* mutants would have to induce protective humoral immune responses. To determine whether infected mice developed humoral immunity, we injected mice with 1×10^3 cfu of WT or mutant *S. Typhimurium*. We expected that activation of the immune system would be detectable by increases in total serum IgG. Therefore, we measured total IgG1 and IgG2a in the sera of mice before infection and 2, 5, 7, and 14 days post-infection. Total serum IgG1 antibody titers increased significantly in mice that were infected with *lppAB/msbB*, *lppB/msbB*, and *lppAB* mutant *S. Typhimurium* (Fig. 4a). Peak levels were reached five days after infection. In contrast, serum IgG1 levels did not increase in mice infected with WT, *msbB* mutant, or *lppB* mutant *S. Typhimurium*. The findings were very different for IgG2a (Fig. 4b). Serum levels of total IgG2a increased in all infected mice, but the strongest and most rapid increase was observed in mice infected with WT *S. Typhimurium*. The *lppB* and *msbB* mutant strains also demonstrated an ability to increase total serum IgG2a with a similar kinetics as observed for WT *S. Typhimurium* (peak level five days post-infection). Importantly, the mutant *S. Typhimurium* strains (*lppAB/msbB*, *lppB/msbB*, and *lppAB*) that elicited the most robust IgG1 response (Fig. 4a) were least potent in inducing a stable IgG2a response (Fig. 4b). These three bacterial strains showed a moderate increase in IgG2a serum levels two days after infection, but five days after infection, serum IgG2a titers were almost back to normal levels.

2.5. Protective effect of sera from mice infected with mutant *S. Typhimurium* strains

It was important to determine whether the increase in total serum IgG1 was associated with protection from WT *S. Typhimurium*. Therefore, we infected/immunized Swiss-Webster mice with 1×10^3 cfu of either WT or *lppB/msbB* or *lppAB/msbB* mutant *S. Typhimurium*. Ten days after the infection when the IgG1 titers were still significantly higher in mutant-infected mice compared to the WT control (Fig. 4a), immune sera were collected. A 120- μ l serum aliquot was injected intravenously into uninfected mice. After 24 h, the passively immunized mice were infected with 1×10^4 cfu of WT *S. Typhimurium*, a dose that killed 100% of unprotected mice within 4–5 days (Fig. 1b). The serum from mice infected with either *lppB/msbB* or *lppAB/msbB* mutant *S. Typhimurium* provided complete protection; 100% of passively immunized mice survived infection with a lethal dose of WT *S. Typhimurium* (Fig. 5). In contrast, 80% of the mice that either did not receive serum or were injected with 120 μ l of pre-immune serum died within 7 days following infection with WT *S. Typhimurium* (indicated as a control group). Sera from animals that were infected with WT *S. Typhimurium* provided 60% protection in naïve mice against subsequent challenge with WT *S. Typhimurium*.

2.6. T cell responses to a primary exposure to WT or mutant strains of *S. Typhimurium*

The SKO (either *lpp* or *msbB*) mutants still induce TNF- α and IL-8 in mouse macrophages and human epithelial cells, respectively, at levels comparable to those induced by WT *S. Typhimurium* [23,25]. Further, the *lppB* SKO mutant induces inflammatory cytokine responses and tissue damage as indicated by histopathology studies, albeit at a much reduced level compared to the WT *S. Typhimurium*-infected mice [23]. Therefore, it was important to determine the ability of the two prospective vaccine strains of *S. Typhimurium* in inducing inflammatory T cell responses during a primary infection. To this end, we cultured splenocytes from uninfected mice with WT, *lppB/msbB*, or *lppAB/msbB* mutant of *S. Typhimurium* at a multiplicity of infection (MOI) of 1 for 16 h. Following this incubation period, T cells were enriched by passage through nylon-wool columns and cultured for 72 h with irradiated splenocytes from uninfected mice. Culture supernatants were collected and IL-4, IL-6, and IFN- γ levels were measured by ELISA (Figs. 6a and b). Culture supernatants from T cells infected with either the *lppB/msbB* or the *lppAB/msbB* mutant contained significantly higher levels of IL-6 than did supernatants from T cells that were infected with WT *S. Typhimurium*.

In contrast, levels of IFN- γ in conditioned media from T cells infected with WT *S. Typhimurium* were significantly higher than those in culture supernatants from T cells infected with either of the two mutant strains (Fig. 6b). In fact, the two mutant *S. Typhimurium* strains did not significantly induce IFN- γ production in infected cultures. The IL-4 levels were in the range of 430 ± 65 and 380 ± 40 pg/ml in *lppAB/msbB* and *lppB/msbB* mutant-infected T cells ($p < 0.0001$) compared to that of WT *S. Typhimurium*-infected T cells (150 ± 30 pg/ml). The latter were not statistically different from IL-4 produced from control T cells (120 ± 14 pg/ml).

To determine whether the differences in splenocyte survival and T cell proliferative responses was dependent on the expression of genes encoding Lpp and LPS of *S. Typhimurium*, we exposed splenocytes from unimmunized mice to WT or mutant strains of *S. Typhimurium* for 16 h (MOI of 1) and then determined splenocyte survival. Splenocyte survival was consistently $>90\%$, independent of the *S. Typhimurium* strain used in the *ex vivo* infection (Fig. 7a, black bars). We then enriched T cells from the *S. Typhimurium*-exposed cultures and incubated them with irradiated splenocytes. Instead of harvesting the supernatants, we added [3 H]-thymidine to the cultures for the last 10 h of incubation. Subsequently, we measured incorporated radioactivity as a measurement of proliferation. Importantly, WT *S. Typhimurium* induced the strongest T cell proliferation in this primary *ex vivo* infection (Fig. 6c).

Because the timeframe of the *ex vivo* infection did not allow for the development of specific T cell responses, the observed T cell proliferation and cytokine secretion were presumably due to the mitogenic stimulation of splenocytes by Lpp and LPS. Interestingly, not only were T cell proliferative responses lower in cultures exposed to *lppB/msbB* or the *lppAB/msbB* mutant of *S. Typhimurium* than in those exposed to WT *S. Typhimurium*, but cytokine secretion also differed: WT *S. Typhimurium*-exposed cultures secreted significantly higher levels of inflammatory IFN- γ than did cultures exposed to either of the mutant strains, suggesting that the mutant strains would induce less tissue damage and thus could possibly serve as vaccine candidates.

2.7. T cell activation in mice infected with WT or mutant strains of *S. Typhimurium*

To determine whether T cells were activated in Swiss-Webster mice immunized with the immunogenic mutant strains of *S. Typhimurium* (i.e., the *lppB/msbB* and the *lppAB/msbB* mutant), we isolated splenocytes from mice that were infected with 1×10^3 cfu of either WT, *lppB/msbB* mutant, or *lppAB/msbB* mutant *S. Typhimurium*. Spleens were collected at 3, 5, 7, or 14 days post-infection and the splenocytes stained with antibodies against CD4 or CD8 in combination with anti-CD44 antibodies. These markers served to distinguish naïve from activated or memory T cells: naïve CD4 $^+$ T cells express little CD44 while activated and memory CD4 $^+$ T cells express high levels of CD44 [26]. Five days post-infection, the percentage of CD44 $^+$ cells was significantly increased in both CD4 $^+$ and CD8 $^+$ T cells from mice infected with *lppB/msbB* or *lppAB/msbB* mutant *S. Typhimurium*, in comparison to either uninfected mice or mice infected with WT *S. Typhimurium* (Fig. 7b).

To further investigate whether the *lppB/msbB* and *lppAB/msbB* mutant *S. Typhimurium* induced specific T cell immunity, we infected Swiss-Webster mice with 1×10^3 cfu of WT, *lppB/msbB* mutant, or *lppAB/msbB* mutant *S. Typhimurium*. Spleens were collected five days post-infection and splenocytes isolated and cultured with WT *S. Typhimurium* at an MOI of 1 for 16 h. Splenocyte survival was determined and T cells were enriched and incubated for another 72 h with irradiated splenocytes. During the last 10 h of incubation, proliferating T cells were labeled with [3 H]-thymidine. Importantly, we detected statistically significant lower cell survival in splenocytes from mice infected with WT *S. Typhimurium* than in splenocytes from mice infected with either the *lppB/msbB* or *lppAB/msbB* mutant strains (Fig 7a, white bars). Along the same lines, we measured significantly increased proliferative responses in T cell cultures from mice that were infected with either the *lppB/msbB* or *lppAB/msbB* mutant

S. Typhimurium (Fig. 7c). In contrast, proliferation of T cells from mice infected with WT *S. Typhimurium* did not differ from the spontaneous proliferation observed in control cultures that contained T cells from uninfected mice and were cultured without bacteria throughout the experiment (Fig. 7c). Earlier studies indicated that mice infected with a live avirulent *S. Typhimurium* SL3235 strain caused more than 90% reduction in the proliferative responses of splenocytes from immunized mice to a panel of B- and T-cell mitogens [27,28]

3. Discussion

We recently reported that the deletion of *lpp* genes from either the WT *S. Typhimurium* 14028 or from the *msbB*-negative background strain resulted in mutants that were highly attenuated in *ex vivo* and *in vivo* models of salmonellosis [23]. In our present study, among several mutants that we generated (e.g., *lppA*, *lppB*, and *msbB* SKO, *lppAB*, *lppA/msbB* and *lppB/msbB* DKO, and the *lppAB/msbB* TKO), we believe that the *lppB/msbB* and *lppAB/msbB* mutants are the best candidates for a new live-attenuated vaccine. An important link between innate and adaptive immune responses is established by the activation of TLRs [29]. Lpp activates TLR-2 [19,24], and in the *lppB/msbB* mutant, one intact copy of the *lpp* gene (*lppA*) remains. LPS acts *via* both TLR-4 and the CD14 receptor [29]. Although the biological potency of lipid A is significantly reduced in the *lppB/msbB* mutant, this mutant still expresses LPS. Therefore, we predicted that the *lppB/msbB* mutant strain of *S. Typhimurium* would induce minimal proinflammatory cytokine responses making it an attractive vaccine candidate with improved safety for human use. A similar argument could be made for the *lppAB/msbB* mutant strain of *S. Typhimurium*. However, our previous studies using *ex vivo* and *in vivo* models of *Salmonella* infections showed that the *lppAB/msbB* mutant could not survive in host cells [23] and, hence, might not trigger sufficiently strong cell-mediated immune responses. Here, we show that despite reduced viability in the host, both the *lppB/msbB* DKO and the *lppAB/msbB* TKO strain of *S. Typhimurium* induced excellent humoral and cellular protective immunity.

Our present study indicated that infection by i.p. injection with either the *lppB/msbB* or the *lppAB/msbB* mutant strains of *S. Typhimurium* did not cause death in mice at doses as high as 1×10^7 cfu (Fig. 1d) whereas *lppB* and *lppAB* mutants killed all mice within 4 days when initially infected at a dose of 1×10^6 cfu (Fig. 1c). We also showed that mice that were first immunized with either of these two mutant strains (*lppB/msbB* and *lppAB/msbB*) at a dose of 1×10^6 cfu before being challenged with WT *S. Typhimurium* at the same dose were protected (Fig. 2b). Further, significant protection was achieved in mice immunized with these mutant strains of *S. Typhimurium* if the mice were subsequently challenged with 1×10^7 cfu of WT *S. Typhimurium* 14028 (Fig. 2b). These data clearly indicated that although our *lpp/msbB* mutants were highly attenuated in their virulence, they still induced protective immunity against subsequent challenges with WT *S. Typhimurium*. We obtained confirming evidence that T cells were activated and may have differentiated to memory T cells from flow cytometric analyses: the percentage of CD4⁺ and CD8⁺ T cells expressing the CD44 cell surface markers was significantly higher in mice infected with mutant *S. Typhimurium* strains than in mice that were infected with WT *S. Typhimurium* (Fig. 7b).

Further, we noted increased proliferation of the T cells from mice infected with the mutants (*lppB/msbB* and *lppAB/msbB*) compared to WT *S. Typhimurium*-infected animals as measured by thymidine uptake (Fig. 7C). Mittrucker *et al.* [30] demonstrated that mice infected with WT *S. Typhimurium* SL1344 had higher percentage of activated T cells in the spleen, however, they did not observe any proliferation of the T cells. In our studies, we noted activation and proliferation of T cells only with the mutant-infected mice. Taken together, our data suggested that immunization of mice with the *lppAB/msbB* or the *lppB/msbB* mutant *S. Typhimurium* induced the activation and proliferation of T cells reactive against WT *S. Typhimurium*.

We chose to infect mice in these studies *via* the i.p. route as salmonellae lead to systemic infection in humans and animals. However, in order to demonstrate efficacy of our vaccine candidates, we plan to perform future studies in which animals will be infected by the oral route and shedding of the bacteria in stool specimens will also be examined. The results presented in this study illustrated first evidence of immune responses generated by *lpp/msbB* mutants in a mouse model and provided proof-of-principle that such mutants could lead to the development of excellent live attenuated vaccine candidates.

Because *S. Typhimurium* is a facultative intracellular pathogen, cellular immune responses are important components of the protective immunity against this organism. This protective cellular immunity is mediated *via* CD4⁺ T cells and causes activation of macrophages and delayed-type hypersensitivity responses [31]. Our data suggested that the tested *lppB/msbB* and *lppAB/msbB* mutants activated antigen-presenting cells to mount cell-mediated and humoral immune responses before they were cleared from the animals (Figs. 3,4, and 7). Protective immune responses against *S. Typhimurium* in mice have been attributed to a correct balance between antibody and cellular immunity with an emphasis on the development of Th1 [32] and Th2 T cell subsets [33]. In order to evaluate the immune response elicited after immunization with the mutant strains of *S. Typhimurium*, we examined sera of immunized mice for the production of IgG2a and IgG1 antibodies as indicative of Th1 and Th2 subsets, respectively. Our results showed lower levels of IgG2a antibodies in mice infected with the *lppAB/msbB* or the *lppB/msbB* mutant strains than in mice that were infected with the WT *S. Typhimurium* (Fig. 4b).

Although SKO mutants such as *msbB* and *lppB* showed higher levels of IgG2a (Fig. 4b) in the serum than the *lppAB/msbB* or *lppB/msbB* mutants, they were not considered for further studies because of their higher virulence in mice (Figs. 1b and c) as compared to *lppB/msbB* and *lppAB/msbB* mutants. Conversely to IgG2a, levels of IgG1, an indicator of Th2 responses, were higher in mice infected with the *lppAB/msbB* or the *lppB/msbB* mutant strains than in mice infected with WT *S. Typhimurium* with a peak at 5 days post-infection and a slow decline thereafter (Fig. 4a). Serum levels of IgG1 were still significantly higher after 14 days, indicating the generation of a strong neutralizing antibody for an appropriate anti-bacterial response. Similarly supporting a predominant influence of Th2 cells on antibody production in mice infected with the *lppAB/msbB* or the *lppB/msbB* mutant strains was our observation that these mice had higher serum levels of IgG2b and IgA than did mice infected with WT *S. Typhimurium* (data not shown).

Further, we examined cytokines such as IFN- γ , IL-4, and IL-6 in T cells. IFN- γ , a major cytokine produced by CD4⁺ Th1 and NK cells, has been demonstrated to play an important role in the protection against *Salmonella* infection in avian hosts and is an appropriate indicator of an adequate inflammatory response [24,34,35]. During infections with intracellular pathogens, IFN- γ derived from activated Th1 cells is critical for host resistance by mediating the activation of effector cells and promoting inflammatory responses. Nevertheless, overproduction of the potent antimicrobial and inflammatory Th1 cytokines can lead to tissue damage [36–39]. One important regulator of LPS-induced pathology is IFN- γ [37,40]. The multi-functional cytokine IL-6 exerts effects on many different target cell types, and the main biological activities include the induction of terminal differentiation in B cells and the activation of T cells [41]. Important for our attempts to limit inflammatory Th1 responses is the inhibitory effect of IL-6 on the differentiation of CD4⁺ T cells into effector Th1 cells. IL-6 induces the expression of suppressor of cytokine signaling (SOCS)1 which inhibits IFN- γ signaling [42]. In addition, IL-6 directs the differentiation of CD4⁺ T cells into IL-4-producing effector Th2 cells [43].

In our experiments, the IFN- γ response was higher in splenic T cells challenged with WT *S. Typhimurium* compared to responses following exposure to the mutant strains *lppB/msbB* or *lppAB/msbB* (Fig. 6). Conversely, IL-6 levels were significantly higher in splenic T cells exposed to the *lppB/msbB* or *lppAB/msbB* mutant strains. Likewise, we noted increased production of IL-4 from T cells exposed to the above two mutants. Important for our study is to recognize that Th1 cells produce preferentially IFN- γ and tumor necrosis factor (TNF)- β , whereas Th2 cells preferentially produce IL-4 and IL-5 [44]. A consequence of this selective cytokine production by Th cells is the differential regulation of immunoglobulin isotype switching where Th1 and Th2 cells specifically induce antigen-specific B cells to secrete IgG2a and IgG1, respectively [45]. We noted that both IL-4 and IL-5 levels were difficult to measure, presumably due to their consumption by activated T cells and their instability. In general, the levels of inflammatory cytokines, as measured by Bio-Plex, were very low in the supernatant of T cells infected with *lppB/msbB* and *lppAB/msbB* mutants compared to WT *S. Typhimurium* exposed T cells (data not shown).

Several vaccines against *Salmonella*, including whole-cell killed and live vaccines, have been developed with variable results [19]. Killed *Salmonella* vaccines induce strong antibody responses but are poor triggers of cell-mediated immunity [19,46,47]. In addition, killed *Salmonella* vaccines require parenteral immunization, whereas live vaccines can be administered orally. Further, T-cell or antibody responses alone confer only a moderate level of protection against virulent *Salmonella* strains [48–51]. Mutants of *S. Typhimurium* deficient in the biosynthesis of aromatic metabolites (e.g. *aroA*, *aroC* and *aroD*) [52–56] or purines (e.g. *purA* and *purE*) [57,58], in the production of adenylate cyclase (*cya*) or the cyclic AMP receptor protein (*crp*) [59], or with mutations affecting the global regulatory system (*phoP/phoQ*) [60] or the gene encoding Cdt (colonization of deep tissues) [61] have been investigated in mice as potential live vaccine candidates. Strains of *S. Typhimurium* with mutations in the *Salmonella* pathogenicity island-2 (SPI-2) are also attenuated in mice [62–65]. Further, the *phoP/phoQ* and the Δ *aroC* Δ *ssaV* *S. Typhimurium* mutant strains have been reported to be safe and elicit specific antibodies and IgA antibody-secreting cell responses to the heterologous antigens as well as to the carriers LPS [56,66,67] in human volunteers. The *ssaV* gene codes for a protein involved in protein secretion via the type 3 secretion system (T3SS) on the SPI-2. However, these mutants were heavily colonized in the tested volunteers, as indicated by prolonged shedding, and induced a significant acute-phase response, indicative of inflammation, at the highest dose levels. Most importantly, these mutants still have intact LPS and Lpp which induce strong proinflammatory cytokine responses. Therefore, further live-attenuated oral vaccine strains generated by mutating genes that contribute to shedding and the generation of proinflammatory cytokines are needed for safer vaccine development.

Importantly, we found that the *lppB/msbB* and *lppAB/msbB* mutant *S. Typhimurium* strains did not persist *in vivo*, and were rapidly cleared from the infected mice. In fact, the *lppAB/msbB* mutant strain could not be cultured from spleens collected two days post-infection, and the *lppB/msbB* mutant strain persisted less than seven days in the spleen of infected mice (Fig. 3a). The time-frame of this bacterial clearance, however, is sufficient for the induction of protective immunity as has been recently demonstrated by Ravindran *et al.* using an *in vivo* system of *Salmonella* infection with subsequent visualization of bacterial antigen and T cell activation [68]. We believe that the new mutant strains of *S. Typhimurium* (*lppB/msbB* and *lppAB/msbB*) that have been tested in this investigation provided three important features that are crucial for a good vaccine candidate: **1)** significantly enhanced specific antibody and cellular responses against WT *S. Typhimurium* in a mouse model, **2)** ability to clear rapidly from infected mice, and **3)** inability to generate significant pro-inflammatory cytokine responses. In future studies, we will compare immunological responses of our *lpp/msbB* mutants with other live attenuated *S. Typhimurium* mutants (e.g., *PhoP/Q*, *AroA*, *HtrA*), which could be crucial for evaluating their potential use as vaccine candidates in humans and animals.

Taken together, we have developed and characterized new mutants of *S. Typhimurium* that could be excellent live vaccine candidates. Because *lpp* genes are found in *S. Typhi* as well, such mutants could also be developed in the *S. Typhi* background strain for their possible use against typhoid.

4. Materials and Methods

4.1. Bacterial culture

S. Typhimurium strains used in this study are listed in Table 1. The organisms were grown in Luria-Bertani (LB) broth and on LB agar plates in the presence of the appropriate antibiotics. For the growth of *msbB* mutants, we used a special MsbB medium as previously described [23]. The MsbB medium/liter consisted of the following: Tryptone 10 g, Yeast Extract 5 g, 1 M MgSO₄ (1 ml), and 1 M CaCl₂ (1 ml). The bacteria were cultivated at 37°C overnight with shaking at 200 rpm. Bacteria were harvested by centrifugation (6,000 rpm for 5 min), washed with PBS, and resuspended in a minimal amount of PBS. Bacteria were enumerated by determining colony-forming units (cfu) in triplicate, and expressed as cfu/ml.

4.2. Animals

We used 6- to 8-week-old Swiss-Webster mice that were purchased from Taconic Farms, Germantown, NY. These mice are susceptible to infection with *S. Typhimurium* [69], and we chose these out-bred mice over in-bred mice to examine true heterogeneity in immune responses to vaccine candidates. We believe that this model better represents the situations to be encountered in human populations. Mice were kept under specific pathogen-free conditions in filter-topped cages with sterile bedding, and were fed sterile food and water.

4.3. Infection of mice

Mice were infected *via* the intraperitoneal (i.p.) route with the following bacterial strains: WT *S. Typhimurium* 14028 and its *msbB*, *lppB*, *lppAB*, *lppB/msbB*, and *lppAB/msbB* mutants as prepared and characterized previously [9,23,25]. We chose *lppB* and *lppB/msbB* mutants over *lppA* and *lppA/msbB* mutants because our earlier studies indicated that the *lppB* mutant, retaining expression of *lppA*, is more potent than the *lppA* mutant in inducing inflammatory cytokines necessary to activate an adaptive immune response [23]. Mice were infected with bacterial doses ranging from 1×10^3 – 1×10^8 cfu, and observed for 30 days. In some cases, mice initially infected with mutant *S. Typhimurium* were rechallenged with WT *S. Typhimurium* (1×10^4 – 1×10^7 cfu) 30 days after the initial infection.

4.4. Enumeration of bacteria

At indicated time intervals after infection/immunization of mice, the spleens were removed, homogenized in PBS, and serial dilutions of the homogenate were plated on LB and *Salmonella-Shigella* agar plates [23,25] for determining bacterial counts. The plates were incubated at 37°C for 24 to 48 h and colonies were counted.

4.5. Serum immunoglobulins

Mice were infected with WT or mutant *S. Typhimurium*. Total serum IgG1 and IgG2a were measured after 2, 5, 7, or 14 days by enzyme-linked immunosorbent assay (ELISA). Highbinding ELISA plates (flat bottom, Evergreen Scientific, Los Angeles, CA) were coated with 1 µg/ml of capture antibody (anti-IgG1 or anti-IgG2a; Bethyl Inc., Montgomery, TX) diluted in 0.05 M carbonate/bicarbonate buffer (pH 9.6) for 1 h at room temperature. Subsequently, the wells of the microtiter plates were washed three times with washing buffer (50 mM Tris, 0.14 M NaCl, and 0.05% Tween 20). Sera from mice were diluted 1:50 in sample buffer (50 mM Tris, 0.14 M NaCl, 1% bovine serum albumin [BSA], and 0.05% Tween 20).

Mouse Reference Serum (Bethyl Inc.) was diluted two-fold starting at a concentration of 250 ng/ml and used for plotting a standard curve. Subsequently, the plates were incubated at room temperature for 1 h and washed three times with washing buffer. The plates were then blocked with blocking buffer (50 mM Tris, 0.14 M NaCl, and 1% BSA). After washing, a 1:150,000 dilution of horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Bethyl Inc.) was added to the wells of the microtiter plate. Finally, 3, 3', 5, 5' tetramethylbenzidine (TMB) substrate was added to visualize antigen-antibody reactions. The reaction was stopped with 2 M H₂SO₄, and the optical density (OD) was measured at 450 nm using an ELISA reader. Serum from uninfected mice was used as a control. The concentration of IgG subclass antibodies was expressed in ng/ml.

4.6. Collection of blood

Blood was obtained by retro-orbital bleeding. Samples were kept at 4°C for 4–6 h, and then centrifuged at 4,000 rpm for 5 min to separate the serum. Sera were stored at –20°C.

4.5. Flow cytometric analysis

Spleen cells were obtained from mice infected with WT or mutant *S. Typhimurium*. Splenocytes ($0.5\text{--}1.0 \times 10^6$) were suspended in PBS and 1% fetal calf serum (FCS), and incubated with mouse CD16/CD32 monoclonal antibodies (0.25 µg/100 µl) (BD Bioscience, Franklin Lakes, NJ) for 15 min at room temperature. These antibodies were added to block antibody binding to mouse Fc-γ receptor-bearing cells. Then, the cells were washed twice with PBS plus 1% FCS. To quantitate activated T cells, the cell suspension was incubated with either anti-CD4 or anti-CD8 antibody conjugated to fluorescein isothiocyanate [FITC] (0.25 µg/100 µl) and anti-CD44 antibody conjugated to phycoerythrin (PE) (0.1 µg/100 µl) for 60 min at 4°C. Subsequent to incubation for 60 min at 4°C, the cells were washed twice with PBS, and then analyzed using a FACScan flow cytometer and CellQuest (Becton Dickinson, Mountain View, CA) software.

4.7. Cytokine assays

Cytokine production from T cells was measured as previously described [9]. Briefly, splenocytes were isolated from naïve mice and plated at a density of 1×10^7 /ml in 24-well tissue culture plates. These cells were then incubated with WT *S. Typhimurium* or the *lppB/msbB* or *lppAB/msbB* mutants at a multiplicity of infection (MOI) of 1 for 16 h. Subsequently, splenocytes were washed extensively with PBS, and T cells were enriched by passing splenocytes through nylon-wool columns (10^8 splenocytes were incubated in the column at 37°C in the presence of 5% CO₂ for 1 h before eluting the cells with 9 ml of RPMI medium) [70]. The T cells were plated at a density of 2×10^5 with irradiated splenocytes from uninfected mice (2×10^4) as feeder cells in 96-well microtiter plates in 200 µl of RPMI medium for 72 h at 37°C in the presence of 5% CO₂. The culture supernatants were then examined by ELISA for the presence of IFN-γ, IL-6, and IL-4.

For the ELISA, the wells of microtiter plates were coated with 100 µl of purified anti-mouse monoclonal capture antibody (eBioscience, San Diego, CA) against either IFN-γ, IL-4, or IL-6 at a concentration of 1 µg/ml in coating buffer (PBS plus 0.1% BSA). The plates were incubated overnight at 4°C. Subsequently, the plates were washed three times with washing buffer (PBS plus 0.05% Tween 20), followed by blocking with 200 µl of blocking buffer (PBS plus 1% BSA) per well. The plates were incubated at room temperature for 60 min, washed three times with washing buffer, and then 100 µl of standard or sample was added to each well. Recombinant mouse IL-4, IL-6, and IFN-γ were diluted two-fold starting at a concentration of 0.1–0.5 µg/ml and used as a standard. Subsequently, the plates were incubated at room temperature for 1 h, washed three times with washing buffer, and incubated with 100 µl of detection antibody (1 µg/ml of biotinylated anti-mouse IL-6/IL-4 or anti-IFN-γ antibody;

eBioscience) prepared in PBS plus 0.1% BSA. The plates were incubated at room temperature for 1 h, washed six times with washing buffer, followed by the addition of streptavidin-conjugated HRP (from eBioscience) at a 1:500 dilution in blocking buffer. The plates were incubated at room temperature for 30 min, washed six times with washing buffer, and then 100 μ l of TMB substrate was added. The color was developed at room temperature for 20–30 min followed by the addition of 50 μ l of 2 M H₂SO₄ to stop the reaction. The OD was measured at 450 nm. The supernatants from T cells were also examined using the multiplex assay with Bio-Plex (Bio-Rad, Hercules, CA), which detects 23 cytokines/chemokines.

4.8. T cell proliferation assay

Mice were infected/immunized with mutant or WT *S. Typhimurium* at a dose of 1×10^3 cfu by the i.p. route. After 2, 5, 7, and 14 days, splenocytes from three infected/immunized mice were isolated, pooled, and incubated with WT *S. Typhimurium* at an MOI of 1 for 16 h. After the incubation period, the number of live splenocytes was determined by the trypan blue exclusion technique and counting with a hemocytometer. Live T cells were enriched using nylon-wool columns, and incubated at a cell density of 2×10^5 /well with irradiated splenocytes (2×10^4) from uninfected mice in 96-well U-bottom microtiter plates for 72 h. Cells were pulsed with [³H]-thymidine for the last 10 h, and incorporation of radioactivity was measured by liquid scintillation counting in triplicate. Data were expressed as arithmetic means $\pm$ standard deviation (S.D.).

In another set of experiments, splenocytes were isolated from naïve mice and incubated with WT or *lppB/msbB* or *lppAB/msbB* mutant *S. Typhimurium* for 16 h at an MOI of 1. Subsequently, live cells were counted by the Trypan Blue exclusion technique, and T cells were enriched using nylon-wool columns and incubated with irradiated splenocytes for proliferation measurements as described above.

4.9. Statistics

At least three independent experiments were performed, and the data were analyzed using Student's *t* test. *p*-values of ≤ 0.05 were considered significant. Animal mortality was analyzed using Fisher's exact test.

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Figure 1.

Survival of Swiss-Webster mice following infection with WT *S. Typhimurium* or various mutant strains of *S. Typhimurium*. **a)** Mice were infected *via* i.p. injection with 1×10^3 cfu, and observed for 30 days; **b)** mice were injected with 1×10^4 cfu.; **c)** mice were injected with 1×10^6 cfu; **d)** mice were injected with 1×10^7 cfu of WT or *lppB/msbB* or *lppAB/msbB* mutant *S. Typhimurium*; The actual *p*-values (Fisher's exact test) are provided. The *p*-values shown for the WT or the mutant(s) *S. Typhimurium* were in comparison to those mutants that showed 100% protection. Asterisks denote statistically significant differences.

Figure 2.

Protection of Swiss-Webster mice following infection with mutant strains of *S. Typhimurium* and challenge with WT *S. Typhimurium*. **a)** mice were injected with 1×10^4 cfu of various mutant strains of *S. Typhimurium* and, after 30 days, rechallenged with 1×10^5 cfu of WT *S. Typhimurium*; **b)** mice were injected with 1×10^6 cfu of *lppB/msbB* or *lppAB/msbB* mutant *S. Typhimurium* and, after 30 days, rechallenged with 1×10^6 or 1×10^7 cfu of WT *S. Typhimurium*. The control groups of mice were given a primary challenge dose of 1×10^7 cfu of WT *S. Typhimurium*. The actual *p*-values (Fisher's exact test) are provided. The *p*-values shown for the WT or the mutant(s) *S. Typhimurium* were in comparison to those mutants that showed 100% protection. Asterisks denote statistically significant differences.

Figure 3.

Bacterial growth in the spleens of infected Swiss-Webster mice. **a)** Mice were infected *via* i.p. injection with 1×10^3 cfu of WT or various mutant strains of *S. Typhimurium* (symbols are defined in the figure). At the indicated times, cfu in the spleens were determined. Data are expressed as mean $\pm$ S.D. and p -values < 0.05 are considered statistically significant (*). **b)** Mice were injected with 1×10^3 cfu of *lppB/msbB* or *lppAB/msbB* mutant *S. Typhimurium*, and rechallenged with 1×10^3 cfu of WT *S. Typhimurium* after 30 days. Seven or 14 days after the rechallenge, cfu in the spleens were determined. The p -values were determined for the comparison between immunized groups vs. a control group given a primary challenge with 1×10^3 cfu of WT *S. Typhimurium*. The p -values were < 0.05 for all immunized groups compared to a control group given a primary challenge with 1×10^3 cfu of WT *S. Typhimurium* for 7 and 14 days. The actual p -values (Student's *t*-test) are provided.

Figure 4.

Swiss-Webster mice were infected with 1×10^3 cfu of WT or various mutant strains of *S. Typhimurium*. **a)** Serum IgG1 and **b)** serum IgG2a concentrations were determined by ELISA at the indicated times after infection. Data are expressed as mean $\pm$ S.D. The p -values of < 0.05 for all mutant strains except *msbB* (only IgG1) compared to WT at all time points were considered statistically significant (*). The actual p -values (Student's t -test) are provided.

Figure 5.

Sera from Swiss-Webster mice immunized once with 1×10^3 cfu of *lppB/msbB* or *lppAB/msbB* mutant *S. Typhimurium* completely protected animals from rechallenge with 1×10^4 cfu of WT *S. Typhimurium*. Mice were injected i.v. with 120 μ l of sera from mice infected with either WT or *lppB/msbB* or *lppAB/msbB* mutant strains of *S. Typhimurium*. Control groups received PBS or pre-immune sera. After 24 h, mice were challenged with 1×10^4 cfu WT *S. Typhimurium* and observed for 30 days. The actual *p*-values (Fisher's exact test) between different groups are provided. Asterisks denote statistically significant differences.

Figure 6.

Cytokine production by T cells following *in vitro* culture of splenocytes from Swiss-Webster mice with WT or mutant *S. Typhimurium* at an MOI of 1 for 16 h. T cells were then isolated and incubated with irradiated splenocytes from uninfected mice for 72 h. **a)** IL-6 and **b)** IFN- γ concentrations in culture supernatants. T cell proliferation following *in vitro* culture of splenocytes from Swiss-Webster mice with WT or mutant *S. Typhimurium* at an MOI of 1 for 16 h. **c)** T cells were then isolated and incubated with irradiated splenocytes from uninfected mice for 72 h. ^3H -thymidine was added during the last 10 h of incubation. Controls were cultured in the absence of *S. Typhimurium*. The *p*-values (student's *t*-test) are given for statistically significant differences.

Figure 7.

T cell activation in infected Swiss-Webster mice. **a)** Survival of splenocytes. Splenocytes were harvested five days post-infection with 1×10^3 cfu of *lppB/msbB*, *lppAB/msbB*, or WT *S. Typhimurium* (white bars). Splenocytes were then incubated with WT *S. Typhimurium* at an MOI of 1 for 16 h, and cell survival was determined. Controls were from uninfected mice and were incubated *in vitro* with *lppB/msbB*, *lppAB/msbB*, or WT *S. Typhimurium* (black bars). **b)** Expression of the activation and memory T cell marker CD44 in T cells from Swiss-Webster mice infected with WT or mutant *S. Typhimurium*. Cells were analyzed by flow cytometry five days post-infection with 1×10^3 cfu. Controls were from uninfected mice. **c)** Swiss-Webster mice were infected with 1×10^3 cfu of WT or mutant *S. Typhimurium* or left uninfected (control). After five days, splenocytes were isolated and incubated with WT *S. Typhimurium* at an MOI of 1 for 16 h. T cells were then isolated and incubated with irradiated splenocytes from uninfected mice for 72 h. T cell proliferation was measured by incorporation of [3 H]-thymidine during the last 10 h of incubation. The *p*-values (Student's *t*-test) are given for statistically significant differences. Asterisks denote statistically significant differences.

TABLE 1
Strains used in this study

Strain	Relevant characteristic(s)	Reference
<i>S. enterica</i> serovar Typhimurium strains		
14028	<i>S. enterica</i> serovar Typhimurium	9
Mutant <i>lppB</i>	Isogenic mutant in which only one copy of the <i>lpp</i> gene (<i>lppB</i>) was deleted; Nal ^r Kn ^r	9
Mutant <i>msbB</i>	14028 in which the <i>msbB</i> gene was truncated; Tc ^r	23
Mutant <i>lppAB</i>	Isogenic mutant in which both copies of the <i>lpp</i> gene (<i>lppAB</i>) were deleted; Nal ^r Kn ^r	9
Mutant <i>lppB/msbB</i>	Isogenic mutant in which the <i>lppB</i> gene was deleted from the <i>msbB</i> minus background strain of 14028; Nal ^r Tc ^r Kn ^r	25
Mutant <i>lppAB/msbB</i>	Isogenic mutant in which the <i>lppAB</i> genes were deleted from the <i>msbB</i> minus background strain of 14028; Nal ^r Tc ^r Kn ^r	23

Nal^r = Nalidixic acid resistance; Tc^r = Tetracycline resistance; Kn^r = Kanamycin resistance