

Research Paper

# Regulated Delayed *Shigella flexneri* 2a O-antigen Synthesis in Live Recombinant *Salmonella enterica* Serovar Typhimurium Induces Comparable Levels of Protective Immune Responses with Constitutive Antigen Synthesis System

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## Abstract

*Shigella flexneri* (*S. flexneri*), a leading cause of bacillary dysentery, is a major public health concern particularly affecting children in developing nations. We have constructed a novel attenuated *Salmonella* vaccine system based on the regulated delayed antigen synthesis (RDAS) and regulated delayed expression of attenuating phenotype (RDEAP) systems for delivering the *S. flexneri* 2a (Sf2a) O-antigen.

**Methods:** The new *Salmonella* vaccine platform was constructed through chromosomal integration of the *araC* P<sub>BAD</sub> *lacl* and *araC* P<sub>BAD</sub> *wbaP* cassettes, resulting in a gradual depletion of WbaP enzyme. An expression vector, encoding Sf2a O-antigen biosynthesis under the control of the *Lacl*-repressible P<sub>trc</sub> promoter, was maintained in the *Salmonella* vaccine strain through antibiotic-independent selection. Mice immunized with the vaccine candidates were evaluated for cell-mediated and humoral immune responses.

**Results:** In the presence of exogenous arabinose, the *Salmonella* vaccine strain synthesized native *Salmonella* LPS as a consequence of WbaP expression. Moreover, arabinose supported *Lacl* expression, thereby repressing Sf2a O-antigen production. In the absence of arabinose *in vivo*, native *Salmonella* LPS synthesis is repressed whilst the synthesis of the Sf2a O-antigen is induced. Murine immunization with the *Salmonella* vaccine strain elicited robust Sf2a-specific protective immune responses together with long term immunity.

**Conclusion:** These findings demonstrate the protective efficacy of recombinant Sf2a O-antigen delivered by a *Salmonella* vaccine platform.

Key words: *Shigella flexneri* 2a, regulated delayed antigen synthesis, O-antigen, attenuated *Salmonella* vaccine.

## Introduction

Recombinant attenuated *Salmonella* strains are attractive vaccine platforms that provide needle-free, low-cost, and highly-versatile antigen delivery systems [1]. *Salmonella*, one of the most extensively studied bacterial genera, is readily manipulated to

construct recombinant attenuated *Salmonella* vaccines (RASVs). Through modification of RASVs and antigen expression systems, host immune responses can be tailored to elicit humoral, cell-mediated, or mucosal immunity bias. One of the key features of

current RASV vaccine candidates is the ability to undergo regulated delayed attenuation. Upon oral immunization, the RASV synthesizes virulent factors essential for host colonization and invasion. Due to the absence of carbohydrate inducers *in vivo*, the RASV strain gradually experienced controlled attenuation. Conversely, the production of antigens maintained by antibiotic-independent balanced lethal plasmids is enhanced in the mammalian system. The combined attributes of delayed attenuation and antigen expression enable productive colonization of the internal effector lymphoid tissues and the robust stimulation of mucosal and systemic humoral and cell-mediated immune responses [2].

The efficiency of recombinant vector vaccines largely depends on the capability to deliver foreign antigens to induce a protective immune response. Many strategies, such as high-copy number expression vectors or strong promoters, have been previously investigated to maximize heterologous antigen expression [3, 4]. Increased energy expenditure dedicating to plasmid replication and maintenance imparts substantial metabolic burden. Furthermore, over-expression of antigens may be detrimental or toxic to the vaccine strain. Therefore, conventional strategies to enhance antigen expression often impair vaccine efficacy [5]. Many alternative approaches, including chromosomal integration of heterologous genes [6], construction of stabilized low-copy-number expression plasmids [7], surface display of heterologous antigen [8], and the use of runaway vectors [9], have been investigated to support antigen synthesis while maintaining vaccine strain immunogenicity. One of the most popular approaches to improve RASV immune responses is the use of *in vivo* inducible promoters, including *pagC* [10], *nirB* [11], *spv* and *dps* [12]. In previous work, in order to reduce the metabolic burden imposed by constitutive antigen gene expression, we utilized the  $P_{trc}$  promoter to develop the regulated delayed antigen synthesis (RDAS) system [13]. In this system, the  $P_{trc}$  promoter is repressed by LacI, which is produced from an *araC*  $P_{BAD}$  *lacI* cassette inserted into the chromosome of the RASV. When a vaccine strain is grown *in vitro* in the presence of arabinose, LacI is synthesized to preclude the  $P_{trc}$ -controlled expression of the antigen gene. When the *Salmonella* vaccine reaches tissues in the host, in the absence of arabinose, LacI is no longer produced and the antigen synthesis is initiated [13]. In order to achieve an effective balance of attenuation and immunogenicity of RASVs, we also developed systems of regulated delayed expression of attenuating phenotypes (RDEAP) to maintain the ability of RASVs to colonize internal lymphoid tissues. One of these systems involved

native RASV LPS synthesis upon *in vitro* growth media supplementation with arabinose [14]. The expression of *Salmonella* LPS at the time of immunization supported invasion of intestinal mucosal cells. Due to the absence of arabinose in the mammalian system, *in vivo* synthesis of the *Salmonella* O-antigen was ceased. The regulated delayed LPS deficiency enabled vaccine strain tissue invasion while sparing energy for the synthesis of heterologous antigens. Additionally, the *in vivo* impairment of native *Salmonella* O-antigen diminished undesired immune responses generated against the RASV vector.

*Shigella*, the etiological agent of shigellosis or bacillary dysentery, is Gram-negative enteroinvasive bacterial pathogens [15]. Largely as a consequence of poor sanitation conditions, *S. flexneri* is a leading cause of disease in the developing world. Shigellosis continues to be a major public health concern among children less than 5 years old in many developing countries [16]. Treatment strategies are limited to post-exposure antibiotics which are compromised by the emergence of antibiotic resistant strains. Therefore, the development of equitable and cost-effective vaccines against *S. flexneri* has been viewed as a high priority by the World Health Organization [17]. Significant efforts have been made in the development of *Shigella* vaccines, including attenuated or inactivated whole-cell bacterial vaccines, subunit or conjugated vaccines, and outer membrane vesicle vaccines (OMV) [18]. However, all these vaccine candidates suffer from poor immunogenicity, are insufficiently attenuated, or require complicated purification procedures. Currently, no licensed vaccine is available against *Shigella* infection in humans. *S. flexneri*, which causes the highest mortality rate among the *Shigella* species [19], encompasses at least 14 serotypes based on the structure of the lipopolysaccharide O-antigen repeats. All *S. flexneri* serotypes, aside from serotype 6, are variants of a conserved, repeated structural unit referred to as serotype Y. The serotype Y O-antigen is comprised of repeating tetrasaccharide *N*-acetylglucosamine-rhamnose-rhamnose-rhamnose units [20]. Differences between classical *S. flexneri* serotypes are generated by temperate bacteriophages through addition of either glucosyl or O-acetyl residues at specific positions on the repeating units. For instance, *S. flexneri* 2a (Sf2a) O-antigen has glycosylation on a specific rhamnose residue of the repeating units (Fig. 1A) [21]. As the host immune responses against *S. flexneri* infection are thought to be serotype specific, which means that immunity generated against a specific *S. flexneri* serotype only provides protection against strains of the same

serotype [22], O-antigen structures are believed to be the primary targets of the immune response. Moreover, there is a substantial body of evidence indicating that antibodies generated against *Shigella* O-antigen play a key role in affording protection [23]. As pure polysaccharides are mostly T-cell independent (TI) antigens and are poorly immunogenic, glycoconjugate vaccines generated by chemical conjugation of polysaccharides to immunogenic proteins were developed in order to stimulate polysaccharide-specific T-cell dependent (TD) immune responses [24]. However, the production of glycoconjugates requires a complicated purification processes, substantially increasing the cost of a vaccine. Strategies have been employed to construct live vector vaccines to synthesize *Shigella* O-antigen in rough *E. coli* or attenuated *Salmonella* strains. However, a hybrid *E. coli* vaccine failed to protect *Shigella* infection in humans because it had no capacity to invade epithelial cells and showed only weak protection even with introduced epithelial invasiveness [25]. A *Salmonella* vaccine strain, using the attenuated *Salmonella* vaccine vector Ty21a expressing the Sf2a O-antigen, required laborious manipulation and maintained all the genes required for native *Salmonella* O-antigen synthesis [26]. The synthesis of both the *Shigella* and *Salmonella* O-antigens throughout the bacterial infectious cycle is believed to result in the excessive consumption of

nutrients and energy, thereby reducing immunogenicity.

In this study, a new RASV system was developed which combines the RDAS and RDEAP features for delivering of exogenous O-antigens. In this system, the *araC* P<sub>BAD</sub> *lacI* and *araC* P<sub>BAD</sub> *wbaP* cassettes were chromosomally integrated in the parental RASV (with a native *wbaP* deletion). The LacI-repressible P<sub>trc</sub> promoter was used to control expression of Sf2a O-antigen biosynthesis genes. During *in vitro* propagation in the presence of arabinose, WbaP was induced to support native *Salmonella* O-antigen synthesis; whereas, LacI was induced to repress Sf2a O-antigen production. In the absence of arabinose *in vivo*, native *Salmonella* LPS was repressed while LacI induction was relieved, thereby Sf2a O-antigen production started. Oral immunization of mice with the new RASV strains delivering Sf2a O-antigen elicited protective immune responses against Sf2a infection together with long term immunity. Taken together, the regulated delayed expression of the Sf2a O-antigen reduced potential toxicity of foreign antigen expression and alleviated RASV metabolic burden. Moreover, the expression of native *S. Typhimurium* O-antigen upon immunization mimicked the early stages of colonization, thereby enhancing RASV immunogenicity.

**Table 1.** Bacterial strains and plasmids used in this study

Strains or Plasmids	Description	Source
<b>Suicide and cloning vectors</b>		
pYA4278	Suicide vector, <i>sacB mobRP4</i> R6K <i>ori</i> Cm <sup>r</sup> , pRE112-T-vector	[28]
pYA5457	Suicide vector, <i>pagL7</i> deletion	pYA4278 [34]
pYA5470	Suicide vector, <i>pagL7</i> deletion and TT <i>araC</i> P <sub>BAD</sub> <i>wbaP</i> insertion	This study
pYA3700	Cloning vector containing TT <i>araC</i> P <sub>BAD</sub> cassette	[29]
<b>Recombinant plasmids</b>		
pG8R184	Asd <sup>+</sup> vector, pSC101 <i>ori</i> , Kan <sup>r</sup>	Lab stock
pG8R190	Asd <sup>+</sup> vector carrying genes involved in the biosynthesis of Sf2a O-antigen ( <i>galF-rfc</i> fused with <i>gtrA-gtrII</i> ), pSC101 <i>ori</i>	Lab stock
pG8R257	Asd <sup>+</sup> vector carrying genes involved in the biosynthesis of Sf2a O-antigen ( <i>rfbB-rfc</i> fused with <i>gtrA-gtrII</i> ) driven by the P <sub>trc</sub> promoter, pSC101 <i>ori</i>	This study
<b><i>E. coli</i> strains</b>		
χ6097	Δ( <i>lac-pro</i> ) <i>rpsL</i> Δ <i>asdA4</i> Δ ( <i>zhf-2::Tn10</i> ) <i>thi</i> 80 <i>dlacZ</i> Δ <i>M15</i>	K-12/F-
χ7213	<i>thi-1 thr-1 leuB6 glnV44 flhA21 lacY1 recA1 RP4-2-Tc::Mu λpir ΔasdA4 Δzhf-2::Tn10</i>	[30]
<b><i>S. enterica</i> serovar Typhimurium strains</b>		
χ3761	Wild-type UK-1	Lab collection
χ9241	Δ <i>pabA1516</i> Δ <i>pabB232</i> Δ <i>asdA16</i> Δ <i>araBAD23</i> Δ <i>relA198::araC</i> P <sub>BAD</sub> <i>lacI</i> TT	[13]
χ11316	Δ <i>pabA1516</i> Δ <i>pabB232</i> Δ <i>asdA16</i> Δ <i>araBAD23</i> Δ <i>relA198::araC</i> P <sub>BAD</sub> <i>lacI</i> TT Δ <i>wbaP45</i>	χ9241 [28]
χ12491	Δ <i>pabA1516</i> Δ <i>pabB232</i> Δ <i>asdA16</i> Δ <i>araBAD23</i> Δ <i>relA198::araC</i> P <sub>BAD</sub> <i>lacI</i> TT Δ <i>wbaP45</i> Δ <i>pagL7</i>	χ11316 This study
χ12514	Δ <i>pabA1516</i> Δ <i>pabB232</i> Δ <i>asdA16</i> Δ <i>araBAD23</i> Δ <i>relA198::araC</i> P <sub>BAD</sub> <i>lacI</i> TT Δ <i>wbaP45</i> Δ <i>pagL7::TT</i> <i>araC</i> P <sub>BAD</sub> <i>wbaP</i>	χ12491 This study
<b>Strains for immunization</b>		
Vaccine strain 1 (Vac1)	χ12514 electroplated with pG8R257	
Vac2	χ9241 electroplated with pG8R257	
Vac3	χ11316 electroplated with pG8R190	
Vac4	χ9241 electroplated with pG8R190	
Vac5	χ9241 electroplated with pG8R184	
<b><i>Shigella flexneri</i> 2a (Sf2a) Strain</b>		
2457T	Wild-type Sf2a strain	Lab collection

## Methods

### Bacterial strains, plasmids, media, and growth conditions.

Strains and plasmids used in this work are listed in Table 1. The wild type *S. Typhimurium* strain and transformants of *S. Typhimurium* and *E. coli* strains containing the *asdA* gene were grown at 37°C in Luria-Bertani (LB) broth or on LB agar. In order to propagate strains with the *asdA* gene deletion, growth medium was supplemented with 50 µg/ml diamminopimelic acid (DAP) [27]. *S. Typhimurium* strains carrying P<sub>trc</sub> promoter sequence used for Sf2a O-antigen detection and animal immunizations were grown at 37°C on LB agar or in LB broth containing 0.1% arabinose. The wild-type Sf2a (2457T) was grown at 37 °C in trypticase soy broth (TSB) or on TSB agar containing 0.01% Congo red.

### Plasmid and mutant strain construction

Primers used in this study are described in Table 2. Genes involved in the biosynthesis of the Sf2a O-antigen were amplified from plasmid pG8R190 and integrated into the plasmid pG8R184 backbone. Amplicons containing 18-20 base pair overlapping regions (Fig. 1B) were assembled via the NEBuilder HiFi DNA Assembly Master Mix (New England Biolabs (NEB), Ipswich, MA, USA) to generate plasmid pG8R257. The vector control plasmid pG8R184 as well as the pG8R190 and pG8R257 plasmids encoding Sf2a O-antigen biosynthesis genes were transferred to *asd*-deficient *S. Typhimurium* by electroporation. The resulting transformants were selected on LB agar plates lacking DAP.

For generating the *pagL* deletion, approximately 350-bp fragments from upstream and downstream of the *pagL* gene were amplified from the  $\chi$ 3761 genome, using primers P7/P8 and P9/P10 (Table 2), respectively. Following agarose gel purification, the two PCR fragments were combined through overlapping PCR. The addition of terminal adenine residues at both ends of the joined PCR products was catalyzed by the GoTaq enzyme (Promega, Madison, WI). The PCR product was ligated to the AhdI treated plasmid pYA4278 [28] to generate plasmid pYA5457.

For construction of the  $\Delta$ *pagL* ::TT *araC* P<sub>BAD</sub> *wbaP* deletion-insertion mutation, the *wbaP* gene was amplified by P11 and P12 (Table 2) from the  $\chi$ 3761 genome and inserted into pYA3700 [29]. The fragment TT *araC* P<sub>BAD</sub> *wbaP* was then amplified (with primers P12 and P13) and digested with NotI and SbfI. The gel purified product was ligated to the NotI-SbfI-digested plasmid pYA5457 to form suicide vector pYA5470.

*E. coli* strain  $\chi$ 7213 [30] harboring the suicide vector of pYA5457 was conjugated with *S.*

*Typhimurium* strain  $\chi$ 11316 [28] in order to generate the  $\Delta$ *pagL* mutation through allelic exchange, resulting in strain  $\chi$ 12491. The  $\Delta$ *pagL*::TT *araC* P<sub>BAD</sub> *wbaP* mutation was similarly introduced into  $\chi$ 12491 to yield  $\chi$ 12514. The genotypes of  $\Delta$ *pagL* and  $\Delta$ *pagL*::TT *araC* P<sub>BAD</sub> *wbaP* mutations were verified by PCR. LPS profiles of *S. Typhimurium* strains were examined as described by Hitchcock and Brown [31].

**Table 2.** Primers used in this study

Primer ID	Sequence (5'-3')
P1	CAGGAAACAGACCATGAAGATACTGTTACTGGTGGC
P2	CATTTCTCTCCTTTATTTTGTCCAGAAGTGAGG
P3	GCAAAATAAAGGAGAAGAAATGTTAAAGTTATTTGTAAGTA
P4	TTCATCCGCCAAAAACAGCCCTAAATATTAATGGAAGCCACC
P5	AAGTATCITCATGGTCTGTTTCTGTGTGAAATG
P6	TAAGGCTGTTTTGGCGGATGAGAGAAG
P7	AGACTATCTTTACTGGCAGG
P8	CACCTGCAGGCGATGCGCGCCCTCCACCACCATTTCAATGTC
P9	GAGCGGCCGATGCCCTGCAGGTGAAGTTGAATAACAATTAGC
P10	TGCGGATGAAGCTGCCGACC
P11	GCAGTCGACAAGGCTCTATATGGATAATATTGATAATAAG
P12	ACGGTACCTGCAGGCTTAATACGCACCATCTCCG
P13	ATCGGCCGAGATCTTTTATTATCTATCC

### Western blotting

Wild-type *S. Typhimurium*  $\chi$ 3761, wild-type Sf2a 2457T, and the mutant strains of *S. Typhimurium* harboring recombinant plasmids were grown overnight at 37 °C in LB broth supplemented with 0.1% arabinose when needed. LPS were extracted and fractionated by Tris-glycine PAGE as described previously [31]. The separated LPS were transferred to a nitrocellulose membrane and immunologically detected with 1:1000 dilutions of rabbit polyclonal antisera against *S. flexneri* type 2a O-antigen (Tianjin Biochip Corporation, Tianjin, China) and *Salmonella* O Group B Antiserum (BD, Sparks, MD, USA) for the identification Sf2a O-antigen and *S. Typhimurium* O-antigen profiles, respectively.

### Animal experiments

All animal experimentation was carried out in compliance with the recommendations of the Animal Welfare Act and US Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training. The protocol was reviewed and approved by the University of Florida Institutional Animal Care and Use Committee.

For immunization, the RASV strains were grown overnight in LB broth at 37 °C. LB broth supplemented with 0.1% arabinose was used for growth of RDAS strains. The overnight cultures of vaccine strains were diluted 1:100 and grown at 37°C to an OD<sub>600</sub> of 0.8 to 0.9. Each group consisting of 16 seven-week-old female BALB/c mice (Charles River



Laboratories, Wilmington, MA) were acclimated for 7 days upon arrival before initiating experiments. Groups of mice were orally inoculated with 20  $\mu$ l of buffered saline with gelatin (BSG) containing approximately  $1 \times 10^9$  CFU of each vaccine strain on day 0 and boosted on day 14 with an identical dose of the same strain. Mice administrated with  $1 \times 10^9$  CFU of  $\chi$ 9241 (pG8R184) or with 20  $\mu$ l of plain BSG were included as control groups. Three mice in each group were euthanized on day 7 and day 75 for detection of cell-mediated immune responses and memory immune responses, respectively. Blood samples collected on day 28 through mandibular vein puncture were allowed to coagulate at 37 °C for 2 h. The serum samples were collected and stored at -80 °C, after centrifugation. Vaginal-wash samples were collected and stored as described previously [13].

The overnight culture of a Congo red positive Sf2a 2457T colony was diluted 1:100 in fresh TSB and grown at 37 °C (up to OD<sub>600</sub> 0.8-0.9). Two weeks after the secondary immunization (day 28), 10 mice in each group were challenged with 100  $\mu$ l of BSG containing 2457T ( $5 \times 10^7$  CFU per mouse) through the intraperitoneal route as described previously [32].

### Antigen preparation and ELISA

*S. Typhimurium* LPS and Sf2a LPS were extracted using the LPS extraction kit (Boca Scientific, Westwood, MA) from strains of  $\chi$ 3761 and 2457T, respectively. An enzyme-linked immunosorbent assay (ELISA) was used to assay antibodies in serum and vaginal washes against Sf2a and *S. Typhimurium* LPS. The 96-well flat-bottom microtiter plates were coated overnight with 100  $\mu$ l/well of poly-L-lysine (MW.260000, 10  $\mu$ g/ml) (Sigma Aldrich, St. Louis, MO) in 0.01 M phosphate-buffered saline (PBS) at pH7.2 at room temperature. The LPS samples suspended in PBS were applied with 100  $\mu$ l volumes in each well. The coated plates were incubated for 1 h at 37°C and blocked with the SEA BLOCK Blocking Buffer (Thermo Fisher Scientific, Waltham, MA) for 1 h at room temperature. A 100  $\mu$ l/well of diluted serum sample (1:100) or vaginal wash (1:10) was added to individual wells in triplicate and incubated for 1 h at 37°C. Plates were then treated with biotinylated goat anti-mouse IgG, IgG1, IgG2a or IgA (Southern Biotechnology Associates, Birmingham, AL). After incubation of wells with a streptavidin-alkaline phosphatase conjugate (Southern Biotechnology) for 1 h at 37°C, the p-nitrophenyl phosphate (PNPP, Thermo Fisher Scientific) was added for color development. The optical density (OD) unit was read at 405 nm using an automated ELISA plate reader (model EL3115X; Biotek, Winooski, VT). Unconjugated IgG

(IgG-UNLB), IgG1(IgG1-UNLB), IgG2a (IgG2a-UNLB) and IgA (IgA-UNLB) derived from normal mice (Southern Biotechnology) were serially diluted in 2-fold steps in PBS (IgG-UNLB, from 5  $\mu$ g/ml to 40 ng/ml; IgG1-UNLB and IgG2a-UNLB, from 1  $\mu$ g/ml to 8 ng/ml; IgA-UNLB, from 62.5 ng/ml to 0.488 ng/ml). The OD values at 405nm were plotted against the representative concentrations of the diluted unconjugated antibody solutions to create the standard curves using linear regression in Excel ( $R^2 \geq 0.98$ ). The antibody concentrations were calculated from the corresponding curve.

### Splenic lymphocytes proliferation

Mice were euthanized 7 days after primary immunization (3 mice per group) and spleens were removed aseptically. Spleen cell suspensions were obtained by passing through a 70  $\mu$ m sterile cell strainer (Fisher brand, Houston, TX; 1 spleen/strainer) and the erythrocytes were lysed with RBC lysis buffer (eBioscience, San Diego, CA). Splenocytes were resuspended in RPMI 1640 supplemented with 5% fetal calf serum (FCS). The suspended cells were inoculated in a 96-well cell culture plate ( $3 \times 10^5$  cells/well) and incubated in a 5% CO<sub>2</sub> incubator at 37 °C. For experimental test groups, cells were cultured with the Sf2a LPS or *S. Typhimurium* LPS (30 ng/well). As a positive control, cells were cultured with phytohemagglutinin (PHA, 10  $\mu$ g/ml). For the negative control, cells were incubated with sterile culture medium. After 72 h of incubation, 20  $\mu$ l of MTT (5 mg/ml in PBS) was added to each well. After 4 h of incubation at 37 °C in the 5% CO<sub>2</sub> incubator, cells were centrifuged at 200 g for 5 min and the supernatants were removed. The colored crystals were dissolved with 100  $\mu$ L per well of DMSO by agitation at 37 °C for 10 min. The OD was read at 490 nm using the microplate reader (ELISA plate reader). Cell proliferation was calculated as stimulation index (SI), which is the ratio of the mean absorbance of triplicate wells with PHA or LPS stimulation to the mean absorbance of triplicate wells of the negative control.

### Cell Stimulation and Flow Cytometry

Single cell suspensions of splenic lymphocytes were stimulated *ex vivo* with the Sf2a LPS or *S. Typhimurium* LPS (50 ng /  $2 \times 10^6$  cells) for 8 h in the presence of Protein Transport Inhibitor Cocktail (eBioscience). Cell surface staining was performed using fluorophore-conjugated antibodies at 4 °C for 30 min. Intracellular staining was performed by eBioscience™ Intracellular Fixation and Permeabilization Buffer Set. All the cells were run on the BD LSRFortessa™ cell analyzer. Dead cells were

excluded by Zombie Red™ (Biolegend, San Diego, CA) for detection of cytokines and IgG<sup>+</sup> memory B (B<sub>M</sub>) cells, or Helix NP™ Blue (Biolegend) for detection of T follicular helper (Tfh) Cells. Cells were stained with the rat anti-mouse CD16/CD32 monoclonal antibody (eBioscience) to block Fc receptors.

Cytokine expression of CD4<sup>+</sup> T cells was detected 7 days after primary immunization with rat anti-mouse CD3-Alexa Fluor 700, CD4-FITC, IFN $\gamma$ -APC/Cy7 and IL-4-Alexa Fluor 647, using APC/Cy7 conjugated rat IgG1 $\kappa$  and Alexa Fluor 647 conjugated rat IgG1 $\kappa$  (Biolegend) as isotype controls. To determine the percentage of antigen-specific cells, the ratio of cytokine-positive cells of unstimulated sample was subtracted from the antigen-stimulated value of the same mouse.

On day 75 (61 days after secondary immunization), Tfh cells were identified as CD3<sup>+</sup>CD4<sup>+</sup>CD44<sup>+</sup>CD62L<sup>+</sup>CXCR5<sup>+</sup>PD-1<sup>+</sup>IL-21<sup>+</sup> lymphocytic cells after staining with anti-mouse CD3-PE, CD4-FITC, CD44-APC/Cy7, CD62L-Alexa Fluor 700, CXCR5-Brilliant Violet 421™, PD-1-PE/Dazzle™ 594, and IL-21-APC / isotype APC conjugated Rat IgG2a $\kappa$  (Biolegend). IgG<sup>+</sup> B<sub>M</sub> cells were identified as IgG<sup>+</sup>B220<sup>low/int</sup>CD80<sup>+</sup>IgD<sup>-</sup> or IgG<sup>+</sup>B220<sup>high</sup>CD80<sup>+</sup>IgD<sup>-</sup> live lymphocytes by staining with anti-mouse B220-Pacific Blue, CD80-FITC, IgD-Alexa Fluor 700, and IgG-APC (Biolegend) / isotype APC conjugated Goat IgG (R&D Systems, Minneapolis, MN). Data from both of the Tfh cells and IgG<sup>+</sup> B<sub>M</sub> assays were further displayed as antigen-specific cells which were calculated by subtracting the number of unstimulated Tfh or B<sub>M</sub> cells from antigen-stimulated values.

### **Shigella-specific serum bactericidal antibody (SBA) assay**

Serum samples in each group were heated at 56 °C for 30 min and pooled. Inactivated serum samples (with an initial dilution of 1:10) were added to 96-well U-bottom plates and serially diluted 2-fold in Muller Hinton broth. The final volume of the reaction mixture was made up to 100  $\mu$ L/well, containing 10  $\mu$ L of diluted serum, 10  $\mu$ L of the Sf2a 2457T cells (1000 CFU), 25  $\mu$ L of baby rabbit complement (BRC, Sigma). The plates containing the reaction mixture were incubated for 1 h at 37°C in a shaker at 200 rpm. At the end of incubation, 10  $\mu$ L of each reaction mixture was plated on the TSB agar and grown overnight. Colonies were counted and the percentage of killed bacteria was calculated using the equation [1- (the average CFU of the tested control / the average CFU of negative control)]. For each sample, the testing and plating were performed in duplicate (four

independent CFUs per sample), the four CFU counts were averaged for each sample to calculate the percentage of killed bacteria.

### **Statistical analyses**

The GraphPad Prism 5 software package (Graph Software, San Diego, CA) was used for statistical analyses. The one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test was used to evaluate differences in antibody concentrations and cell numbers of antigen-specific B<sub>M</sub> and Tfh cells. Bonferroni's multiple comparison test in a two-way ANOVA was used to analyze the differences in percentages of antigen-specific cells and stimulation indexes of proliferation. All data mentioned above were expressed as means with deviations. The Kaplan-Meier method was applied to analyze the survival data. The survival differences were assessed by the log-rank (Mantel-Cox) test. The significance levels were set at 0.05, 0.01 and 0.001.

## **Results**

### **RDAS system was constructed for delivering Sf2a O-antigen by *S. Typhimurium***

In order to reduce the adverse effects of RASV strains caused by antigen overexpression, an RDAS system was previously established [13]. Moreover, this RDAS system was utilized to facilitate the controlled synthesis of LPS containing exogenous O-antigens in a  $\Delta wbaP$  mutant [28]. Previous studies demonstrated that the  $\Delta wbaP$  deletion impaired synthesis of O-antigen O-unit (undecaprenyl-phosphate-O-unit), thereby preventing *S. Typhimurium* LPS production.

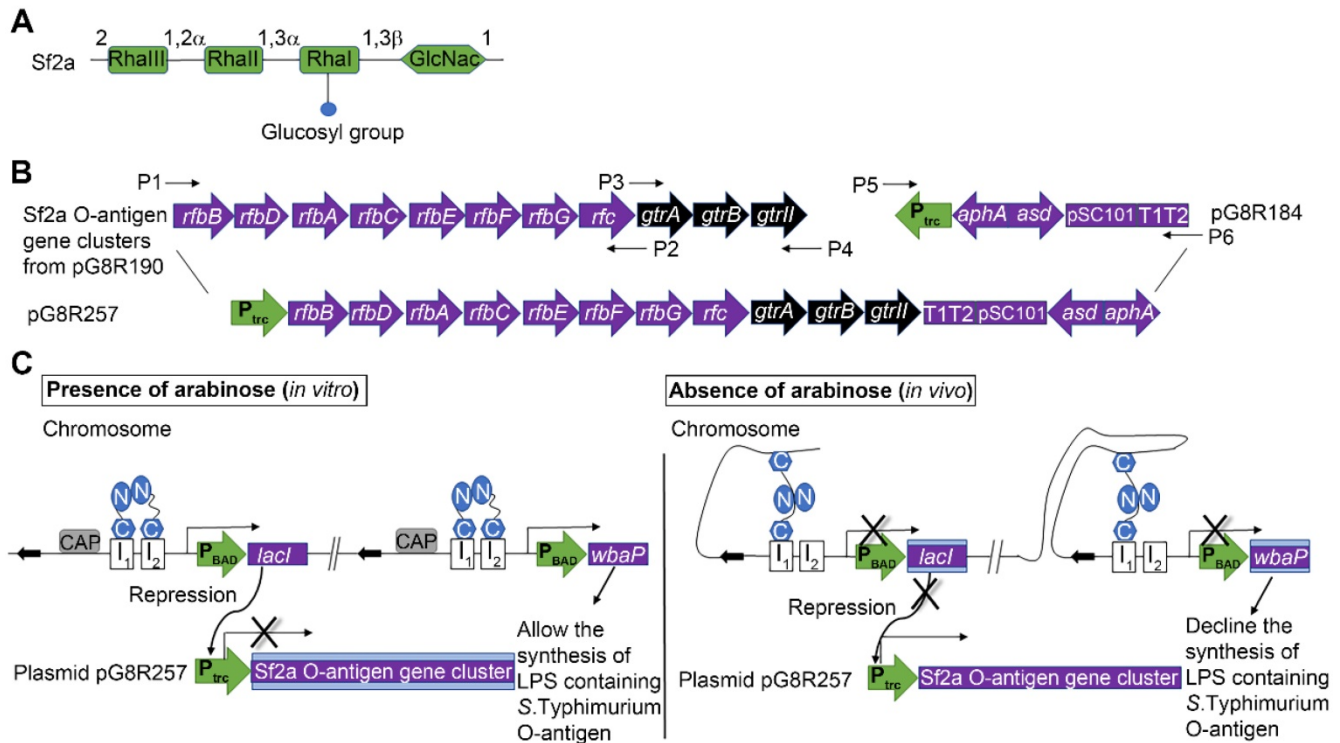
In this study, in order to achieve the regulated delayed synthesis of Sf2a O-antigen in RASV strains while maintaining the cell invasive capacity as wild-type *Salmonella*, we developed a new RDAS system by chromosomally integrating the TT *araC* P<sub>BAD</sub> *wbaP* cassette in place of the *pagL* gene of  $\chi$ 11316 [28] (Fig. 1C) to generate RDAS strain  $\chi$ 12514. In this system, both *lacI* and *wbaP* are placed under the control of the arabinose-inducible *araC* P<sub>BAD</sub> promoter. When RDAS strain  $\chi$ 12514 is cultured *in vitro* in the presence of arabinose, LacI is stimulated to repress the transcription of P<sub>trc</sub>-driven exogenous O-antigen genes and WbaP is expressed to facilitate the synthesis of *S. Typhimurium* native LPS. Upon *in vivo* inoculation, due to the absence of free arabinose, the expression of LacI and WbaP are repressed in strain  $\chi$ 12514. Therefore, *S. Typhimurium* native LPS is gradually suppressed in a mammalian system. PagL is capable of modifying the lipid A structure of *S. Typhimurium* through removal of the acyl chain [33].

Here, *pagL* was chosen as the integration site for the  $\Delta pagL::TT\ araC\ P_{BAD}\ wbaP$  deletion/insertion mutation, because deletion of *pagL* did not alter the virulence of the *Salmonella* and maintaining the immunogenicity and capability of colonization [34].

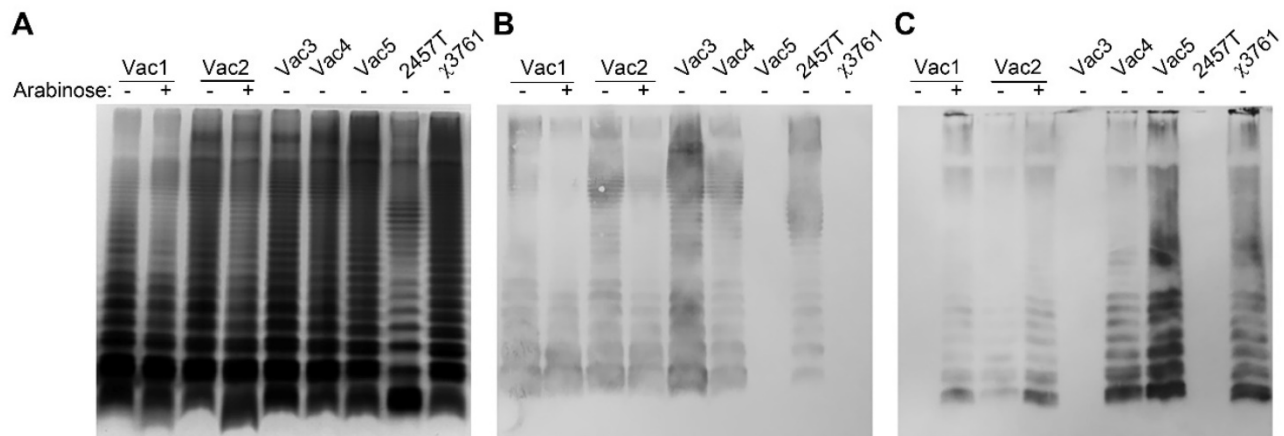
The Asd<sup>+</sup> recombinant plasmid pG8R257 (Fig. 1B) was constructed by integrating genes involved in the biosynthesis of the basic O-antigen (*rfbB-rfc*) and the Sf2a serotype-specific modification genes (*gtrA-gtrII*) under the control of P<sub>trc</sub> promoter. Plasmid

pG8R257 was introduced into RDAS strains  $\chi 12514$  (with *araC* P<sub>BAD</sub>-regulated *wbaP*) and  $\chi 9241$  (without mutation in *wbaP*), yielding Vac1 and Vac2, respectively. The pG8R190 plasmid carrying original Sf2a O-antigen gene cluster was also introduced into strains  $\chi 11316$  and  $\chi 9241$  respectively, referred to as Vac3 and Vac4. Vac5 reflects strain  $\chi 9241$  containing the pG8R184 empty control vector.

When cultured in the presence of 0.1% arabinose, both Vac1 and Vac2 indicated unique LPS banding



**Figure 1. The RDAS system for delivering Sf2a O-antigen by attenuated Salmonella strains. (A)** Structure of the Sf2a O-antigen. **(B)** Construction of plasmid pG8R257. Plasmid sequences include genes involved in the biosynthesis of Sf2a O-antigen (*rfbB-rfc* fused with *gtrA-gtrII*) under the control of P<sub>trc</sub> promoter; the *aphA* and *asdA* genes. **(C)** Diagram of the RDAS system, modified from Shifeng et al, 2010. The RDAS strain  $\chi 12514$  could express Lacl and WbaP under the control of the arabinose-inducible *araC* P<sub>BAD</sub> promoter in the presence of arabinose *in vitro*. Therefore, the transcription of P<sub>trc</sub>-driven Sf2a O-antigen genes was repressed by Lacl and synthesis of *S. Typhimurium* native LPS initiated at the participation of WbaP. In animal tissues, in the absence of free arabinose, Lacl and WbaP could not be produced. Thus, the transcription of Sf2a O-antigen genes started and the *S. Typhimurium* native LPS could no longer be synthesized. Rha, rhamnose; GlcNAc, N-acetylglucosamine.



**Figure 2. LPS synthesis in RDAS strains and constitutive antigen synthesis strains. (A)** LPSs from transfected strains grown in nutrient broth with (+) or without (-) 0.1% arabinose were separated by 12% SDS-PAGE, and detected by silver staining method. **(B)** Detection of Sf2a O-antigen synthesis with the antisera against *S. flexneri* type 2a O-antigen by western blot. **(C)** Identification of *S. Typhimurium* O-antigen with the rabbit polyclonal *Salmonella* group B antisera by western blot.



patterns compared with the synthesis pattern when cultured in the absence of arabinose (Fig. 2A). As expected, Vac1 ( $\chi$ 12514 with pG8R257) primarily synthesized LPS recognized by antisera generated against *S. flexneri* type 2a O-antigen (Fig. 2B) in the absence of arabinose. The detection of Sf2a O-antigen indicated that all the genes from P<sub>trc</sub> to T1T2 in the pG8R257 were transcribed and expressed successfully. When 0.1% arabinose was supplemented in the culture medium, *S. Typhimurium* LPS was detected in the Vac1 ( $\chi$ 12514 with pG8R257) strain (Fig. 2C). The Vac2 strain ( $\chi$ 9241 with pG8R257), which retained all the genes required for native *S. Typhimurium* LPS synthesis, produced a combination of Sf2a O-antigen and *S. Typhimurium* O-antigen when cultured in the absence of arabinose. When 0.1% arabinose was added to the growth medium, the native *Salmonella* LPS was primarily produced by Vac2. Only the Sf2a O-antigen could be detected in strains Vac3 ( $\chi$ 11316 with pG8R190) and both Sf2a O-antigen and *Salmonella* O-antigen could be detected in Vac4 ( $\chi$ 9241 with pG8R190). Conversely, strain Vac5 ( $\chi$ 9241 with pG8R184) solely synthesized LPS recognized by anti-*S. Typhimurium* O-antigen antibody.

#### **RDAS strains elicited lower Sf2a LPS-specific CD4<sup>+</sup>-T-Cell responses compared to strains that constitutively produce Sf2a O-antigen**

T cell activation is believed to be a key element for the development of efficient humoral and cell-mediated immune responses, along with the priming of memory immunity. However, natural infection of *Shigella* has been described to inhibit CD4<sup>+</sup>-T-cell migration, therefore compromising host immune responses [35]. In our previous study, it was demonstrated that immunization of mice with RASV strains can increase CD4<sup>+</sup> cytokine responses, thereby stimulating a Th1-type response [36]. In this study, the Sf2a LPS-specific cytokine expression of CD4<sup>+</sup> T cells was detected using intracellular staining combined with flow cytometry 7 days after primary immunization. The RDAS strains of Vac1 ( $\chi$ 12514 with pG8R257) and Vac2 ( $\chi$ 9241 with pG8R257) significantly increased IFN $\gamma$  stimulation relative to the Vac5 (empty plasmid control) and BSG treated groups. However, the levels of IFN $\gamma$  produced in response to RDAS strains Vac1 and Vac2 were significantly less pronounced compared to strains Vac3 ( $\chi$ 11316 with pG8R190) and Vac4 ( $\chi$ 9241 with pG8R190), which constitutively synthesized Sf2a O-antigen (Fig. 3A). Both RDAS strains Vac1 and Vac2 failed to elicit robust Sf2a LPS-specific IL-4 on day 7 post primary immunization. To the contrary, the Vac3 and Vac4 strains which constitutively produced Sf2a

O-antigens induced significantly increased levels of IL-4 production relative to the RDAS strains as well as the Vac5 and BSG controls (Fig. 3B). *S. Typhimurium* LPS-specific cytokines production was significantly lower in the groups immunized with strains Vac1 (synthesizing Sf2a O-antigen *in vivo* and *S. Typhimurium* O-antigen *in vitro*) and Vac3 (constitutively producing Sf2a O-antigen only) compared to the Vac2, Vac4 and Vac5 strains which synthesized *S. Typhimurium* O-antigen constitutively.

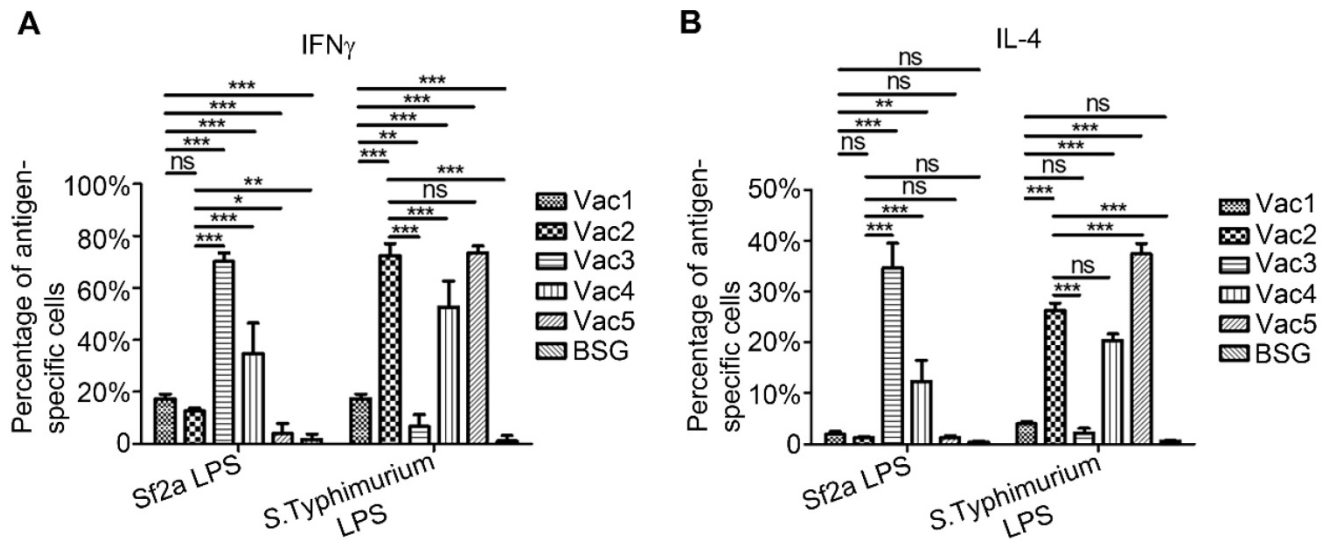
#### **RDAS strains delivering Sf2a O-antigen induced robust humoral immune responses**

Serum IgG and mucosal IgA responses against Sf2a LPS and *S. Typhimurium* LPS were measured by ELISA 2 weeks after secondary immunization. Both of the Vac1 and Vac2 RDAS strains induced significantly increased levels of Sf2a LPS-specific IgG relative to the Vac5 and BSG control groups (Fig. 4A). Moreover, the RDAS strain Vac1 ( $\chi$ 12514 with pG8R257), which synthesized native *S. Typhimurium* LPS in the presence of arabinose *in vitro* and produced LPS containing Sf2a O-antigen *in vivo*, triggered Sf2a LPS-specific IgG production comparable to the Vac3 strain ( $\chi$ 11316 with pG8R190) constitutively producing Sf2a O-antigen. The Vac2 strain ( $\chi$ 9241 with pG8R257), which synthesized mainly *Salmonella* native LPS *in vitro* but produced LPS containing both of *S. Typhimurium* O-antigen and Sf2a O-antigen *in vivo*, induced similar levels of IgG compared to Vac4, which constitutively produced both native *S. Typhimurium* and foreign Sf2a O-antigens. The levels of *S. Typhimurium* LPS-specific IgG induced by the Vac1 and Vac2 RDAS strains were significantly enhanced relative to Vac3 (producing only Sf2a O-antigen) immunized and BSG treated groups, but lower than Vac5 (producing only *S. Typhimurium* O-antigen) immunized group (Fig. 4E). Further analysis revealed that IgG2a was the predominant subtype of LPS-specific IgG elicited by the RASV strains (Fig. 4B, 4C, 4F and 4G). Both of the Vac1 and Vac2 RDAS strains stimulated similar levels of Sf2a LPS-specific vaginal IgA compared to the Vac3 and Vac4 strains that constitutively synthesized Sf2a O-antigen (Fig. 4D). The Vac2 strain (with Sf2a O-antigen *in vivo* and constitutively synthesizing *S. Typhimurium* LPS) stimulated the production of *S. Typhimurium* LPS-specific vaginal IgA; whereas, Vac1 (synthesizing Sf2a O-antigen *in vivo* and *S. Typhimurium* O-antigen *in vitro*) failed to elicit vaginal mucosal IgA responses (Fig. 4H). The level of vaginal IgA generated against *S. Typhimurium* LPS in Vac2 immunized group was comparable to that detected in the Vac4 (constitutively producing both of

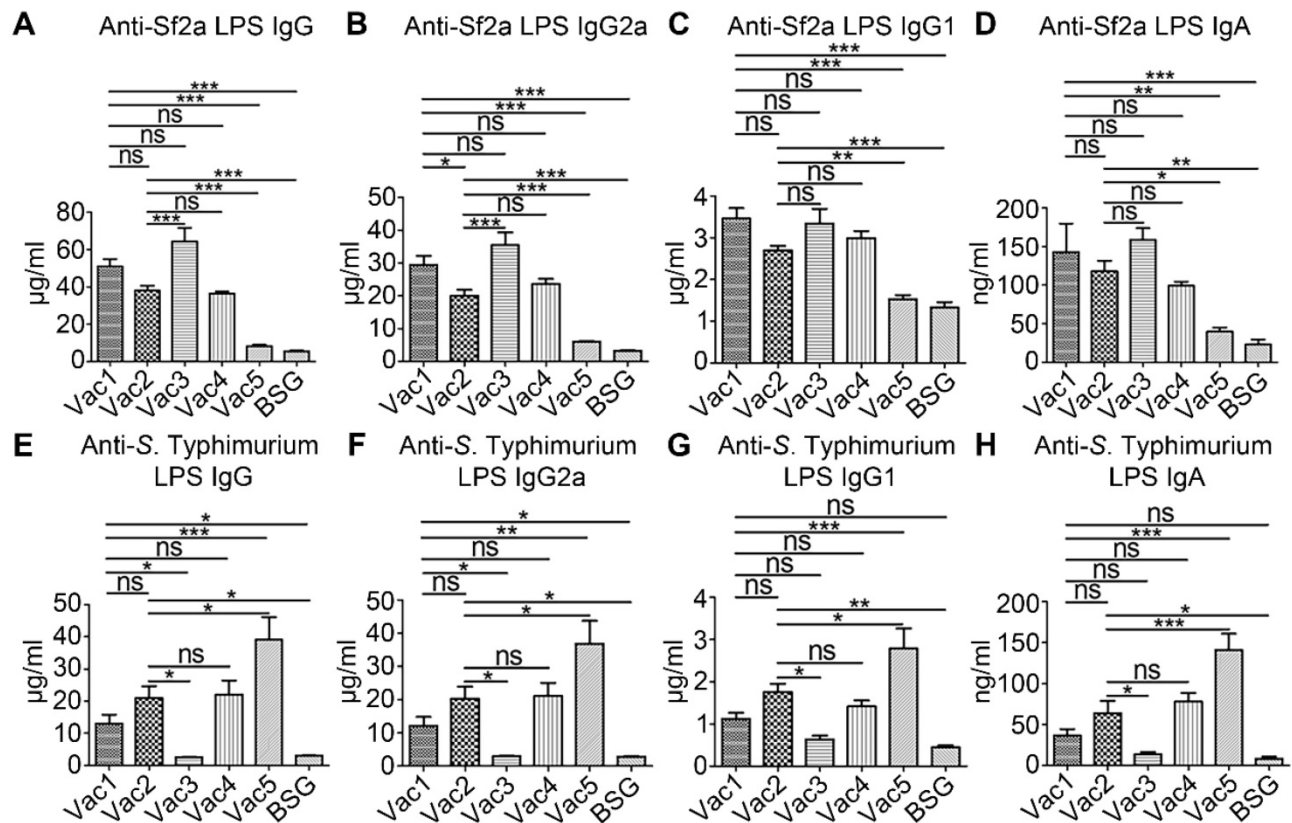


Sf2a and *S. Typhimurium* O-antigen) immunized group. The Vac5 (producing only *S. Typhimurium* O-antigen) group indicated significantly increased

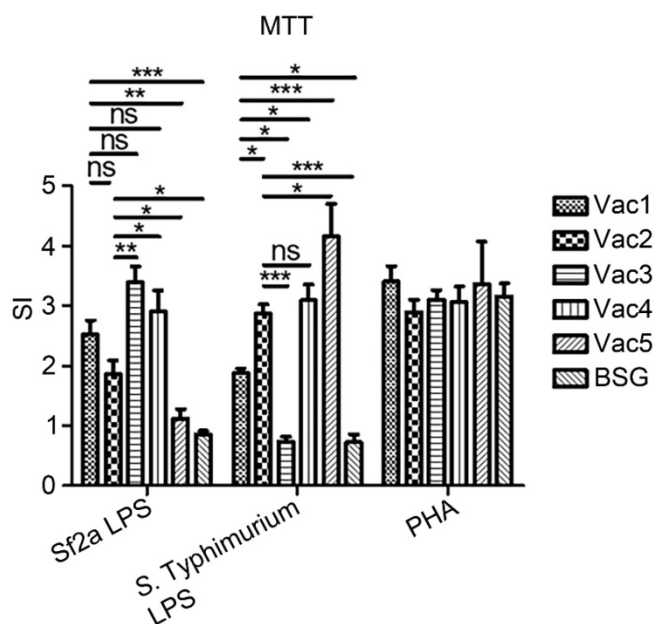
vaginal IgA against *S. Typhimurium* LPS relative to all other treatment groups.



**Figure 3.** Cytokine expression of CD4<sup>+</sup> T cells stimulated with Sf2a or *S. Typhimurium* LPS *ex vivo* at 7 days after primary immunization. Bar graphs depicted the proportions of antigen-specific cells expressing IFN $\gamma$  (A) and IL-4 (B), which were calculated by deducting the percentages of cytokine-positive cells of unstimulated samples from the antigen-stimulated values of corresponding samples. Data were summarized from two (n=6) independent experiments. \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ .



**Figure 4.** Systemic and mucosal antibody concentrations against Sf2a and *S. Typhimurium* LPS. Serum IgG, IgG2a, IgG1 and mucosal IgA against Sf2a LPS (A-D) and *S. Typhimurium* LPS (E-H) were measured by ELISA at 14 days after secondary immunization. The RDAS strains (Vac1 and Vac2) could stimulate comparable levels of Sf2a LPS-specific antibody responses with the constitutive antigen synthesis strains (Vac3 and Vac4). \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ .



**Figure 5. Spleen cell proliferation in response to Sf2a and *S. Typhimurium* LPS.** Spleen cell suspensions were prepared from mice immunized with indicated *Salmonella* strains or BSG at 7 days after primary immunization. Cells ( $3 \times 10^5$  cells/well) were stimulated by Sf2a LPS, *S. Typhimurium* LPS, PHA (positive control), or culture medium (negative control) for 72 h. Cell proliferation was measured with the MTT based colorimetric assay. \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ .

### RDAS strains stimulated Sf2a LPS-specific splenic lymphocyte proliferation

Priming of cell-mediated immune responses plays important roles in inducing protective immunity against *S. flexneri* infections [35]. The proliferative responses of spleen cells in response to Sf2a LPS and *S. Typhimurium* LPS stimulation were measured 7 days after primary immunization (Fig. 5). Both of the Vac1 and Vac2 RDAS strains stimulated significantly elevated Sf2a LPS-specific proliferation compared to the Vac5 and the BSG control groups. The Vac1 strain, synthesizing Sf2a O-antigen *in vivo* and *S. Typhimurium* O-antigen *in vitro*, demonstrated comparable capability in stimulating Sf2a LPS-specific splenocyte proliferation to the strains constitutively producing Sf2a O-antigen (Vac3 and Vac4). The Vac2 strain, which produced Sf2a O-antigen *in vivo* and constitutively synthesized *S. Typhimurium* LPS, induced equivalent splenocyte proliferation upon Sf2a LPS stimulation compared to Vac1. However, the splenocyte proliferation of the Vac2 groups was significantly lower than that of the Vac 3 (constitutively producing Sf2a O-antigen only) and Vac4 (constitutively producing both of Sf2a and *S. Typhimurium* O-antigen) groups. When stimulated with *S. Typhimurium* LPS, the splenocyte proliferation in Vac1 group was significantly higher than the Vac 3 immunized and the BSG treated groups. The *S. Typhimurium* LPS-specific splenocyte

proliferation in the Vac2 group was greater than Vac1, but markedly lower than that of Vac5 (empty plasmid control, producing only *S. Typhimurium* LPS).

### RDAS strains elicited Sf2a LPS-specific B<sub>M</sub> and Tfh cell responses

Memory B cell (B<sub>M</sub>) responses to *Shigella* antigens, including LPS, have been indicated to be a suitable surrogate of protection in shigellosis [37]. Memory B cells are believed to be differentiated from germinal center (GC) B cells, with the help of Tfh cells, which may provide support for GC induction, B cell affinity maturation, and generation of memory B cells [38]. Memory Tfh cells may also provide rapid assistance to B<sub>M</sub> cells in the spleen and lymph nodes (LNs) [39]. In this study, we measured both the Sf2a LPS-specific IgG<sup>+</sup> B<sub>M</sub> and Tfh cell responses by flow cytometry on Day 75 (61 days after secondary immunization) (Fig. 6). Upon stimulation with Sf2a LPS, the ratio of IgG<sup>+</sup>B220<sup>low/int</sup>CD80<sup>+</sup>IgD<sup>-</sup> B<sub>M</sub> cells [40, 41] in groups immunized with the RDAS strains (Vac1 and Vac2) was significantly increased relative to the Vac5 (empty plasmid control) and BSG treated groups. Moreover, the IgG<sup>+</sup>B220<sup>low/int</sup>CD80<sup>+</sup>IgD<sup>-</sup> B<sub>M</sub> cell responses in the RDAS treated groups were comparable to the groups treated with RASV strains constitutively producing Sf2a O-antigen (Fig. 6A and 6B). The ratio of IgG<sup>+</sup>B220<sup>high</sup>CD80<sup>+</sup>IgD<sup>-</sup> cells showed no difference among groups (data not shown). Numbers of Tfh cells (CD3<sup>+</sup>CD4<sup>+</sup>CD44<sup>+</sup>CD62L<sup>+</sup>CXCR5<sup>+</sup>PD-1<sup>+</sup>IL-21<sup>+</sup>) with or without Sf2a LPS stimulation were also detected and analyzed on Day 75. These findings indicated that Sf2a LPS-specific Tfh cell responses were significantly increased in the RDAS treated groups relative to the empty vector and negative control groups. The level of Tfh cell responses were comparable among the groups immunized with the RDAS strains (Vac1 and Vac2) and the groups administered with strains constitutively synthesizing Sf2a O-antigen (Vac 3 and Vac 4) (Fig. 6C and 6D).

### Serum bactericidal assay

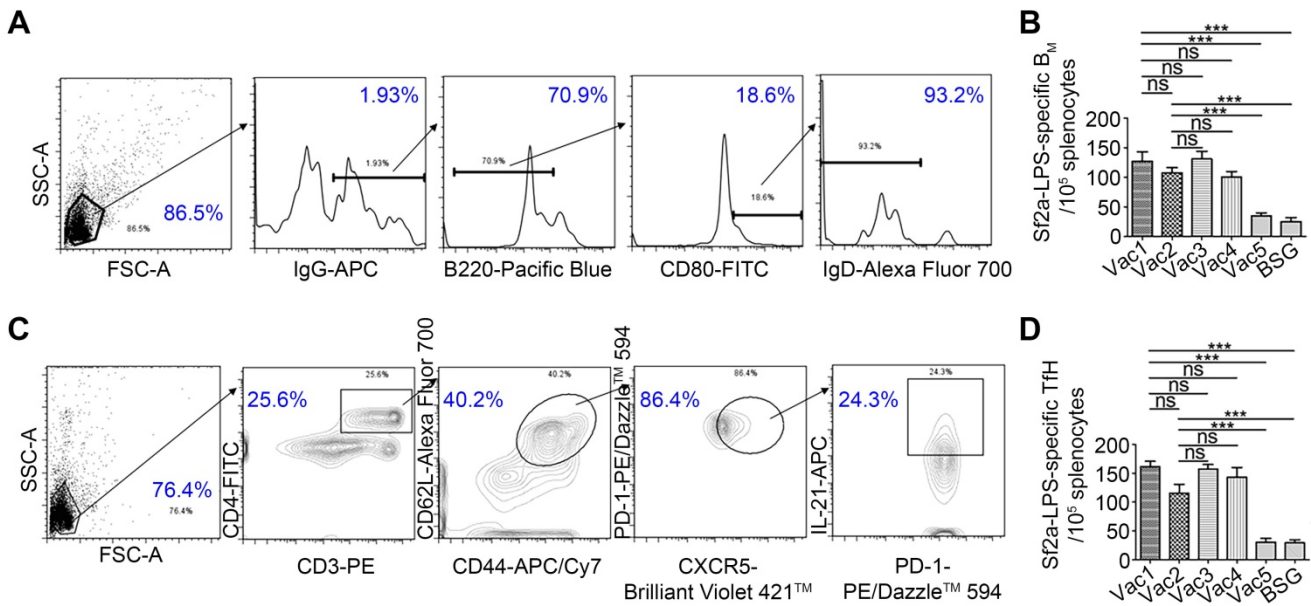
Serum bactericidal assays were performed to determine the functional capability of Sf2a-specific antibodies in killing 2457T through the complement-mediated pathway (Fig. 7A). Pooled serum samples collected from groups, which received the Vac1 strain (synthesizing Sf2a O-antigen *in vivo* and *S. Typhimurium* O-antigen *in vitro*) and the Vac3 strain (constitutively producing only Sf2a O-antigen) showed similar levels of killing capacities, which appeared to be more efficient than the other four groups in killing 2457T. The level of serum killing activity of the group immunized with Vac2

(producing Sf2a *in vivo* and constitutively synthesizing *S. Typhimurium* LPS) was equivalent to that of Vac4 (constitutively producing both of Sf2a and *S. Typhimurium* O-antigen) immunized group. The serum killing capacity of all experimental groups surpassed that of Vac5 (empty plasmid control) and BSG treated groups.

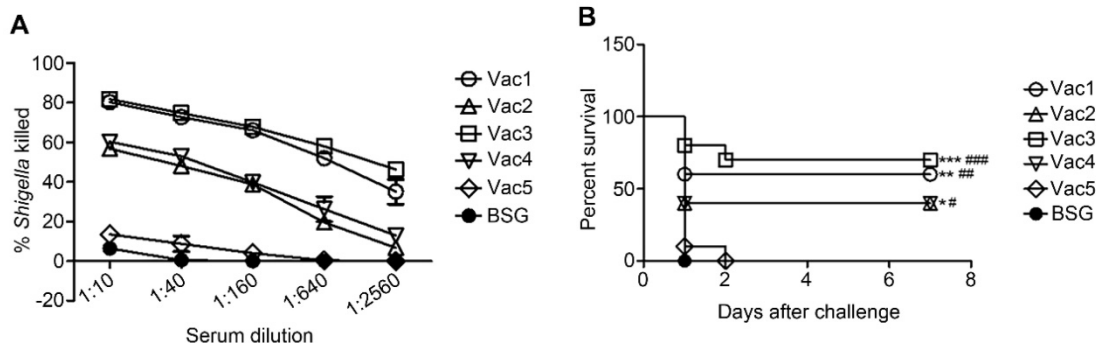
**Protection conferred by RDAS strains against Sf2a challenge**

A murine model system was utilized in order to evaluate the protective efficacy conferred by *Salmonella* strains synthesizing Sf2a O-antigen against virulent Sf2a challenge. BALB/c mice were orally immunized with Sf2a O-antigen-synthesizing strains administered in two identical doses, with the booster delivered two weeks following the primary immunization event. Two weeks after receiving the

second dose, mice were intraperitoneally challenged with  $5 \times 10^7$  CFU of virulent Sf2a strain 2457T. All RASV strains producing Sf2a O-antigen provided significant protection against Sf2a challenge compared to the empty-plasmid and BSG controls (Fig. 7B). Immunizations with the Vac1 and Vac3 strains (synthesizing only Sf2a O-antigen *in vivo*) demonstrated slightly enhanced levels of protection relative to the Vac2 and Vac4 strains (producing both of Sf2a O-antigen and *S. Typhimurium* O-antigen *in vivo*), although this trend was not statistically significant. The RDAS strain Vac1 (synthesizing Sf2a O-antigen *in vivo* and *S. Typhimurium* O-antigen *in vitro*) provided a comparable level of protection to the Vac3 strain (constitutively producing Sf2a O-antigen only).



**Figure 6.** B<sub>M</sub> cells and Tfh cells were determined by flow cytometry *ex vivo* on day 75 (61 days after secondary immunization). (A) Gating strategy used to identify B<sub>M</sub> cells. (B) The antigen-specific B<sub>M</sub> cells were calculated by subtracting the number of IgG<sup>+</sup>B220<sup>low/int</sup>CD80<sup>+</sup>IgD<sup>-</sup> B cells in the sample without stimulation from that stimulated with Sf2a LPS. (C) Tfh cells were gated as the CD3<sup>+</sup>CD4<sup>+</sup>CD44<sup>+</sup>CD62L<sup>+</sup>CXCR5<sup>+</sup>PD-1<sup>+</sup>IL-21<sup>+</sup> cells. (D) The Sf2a-LPS-specific Tfh cells were counted by subtracting the number of CD3<sup>+</sup>CD4<sup>+</sup>CD44<sup>+</sup>CD62L<sup>+</sup>CXCR5<sup>+</sup>PD-1<sup>+</sup>IL-21<sup>+</sup> cells in the unstimulated sample from that stimulated by Sf2a LPS. Data from two independent experiments (n=6) were statistically analyzed. \* P < 0.05, \*\* P < 0.01, \*\*\* P < 0.001.



**Figure 7.** Sf2a-specific serum bactericidal assays (A) and protection of mice against the challenge of virulent Sf2a strain 2457T (B). (A) Serially diluted serum from each group collected on Day 28 (two weeks after secondary immunization) were used to determine and assay the percentages of killed 2457T. (B) Female BALB/c mice immunized with 2 doses of vaccine strains ( $1 \times 10^9$  CFU per dose) expressing Sf2a O-antigen showed significantly greater protection against the challenge of 2457T ( $5 \times 10^7$  CFU per mouse) through the intraperitoneal route two weeks after the secondary immunization, compared to the empty-plasmid and BSG controls. \* P < 0.05 compared to BSG, \*\* P < 0.01 compared to BSG, \*\*\* P < 0.001 compared to BSG, # P < 0.05 compared to Vac5, ## P < 0.01 compared to Vac5, ### P < 0.001 compared to Vac5.



## Discussion

The key aspects of an ideal RASV system include complete attenuation of the live vaccine strain whilst retaining robust invasive capability, thereby supporting a robust immune response without compromising safety [1]. To achieve this objective, we combined the RDAS and RDEAP attributes to develop vaccine strains which exhibit controlled expression of the Sf2a O-antigen. In this system, during *in vitro* propagation in the presence of arabinose, the vaccine strain synthesizes native *Salmonella* LPS but represses the production of exogenous Sf2a O-antigen, and after invasion of the host tissues, in the absence of arabinose, the synthesis of native LPS is repressed and the transcription of exogenous Sf2a O-antigen biosynthesis is induced.

Both the Vac1 and Vac2 strains, which have the *araC* P<sub>BAD</sub> *lacI* cassette inserted into the chromosome, efficiently synthesize the Sf2a O-antigen when cultured in the absence of arabinose. It is important to note that the Sf2a O-antigen was still detected in Vac1 and Vac2 when cultured in the presence of 0.1% arabinose, indicating that the expression of a single copy of *lacI* partially represses the transcription of the Sf2a O-antigen genes under the control of the P<sub>trc</sub> promoter (Fig. 2B). Increased copy numbers of *araC* P<sub>BAD</sub> *lacI* may suppress the expression of exogenous antigen more efficiently. However, overexpression of *lacI* was reported to disrupt *S. Typhimurium* colonization in host tissues [42]. In this study, the level of LacI expressed from the single copy in our new RDAS system induced an efficient humoral immune response. The other approach to achieve the tight regulation of transcription of the Sf2a O-antigen gene cluster is via modification of a Shine-Dalgarno (SD) sequence and/or start codon of P<sub>trc</sub> promoter [14]. As this promoter needs to drive a 13kb gene cluster in our construct, we did not investigate the effects of alteration of SD sequence or start codon of P<sub>trc</sub> on the transcription of the Sf2a O-antigen gene cluster. Therefore, the use of single copy of *araC* P<sub>BAD</sub> *lacI* and a P<sub>trc</sub> carrying an appropriate SD sequence and/or start codon may be pursued in other RASV vaccine candidates in order to maintain a balance between attenuation and immunogenicity.

In this study, immunization with the RDAS strains (Vac1 and Vac2) induced significantly decreased production of IFN $\gamma$  and IL-4 in response to Sf2a LPS-stimulation compared to strains synthesizing Sf2a O-antigen constitutively (Vac3 and Vac4). This result is consistent with a previous study in which impaired cytokine immune responses were attributed to the expression level of LacI in the RDAS system [43]. Our findings suggest that the decreased

cytokine expression in this study may result from a delay in Sf2a O-antigen production at the early stage of bacteria colonization. The O-antigen of Gram-negative bacteria is synthesized by three groups of proteins, including proteins for nucleotide sugar biosynthesis, sugar transferases and proteins involved in O-antigen assembly and export [44]. Genes involved in nucleotide sugar biosynthesis and sugar transfer were cloned under the control of the regulated promoter in our system. As O-antigen biosynthesis is a multifactorial process, the controlled stimulation of O-antigen production may not be as efficient as regulated protein antigen expression. Thus, when colonizing the mammalian system, the induction of O-antigen biosynthesis by the RDAS candidates may suffer a detrimental lag phase. The potential lapse in O-antigen production may be responsible for the reduced stimulation of host immune responses relative to the constitutive antigen synthesis systems. This reminds us of the importance to consider about the lag phase in the initiation of adaptive immunity, when the RDAS vaccine strains are applied for delivering O-antigens in the future.

The synthesis of native *Salmonella* LPS O-antigen was controlled in this study through arabinose-regulated expression of WbaP. This RDAS system (Vac1) solely produced *Salmonella* native LPS *in vitro* in the presence of 0.1% arabinose, and gradually suppressed the synthesis of *Salmonella* native LPS upon host tissue invasion where free arabinose was no longer available. Moreover, due to the absence of arabinose in the mammalian system, inducible LacI expression was alleviated, thereby enabling synthesis of LPS containing exogenous Sf2a O-antigen. The Vac1 strain elicited increased anti-Sf2a-LPS IgG2a and serum bactericidal activity relative to the Vac2 strain, which lacked modified WbaP regulation and produced LPS containing both *Salmonella* native O-antigen and Sf2a O-antigen *in vivo*. This result indicated that depletion of native LPS synthesis *in vivo* may benefit the RASV by sparing nutrients and energy for producing exogenous antigens, thereby increasing antigen-specific immune responses. The Vac3 strain carrying a  $\Delta wbaP$  mutation (producing only Sf2a O-antigen constitutively) induced comparable protective immune responses to the Vac1 strain (synthesizing *S. Typhimurium* O-antigen *in vitro* and Sf2a O-antigen *in vivo*) upon 14 days after secondary immunization. This result suggests that constitutive expression of LPS containing the Sf2a O-antigen does not negatively influence the ability of RASV strain of Vac3 to invade the host and induce an efficacious immune response, indicating that smooth LPS of RASV is essential for initiating the colonization and invasion of host cells and later inducing immune

responses regardless of its mixed or solely O-antigen presence on the surface of RASV [45].

Natural protective immunity against *Shigella* infection is developed after several rounds of re-infection and is notably short-lasting [35]. Moreover, protective immunity elicited by natural infection is poorly established in young patients [46]. In this study, the RDAS strains producing LPS containing Sf2a O-antigen induced efficient IgG<sup>+</sup> B<sub>M</sub> and memory Tfh cell responses, of which the levels were comparable to those of the constitutive antigen synthesis strains. Furthermore, the RDAS strains induced robust humoral immune responses and provided significant protection against the challenge of wild-type Sf2a 2457T in a murine model system.

Although the RDAS system developed in this study shows promise for prevention of *Shigella* infection, additional refinement should be pursued before progressing to human trials. One major issue is the lack of clinically relevant non-primate animal models strictly mimicking human infection with *Shigella*. Previous studies have attempted to develop *Shigella* infection models using rabbits, guinea pigs and mice. As infection of the rabbit ileal loop can induce acute inflammation and tissue destruction, this model is more suitable for studying *Shigella* interactions with the intestinal barrier and subsequent induction of an innate immune response [47, 48]. In the guinea pig keratoconjunctivitis model, *Shigella* invades the corneal epithelia and spreads to adjacent cells, causing ulcerative conjunctivitis. However, due to the limited availability of immunological tools, characterizing the immune response induced in this model remains a challenging limitation [49]. Thus, mouse models are widely used due to the low cost, ease of handling, and availability of immunological research tools. Since oral infection with *Shigella* in adult mice does not cause diarrheal disease, the mouse pulmonary pneumonia infection model is commonly used to examine protective efficacy [50, 51]. However, the clinical irrelevance of the pulmonary pneumonia infection model remains a serious concern. A mouse model of shigellosis by intraperitoneal (IP) infection was successfully developed by Jin-Young Yang et al. to produce clinical symptoms that mimic human shigellosis [32] and this model was applied to predict protective efficacy of vaccine candidates targeting *Shigella flexneri* 2a and 3a infection [26]. In this study, we applied the IP infection model. We acknowledge that the immune responses elicited by this IP infection model do not entirely reflect natural human infection. Therefore, further studies are necessary in higher-level organisms, such as application of the intragastrically infected macaque monkey model, in order to fully

characterize the protective efficacy and immune responses elicited by the RASV candidates [52].

Another concern with live vaccine candidates pertains to the inherent risk of insufficient attenuation or virulence reversion, thereby causing adverse events. For the RDAS strains used in this study, attenuation was achieved through complete deletion of virulence genes, as opposed to introducing inactivating point mutations, which minimizes the likelihood of reversion. Furthermore, there are two well-established attenuating deletions ( $\Delta pabA1516$  and  $\Delta pabB232$ ) introduced to the candidate strains to further reduce the probability of reversion. It is important to note that wild-type *S. Typhimurium* generally causes a self-limited infection in humans [53], and prior studies have shown that an attenuated *S. Typhimurium* strain (WT05) elicits negligible clinical symptoms in a clinical trial [54]. Thus, the RDAS system has great potential for development of a safe, live-attenuated vaccine platform. The system we developed in this study to reduce the side effects of overexpressed foreign antigens in the vaccine strains provides an alternative to the constitutive antigen synthesis system in *Shigella* vaccine development based on the attenuated *Salmonella* strains producing exogenous O-antigen.

## Abbreviations

Sf2a: *Shigella flexneri* 2a; RDAS: regulated delayed antigen synthesis; RDEAP: regulated delayed expression of attenuating phenotype; RASV(s): recombinant attenuated *Salmonella* vaccine(s); WbaP: the Und-P galactose phosphotransferase; BSG: buffered saline with gelatin; Tfh cells: T follicular helper cells; B<sub>M</sub> cells: memory B cells; Rha: rhamnose; GlcNac: N-acetylglucosamine.

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## Competing Interests

The authors have declared that no competing interest exists.

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