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Mouse models to assess the efficacy of non-typhoidal *Salmonella* vaccines: revisiting the role of host innate susceptibility and routes of challenge

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Abstract

Non-typhoidal *Salmonella enterica* (NTS) serovars Typhimurium and Enteritidis are important causes of bacterial gastroenteritis in the USA and worldwide. In sub-Saharan Africa these two serovars are emerging as agents associated with lethal invasive disease (e.g., bacteremia, meningitis). The development of NTS vaccines, based on mucosally-administered live attenuated strains and parenteral non-living antigens, could diminish the NTS disease burden globally. Mouse models of *S.* Typhimurium and *S.* Enteritidis invasive disease can accelerate the development of NTS vaccines. Live attenuated NTS vaccines elicit both cellular and humoral immunity in mice and their efficacy is well established. In contrast, non-living vaccines that primarily elicit humoral immunity have demonstrated variable efficacy. An analysis of the reported studies with non-living vaccines against *S.* Typhimurium and *S.* Enteritidis reveals that efficacy is influenced by two important independent variables: 1) the innate susceptibility to NTS infection that differs dramatically between commonly used mouse strains and, 2) the virulence of the NTS strain used for challenge. Protection by non-living vaccines has generally been seen only in host-pathogen interactions where a sub-lethal infection results, such as challenging resistant mice with either highly virulent or weakly virulent strains or susceptible mice with weakly virulent strains. The immunologic basis of this discrepancy and the implications for human NTS vaccine development are reviewed herein.

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Keywords

Salmonella Typhimurium; *Salmonella* Enteritidis; mouse model; vaccine efficacy; live attenuated vaccine; conjugate vaccine

1. Introduction

Certain serovars of *Salmonella enterica* sub-species *enterica* that cause invasive infections in humans pose a public health burden worldwide [1]. Among the most important are the etiologic agents of typhoid and paratyphoid fever, *Salmonella* Typhi and *Salmonella* Paratyphi A and B (and occasionally C), often referred to collectively as the enteric fever serovars. *S.* Typhimurium and *S.* Enteritidis are the two most common NTS serovars associated with gastroenteritis [2] and invasive disease [3]. Whereas in industrialized countries it has long been recognized that NTS serovars can occasionally cause severe invasive infections accompanied by high case fatality rates when they infect young infants or the elderly, only recently has systematic, blood culture-based surveillance of febrile infants and young children in sub-Saharan Africa revealed an enormous burden of invasive disease associated with NTS. Although many of the surveillance activities in Africa were originally undertaken to quantify the burden of invasive *Haemophilus influenzae* type b (Hib) and *Streptococcus pneumoniae* infections, the studies unexpectedly revealed that invasive NTS were as commonly isolated from bacteremic patients as Hib and pneumococcus [4–13]. Notably, 75–95% of these invasive NTS infections in Africa are due to *S.* Typhimurium and *S.* Enteritidis [4–14]. Four features differentiate the pediatric invasive NTS infections in sub-Saharan Africa from the invasive pediatric NTS infections observed in the USA and Europe. First, the sub-Saharan Africa infections are clinically severe and accompanied by case fatality rates of ~15–20%. Second, two-thirds of the African invasive NTS cases neither present with gastroenteritis nor have a history of gastroenteritis [15]. Third, full genome sequencing of an invasive prototype *S.* Typhimurium strain from Malawi surprisingly revealed that the strain manifested considerable genomic degradation, including complete loss of some genes and the presence of multiple pseudogenes, some of which are also found in *S.* Typhi or *S.* Paratyphi A [16]. Lastly, the few epidemiological investigations carried out so far have failed to identify an animal reservoir for these emerging “invasive” NTS strains [17].

Oral challenge of mice with the human host-restricted serovars *S.* Typhi and *S.* Paratyphi does not lead to a productive invasive infection. In contrast, oral challenge of mice with *S.* Typhimurium and *S.* Enteritidis generates an invasive, generalized infection of the gut-associated lymphoid tissue and reticuloendothelial system (RES) that recapitulates many aspects of typhoid fever in humans [18, 19]. Accordingly, these “mouse typhoid” models have been used to study *Salmonella* pathogenesis, to identify attenuating genetic lesions possibly applicable to *S.* Typhi, and to assess the immunogenicity and efficacy of different candidate vaccines.

The burgeoning interest in developing NTS vaccines to control invasive NTS disease in sub-Saharan Africa has renewed interest in mouse models of *S.* Typhimurium and *S.* Enteritidis, since such pre-clinical models may be invaluable for examining the efficacy of candidate NTS vaccines intended for human use.

2. Mouse Models of *Salmonella* Pathogenesis

Since the pathogenesis of *Salmonella* infection is reviewed extensively elsewhere [18–23], it will only be briefly summarized here. Following oral inoculation in mice, *Salmonella*

bacteria initiate infection in the small intestine through penetration of the Peyer's patches [24, 25]. Invasion and colonization of the Peyer's patches is soon followed by migration within phagocytic cells to mesenteric lymph nodes, followed by a primary bacteremia and dissemination to the organs of the reticuloendothelial system (RES) (spleen, liver, bone marrow, etc.), onset of a systemic febrile illness, and, finally death [19, 23]. Mice can also be infected by parenteral inoculation via the intraperitoneal (IP), intravenous (IV) and subcutaneous (SC) routes, resulting in similar patterns of infection of the RES and death [26–28]. Of these various routes, inoculation with *Salmonella* by the oral route most closely resembles the natural route of infection in humans [18, 26], but requires the highest number of viable bacteria in order to induce lethal infection. However, oral infection allows more accurate control of the administered dose, since orally administered *Salmonella* do not immediately enter a rapid growth phase following inoculation, as can happen with IP infections [18, 20, 26, 28].

3. Literature Search

3.1. Methods

A review of the literature was performed by searching the PubMed database with no time limits, using search terms including "*Salmonella*", "Typhimurium", "Enteritidis", "vaccine", "mice", "porin", "conjugate", "live attenuated", "killed", "humoral", "cell mediated", as well as the references of included papers. Our search was limited to studies published in the English language. To be selected for inclusion, a publication had to provide detailed information on 1) well characterized vaccine candidates; 2) route and schedule of immunization; 3) the degree of virulence of the wild-type *Salmonella* challenge strain; 4) the interval from last vaccine dose until challenge; 5) size of the challenge inoculum; 6) route of challenge; and 7) genetic background of the mouse strain with defined or evident NTS susceptibility.

4. The Vaccines Used to Prevent Fatal *Salmonella* Typhimurium and *Salmonella* Enteritidis Infections in Mice

4.1 Live attenuated vaccine strategies

Live attenuated strains based on lesions in genes encoding products in bacterial metabolic, signaling, and gene regulation pathways have formed the basis of several live attenuated *Salmonella* vaccine strains that have shown marked vaccine efficacy in mouse challenge experiments [29]. The mutated genes encode aromatic amino acid biosynthesis (*aroA*, *aroC*, *aroD*), galactose metabolism (*galE*), heat shock proteins (*htrA*), guanine nucleotide biosynthesis (*guaA*, *guaB*), purine nucleotide biosynthesis (*purA*, *purB*, *purE*, *purH*), adenylate cyclase signaling (*cya*, *crp*), two-component regulatory systems (*phoP*, *phoQ*), regulators of protein metabolism (*clpP*, *clpX*), outer membrane proteins (*ompC*, *ompF*, *ompR*), DNA recombination and repair pathways (*recA*, *recB*, *recC*), dissemination to deep organs of the reticuloendothelial system (*cdt*) and transcriptional regulators of gene expression (*dam*) [29–38].

4.2 Inactivation and subunit strategies

Killed whole cell vaccines prepared by a variety of methods, including thermal inactivation, or chemical inactivation with acetone, deoxycholate, or formalin, have been tested in mice. In an effort to target individual *Salmonella* antigens to avoid the reactogenicity associated with parenteral administration of inactivated whole cell *Salmonella* vaccines, candidate subunit vaccines have been developed. These include protein (purified porins, bulk outer membrane proteins, flagellin protein), lipopolysaccharide or O-polysaccharide and *Salmonella* O-polysaccharide-protein conjugate vaccines. Conjugate vaccines based on

chemical covalent linkage of the *Salmonella* O-polysaccharide (OPS) with a protein carrier have been utilized to enhance the immunogenicity of the otherwise weakly immunogenic polysaccharide hapten, and provide functional immunological memory [39].

5. Sources of Variability in Murine Models that Test the Efficacy of *Salmonella* Typhimurium and *Salmonella* Enteritidis Vaccines

5.1 Importance of the strain of mouse

The development of the mouse model of lethal *S.* Typhimurium and *S.* Enteritidis infections has provided a robust platform for testing experimental vaccines against these two serovars and allowed considerable progress to be made towards understanding the immune responses that can mediate protection against these pathogens [19, 23, 40–42]. Nevertheless, the systematic analysis that we undertook of the published scientific literature on *S.* Typhimurium and *S.* Enteritidis vaccines evaluated in murine models reveals considerable variability and often contradictory results (Table 1, 2). We found that the genetic background of the mouse strain and the virulence of the wild-type *Salmonella* challenge strain stood out, strikingly, as the two most important fundamental variables [43–48], aside from expected differences in efficacy stemming from the type of vaccine. Indeed, since several commonly used mouse strains differ so markedly in their natural susceptibility to *Salmonella* infection, they are classified as either inherently “resistant” or “susceptible” [46, 49–53]. A defining phenotypic trait of susceptible mouse strains is the dramatic reduction in the lethal dose of a given *Salmonella* strain required to kill 50% of infected mice (LD_{50}), which can be > 1,000-fold lower as compared to resistant mice (Table 3).

The use of susceptible mice in NTS vaccine development offers several advantages, as well as disadvantages. Of notable importance, the significantly lowered LD_{50} with virulent NTS in these mice allows a wider range of challenge inocula to be tested. This is particularly pertinent to the oral challenge model, which necessitates a much larger inoculum size that can be on the order of 1,000-fold greater than that required to achieve similar lethality with parental challenge (Table 3). Moreover, in resistant mice it may not be possible to achieve a lethal dose following oral challenge if a weakly virulent NTS strain is given. On the other hand, a sub-lethal infection with highly virulent strains of *S.* Typhimurium and *S.* Enteritidis administered parenterally cannot be established in susceptible mice, as the LD_{50} for parenteral challenge is frequently less than 20 colony forming units (cfu) [46, 49, 50, 52–54]. A final point to consider is that inbred susceptible mice do not model accurately the genetic heterogeneity of human populations.

5.2 Genetic basis of mouse susceptibility to Salmonella

Mutations in several important genetic loci in mice have identified susceptibility factors for infection with *Salmonella*, and are associated with significant lowering of the LD_{50} [23, 49–51, 53]. Of these, NTS susceptibility loci originally referred to as Immunity to Typhimurium (*Ity*) and resistance to Lipopolysaccharide (*Lps*), involve genes associated with innate immunity. The *Ity* locus contains the Natural resistance associated macrophage protein 1 gene (Nramp1) which encodes an intracellular endosome-associated pH-controlled ion transport protein, present within resident peritoneal and splenic macrophages. A mutation in this gene found in susceptible mice leads to defects in phagocyte endolysosome function that impairs control of intracellular infections and increases susceptibility to lethal *Salmonella* infection [55, 56]. Mutations in the *Lps* locus were originally identified in the C3H/HeJ mouse strain, a spontaneous mutant derived from the C3H line, that is insensitive to the toxic effects of bacterial lipopolysaccharide (LPS) [57]. The inability to respond to LPS, an important *Salmonella* pathogen associated molecular pattern, causes these mice to become highly susceptible to *Salmonella* infection, as they are unable to mount an adequate innate

immune response to control the early stages of infection. The mouse *Lps* locus is associated with a mutation in the gene for Toll-like Receptor 4 (TLR4), the innate immune receptor for LPS, that renders it nonfunctional for signaling [58–60]. The broad importance of LPS recognition in natural *Salmonella* immunity has been further confirmed, as defects in the endotoxin recognition system including LPS Binding Protein (LBP), and CD-14 have also been associated with *Salmonella* susceptibility [23, 61, 62].

6. Immunization of Susceptible Mouse Strains with Live Attenuated NTS Vaccines and Protection against NTS Challenge

6.1 Oral immunization and oral challenge

As summarized in Table 1, every report of susceptible mice (e.g., BALB/c, C57Bl/6) immunized orally with *S.* Typhimurium attenuated mutants has demonstrated a high level of protection when the mice were subsequently challenged orally with wild type *S.* Typhimurium [30–33, 63, 64]. This is true for an array of live vaccines carrying different attenuating mutations. Moreover, in most studies the oral challenge was rigorous and contained > 10,000 × LD_{50} of virulent *S.* Typhimurium [30, 32, 33].

6.2 Oral immunization and parenteral challenge

The literature search was unable to identify reports describing susceptible mice immunized orally with attenuated *S.* Typhimurium or *S.* Enteritidis vaccines that were then challenged parenterally with wild type organisms.

6.3. Parenteral immunization and oral challenge

There are multiple reports of experiments with susceptible mice immunized with a variety of attenuated *S.* Typhimurium or *S.* Enteritidis vaccines by parenteral routes (IP, IV or SC) and were then challenged orally [31, 57, 63, 65–67]. In all instances a high level of vaccine efficacy was observed (Table 1).

6.4. Parenteral immunization and parenteral challenge

There are also several reports of susceptible strains of mice that were immunized parenterally (IP or IV) with attenuated *S.* Typhimurium or *S.* Enteritidis vaccines and were then shown to be highly protected when subsequently challenged with wild type NTS administered IP (Table 1) [47, 57, 68].

7. Immunization of Susceptible Mouse Strains with Non-Living NTS Vaccines and Protection against Challenge with Virulent NTS

7.1. Oral immunization followed by oral or parenteral challenge

Our search uncovered no reports of susceptible mice immunized orally with non-living *S.* Typhimurium or *S.* Enteritidis vaccines who were then challenged with the homologous wild type serovar given either orally or parenterally.

7.2. Parenteral immunization followed by oral challenge

Several reports describe the parenteral immunization of susceptible strains of mice (e.g., BALB/c, C57Bl, C57Bl/6) with chemical or heat-inactivated whole cell *S.* Typhimurium or *S.* Enteritidis vaccines or with a subunit vaccine (flagellin) followed by oral challenge with low doses of virulent organisms (Table 1). The only vaccine or regimen that achieved a high level of protection against oral challenge was when inactivated *S.* Enteritidis were administered with Complete Freunds Adjuvant (CFA) and the challenge dose was low and

near the LD_{50} [69, 70]. The only other hint of protection in the face of oral challenge was observed following SC immunization of C57Bl/6 mice with *S.* Typhimurium flagellin protein FliC; weak protection was recorded against oral challenge with a low dose of *S.* Typhimurium [64].

7.3. Parenteral immunization followed by parenteral challenge

As summarized in Table 1, non-living *S.* Typhimurium vaccines have provided generally disappointing and inconsistent results in instances where both the vaccine and the challenge were administered parenterally to susceptible strains of mice (Table 1). With only one exception [46], parenteral immunization with inactivated whole cell vaccines have failed to protect susceptible mice against parenteral challenge with virulent NTS. Those few inactivated parenteral vaccines that did confer measurable protective efficacy against wild type challenge involved immunization with subunit vaccines, followed by challenge with weakly virulent *Salmonella* strains (parenteral LD_{50} > 10,000 cfu) or low inocula of virulent strains [46, 54, 71–76].

8. Immunization of Resistant Mouse Strains with Live Attenuated NTS Vaccines and Protection against Challenge

8.1. Oral immunization followed by oral or parenteral challenge

We did not find any reports of resistant strains of mice immunized orally with attenuated NTS vaccines and then challenged, either orally or parenterally.

8.2 Parenteral immunization followed by parenteral challenge

Our search revealed that resistant mice immunized by the IP route with live attenuated *aroA* and a rough LPS mutant of *S.* Typhimurium, were protected against subsequent IP challenge with 1,000 × LD_{50} of virulent *S.* Typhimurium (Table 2) [47, 77].

9. Immunization of Resistant Mouse Strains with Non-Living NTS Vaccines and Protection against Challenge

9.1 Oral immunization followed by either oral or parenteral challenge

Our search failed to reveal any reports of oral immunization of resistant mice with non-living *S.* Typhimurium or *S.* Enteritidis vaccines followed by either oral or parenteral challenge with wild type organisms where mortality was reported. In the one study, CD-1 mice immunized orally or IM with heat-killed *S.* Enteritidis showed a slight decrease in tissue cfu burden following oral challenge with highly virulent *S.* Enteritidis. However, this would be unlikely to translate to a decrease in mortality [26].

9.2. Parenteral immunization and parenteral challenge

Resistant mice have proven to be a robust model for testing the efficacy of a panoply of non-living *S.* Typhimurium and *S.* Enteritidis vaccines, including inactivated whole cell, subunit and polysaccharide-protein conjugates, in preventing mortality following challenge with NTS (Table 2). Several publications describe parenteral (IP or IV) immunization of resistant mice (e.g., C3H/HeNCrIBR, CD-1, CF1, ddy, White Swiss), followed by parenteral (primarily IP) challenge with virulent *S.* Typhimurium or *S.* Enteritidis (Table 2) [27, 39, 43, 46, 54, 77–83]. In all reports a high level of protection was observed, even with challenge inocula containing wild type organisms equivalent to 1,000 × LD_{50} (Table 2). These reports contrast with the highly variable protection reported when susceptible mice were challenged after being immunized with non-living vaccines.

10. Role of Mouse *Salmonella* Susceptibility Gene Alleles and Vaccine Efficacy

The variability in protective efficacy seen with non-living vaccines in different strains of mice led to considerable confusion in the field, for a period of time. The situation was clarified when studies to address this conundrum established, unequivocally, that the background murine genetic *Salmonella* susceptibility locus exerts a powerful influence on the observed efficacy of non-living vaccines [46, 54]. In one key study conducted by Robson and Vas, inbred mouse strains that vary in innate susceptibility to infection with NTS were vaccinated IP with phenol-inactivated *S.* Typhimurium and then challenged by the IP route with graded inocula of *S.* Typhimurium ranging from 10^1 to 10^7 cfu [54]. Strikingly, highly susceptible (C57Bl/6J, BALB/cJ, C3H/HeJ) and moderately susceptible (DBA/2/J) mice immunized with whole-cell killed-vaccine were not significantly protected against fatal *S.* Typhimurium infection at any challenge dose tested. In contrast, resistant (A/J) mice were highly protected against lethal *Salmonella* challenge at all levels tested. Notably, both naïve and vaccinated highly susceptible mice succumbed to infection with less than 10 *S.* Typhimurium cfu, whereas infection in naïve resistant A/J mice was consistently fatal only at 10^5 cfu, and immunized mice were fully protected at 10^7 cfu.

Eisenstein et al. immunized mice of the C3H lineage that vary in inherent susceptibility to *Salmonella* by the IP route with either whole-cell killed vaccine or purified LPS and then challenged the mice by IP inoculation with ~ 20 × LD_{50} of virulent *S.* Typhimurium [46]. Naturally resistant C3H/HeNCrlBR mice were fully protected, whereas highly susceptible C3H/HeJ mice (that are non-responsive to bacterial LPS) showed no significant protection against challenge. C3HeB/FeJ mice that are innately responsive to LPS but nonetheless more highly susceptible to *S.* Typhimurium, as compared to traditionally resistant mice, displayed an intermediate protection phenotype; partial protection was observed compared to resistant C3H/HeNCrlBR mice. This study was the first to demonstrate that the differential performance of non-living vaccines in different mouse strains that vary in natural susceptibility to wild-type *Salmonella* infection is consistent across different types of vaccine preparations.

11. Mechanisms of protective immunity to *S.* Typhimurium and *S.* Enteritidis in mice

11.1 Humoral immunity

The extensive work conducted towards understanding the mechanistic basis of the protective adaptive immune response to *Salmonella* has been reviewed elsewhere [23, 34, 84] and will be discussed here only briefly, with an emphasis on mechanisms of immunity in susceptible versus resistant strains of mice. Immunization with non-living vaccines can stimulate the production of humoral immunity, including antibody against *Salmonella* outer membrane antigens and flagellin that can provide protection against extracellular bacteria. Non-living vaccines, in contrast, stimulate comparatively negligible cytotoxic T-cell mediated cellular immunity [84]. Mucosal IgA plays an important role against oral infection with *Salmonella* as a first line of defense at the mucosal epithelial surface, through inhibition of bacterial penetration into the Peyer's patches [85, 86]. Protection by circulating serum antibody can potentially play a role in inhibiting cell-to-cell transmission of *Salmonella* following exit from infected cells, as well as a reduction in overall systemic bacterial cell burden [68, 87–89].

The systemic immune responses mediated by antibody to *Salmonella* include direct complement mediated cytocidal activity by IgM and IgG, as well as opsonophagocytic

uptake into professional phagocytes such as macrophages, neutrophils, and dendritic cells [88]. Of these, antibody-mediated opsonophagocytic uptake, which is associated with secretion of pro-inflammatory cytokines and intracellular oxidative burst, is likely more important *in vivo* as natural resistance to serum and complement-mediated bactericidal activity may be common among invasive non-typhoidal *Salmonella* [90]. Killing by antibody-mediated opsonophagocytosis is effective against complement-resistant strains, and opsonophagocytosis is important for presentation of antigenic peptides and activation of CD4+ T-helper cells [79, 90]. Surface bound antibody on B-cells can also mediate internalization and processing of bacterial cells, for subsequent presentation of *Salmonella*-specific peptide epitopes on MHC-II molecules to T-cells [91]. Presentation of *Salmonella* antigens by antibody mediated opsonophagocytosis can aid in the development of cell-mediated immunity, that may not progress normally otherwise, as *Salmonella* outer membrane polysaccharides are known to interfere with non-specific opsonophagocytic uptake [92, 93]. The importance of antibody-mediated protection against *Salmonella* has been demonstrated in genetic models of B-cell deficiency. In these studies, transgenic derivatives of the susceptible C57Bl/6 mouse strain, defective for the generation of B-cells, were unable to mount a protective immune response against virulent *Salmonella* Typhimurium following vaccination with a live attenuated *aroA* mutant. Furthermore, greatly diminished T-cell activation was observed in these B-cell deficient mice, and full protection was not restored by passive immunization with anti-Typhimurium sera, further underscoring the important role that antibody and B-cells play in the generation of cellular immunity [67, 94].

11.2. Cellular immunity

Immunization with live attenuated non-typhoidal *Salmonella* vaccine strains elicits robust both humoral immunity and cellular immunity that is important for the control of intracellular infection [84]. Cellular immunity against *Salmonella* involves the coordinated action of CD4+ T helper and CD8+ Cytotoxic T lymphocytes (CTL) against *Salmonella* proteins [84, 95, 96]. Cellular responses against *Salmonella* proteins have been demonstrated for several *Salmonella* protein antigens including, for example, flagellin FliC [34, 64, 97–99]. T-helper type I immunity, mediated by tumor necrosis factor-α, (TNF-α), interleukin-12 (IL-12) and interferon-γ (IFN-γ), that includes the action of activated macrophages, subsets of CD8+ and CD4+ T-cells, and opsonophagocytic antibodies, is strongly associated with the protective immune response to *Salmonella* [95, 100–103]. Vaccination of mice with live attenuated strains of *Salmonella* has been demonstrated to elicit potent cellular immunity as characterized by the production of high levels of IFN-γ upon *ex-vivo* restimulation of immune T-cells with *Salmonella* antigens [64, 98, 104]. Indeed, observations from both mouse and human studies have highlighted the overt susceptibility associated with perturbation of the IL-12/IFN-γ axis towards infection with *Salmonella* [61, 105–108]. Allelic specificity of the Major Histocompatibility Complex-II (MHC-II) has also been implicated as important for clearance of *Salmonella* infection in both mice and humans [34, 109, 110].

11.3. Importance of antibody and T-cells to immunity against NTS

Elucidation of the comparative contribution of humoral and cellular immunity in functional protection against *Salmonella* infection in highly susceptible mice has been demonstrated in several published reports. Adoptive transfer models of immunity with immune serum and T-cells from mice immunized with live attenuated *aroA Salmonella* Typhimurium, into susceptible naïve BALB/c mice, has provided evidence that both humoral and cellular immunity are necessary for immunity to oral challenge with virulent *Salmonella* Typhimurium. In this model, naïve mice receiving either immune serum or T-cells separately, succumbed to oral infection, whereas mice receiving both were protected [66].

Further studies with this model have highlighted the importance of O-specific antibody against bacterial polysaccharide as a functional correlate of protection [65]. Separate studies with susceptible BALB/c mice genetically ablated for B-cell responses, has further confirmed the dual requirement for both antibody and T-cells in the IP model of *Salmonella* Typhimurium challenge. In these experiments, partial protection against challenge was seen following passive transfer of anti-*Salmonella* Typhimurium immune serum into B-cell deficient BALB/c mice that had been previously immunized with live attenuated *aroA Salmonella* Typhimurium. Protection however was not seen following transfer of immune serum into naïve mice, or in *aroA Salmonella* Typhimurium vaccinated B-cell deficient mice [68].

Evidence for antibody-mediated protection against parenteral challenge with highly virulent NTS in resistant mice is provided in several published reports. Protection from parenteral challenge mediated by immunization with killed whole cell *Salmonella* Typhimurium in resistant White Swiss mice is mirrored by the rise in titer of functional bactericidal antibody [81, 82]. In this model, the substantial protection observed following challenge is similarly associated with a large reduction of *Salmonella* CFU in the liver and spleen as compared to unvaccinated mice and can be seen as early as six hours after challenge. Resistant CD-1 and NMRI mice immunized with O-polysaccharide based conjugate vaccines were also shown to produce high levels of functional opsonophagocytic antibody that could transfer protection by passive immunization into naïve hosts [39, 80, 111–113]. As further evidence for the protective role of antibody in resistant mice, passively transferred monoclonal IgG and IgM specific for *Salmonella* Typhimurium OPS mediated significant protection against IP challenge with LD_{100} levels of *Salmonella* Typhimurium in resistant C3H/HeN mice [114]. By comparison, the failure of passive transfer of these same monoclonal IgG and IgM anti-OPS antibodies to provide protection against IP challenge with LD_{100} levels of *S.* Typhimurium in susceptible C3H/HeJ mice [114], underscores the limitations of antibody-mediated protection alone in susceptible mice.

Based on results from these mechanistic and functional immunological studies in susceptible and resistant mice immunized with living as compared to non-living NTS vaccines, several important points emerge. In susceptible mice challenged by either the parenteral or oral route with highly virulent NTS strains, the coordinated action of both humoral as well as cellular immunity is required for effective protection from *Salmonella* Typhimurium challenge. This is likely a result of the failure to adequately control intracellular infection for example in Nramp1 deficient mice [23, 56]. The contribution of humoral immunity alone to protection by vaccines against NTS in mice is generally only seen in host-pathogen interactions that can result in a sub-lethal infection, such as can be produced in either resistant mice challenged with either highly or weakly virulent NTS strains, or susceptible mice challenged with weakly virulent NTS strains. Under these conditions, functional bactericidal and opsonophagocytic antibody directed against NTS likely causes an immediate reduction in *Salmonella* challenge inoculum present extracellularly, that functions to lower a fatal *Salmonella* dose to a level that is sub-lethal [81, 82]. This sub-lethal infecting population becomes disseminated to secondary sites of infection, and may also potentially generate cell-mediated immunity akin to that elicited during a natural sub-lethal infection in naïve animals [26, 43, 69, 81, 82, 97]. As highly susceptible mice are impaired for control of *Salmonella* infection, and succumb to very low parenteral challenge doses, they are virtually unable to be infected at sub-lethal levels with fully virulent strains, and thus protection by antibody is likely not significant [54].

12. Conclusions

The mouse model is expected to play an invaluable role in accelerating the development of safe and effective vaccination strategies against recently emerging NTS that are capable of causing invasive infections in humans. As emphasized in this review, choice of a mouse strain is of fundamental importance among the variables that affect the experimental outcome of investigational NTS vaccines.

The choice of mouse strain used for testing NTS vaccines should be tailored to the expected mechanism of protective immunity generated and the background virulence of the proposed NTS challenge strain. For vaccines designed to elicit both humoral and cellular immunity, such as live attenuated strains, both resistant as well as susceptible mice can be used to test for protection against highly virulent (as well as weakly virulent) NTS challenge strains. Efficacy against highly virulent NTS strains by vaccines for which the correlate of protection is expected to be antibody may be better studied in resistant mice. Thus, parenteral NTS conjugate vaccines, for example, should be tested for efficacy in resistant strains of mice.

Based on the findings revealed in our review, another consideration in selecting the appropriate mouse model for testing the efficacy of NTS vaccines is the selection of the route of challenge. Oral challenge, which resembles the natural route of infection in humans, can be accomplished in either susceptible or resistant mice, in testing live oral vaccines, with the caveat that the LD_{50} in resistant mice challenged with weakly virulent NTS strains may be so high as to pose a practical obstacle to achieving lethality in control animals.

Our review uncovered no examples where parenteral non-living NTS vaccines found to protect resistant mice against mortality when challenged parenterally were also tested for their ability to protect against oral challenge. We identify this as an important knowledge gap to be filled. If parenteral NTS vaccines under development, such as conjugates, porins and common proteins, can be shown to protect resistant mice against oral challenge, this will be an important breakthrough for generating relevant pre-clinical data for regulatory agencies.

Two vaccine strategies that have proved useful for developing successful human vaccines against *S*. Typhi are attenuated strains that serve as live oral vaccines [35, 115] and parenteral conjugates consisting of capsular Vi polysaccharide linked to a carrier protein [116–118]. Not surprisingly, these same strategies are being followed to develop safe and effective vaccines against invasive *S*. Typhimurium and *S*. Enteritidis disease. Carefully selected mouse models will be particularly critical for non-living NTS vaccine development in order to generate relevant pre-clinical data for regulatory agencies that includes evidence of vaccine efficacy.

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Abbreviations

NTS	Non-Typhoidal *Salmonella*
LPS	Lipopolysaccharide
IP	intraperitoneal

IV	intravenous
SC	subcutaneous
IM	intramuscular

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Table 1

Vaccine efficacy mediated by living and non-living NTS vaccines in susceptible mice

Vaccine type	Mouse strain	Vaccine (dose)	Route of vaccination	Number of doses	Duration till challenge	Route of challenge	Challenge strain	Reported LD_{50}	Challenge Inoculum size	Vaccine Efficacy[a]	Reference
Live	BALB/c	Live attenuated *dam S.* Typhimurium (10^9 cfu)	Oral	1	5 weeks	Oral	*S.* Typhimurium 14028	1×10^5	10,000 × LD_{50}	100%	[32, 33]
Live	BALB/c	Live attenuated *crp,cdt S.* Typhimurium SL1344 (10^9 cfu)	Oral	1	30 days	Oral	*S.* Typhimurium SL1344	6×10^4	> 10,000 × LD_{50}	100%	[30]
Live	BALB/c	Live attenuated *crp,cdt S.* Typhimurium UK-1 (10^9 cfu)	Oral	2	30 days	Oral	*S.* Typhimurium UK-1	8.5×10^3	>10,000 × LD_{50}	100%	[30]
Live	BALB/c	Delayed attenuated *phoPQ,crp,rpoS,fur S.* Typhimurium (~10^9 cfu)	Oral	1	30 days	Oral	*S.* Typhimurium UK-1	1.2×10^4	10,000 × LD_{50}	100%	[31]
Live	BALB/c	Live attenuated *aroA S.* Typhimurium SL3261 (10^{10} cfu)	Oral	1	8 weeks	Oral	*S.* Typhimurium C5	1×10^6	10,000 × LD_{50}	100%[c]	[63]
Live	C57Bl/6	Live attenuated *aroA S.* Typhimurium SL3261 (10^9 cfu)	Oral	1	6 weeks	Oral	*S.* Typhimurium SL1344	NR[b]	1×10^5 cfu	100%	[64]
Live	BALB/c	Delayed attenuated *phoPQ,crp,rpoS,fur S.* Typhimurium (10^4 cfu)	IP	1	30 days	Oral	*S.* Typhimurium UK-1	1.2×10^4	10,000 × LD_{50}	100%	[31]
Live	BALB/c	Live attenuated *aroA S.* Typhimurium SL3261 (10^6 cfu)	IV	1	8–12 weeks	Oral	*S.* Typhimurium C5	1×10^6	10,000 × LD_{50}	100%[c]	[63, 65, 66]
Live	BALB/c	Live attenuated *aroA* Se795 *S.* Enteritidis (10^6 cfu)	IV	1	3 months	Oral	*S.* Enteritidis Thirsk	1×10^6	10,000 × LD_{50}	100%[c]	[65]
Live	C57Bl/6	Live attenuated *aroA S.* Typhimurium SL3261 (5×10^5 cfu)	IV	1	4 months	Oral	*S.* Typhimurium C5	1×10^7	250 × LD_{50}	100%	[67]
Live	BALB/c	Live attenuated *aroA S.* Typhimurium SL3261 (10^6 cfu)	SC	1	8 weeks	Oral	*S.* Typhimurium C5	1×10^6	1,000 × LD_{50}	100%[d]	[63]
Live	BALB/c	Live attenuated *aroA S.* Typhimurium SL3261 (5×10^5 cfu)	IV	1	6 weeks	IP	*S.* Typhimurium SL1344	<10	10^4 cfu	100%	[68]

Vaccine type	Mouse strain	Vaccine (dose)	Route of vaccination	Number of doses	Duration till challenge	Route of challenge	Challenge strain	Reported LD_{50}	Challenge Inoculum size	Vaccine Efficacy[a]	Reference
Live	C3HeB/FeJ	Live attenuated *aroA S.* Typhimurium SL3235 (7×10^4 cfu)	IP	1	21 days	IP	*S.* Typhimurium W118-2	1 cfu	> 1,000 × LD_{50}	100%	[57]
Live	C3H/HeJ	Live attenuated *aroA S.* Typhimurium SL3235 (2.5×10^5 cfu)	IP	1	21 days	IP	*S.* Typhimurium W118-2	<7 cfu	> 1,000 × LD_{50}	100%	[47, 57]
Non-living	BALB/c	Acetone-inactivated attenuated *aroA S.* Typhimurium SL3261 (10^8 cfu)	SC	2	8 weeks	Oral	*S.* Typhimurium C5	1×10^6	10 × LD_{50}	25%	[63]
Non-living	C57Bl	Heat-inactivated *S.* Enteritidis 5694 (10^8 cfu) +CFA	SC	2	2 weeks	Oral	*S.* Enteritidis 5694	5×10^3	2 × LD_{50}	100%[e]	[69, 70]
Non-living	C57Bl	Heat-inactivated *S.* Enteritidis 5694 (10^8 cfu)	SC	2	2 weeks	Oral	*S.* Enteritidis 5694	5×10^3	2 × LD_{50}	0%[f]	[69, 70]
Non-living	C57Bl/6	Flagellin (200 μg)	SC	2	1 week	Oral	*S.* Typhimurium SL1344	NR	1×10^5 cfu	70%	[64]
Non-living	BALB/c	Porin (50 μg)	IP	1	3 weeks	IP	*S.* Typhimurium LT2	1×10^5	1,000 × LD_{50}	100%[d]	[71, 72]
Non-living	BALB/c	OPS-Tetanus Toxoid Conjugate (10 μg)	SC	3	1 week	IP	*S.* Typhimurium PTCC 1733	1.3×10^4	77 × LD_{50}	50%	[73]
Non-living	BALB/c	OPS (10 μg)	SC	3	1 week	IP	*S.* Typhimurium PTCC 1733	1.3×10^4	77 × LD_{50}	12.5%	[73]
Non-living	BALB/c	Heat-inactivated *S.* Typhimurium PTCC 1735 (10^8 cfu)	SC	3	1 week	IP	*S.* Typhimurium PTCC 1733	1.3×10^4	77 × LD_{50}	37.5%	[73]
Non-living	C3H/HeJ	Acetone-inactivated *S.* Typhimurium W118-2 (60 μg)	IP	2	21 days	IP	*S.* Typhimurium W118-2	<7 cfu	6 cfu	36%	[46]
Non-living	C3H/HeJ	Crude LPS (100 μg)	IP	2	21 days	IP	*S.* Typhimurium W118-2	<7 cfu	6 cfu	21%	[46]
Non-living	C3H/HeJ	Purified LPS (100 μg)	IP	2	21 days	IP	*S.* Typhimurium W118-2	<7 cfu	6 cfu	0%	[46]
Non-living	C3HeB/FeJ	Acetone-inactivated *S.* Typhimurium W118-2 (60 μg)	IP	2	21 days	IP	*S.* Typhimurium W118-2	1 cfu	24 cfu	100%	[46]
Non-living	C3HeB/FeJ	Crude LPS (100 μg)	IP	2	21 days	IP	*S.* Typhimurium W118-2	1 cfu	24 cfu	50%	[46]
Non-living	C3HeB/FeJ	Purified LPS (100 μg)	IP	2	21 days	IP	*S.* Typhimurium W118-2	1 cfu	24 cfu	10%	[46]
Non-living	C57Bl/6	Porin (20 μg)	IP	2	35 days	IP	*S.* Typhimurium SL1344	NR	3×10^3 cfu	0%[g]	[74]

Vaccine type	Mouse strain	Vaccine (dose)	Route of vaccination	Number of doses	Duration till challenge	Route of challenge	Challenge strain	Reported LD_{50}	Challenge Inoculum size	Vaccine Efficacy[a]	Reference
Non-living	C57Bl/6J	Phenol-inactivated *S.* Typhimurium (3×10^7 cfu)	IP	6	7 days	IP	*S.* Typhimurium C5	<10	$10^1 – 10^7$ cfu	0%	[54]
Non-living	BALB/cJ	Phenol-inactivated *S.* Typhimurium (3×10^7 cfu)	IP	6	7 days	IP	*S.* Typhimurium C5	<10	$10^1 – 10^7$ cfu	0%	[54]
Non-living	C3H/HeJ	Phenol-inactivated *S.* Typhimurium (3×10^7 cfu)	IP	6	7 days	IP	*S.* Typhimurium C5	<10	$10^1 – 10^7$ cfu	0%	[54]
Non-living	DBA/2J	Phenol-inactivated *S.* Typhimurium (3×10^7 cfu)	IP	6	7 days	IP	*S.* Typhimurium C5	<10	$10^1 – 10^7$ cfu	0% – 10%	[54]
Non-living	BALB/c	Porin (100 μg)	SC	2	10 days	IV	*S.* Typhimurium C5	1×10^5	$10 \times LD_{50}$	25%	[76]
Non-living	BALB/c	LPS (50 μg)	SC	2	10 days	IV	*S.* Typhimurium C5	1×10^5	$10 \times LD_{50}$	35.7%	[76]
Non-living	BALB/c	Porin (100 μg) + LPS (50 μg)	SC	2	10 days	IV	*S.* Typhimurium C5	1×10^5	$10 \times LD_{50}$	100%	[76]
Non-living	BALB/c	Outer-membrane Protein (50 μg)	SC	2	10 days	IV	*S.* Typhimurium C5	1×10^5	$50 \times LD_{50}$	100%	[75]

[a] ((Mortality in controls–Mortality in vaccine group) / Mortality in controls) × 100

[b] Not reported

[c] The reported LD_{50} is increased > 10,000 fold as compared to unimmunized mice, it is assumed then that 100% mortality in controls, and 0% mortality in vaccinated animals at 10,000 × LD_{50} challenge

[d] The reported LD_{50} is increased > 1,000 fold as compared to unimmunized mice, it is assumed then that 100% mortality in controls, and 0% mortality in vaccinated animals at 1,000 × LD_{50} challenge

[e] Mortality not reported, however of the remaining mice not sacrificed for analysis by day 15, none had died and a significant decrease in NTS tissue burden was reported

[f] Mortality not reported, however, of the mice not sacrificed for analysis by day 15, several had died, and the NTS tissue burden reported was comparable to unvaccinated controls

[g] Mortality not reported, the lack of protection from mortality however was stated in the results.

Table 2

Vaccine efficacy mediated by living and non-living NTS vaccines in resistant mice

Vaccine type	Mouse strain	Vaccine (dose)	Route of vaccination	Number of doses	Duration till challenge	Route of challenge	Challenge strain	Reported LD_{50}	Challenge Inoculum size	Vaccine Efficacy[a]	Reference
Live	C3H/HeNCrlBR	Live attenuated *aroA* *S.* Typhimurium SL3235 (7×10^4 cfu)	IP	1	21 days	IP	*S.* Typhimurium W118-2	1×10^3	1,000 × LD_{50}	100%	[47]
Live	CF1	Live attenuated rough mutant *S.* Typhimurium SL1004 (6×10^6 cfu)	IP	1	14 days	IP	*S.* Typhimurium LT2	5×10^2	1,000 × LD_{50}	100%	[77]
Live	ddY	Live attenuated rough mutant *S.* Typhimurium SL1004 (6×10^6 cfu)	IP	1	14 days	IP	*S.* Typhimurium LT2	1×10^3	1,000 × LD_{50}	100%	[77]
Non-living	A/J	Phenol-inactivated *S.* Typhimurium (3×10^7 cfu)	IP	6	7 days	IP	*S.* Typhimurium C5	~ 10^4	10^7 cfu	90%	[54]
Non-living	C3H/HeNCrlBR	Acetone-killed *S.* Typhimurium W118-2 (60 μg)	IP	2	21 days	IP	*S.* Typhimurium W118-2	1.2×10^3	19 × LD_{50}	100%	[46]
Non-living	C3H/HeNCrlBR	Crude LPS (100 μg)	IP	2	21 days	IP	*S.* Typhimurium W118-2	1.2×10^3	19 × LD_{50}	100%	[46]
Non-living	C3H/HeNCrlBR	Purified LPS (100 μg)	IP	2	21 days	IP	*S.* Typhimurium W118-2	1.2×10^3	19 × LD_{50}	89%	[46]
Non-living	CBA x C57Bl/6	Porin (10 μg)	IP	2	10 days	IP	*S.* Typhimurium SH6739	1×10^5	27 × LD_{50}	86%	[78]
Non-living	CD-1	Acetone-inactivated *S.* Typhimurium W118-2 (10^8 cfu)	IP	1	21 days	IP	*S.* Typhimurium W118-2	1×10^4	1,000 × LD_{50}	100%[b]	[43]
Non-living	CD-1	LPS (25 μg)	IP	1	21 days	IP	*S.* Typhimurium W118-2	1×10^4	500 × LD_{50}	100%[b]	[43]
Non-living	CF1	LPS (100 μg)	IP	1	14 days	IP	*S.* Typhimurium LT2	5×10^2	1,000 × LD_{50}	0%	[77]
Non-living	CF1	Formalin-inactivated *S.* Typhimurium P173C (10^9 cfu)	IP	4	21 days	IP	*S.* Typhimurium P173C	< 10^2	1,000 × LD_{50}	100%	[79]
Non-living	CF1	Formalin-inactivated *S.* Enteritidis Br1203bM (10^9 cfu)	IP	4	21 days	IP	*S.* Enteritidis 203	10^2	> 1,000 × LD_{50}	100%	[79]
Non-living	ddY	LPS (100 μg)	IP	1	14 days	IP	*S.* Typhimurium LT2	1×10^3	1,000 × LD_{50}	0%	[77]
Non-living	NMRI	OPS-Porin Conjugate (5 μg)	IP	4	6 days	IP	*S.* Typhimurium SH 2201	4.4×10^4	200 × LD_{50}	90%	[80]

Vaccine type	Mouse strain	Vaccine (dose)	Route of vaccination	Number of doses	Duration till challenge	Route of challenge	Challenge strain	Reported LD_{50}	Challenge Inoculum size	Vaccine Efficacy[a]	Reference
Non-living	NMRI	Porin (5ug)	IP	4	6 days	IP	*S.* Typhimurium SH 2201	4.4×10^4	$200 \times LD_{50}$	70%	[80]
Non-living	White Swiss	Heat-inactivated *S.* Typhimurium Suc EE (10^8 cfu)	IP	1	10 days	IP	*S.* Typhimurium Suc LL	5×10^4	$100 \times LD_{50}$	100%	[81]
Non-living	White Swiss	Heat-inactivated *S.* Typhimurium Suc EE (250 μg)	IP	3	NR[c]	IV	*S.* Typhimurium Suc LL	1×10^6	$100 \times LD_{50}$	100%	[82]
Non-living	CD-1	OPS-Tetanus Toxoid conjugate (2.5 μg)	SC	3	1 week	IP	*S.* Typhimurium W118	1.9×10^4	$100 \times LD_{50}$	100%	[39]
Non-living	White Swiss	Porin (50 μg)	SC	2	10 days	IP	*S.* Typhimurium LT2-71	1.9×10^3	$100 \times LD_{50}$	100%	[83]
Non-living	White Swiss	LPS (20 μg)	SC	2	10 days	IP	*S.* Typhimurium LT2-71	1.9×10^3	$100 \times LD_{50}$	50%	[83]
Non-living	White Swiss	Ethyl-inactivated *S.* Enteritidis 5694 (3×10^6 cfu)	IP, IV, SC	3	1 week	IP, IV, SC	*S.* Enteritidis 5694	3.5×10^2 (IV)	$100 – 1{,}000 \times LD_{50}$	11% – 60%	[27, 28]

[a] ((Mortality in controls – Mortality in vaccine group) / Mortality in controls) × 100

[b] Mortality in controls not reported, 100% mortality in unvaccinated controls is assumed however at the high challenge dose reported

[c] Not reported

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Table 3

Reported comparative natural resistance to individual NTS isolates in commonly used mouse strains[a]

Reported Mouse NTS susceptibility phenotype	Mouse Strain	Reference(*Salmonella* strain used)				
		Hormaeche, 1979 [50] (*S.* Typhimurium C5)		Plant and Glynn, 1976 [53] (*S.* Typhimurium C5)	Collins, 1972 [52] (*S.* Enteritidis 5694)	
		IV LD_{50} (LOG 10)	SC LD_{50} (LOG 10)	SC LD_{50}	IV LD_{50}	Oral LD_{50}
Susceptible	BALB/c	1.27	< 1	< 10		
Susceptible	DBA/1			< 10		
Susceptible	C57Bl			2×10^1	5 – 10	5×10^3
Susceptible	B10	1.26	< 1			
Susceptible	B10.D2	< 1	< 1	5×10^1		
Susceptible	B10.A	1.5	< 1			
Susceptible	B10.Br	< 1	< 1			
Resistant	DBA/2	1.6	ND[b]	2×10^5		
Resistant	C3H	2.1	ND	1×10^6		
Resistant	CBA	3.0	6.9	1×10^7		
Resistant	A/J	4.26	ND		4×10^2	5×10^5
Resistant	A/JAX			2×10^6		
Resistant	F1 (BALB/C x CBA)			3×10^6		
Resistant	F1 x CBA			9×10^6		
Resistant	CD-1				3×10^3	2×10^6
Resistant	CF-1				5×10^3	6×10^6

[a] Reproduced from indicated reports

[b] Not done