Evaluation of Regulated Delayed Attenuation Strategies for *Salmonella enterica* Serovar Typhi Vaccine Vectors in Neonatal and Infant Mice

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We developed regulated delayed attenuation strategies for *Salmonella* vaccine vectors. In this study, we evaluated the combination of these strategies in recombinant attenuated *Salmonella enterica* serovar Typhi and *Salmonella enterica* serovar Typhimurium vaccine vectors with similar genetic backgrounds in vitro and in vivo. Our goal is to develop a vaccine to prevent *Streptococcus pneumoniae* infection in newborns; thus, all strains delivered a pneumococcal antigen PspA and the impact of maternal antibodies was evaluated. The results showed that all strains with the regulated delayed attenuated phenotype (RDAP) displayed an invasive ability stronger than that of the S. Typhi vaccine strain, Ty21a, but weaker than that of their corresponding wild-type parental strains. The survival curves of different RDAP vaccine vectors in vitro and in vivo exhibited diverse regulated delayed attenuation kinetics, which was different from S. Typhi Ty21a and the wild-type parental strains. Under the influence of maternal antibody, the persistence of the S. Typhimurium RDAP strain displayed a regulated delayed attenuation trend in nasal lymphoid tissue (NALT), lung, and Peyer’s patches, while the persistence of S. Typhi RDAP strains followed the curve only in NALT. The bacterial loads of S. Typhi RDAP strains were lower in NALT, lung, and Peyer’s patches in mice born to immune mothers than in those born to naïve mothers. In accordance with these results, RDAP vaccine strains induced higher titers of IgG antibodies against PspA and against *Salmonella* lipopolysaccharides. Immunization of mothers with S. Typhi RDAP strains enhanced the level of vaginal mucosal IgA, gamma interferon (IFN-γ), and interleukin 4 (IL-4) and resulted in a higher level of protection against *S. pneumoniae* challenge.

The characteristics of the poor immune responses and the potentially suppressive effects of maternally derived antibodies in early life favor infection by various pathogens (1–4) and complicate developing safer efficacious vaccines for newborns. Vaccines that can rapidly and effectively induce protective immunity in this population are required. Recombinant attenuated *Salmonella* vaccines (RASVs), delivering protein antigens from different pathogens through mucosal routes, can colonize internal lymphoid tissues, stimulate the innate immune system, and induce systemic and mucosal immune responses, including serum antibodies, mucosally secreted antibodies, and a panoply of cell-mediated immune responses at local and distal sites (5–9). Although attenuated *Salmonella enterica* serovar Typhi vaccines hold great promise as live vectors for presentation of foreign antigens from many pathogens to the immune system, results have been disappointing for clinical trials carried out thus far (10), and there are currently no licensed live recombinant attenuated bacterial vector vaccines available to humans. One of the potential pitfalls of live bacterial vectors is balancing immunogenicity and attenuation of vectors. Excessive attenuation of the bacterial vector can render it poorly immunogenic (11), whereas inadequate attenuation may result in systemic dissemination and disease from the vaccine. So, we believed that ideally attenuated *Salmonella* vaccine vectors should be fully attenuated with respect to the animal or human host so as not to impair physiological well-being while being able to exhibit a high degree of immunogenicity against the carried heterologous antigen.

Achieving a balance between adequate attenuation/safety and maximal immunogenicity in vaccine construction is a challenge. After several years of endeavor, we recently designed and developed new-generation vaccine vector strains with regulated delayed attenuation strategies that display many features of wild-type virulent strains of *Salmonella* that enable vaccine strains to effectively colonize lymphoid tissues without inducing disease symptoms, while stimulating both strong primary and lasting memory immune responses in mice (12–15). As described previously (12), these strategies are composed of a smooth-to-rough phenotypic change in lipopolysaccharides (LPS) in vivo (Δpmi) (16) and a tightly regulated araC P_BAD cassette for the promoters of the fur, crp, and rpoS genes such that expression of these genes is dependent on arabinose provided during growth in vitro. Following colonization of lymphoid tissues, the Fur, Crp, and/or RpoS proteins cease to be synthesized due to the absence of arabinose such that attenuation is gradually manifested in vivo to preclude induction of disease symptoms (12, 17).

We previously reported manipulating the genes of *Salmonella* to establish the regulated delayed attenuation system in *Salmonella enterica* serovar Typhimurium (12, 17), evaluating the immunogenicity of live *S. Typhimurium* vaccine vectors in adult and newborn mice (13, 14) and that of *S. Typhi* vaccine vectors in adult mice (15). The aims of the present study were to further evaluate the combination of regulated delayed attenuation pheno...
typhus vaccine (12, 17). Strain S. Typhi ISP1820, which is an FDA-approved live attenuated vaccine, has been extensively tested and proven to be protective (14, 20, 21).

Plasmids

pYA3493

Asd⁺, pBR ori, β-lactamase signal sequence-based periplasmic secretion plasmid

pYA4088

pBR ori, 852-bp DNA encoding the α-helical region of PspA from amino acid 3 to 285 in pYA3493

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, and growth conditions.** The bacterial strains and plasmids used in this study are listed in Table 1. The strains with similar genetic backgrounds of regulated delayed attenuated phenotypes were Shi et al. 2018. Strain 5833 was used as an attenuation control (22). The genetic characterization of the strains has been reported (15). Plasmid pYA3493 is an Asd⁺ empty vector, and plasmid pYA4088, derived from pYA3493, carries a portion of the pspA gene, encoding the α-helical immunogenic domain from Streptococcus pneumoniae. LB broth and agar were used as complex rich media for propagation of all bacterial strains (23). When required, media were supplemented with 2,6-diaminopimelic acid (DAP) (50 μg/ml), L-arabinose (0.05% [wt/vol]), α-mannose (0.2% [wt/vol]), α-lactose (1% [wt/vol]), and galactose (0.05% [wt/vol]). Selenite broth and tetrathionate broth (Difco), with or without supplements, were used for enrichment of S. Typhimurium and S. Typhi from animal tissues. Strains were grown and prepared as previously described (13, 14). Briefly, S. Typhi and S. Typhimurium vaccine strain harboring plasmid pYA4088 (pspA expression vector) or pYA3493 (empty vector) were grown in LB broth with 0.05% arabinose and 0.2% mannose overnight at 37°C asstanding cultures that were diluted 1:100 in the same prewarmed medium and grown with aeration at 37°C to an optical density at 600 nm (OD600) of 0.8 to 0.9. Bacteria were collected and resuspended in buffered saline with gelatin (BSG) to densities appropriate for the inoculation. Bacterial growth was monitored spectrophotometrically, and titers were determined by plating serial dilutions of the vaccine strains on MacConkey agar supplemented with 1% lactose, 0.05% arabinose, and 0.2% mannose.

**Invasion and persistence assays.** The human epithelial cell line INT-407 (ATCC CCL6) (24, 25) and human monocytic cell line THP-1 (ATCC TIB-202) (26) were obtained from ATCC and maintained in Dulbecco modified Eagle medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 0.45% glucose, 2 mM glutamine, 10% heat-inactivated fetal bovine serum (Gibco), and 50 μM β-mercaptoethanol. To obtain monolayers for invasion and persistence assays, 10⁵ cells in 1 ml of complete DMEM were seeded into 24-well culture plates. Confluent monolayers were obtained after 24 h. THP-1 cells were differentiated by addition of 10⁻⁷ M phorbol 12-myristate 13-acetate for 48 h. The medium was replaced with 1 ml of fresh medium per well immediately before invasion and persistence assays.

Bacteria were grown and resuspended in BSG as described above and added to each of three duplicate wells containing monolayers of human monocytic THP-1 cells or human epithelial INT-407 cells at a multiplicity of infection (MOI) of about 20. The plate was centrifuged at 800 × g for 10 min to promote the interaction between bacteria and monolayer cells. After incubating at 37°C in a CO₂ (5%) incubator for 60 min, each well was rinsed three times with 1 ml of complete DMEM per wash. To obtain monolayers for invasion and persistence assays, 10⁷ cells in 1 ml of complete DMEM were seeded into 24-well culture plates. Confluent monolayers were obtained after 24 h. THP-1 cells were differentiated by addition of 10⁻⁷ M phorbol 12-myristate 13-acetate for 48 h. The medium was replaced with 1 ml of fresh medium per well immediately before invasion and persistence assays.

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To determine the CFU, serial dilutions of the lysed cells were plated on LB agar plates. The LB plates were incubated overnight at 37°C, and colonies were quantitatively compared to the wild-type strain.
were counted the next day. For the persistence assay, following three times with 1 ml of complete DMEM, the infected cells were incubated continuously with DMEM containing gentamicin (10 μg/ml) at 37°C in a CO₂ (5%) incubator, which limited extracellular bacterial growth (27). Monolayers of cells were treated and plated as above at various times (2, 24, 48, and 72 h). The data presented were means of duplicate plate counts from each of three culture wells and are representative of three to five experiments.

Mice. All animal procedures were approved by the Arizona State University Animal Care and Use Committee. Animal work was performed as described previously (13). Briefly, BALB/c mice (8 weeks old) purchased from Charles River Laboratories (Wilmington, MA) were bred to produce pups. Groups of female BALB/c mice (8 weeks old) were immunized intranasally (i.n.) with 10 μl containing 1 × 10⁷ CFU of strains χ9633(pYA4088) RpoS⁺, χ9639(pYA4088) RpoS⁻, χ9640(pYA4088) RpoS⁻, and χ9558(pYA4088) and Ty21a 2 weeks before breeding. Approximately 1 week before delivery, sera were collected from pregnant mice. All immunized mothers had reciprocal PsP and Salmonella LPS-specific serum IgG titers ranging from 800 to 1,200 for individual mice. Male and female mice from each litter were placed in separate cages after weaning. Mouse breeding cages were checked daily with new births recorded, and the pups were kept with their mothers until weaning at the age of 3 weeks. Experimental groups contained two litters for persistence colonization experiments (with or without maternal antibody) and four litters for immunogenicity experiments (7-day-old [7d] and 21-day-old [21d] mice with or without maternal antibody).

Dynamics of regulated delayed attenuation in vivo in 4-week-old mice. For kinetics experiments, 4-week-old mice (24/group; note that Peyer’s patches are difficult to detect in mice younger than 4 weeks) born to either naive or immunized mothers were intranasally inoculated with 1 × 10⁸ CFU of various strains as described above. Mice were euthanized by CO₂ asphyxiation at 0.25, 1, 24, and 72 h (5 mice/time point) postinfection, and samples of the nasal lymphoid tissue (NALT), lungs, and Peyer’s patches were collected. Tissues were weighed and homogenized in a total volume of 1 ml BSG. Serial dilutions were plated to determine the number of viable bacteria. We also inoculated 900 μl of homogenized tissues into 5 ml selenite or tetrathionate broth (Difco) for S. enterica.

RESULTS

Invasion assays. The RDAP vaccine strains used here contain a ΔpspA, a ΔPcrp::TT araC P BAD crp (P stands for promoter and TT for transcription terminator) (hereinafter abbreviated ΔP crp), and a Δfur::TT araC P BAD fur (hereinafter abbreviated ΔP fur) mutation, which represented three main strategies for regulated delayed attenuation to increase the safety and efficacy of the vaccines (12). The gene pmi encodes an isomerase involved in the integration of mannose into LPS O-antigen side chains (30). The gene crp encodes a global regulator responsible mainly for energy metabolism. The gene fur encodes another global regulator, responsible for a diversity of cellular functions, mainly iron uptake. A mutation in either pmi, crp, or fur attenuates strains, but all mutations make strains more susceptible to being killed/inhibited by host defense strategies after oral inoculation, whereas the RDAP strains do not have these problems (12). They also contain a constellation of additional mutations that aid in further enhancing the safety and efficacy. The mutation ΔpspB reduces disease symptoms through reduction of fluid secretion and inflammation in the gut (31, 32) and enhances the immunogenicity of Salmonella-delivered antigen (33). ΔrelA::araC P BAD lacI TT allows regulated delayed synthesis of a recombinant protective antigen in vivo to induce better immune responses, and Δsda enables establishment of a drug-free balanced-lethor vector-host system (14, 17, 18, 34–36). The FDA-approved S. Typhi Ty21a vaccine strain was isolated

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by mutagenesis (22). It represented a traditional way to generate mutations for attenuated vaccine use and was used as a control in the current research. Vaccines generated by this concept display the dysfunction of the genes related to virulence, nutrient absorption, and global regulation, as well as other genes disturbing the normal growth of bacteria in certain environments. Once the mutation is generated, the effect of the mutation will not change, and thus they are nonregulated attenuations.

An important step in Salmonella infection is the invasion of the intestinal epithelium. Cultured mammalian cells provided a simple in vitro system that mimics the invasion of enterocytes in vivo (37) and were widely used as a model to study bacterial invasiveness (37–41). One of the goals of regulated delayed attenuation is to create vaccine strains that display wild-type abilities to invade the mucosal epithelium (12). We used human epithelial INT-407 cells to compare the invasion abilities of Ty21a and that of their correspondent wild type (Fig. 1). Invasive cine strains were intermediate between that of the reference strain /H9273 and the mucosal epithelium (12). We used human epithelial INT-407 cells to compare the invasion abilities of Ty21a and that of their correspondent wild type (Fig. 1). The invasive ability of Ty21a was 210-fold lower than that of wild-type Ty2 RpoS

<0.0001) and significantly higher than that of Ty21a (###, P < 0.0001), respectively.

Persistence dynamics of regulated delayed attenuated phenotype in human monocytic cell line THP-1 cells. We further evaluated the virulences of different S. Typhi strains in an in vitro experimental model with human monocytic THP-1 cells (44). Ideally, the survivability of a strain with regulated delayed attenuation strategies will be expected to be similar to that of the wild-type strains, instead of low persistence from a strain with nonregulated delayed attenuation in the early stages of the infection process. Thus, the attenuation process is expected to be gradual for the strains with RDAP but to be immediate for the strains without RDAP. In other words, our delayed attenuation strains should typically display higher bacteria loads initially after inoculation into hosts and then steadily decrease their numbers. In this study, S. Typhi-derived RDAP strains showed higher titers than that of Ty21a in the human monocytic cell line THP-1 cells from 2 h to 48 h after inoculation (Fig. 2A to C; P < 0.01 for χ9633(pYA4088) and χ9640(pYA4088); P < 0.05 for χ9639(pYA4088)) but less than those of their correspondent parental strains (Fig. 2A to C) (P < 0.01), except χ9640(pYA4088) RpoS⁺, in which persistent ability was identical to that of its wild type, S. Typhi RpoS⁺, at the 2-h time point (Fig. 2C). The persistent ability of the S. Typhimurium-derived χ9558(pYA4088) RpoS⁺ strain was lower than that of its wild type, S. Typhimurium UK-1, after inoculation (Fig. 2D) (P < 0.01). In comparison to the primary survival level (2 h), RDAP vaccines and Ty21a persisted at progressively lower numbers at each sequential time. However, there were no such trends for any wild-type parental strain. The wild-type parental strains replicated to higher titers at 2 h than at 2 h except for S. Typhi ISP1820 RpoS⁺, which gradually kept similar but reduced survivability from 2 h to 72 h (Fig. 2A) (P < 0.01). Compared with Ty21a, the survivability of RDAP S. Typhi strains was higher at 2 h and 24 h postinfection (Fig. 2A to C) (P < 0.01) but lower at 72 h postinfection except for χ9640(pYA4088) (Fig. 2C). This experiment demonstrated that the RDAP vaccine vector strains gradually became attenuated, in contrast to the Ty21a strain, which exhibited an attenuated phenotype instantly after entering human monocytic THP-1 cells.

Similar results were observed in another in vitro experimental model using human intestinal INT-407 cells (see Fig. S1 in the supplemental material). In human intestinal INT-407 cells, the S. Typhi-derived RDAP strain χ9633(pYA4088) RpoS⁺ showed
higher titers than Ty21a from 2 h to 72 h after inoculation (see Fig. S1A) (P < 0.01); χ9639(pYA4088) RpoS− exhibited higher titers than Ty21a at 2 h and 24 h postinoculation (see Fig. S1B) (P < 0.01), and the persistent ability of χ9640(pYA4088) RpoS− was similar to that of Ty21a during 2 h and 72 h after infection (see Fig. S1C), but all RDAP strains showed a less-persistent bacteria load than their correspondent wild-type parental strains (see Fig. S1A to D) (P < 0.01).

The behaviors of persistence curves in different strains with the same regulated delayed attenuation strategies were varied (Fig. 2). In THP-1 cells, strain χ9640(pYA4088) RpoS− showed a bacterial load similar to that for its correspondent parental strain at 2 h, while that for the other three strains, χ9633(pYA4088) RpoS−, χ9639(pYA4088) RpoS−, and χ9558(pYA4088) RpoS−, were less (Fig. 2A to D) (P < 0.01). In INT-407 cells (see Fig. S1 in the supplemental material), all the RDAP strains showed lower bacterial persistence than those of their correspondent wild-type strains during infection. More bacterial loads were shown for strain χ9633(pYA4088) RpoS+ from 2 h to 72 h and for strain χ9639(pYA4088) RpoS− from 2 h to 24 h postinoculation than for Ty21a (see Fig. S1A and B), while vaccine strain χ9640(pYA4088) RpoS+ had a persistence ability similar to that of Ty21a (see Fig. S1C).

These results suggested that the persistence of Salmonella RDAP vaccine vectors in epithelial and macrophage cells was better than that of Ty21a but lower than those of their wild-type parental strains. They also indicated that the regulated delayed attenuation strategies used to attenuate wild-type strains achieved attenuation levels comparable to that of Ty21a, which was attenuated by traditional methods and proved to be safe for human use. The RDAP strains were basically fulfilling our primary design goal.

Kinetics of survival of strains with RDAP in 4-week-old mice born to naive mothers. Intranasal inoculation of mice has been used as an experimental model to study S. Typhi-based live vector vaccines for their attenuation and immunogenicity (28, 45, 46). We used this model to evaluate the survival kinetics of our RDAP S. Typhi vaccine strains. Four-week-old mice born to naive mothers were intranasally inoculated with designated strains. The maturity of the immune system in 4-week-old mice is equal to that of a 12- to 24-month-old human (47). We compared the persistences of the RDAP S. Typhi strains, their wild-type parental strains, and non-RDAP attenuated vaccine strain Ty21a in NALT, lungs, and Peyer’s patches of mice at 0.25, 1, 24, and 72 h postinoculation. At the initial inoculation time (0.25 h), all RDAP S. Typhi and S. Typhimurium strains colonized at levels similar to those of their wild-type parental strains, except for S. Typhi Ty2 RpoS−-derived strain χ9639(pYA4088) in the lung, in which the bacterial load was significantly lower than that of its wild-type parental strain (Fig. 3F) [P < 0.01 for χ9639(pYA4088) compared to S. Typhi Ty2 RpoS−]. Bacterial numbers of all RDAP S. Typhi strains were lower than (Fig. 3B) [P < 0.05 for χ9639(pYA4088)], similar to (Fig. 3A, C, F, and G), and higher than (Fig. 3E and I to K) [P < 0.01 for χ9633(pYA4088), χ9639(pYA4088), and χ9640(pYA4088)] that of Ty21a at 0.25 h after inoculation. Then, the numbers of all RDAP strains steadily decreased at subsequent...
To explore the influence of immunization of mothers on a regulated delayed attenuated phenotype in 4-week-old mice born to immune mothers, we designed similar experiments that evaluated the survival kinetics of RDAP strains in 4-week-old mice born to naive mothers instead of 4-week-old mice born to naive mothers. Mice born to immune mothers had a reduced initial bacterial load of RDAP strains \( \chi 9633(pYA4088) \) in lung and Peyer’s patches, \( \chi 9639(pYA4088) \) in NALT and \( \chi 9640(pYA4088) \) in lung and Peyer’s patches compared to those of mice born to naive mothers at 0.25 h (Fig. 4A to C, E to G, and I to K). Ty21 showed reduced numbers in lung and Peyer’s patches at 2.5 h in mice born to immune mothers. In contrast to survival curves of RDAP vaccine strains in 4-week-old mice born to naive mothers (Fig. 3), all RDAP vaccine strains, including S. Typhi-derived and S. Typhimurium-derived strains, showed similar or higher survivability in 4-week-old mice born to immune mothers at the 1-h time point compared to those at the 0.25-h time point after inoculation (Fig. 4A to D, E, G, and I to L) (P < 0.01), except for \( \chi 9639(pYA4088) \) in the lung. The survival of Ty21a in lung and Peyer’s patches was reduced to 55-fold and 91.5-fold at the 1-h time point compared to that at the 0.25-h time point in 4-week-old mice born to immune mothers (Fig. 4E and I), while the survivability of Ty21a was 160-fold and 150-fold higher at the 1-h time point than that at 0.25-h time point in 4-week-old mice born to naive mothers (Fig. 3I and K). Ty21a showed reduced numbers in lung and Peyer’s patches, and Ty21a showed lower survivability in 4-week-old mice born to naive mothers than in mice born to naive mothers at 24-h time point (Fig. 4A, C, E, G, and I to K) (P < 0.05; P <

| Strain | NALT | Lung | Peyer’s Patches |
|--------|------|------|-----------------|
| Ty21a  | 9633 | 9640 | 9558            |
| Ty21a  | 9639 | 9640 | 9558            |
| Ty21a  | 9640 | 9640 | 9558            |

Fig 3: Survival curves of the RDAP vaccine strains in 4-week-old mice born to naive mothers. To assess bacterial growth in NALT (A to D), lung (E to H), and Peyer’s patches (PP) (I to L), the numbers of bacterial cells remaining at 0.25 h, 1 h, 24 h, and 72 h after initial intranasal inoculation were determined. Data were the means ± standard errors for two separate experiments with 6 mice for each time point. Numbers were percentages of bacteria at 1 h, 24 h, and 72 h calculated with respect to inocula at 0.25 h (which served as the initial time point), respectively. The persistence of all RDAP vaccine strains compared to that of their wild-type parental strains (\( * P < 0.05; ** P < 0.01 \) and to Ty21a (\( ##, P < 0.01 \) is shown. Solid circle, S. Typhi ISP1820; open circle, \( \chi 9633(pYA4088) \); inverted open triangle, Ty21a; solid square, \( \chi 9639(pYA4088) \); solid triangle, \( \chi 9640(pYA4088) \); solid rhombus, \( \chi 9640(pYA4088) \); open rhombus, \( \chi 9640(pYA4088) \); solid triangle, S. Typhimurium UK-1; open triangle, \( \chi 9558(pYA4088) \). Panels show \( \chi 9633(pYA4088) \) (A, E, and I), \( \chi 9639(pYA4088) \) (B, F, and J), \( \chi 9640(pYA4088) \) (C, G, and K), or \( \chi 9558(pYA4088) \) (D, H, and L) in NALT, lung, and Peyer’s patches, respectively.

**Table 1:** Summary of Survival Kinetics of RDAP Vaccine Strains in 4-Week-Old Mice

| Strain   | NALT Survival | Lung Survival | Peyer’s Patches Survival |
|----------|----------------|---------------|--------------------------|
| Ty21a    | 9633           | 9640          | 9558                     |
| Ty21a    | 9639           | 9640          | 9558                     |
| Ty21a    | 9640           | 9640          | 9558                     |

**Fig 4:** Survival curves of RDAP vaccine strains in 4-week-old mice born to immune mothers. To assess bacterial growth in NALT (A to D), lung (E to H), and Peyer’s patches (PP) (I to L), the numbers of bacterial cells remaining at 0.25 h, 1 h, 24 h, and 72 h after initial intranasal inoculation were determined. Data were the means ± standard errors for two separate experiments with 6 mice for each time point. Numbers were percentages of bacteria at 1 h, 24 h, and 72 h calculated with respect to inocula at 0.25 h (which served as the initial time point), respectively. The persistence of all RDAP vaccine strains compared to that of their wild-type parental strains (\( * P < 0.05; ** P < 0.01 \) and to Ty21a (\( ##, P < 0.01 \) is shown. Solid circle, S. Typhi ISP1820; open circle, \( \chi 9633(pYA4088) \); inverted open triangle, Ty21a; solid square, \( \chi 9639(pYA4088) \); solid triangle, \( \chi 9640(pYA4088) \); solid rhombus, \( \chi 9640(pYA4088) \); open rhombus, \( \chi 9640(pYA4088) \); solid triangle, S. Typhimurium UK-1; open triangle, \( \chi 9558(pYA4088) \). Panels show \( \chi 9633(pYA4088) \) (A, E, and I), \( \chi 9639(pYA4088) \) (B, F, and J), \( \chi 9640(pYA4088) \) (C, G, and K), or \( \chi 9558(pYA4088) \) (D, H, and L) in NALT, lung, and Peyer’s patches, respectively.
human neonates and infants (47). Interestingly, at 2 weeks and 21-day-old mice yielded observations very similar to those for route. Siegrist has reported that antibody responses elicited in 7-nurized mother, immunized at day 21) mothers via the intranasal rearmisized mother, immunized at day 7; I 21d, pups born to immu- namely immunized at day 7; I 21d, pups born to immunizelike immu- or immunized (I 7d, pups born to immu- infant (21-day-old) mice born to naive (N 7d, pups born to naive mice) or immunized (I 7d, pups born to naive mothers). The persistence of all RDAP vaccine strains in mice born to immune mothers compared to that in mice born to naive mothers (*, P < 0.05; **, P < 0.01) is shown. Open circle, χ9633(pYA4088); inverted open triangle, Ty21a; open square, χ9639(pYA4088); open rhombus, χ9640(pYA4088); open triangle, χ9558(pYA4088). Red and green colors represent mice born to immune mothers; black color represents mice born to naive mothers. χ9633(pYA4088) (A, E, and I), χ9639(pYA4088) (B, F, and J), χ9640(pYA4088) (C, G, and K), or χ9558(pYA4088) (D, H, and L) in NALT, lung, and Peyer’s patches, respectively, is shown.

FIG 4 Survival curves of the RDAP vaccine strains in 4-week-old mice born to immunized mothers (mothers immunized 2 weeks prior to breeding). To assess bacterial persistence in NALT (A to D), lung (E to H), and Peyer’s patches (I to L), the numbers of bacterial cells remaining at 0.25 h, 1 h, 24 h, and 72 h after initial intranasal inoculation were determined for mice born to immune mothers. Data were the means ± standard errors for two separate experiments with 6 mice for each time point. Numbers were percentages of bacteria at 1 h, 24 h, and 72 h calculated separately with respect to inocula at the 0.25 h time point. All continuous bacterial loads at the 72-h time point in NALT tissue (Fig. 4B) (P < 0.01), except for χ9639(pYA4088) in NALT and lung (Fig. 4B and F). Strain χ9639(pYA4088) showed a reduced bacterial load at the 72-h time point in NALT tissue (Fig. 4B) (P < 0.01) and at the 1-h time point in lung tissue (Fig. 4F) (P < 0.05). However, S. Typhimurium strain χ9558(pYA4088) exhibited similar or higher survivability in 4-week-old mice born to immune mothers compared to that in mice born to naive mothers after inoculation (Fig. 4D, H, and L) (P < 0.05; P < 0.01). This difference between S. Typhi and S. Typhimurium in mice born to immune mothers may result from mice being the natural host for S. Typhimurium but not for S. Typhi. These results indicated that the immunization of mothers with RDAP strains can enhance the persistent ability of most vaccine strains in tissues at early inoculated stages.

Strains with RDAP were immunogenic in neonatal and infant mice from naive or immunized mothers. The immunogenicity of RDAP strains was evaluated in neonatal (7-day-old) and infant (21-day-old) mice born to naive (N 7d, pups born to naive mothers, immunized at day 7; N 21d, pups born to naive mothers, immunized at day 21) or immunized (I 7d, pups born to immunized mother, immunized at day 7; I 21d, pups born to immunized mother, immunized at day 21) mothers via the intranasal route. Siegrist has reported that antibody responses elicited in 7- and 21-day-old mice yielded observations very similar to those for human neonates and infants (47). Interestingly, at 2 weeks postimmunization, all RDAP S. Typhi strains induced anti-PspA antibodies in mice born to immunized mothers (Fig. 5A to D; P < 0.01). However, the anti-PspA antibody induced by RDAP S. Typhi strain χ9633(pYA4088) RpoS+ cannot be detected either in N 7d or in N 21d mice at 2 weeks (Fig. 5A and C) (P < 0.01). S. Typhi strain χ9639(pYA4088) RpoS− induced anti-PspA antibody only in group N 7d mice born to naive mothers at 2 weeks (Fig. 5A) (P < 0.01), and S. Typhi strain χ9640(pYA4088) RpoS− induced anti-PspA antibody in both group N 7d and N 21d mice (Fig. 5A to D) (P < 0.01). At 8 weeks, all RDAP strains carrying plasmid pYA4088 induced strong anti-PspA IgG responses in immunized mice (Fig. 5A to D). Strain χ9639(pYA4088) RpoS− induced the highest antibody titers compared to those of the other two S. Typhi strains in I 7d group mice (Fig. 5B) (P < 0.01). However, in the I 21d mouse group, χ9639(pYA4088) RpoS− induced a poorer antibody titer to PspA than those of χ9633(pYA4088) RpoS+ and χ9640(pYA4088) RpoS− (Fig. 5C and D) (P < 0.01).

The overall anti-PspA IgA responses in vaginal washes were low for all the vaccine groups. At 8 weeks, the anti-PspA IgA titers induced by four RDAP strains in mice born to immunized mothers were always higher than those in mice born to naive mothers (Fig. 5E to H) (P < 0.01), except in the case of strain χ9639(pYA4088) RpoS+, for which the IgA titer in I 21d mice was only slightly higher than that in N 21d mice (Fig. 5G and H) (P > 0.05). In addition, χ9633(pYA4088) RpoS− in-
duced better mucosal IgA antibody responses than those of \( \chi_9639(pYA4088) \) RpoS\(^{-}\) and \( \chi_9640(pYA4088) \) RpoS\(^{+}\) in 7d and 21d mice born to either naive mothers or immune mothers. In 7d and 21d mice born to immune mothers, the serum IgA titers induced by \( \chi_9558(pYA4088) \) RpoS\(^{+}\) were similar to those of \( \chi_9633(pYA4088) \) RpoS\(^{+}\) but stronger than those of \( \chi_9639(pYA4088) \) RpoS\(^{-}\) and \( \chi_9640(pYA4088) \) RpoS\(^{+}\) (Fig. 5E to H) \((P < 0.01)\). Although all the strains induced high anti-LPS titers at 8 weeks in mice, S. Typhi RDAP vectors with plasmid pYA4088 induced early anti-LPS antibody responses at
2 weeks in pups from immune mothers (Fig. 6B and D), which is in contrast to those at 4 weeks in pup from naive mothers (Fig. 6A and C). However, S. Typhimurium χ9558(pYA4088) RpoS+ induced anti-LPS immune response starting at 2 weeks in mice born to either naive mothers or immune mothers (Fig. 6A to D). By week 8, χ9558 RpoS+ carrying plasmid pYA4088 or pYA3493 in both 7d and 21d mice induced the highest anti-LPS antibody titers among four RDAP vaccine strains carrying
the corresponding plasmid (Fig. 6A to F) \( (P < 0.01)\). Notably, the anti-PspA responses in pups born to either naive mothers or immunized mothers were stronger than anti-LPS reactions by week 8 for three RDAP S. Typhi strains (Fig. 5 and 6). The anti-LPS antibody titers induced by RDAP vectors χ9633 RpoS+ and χ9639 RpoS+ with empty plasmid pYA3493 were higher than those induced by χ9640(pYA3493) RpoS+ in N 7d and N 21d pups at 8 weeks (Fig. 6E and F) \( (P < 0.01)\).

We further analyzed the IgG1 and IgG2a responses induced by the four RDAP strains (see Fig. S2 in the supplemental material). Since the 7d and 21d mice become adult mice after 4 weeks, we report only the IgG1 and IgG 2a isotypes at 2 weeks, when the maternal antibody still exists. The results showed that there were significantly increased IgG2a responses, which favored a Th1 response, at 2 weeks in mice from mothers immunized with RDAP S. Typhi χ9633(pYA4088) RpoS+ and χ9640(pYA4088) RpoS+ compared to those from naive mothers in both 7d groups and 21d groups. At 2 weeks, we also observed the same phenomenon in mice immunized with S. Typhimurium χ9558(pYA4088) RpoS+, although the IgG1 response was increased as well. However, we did not observe this change in mice immunized with RDAP S Typhi χ9639(pYA4088) RpoS−.

**Splenocyte cytokine secretion in neonatal (7d) and infant (21d) mice after immunization with RDAP vaccines.** We further evaluated the PspA-specific T-cell responses induced by the four RDAP vaccines in 7d and 21d mice born to naive and immune mothers by measuring the frequencies of IFN-γ- and IL-4-producing cells in the spleens at 7 weeks after initial immunization (Fig. 7). For IFN-γ responses, there were no significant differences between groups immunized with three S. Typhi RDAP vaccines [χ9633(pYA4088) RpoS+, χ9639(pYA4088) RpoS−, and χ9640(pYA4088) RpoS+] and a control group immunized with BSG in 7d mice born to either naive mothers or immunized mothers, except in the case of χ9633(pYA4088) RpoS+, which induced a higher IFN-γ response in 17d mice than that in the control group (Fig. 7A and B) \( (P < 0.01)\), which was also higher than that of χ9633(pYA4088) RpoS+ in the N 7d group (Fig. 7A and B) \( (P < 0.01)\). S. Typhimurium χ9558(pYA4088) RpoS− developed a significantly higher IFN-γ response in 7d mice born to either naive or immune mothers compared to that in mice inoculated with BSG (Fig. 7A and B) \( (P < 0.01)\). In 21d mice born either to naive or to immune mothers, the four RADP vaccine vectors with pYA4088 were associated with significant PspA-specific IFN-γ responses compared to results for the BSG group (Fig. 7C and D) \( (P < 0.01)\). In 7-day- and 21-day-age mice, S. Typhimurium χ9558(pYA4088) RpoS− developed higher levels of IFN-γ than those of the control group and three S. Typhi vaccine strains [χ9633(pYA4088) RpoS+, χ9639(pYA4088) RpoS−, and χ9640(pYA4088) RpoS+] (Fig. 7A to D) \( (P < 0.01)\).

Compared to the BSG group, neonates (7d) born to either naive or immune mothers exhibited potently IL-4 secretion in response to χ9633(pYA4088) RpoS+ and χ9639(pYA4088) RpoS− vaccines (Fig. 7E and F) \( [P < 0.01 \text{ for } \chi9633(pYA4088) \text{ RpoS}+ \text{ and } P < 0.05 \text{ for } \chi9639(pYA4088) \text{ RpoS}−] \). For S. Typhimurium strain χ9558(pYA4088) RpoS+, only neonate mice born to immune mothers developed significantly higher levels of IL-4 than those in the BSG group (Fig. 7F) \( (P < 0.01)\). Infant (21-day) mice born to naive mothers generated significantly higher levels of IL-4 secretion in response to four RDAP vaccine strains than those in the BSG group (Fig. 7G and H) \( (P < 0.01)\), and more IL-4 was generated in mice born to immune mothers for the four RDAP vaccine vectors (Fig. 7E to H).

It is notable that poor IL-4 responses were observed for strain χ9640(pYA4088) RpoS− in neonatal mice born to either naive or immunized mothers compared to results for the BSG group (Fig. 7E and F), and lower IL-4 responses were observed in infant mice born to naive or immune mothers than was seen with use of strains χ9633(pYA4088) RpoS+ and χ9639(pYA4088) RpoS− (Fig. 7G and H) \( (P < 0.01)\).

**Evaluation of protective immunity.** To evaluate the ability of RDAP vaccines to protect against pneumococcal infection, the immunized mice were challenged intraperitoneally with 4.0 \( \times 10^5 \) CFU (40 times the LD50) of S. pneumoniae WU2 at 10 weeks after the first immunization. All the groups immunized with vaccines synthesized PspA showed significant protection compared with control groups immunized with strains with the empty plasmid (pYA3493) or with BSG (Table 2) \( (P < 0.001)\). The protection of χ9558(pYA4088) RpoS− against S. pneumoniae WU2 has been reported previously \( (13)\). The protection induced by three S. Typhi RDAP vaccine strains in mice born to immune mothers showed a better survival rate than that for the animals born to naive mothers in mice initially immunized at either 7 days or 21 days (Table 2). However, only the protection induced by strain χ9639(pYA4088) RpoS− in 21d mice showed a significant difference between mice born to immune mothers and those born to naive mothers (Table 2). We observed slightly greater survival in mice immunized with χ9633(pYA4088) RpoS− (survival rates of 11/18 for 17d and 15/20 for 21d) and χ9639(pYA4088) RpoS− (13/20 for 17d and 14/20 for 21d) than in those immunized with χ9640(pYA4088) RpoS+ (8/20 for 17d and 14/23 for 21d) in 7d and 21d mice born to immune mothers, but the difference was not statistically significant (Table 2). Overall, our RDAP S. Typhi vaccine vectors displayed good protective efficacy against S. pneumoniae WU2 in neonatal and infant mice with the intraperitoneal challenge method.

**DISCUSSION**

Our lab has described the development of regulated delayed attenuation strategies in S. Typhimurium, which make the Salmonella vaccine vectors safe and immunogenic for delivery of heterologous antigens to adult, newborn, and infant mice \( (13–15)\). The S. Typhi strains with RDAP strategies also proved effective in adult mice \( (15)\). To further expand the knowledge and application of regulated delayed attenuation strategies, we tested RDAP S. Typhi vaccines in this study by quantifying their invasion into and persistence in tissue culture cells and their immunogenicity in neonatal and infant mice. The goal of the regulated delayed attenuation system is to create vaccine vector strains displaying sufficient invasive and persistence abilities to stimulate both strong primary and lasting memory immune responses. Therefore, we validated the four strains with RDAP in vitro and in vivo and compared them to their wild-type parental strains and Ty21a, a live attenuated S. Typhi vaccine licensed for humans.

We used an arabinose-regulated promoter to achieve the goal of regulated delayed attenuation. Arabinose is produced in plant and yeast \( (48)\) and could be consumed by commensal bacteria. It is a poorly absorbed sugar and can be tolerated to 4% without any gastrointestinal symptoms in humans \( (49)\). In human blood plasma, the concentration of arabinose is less than 5 μmol/liter \( (50)\). The arabinose present in cereal nutri-
Functional components, like rodent chow, chicken feed, and chicken breast meat, is insufficient to support the function of the arabinose-regulated promoter (51). In humans, 90% of the arabinose was fermented by 24 h in the ileum (52) and 17.5% of the arabinose will be excreted in urine after drinking a solution containing arabinose (53). Thus, as a single promoter, the arabinose-regulated promoter has the advantage of being active in vitro and inactive in vivo. It thus has been widely

FIG 7 PspA-specific cytokine stimulation in mice immunized with RDAP strains carrying plasmid pYA4088 or with BSG. Numbers of IFN-γ-producing (A to D) or IL-4-producing (E to H) cells were determined by ELISPOT assay. Splenectomies were performed on euthanized mice 7 days after the final immunization. Mice mock immunized with BSG were included as controls. Splenocytes were harvested from 6 mice per group, and cells from each spleen were assayed in triplicate. N 7d mice and N 21d mice were born to naive mothers; I 7d mice and I 21d mice were born to immunized mothers. The results from each well are presented as spots per million splenocytes minus any background spots from unpulsed mock controls. There is no spot from the negative control. Significant differences between mice immunized with RADP vaccine vectors with plasmid pYA4088 and BSG groups (*, P < 0.05; **, P < 0.01) and for mice born to naive mothers (#, P < 0.05; ##, P < 0.01) are indicated.
used as a promoter to achieve regulated delayed attenuation, regulated delayed antigen synthesis, and regulated delayed lysis (12, 51, 54, 55). The collective results of the invasion assay in the human epithelial INT-407 cells indicated that all strains with the regulated delayed attenuation system using the arabinose-regulated promoter displayed invasive abilities stronger than that of Ty21a but weaker than those of their wild-type parental strains (Fig. 1). These results indicated that we still have room to improve our system to make it better. Notably, parental strains (Fig. 1). These results indicated that we still have room to improve our system to make it better. Notably, parental strains (Fig. 1). These results indicated that we still have room to improve our system to make it better. Notably, parental strains (Fig. 1). These results indicated that we still have room to improve our system to make it better. Notably, parental strains (Fig. 1).

Another goal of the regulated delayed attenuation strategies is that the attenuated vaccine should be able to sufficiently colonize and persist within the gut-associated and internal lymphoid tissues to stimulate both strong primary and lasting memory immune responses. Our results suggest that the vaccine strains χ9633(pYA4088) RpoS⁺ and χ9640(pYA4088) RpoS⁺ exhibit a more ideal regulated attenuated phenotype. All RDAP strains showed more sharply decreased persistence in THP-1 cells than in INT-407 cells. This phenotype may be caused by the defensive mechanism present in the human monocytoid THP-1 cell line (56). Considering that the bacteria will fight against human monocytic cells in vivo, the final titers of χ9633 and χ9639 are lower than that of Ty21a in THP-1 cells at 72 h (see Fig. S1 and Fig. S2 in the supplemental material). These results indicated that these two strains are as safe as Ty21a but are likely overattenuated. Strain χ9640 reached the same amount of bacteria as Ty21a at 72 h, although it started with 10 times more bacteria. This strain may therefore represent an ideal balance between immunogenicity and attenuation.

Because S. Typhi cannot infect mice, we used the RDAP S. Typhimurium χ9558(pYA4088) RpoS⁺ strain in parallel with RDAP S. Typhi strains to evaluate the regulated attenuated phenotype in mice. The strain χ9558(pYA4088) RpoS⁺ has the same genotype as RDAP S. Typhi strains. The collective data further confirmed that the regulated delayed attenuated phenotype was displayed in NALT, lungs, and Peyer’s patches (Fig. 3). Although S. Typhi cannot infect mice, the higher persistence ability of RpoS⁺ strains than of RpoS⁻ strains at each time point (Fig. 3G to K) (P < 0.01) indicated that S. Typhi strains with rpoS mutations also have markedly diminished abilities to colonize Peyer’s patches in mice, which was also reported for S. Typhimurium with the rpoS mutation (27). In immune-related tissue, NALT and Peyer’s patches, strains χ9633 and χ9640 have better persistence than χ9639. These results implied that χ9633 and χ9640 might give a better interaction with the immune system than χ9639 (Fig. 3). Our results are roughly consistent with Pickett et al.’s report that progressively lower numbers of S. Typhi with time were isolated from different tissues via intranasal immunization (28). However, compared with their report that only 10⁴ or 10⁶ CVD 908-htrA bacteria were isolated from different tissues at 0.25 h (28), we isolated around 10⁴ or 10⁵ bacteria in different tissues from mice born to naïve mothers. Further, we still isolated RDAP S. Typhi vaccine strains at 72 h, except for χ9639(pYA4088) RpoS⁻ in lung and Peyer’s patches and χ9640(pYA4088) RpoS⁺ in Peyer’s patches from mice born to naïve mothers (Fig. 3). In mice born to immunized mothers, we isolated RDAP vaccine strain χ9633(pYA4088) RpoS⁻ and χ9640(pYA4088) RpoS⁺ (Fig. 4) from NALT at 72 h. Thus, the regulated delayed attenuation strategies enable vaccine strains to continuously reseed to Peyer’s patches and lung at a low level via aspiration and ingestion to stimulate immune responses. It is a superior construction strategy to that of vaccines, like Ty21a, generated by traditional means. CVD 908-htrA, with deletions of the arSc, arDC, and htrA genes to make strains auxotrophic and susceptible to oxidative stress, is another promising vaccine generated by traditional means. CVD 908-htrA, with deletions of the arSc, arDC, and htrA genes to make strains auxotrophic and susceptible to oxidative stress, is another promising vaccine generated by traditional means. CVD 908-htrA, with deletions of the arSc, arDC, and htrA genes to make strains auxotrophic and susceptible to oxidative stress, is another promising vaccine generated by traditional means. CVD 908-htrA, with deletions of the arSc, arDC, and htrA genes to make strains auxotrophic and susceptible to oxidative stress, is another promising vaccine generated by traditional means. CVD 908-htrA, with deletions of the arSc, arDC, and htrA genes to make strains auxotrophic and susceptible to oxidative stress, is another promising vaccine generated by traditional means (57). Direct comparison of the CVD 908-htrA strain with our RDAP strains will further clarify whether this reflects the different coloniza-

### TABLE 2 Immunization with RDAP strains carrying plasmid pYA4088 protects BALB/c mice against i.p. challenge with S. pneumoniae WU2

| Vaccine strain | Groups | No. of challenged mice | % survival |
|---------------|--------|------------------------|------------|
| BSG           | N 7d   | 16                     | 0 (0/16)   |
|               | N 21d  | 16                     | 0 (0/15)   |
| χ9633(pYA3493)| N 7d   | 15                     | 0 (0/15)   |
|               | N 21d  | 15                     | 0 (0/15)   |
| χ9633(pYA4088)| N 7d   | 22                     | 41 (9/22)  |
|               | I 7d   | 18                     | 61 (11/18) |
|               | N 21d  | 20                     | 55 (11/20) |
|               | I 21d  | 20                     | 75 (15/20) |
| χ9639(pYA4088)| N 7d   | 21                     | 52 (11/21) |
|               | I 7d   | 20                     | 65 (13/20) |
|               | N 21d  | 20                     | 40 (8/20)  |
|               | I 21d  | 20                     | 70 (14/20) |
| χ9640(pYA4088)| N 7d   | 18                     | 33 (6/18)  |
|               | I 7d   | 20                     | 40 (8/20)  |
|               | N 21d  | 21                     | 57 (12/21) |
|               | I 21d  | 23                     | 61 (14/23) |

| Groups | No. of challenged mice | % survival |
|--------|------------------------|------------|
|        | 0 (0/16)               |
|        | 0 (0/15)               |
|        | 0 (0/15)               |
|        | 0 (0/15)               |
|        | 41 (9/22)              |
|        | 61 (11/18)             |
|        | 55 (11/20)             |
|        | 75 (15/20)             |
|        | 52 (11/21)             |
|        | 65 (13/20)             |
|        | 40 (8/20)              |
|        | 70 (14/20)             |
|        | 33 (6/18)              |
|        | 40 (8/20)              |
|        | 57 (12/21)             |
|        | 61 (14/23)             |

鼻状 Nasal

* Mice were orally immunized with four doses of the indicated vaccine strains, at 0, 2, 4, and 6 weeks.
* N 7d or N 21d, pups born from naïve mothers, immunized at day 7 or 21; I 7d or I 21d, pups born from immunized mothers, immunized at day 7 or 21.
* Ten weeks after the primary oral immunization, mice were challenged with approximately 4 × 10⁸ CFU of S. pneumoniae WU2. The LD₅₀ₐ₀ of WU2 in nonimmunized BALB/c mice is 1 × 10⁷ (14).
* No. of survivors/total is given in parentheses. The survival data were analyzed using the Kaplan-Meier method, and survival comparisons were done by the Mantel-Cox test method. All RDAP vaccine vectors carrying pYA4088 groups were significantly different from the RDAP with pYA3493 (vector control) and BSG controls (P < 0.01).
* Compared to χ9639(pYA4088)-immunized N21 mice, P < 0.05.
can enhance the persistence ability of vaccine strains in tissues at early stages after inoculation. Meanwhile S. Typhi RDAP strains c9633(pYA04088) RpoS−, c9639(pYA04088) RpoS−, and c9640(pYA04088) RpoS+ displayed regulated delayed attenuated curves in NALT and in lung, but not in Peyer’s patches (Fig. 4).

RDAP vaccine vectors carrying pYA04088 developed immune responses against PspA and Salmonella antigens in neonatal (day 7) and infant (day 21) mice immunized by the intranasal route. Notably, immunization of mothers induced quicker antibody responses at 2 weeks after immunization. Since the newborns are prone to infection by S. pneumoniae, an early antibody response is important to an efficient S. pneumoniae vaccine for newborns. It is worthwhile to try different immunization schedules, such as immunization at day 1 or 3, to see if we can induce even earlier immune responses. In accordance with a greater persistence primarily in INT-407 cells, in NALT tissue, and in Peyer’s patches, four RDAP vaccine strains induced high titers of IgG antibodies against PspA and LPS, but maternal immunization decreased the IgG levels of anti-PspA and anti-LPS antibody induced by vaccine strains at 4 or 6 weeks after first immunization, except in the case of S. Typhimurium χ9558(pYA04088) RpoS−, for which maternal immunization did not affect the survival rate of χ9558(pYA04088) RpoS+ in NALT, lung, and Peyer’s patches and enhanced immune responses against PspA and Salmonella LPS (Fig. 6A to D). In contrast to humoral immunity of S. Typhi RDAP strains, maternal immunization of S. Typhi RDAP strains had a potentially stronger trend to make immunized mice secrete higher vaginal mucosal IgA, IFN-γ, and IL-4 (Fig. 7) and resulted in a higher level of protection against S. pneumoniae WU2 challenge in mice born to immune mothers than in mice born to naive mothers (Table 2).

It was reported that the IgG2a production is lower in early life because murine T-cell responses to viral/protein vaccines toward the default Th2 developmental pathway due to adaption for early persistence primarily in INT-407 cells, in NALT tissue, and in Peyer’s patches, four RDAP vaccine strains induced high titers of IgG antibodies against PspA and LPS, but maternal immunization decreased the IgG levels of anti-PspA and anti-LPS antibody induced by vaccine strains at 4 or 6 weeks after first immunization, except in the case of S. Typhimurium χ9558(pYA04088) RpoS−, for which maternal immunization did not affect the survival rate of χ9558(pYA04088) RpoS+ in NALT, lung, and Peyer’s patches and enhanced immune responses against PspA and Salmonella LPS (Fig. 6A to D). In contrast to humoral immunity of S. Typhi RDAP strains, maternal immunization of S. Typhi RDAP strains had a potentially stronger trend to make immunized mice secrete higher vaginal mucosal IgA, IFN-γ, and IL-4 (Fig. 7) and resulted in a higher level of protection against S. pneumoniae WU2 challenge in mice born to immune mothers than in mice born to naive mothers (Table 2).

It was reported that the IgG2a production is lower in early life because murine T-cell responses to viral/protein vaccines toward the default Th2 developmental pathway due to adaption for early postnatal life (47). By analysis of antibody isotypes, we found that mice born to mothers immunized with RDAP S. Typhi χ9633(pYA04088) RpoS− and χ9640(pYA04088) RpoS+ produced more IgG2a than the mice born to naive mothers in 7d groups (see Fig. S2 in the supplemental material), but this was not the case with S. Typhi χ9639(pYA04088) RpoS− at 2 weeks. Although some methods (47), such as using a DNA vaccine, low doses of a replicating live virus vaccine, and adjuvants, have been used to increase the Th1 response in early life, our results indicate a new method by using immunization of mothers to increase the Th1 response. In this way, we can prime the immune system to be better prepared for future responses.

The regulated delayed attenuation strategies have been proven to be safe and effective by using S. Typhimurium, S. Typhi, and Yersinia pestis as models against homologous or heterologous challenge in adult and/or baby mice (12, 13, 15, 55). We further evaluated the behaviors of three recombinant attenuated S. Typhi vaccine vectors and one recombinant attenuated S. Typhimurium vaccine vector with regulated delayed attenuation strategies in vitro and in vivo by quantification and the immunogenicity of these strains in neonatal and infant mice by using the intranasal immunization route. The RDAP by a combination of these strategies in strains derived from different parental wild-type strains displayed various kinetics of attenuation in different mouse tissues. They also induced different levels of immune responses. It appears that χ9633 induced immunity that was more protective in mice; considering the differences between humans and mice and the different behaviors of these strains, we expect that different human test results will be achieved; the best candidate might be one of the two S. Typhi RpoS+ strains.

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