Original article

Oral immunization of rat with chromosomal expression LipL32 in attenuated *Salmonella* vaccine induces immune respond against pathogenic *Leptospira*

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Purpose: Leptospirosis caused by *Leptospira* spp. remains a global health problem. Available commercial leptospiral vaccines have shown an ineffective prevention for leptospiral infection. The aim of this study was to develop leptospirosis vaccine using recombinant attenuated *Salmonella* vaccine (RASV) as a platform. We expected that this vaccine has ability to continuous and strongly stimulate immune systems including protective mucosal, humoral, and cell mediated immunity in rat model.

Materials and Methods: In this study, we engineered RASV, NRSL32 strain containing chromosomal fusion between nucleotides encoding secretion signal of SPI-2 effector protein, SspH2 and gene encoding major pathogenic leptospiral outer membrane lipoprotein, LipL32. Subsequently, our modified RASV was oral vaccination to rat and blood samples were taken for assessment of immune responses.

Results: Our *Salmonella* NRSL32 strain showed expression and secretion of $SspH2_{1-215}$-LipL32 recombinant protein via SPI-2 T3SS. After oral administration of NRSL32 strain to rats, significant titers of total immunoglobulin G (IgG) and immunoglobulin A against rLipL32 were observed in long period up to 77 days after vaccination. The stimulated antibody showed ability to specific bind with LipL32 protein on surface of pathogenic *Leptospira* spp. Additionally, the balance level of IgG2a/IgG1 ratio and level of interferon-γ and interleukin-4 secretion were detected.

Conclusion: The results showed that our RASV platform with chromosomal expression elicited effective immune responses to leptospiral antigen. Moreover, this platform was capable for simultaneous stimulation of Th1 and Th2-biased responses. Further investigation is necessary study of protective efficacy against leptospiral infection in animal models.

Keywords: Recombinant attenuated *Salmonella* vaccine, Leptospirosis, LipL32

Introduction

Leptospirosis is a worldwide serious zoonotic disease. It causes by a gram-negative spiral bacterium.

Leptospira is classified as a pathogenic and saprophytic species. The pathogenic *Leptospira interrogans* (*L. interrogans*) is the most prevalent genospecies in the world [1]. The infection is predominantly found in developing countries with tropical climate regions. High incidence and mortality rates have been frequently recorded in Oceania,

Latin America, and South East Asia especially Thailand [2]. Pathogenic leptospiral can infect both human and vertebrate animals. The spirochetes are transmitted to humans by directly contacting with contaminated urine or indirectly contacting with an environment contaminated with urine from infected animals [3]. An infected person presented various severity from no symptoms to severe complications such as respiratory distress syndrome, acute interstitial nephritis, and even fatal death [4,5]. Effective host immunity is a key mechanism to inhibit and eliminate their infection [6]. Vaccination is a method to trigger stronger and persistent host immune responses to protect against leptospiral infection. The important consideration about the leptospirosis vaccine is how to develop a vaccine that protected all more than 250 pathogenic serovars. Although, currently available killed whole cell leptospiral vaccines have been accepted to protect only homologous *Leptospira* serovars infection. Furthermore, these vaccines also revealed side effects and short-term immunological responses [7,8]. Several potential recombinant subunit vaccine candidates have been developed to overcome their cross-serovars protection and stimulated long lasting immunity. Many of these target antigens have focused on leptospiral outer membrane proteins. The most abundant leptospiral outer membrane protein is 32 kDa lipoprotein, LipL32, or its synonym, hemolysis-associated protein-1 [9]. This lipoprotein is constitutively expressed in all pathogenic *Leptospira* spp. during acute or convalescent mammalian infection [10]. Contrasting, this protein is not available in saprophytic *Leptospira*, *L. biflexa* [11]. Thus, LipL32 was considered to be a potent immunogen that elicited outstanding protective immune responses during infection. In addition, a highly conserved at both genetic and protein levels of LipL32 is prominent factor of pathogenic strain to instantly regarded for candidate vaccine antigen development [12].

An improved recombinant antigen deliver system using *Salmonella* based vaccine carrier has been developed to export foreign antigen from several viruses, parasites and bacterial pathogens to enhance host immunity against infectious diseases [13]. Asymptomatic *Salmonella* strain has several advantages over other vaccine delivery systems including completely capability to invade mucosal lymphoid tissue and entering systemic compartments of the immune system to elicit fully potency of mucosal, humoral and cellular responses [14,15]. A well-known genomic and characteristic resulting in easy for genetic modification and manipulation [16]. These properties supported the rational design and generation of recombinant attenuated *Salmonella* vaccine (RASV).

The antigen transporting platform of RASV mimics natural function of infection of *Salmonella* to translocate virulence effector proteins into host cell depending on two distinct type III secretion systems (T3SS). During the infection, the T3SS encoded in *Salmonella* pathogenicity island 1 (SPI-1 T3SS) is required for initial invasion into eukaryotic cell. After that, intracellular *Salmonella* required second type of T3SS that located in *Salmonella* pathogenicity island 2 (SPI-2 T3SS) for survival and systemic infection [17-19]. Oral administration of RASV allows infection at gut-associated lymphoid tissue (GALT) through M cells of Peyer's patches. After bacteria invade into host cells, these bacteria colonize in deeper tissues. The bacteria are capable to reach the mesenteric lymph nodes and spleen without causing symptoms when carrying heterologous antigens [20]. Following the bacterial invasion into antigen presenting cells (APCs), *Salmonella* spp. remains permanently localization in a membrane-bound vacuole also known as the *Salmonella*-containing vacuole (SCV). In this stage, RASV regularly produced recombinant protein for adequate period under control of SPI-2 conditions and then translocated into cytosol of APC via SPI-2 T3SS. Secreted peptide are processes and presents with major histocompatibility complex (MHC) class I and MHC class II on APC to stimulated T cell responses [17]. Persistent and continuous motivation by *Salmonella* carrier is considerable as the advantage of strategy for long term protection to combat pathogens.

Several previous studies illustrated that live attenuated bacterial vaccines harboring recombinant multi-copies plasmids are widely used for vaccination. However, the main argument about genetic instability has been reported [21]. One technical capability to stable delivery of heterologous antigens has been an integration of recombinant antigen genes into the live attenuated vector chromosome. In this study, we engineered attenuated *S. enterica* serovar Typhimurium *ΔaroA* harboring *ssph2-lipl32* gene integration in the bacterial chromosome. This recombinant stain potentially expressed, secreted, and translocated the SspH2-LipL32 fusion antigen. After oral immunization, RASV carrying chromosomally encoding recombinant antigen accomplished to induce effective mucosal and systemic immune responses against *Leptospira* infection. Thereby, this article has been illustrated the usefulness of RASV as a candidate orally-delivered leptospirosis vaccine.

Material and Methods

Bacterial strains, plasmids, and growth conditions

Salmonella, Escherichia coli strains and plasmids are listed in Table 1. *S.* Typhimurium SL3261 stain was purchased from *Salmonella* genetic stock center, University of Calgary, Canada. For SPI-2 induction condition [22], *S.* Typhimurium were grown in Luria-Bertani (LB) medium for overnight, and diluted 1:100 into M9 minimal medium, pH 4.5 with aromix solution (40 μg/mL each of L-phenylalanine, L-tryptophan, and L-tyrosine, and 10 μg/mL each of 4-para-amino benzoic acid and 2, 3-dihydroxybenzoic acid). Bacterial cells were grown for overnight at 37°C and 200 rpm *E. coli* strain BL21 was used for protein overexpression. *E. coli* was grown in LB medium containing appropriate antibiotic at 37°C, 200 rpm. The expression of recombinant proteins was induced with 0.5 mM IPTG and left for 3 hours at 37°C.

Plasmids and chromosomal fusion in SL3261 strain constructions

All recombinant DNA techniques used were based on standard protocols [23]. The primers were synthesized by Macrogen (Seoul, Korea). The primers used in this work are listed in Table 2. The polymerase chain reactions (PCRs) were carried out using Phusion DNA Polymerase (Finnzymes, Woburn, MA, USA), and ligations were performed using T4 DNA ligase (Fermentas, Waltham, MA, USA).

DNA fragments encoding kanamycin resistant gene were amplified using pKD13 as template and a set of KFR primers. The PCR products were digested with *EcoR*I and cloned into pGRK32 by utilizing the same restriction enzyme to produced pGRK32K recombinant vector [24]. This vector containing kanamycin resistant gene (kan^R) was downstream from the DNA fragments encoding secretion-signal less LipL32 (LipL32 $_{\Delta 1-20}$).

S. Typhimurium NRSL32 strain was engineered from SL3261 strain. This strain was chromosomally in-frame fusion between gene encoding amino acids 1–215 of SspH2 and secretion signal less LipL32 with strep-tag epitope on C-terminus protein using λ Red recombination system as described by Datsenko and Wanner [25]. Genes fusion cassettes were amplified using pGRK32K plasmid as a template and a set of SH32 primers. This primer set had 40 nucleotides complementary to nucleotides encoding SspH2 residues 203–215 and nucleotides locating downstream stop-codon of *ssph2* gene of the *Salmonella* chromosome followed 20 nucleotides

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Properties	Reference or source
Salmonella Typhimurium strains		
SL3261	*ΔaroA*	*Salmonella* genetic stock center
NRSL32	*ΔaroA, sspH2₁₋₆₄₅::lipl32* strep-tag	This study
Escherichia coli strains		
BL21 (DE3)	F–, *omp*T, *hsd*SB (rB–, mB–), *gal, dcm* (DE3)	Novagen
Plasmids		
pKD46	Red recombinase expression plasmid	Datsenko et al. [25] (2000)
pKD13	Template plasmid for kanamycin cassette	Datsenko et al. [25] (2000)
pCP20	Flp recombinase expression plasmid	Datsenko et al. [25] (2000)
pGRK32	pGEX-2T derivative encoding secretion signal less LipL32	Thongsukkaeng et al. [24] (2018)
pGRK32K	pGRK32 derivative containing kanamycin resistance gene	This study

Table 2. Primer sets and primers used in this study

Primer set or primer	Forward primer sequence (5´-3´)[a]	Reverse primer sequence (5´-3´)[a]
KFR	CGGgaattcAGATTGCAGCATTACACGTCT	GATgaattcTCAAACATGAGAATTAATTCCG
SH32	CAATGCAGTGCTTAACGTGGGA	GGAATATCTTTGTCGCACCGCACCT
	GAATCAGGTCTTACCACCT*GTGG*	CATTCACCTGGTGCAT**CAAACATGA**
	TGCTTTCGGTGGACT	**GAATTAATTC**
ssph checking, (ssphF and ssphR)	CGCACCAGACTGAAGCGCTGGAGTG	GGCCTGAACAATACAACGCCC

Underlined-case type indicates complementary sequences to *Salmonella ssph2* gene loci. Italic-case type indicates complementary sequences to *lipl32* gene. Bold-case type indicates complementary sequences to *kan^R* gene.
[a] Lower-case type indicates an engineered restriction enzyme site.

complementary to the *lipl32* and *kan*[R] fragments in pGRK32K, respectively. DNA fragments of genes fusion cassettes were purified on Qiagen column and used for electroporation of *S.* Typhimurium SL3261 harboring pKD46 for expression of red recombinase. Kanamycin-resistant transformants were selected at 37°C. Then, the kanamycin-resistant gene was eliminated after transformation with pCP20. The proper chromosomal insertion in mutant strains was confirmed by PCR and a set of ssph checking primer for checking the insertion of fusion cassette into *ssph2* gene. The sequence of genes fusion was verified by DNA sequencing (Macrogen).

Protein expression and secretion analysis

To evaluate protein secretion, *S.* Typhimurium NRSL32 strain was grown in SPI-2 T3SS inducing condition as described above. Bacterial cells and culture supernatants were separated by centrifugation at 12,000 ×g for 20 minutes. Culture supernatants were filter-sterilized (0.22 μm pore-size), and proteins were precipitated with 10% trichloroacetic acid (TCA) and acetone. Bacterial cells and culture supernatant samples were electrophoresed on 12% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by Western blotting.

Determination of bacterial protein expression and translocation in HeLa cells

HeLa cell lines were purchased from the American Type Culture Collection and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum. The cells were grown in 24-well culture plates or on glass cover slips. Bacteria were grown in LB medium at 37°C to optical density (OD)$_{600}$ approximately 0.5 and washed once with phosphate-buffered saline (PBS). Bacteria were diluted in DMEM and added into the HeLa cells at multiplicity of infection of about 10. After infection for 30 minutes, uninfected bacteria were killed with DMEM supplemented with 150 μg/mL gentamycin. Protein expression or translocated protein was performed by Western blotting or immune-fluorescence analysis after intracellular infection for 16 hours.

For protein expression analysis, the cells were lysed with lysis buffer containing 1% Triton-X 100 for 30 minutes at room temperature. Cellular lysis supernatants were filter-sterilized (0.22 μm pore-size), and then the filtrates were precipitated with 10% TCA and acetone. The precipitated samples were analyzed by SDS-PAGE and Western blotting with polyclonal antibody against strep-tag (IBA Lifesciences

GmbH, Göttingen, Germany).

Protein translocation was determined using immune-fluorescence analysis. The cells on coverslips were fixed with 0.05% glutaraldehyde for 30 minutes and then permeabilized with 0.5% Triton-X 100 for 30 minutes at room temperature. The samples were blocked with blocking solution containing 5% bovine serum albumin (BSA) for 2 hours. The fixing cells were stained goat anti-*Salmonella* CSA-1 polyclonal antibody conjugated with fluorescein isothiocyanate (FITC; SeraCare, Milford, MA, USA) at dilution of 1:10,000. The SspH2$_{1-215}$-LipL32 tagged with strep-tag epitope was detected indirectly with polyclonal antibody against strep-tag (IBA Lifesciences GmbH) at a dilution 1:20,000. Secondary antibody, goat anti-rabbit antibody Rhodamine conjugation (IBA Lifesciences GmbH) were used for detection of strep-tag epitope at a dilution of 1:10,000. All antibodies were performed for 2 hours at room temperature. Eukaryotic nucleus was counterstained with DAPI for 30 minutes at room temperature. The samples were analyzed using Zeiss LSM700 fluorescence microscope (Zeiss, Oberkochen, Germany).

Immunization experiments

Female Sprague Dawley rat (8 weeks old) were purchased from Nomura Siam International Co. Ltd., Bangkok, Thailand. They were housed at the Department of Experimental Animal, National Institute of Animal Health, Thailand. For vaccination, bacterial strains were grown overnight in LB broth at 37°C. These bacterial cultures were diluted 1:100 into fresh LB medium. Bacterial cells were grown to approximately OD$_{600}$=0.5 at 37°C with shanking. Bacterial pellets were resuspended in PBS. Groups of 5 female rats were immunized with a dose of 1×10^7 colony-forming unit (CFU)/rat of various *S.* Typhimurium strains or PBS alone at day 0, 14, and 28. Blood samples were collected from lateral tail veins before vaccination and at day 7, 21, 35, and 77 post-infection. Blood samples were centrifuged at 3,000 ×g for 10 minutes. The serums were removed and stored at -70°C.

Assessment of antibodies response against vaccination
Antigen preparation

The GST-LipL32 recombinant protein was produced and purified as describes [24]. The GST-tag was removed from rLipL32 protein by Thrombin protease. The concentration of purified protein was determined using Bradford protein assay.

Determination of antigen specific antibody titer and Isotyping

Antibody against LipL32 protein from rat serum were determined using immunoglobulin (Ig) Isotyping Rat enzyme-linked immunosorbent assay (ELISA) kit (Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturer's protocol. Briefly, the purified LipL32 (5 μg/mL) was coated on 96-well immunoplate and blocked with blocking buffer containing 5% BSA at 37°C for 2 hours. For determination of the immunoglobulin G (IgG) specific antibody titer against LipL32 protein, rat serums at day 21, 35, and 77 post-infection were prepared two-fold serial dilution in blocking buffer. Then, diluted rat serum was added into ELISA wells containing antigens. Antigen-antibody interaction were reacted with mouse anti-rat IgG monoclonal antibody. The detecting antibody; horseradish peroxidase (HRP)-conjugated anti-mouse Ig (H+L) polyclonal antibody was added and incubated at 37°C for 1 hour. The substrate TMB solution was added in reaction wells and incubated for 20 minutes at room temperature. Then the reaction was stopped with 1 M HCl. The absorbance was read at 450 nm with EnSpire Plate Reader (PerkinElmer, Waltham, MA, USA). For isotyping analysis, rat serum at day 77 was used as samples. Isotyping were analyzed were according to steps mentioned above. Antibody interaction with LipL32 antigen was classified into isotypes with mouse anti-rat monoclonal antibody against rat IgG1 or IgG2a or immunoglobulin A (IgA).

Analysis of serum specific-reactivity

Rat serum were detected for specific antibodies against LipL32 proteins on leptospiral membranes by indirect immunofluorescent assay (IFA). Formalin killed pathogenic and non-pathogenic *Leptospira* spp. 24 strains were used in this experiment shown in Supplement 1. These killed bacteria were air dried on glass slides and then fixed with 4% paraformaldehyde for 30 minutes at room temperature. Slides were washed twice with PBS and stained with FITC-conjugated anti-rat IgG (H-L) polyclonal antibody (IBA Lifesciences GmbH). The samples were analyzed by using Zeiss LSM700 fluorescence microscope (Zeiss).

Measurement of cytokine profiles

The level of interferon-γ (IFN-γ) and interleukin-4 (IL-4) in rat serum or culture medium from re-stimulation of rat splenocytes were directly measured using a rat cytokine ELISA kit (Sigma Aldrich, Burlington, MA, USA) according to the manufacture's protocol. For preparation of sample from antigen-specific cytokine responses upon re-stimulation *in vitro*, rat splenocytes approximately 1×10^6 cells/mL were seed in 12-well culture plate supplement with DMEM medium. The cells were stimulated with 20 μg/mL of rLipL32 antigen or medium alone for 72 hours at 37°C in 5% CO_2 condition. The culture mediums were collected for determination of cytokines production. For cytokines detection, the rat serum or culture supernatants were added into detection wells coating with specific cytokine antibody and incubated overnight at 4°C. Antibody-cytokine complexes were bound with biotinylated detection antibody. Subsequently, HRP-Streptavidin solution was added and incubated at room temperature for 1 hour. Then, the reaction was stopped with 1 M HCl. The absorbance was read at 450 nm with EnSpire Plate Reader (PerkinElmer).

Statistical analysis

All statically analyses were performed using GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA). The data were presented as mean and standard deviation. One-way analysis of variance with Turkey-Kremer's *post-hoc* was used to consider the statistical significance at a p-value of <0.05.

Ethics statement

The animal studies were performed after receiving approval of the National Institute of Animal Health Ethics Committee (no., EA-004/62(R)).

Results

Integration of genes fusion cassettes into *Salmonella* chromosome for secretion of fusion proteins

Several studies reported that fusion proteins between N-terminus of SPI-2 effector protein with heterologous protein was able translocation from SCV into cytoplasm of host cells [26]. In this study, we engineered *S.* Typhimurium NRSL32 strain harboring chromosomally in-frame fusion between gene encoding N-terminus (residues 1–215) of SPI-2 T3SS effector protein, SspH2 and secretion signal less LipL32 with strep-tag epitope on C-terminus protein using λ red recombination system. Genes fusion cassettes were amplified using pGRK32K plasmid as template. The genes fusion cassettes were exchanged to chromosomal *sspH2* loci of *Salmonella*. Schematics for construction of Typhimurium NRSL32 strain was shown in Fig. 1A. After kanamycin-resistant gene was cured by pCP20, the resulting mutant strains carrying insertions of
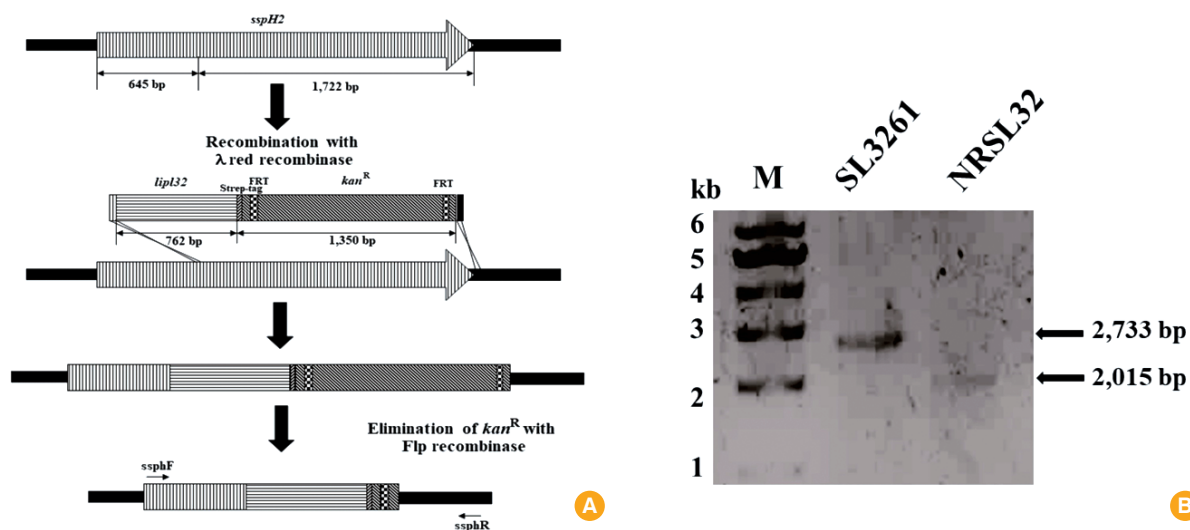
Fig. 1. Recombineering of *Salmonella* Typhimurium NRSL32 strain containing nucleotides encoding secretion signal less LipL32 using λ red recombination system. (A) Schematic diagram of *S.* Typhimurium NRSL32 strain construction. (B) Polymerase chain reaction products from *Salmonella* parental SL3261 and NRSL32 strains using primers, ssphF and ssphR.

genes fusion cassettes in the chromosome were confirmed by PCR with ssph checking primers. Successful genes fusion cassettes recombination bacteria generated PCR product of 2,015 bp after amplification with ssph checking primers set, while *S.* Typhimurium SL3261 parental strain produced approximately a 2,700 bp of DNA fragment (Fig. 1B).

Protein expression, SPI-2 T3S, and translocation of *S.* Typhimurium NRSL32 strain

To investigate the ability of expression, SPI-2 T3S and translocation of recombinant proteins from chromosome in *Salmonella* NRSL32 strain. Bacterial strains were cultured in SPI-2 inducing medium. The bacterial cells and culture supernatant were analyzed for recombinant $SspH2_{1-215}$-LipL32 protein. Results from Western blotting demonstrated that fusion proteins were expressed and secreted into the supernatant of the NRSL32 mutant strain. To rule out cell lysis, we also detected for the intracellular protein, DnaK, which was not detected in the same supernatant fractions (Fig. 2A).

In cell culture experiment, HeLa epithelial cells were invaded with the SL3261 parent or NRSL32 mutant strains for 30 minutes. Cells were lysed, subsequently, filtered cytoplasmic fraction was analyzed by Western blotting. Protein $SspH2_{1-215}$-LipL32 was detected in cytoplasmic sample of the NRSL32 mutant strain, but was undetectable in the sample from the SL3261 parent strain (Fig. 2B). For translocation investigation, immune-fluorescence staining techniques was performed to analyze the recombinant protein translocation

in cells. Results from immunofluorescence illustrated that the fluorescent signals were exhibited from NRSL32 mutant strains infected HeLa cells. The fluorescent spots were located in the cytoplasm of host cells. Whereas, fluorescent spots from chimeric proteins were not detected in HeLa cells infected with the SL3261 parent strain (Fig. 2C).

These data indicated that RASV engineering strain, NRSL32 is capable in expression, secretion, and translocation of fusion protein consisting of N-terminus of SPI-2 effector protein, SspH2 and LipL32 leptospiral antigen to cytosol of host cells.

Determination of rat specific humoral immune response and IgG isotype after oral immunization with *S.* Typhimurium NRSL32 strain

To determine the antibody response against leptospiral protein, LipL32. Sprague Dawley rats were orally immunized 3 times at 14-day intervals with PBS, 1×10^7 CFU of parental SL3261 strain, or NRSL32 vaccine strain. Rat sera was collected on day 0, 7, 21, 35, and 77 after vaccination. All serum samples were analyzed for LipL32-specific IgG antibodies by ELISA. As shown in Fig. 3A, any immune responses were not observed in all group of pre-immunized rats on day 0. Total IgG antibody titers against LipL32 were detected 7 days after the primary immunization in rat inoculated with NRSL32 strain. After the third immunization at day 28, anti-LipL32 titers of rats immunized with NRSL32 strain were significantly higher than rats immunized with PBS or SL3261 strain. Additionally, the
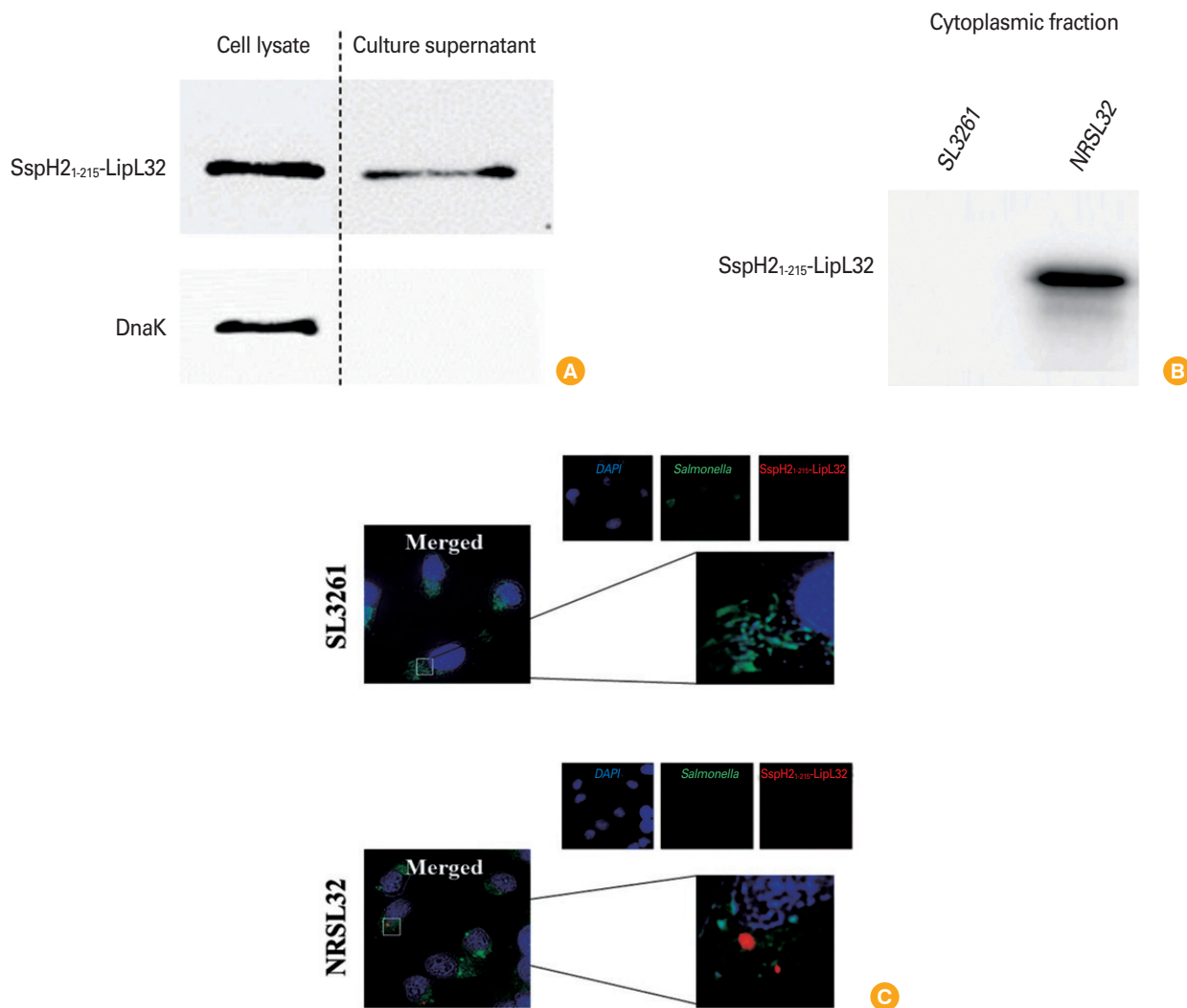
Fig. 2. NRSL32 strain is capable expression, secretion, and translocation of SspH2$_{1-215}$-LipL32 protein in SPI-2 induction condition. (A) Evaluation of expression and secretion of recombinant SspH2$_{1-215}$-LipL32 protein in NRSL32 strain. The control protein DnaK was not secreted in culture supernatant. Results from Western blotting (B) and immuno-staining (C) indicated that the experimental vaccine strain NRSL32 was able to translocate recombinant SspH2$_{1-215}$-LipL32 protein in cytoplasm of HeLa cells. Cells were fixed and stained using DAPI for nucleus (blue), anti-*Salmonella*-fluorescein isothiocyanate antibody to detect bacteria (green), the SspH2$_{1-215}$-LipL32 strep-tag (red). Area in the white box has been magnified.

serum IgG titers against LipL32 were still observed by day 77 in vaccinated group. This observation indicated that *Salmonella* NRSL32 strain was able to stimulate humoral immunological responses against LipL32 in long lasting up to 77 days.

We further analyzed anti-LipL32 IgG antibody subclasses. IgG1 and IgG2a isotypes against LipL32 protein were determined in rat sera day 77 after the final vaccination. As shown in Fig. 3B, high serum IgG1 and IgG2a titers against LipL32 were observed at day 77 in rat immunized with NRSL32 strain. There was a significant difference between IgG1 and IgG2a titers from NRSL32 group and PBS or SL3261 group. From previous study showed that RASV was able to invade

into GALT and sufficient to induce mucosal immune response. We analyzed the mucosal immune responses to LipL32 by IgA titer determination. High titers of anti-LipL32 IgA antibody from NRSL32 group were detected at day 77. The levels of anti-LipL32 IgA antibody in rat vaccinated with NRSL32 strain were significantly higher than IgA level in the rat immunized by PBS or SL3261 strain (Fig. 3C).

To verified the septicity of induced antibody by NRSL32 strain in rat. Immunized rat sera containing anti-LipL32 antibody were tested specifically binding to natural LipL32 protein on leptospiral membranes. Results from IFA showed that there were fluorescent signals from interaction between anti-
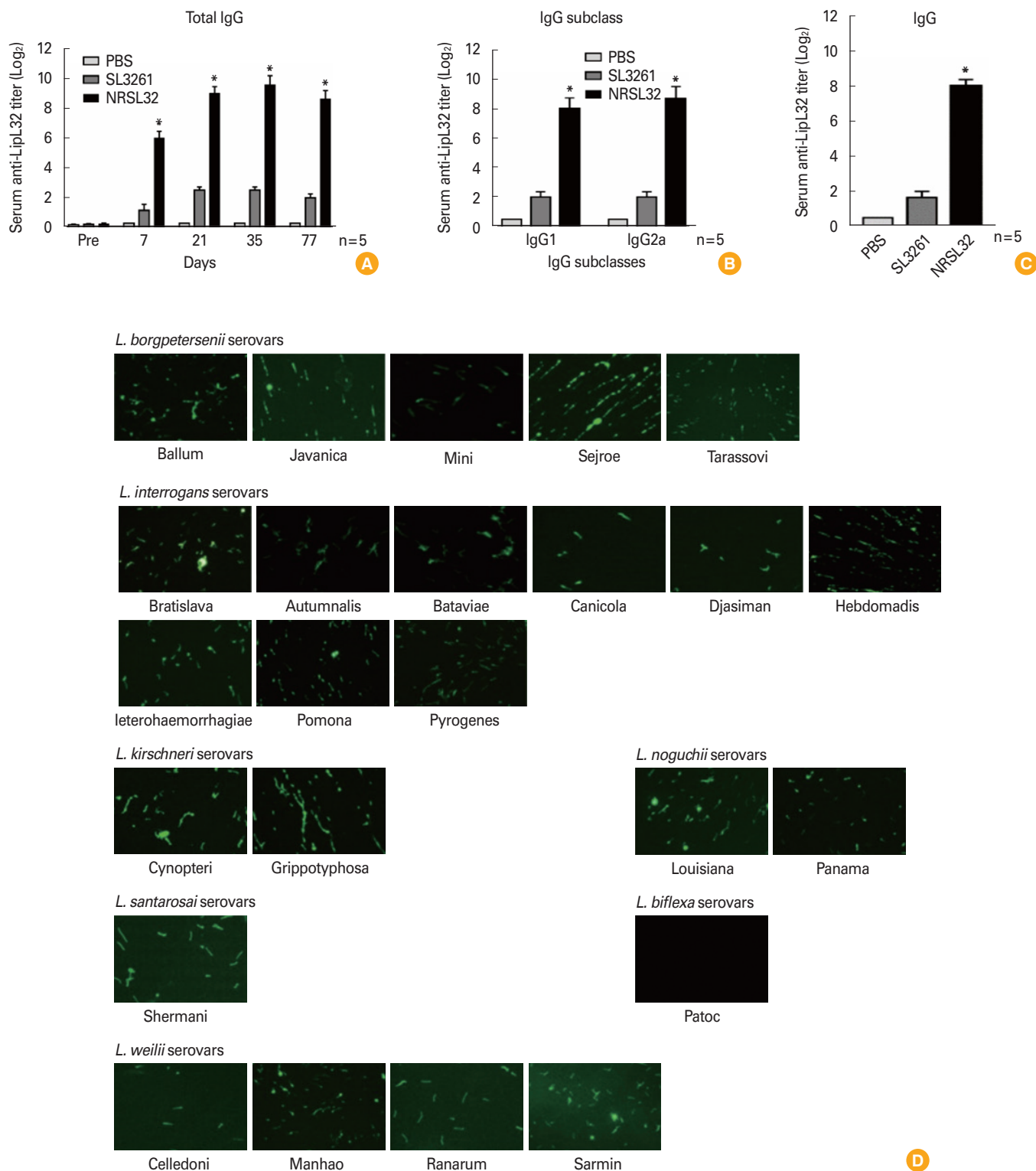
Fig. 3. Antibody stimulation in rats after oral vaccination with NRSL32 strain. (A) Serum immunoglobulin G (IgG) reponses to LipL32 protein in phosphate-buffered saline (PBS), parental SL3261 strain, or NRSL32 strain on day 0, 7, 21, 35, and 77. Subclasses IgG1 and IgG2a (B) or immunoglobulin A (IgA) (C) were measured in rat serums at 77 days after first immunisation. The standard deviations from five individual rats per group are indicated. Asterisks indicate significantly different (p<0.05) with rats immunized with PBS and SL3261 strain in each time. (D) Induced antibody is specific binding to native LipL32 protein on pathogenic leptopira: *Leptospira borgpetersenii*, *L. interrogans*, *L. kirschneri*, *L. noguchii*, *L. santarosai*, and *L. weilii*.
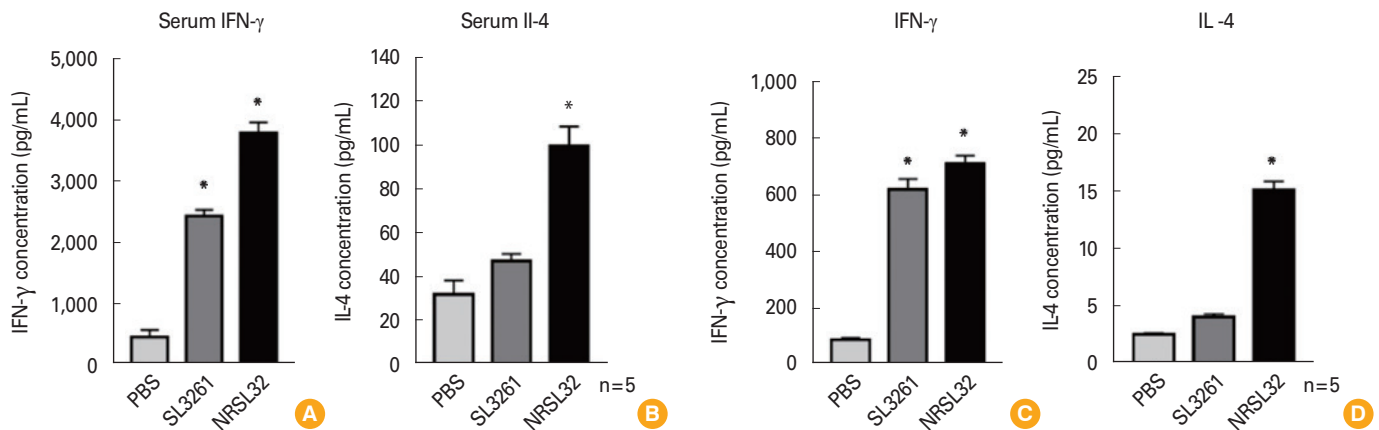
Fig. 4. Antigen-specific cytokines responses in serum (A, B) and spleen cells (C, D) from rats vaccinated with phosphate-buffered saline (PBS), SL3261, or NRSL32 strain. The standard deviations from five individual rats per group are indicated. IFN-γ, interferon-γ; IL-4, interleukin-4. *p<0.05; Asterisks indicate significantly different with rats immunized with PBS and SL3261 strain in each time.

LipL32 in rat sera with LipL32 protein on all 23 pathogenic *Leptospira* outer membranes, but was not detected in non-pathogenic species, *Leptospira biflexa* (Fig. 3D). It demonstrated that anti-LipL32 antibody from vaccinated rats showed ability to bind with native LipL32 protein located on outer membrane of pathogenic *Leptospira* spp.

These data indicated that immunization with NRSL32 strain was able to promote mixing of Th1 and Th2 biased responses. A balance proportion of LipL32 specific IgG2a/IgG1 antibody ratio showed that NRSL32 strain induced equal level of two-way stimulation between humoral and cellular immune responses. Moreover, NRSl32 stain was capable to induce anti-LipL32 IgA mucosal antibody response in rat models.

Assessment of cytokine response
In previous experiment, we demonstrated an elevation of IgG isotype responses in group of rats immunized with NRSL32 strain. Next, Th-1 cytokine (IFN-γ) and Th-2 cytokine (IL-4) were investigated in rat serum day 77 after final vaccination. Vaccinated rats with NRSL32 strain had significantly higher serum IFN-γ and IL-4 levels than the cytokine levels from the rat serum with oral vaccination by PBS or SL3261 strain (Fig. 4A, B).

To further evaluation of LipL32 specific cytokines production, splenic lymphocyte responses from vaccination rats were re-stimulated to determine IFN-γ and IL-4 production. There was significant increasing of LipL32-specific IFN-γ and IL-4 productions from splenocytes of NRSL32 group comparing with PBS-treated control. IFN-γ level was also found to be increased from splenic cells in SL3261 vaccinated rat group, while the IL-4 level in this group remained unchanged (Fig.

4C, D). This observation confirmed that vaccination with NRSL32 strain was able to sufficiently stimulate both Th1 and Th2 responses.

In summary, oral vaccination with *S.* Typhimurium NRSL32 strain harboring chromosomally in-frame fusion genes have ability to deliver chimeric LipL32 protein by SPI-2 T3SS for stimulation of adaptive humoral, cell mediated, and mucosal immune responses.

Discussion

Vaccination is one of the best efficiently strategy for prevention of infectious diseases causing from viruses, bacteria or parasites [27,28]. Inactivated vaccine and subunit vaccine are widely used and registration. However, both types of vaccines still have some limitations. For example, these vaccines stimulate only humoral immunity but not for mucosal and cell-mediated immunity [29], adjuvants are essential for successful vaccination, and boosting immunization is required for long-lasting immunity [30]. From these above reasons, it is necessary to generate a novel vaccine platform which able to overcome limitations. In recent decades, several bacterial based vaccine carriers had been widely used for homologous and heterologous antigen delivery [31]. RASV is an interesting choice for oral bacterial vector development. The antigen protein delivery platform of RASV bases on function of natural infection of intracellular *Salmonella* to translocate virulence effector proteins into host cell depending on T3SS. Several studies have been generated *Salmonella* based vaccine with plasmid-based systems to trigger immunity *in vivo* study. Although the protective mediated by *Salmonella* har-

boring plasmid expression was higher than *Salmonella* with chromosomal expression, the plasmid-based system has physical problems with stability, and antibiotic resistance gene [21]. In this study, we decided using chromosomal expression approach for continuous antigen secretion and without using antibiotic-resistance markers. We engineered chromosomal of RASV platform by in-frame fusion between nucleotides encoding N-terminal segment of SspH2 effector protein containing T3S signal and leptospiral antigen, LipL32. Our strategy provided consecutive heterologous antigen expression without require a selective antibiotic marker.

In SPI-2 T3SS, more than 20 proteins of SPI-2 effector proteins may have a potentiality for carrying recombinant protein [32]. In this experiment, we selected N-terminal of SspH2 for carrier protein. SspH2 protein was restricted to the vacuole membrane associated F-actin meshwork to supply actin filament for cross-link formation. Additionally, SspH2 protein containing leucine rich repeats which prominent for protein-protein interaction and mimic as E3 ligase in ubiquitin system [33,34]. Previous study demonstrated that the SspH2 protein containing secretion signal was able to translocate p60 protein of *Listeria monocytogenes* into cytoplasm of macrophage. Moreover, this study showed that SspH2 fusion construction was able to induce concurrent antigen-specific CD4 and CD8 T-cell responses against *Listeria* infection in challenge study [35]. In this study, we integrated gene encoding of LipL32 antigen downstream nucleotides encoding for amino acid residue 215 of SspH2 in *Salmonella* chromosome. Our result showed the secretion signal of SspH2 protein potentially persuaded LipL32 antigen into supernatant fraction under *in vitro* study (Fig. 2A). In translocation experiment, the secretion signal of SspH2 mediator potentially translocated recombinant LipL32 antigen into cytoplasmic of HeLa cell depended on SPI-2 T3SS machinery (Fig. 2B, C). It demonstrated that SspH2 mediator was suitable for our chromosomal integrated RASV platform.

In leptospirosis, the humoral immune response is a major protective immunity against infection [36]. Our data showed that RASV was also stimulation of antibody responses by production of LipL32 specific IgG antibodies in NRSL32 immunized rats. We noticed steadily rising of anti-LipL32 IgG antibodies response on days 21 and 35 after each boosting vaccination in NRSL32 group. Five weeks after final immunization, it still observed the satisfied levels of anti-LipL32 IgG antibody in NRSL32 immunized group (Fig. 3A). The persistence of antibodies may cause from continuous expression and

translocation of leptospiral antigens from RASV inside APC. Then, these antigens were processed and displayed antigen complexes on surface of APC. Antigen complexes on surface stimulate immature T cells to become mature Th1 or Th2 helper CD4+ cells. Mature Th2 helper T cells secreted cytokines to activate B cells. These B lymphocytes have ability to produce continuously specific LipL32 antibody response for protection of recurrent infection. Furthermore, our data showed that there was elevation of specific IgA antibodies after vaccination of RASV (Fig. 3C). It suggested that *Salmonella* NRSL32 strain may be able to stimulate of mucosal immunity for prevention of leptospiral infection through mucosal routes.

The goal for leptospirosis vaccine development is to induce cross-protective immunity against pathogen species. Previous study supposed that the antigens targeted for protection during challenge are proteins. Contrastingly, leptospiral lipopolysaccharide (LPS) are not capable of inducing cross-immunity among species-related serovars [8,37]. From these reasons, we manipulated RASV with gene encoding leptospiral LipL32 protein, highly conserved up to 98% among pathogenic species [11]. Our data illustrated that RASV expressing LipL32 fusion protein was able to induce specific antibody to bind natural LipL32 antigens among 23 serovars of pathogenic *Leptospira* spp. (Fig. 3D). Therefore, it may assume that the induced antibody recognized cross-immunogen of pathogenic *Leptospira* species. The opsonization between antibody and *Leptospira* stimulate phagocytosis and complement-dependent killing [38].

In our *in vivo* experiments, we observed a remarkable level of anti-LipL32 IgG1 and IgG2a antibodies as well as strong IFN-γ responses from Th1 cells and IL-4 responses from Th2 cells after vaccination with RASV (Figs. 3B and 4A, B). Moreover, results from antigen specific cytokine secretion illustrated splenocytes from NRSL32 immunized group were produced a notable level of LipL32 specific IFN-γ responses as well as LipL32 specific IL-4 responses after restimulated with purified rLipL32 (Fig. 4C, D). We concluded that *Salmonella* NRSL32 strain expressing LipL32 has ability to stimulate full coverage systemic responses with a balanced Th1–Th2 type immune responses pattern. Additionally, NRSL32 immunized group was able to induced activated Th1 and Th2 CD4 T lymphocyte to secrete LipL32 specific cytokine responses. However, we noticed remarkable levels of specific cytokines in SL3261 immunized group after re-stimulation with purified rLipL32 protein. The increasing level of specific IFN-γ in

these group may cause of trace residuals of LPS in rLipL32 purification process. LPS is generally component in outer membrane of Gram-negative bacteria. Previous publications have been reported the remaining of bacterial LPS in prokaryotic recombinant protein purification. Bacterial LPS can bind and stimulate TLR4 on APC [39]. The activated TLR4 could stimulate APC, subsequently it may stimulate spleen cells for IFN-γ cytokines production in SL326 group.

This study, we provided a platform for needle-free leptospiral vaccine development that uses RASV as live vaccine vector to induce a balanced Th1–Th2 type immune responses pattern both humoral immune response, cell-mediated immune responses, and mucosal immune responses. Although our data demonstrated the successful stimulation of immunological responses in animal experiment after immunization with RASV. We were still unable to entirely confirm that generated mucosal and systemic immunity from NRSL32 vaccine immunization were completely effective until further lethal challenge studies are undertaken.

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Supplementary Materials

Supplementary materials are available at Clinical and Experimental Vaccine Research website (http://www.ecevr.org).

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