

Original Article



OPEN ACCESS

Received: Mar 16, 2020

Revised: Oct 12, 2020

Accepted: Nov 2, 2020

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# Intranasal Vaccination with Outer-Membrane Protein of *Orientia tsutsugamushi* induces Protective Immunity Against Scrub Typhus

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## ABSTRACT

Scrub typhus develops after the individual is bitten by a trombiculid mite infected with *Orientia tsutsugamushi*. Since it has been reported that pneumonia is frequently observed in patients with scrub typhus, we investigated whether intranasal (i.n.) vaccination with the outer membrane protein of *O. tsutsugamushi* (OMPOT) would induce a protective immunity against *O. tsutsugamushi* infection. It was particular interest that when mice were infected with *O. tsutsugamushi*, the bacteria disseminated into the lungs, causing pneumonia. The i.n. vaccination with OMPOT induced IgG responses in serum and bronchoalveolar lavage (BAL) fluid. The anti-*O. tsutsugamushi* IgA Abs in BAL fluid after the vaccination showed a high correlation of the protection against *O. tsutsugamushi*. The vaccination induced strong Ag-specific Th1 and Th17 responses in the both spleen and lungs. In conclusion, the current study demonstrated that i.n. vaccination with OMPOT elicited protective immunity against scrub typhus in mouse with *O. tsutsugamushi* infection causing subsequent pneumonia.

**Keywords:** Intranasal administration; *Orientia tsutsugamushi*; Protective immunity; Pneumonia

## INTRODUCTION

*Orientia tsutsugamushi* is an obligate intracellular pathogen that is the causative agent of scrub typhus, an acute febrile and fatal disease transmitted by larval trombiculid mites or chiggers (1,2). It is characterized by fever, rash, eschar, meningitis, and pneumonitis in humans and is one of the major health problems in the Asia-Pacific region referred to as the “*tsutsugamushi* triangle.” Approximately one billion people are at risk and about 1 million cases occur each year (3). The endemic area is extended to Siberia, Russia, the South Pacific islands, and Afghanistan (4), and antibiotic-resistant strains have also been reported in these endemic

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#### Conflict of Interest

The authors declare no potential conflicts of interest.

#### Abbreviations

ARDS, acute respiratory distress syndrome; ASC, Ab-secreting cell; BAL, bronchoalveolar lavage; ICU, infected cell-counting unit; i.d., intradermal; i.m., intramuscular; i.n., intranasal; i.p., intraperitoneal; i.v., intravenous; KCDC, Korea Centers for Disease Control and Prevention; LB, Luria Bertani; OMPOT, outer membrane protein of *O. tsutsugamushi*; s.c., subcutaneous; SNU, Seoul National University.

#### Author Contributions

Conceptualization: Park SM, Yun CH; Data curation: Park SM, Ju YJ; Formal analysis: Ju YJ; Funding acquisition: Yun CH; Investigation: Park SM, Cheon IS, Son YM, Jeung W, Chu H; Methodology: Park SM, Hwang KJ, Shim BS; Resources: Hwang KJ, Gill B, Shim BS, Jeong HJ, Choi S; Supervision: Han SH, Chu H, Yun CH; Writing - original draft: Park SM, Gu MJ; Writing - review & editing: Gu MJ, Han SH, Chu H, Yun CH.

areas (5,6), thereby demanding attention and an urgent need for vaccine development. Although various efforts have been made to develop a safe and effective prophylactic vaccine against scrub typhus (7,8), no licensed vaccine is available currently.

Scrub typhus presents various clinical symptoms ranging from mild to lethal; without appropriate treatment, complications such as pneumonitis, disseminated intravascular coagulation, acute renal failure, and meningitis can develop, often resulting in death (9). Although *O. tsutsugamushi* first infects the skin, it disseminates to various organs such as the lymph nodes, gastrointestinal tract, brain, and lungs through blood, body fluids, and phagocytic cells (10-12). Patients typically develop mild interstitial pneumonitis during self-resolving or promptly treated scrub typhus (13). But, some patients with scrub typhus have shown severe pulmonary dysfunctions such as pneumonia, pleural effusion, interstitial pneumonitis, and acute respiratory distress syndrome (ARDS), most of which are life-threatening complications (14,15). However, no vaccine is available to overcome the life-threatening lung pathologies such as ARDS and pneumonitis induced by scrub typhus.

Most, if not all, vaccine trials for scrub typhus in human and mouse models used parenteral administration, usually by the subcutaneous (s.c.), intraperitoneal (i.p.), or intramuscular (i.m.) route (16,17). Although immunization via the systemic route induces relatively strong systemic immune responses, it is very weak at mucosal effector sites. In contrast, mucosal immunization can elicit effective adaptive immune responses in mucosal and systemic compartments. There is a need for developing new vaccine against scrub typhus, and mucosal immunization could be a strategy for preventing morbidity, such as pneumonia and pneumonitis, and mortality following *O. tsutsugamushi* infection. Recently, *O. tsutsugamushi* infection has been found to cause inflammation often followed by endothelial malfunction in the lung (18,19). Nevertheless, most of vaccination research have focused the protective effect against mainly systemic pathologies including spleen, blood and liver (20-22), rarely spotlighting on pulmonary immune response. Vaccination against scrub typhus via the intranasal (i.n.) route could induce an effective immune response in the lungs and systemic compartments, resulting in efficient protective immunity.

The outer membrane protein of *O. tsutsugamushi* (OMPOT), one of the major Ags, has been used as a vaccine candidate and diagnostic target molecule (23,24). It is highly conserved among *O. tsutsugamushi* strains and contains group-reactive and strain-specific B cell epitopes as well as helper T cell epitopes (25,26). Immunization with OMPOT provided partial protection against infection with various *O. tsutsugamushi* strains (27). Therefore, we hypothesized that i.n. vaccination with OMPOT will elicit vaccine-induced immune responses by blocking *O. tsutsugamushi* dissemination into the lungs and inhibiting pneumonia and systemic infection by scrub typhus. Here, we found that severe lung damage was caused by *O. tsutsugamushi* infection, and that the protective immunity in the lung is essential for survival of mice against *O. tsutsugamushi* infection.

## MATERIALS AND METHODS

### Mice

Female BALB/c mice, 6–10 wk old, were purchased from Orient Bio Inc. (Seongnam, Korea). Mice were maintained under pathogen-free conditions in animal facility at Seoul National University (SNU), where they received food and water ad libitum. The experiments to evaluate

vaccine efficacy were approved by the Institutional Animal Care and Use Committee of the SNU (SNU-130111-4-2). All challenge experiments were carried out in the animal biosafety level 3 facility of Korea Centers for Disease Control and Prevention (KCDC) with the approval of KCDC's Institutional Animal Welfare Committee (KCDC-023-11). Animal care and use protocol for the present study adhere the guideline set up by the Korea Association for Laboratory Animal Sciences.

### Propagation of *O. tsutsugamushi*

*O. tsutsugamushi* Boryong strain was propagated in the monolayer of L929 cells as previously described (28). Briefly, bacteria were inoculated into confluent monolayer of the cells in fetal bovine serum (FBS)-free Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen, Carlsbad, CA, USA) and incubated for 2 h at 34°C, at the end of which DMEM was supplemented with 1% FBS (Invitrogen) and 1% HEPES (Invitrogen). The levels of infectivity were determined by indirect immunofluorescence Ab assay after the cells expressed extensive cytopathic effect. When more than 90% of the cells were infected, the cells were collected, homogenized using a glass Dounce homogenizer (Wheaton Industries Inc., Millville, NJ, USA) and centrifuged at 500×g for 5 min. The supernatant was stored in liquid nitrogen tank until use. The infected cell-counting unit (ICU) was calculated microscopically according to the following formula: ICU = total number of cells used infection × ratio of infected cells to counted cells × dilution fold of *O. tsutsugamushi* Boryong inoculums (29-31). For infection, 100% lethal dose of *O. tsutsugamushi* was determined as  $2.4 \times 10^5$  ICU in wild type BALB/c mice.

### Indirect immunofluorescence assay

The *O. tsutsugamushi* stock was serially diluted and inoculated into a L929 cell layer on a 12 mm glass coverslip in a 24-well tissue culture plate (Nunc). After 6 h at 34°C, the culture medium was removed. The cells were washed with PBS, fixed in cold 100% acetone (Sigma-Aldrich, St. Louis, MO, USA) for 15 min at -20°C. The cells were blocked using PBS containing 1% BSA (Invitrogen) for 30 min and washed with PBS containing 0.5% Tween-20 (Sigma-Aldrich). Rabbit anti-*O. tsutsugamushi* Ab was added into the infected cells and incubated for 1 h. After washing, goat anti-rabbit Ab conjugated with FITC was added into the cells and incubated for 30 min and washed as above. The number of bacteria was counted using inverted fluorescent microscopy (Olympus, Tokyo, Japan).

### Cloning of the gene, coding fragment of outer-membrane protein of *O. tsutsugamushi*

A pair of primers (forward primer: 5'-GTGGATCCATGGTATTACCTCAACAAAAATC-3', reverse primer: 5'-GTCTCGAGTTACTTATTAATATTAGGTAAAGC-3') were designed for the cloning of 47 kDa Ag from OMPOT Boryong strain. The coding sequence for amino acids from 31 to 466 of OMPOT was amplified by PCR, using genomic DNA purified from *O. tsutsugamushi* Boryong strain using QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany). The PCR product was digested with BamH1 and Xho I and inserted into the bacterial expression vector pRSET (Invitrogen). The final sequence was confirmed by PCR and DNA sequencing.

### OMPOT purification

The pRSET plasmid containing DNA product derived from 47 kDa OMPOT gene was prepared. In brief, the gene was transformed into *Escherichia coli* BL21 (DE3) strain-RIPL (Novagen) and cultured overnight at 37°C in Luria Bertani (LB) medium supplemented with 100 µg/ml of ampicillin. The culture was transferred into fresh LB medium for 2 consecutive times and cultured until OD<sub>600</sub> 0.4–0.6. Protein expression in transformed *E.*

*coli* was enhanced by adding 0.5 M Isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG) for 3 h, and harvested by centrifugation at 4,000 $\times$ g for 10 min. The pellet was suspended in binding buffer (20 mM Tris pH 8.0, 0.5 M NaCl, pH 7.9) and disturbed by sonication on ice. After sonication, the soluble fraction was separated by centrifugation for 40 min at 20,000  $\times$ g and applied to a NTA resin-charged (immobilized metal affinity) affinity column, washed with binding buffer containing 20 mM imidazole, and the proteins were eluted by using elution buffer (250 mM imidazole, 20 mM Tris, 0.5 M NaCl, pH7.4). The eluted proteins were dialyzed with 1 $\times$  PBS. The endotoxin was removed by using Triton X-114 as previously described (32), which was confirmed by the limulus amoebocyte lysate assay kit (Lonza, Basel, Switzerland) according to the manufacturer's instruction. The purified proteins were examined by SDS-PAGE gel electrophoresis and visualized by staining with Coomassie Brilliant Blue (Bio-Rad, Hercules, CA, USA). The protein concentration was determined by BCA protein assay kit (Thermo Scientific, Waltham, MA, USA). The purified proteins were stored at  $-80^{\circ}\text{C}$  until use.

### Immunization & challenge

BALB/c mice were immunized with 5  $\mu\text{g}$  of OMPOT with cholera toxin (List Biological Laboratories, Inc., Campbell, CA, USA) through i.n. route (Thermo Scientific) 3 times at 2-wk interval. Mice injected with PBS were used as a negative control. For challenge experiments, mice were infected via i.p. route with  $2.4 \times 10^5$  ICU (100% lethal dose) of *O. tsutsugamushi* Boryong strain in 200  $\mu\text{l}$  of PBS, 28 days or 6 months (to evaluate long-term protection) after the last immunization. Mice were monitored daily for sign of morbidity (body weight changes) and mortality for 15 days. Body weight loss over 30% was considered as experimental end point.

### Bacterial dissemination

Bacterial disseminations were determined by nested PCR (33). Mice were challenged with  $1.2 \times 10^5$  (sub-lethal dose) or  $2.4 \times 10^5$  (lethal dose) ICU of *O. tsutsugamushi* strain via i.p. route. Lung, liver, heart and spleen were isolated from infected mice 7 days after the challenge. DNA was extracted from each tissue using a DNeasy Kit (Qiagen). Bacterial dissemination was identified by amplification of OMPOT gene using the forward primer (5'-GTGAATTCGTCGACAGAGCAGAGCTAGGT-3') and reverse primer (5'-GTAAGCTCTCGAGTCAATACCCTTTAACATCC-3'). The PCR products were verified by using agarose gel electrophoresis.

### ELISA for detection of specific Abs against OMPOT or *O. tsutsugamushi*

Serum and bronchoalveolar lavage (BAL) fluid were collected 7 days after the last vaccination and specific Ab titers against OMPOT or *O. tsutsugamushi* were measured by ELISA. Briefly, 96-well Maxisorp plates (Nunc) were coated with 1  $\mu\text{g}/\text{ml}$  of recombinant OMPOT or  $1.2 \times 10^4$  ICU/ml of *O. tsutsugamushi* and incubated overnight at  $4^{\circ}\text{C}$ . After blocking with PBS containing 1% BSA (Invitrogen) for 1 h at  $37^{\circ}\text{C}$ , sera and BAL fluid were serially diluted in blocking buffer to each well and incubated for 2 h at  $37^{\circ}\text{C}$ . HRP-conjugated goat anti-mouse IgG and IgA Abs (Southern Biotechnology Associates, Birmingham, AL, USA), diluted in a ratio of 1:3,000 in 0.1% BSA in PBS plus 0.05% Tween-20, were added and incubated for 1 h at room temperature. 3,3',5,5'-Tetramethylbenzidine (Millipore, Burlington, MA, USA) were added for the color reaction and stopped by adding 50  $\mu\text{l}$  of 2N  $\text{H}_2\text{SO}_4$ . The absorbance at wavelength 450 nm was measured by a microplate reader (Molecular Devices, San Jose, CA, USA) and endpoint titers were determined by the value of 0.2 and expressed as reciprocal  $\log_2$  titer by using Softmax pro version 5.4.1 (Molecular Devices).

### ELISPOT assay

To determine the number of Ab-secreting cells (ASCs), mononuclear cells were isolated from spleen and lung as previously described (34). Ag-specific ASCs were measured by ELISPOT. In brief, the 96-well ELISPOT MultiScreen<sup>®</sup><sub>HTS</sub> filter plate (Millipore) were coated with 5 µg/ml of recombinant outer-membrane protein in 50 mM sodium bicarbonate solution (pH 9.6), and incubated overnight at 4°C. After blocking with RPMI-1640 (Invitrogen) supplemented with 10% FBS, serially diluted mononuclear cells were added into each well and incubated for 4 h at 37°C in a humidified incubator providing 5% CO<sub>2</sub>. HRP-conjugated goat-anti-mouse IgG and IgA Abs (Southern Biotechnology Associates) were added into each well and incubated for 2 h at room temperature. For color development, peroxidase substrate (amino ethyl carbazole Peroxidase Chromogen substrate kit; ImmunoBioScience Corp., Mukilteo, WA, USA) was used. The number of ASCs was counted using a stereomicroscope (Olympus).

### Intracellular cytokine staining

Spleen, draining lymph nodes and lungs were isolated from immunized mice 7 days after the last immunization. To identify cytokine-producing cells, single cell suspension was prepared from each organ as previously described (35) and re-stimulated with 5 µg/ml of OMPOT for 6 h. To accumulate cytokines, Golgi plug (BD Biosciences, San Joes, CA, USA) was added and incubated for additional 10 h. The cells were stained with anti-mouse CD4-PerCP and CD44-FITC and stained with anti-mouse IFN-γ-APC, IL-17-PE, TNF-α-Pe-Cy7, or IL-2-Pe-Cy7 (BD Biosciences) after the fixation and permeabilized using Cytofix/Cytoperm solution. The cells were acquired by using flow cytometry, LSR II (BD Biosciences). Cytometry data were analyzed by using FlowJo software (Tree Star, Ashland, OR, USA). Comparison of cytokines combination and Pie graphs were analyzed using Simplified Presentation of Incredibly Complex Evaluations (Spice) software version 5.3 (National Institutes of Health, Bethesda, MD, USA).

### Cytokine profiles by cytometric bead array

Cytokine profiles were determined from BAL fluid, lung lysate and cell culture supernatant of spleen or draining lymph node. BAL fluid was harvested 7 days after the last immunization using 600 µl of PBS and centrifuged at 400×g for 10 min at 4°C. Lungs were isolated and homogenized through 70 µm cell strainer (BD Biosciences) using 2 ml of RPMI (Invitrogen) for obtaining lysate and centrifuged at 400×g for 10 min at 4°C. Single cell suspension from spleen and draining lymph node was re-stimulated with 5 µg/ml of OMPOT for 24 h. The supernatants were centrifuged and collected. All samples were stored at -70°C before analysis. Cytokine profiles were measured by mouse Th1/Th2/Th17 Cytometric Bead Array kit (BD Biosciences) according to manufacturer's instruction. The cytokine concentrations were calculated using the FCAP software array version 1.0.2 (BD Biosciences).

### Histology

For histology analysis, lungs were isolated and fixed with 4% formaldehyde at 7 days after the last immunization with OMPOT and/or challenged with *O. tsutsugamushi* strain. The fixed lungs were embedded in paraffin and dissected at a thickness of 4 µm, and stained with H&E to examine the trace of inflammation and damage.

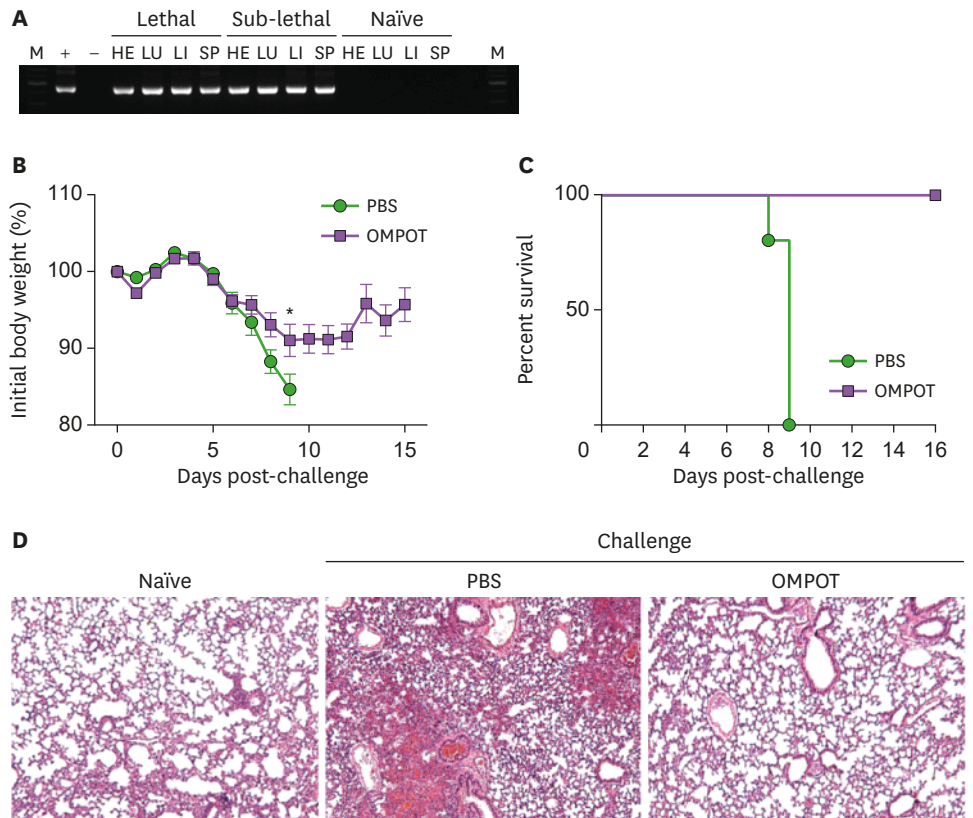
### Statistical analysis

Statistical differences were analyzed by 1-way ANOVA using GraphPad Prism Software (GraphPad Software, Inc., San Diego, CA, USA). Each experiment was repeated 3 times using at least 5 mice per group. Statistically significant values were expressed as p-values of <0.05.

RESULTS

Lung pathology in mice i.p. infected with *O. tsutsugamushi*

Pneumonia and pneumonitis occur in patients with *O. tsutsugamushi* infection when appropriate medical care and hospitalization are not provided (36,37). To ascertain whether *O. tsutsugamushi* is disseminated into tissues, including lungs, after the infection via i.p. route, we first examined the persistence of *O. tsutsugamushi* in each tissue. *O. tsutsugamushi* DNA was detected in the lungs and other organs including spleen, liver, and heart (Fig. 1A). *O. tsutsugamushi* infection had a significant loss of body weight (Fig. 1B), accompanied by severely hunched posture and swollen abdomen (data not shown), and did not survive at 10 days after the challenge (Fig. 1C). Furthermore, *O. tsutsugamushi*-infected mice showed a lethal acute lung injury accompanied by cell recruitment with hemorrhage (Fig. 1D). These results indicated that *O. tsutsugamushi* infection via the i.p. route induced pneumonia in mice, which could therefore be useful model to study the pathogenesis of *O. tsutsugamushi* infection and the protective response against scrub typhus.



**Figure 1.** Lung pathology and protective efficacy in mice against *O. tsutsugamushi* infection. Naïve BALB/c mice were infected with sub-lethal or lethal dose of *O. tsutsugamushi* Boryong strain via i.p. route. The lungs, liver, heart, and spleen were obtained at 7 days after the infection. (A) Purified DNA, obtained from each organ, was used as a template for PCR amplification of OMPOT gene sequence. DNA amplification products from each organ are shown. (B, C) Mice were immunized 3 times with OMPOT or PBS as a negative control. Twenty-eight days after the last immunization, mice were challenged with *O. tsutsugamushi* Boryong, and then the (B) body weight and (C) survival were monitored daily for 15 days. Body weight was expressed as the percentage of the initial body weight on day 0, before the challenge infection (n=6). (D) Lungs were isolated at 7 days after the challenge and stained by using H&E. Representative H&E-stained sections are shown (PBS-treated [PBS] or immunized [OMPOT],  $\times 10$ ). M, DNA ladder; +, positive control (DNA from *O. tsutsugamushi*); -, negative control (without DNA); HE, heart; LU, lung; LI, liver; SP, spleen. \*p<0.05.

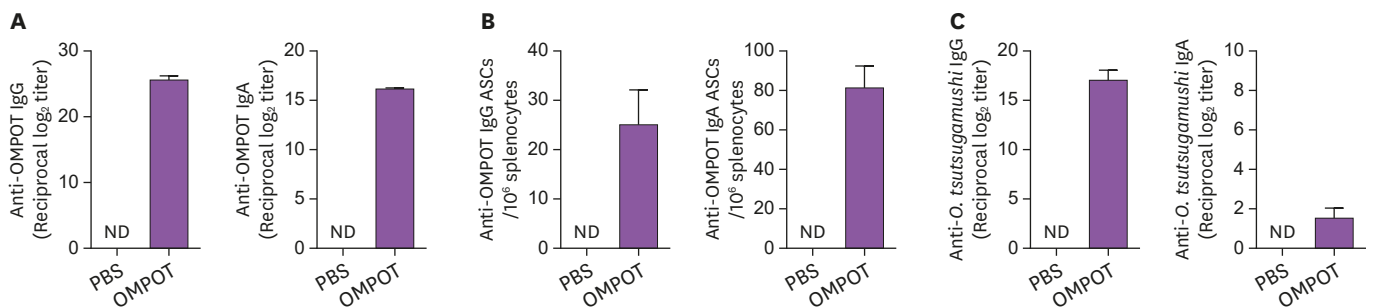
**The i.n. immunization with OMPOT induces protective immunity against *O. tsutsugamushi* infection**

To determine the effect of mucosal immunization with OMPOT on host protective immunity against lethal doses of *O. tsutsugamushi* infection, we purified the 47 kDa Ag from the outer-membrane protein from *O. tsutsugamushi* Boryong and immunized mice with OMPOT via i.n. route for 3 times at 2-wk interval (22,23). We chose cholera toxin as an adjuvant because it has been reported as effective for i.n. vaccination that acts through stimulating Th1- as well as Th2-mediated response (6,38). A 4 wk after the last immunization, the mice were challenged with a lethal dose ( $2.4 \times 10^5$  ICU) of *O. tsutsugamushi*. The immunized mice showed recovery of body weight loss and 100% survival (Fig. 1B and C) with no signs of disease (data not shown). The next question was whether i.n. immunization alleviates symptoms of pneumonia caused by *O. tsutsugamushi*. Lungs from the immunized mice did not show any sign of inflammation, and maintained a normal lung architecture (Fig. 1D). Taken together, these results clearly demonstrated that i.n. immunization with OMPOT provided protective immunity against lethal dose of *O. tsutsugamushi*.

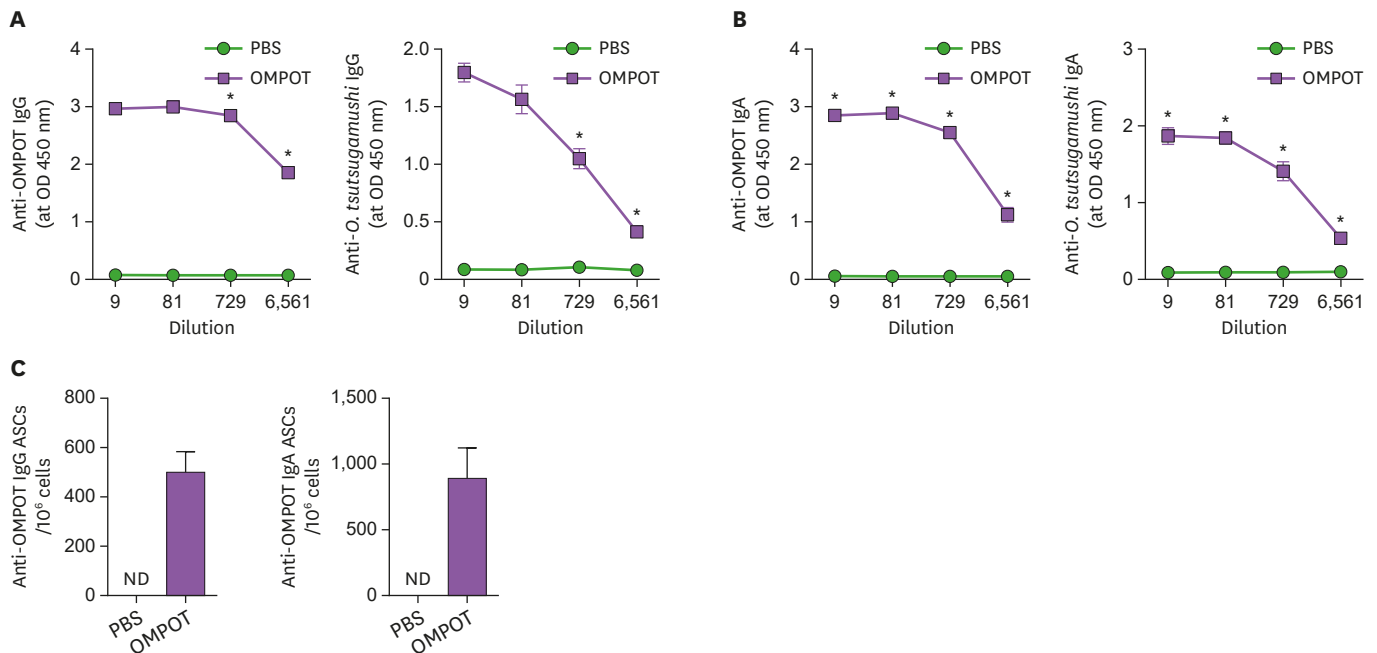
**Humoral immune responses in serum, induced by OMPOT immunization**

Earlier reports suggested that vaccine-induced Abs play an important role in inducing protective immunity against *O. tsutsugamushi* infection (39). We investigated whether i.n. vaccination induced Ag-specific Ab responses. We first examined the levels of OMPOT-specific Ab after the vaccination. Compared to the PBS group, the immunized mice elicited significantly high levels of OMPOT-specific serum IgG at 7 days after the last immunization (Fig. 2A). Moreover, the i.n. immunization also showed significantly high OMPOT-specific IgA in serum (Fig. 2A). The number of OMPOT-specific IgA ASCs in the spleen also elevated in the immunized group (Fig. 2B).

To investigate whether vaccine-induced Abs recognize not only OMPOT but also *O. tsutsugamushi*, we examined *O. tsutsugamushi*-specific IgG and IgA responses in the serum from immunized mice. Consistent with OMPOT-specific IgG response, serum IgG induced by immunization recognized *O. tsutsugamushi* (Fig. 2C). It was evident that *O. tsutsugamushi*-specific serum IgA responses were not strong. Taken together, these results suggested that both OMPOT and *O. tsutsugamushi*-specific serum Abs were greatly induced by OMPOT immunization.



**Figure 2.** Humoral immune responses in serum and spleen from mice immunized with OMPOT. Mice were immunized 3 times with OMPOT via the i.n. route (n=5). (A) Titers of anti-OMPOT-specific IgG and IgA in serum from PBS and immunized groups were evaluated by ELISA at 7 days after the last immunization. (B) OMPOT-specific IgG and IgA secreting cells in spleen from PBS and immunized groups were determined by ELISPOT. (C) Titers of anti-*O. tsutsugamushi*-specific IgG and IgA in serum from PBS and immunized groups were evaluated by ELISA at 7 days after the last immunization. ND, not detected.



**Figure 3.** Humoral immune responses in BAL fluid and lungs from mice immunized with OMPOT. Mice were immunized 3 times with OMPOT. PBS group was used as the negative control. Titers of (A) OMPOT-specific and *O. tsutsugamushi*-specific IgG and (B) OMPOT-specific and *O. tsutsugamushi*-specific IgA in BAL fluids from PBS or immunized groups were evaluated by ELISA at 7 days after the last immunization. (C) Anti-OMPOT IgG and IgA secreting cells were determined by ELISPOT in lung cells from PBS and immunized groups at 7 days after the last immunization. Data represent the means of triplicate experiments, with a minimum of 4 mice per group.

ND, not detected.

\* $p < 0.05$ .

### Humoral immune responses in the respiratory tract, induced by OMPOT immunization

Next, we investigated the humoral immune responses in the lungs after the immunization. Seven days after the last immunization, the vaccine-induced Ab responses in the respiratory tract was examined. The i.n. immunization with OMPOT elicited significantly high levels of OMPOT- and *O. tsutsugamushi*-specific IgG responses in BAL fluids (Fig. 3A). Furthermore, the level of OMPOT- and *O. tsutsugamushi*-specific IgA in BAL fluids increased in the immunized mice (Fig. 3B). High numbers of OMPOT-specific IgG and IgA secreting cells were also detected in lungs from the immunized mice (Fig. 3C). These results suggested that OMPOT- and *O. tsutsugamushi*-specific Abs in the respiratory tract were greatly induced against *O. tsutsugamushi* infection in mice immunized with OMPOT.

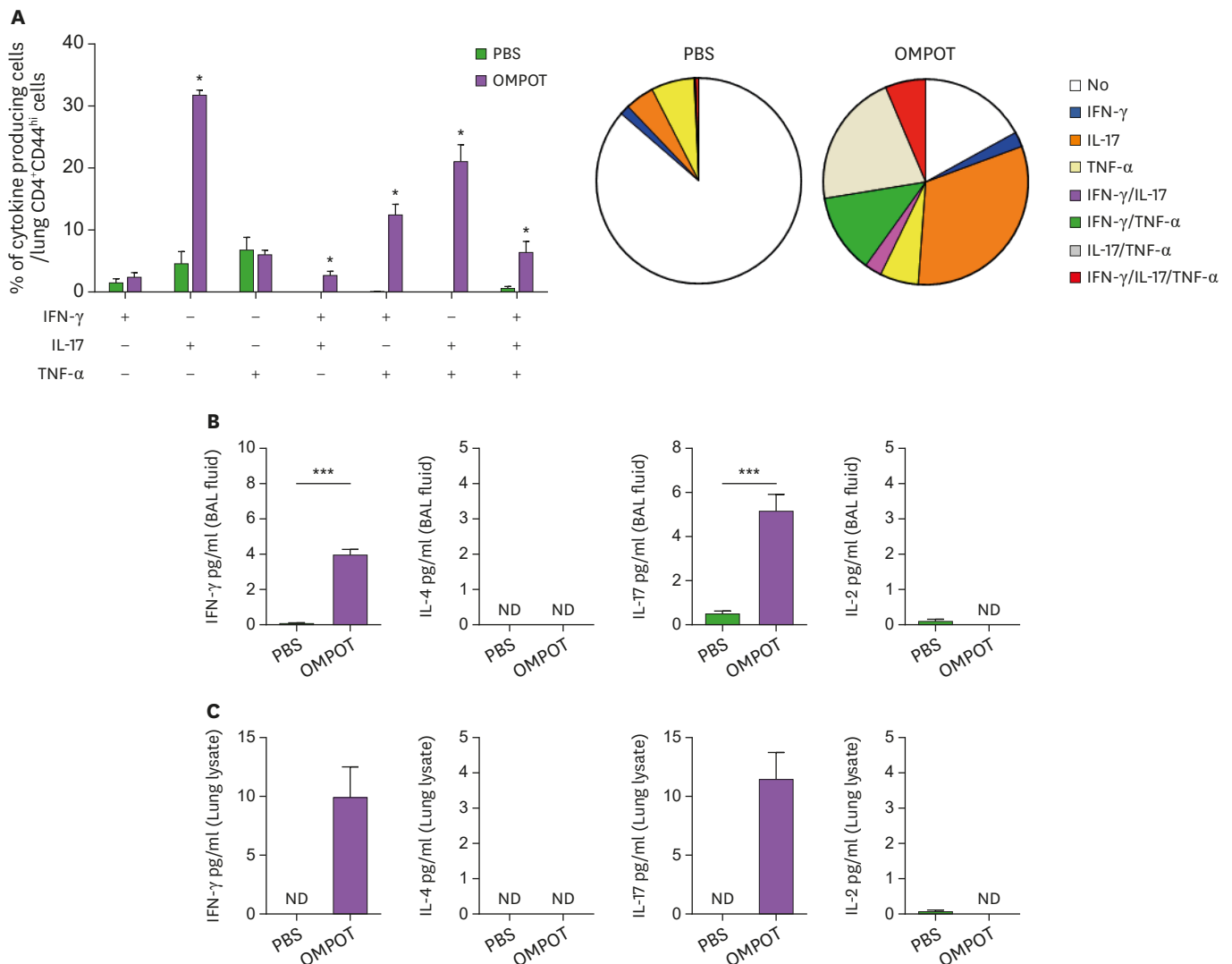
### Cell-mediated immunity induced by OMPOT immunization

Previously, several reports have shown that T cell-mediated immunity is associated with the development of protective immunity against scrub typhus (40,41). T cell quality has been suggested to play an important role in protective immunity against infectious pathogens (42,43). Therefore, we compared the cellular immune responses in mice immunized with OMPOT by using the intracellular cytokine staining assay. For this, 7 days after the last immunization, splenocytes were collected and re-stimulated with OMPOT for 16 h to determine the OMPOT-specific CD4<sup>+</sup> T cell response. There were no significant differences in IFN- $\gamma$ -producing CD4<sup>+</sup> T cells in spleen between PBS and immunized mice. Interestingly, the immunized group induced significantly higher IL-17- and IL-2-producing CD4<sup>+</sup> T cells than those of PBS groups. Furthermore, the levels of IFN- $\gamma$ , IL-2, and IL-17 were significantly higher in OMPOT-re-stimulated splenocytes from immunized mice than PBS mice. These

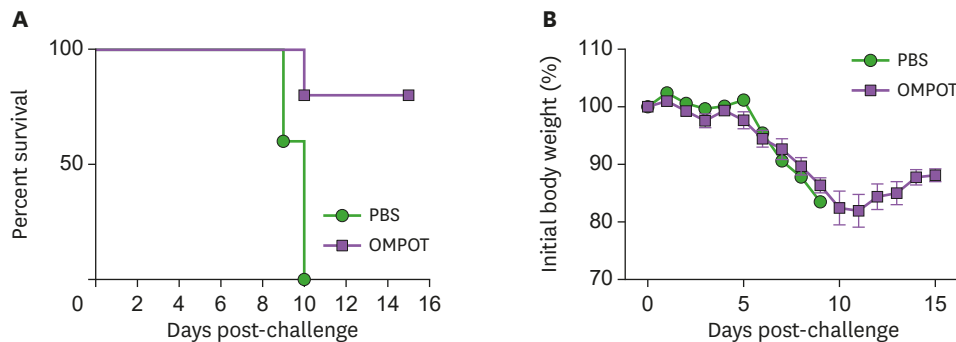


results suggested that OMPOT vaccination induced systemic IL-2 and IL-17 with mixed Th1 and Th17 responses (**Supplementary Fig. 1**).

Next, we investigated the cellular immune responses in the lung CD4<sup>+</sup> T cells of mice immunized with OMPOT. The OMPOT-specific single, double and triple cytokine-producing T cells based on the frequency of intracellular IFN- $\gamma$ , IL-17, and TNF- $\alpha$  were examined. The frequency of IL-17-producing CD4<sup>+</sup> T cells in the immunized mice was significantly higher than those in PBS group (**Fig. 4A**). In addition, the frequency of IFN- $\gamma$ /IL-17 and IL-17/TNF- $\alpha$  double-positive and IFN- $\gamma$ /IL-17/TNF- $\alpha$  triple positive CD4<sup>+</sup> T cells also significantly increased in the lungs from immunized mice (**Fig. 4A**). Elevation of multifunctional effector CD4<sup>+</sup> T cells was also observed in the cervical lymph nodes of immunized mice although the



**Figure 4.** Cellular immune responses in the respiratory tract of mice immunized with OMPOT. Mice were immunized 3 times with OMPOT. Lungs were taken at 7 days after the last immunization (n=5). (A) Single cell suspension from the lungs was re-stimulated with 5  $\mu$ g/ml of recombinant OMPOT for 16 h. Intracellular cytokine staining for IFN- $\gamma$ , IL-17, and TNF- $\alpha$  in CD4<sup>+</sup>CD44<sup>hi</sup> T cells was measured by flow cytometry. Comparison for the combination of cytokines and pie graphic presentations were analyzed using Spice software. (B) BAL fluid and (C) lung lysates were harvested at 7 days after the last immunization and examined for the levels of IFN- $\gamma$ , IL-4, IL-17, and IL-2 by using a mouse CBA Th1/Th2/Th17 cytokine kit (n=5). The concentration of cytokines was expressed as mean $\pm$ SEM for groups. ND, not detected. \*p<0.05, \*\*\*p<0.001.



**Figure 5.** Long-term protective immunity in mice immunized with OMPOT against *O. tsutsugamushi* lethal infection. Mice were immunized 3 times with OMPOT. PBS group was used for the negative control. Six months after the last immunization, the mice were challenged with *O. tsutsugamushi* Boryong strain. Subsequently, (A) their survival rate and (B) body weight changes were monitored daily for 15 days. Body weight was expressed as the percentage of the initial body weight on day 0, before the challenge infection (n=5).

frequency was lower than those in the lungs (**Supplementary Fig. 2**). In BAL fluid and lung lysate from immunized mice, enhanced IFN- $\gamma$  and IL-17 production was observed (**Fig. 4B and C**). These results indicated that the immunization with OMPOT elicited higher Th1 and Th17 responses in the respiratory tract.

### The i.n. immunization with OMPOT induced a long-term protective immunity

Since one of the main goals of vaccination is to induce long-term protective immunity against target infectious disease (44,45), mice were immunized with OMPOT and then challenged with lethal dose of *O. tsutsugamushi* strain 6 months later. Immunized mice showed 100% survival by day 10 and 80% by day 15 post-challenge (**Fig. 5A**). Immunized mice showed a transient decrease of body weight for the first 10 days and then recovered thereafter (**Fig. 5B**). Taken together, these results showed that i.n. immunization with OMPOT effectively induced a long-term protective immunity against lethal challenge of *O. tsutsugamushi*.

## DISCUSSION

*O. tsutsugamushi* disseminates systemically and targets multiple organs such as the lung, kidney, liver, brain, and spleen (11). Without appropriate treatment, the bacteria can spread and cause interstitial pneumonia, myocardial and hepatic lesions, meningoencephalitis, ARDS, and multi-organ failure (46). Lung is one of the main target organs during scrub typhus, and patients typically suffer from interstitial pneumonitis and severe pulmonary pathology including lung hemorrhage, pulmonary edema, and vasculature damage (13). Here, we hypothesized that mucosal vaccination against scrub typhus may prevent systemic spread of *O. tsutsugamushi* coincident with alleviation of severe lung dysfunction.

Our study aimed to investigate the effect of OMPOT vaccination through i.n. route on the protection against *O. tsutsugamushi* in both mucosal and systemic compartments. We found that the group immunized via systemic routes (s.c. or i.p.) showed a significant loss of body weight, low survival rate, and severe lung pathology similar to PBS group (**Supplementary Fig. 3**), indicating insufficient protection against i.p. infection. Meanwhile, systemic immunization induced sufficiently high Ag-specific IgG in the serum and BAL fluid, coincident with high number of IgG-secreting cells in the spleen and lung, similar as i.n. route, while poor OMPOT-specific IgA response in the lung was found (**Supplementary Figs. 4 and 5**). Thus, i.n. immunization was superior to systemic routes for inducing OMPOT-specific IgA responses

and protection in the lung. Further studies are required to confirm the functional roles of Abs, induced by i.n. vaccination with OMPOT, that neutralize and inhibit *O. tsutsugamushi* infection.

On the other hand, cell-mediated immunity also plays an important role in protective immunity, especially the Th1 response, against scrub typhus (47). Our study demonstrated that i.n. vaccination with OMPOT enhanced cell-mediated immunity, especially Th1 and Th17 responses in lungs and lymph nodes. Th1 cells appeared to play a central role in inhibiting *O. tsutsugamushi* growth in not only endothelium, but also macrophages and fibroblasts (48-50). Adoptive transfer of IFN- $\gamma$  producing T cells into naïve mice showed a critical role of Th1 cells for the protective response (51). However, there are no reports on the functional role of Th17 in modulating *O. tsutsugamushi* infection although the action of IL-17 against other intracellular pathogens such as *Francisella tularensis* and *Chlamydia muridarum* is indispensable, as a part of protective immunity (52,53). Further studies should examine whether vaccine-induced Th17 responses are required for protection against scrub typhus. Our data highlighted the possibility that Th1 and Th17 immunity induced by OMPOT vaccination played an important role in improving protective immunity against scrub typhus.

Here, we observed the improvement of Th1 and Th17 responses in the pulmonary compartments at days 7 after the last immunization. It has been reported that i.n. immunization enhanced mucosal IgA or Th17 response at the early phase after the last vaccination leading to sufficient protection against infection with other pathogens (54,55). Although we confirmed long-term protection by analyzing survival against challenge infection, whether Ag-specific IgG/IgA or Th1/Th17 response are functionally maintained in the lung are remained to be investigate. It will be interesting to examine in future study how such improved mucosal immunity is sustained after the challenge infection to strengthen the protective effect of i.n. OMPOT immunization.

The intravenous (i.v.) or intradermal inoculation of *O. tsutsugamushi* has been considered as a valid model for scrub typhus by inducing systemic and pulmonary endothelial infection, which is the hallmark of *O. tsutsugamushi* infection in human (19,56). Although i.p. inoculation of *O. tsutsugamushi* into mice has been mainly used to investigate protective immunity against *Orientia* infection (1,57), it did not reflect the scrub typhus-like pathology because it give rise to mainly peritonitis and not to induce systemic endothelial infection (58). In the present study, we used i.p. challenge to develop scrub typhus in mice and found that sub-lethal and lethal dose of *O. tsutsugamushi* induced a lung pathology. It was supported by the findings that *Orientia* Ag was detected in endothelial cells in the lung from i.p.-inoculated mice that induced less pulmonary cellular infiltrate than i.v.-inoculated mice (58). Though, this has to be further clarified for the pulmonary protective effect in mice with i.n. immunization followed by i.v. challenge.

Our results strongly advocate the development of a mucosal vaccine against scrub typhus that would target outer-membrane protein Ags, including OMPOT, on the surface of *O. tsutsugamushi*. Vaccination study using outer membrane proteins of *O. tsutsugamushi* has been previously reported the cellular and humoral responses in the spleen (22), while the present study focused on the mucosal immune responses in the lung that are the one of the main infectious sites of *O. tsutsugamushi*. The most interesting finding of the present study was that i.n. immunization with OMPOT uniquely induced protective immunity against a lethal dose of *O. tsutsugamushi*. The i.n.-immunized group showed high frequency of OMPOT-specific T cell cytokine responses in the systemic and mucosal organs, whereas s.c.- and i.p.-immunized group was not able to produce multi-functional cytokines as same as control

group (**Supplementary Fig. 6**). Therefore, i.n. vaccination induced a high magnitude of cell-mediated immunity in both mucosal and systemic compartments when compared to those of systemic vaccination (45,59).

In conclusion, we demonstrated that severe lung damage and mortality were induced by the challenge with *O. tsutsugamushi* strain in a mouse model. Moreover, we showed that i.n. immunization with OMPOT enhanced cell-mediated and protective immunity in the pulmonary compartments. Overall, our study suggested that i.n. vaccination strategy using OMPOT could provide an effective protection against scrub typhus.

## ACKNOWLEDGEMENTS

This work was carried out with the support of Cooperative Research Program for Agriculture Science and Technology Development (PJ01336401), Rural Development Administration, and National Research Foundation of Korea (2018R1A2B2006793), Republic of Korea. This research was also supported by a fund (2010-NI52001-00) from Research of Korea Centers for Disease Control and Prevention, and Korea Institute of Planning and Evaluation for Technology in Food, Agriculture and Forestry (IPET) through Animal Disease Management Technology Development Program, funded by Ministry of Agriculture, Food and Rural Affairs (MAFRA) (319081-03). The current work was also partially supported by the BK21 FOUR Program of the Department of Agricultural Biotechnology, Seoul National University, Seoul, Korea.

## SUPPLEMENTARY MATERIALS

### Supplementary Figure 1

Cytokine responses in the spleen of mice immunized with OMPOT. Mice were immunized 3 times with OMPOT (n=5). (A) Spleens were obtained 7 days after the last immunization. A single cell suspension from the spleen was re-stimulated with 5 µg/ml recombinant OMPOT for 16 h. Intracellular staining for IFN-γ, IL-4, IL-17, and IL-2 in CD4<sup>+</sup>CD44<sup>hi</sup> T cells was measured by flow cytometry. (B) After 72 h, cell supernatants were harvested. IFN-γ, IL-4, IL-17, and IL-2 levels were evaluated using a mouse CBA Th1/Th2/Th17 cytokine kit. Five mice per group were analyzed individually, and cytokine concentrations were expressed as mean ± SEM for groups. Data were analyzed using 1-way ANOVA.

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### Supplementary Figure 2

T cell quality in cervical lymph nodes from mice immunized with OMPOT. Mice were immunized 3 times with OMPOT (n=5). Cervical lymph nodes (CLNs) were obtained 7 days after the last immunization. A single cell suspension from the cervical lymph nodes was re-stimulated with 5 µg/ml recombinant OMPOT for 16 h. Intracellular staining for IFN-γ, IL-17, and TNF-α in CD4<sup>+</sup>CD44<sup>hi</sup> T cells was measured by flow cytometry. The bar graph and pie charts showed the cytokine combinations after OMPOT re-stimulation. Five mice per group were analyzed individually. Results are expressed as mean±SEM for groups. Data were analyzed using 1-way ANOVA.

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### Supplementary Figure 3

No or low protective effect of systemic immunization against *O. tsutsugamushi* Boryong infection. Mice were immunized 3 times with OMPOT through the s.c. or i.p. route. PBS group was used as the negative control. Twenty-eight days after the last immunization, mice were challenged with *O. tsutsugamushi* Boryong strain, and the survival and body weight change were monitored daily for 15 days. Body weight was expressed as the percentage of the initial body weight on day 0, before the challenge infection (n=6).

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### Supplementary Figure 4

Humoral immune responses in serum and spleen from mice immunized with OMPOT via systemic route. Mice were immunized 3 times with OMPOT via the s.c. and i.p. route (n=5). (A) Titers of anti-OMPOT-specific IgG and IgA in serum were evaluated by ELISA at 7 days after the last immunization. (B) OMPOT-specific IgG and IgA secreting cells in spleen were determined by ELISPOT.

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### Supplementary Figure 5

Humoral immune responses in BAL fluid and lungs from mice immunized with OMPOT via systemic route. Mice were immunized 3 times with OMPOT via the s.c. (▲) and i.p. (▼) route. PBS group (○) was used as the negative control. Titers of (A) OMPOT-specific and *O. tsutsugamushi*-specific IgG and (B) OMPOT-specific and *O. tsutsugamushi*-specific IgG and IgA in BAL fluids were evaluated by ELISA at 7 days after the last immunization. (C) Anti-OMPOT IgG and IgA secreting cells were determined by ELISPOT in lung cells at 7 days after the last immunization. Data represent the means of triplicate experiments, with a minimum of 4 mice per group.

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### Supplementary Figure 6

Cellular immune responses in the respiratory tract of mice immunized with OMPOT via systemic route. Mice were immunized 3 times with OMPOT via the s.c. and i.p. route. Lungs were taken at 7 days after the last immunization (n=5). (A) Single cell suspension from the lungs was re-stimulated with 5 µg/ml of recombinant OMPOT for 16 h. Intracellular staining for IFN-γ, IL-17, and TNF-α in CD4<sup>+</sup>CD44<sup>hi</sup> T cells was measured by flow cytometry. The pie charts showed the cytokine combinations after OMPOT re-stimulation (B) BAL fluid and (C) lung lysates were harvested at 7 days after the last immunization and examined for the levels of IFN-γ, IL-4, IL-17, and IL-2 by using a mouse CBA Th1/Th2/Th17 cytokine kit (n=5). The concentration of cytokines was expressed as mean±SEM for groups.

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