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Protective Immunity of 56-kDa Type-Specific Antigen of *Orientia tsutsugamushi* Causing Scrub Typhus

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Scrub typhus, caused by infection with *Orientia tsutsugamushi*, is a mite-borne zoonotic disease endemic to the Asian-Pacific region. In Korea, the incidence of this disease has increased with climate changes, and over 10,000 cases of infection were reported in 2013. Although this infection is treatable with antibiotics such as doxycycline and azithromycin, an effective prophylactic vaccine against *O. tsutsugamushi* would be more desirable for preventing scrub typhus in endemic areas. In this study, we investigated the 56-kDa type-specific antigen (TSA56), which is a major outer membrane protein of *O. tsutsugamushi*, as a vaccine candidate. Intranasal immunization of recombinant TSA56 (rec56) induced a higher level of TSA56-specific IgG than that induced by intramuscular immunization of *tsa56*-expressing DNA (p56). Both types of immunization induced a cell-mediated immune response to TSA56, as demonstrated by the splenic cell proliferation assay. Mice immunized with p56, followed by rec56 plus heat-labile enterotoxin B subunit from *E. coli*, had a stronger protection from a homologous challenge with the *O. tsutsugamushi* Boryong strain than with other combinations. Our preliminary results suggest that an effective human vaccine for scrub typhus can include either recombinant TSA56 protein or *tsa56*-expressing DNA, and provide the basis for further studies to optimize vaccine performance using additional antigens or different adjuvants.

Keywords: Scrub typhus, *Orientia tsutsugamushi*, 56-kDa type-specific antigen (TSA56), vaccination

Introduction

Scrub typhus is an acute febrile disease caused by infection with an obligate intracellular bacterium, *Orientia tsutsugamushi*. It is characterized by fever, rash, eschar, pneumonitis, myocarditis, and disseminated intravascular coagulation [8, 35]. It is a chigger-borne zoonotic disease that is one of the critical concerns in public health for tropical and rural areas of the Asian-Pacific region, including Korea, China, Japan, India, and Southeast Asian countries [35]. It has been estimated that 1 billion people are at risk of infection, and 1 million cases are reported annually in the Asia-Pacific region [31, 40].

O. tsutsugamushi strains are antigenically diverse and divided into several serotypes, including Karp, Gilliam, Kato, and other regional strains [41]. The endemic isolate most frequently found in Korea is the Boryong strain, which is a relative of the Karp serotype [6, 27]. In Korea, disease incidence has increased with environmental changes and increased outdoor activity, leading to frequent exposure to the vector of this pathogen, chigger mites [18]. Data suggest a temporal correlation to the previous or following year, as well as a spatial correlation to adjacent regions, in the transmission and spread of scrub typhus. In 2012 and 2013, 8,604 and 10,365 infection cases of scrub typhus were reported, respectively [24] (Disease Web Statistics System in Korea, http://is.cdc.go.kr).

Although scrub typhus can be effectively treated with antibiotics such as doxycycline, tetracycline, and azithromycin, re-infection and relapses occur frequently because of poor cross-reactive immunity and a short duration for protective immunity [14]. Moreover, chronic infections are established

in an unknown number of patients [38]. Since World War II, continuous efforts have been invested for developing a prophylactic vaccine, and laboratory-based and clinical trials for vaccine development have been performed using formalin-fixed homogenized lung from *Orientia*-infected cotton rats, formalin-killed or low-virulent live bacteria, and live irradiated strains [2, 3, 5, 10–12, 32]. Recombinant proteins have been used as vaccine antigens. Several recombinant proteins were tested for protective immunity against homologous and/or heterologous strains in mice and non-human primates [7, 23, 36, 37, 41]. However, an effective vaccine for humans has not yet been made available, and the development of an effective vaccine against *O. tsutsugamushi* is still considered an attractive and desirable tool for the control of scrub typhus in endemic countries. Vaccination could overcome difficulties in early clinical diagnosis, high mortality in untreated cases, and commercially unavailable on-site laboratory diagnosis, and it could limit the potential for developing antibiotic resistance [19, 25, 33, 39].

In the present study, 56-kDa type-specific antigen (TSA56), a major outer membrane protein of *O. tsutsugamushi*, was investigated for the development of an effective vaccine against scrub typhus. In mice, we used intranasal and intramuscular immunization of recombinant TSA56 (rec56) and *tsa56*-expressing DNA (p56), respectively. The immunization efficacy was observed, including humoral and cellular immune responses as well as protection against a homologous challenge. The preliminary results obtained in this study demonstrate that both the recombinant protein and the DNA vaccines ectopically expressing *tsa56* are capable of inducing immune responses and some protection against *O. tsutsugamushi* infection. However, there seem to be different immune mechanisms involved, and there is a need for enhancing the protective immunity conferred by these vaccines.

Materials and Methods

Bacterial Strains, Plasmids, and Recombinant TSA56

The pathogenic *O. tsutsugamushi* Boryong strain, an endemic isolate in Korea, was used in this study. The strain was propagated in L929 cells (ATCC NTCT929), murine aneuploid fibroblasts, as described previously [26]. Infected cells were incubated at 34°C in 5% CO_2. At 3 to 4 days post-infection, infection was determined using an indirect immunofluorescence assay (IFA) with human sera positive for scrub typhus. Partial purification of the bacteria was performed as described elsewhere and then used for homologous challenge in immunized mice; genomic DNA was extracted with a QIAamp genomic DNA kit (Qiagen, Hilden, Germany). *Escherichia coli* TOP10 and BL21 DE3 strains (Invitrogen, Carlsbad, CA, USA) were used for gene cloning and prokaryotic expression, respectively. Plasmids pRSET A and pVAX1 (Invitrogen) were used for prokaryotic and mammalian expression, respectively.

The primers OTBS0602-88-F (5′-GTGAATTCGTCGACAGAG CAGAGCTAGGT-3′) and OTBS0602-479-R (5′-GTAAGCTTCTCG AGTCAATACCCTTTAACATCC-3′) were used for amplification of the truncated form of the *tsa56* gene encoding amino acids 88–479, using a conventional PCR. The amplified PCR product was digested with *Eco*RI and *Xho*I restriction enzymes and then cloned into pRSET A to generate a TSA56 clone for bacterial expression. The plasmid expressing recombinant TSA56 protein (rec56) was transformed into *E. coli* BL21 (DE3) for overexpression. The culture conditions and procedures for purification of rec56 were as described elsewhere [21].

The nucleotides for a DNA vaccine of *tsa56* were synthesized with a codon optimization process for mammalian expression (Bioneer Co., Daejeon, Korea). They were cloned into pVAX1 to generate a recombinant plasmid for mammalian *tsa56* expression, p56. Mammalian expression of *tsa56* was confirmed by transient expression of p56 in a murine fibroblast cell line, L929, using Lipofectamine 2000 Reagent (Invitrogen) according to the manufacturer's instructions.

Immunization and Challenge in Mice

The animal experiments were approved by the Institutional Animal Care and Use Committee of the Korea Centers for Disease Control and Prevention and performed in accordance with the laboratory's animal ethics guidelines (approved document no. KCDC-005-13-1A). At the end of the experiments, the animals were euthanized.

Seventy-five 6-week-old female BALB/c mice (Charles River Laboratories, MA, USA) were randomly divided into five groups of 15 mice each and used for immunization. In recombinant protein administration groups, mice were intranasally injected with rec56 (10 μg) alone, or rec56 with 1 μg of heat-labile enterotoxin B subunit from *E. coli* (LTB) as an adjuvant. In DNA administration groups, mice were intramuscularly injected with p56 plasmid (100 μg) alone, or p56 with an equivalent amount of pBOOST2-samIRF7/3 (pB), a plasmid expressing a super-activated mouse *IRF7/3* chimeric gene as an adjuvant (Table 1). The immunizations were performed three times with 2-week intervals. On the 10th day after the first two immunizations, blood was collected from the immunized mice by tail bleeding; the serum was recovered by centrifugation and then stored at −20°C for further assays. On the 7th day after the third immunization, three mice in each group were euthanized and their spleens were isolated for cell proliferation analyses.

For the protection study, the immunized mice were intraperitoneally challenged with a dose 100 times the median lethal dose (LD_{50}) of the homologous *O. tsutsugamushi* Boryong strain 2 weeks after the third immunization. The mortality rates of

Table 1. Experimental groups for mouse immunization.

Group[a]	Administration route	Number of mice
PBS	Intranasal	15
rec56 (10 μg)	Intranasal	15
rec56 (10 μg) plus LTB (1 μg)	Intranasal	15
p56 (100 μg)	Intramuscular	15
p56 (100 μg) plus pB (100 μg)	Intramuscular	15

[a]rec56, recombinant 56-kDa protein; p56, mammalian expression vector for 56-kDa protein; LTB, heat-labile enterotoxin B subunit from *E. coli*; pB, plasmid pBOOST2-samIRF7/3 expressing a super-activated mouse *IRF7/3* chimeric gene.

the challenged mice were monitored for 2 weeks, and their survival rates were recorded. The results are expressed as percent survival of the immunized mice.

Determination of TSA56-Specific Antibodies

TSA56-specific IgG and IgA in the sera were titrated by indirect ELISA. The antigen used was rec56, and the procedures were as described elsewhere [20]. Briefly, a microtiter plate was coated with 100 ng/well of rec56, sealed, and incubated for 1 h at 37°C. After washing three times with PBS, the plate was recoated with 150 μl of blocking solution containing 0.5% skim milk in PBS. After 1 h incubation at 37°C, the plate was again washed three times with PBS and incubated with 100 μl of diluted mouse sera (1:100 in PBS with 0.05% skim milk) for 1 h at 37°C. Thereafter, the plate was then washed three times with PBS containing 0.05% Tween 20 (PBS-T), and 100 μl of diluted secondary antibody (horseradish peroxidase-conjugated goat anti-mouse IgG or IgA antibody (Abcam, Cambridge, UK) diluted 1:1,000 in PBS-T with 0.05% skim milk) was added to the wells. After 30 min incubation at 37°C, the plate was rewashed four times with PBS-T. For colorimetric detection, 100 μl of a substrate solution (1:1 mixture of H_2O_2 and 3,3′,5,5′-tetramethylbenzidine) and 50 μl of stop solution were used. Absorbance was measured at 450 nm by using a microplate reader. All the samples were subsequently tested in triplicate, and the absorbance value was presented as a relative unit.

Splenic Cell Proliferation Assay

For the measurement of the TSA56-specific cell-mediated immune response, a cell proliferation assay was performed *in vitro*. Splenic cells were isolated from the spleen of immunized mice and seeded onto 96-well plates (1×10^5 cells per well). For re-stimulation, 10 μg/ml of rec56 was added to each well, and the plate was incubated at 37°C in 5% CO_2 for 72 h. Cell proliferation was checked using an EZ-CYTOX assay kit (Daeil Lab Service Co., Seoul, Korea) according to the manufacturer's instructions.

Statistical Analysis

All laboratory assays were repeated at least three times independently. The values are expressed as the mean ± standard deviation. The statistical significance of variation among different groups was determined by one-way analysis of variance. Student's two-tailed *t*-test was used to estimate the variance parity between the experimental and control groups. Differences were considered statistically significant at p values of < 0.05.

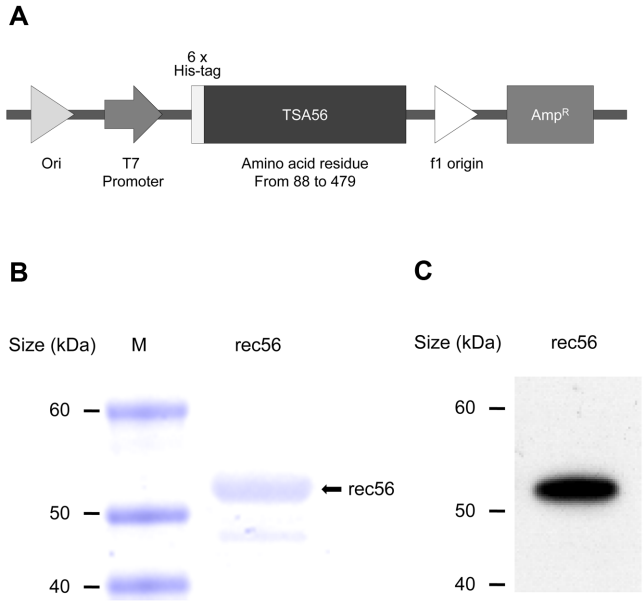

Fig. 1. SDS-PAGE and western blot analysis of the recombinant TSA56 protein (rec56).
(**A**) Schematic diagram of the expression vector for rec56 production in an *E. coli* expression system. The truncated form of the recombinant TSA56 (amino acids 88–479) was produced. (**B**) SDS-PAGE analysis of the purified recombinant protein. (**C**) Western blot analysis of rec56 using antisera from scrub typhus patients confirmed by IFA (IgG titer >1:2,048).

Results

Preparation of rec56 and p56

For the construction of a prokaryotic expression vector for TSA56, a truncated form of the *tsa56* gene encoding amino acids 88–479 was amplified by PCR and cloned into pRSET A (Fig. 1A). This vector was used with an *E. coli* BL21 (DE3) expression system to produce the TSA56 recombinant protein (rec56) as an insoluble protein with six N-terminal histidines added. The insoluble rec56 was purified by Ni-NTA affinity column chromatography under denaturing conditions and then refolded (Fig. 1B). The expressed rec56 was estimated to be over 30% of the total cellular protein (data not shown). The recombinant protein was clearly detected in western blot analysis with serum

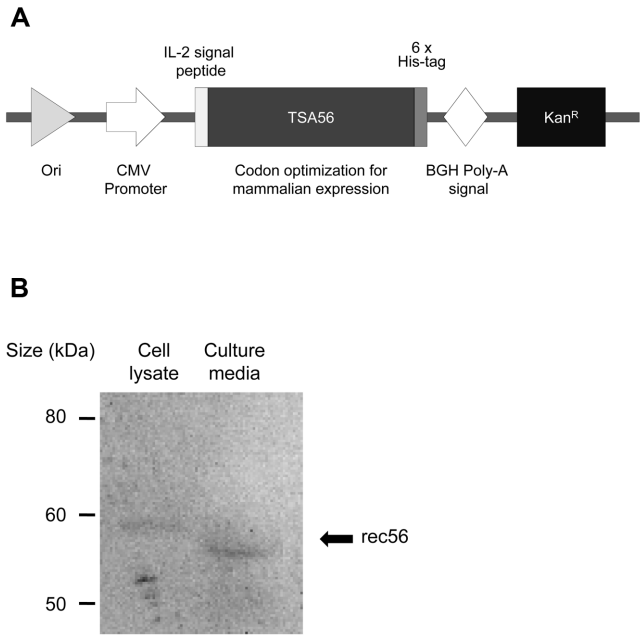

Fig. 2. Construction and expression of *tsa56* in a mammalian system.

(**A**) Schematic diagram of expression vector p56 for mammalian *tsa56* expression. The nucleotides for *tsa56* were optimized for mammalian expression. (**B**) Western blot analysis of the transient expression of recombinant TSA56 protein in L929 cells after transfection with p56 plasmid. TSA56 protein expression was detected using an anti-His-tag antibody.

from a scrub typhus patient (IgG titer >1:2,048 in IFA) (Fig. 1C).

For DNA-based immunization with *tsa56*, we used the mammalian codon-optimized *tsa56* sequence inserted into the mammalian expression vector pVAX1 to generate p56. The cloned insert was confirmed by sequencing. The CMV promoter and a BGH poly-A signal were used to control the transcription of *tsa56* from p56, and an IL-2 signal peptide and six histidines were added into the construct for secretion and detection, respectively (Fig. 2A). In order to evaluate the expression of TSA56 from p56 in mammalian cells, p56 was transiently transfected into L929 cells. Expression of the recombinant protein was detected in cell lysates within 2 days after transfection (Fig. 2B).

Humoral Immune Response in Mice

The schedule for immunization, bleeding, and specimen collection is presented in Fig. 3. Following the immunization of mice, the titers of the anti-TSA56 antibody were measured by indirect ELISA by using rec56 as the TSA56 antigen. TSA56-specific serum IgG levels in all experimental groups, except for the p56-immunized group, were significantly higher than the negative control after the second immunization (Fig. 4A). Both the rec56-immunized groups, with or without LTB, showed remarkably higher levels of IgG production than the p56-immunized groups after the third immunization ($p < 0.01$). In IgG subclass analysis, TSA56-specific IgG1 significantly increased in rec56 alone and in rec56 plus LTB groups after the second immunization, and these groups also had much higher levels of IgG1 after the third immunization ($p < 0.01$; Fig. 4B). In contrast, after the second immunization, a high level of IgG2a production was observed in the p56-immunized groups as well as in the rec56-immunized groups ($p < 0.01$; Fig. 4C). Mice immunized with rec56 plus LTB showed much higher titers of TSA56-specific serum IgA than did the other immunized groups after the third immunization ($p < 0.01$; Fig. 4D).

Cellular Immune Response in Mice

Splenic cells obtained from the immunized mice were stimulated for 48 or 72 h with 10 μg/ml rec56. The proliferative responses of splenic cells were significantly higher than PBS-immunized negative controls after 72 h stimulation for all immunization groups ($p < 0.01$), except for the group immunized with rec56 but no adjuvant ($p < 0.05$; Fig. 5).

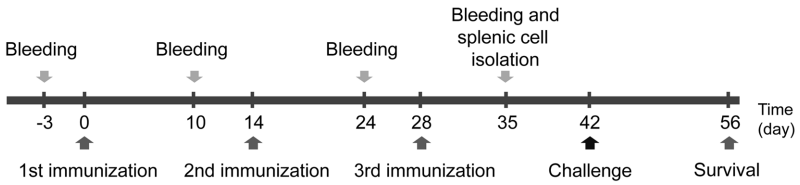

Fig. 3. Timeline of immunization and specimen in mice.

Seventy-five 6-week-old female mice were randomly divided into five groups: negative control with PBS treatment, rec56 alone, rec56 plus LTB, p56 alone, and p56 plus pB. Mice were acclimatized for a week. The immunizations were performed by intranasal administration with rec56 (10 μg) alone or with rec56 plus LTB (1 μg) or by intramuscular injection with p56 plasmid (100 μg) alone or with p56 plus an equivalent amount of pB plasmid.

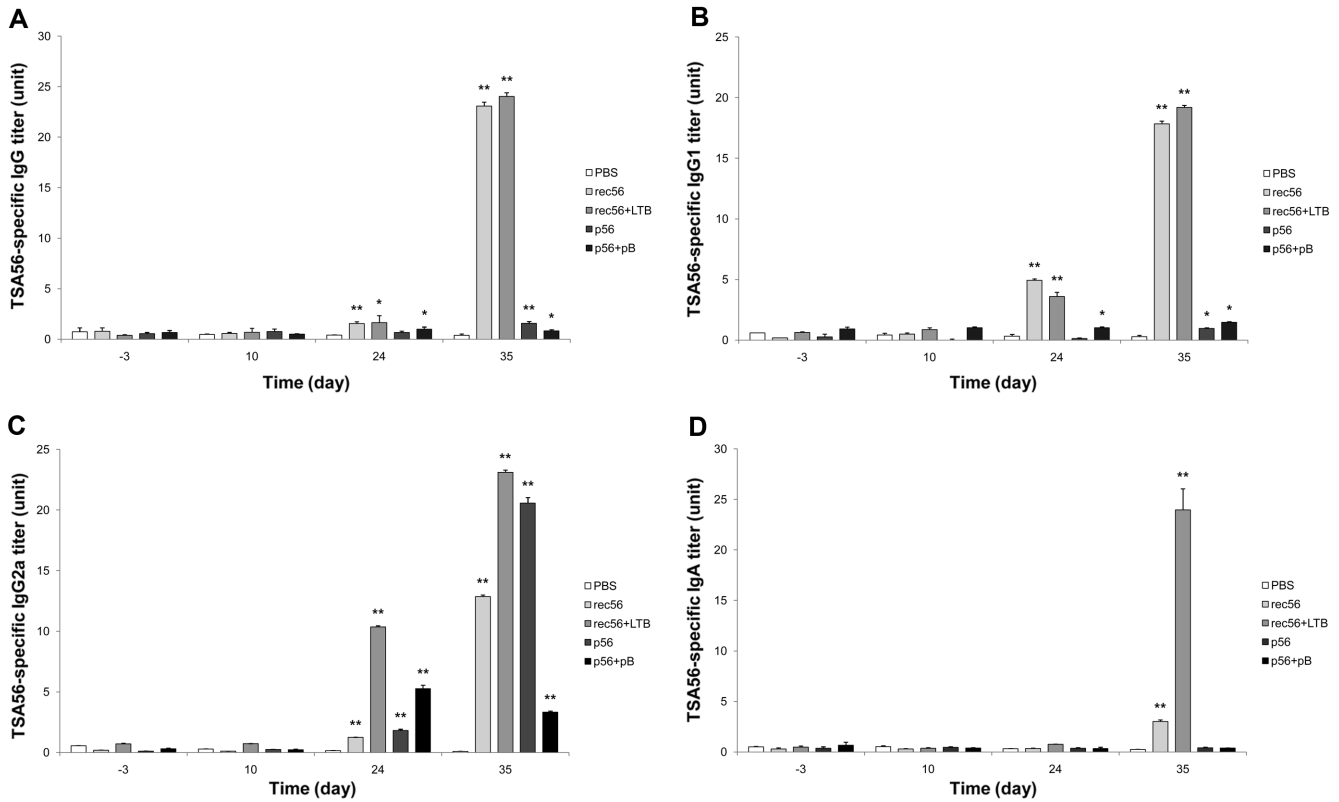

Fig. 4. Humoral immune responses in sera from mice immunized with rec56 and p56.
TSA56-specific immunoglobulins, including total IgG (**A**), IgG1 (**B**), IgG2a (**C**), and total IgA (**D**) were measured by indirect ELISA after every immunization. The titer is presented as a relative unit. Statistical significance between the PBS control and each group is indicated with an asterisk ($p < 0.05$) or double asterisks ($p < 0.01$).

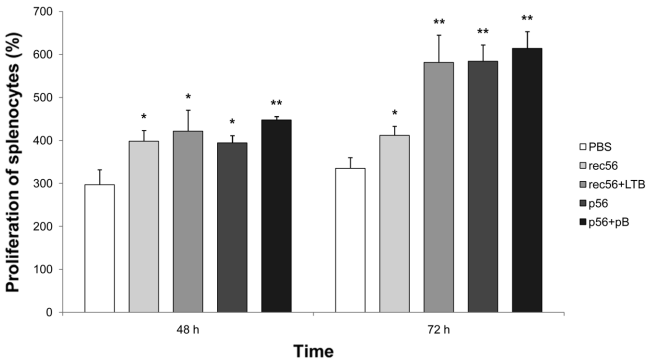

Fig. 5. TSA56-specific splenic cell proliferation.
The isolated splenic cells were re-stimulated with 10 μg of rec56 protein. Viable cells were measured at 48 and 72 h after seeding, and re-stimulation and data are expressed as percent cell viability. Statistical significance between the PBS control and each group is indicated with an asterisk ($p < 0.05$) or double asterisks ($p < 0.01$).

Protective Immunity Conferred by TSA56 in Mice

In a protection study, the p56-immunized mice had the highest survival rate (58.3%), followed by the mice immunized with rec56 plus LTB (41.6%; Fig. 6). A survival rate of 25% was observed in mice groups immunized with rec56 alone or p56 plus pB. The mice in the PBS-immunized negative control group had only a 17% survival rate. The survival rates of mice immunized with p56 alone or rec56 plus LTB were statistically significant compared with the negative controls ($p < 0.05$); however, the difference in survival between these two groups was not statistically significant.

Discussion

Scrub typhus is an endemic disease in the Asia-Pacific region, because various chigger mites belonging to the family Trombiculidae extensively inhabit these areas and serve as vectors for the causative agent of scrub typhus, *O. tsutsugamushi*. Two of the principal vector species in Korea and Japan are *Leptotrombidium pallidum* and *L. scutellare* [29]. The incidence of scrub typhus can be influenced by

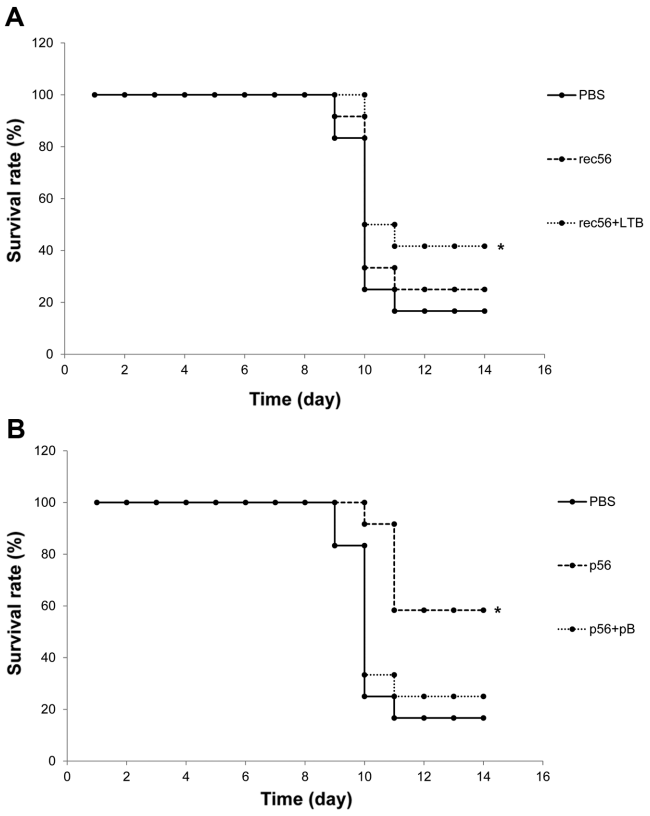

Fig. 6. Evaluation of protection from a homologous strain challenge conferred to immunized mice.

Live *O. tsutsugamushi* Boryong strain was intraperitoneally injected into mice immunized with rec56 or rec56 plus LTB (**A**) and mice immunized with p56 or p56 plus pB (**B**). The mortality rates of the challenged mice were monitored for 2 weeks; the survival rate was calculated as the ratio of living mice to total mice challenged in a group. Statistical significance ($p < 0.05$) compared with the negative control at the 14th day after challenge is indicated with an asterisk.

environmental changes, especially relative humidity and changes in temperature, which are reflected in the population density of these dominant vector species. The prevalence of scrub typhus in Korea has been on a steady rise over the past several years; cases of infection increased more than three times during the period from 2001 to 2012 [9], and over 10,000 cases of infection were reported in 2013. Therefore, more active and comprehensive control and prevention strategies, including implementation of prophylactic vaccination, should be considered for reducing the incidence of this disease.

In this study, we preliminarily investigated the feasibility of using the TSA56 protein, which originated from the *O. tsutsugamushi* Boryong strain, as a vaccine candidate. Protective immunity was observed in mice immunized with intranasal administration of rec56 as a subunit vaccine and with intramuscular administration with p56 as a DNA vaccine expressing *tsa56*. The parameters used to measure the immune responses were antigen-specific antibody production, splenic cell proliferation, and the survival rate of the immunized mice after homologous challenge.

The TSA56 protein is known to have a strong immunogenicity and is expected to play a significant role in generating protective immunity against scrub typhus. In previous studies, immunization with TSA56 derivatives could elicit significant levels of protection in animal experiments. Mice immunized with a partial recombinant 56-kDa protein, fused with the maltose-binding protein as a carrier protein, showed increased resistance to *O. tsutsugamushi* infection [36]. Four consecutive immunizations with a plasmid carrying native nucleotide sequences based on *tsa56* without any adjuvant raised protection by 60% against a 1,000× LD_{50} challenge of live *O. tsutsugamushi* Karp strain in mice [23]. In our study, we used different strategies compared with these previous reports; the N-terminal truncated recombinant TSA56 protein without any carrier, which is a more intact form, was used as the antigen for a subunit vaccine that was intranasally administered to induce mucosal immunity as well as systemic immunity. For immunization with plasmid DNA, we used the mammalian codon-optimized *tsa56* sequence to increase the expression of TSA56 in mice. LTB and pB were used as an mucosal adjuvant and DNA vaccine adjuvant, respectively. LTB is known to induce strong serum and mucosal immune responses against heat-labile enterotoxin, indicating that LTB could be used as a potent mucosal adjuvant [13, 30]. In this study, comparing with rec56 alone, the LTB adjuvant used in intranasal administration did not produce a noticeable effect in total IgG and IgG1 production (Figs. 4A and 4B); however, distinct increases of IgG2a and IgA production as well as splenic cell proliferation were induced by LTB (Figs. 4C, 4D, and 5). The DNA vaccine adjuvant, a pB plasmid, is commercially developed as a genetic adjuvant for DNA vaccines to potentiate the immune response to a specific antigen. This plasmid encodes a chimeric protein from the interferon regulatory factor family, which can enhance both Th1 and Th2 responses in T cells, leading to the activation of cytotoxic T cells and/or the production of antibodies [4, 34]. However, in our study, this adjuvant showed no positive effect in intramuscular administration, because results for the experiments performed without using this adjuvant, showing lower antibody induction, were balanced by the relatively higher induction of cell-

mediated immune responses (Figs. 4 and 5).

In general, recombinant protein immunization with rec56 or rec56 plus LTB showed more TSA56-specific antibody production than did DNA immunization with p56 or p56 plus pB. However, in the splenic cell proliferation assay, DNA immunization appeared to be relatively effective (Fig. 5). Cellular immune responses to rickettsial infections have been shown to coincide with the development of protection from infection. The murine model of scrub typhus suggested that the lymphocyte proliferation level is associated with resistance to rickettsial infection [16]. In our study, p56 alone and rec56 plus LTB respectively induced 58.3% and 41.6% increases in protection efficacy against a homologous strain challenge. These survival rates corresponded to high IgG2a levels in rec56 plus LTB administration, as well as high cell proliferation levels in p56 and rec56 plus LTB immunizations. However, other immunizations also had similar effects, which did not correlate with increased survival. Other studies have suggested that protective immunity to scrub typhus is due to the development of cell-mediated immunity [17], and that the humoral immune response plays a prominent role in protective immunity by inhibiting an event required for the attachment and/or the penetration of the pathogen [15]. In this study, we did not find a clear correlation between the antibody level and protection, and we observed a discordance between cell-mediated immunity and protection. It is likely that different protection mechanisms are involved in intranasal immunization with the recombinant TSA56 and intramuscular immunization with the plasmid DNA expressing *tsa56*.

DNA vaccines have many advantages over other types of vaccines, such as induction of strong cell-mediated immune responses, elimination of the safety concerns associated with live vaccine organisms, convenience of construction in various forms, high productivity and stability, and economic feasibility [1, 22, 28]. The value of p56 as a DNA vaccine may be significantly improved by several approaches, including improving the expression and secretion of the protein, co-immunization with appropriate adjuvants, cloning the gene into dendritic cell-attracting vectors to enhance TSA56 antigen presentation, and using liposomes for a more efficient delivery system [23].

In conclusion, rec56 and p56 induced cell-mediated immune responses and antibody production specific to TSA56; however, protective immunity that completely overcomes a homologous challenge was not observed in this study. It is uncertain whether this antigen could induce heterologous protection as well. Although TSA56 alone may have low efficacy in inducing heterologous protection, as was shown in other early cross-protection studies [11, 12], a multisubunit or multivalent vaccination including TSA56 with other immunogenic components may be a promising approach for overcoming the strain-specific immunity and low protection efficacy of this vaccine [41].

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