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*Orientia tsutsugamushi*의 자가수송단백을 이용한 Scrub typhus 백신개발 연구

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Development of scrub typhus vaccine using an autotransporter protein of *Orientia tsutsugamushi*

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Development of scrub typhus vaccine using an autotransporter protein of *Orientia tsutsugamushi*

by

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ABSTRACT

Introduction: Scrub typhus is an acute febrile illness caused by *O. tsutsugamushi* infection. Recently, the rapid increase of scrub typhus incidence and sporadic outbreaks in several countries of endemic Asian-pacific region has become a serious public health issue. Despite the wide range of preventative approaches that have been attempted in the past 70 years, all have failed to develop an effective prophylactic vaccine. Currently, the selection of the proper antigens is one of the critical barriers to generating cross-protective immunity against antigenically-variable strains of *O. tsutsugamushi*.

Methods: Based on the functional genomic analysis, a group of genes encoding autotransporter proteins has been identified and characterized in the genome of *O. tsutsugamushi*. The sequences of surface cell antigen (*sca*) genes were compared among diverse genotypes of *O. tsutsugamushi* and the potential role of Sca proteins in the bacterial interaction with host cells was examined. In addition, the antigenicity of them was investigated using scrub typhus patients' sera. Finally, I screened Sca antigens for vaccine development after immunization and subsequent lethal challenge of diverse strains in mice infection model.

Results: Five *sca* genes (*scaA-E*) encoding autotransporter proteins were identified in the genome of *O. tsutsugamushi*. Among them, ScaA and ScaC have been well conserved in diverse genotype of O. *tsutsugamushi*. I found

that these two bacterial proteins are involved in the bacterial adhesion to host cells and induced specific immune responses in scrub typhus patients. Furthermore, immunization of ScaA could provide significant protective immunity not only against to homologous strain infection but also to the lethal challenge of heterologous strains.

Conclusions: Immunization of ScaA proteins provides protective immunity in mice when challenged with the homologous strain and significantly enhanced protective immunity against infection with heterologous strains. To our knowledge, this is the most promising result of scrub typhus vaccination trials against infection of heterologous strains in mouse models thus far.

Keywords*:* *O.tsutsugamushi*, Scrub typhus, Autotransporter protein , Vaccine

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LIST OF ABBREVIATIONS

AT : Autotransporter

BSA : Bovine serum albumin

CFU : Colony-forming unit

E.coli : Escherichia coli

EDAC: 1-Ethyl-3-(3-Dimethylaminopropyl)carbodiimide

EDTA : Ethylenediamine tetraacetic acid

ELISA : Enzyme-linked immunoabsorbance assay

FITC : Fluorescein isothiocyanate

GS4B : Glutathione-Sepharose 4B

HRP : Horseradish peroxidase

ICT : Immune-chromatographic technique

ICU : Infected-cell−counting units

IFA : Immunofluorescence assays

IPTG : Isopropyl -D-thiogalactoside

IRB : Institutional Review Board

LB : Luria-Bertani

MatGAT : Matrix Global Alignment Tool

MFI : Mean Fluorescence Intensity

NTA : Ni-nitrilotriacetic acid

PBS : Phosphate buffered saline

PCR : Polymerase chain reactions

PFA : Paraformaldehyde

RFLP : Restriction fragment-length polymorphism

PVDF : Polyvinylidene difluoride

RTase : Reverse transcriptase

RT-PCR : Reverse transcriptase PCR

rOmpA : Rickettsial Outer membrane protein A

rOmpB : Rickettsial Outer membrane protein B

Sca : Surface cell antigen

SDS : Sodium dodecyl sulphate

SDS-PAGE : SDS–polyacrylamide gel electrophoresis

SNU : Seoul National University

STIC : Scrub typhus infection criteria

TMB : 3,3',5,5'-tetramethylbenzidine

TSA : Type-specific antigen

TS buffer : Tris-sucrose buffer

O. tsutsugamushi : *Orientia tsutsugamushi*

INTRODUCTION

Orientia tsutsugamushi, an obligate intracellular bacterium belongs to Rickettsia family, is the causative agent of scrub typhus [1, 2]. The infectious disease is endemic in south-eastern Asia, northern Australia, and the western Pacific islands [3]. It has been estimated that one million new cases of scrub typhus arise annually in the endemic region [4]. Although there is no data available on the clinical epidemiology covering the whole endemic region, recent emergence and continuous local outbreaks of scrub typhus have drawn much attention from the public health agencies in the endemic countries [5-9]. In addition, scrub typhus is one of the frequent travel-associated vector-borne infections [10-12], reminding the tremendous impact of the disease on Allied forces in South-eastern Asia during World War II when the number of scrub typhus victims surpassed the number of direct casualties due to the war [13].

The natural host for *O. tsutsugamushi* is the trombiculid mites in which the bacterial pathogen has maintained via transovarial or transstadial transmission in wild nature [14]. Since the pathogen is also associated with the salivary glands of chigger mites, it could be transmitted to the vertebrate host while the infected chiggers feeding the tissue fluid for their development. Small rodents and wild birds are the primary targets of chigger mites, and humans could be an accidental host [14]. The chigger bite is painless but an eschar often forms at the bite site. The disease symptoms are quite variable

from mild to a fatal illness in human infection. Early clinical manifestations of scrub typhus begin with regional lymphadenopathy, followed by fever, headache, myalgia, and rashes. Due to the lack of specificity of its early clinical presentation compared to other acute febrile illness and unavailability of rapid and effective diagnostic tests in local clinics, delayed treatment with suitable antibiotics, such as doxycycline and chloramphenicol, is common and often leads to severe complications including respiratory distress syndrome, acute renal failure, meningitis, gastrointestinal tract bleeding, and multi-organ failures [15-18]. The burden of disease is still quite large, accounting for up to 20% of febrile hospital admissions in rural areas of south eastern Asia, and the mortality rate reaches up to 10% depending on the area of endemicity [15, 19-21]. Moreover, recurrent infection has been often observed in highly endemic region [22] potentially due to the antigenic diversity of *O. tsutsugamushi* [3] and the failure of generating long-lived immunity after primary infection [23].

Despite the wide range of preventative approaches that have been attempted in the past 70 years, all have failed to develop an effective prophylactic vaccine [24]. Approaches have included the use of formalin-killed bacteria [25, 26], inoculation with viable organisms followed by antimicrobial treatment [27], irradiated *O. tsutsugamushi* [28], subunit vaccines [29, 30], and DNA vaccine [31]. Most of the vaccine trials resulted in short-term protection (generally less than one year), immunity to only the homologous strain, or no significant outcomes, especially in human infections. Immunity generated by the vaccine trials, or even after natural infections, does not last long and is poorly cross-reactive among numerous strains [3, 24],

thus reinfection with scrub typhus is relatively common in highly endemic areas [22]. To date, more than 20 strains have been reported, including the prototype strains Karp, Kato, and Gilliam [3]. Genetic analysis of the major outer membrane protein, the 56 kDa type-specific antigen (TSA56) unique to *O. tsutsugamushi*, revealed at least 11 definable genotype strains [3]. Although the TSA56 protein is an immunodominant antigen and has long been considered as a vaccine target, remarkable genetic heterogeneity among strains of *O. tsutsugamushi* limits cross-protective immunity against heterologous strains [24]. Selecting conserved antigens among different strains of *O. tsutsugamushi* is one of the critical issues to generating a clinically effective vaccine that produces cross-protective immunity against scrub typhus. Utilization of genome sequences obtained from bioinformatics through genomics and proteomics can expedite the vaccine discovery process by rapidly providing a set of potential candidates for vaccine antigen targets [32, 33].

Bacterial invasion of host cells is mediated primarily by interactions between bacterial surface components and complementary host receptors. Therefore, outer membrane proteins of pathogenic bacteria have long been targeted as vaccine antigens if they were involved in the interaction with host cells. As an obligate intracellular organism, *O. tsutsugamushi* must be internalized into host cells in order to survive and replicate. The bacterium infects several types of non-phagocytic cells, such as endothelial cells and fibroblasts, as well as macrophages and polymorphonuclear cells [34-37]. After the entry into the host cells, the intracellular pathogens escape from

vacuoles and move to the perinuclear region where they replicate [38]. However, the molecular basis of intracellular invasion by *O. tsutsugamushi* is poorly characterized. Previously, it was reported that *O. tsutsugamushi* could bind to host fibronectin and utilize it for internalization via interactions with the outer membrane protein, TSA56 [39, 40]. Fibronectin is known to facilitate bacterial entry into host cells, potentially via its interaction with integrins. *O. tsutsugamushi* exploits integrin-mediated signaling and rearrangement of the actin cytoskeleton, which mediate "induced phagocytosis" in non-phagocytic host cells [40].

Bacterial entry into host cells can be divided into two distinct stages: adherence and invasion. Recently, it was reported that *Rickettsia* utilizes multiple outer membrane proteins to adhere to and invade non-phagocytic host cells. The *R. conorii* autotransporter protein, Sca1, mediates bacterial adherence to, but not invasion of, a panel of epithelial and endothelial cells [41], whereas the Sca2 autotransporter protein can mediate both adherence to, and invasion of, non-phagocytic host cells [42]. Also, two Rickettsial surface proteins, Rickettsial outer membrane protein A (rOmpA) and Rickettsial outer membrane protein B (rOmpB), participate in adhesion to, and invasion of, mammalian cells *in vitro* [43-45]. rOmpB mediates bacterial adhesion to mammalian cells by binding to its mammalian receptor, Ku70, and subsequently activating host cell signaling pathways that may ultimately induce actin polymerization at the site of bacterial contact [43, 46]. Therefore, Rickettsial entry into host cells occurs sequentially via the initial interaction

between bacterial adhesins and host receptors, the activation of down-stream host signaling, and, finally, active invasion (defined as induced phagocytosis).

Interestingly, all of the identified outer membrane proteins involved in Rickettsial entry belong to a family of autotransporter proteins that contain an N-terminal signal sequence and a highly conserved C-terminal β-barrel, or autotransporter, domain [47, 48]. The signal sequence targets the protein to the bacterial periplasm, where the autotransporter domain inserts itself into the outer membrane to form a conduit through which the central passenger domain is transported and exposed to the extracellular surface. A recent bioinformatic analysis of the Rickettsial genome showed that at least 15 autotransporter genes, denoted "surface cell antigen (*sca*)" genes, are present in the bacterial genome, and that some of them appear to have evolved under positive selection and are conserved within the genome of most Rickettsial species [48]. This suggests that the positively selected passenger domains may be involved in host cell interactions and may have conserved functions. Even though accumulating evidences have supported the hypothesis that Rickettsal Sca proteins may play a critical role in the bacterial-host interactions, there is no study on the role of Sca proteins in the pathogenesis of *O. tsutsugamushi* thus far. Here, I identified multiple *sca* genes in the genomes of two *O. tsutsugamushi* strains, Boryong [49] and Ikeda [50] and characterized them through bioinformatic approaches. Previously, it was reported the whole genome sequence of the *O. tsutsugamushi* Boryong strain [49] and profiled its global gene expression using a microarray system and proteomic approaches

[51]. From the proteomic analysis, it was predicted that 10 outer membrane proteins are unique to *O. tsutsugamushi* and two of which encode autotransporter proteins [51]. Analysis of two sequenced genomes, the *O. tsutsugamushi* Boryong and Ikeda strains [50], revealed four conserved genes (*scaA, C, D,* and *E*) encoding autotransporter proteins and *scaB* is duplicated in the Boryong strain but absent from the Ikeda strain [52].

Base on the hypothesis that the conserved Sca proteins may play a role in bacterial pathogenesis and represent conserved targets for vaccine development, I examined the potential role of Sca proteins in *Orientia* pathogenesis and evaluate the protective attributes of Sca protein administration against lethal *O. tsutsugamushi* infection in mice. I demonstrated that ScaA protein participates in bacterial adhesion. In addition, immunization with ScaA not only provides protective immunity against lethal challenge of the homologous strain, but also confers significant protection against heterologous strains when combined with TSA56. These results indicate that ScaA proteins could be a novel vaccine target for scrub typhus.

MATERIALS AND METHODS

Ethics statement

Animal experiments were approved by the Seoul National University Hospital Institutional Animal Care and Use Committee (SNUH IACUC No.12-0331-C1A03) and Institutional Biosafety Committee (IBC no.1210-042-004) were performed in strict accordance with the recommendations in the National Guide Line for the care and use of laboratory animals. Ethical approval for this work was granted by the Institutional Review Boards of Seoul National University Hospital (IRB no. C-1308-058-513)

Subjects and sample collection

Human peripheral blood was drawn from healthy volunteers (n = 20) and scrub typhus patient (n = 100) after obtaining informed consent at Chungnam National University Hospital (CNUH). Primary diagnosis of scrub typhus was performed by *O. tsutsugamushi*-specific immunofluorescence assays (IFA) or immune-chromatographic technique (ICT). The positive serology was as a four-fold or greater change in the titer of paired sera, or a single cut-off titer of IgM antibody ≥ 1:160 from an indirect immunofluorescence antibody assay against a mixture of *O. tsutsugamushi* antigens (Gilliam, Karp, Kato and Boryong) in clinical scrub typhus patients [19].

Cell culture

HeLa cells (ATCC CCL-2; American type culture collection, Manassas, VA), L929 cells (ATCC ; NCTC929), ECV304, an endothelial-like cell line, Vero (ATCC CCL-81) were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Welgene, Daegu, Republic of Korea) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Welgene), 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco BRL, Gaithersburg, MD, USA) at 37°C in 5% CO_2.

Antibodies and reagents

Both preimmune mouse sera and anti-Sca polyclonal mouse sera, produced from Balb/c mice immunized with purified Sca proteins (Cosmogenetech, Seoul, Republic of Korea), were used for the experiments. Horseradish peroxidase (HRP)-conjugated anti-mouse, anti-human IgG secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used for immunoblotting and HRP-conjugated anti-mouse IgG_1, IgG_{2c} (Santa Cruz Biotechnology) were used for ELISA. The Alexa Fluor 488- or Alexa Fluor 594-conjugated anti-mouse and -human antibodies used in the immunofluorescence assays were purchased from Molecular Probes (Invitrogen, Carlsbad, CA, USA). For the bead-binding assay, Fluoresbrite microparticles (1 μm; Polyscience Inc., Warrington, PA, USA) containing rhodamine were conjugated to GST or GST-ScaA proteins by using a

PolyLink protein coupling kit (Polyscience Inc., Warrington, PA, USA) in accordance with the manufacturer's instructions.

Preparation of *O. tsutsugamushi* and its genomic DNA

The Boryong, Karp, Kato, Gilliam strains of *O. tsutsugamushi* were purified using a modified Percoll gradient purification method [53]. *O. tsutsugamushi* was propagated in L929 cells. At 3 to 4 days post-infection, infectivity was determined using an indirect immunofluorescence assay (see below). When an infection rate of > 90% was achieved, the cells were harvested by centrifugation at 6,000 × *g* for 20 min. The cell pellet was resuspended with Tris-sucrose (TS) buffer (33 mM Tris-Cl (pH 7.4) and 0.25 M sucrose) and homogenized using 100 strokes of a Polytron homogenizer (Wheaton Inc., Millville, NJ, USA) followed by centrifugation at 200 × *g* for 5 min. The supernatant was then mixed with 40% Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden) in TS buffer and centrifuged at 25,000 × *g* for 60 min. The bacterial band was collected and centrifuged at 77,000 × *g* for 30 min. The bacterial pellet was washed 3 times in TS buffer, resuspended in DMEM and stored in liquid nitrogen until use. The infectivity titer of the inoculum was determined as previously described [54], with minor modifications. Infected-cell–counting units (ICU) were calculated as follows: [(total number of cells used for infection) × (percentage of infected cells) × (dilution of the *O. tsutsugamushi* suspension)]/100 [54]. *O. tsutsugamushi* was collected and used for genomic DNA extraction by use of an RBC

genomic DNA extraction kit (RBC Bioscience Co., Taipei, Taiwan) according to the manufacturer's instructions.

Cloning, expression, and purification of proteins

The *tsa56*, *p47*, and *sca* genes were PCR amplified from *O. tsutsugamushi* genomic DNA by use of the specific primer pairs presented in Table 2. The amplified region of each gene is summarized in Table 2 and Fig. 1A. The amplified DNAs were then cloned into a pET-28a or pGEX4T-1 vector (Novagen, Gibbstown, NJ, USA) for sequence analysis and protein expression. For the bacterial adhesion and invasion assay, full length of *scaA* gene, except signal sequence region was cloned into a pET-28a plasmid which is containing pelB signal sequence [55]. For the expression and purification of TSA56, 47KDa, Sca proteins, each gene containing pET-28a and pGEX4T-1 constructs were transformed into *Escherichia coli* BL21(DE3) strain (RBC Bioscience Co., Taipei, Taiwan). The strain were grown into Luria-Bertani (LB) broth supplemented with 50 μg/ml kanamycin at 37°C for overnight. Cultures were inoculate in fresh LB broth and grown at 37°C until the optical density at 600 nm (OD_{600}) reach to 0.5. Protein expression was induced by adding 0.1 mM isopropyl-D-thiogalactoside (IPTG; Duchefa, Zwijndrecht, Netherlands) for 16 h at 16°C. Bacteria were harvested by centrifugation at 1,000 × *g* for 10 min, resuspended in binding buffer (300 mM NaCl, 50 mM sodium phosphate buffer) containing 1 mg/ml of lysozyme, incubated at 4°C for 30 min, and disrupted by sonication on ice for 5 min. The sonicated lysates

were centrifuged at 1,600 × *g* for 20 min at 4°C and the supernatants applied to a Ni-nitrilotriacetic acid (NTA) His-resin (Qiagen, Calrsbad, CA, USA) or glutathione-Sepharose 4B (GS4B) columns (GE Healthcare, Piscataway, NJ, USA). His-tagged proteins bound to the Ni-NTA resin were eluted with elution buffer (300 mM NaCl, 50 mM sodium phosphate buffer, 250 mM imidazole) and serially dialyzed against elution buffer to remove any free imidazole. The identity and purity of proteins were assessed by Western blotting and Coomassie blue staining, respectively.

Sequence analysis

The passenger domains of *sca* genes and full-length of *scaA* genes were amplified from the genomes of Boryong, Gilliam, Karp, and Kato strains and sequenced (Macrogen Co., Seoul, Republic of Korea). DNA sequence information for *sca* and *tsa56* genes were found in the genomes of *O. tsutsugamushi* strains Boryong (GenBank accession no. AM494475.1) and Ikeda (GenBank accession no. AP008981.1). Sequences of *tsa56* genes from each strain (AM494475.1 for Boryong, AY956315.1 for Gilliam, AY836148.1 for Karp, and GU120147.1 for Kato strain) were used for comparative analysis. The sequences of *sca* passenger domains were deposited in GenBank (Accession No. JQ996623-JQ996633). The full ORF sequences of *scaA* genes amplified from Gilliam, Karp, and Kato strains were deposited to GenBank under accession no. KM591910, KM591911, and KM591912, respectively.

Nucleotide sequence alignments for constructing phylogenetic trees were processed by Clustal W with maximum likelihood method implemented in MEGA6 software [56]. The similarity and identity of those nucleotides and amino acids was calculated through Matrix Global Alignment Tool (MatGAT) version 2.03 [57]. The aligned nucleotide sequences were evaluated in SimPlot version 3.5.1 with Kimura (2-parameter) and Empiric Ts/Tv ratio settings [57]. The aligned amino acid sequences were analyzed through the BLOSUM62-referenced 100 amino acid sliding window analysis. The output values were calculated from R-Project (http://www.r-project.org/). Line graphs were visualized by GraphPad Prism software version (Graph-Pad Software Inc., La Jolla, CA, USA). Repeat sequences within a *scaA* gene were also analyzed using Tandem Repeat Finder software [58].

Immunofluorescence microscopy

Immunofluorescence microscopy was used to visualize *O. tsutsugamushi*. HeLa cells infected with *O. tsutsugamushi* were washed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde (PFA), and then incubated with pooled scrub typhus human serum or anti-ScaA immune serum for 1 h at room temperature, followed by incubation with Alexa Fluor 488-conjugated goat anti-mouse IgG and Alexa Fluor 594-conjugated mouse anti-rabbit IgG (Invitrogen) [59]. In some experiments, recombinant *E. coli* was stained with preimmune mouse serum, anti-ScaA serum, or anti-*E. coli* serum, followed by incubation with Alexa Fluor 488-

conjugated mouse anti-rabbit IgG (Invitrogen). Cells were examined under an Olympus FV1000 laser scanning confocal microscope (Olympus, Tokyo, Japan). Images of cell sections were analyzed and processed using the Olympus Fluoview software (Olympus, Tokyo, Japan).

Western blot

To confirmed the expression of ScaA in *O. tsutsugamushi* or determined the reactivity of the anti-ScaA antibody, Western blotting has been performed. L929 cells were infected with *O. tsutsugamushi* as described above and cells were collected from uninfected cells or from infected cells at 24 h post-infection. Cells were washed three times with ice-cold PBS and then lysed in 0.5% NP-40 lysis buffer (0.5% NP-40, 20 mM Tris [pH 7.4], 150 mM NaCl, Complete protease inhibitor cocktail (Sigma-Aldrich). Lysates were centrifuged at 16,000 × *g* for 15 min to pellet insoluble matter. Samples were adjusted for equal protein content, boiled in SDS sample buffer, resolved by SDS-PAGE, and transferred to a polyvinylidene difluoride (PVDF) membrane using a Trans-Blot SD semidry transfer cell (Bio-Rad, Hercules, CA, USA). Immunoblots were developed using the Super Signal West Pico chemiluminescent reagent (Pierce Biotechnology, Rockford, IL, USA).

Bead-binding assay

Fluoresbrite microparticles (Polyscience Inc., Warrington, PA, USA) were covalently bound to GST or GST-ScaA$_{33\text{-}1000}$ proteins by using the Polylink protein coupling kit (Polyscience). Fluoresbrite microparticles were resuspended in 100 μl coupling buffer (50 mM MES, pH 5.2, 0.05% Proclin-300) and incubate with EDAC (1-Ethyl-3-(3-Dimethylaminopropyl) carbodiimide) for 15 min at RT. 100 μg of GST or GST-ScaA$_{33\text{-}1000}$ proteins were added and incubated at room temperature for 1 h with gentle mix. After extensive washing with PBS, fluoresbrite microparticles (416 μg/well) were added to HeLa cells (2.4×10^5 cells in a 24-well plate). Then, cells were incubated for 1 h, washed extensively with PBS, and fixed with 4% paraformaldehyde for 15 min. Cells were subsequently stain with ToPro-3 (Molecular Probes, Eugene, OR, USA) for 20 min at RT and observed under a FV1000 confocal microscope (Olymphus, Tokyo, Japan) or analyzed using a FACScan (Becton Dickinson, San Jose, CA, USA).

Cellular adhesion and invasion assays

Bacterial adhesion and invasion assays were performed as previously described [59]. Briefly, *E. coli* strains harboring a vector or pET28a encoding *scaA* gene were induced with IPTG and added to confluent monolayers of ECV304, HeLa, or Vero cells in serum-free media. Portions of the bacterium-containing media were plated to determine the number of colony-forming unit

(CFU) added to each host cell monolayer. Contact between bacteria and the mammalian cells was synchronized by centrifugation at 200 × *g*, and the preparations were incubated at 37°C for either 20 min or 60 min for the adherence and invasion assays, respectively. For the invasion assays, infected cells were washed extensively with PBS and incubated for 2 h with complete medium supplemented with 100 μg/ml of gentamicin to kill any extracellular bacteria [60]. For all *E. coli* assays, infected cells were washed extensively with PBS and the bacteria liberated by incubation with 0.1% Triton X-100 in sterile water. The lysate was then plated on LB agar to enumerate the cell-associated bacteria. The results were expressed as the percentages of bacteria recovered relative to the number of bacteria in the initial inoculum [60]. For antibody neutralization assays, HeLa cells or ECV304 cells were grown in a 24-well plate (2.4×10^5 cells/well) and infected with *O. tsutsugamushi* or *E. coli* expressing ScaA in the presence of 1:100-diluted preimmune or anti-Sca polyclonal mouse serum. Association of *E. coli* with host cells were measured by CFU assays as mentioned above. To detect intracellular *O. tsutsugamushi*, infected cells were stained by differential immunofluorescence assay [61]. First, cells were washed three times with PBS, fixed with 4% paraformaldehyde, and incubated with an anti-TSA56 antibody, followed by Alexa Fluor 488-conjugated goat anti-mouse IgG to stain the cell-surface associated-bacteria. Next, cells were permeabilized in a 0.2% Triton X-100 solution for 15 min and incubated with scrub typhus patients' sera for 1 h, followed by Alexa Fluor 633-conjugated goat anti-human IgG to stain intracellular bacteria. Cells were observed using an Olympus FV1000 laser

confocal microscope (Olympus, Tokyo, Japan) and analyzed using the Fluoview software (Olympus, Tokyo, Japan).

Enzyme linked immunosorbent assay (ELISA)

To determine the antibody titers of anti-TSA56, 47 kDa protein, and Sca antibody in the scrub typhus patient samples or immunized mice, immunoassay plates (96-well plates; Nunc, Rochester, NY, USA) were coated with 100 μl of purified antigen at a final concentration of 1 μg/ml at 4°C overnight. The plates were then blocked for 2 h at room temperature with PBS containing 5% skim milk. 100 μl of serum samples serially diluted in 2-fold were incubated at room temperature for 2 h. After washing with PBS containing 0.05% Tween 20 (0.05% PBST), 100 μl of 1:10,000 diluted HRP-conjugated goat anti-mouse IgM, IgG_1, or IgG_{2c} (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was added and incubated at room temperature for 1h. For human serum titration, 100 μl of 1:10000 diluted HRP-conjugated mouse anti-human total IgG (Southern Biotechnology Associates, Birmingham, AL. USA) were incubated at room temperature for 1 h. Wells were washed with 0.05% PBST and incubated with 3,3',5,5'-tetramethylbenzidine (TMB) peroxidase substrate solution (KPL, Gaithersburg, MD, USA) for 10 min. The reactions were stopped by addition of 1 M phosphoric acid (H_3PO_4) solution. Absorbance was measured at 450 nm using a microplate reader (Beckman Coulter Inc., Fullerton, CA).

Immunization of mice and challenges

For immunization experiments, 6- to 8-week-old female C57BL/6 mice (Orient Bio Inc., Seongnam, Republic of Korea) were used. Groups (n = 5/group) of mice were immunized subcutaneously three times at two weeks interval. 20 μg of purified ScaA, ScaC, or TSA56 proteins in PBS emulsified 1:1 with 2% alhydrogel adjuvant (Invitrogen, CA, USA) was used for each immunization. Blood samples were collected with retro-orbital puncture 1 week after each injection and used to determine the serum antibody titer. One week after the final immunization, mice were challenged intraperitoneally with 10 × or 100 × LD_{50} of different *O. tsutsugamushi* strains. Body weight and mice survival was monitored for one month after bacterial challenge.

Statistical analysis

The data was analyzed using the Graph Pad Prism 5.01 software and SigmaPlot (Jandel, San Rafael, CA, USA). Statistical analysis of all the experimental data except survival rate was performed using the two-tailed Student's t-test with 95% confidence interval, One-way ANOVA and Bartlette's test were performed and nonlinear regression module. Data are expressed as the mean ± standard deviation. Statistical analysis on survival rates were performed using the Mantel-Cox Log Rank test. A p-value of < 0.05 was considered statistically significant.

RESULTS

Genetic analysis of the *sca* genes from *O. tsutsugamushi* Boryong strain

According to the global gene expression profiling data of *O. tsutsugamushi* Boryong strain, 6 genes were identified to encode conserved autotransporter domain (Table 1). Among them, *scaB* (OTBS_0864 and OTBS_2137 [GenBank accession no.CAM79930.1 and CAM81232.1, respectively]) gene was duplicated in Boryong strain but absent from the Ikeda strain. Therefore, five *sca* genes exists in the genome of Boryong strain. To predict the Sca protein domain and structure, I applied the Sca amino acids sequence derived from Boryong strain to the SMART (Simple Modular Architecture Research Tool) and I-TASSER (Iterative Threading ASSEmbly Refinement) protein analyzing software (Figure 1). As shown in Figure 1, all the Sca protein contains about 30 amino acids length of signal peptide in N-terminus and about 300 amino acids length of conserved autotransporter (AT) domain in C-terminal region. Each gene has unique passenger domain with different sequence length. Among the Sca proteins, ScaC protein contains a transmembrane domain close to the AT domain and only ScaD protein contains 2 conserved internal repeated domain whereas other Sca proteins had no distinct functional motifs in the sequence. Prediction of 3D structures based on the amino acid sequences revealed

representative β-barrel domain similar to other Gram negative bacterial autotransporter proteins [62, 63]

Comparisons of the *sca* gene sequences in the different strains of *O. tsutsugamushi*

Since AT domains are highly conserved between *Rickettsia* Sca proteins [48], I further examined the degree of sequence similarity between the passenger domains of the *Orientia* Sca proteins found in the genomes of Boryong and Ikeda strain [64, 65]. The passenger domains, including the signal peptide sequences, of the *Orientia* Sca proteins showed 9–23% amino acid sequence identity between paralogs (Table 2). Similar levels of identity were observed between *Rickettsia* Sca proteins [48]. However, the amino acid sequences of *Orientia* Sca proteins are also highly conserved between orthologs (73–84% identical at the amino acid level), implying functional conservation. Of the *Orientia* Sca paralogs, the ScaC protein showed the highest sequence identity (84%) between the Boryong and Ikeda strains.

By using the specific primer sets for each *sca* gene (Table 3), I was also able to amplify *scaA*, *scaC*, and *scaD* genes from the genomes of three prototype strains (Karp, Kato, and Gilliam strains) as well as the Boryong strain (Figure 2A). Interestingly, the sizes of amplified *scaD* genes were different in all four strains. In the case of *scaB*, the gene was detected only in the Boryong strain. On the other hand, I could not amplify *scaE* only from the Kato strain. When I compared the sequences of the amplified *sca* genes, each gene group formed

specific clusters in a phylogenetic tree (Figure 2B). However, nucleotide sequence distances among the strains did not show any consistency among the different Sca groups: i.e. the sequence variation among the strains showed different patterns within each *sca* group. The identities of translated amino acids sequences (Figure 3) among the *sca* genes of different strains are also quite variable: 65.8 ~ 81.8% for ScaA, 77.4 ~ 97.5% for ScaC, 69.7 ~ 93.8% for ScaD, and 62.8 ~ 85.3% for ScaE. ScaC proteins are the most conserved and ScaE proteins show the highest variation among the different strains. The size variation observed among *scaD* genes of different strains is mainly due to the presence of different numbers of internal repeat sequences (Figure 3). There are two types of amino acid repeat sequences. One is comprised of around 70 amino acids that repeat five times in the ScaD of the Boryong strain and three times in the Gilliam strain. The other one consists of 16 amino acids that were detected five times in the gene of the Boryong strain and once in that of the Gillam strain. It is also interesting to note that the phylogenetic distribution of *scaC* genes of different strains is most similar to that of *tsa56* genes (Figure 2B), which has 81.5 ~ 90.9% of amino acid sequence identity.

Detection of antibodies against Sca proteins in Scrub typhus patients.

In order to examine anti-Sca protein antibody responses in scrub typhus patients, we screened one hundred sera from potential scrub typhus

patients during Korea's endemic season. Based on immunofluescence assay (IFA) using L929 cells infected with *O. tsutsugamushi*, seventy sera with varying IFA titers (from 0 to 1,280), were randomly selected and used for ELISA using purified Sca proteins (Figure 4A). IFA-negative sera from ten healthy volunteers were used to determine the cutoff values. The cutoff values were defined as the mean OD_{490} + 2 standard deviation in each experimental condition [66]. To determine the optimal dilution of serum in our ELISA condition, sera from ten healthy volunteers, ten IFA-negative patients, and ten patients with scrub typhus (IFA titer ≥ 1,280) were serially diluted (1:200 ~ 3,200) and examined by ELISA using the different antigens. As shown in Figure 4B, ELISA signals were generally reduced as the sera were diluted, indicating that the antigen-antibody reactions were not saturated within the dilution range. The cutoff values, which indicate the presence of non-specific antibodies in the sera, are relatively higher in ELISAs using 47 kD protein, ScaB, or ScaE as antigens. Non-linear regression analysis was performed to compare IFA titers of patient sera after 1:1000 dilution in PBS with the OD_{490} values from ELISAs of different *O. tsutsugamushi* antigens (Figure 4C). The best correlation of ELISA data with IFA titer was observed when ScaA (r^2 = 0.775, $p < 0.05$) used as an antigen, whereas ScaB showed the lowest correlation (r^2 = 0.082). It is notable that the OD_{490} values from ELISA using TSA56 as an antigen against patients' sera with 1:40 of IFA titer rapidly saturated, although there was a relatively low correlation (r^2 = 0.325, $p <$ 0.001) when compared to those of ELISAs using ScaA or ScaC. These results suggest that specific antibody responses against TSA56 are most robustly

induced in scrub typhus patients, and anti-ScaA and ScaC antibody responses are significant only in patients with high IFA titers (≥ 1:640). In addition, specific antibody responses against 47 kD protein, ScaB, and ScaE are relatively weaker in scrub typhus patients.

Expression of *scaA* gene in *O. tsutsgamushi*

Previously, I found that ScaC protein is actively expressed on the bacterial surface during infection and involved in the bacterial adhesion to host cells, potentially via the interaction with fibronectin molecule [59]. In order to examine whether the ScaA is expressed in *O. tsutsugamushi*, I generated a polyclonal anti-ScaA antiserum by immunizing mice with purified ScaA passenger domain (amino acids 30 to 1,000). The specificity of this antiserum was confirmed by ELISA and immunoblot analysis using the recombinant Sca proteins (Figure 5). To identify endogenous ScaA protein in *O. tsutsugamushi*, the anti-ScaA serum was reacted with the cell lysates of *O. tsutsugamushi*-infected cells. Immunoblot analysis showed that a ~ 150 kDa protein was recognized by the anti-ScaA serum in infected cells but not in uninfected control (Figure 6A). The full-length ScaA protein was predicted to have a mass of 156 kDa. Anti-ScaA serum was also weakly reacted with a few bands lower than ~ 150 kDa, suggesting a cross-reactive antigens or fragmented ScaA protein in infected cells. The TSA56 protein, a major outer membrane protein of *O. tsutsugamushi*, was used as a positive control [52]. To further confirm the specificity of the anti-ScaA antiserum for *O.*

tsutsugamushi, intracellular bacteria were stained using the pooled sera of scrub typhus patients together with anti-ScaA serum or preimmune mouse serum. As shown in Figure 6B, anti-ScaA serum readily detected the bacteria within the host cells, whereas the preimmune serum did not. In addition, we found that the ScaA proteins were located on the periphery of bacterial cells (Figure 6B. lower panels, inset boxes). Taken together, these results confirm that the *scaA* gene is actively translated in *O. tsutsugamushi* within eukaryotic host cells and that the protein might be expressed on the outer membrane of the bacteria.

ScaA mediates bacterial adhesion to host cells

Recently, several studies reported that rickettsial Sca proteins mediate bacterial adherence to and/or invasion into mammalian host cells [52, 67]. Therefore, we examined whether the *O. tsutsugamushi* ScaA protein could function as a virulence factor for bacterial adhesion and/or invasion. First, we performed a bead-binding assay using fluorescent microbeads (1 μm in diameter) covalently conjugated to either purified GST or GST-ScaA. Incubation of HeLa cells with GST-ScaA-conjugated beads resulted in marked binding to the host cells, even after extensive washing. The control beads linked to GST alone interacted only weakly with the HeLa cells (Figure 7A) [52]. The interaction of the fluorescent beads with the host cells was quantified using flow cytometry (Figure 7B). After fixation, the mean fluorescence intensity (MFI) of the HeLa cells incubated with beads

conjugated to GST-ScaA dramatically increased (MFI = 50.1) compared to that of cells incubated with beads conjugated to GST (MFI = 13.6) or that of untreated cells [52].

To further verify the role of the *scaA* gene in bacterial adherence to host cells, we utilized a heterologous *E. coli* expression system [52]. The entire *O. tsutsugamushi scaA* open reading frame was cloned into the IPTG-inducible expression vector, pET-28a, to yield the plasmid pScaA as previously described (Figure 8A) [52]. ScaA was expressed in the *E. coli* strain BL21 (DE3) and analyzed using anti-ScaA serum and confocal microscopy after fixation with 4% paraformaldehyde. As shown in Figure 8B, ScaA was readily detectable on the surface of all the recombinant *E. coli* cells by anti-ScaA serum (lower panels) but not on bacteria harboring empty vector (upper panels). We next examined the ability of ScaA-expressing *E. coli* to adhere to monolayers of nonphagocytic host cells [52]. Epithelial (HeLa and Vero) and endothelial (ECV304) cells were incubated with recombinant *E. coli* harboring an empty vector or pScaA. The cells were then washed extensively to remove non-adherent bacteria, fixed, and analyzed under a confocal microscope after staining with an anti-*E. coli* antibody and the nuclear stain ToPro-3. Immunofluorescence analysis revealed that ScaA expression resulted in an increase in the number of adherent *E. coli* bacteria (Figure 8C). This ScaA-mediated enhanced adhesion was verified by removing the adherent bacteria from the live host cells and counting them using a CFU-based quantification assay [52]. The assay confirmed that ScaA expression significantly increased bacterial adherence to various types of cells

(Figure 8D). Therefore, the expression of *O. tsutsugamushi* ScaA on the outer surface of *E. coli* enhances bacterial adherence to nonphagocytic host cells. We also examined whether our anti-ScaA antibody could neutralize the adhesion of bacteria expressing ScaA to host cells. Treatment of the recombinant bacteria with the anti-ScaA antibody for 1 h before adding it to host cells reduced bacterial adhesion by approximately four fold (Figure 8E) when compared to treatment with nonimmune serum, indicating that the anti-SacA antibody can block ScaA-mediated bacterial adhesion to host cells.

Inhibition of *O. tsutsugamushi* adhesion to host cells by anti-ScaA antibody

To evaluate the functional characteristics of various anti-Sca antibodies against *O. tsutsugamushi* infection, various anti-Sca antibodies were generated by immunization of Balb/c mice with purified Sca proteins and one week after third immunization, antibody production were confirmed by ELISA (Figure 5). In order to confirm the neutralizing effect of anti-Sca antibodies on *O. tsutsugamushi* infection, HeLa cells were infected with the pathogen in the presence of various anti-Sca antibodies or nonimmune serum. At 4 h after infection, bacterial infection was examined by confocal microscopy after differential immunoflourescent staining and the *O. tsutsugamushi*/host cell ratio was determined (Figure 9). Presence of anti-ScaA antibody in the infection media significantly inhibited *O. tsutsugamushi* infection of host cells. The number of bacteria per cell was reduced by

approximately 50% compared with the control group treated with nonimmune serum, whereas other anti-Sca antibodies failed to significantly inhibit bacterial infection, indicating that the anti-ScaA antibody could provide specific protective effect against bacterial infection.

ScaA vaccination provides protective immunity against *O. tsutsugmaushi* infection

Protective effect of the anti-ScaA antibody against *O. tsutsugamushi* infection was further investigated *in vivo* by challenging mice with 100 × LD_{50} of *O. tsutsugamushi* at 7 d after immunization. All the vaccine antigens were derived from the *O. tsutsugamushi* Boryong strain and mice were challenged with the same bacterial strain. As shown in Figure 10, a significant level of protection against the homologous strain was observed in the ScaA-immunized group as well as in the TSA56-immunized mice. In contrast, ScaC immunization did not provide any significant protection. Therefore, vaccination with a ScaA antigen could provide protective immunity against homologous strain infection as efficiently as TSA56, a dominant membrane antigen of *O. tsutsugamushi*.

Next, I tested whether the candidate bacterial antigens could provide protective immunity against heterologous strain infection. Each group of mice were immunized with the indicated antigens derived from the *O. tsutsugamushi* Boryong strain and then challenged with a low (10 × LD_{50}) or high (100 × LD_{50}) dose of Boryong, Karp, or Kato strains (Figure 11 and

Table 5). I confirmed significant increases of both type 1 (IgG_{2C}) [68] and type 2 (IgG_1) antibodies against ScaA and/or TSA56 after third immunization (Figure 12). Following infection with *O. tsutsugamushi*, mock-immunized mice began to lose body weight between 8 – 12 d after inoculation, depending on the bacterial doses and strains challenged, and lost 10 – 25% of body weight before they expired (Figure 13). All the unimmunized mice had expired by 10 – 17 d after infection. The immunized mice survived after infection with *O. tsutsugamushi* maintained normal body weight during the experiment, but the expired ones rapidly lost their body weight from 4 – 8 d before death. All the mice immunized with ScaA or TSA56 were protected from the homologous Boryong strain regardless of infection dose, and were also protected against low dose ($10 \times LD_{50}$) Karp strain infection. When mice were challenged with high dose ($100 \times LD_{50}$) Karp strain infection, all the mice immunized with both ScaA and TSA56 survived and 80% of ScaA-immunized mice were protected. Although TSA56 immunization also provide significant protection (40% survival, $p = 0.017$) compared to mock-immunized control group, the protection level was significantly ($p = 0.049$) lower than that afforded by vaccination with both ScaA and TSA56 antigens. In the groups challenged with low dose Kato strain, groups immunized with both ScaA and TSA56 showed the best protective effect (60% survival) and TSA56 immunization provided only 20% survival. Immunization with ScaA also provided significant protection (40% survival). Although the level of protection afforded by ScaA (median survival = 22 d) was higher than that of TSA56 (median survival = 19 d), the difference was not statistically

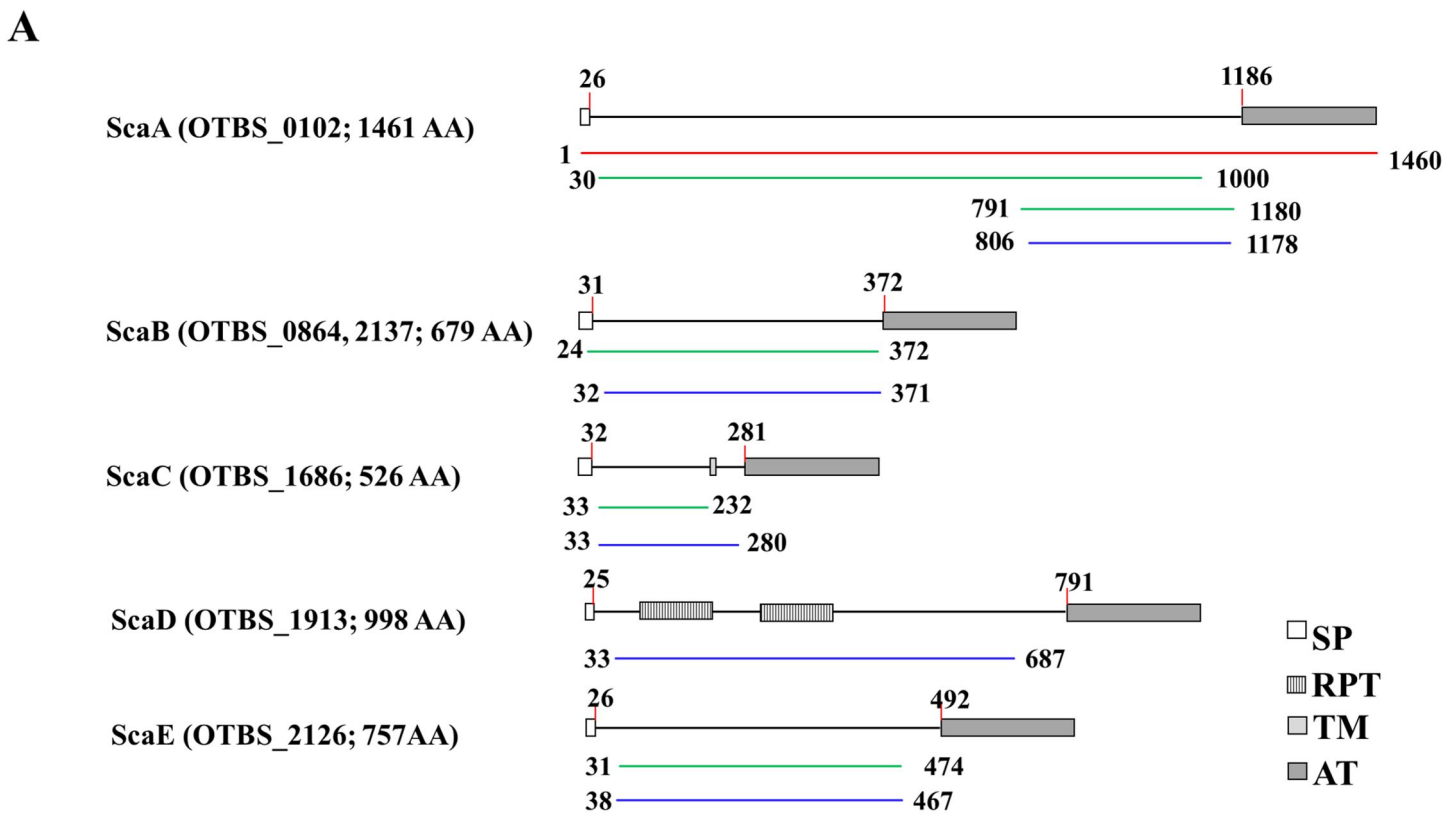
significant ($p > 0.05$). In contrast, ScaA vaccination (median survival = 18 d) significantly prolonged the survival of mice compared to TSA56 and mock-immunization ($p < 0.01$, median survival = 15 d in both groups) when mice were challenged with high dose of Kato strain. Immunization of TSA56 together with ScaA provided a similar level of protection as that observed in the ScaA immunization group even though all the challenged mice ultimately succumbed to pathogen infection.

	CDS (Boryong)	CDS (Ikeda)	
	OTBS_0021	OTT_1548	22kDa antigen
	OTBS_0047	OTT_1511	repeat-containing protein G
*	OTBS_0102	OTT_1215	outer membrane protein, scaA
	OTBS_0135	OTT_1334	hypothetical protein
	OTBS_0137	OTT_1336	hypothetical protein
	OTBS_0161	OTT_1358	ankyrin repeat-containing protein, ank2A2
	OTBS_0170	OTT_1366	hypothetical protein
	OTBS_0199	OTT_1784	hypothetical protein
	OTBS_0227	OTT_0111	hypothetical protein
	OTBS_0240	OTT_1222	repeat-containing protein F
	OTBS_0275	OTT_1396	hypothetical protein
	OTBS_0398	OTT_0078	hypothetical protein
	OTBS_0576	OTT_0970	hypothetical protein
	OTBS_0601	OTT_0946	56 kDa type-specific antigen-like protein
	OTBS_0602	OTT_0945	56 kDa type-specific antigen
	OTBS_0639	OTT_1603	hypothetical protein
	OTBS_0658	OTT_0169	hypothetical protein
	OTBS_0664	OTT_0849	hypothetical protein
	OTBS_0671	OTT_0894	hypothetical protein
	OTBS_0674	OTT_0896	hypothetical protein
	OTBS_0691	OTT_0049	ankyrin repeat-containing protein, ank1U5
	OTBS_0732	OTT_0347	hypothetical protein
	OTBS_0758 & OTBS_1561	OTT_0622	TPR and ankyrin repeat-containing protein, tpr1B10
	OTBS_0782	OTT_0378	hypothetical protein
	OTBS_0787	OTT_0836	hypothetical protein
	OTBS_0920	OTT_0262	hypothetical protein
	OTBS_0922	OTT_0264	hypothetical protein
	OTBS_0923	OTT_0265	hypothetical protein
	OTBS_0999	OTT_1962	hypothetical protein
	OTBS_1045	OTT_0839	hypothetical protein
	OTBS_1096	OTT_0330	hypothetical protein
	OTBS_1097	OTT_0331	hypothetical protein
	OTBS_1110	OTT_0578	hypothetical protein
	OTBS_1271	OTT_0304	hypothetical protein
	OTBS_1306	OTT_1000	hypothetical protein
	OTBS_1311	OTT_0584	hypothetical protein
	OTBS_1376	OTT_0161	hypothetical protein
	OTBS_1395	OTT_0724	hypothetical protein
	OTBS_1402	OTT_0720	repeat-containing protein C
	OTBS_1418	OTT_0826	hypothetical protein
	OTBS_1456 & OTBS_1749	OTT_0440	repeat-containing protein B
	OTBS_1492	OTT_0630	hypothetical protein
	OTBS_1546	OTT_0690	hypothetical protein
	OTBS_1613	OTT_0904	hypothetical protein
	OTBS_1617	OTT_0907	hypothetical protein
	OTBS_1626	OTT_0910	hypothetical protein
	OTBS_1633	OTT_0917	hypothetical protein
	OTBS_1646	OTT_0931	hypothetical protein
	OTBS_1682	OTT_0464	hypothetical protein
	OTBS_1684	OTT_0462	hypothetical protein
*	OTBS_1686	OTT_0460	cell surface antigen, scaC
	OTBS_1689	OTT_0459	ankyrin repeat-containing protein, ank2A10
	OTBS_1690	OTT_0458	hypothetical protein
*	OTBS_1913	OTT_1741	cell surface antigen, scaD
	OTBS_1984	OTT_1539	hypothetical protein
	OTBS_2008	OTT_0664	hypothetical protein
	OTBS_2051	OTT_1232	ankyrin repeat-containing protein, ank2A12
*	OTBS_2126	OTT_1766	outer membrane protein, scaE

Table 1. *O. tsutsugamushi*-specific genes shared by the Boryong and Ikeda strains[a]

[a] *O. tstusgamushi*-specific genes shared by the two strains were reported in a previous study [50].

[b] Genes with a gray background were detected in proteomic analysis in a previous study [51].


A
ScaA (OTBS_0102; 1461 AA)
26
1186
1
1460
30
1000
791
1180
806
1178
ScaB (OTBS_0864, 2137; 679 AA)
31
372
24
372
32
371
ScaC (OTBS_1686; 526 AA)
32
281
33
232
33
280
ScaD (OTBS_1913; 998 AA)
25
791
33
687
ScaE (OTBS_2126; 757AA)
26
492
31
474
38
467
SP
RPT
TM
AT

B

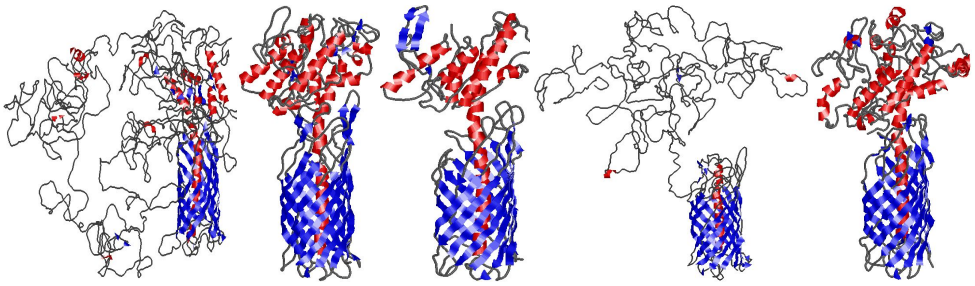


Figure 1. Schematic representation of the five Sca proteins whose genes were identified in the *O. tsutsugamushi* genome and demonstration of purified bacterial antigens.

(A) Domain structures were predicted using SMART (simple modular architecture research tool [http://smart.embl-heidelberg.de/]). The amino acid positions (indicated by numbers) are based on the sequences from the Boryong strain. (Green lines : Cloned region for protein expression, Blue lines : Passenger domains used for sequence analysis, Red line : Full-length used for sequencing analysis) (SP, signal peptide; RPT, internal repeat sequence; TM, transmembrane domain; AT, autotransporter domain; AA, amino acids) (B) 3D Structure model of the autotransporter proteins predicted by I-TASSER program (http://zhanglab.ccmb.med.umich.edu/I-TASSER)

Table 2. The degree of identity and divergence among Sca sequences[a].

		Percent identity								
		ScaA (BR) 1185 aa	ScaB (BR) 374 aa	ScaC (BR) 280 aa	ScaD (BR) 718 aa	ScaE (BR) 494 aa	ScaA (IK) 1232 aa	ScaC (IK) 274 aa	ScaD (IK) 552 aa	ScaE (IK) 492 aa
Divergence	ScaA (BR)		12.3	16.1	12.7	11.7	**75.3**	19.7	16.8	11.4
	ScaB (BR)	386.0		10.7	17.4	19.8	11.2	9.9	15.5	23.0
	ScaC (BR)	159.9	323.0		15.4	9.3	18.9	**83.6**	18.6	10.7
	ScaD (BR)	284.0	311.0	323.0		12.8	13.5	17.2	**79.5**	13.4
	ScaE (BR)	422.0	198.0	313.0	357.0		11.5	13.9	11.9	**73.2**
	ScaA (IK)	**27.1**	426.0	174.6	282.0	466.0		16.4	15.8	10.4
	ScaC (IK)	155.9	313.0	**18.6**	332.0	298.0	165.2		14.6	15.3
	ScaD (IK)	296.0	325.0	292.0	**23.2**	362.0	305.0	285.0		11.6
	ScaE (IK)	430.0	186.5	292.0	317.0	**26.9**	482.0	285.0	335.0	

[a] The amino acid sequences of the signal peptide and passenger domain from each of the Sca proteins were used for analysis and the number of amino acids of each protein is presented.

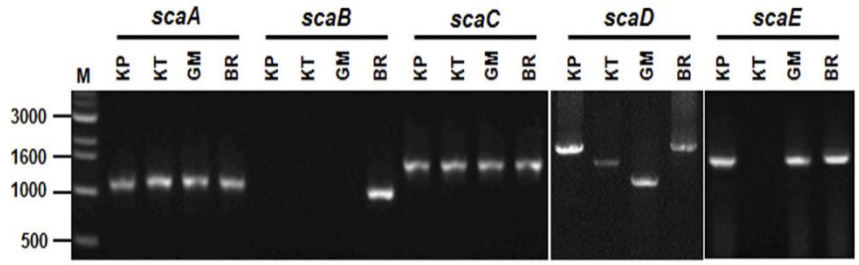
Table 3. Primer sequences used in this study

Gene	Primer direction	Primer sequence[a]	Product size (bp) (amplified region [nt positions])[b]
tsa56	Forward	GGCGGATCCTAATAATCCTGGGCATATG	1,344 (253-1596)
	Reverse	CGGTCGACTAGGTCAGCATAGAGTTT	
p47	Forward	GGCGGATCCATTAGTGTAAATAGTTTATCCG	1,278 (121-1398)
	Reverse	CGGTCGACTTACTTATTAATATTAGGTAAAGC	
scaA	Forward	CGGGATCCTCATCTGCTAGAGGTGAGATG	1,169 (2371-3540)
	Reverse	CGGTCGACATCGCCTTTTAAACCCGGGTTACT	
	Forward	GGCGGATCCTTACTTGGAGATTATACTG	1,121 (2414-3534)
	Reverse	CGCTCGAGTTGTTTATTGATATTAGATCC	
	Forward	CGGGATCCGATCCATCAGCTTCATCA	2,913 (88-3000)
	Reverse	CGGTCGACTATATCTTCGTCTTTGCC	
	Forward	AAGAATTCATGAAAAACTCAAGAAAG	4,383 (1-4383)
	Reverse	GCCTCGAGAAA GCT AGC CTT TGC ATA	
scaB	Forward	GGCGGATCCAGTACAACTCAAAGGATATTAGG	1,047 (70-1116)
	Reverse	CGGTCGACACTACTACAAATGTTTGATCC	
scaC	Forward	GGCGGATCCAAAAGTATAACTCCAGAAAAGTG	600 (97-696)
	Reverse	CGGTCGACGTTTAATTTAGCACGATTTAT	
	Forward	GGCGGATCCATGTACCAATCTAAT	1,578 (1-1578)
	Reverse	CGGTCGACAAAATTAGTTCCTATATG	
scaD	Forward	AAGGATCCCAATTAAGTGAGCGACTA	1,926 (136-2061)
	Reverse	CGGAATTCTACCGTAGCAACAGTAACAGC	
scaE	Forward	GGCGGATCCAATGTAAATGCACAGCCCAATAG	1,332 (91-1422)
	Reverse	CGGTCGACCTGTACTGTTGCTATTTTAGA	
	Forward	GGCGGATCCAATGTAAATGCACAGCCCAA	1,291 (111-1401)
	Reverse	CGGTCGACCTGTACTGTTGCTATTTTAGA	

[a] Restriction enzyme sites are underlined.

[b] For Boryong strain gene

A



B

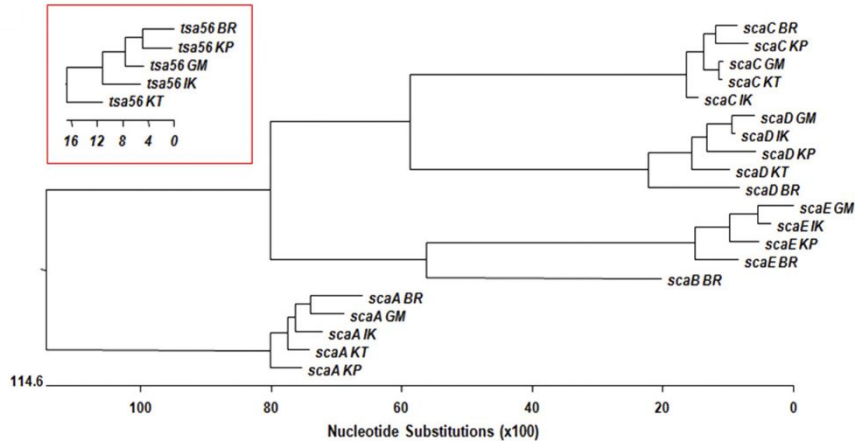
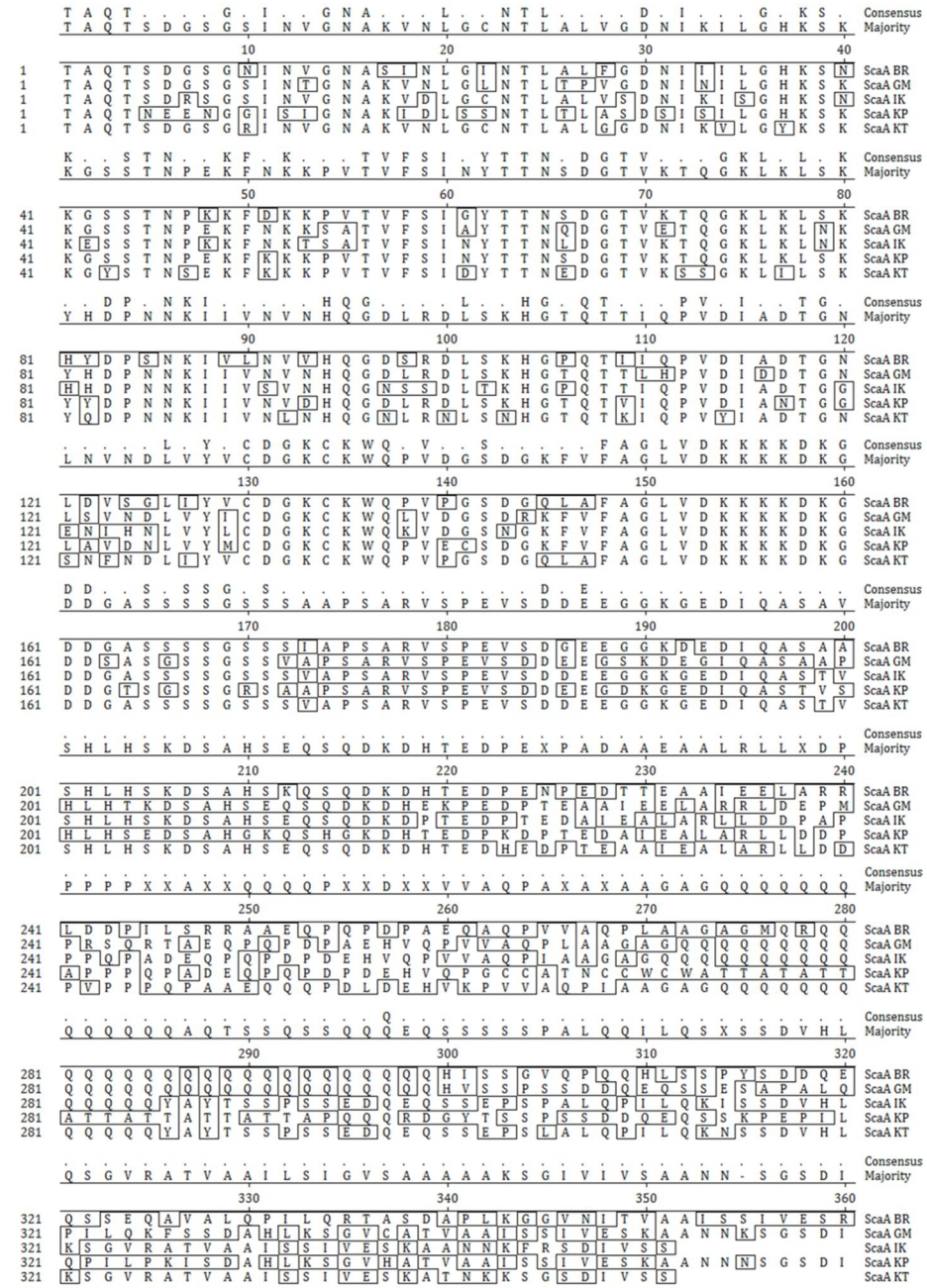


Figure 2. Genetic analysis of the *sca* genes of different strains of *O. tsutsugamushi*.

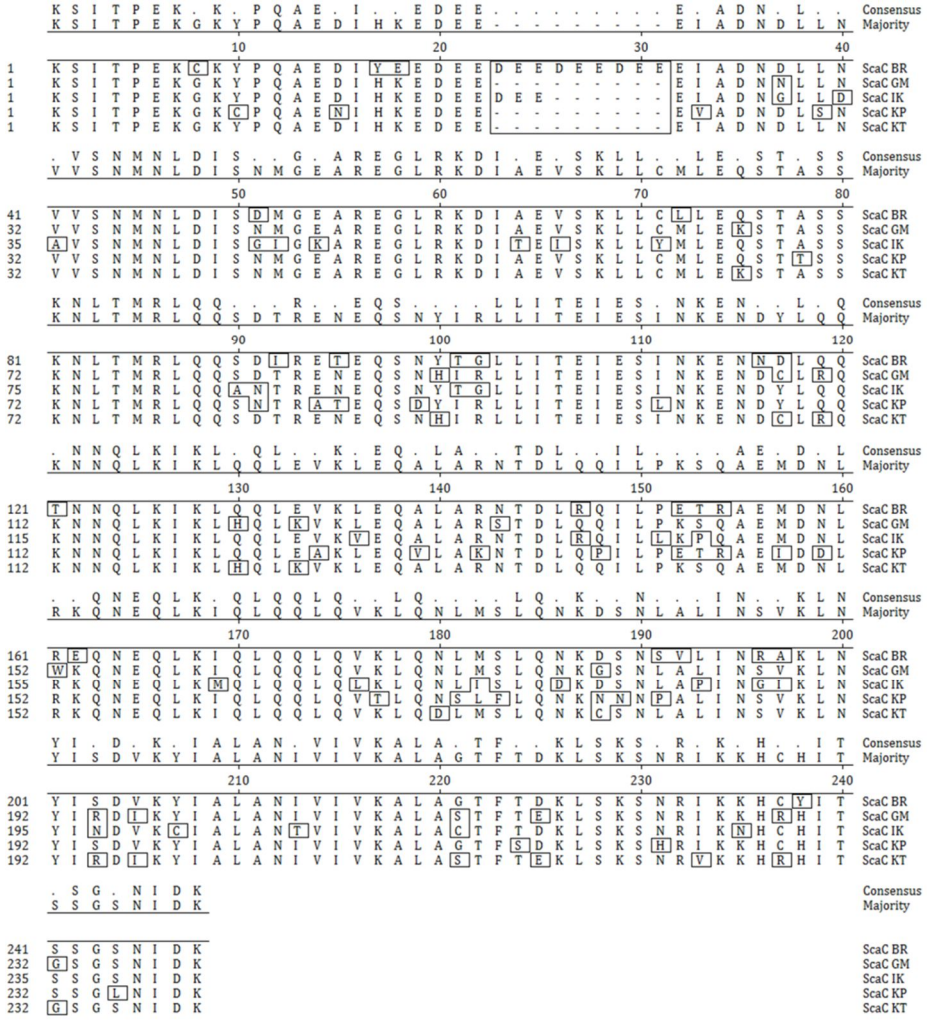
(A) Purified *O. tsutsugamushi* genomes were used as templates, and *sca* genes were amplified by PCR using specific sets of primers (Table 1). (KP, Karp; KT, Kato; GM, Gilliam; BR, Boryong)

(B) Phylogenetic tree of the *sca* genes identified in the genomes of the indicated strains. The red inset box shows a phylogenetic tree for the *tsa56* genes of the indicated strains.

A

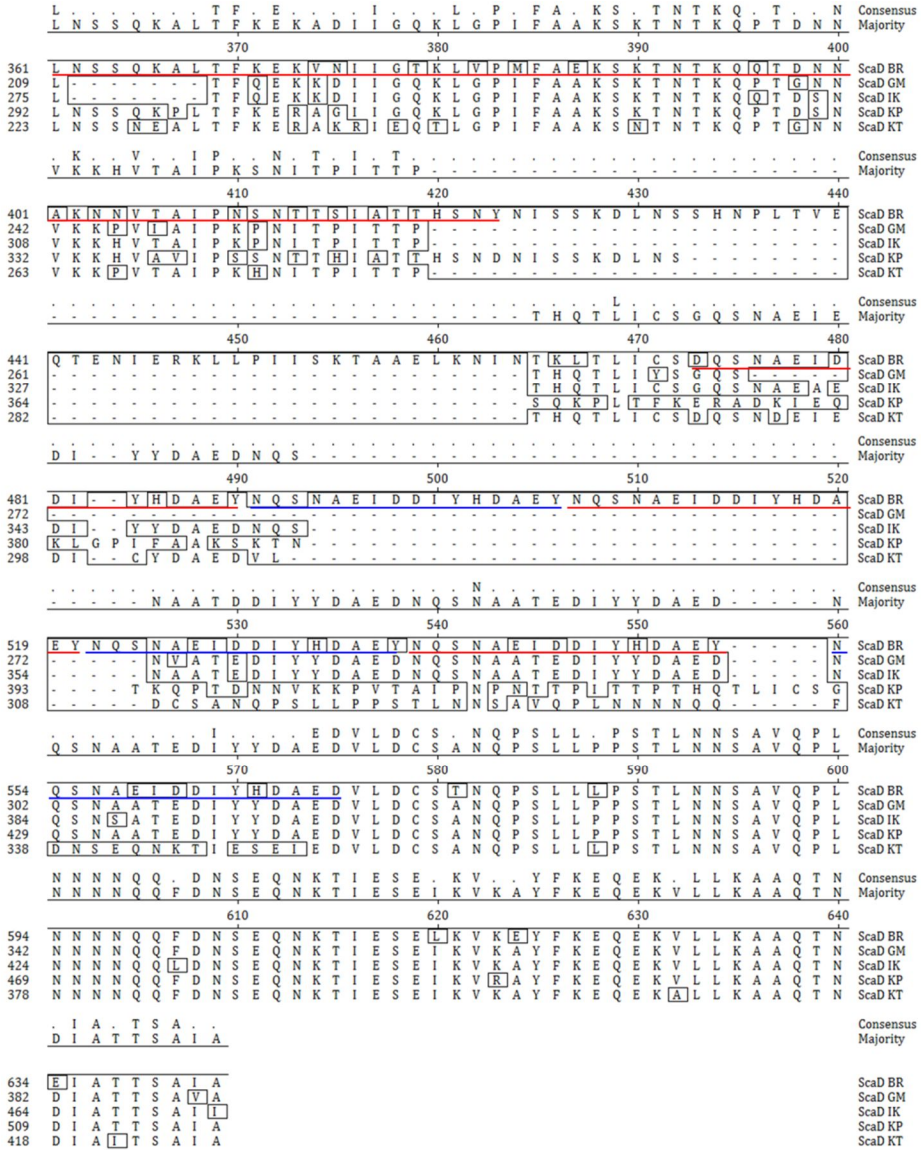


B



C

C

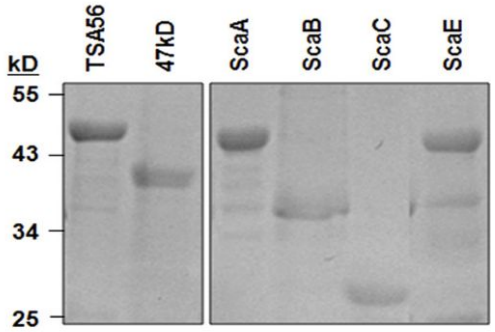


D

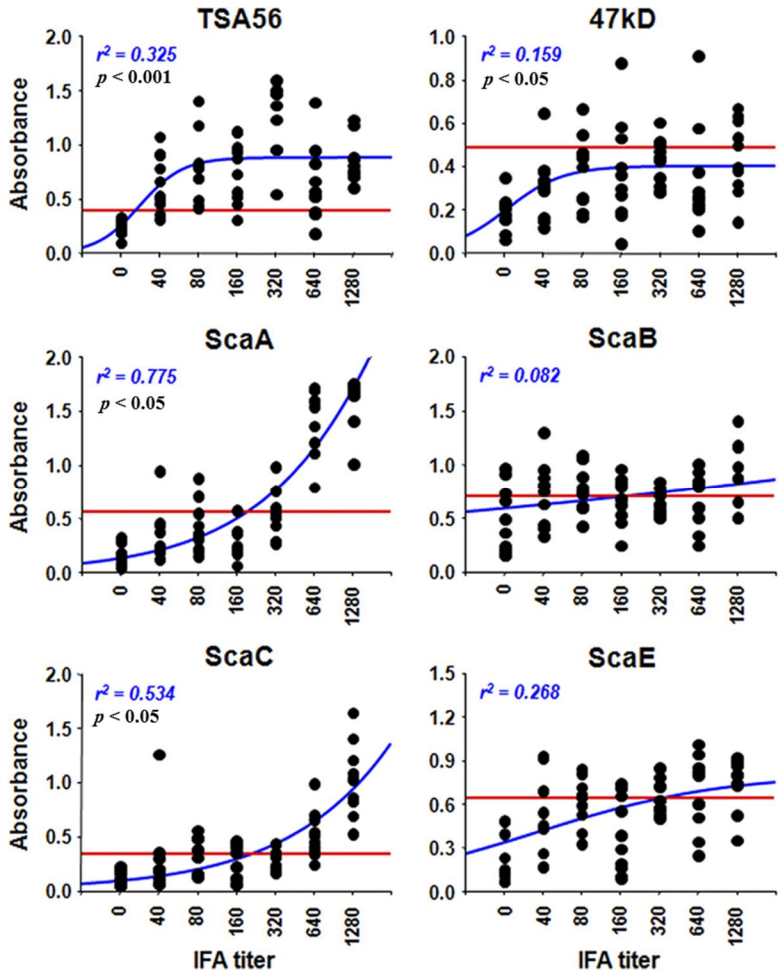
Figure 3. Alignment of aminoacid sequence of Sca proteins detected in the genome of *O.tsutsugamushi* strains.

The passenger domains of the genes identified in the indicated strains were compared (A) ScaA; (B) ScaC; (C) ScaD; (D) ScaE. The diffrent sequences are boxed, and the internal repeat sequences detected in ScaD are underlined with red and blue lines.

A


kD
TSA56
47kD
ScaA
ScaB
ScaC
ScaE
55
43
34
25

B


TSA56
r² = 0.325
p < 0.001
47kD
r² = 0.159
p < 0.05
ScaA
r² = 0.775
p < 0.05
ScaB
r² = 0.082
ScaC
r² = 0.534
p < 0.05
ScaE
r² = 0.268
Absorbance
IFA titer
0
40
80
160
320
640
1280

C

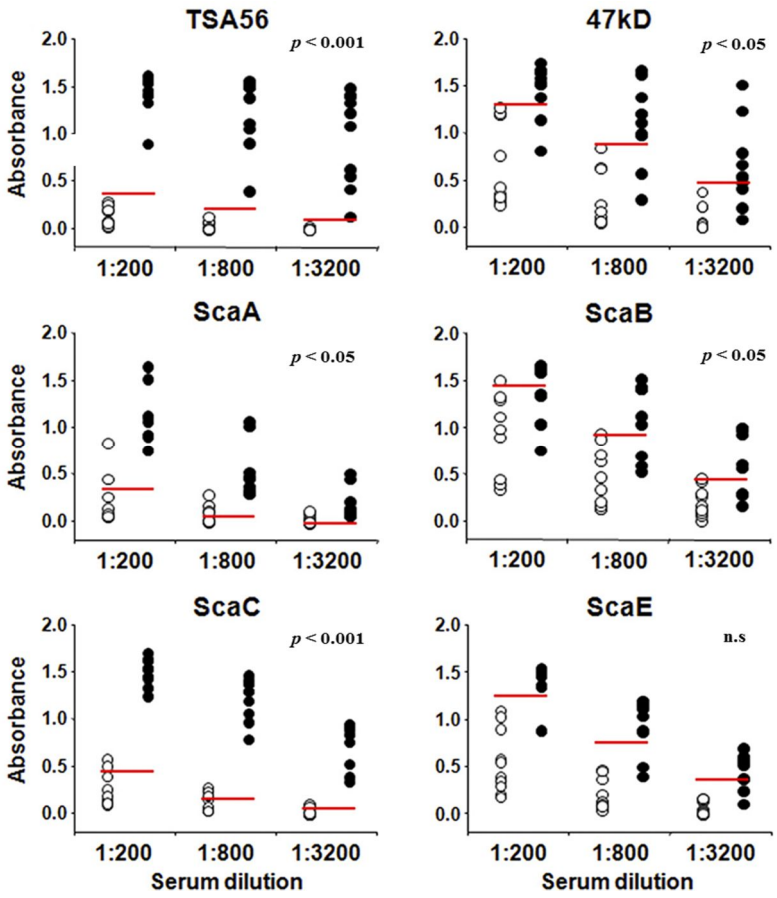


Figure 4. Antibody responses against the indicated *O. tsutsugamushi* antigens were analyzed by ELISA

(A) The bacterial antigens were cloned into the pET28a expression vector by use of the specific primers summarized in Table 1 and were expressed in *E. coli*. The His-tagged proteins were purified using Ni-NTA His-binding resin and then visualized by SDS-PAGE and Coomassie brilliant blue staining.

(B) Comparison of ELISA data obtained using the indicated antigens and IFA titers. The reactivities of the sera (10 samples per IFA titer) were assessed by

ELISA and plotted against IFA titers. The cutoff values (red lines) were determined using IFA-negative sera from 10 patients with acute febrile illness (mean plus 2 standard deviations). Correlations between ELISA results and IFA titers were assessed by nonlinear regression analysis (blue lines), and the r^2 , *p*-values are shown.

(C) Sera from 10 scrub typhus patients (filled circles; IFA titers of ≥1:1,280) and IFA-negative sera from 10 patients with acute febrile illness (open circles) were diluted as indicated and used for ELISAs IFA-negative sera from 10 patients with acute febrile illness were used to determine the cutoff values (red lines; mean plus 2 standard deviations).

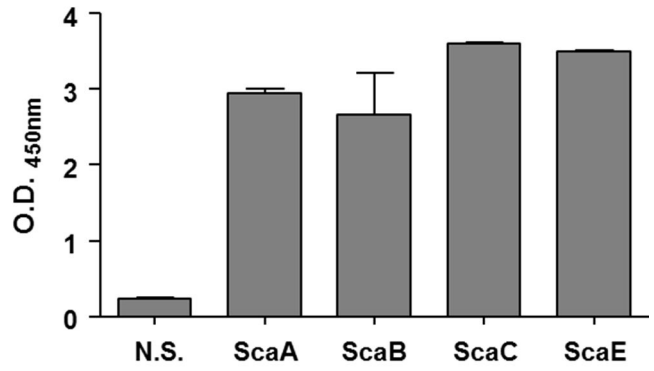


Figure 5. Antibody responses against Sca antigens in immunized mice.

Sera from mice immunized with the indicated antigen were diluted 1:100 and used for ELISAs. Data are presented from triplicate assays.

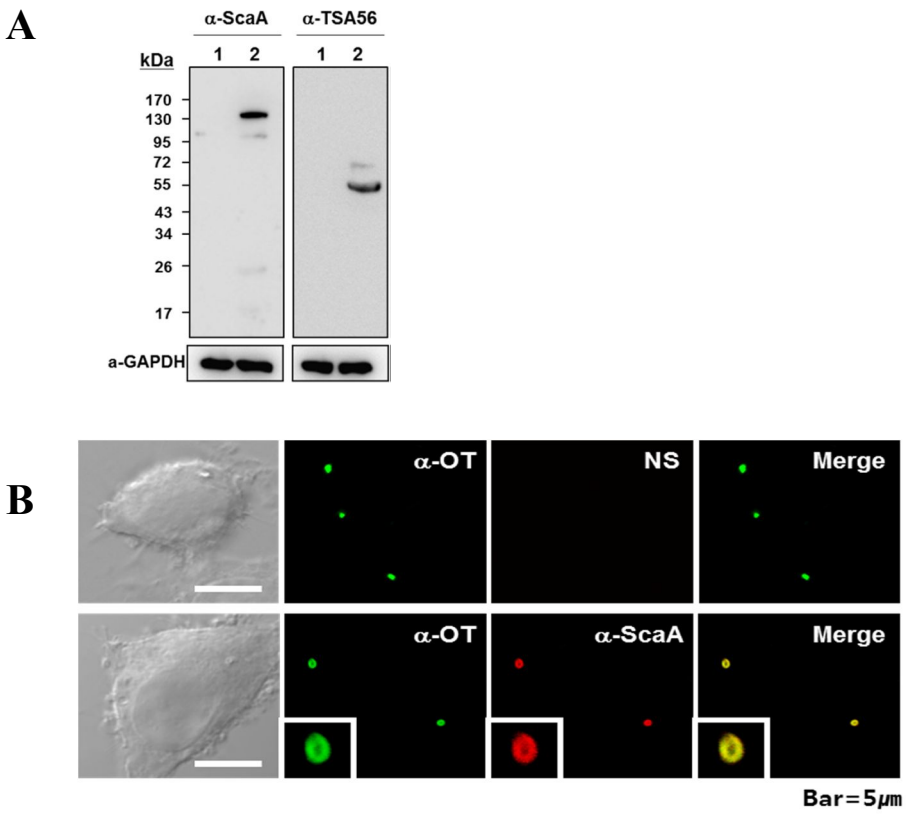


Figure 6. Expression of ScaA by *O. tsutsugamushi*.

(A) Immunoblot analysis of whole proteins from L929 cells infected with *O. tsutsugamushi* proteins by using anti-ScaA serum (right panel). Anti-ScaA serum detected a protein with a molecular mass of approximately 150 kDa. Immunoblotting using anti-TAS56 was performed as a control (left panel).

(B) Immunofluorescence confocal microscopy using preimmune serum or anti-ScaA serum (α-ScaA) showed ScaA in the *O. tsutsugamushi*-infected L929 cells. The left panels show bacteria stained with the pooled sera of scrub typhus patients (α-OT). Magnified images are shown in the lower panels (inset boxes). Scale bars, 5 μm.

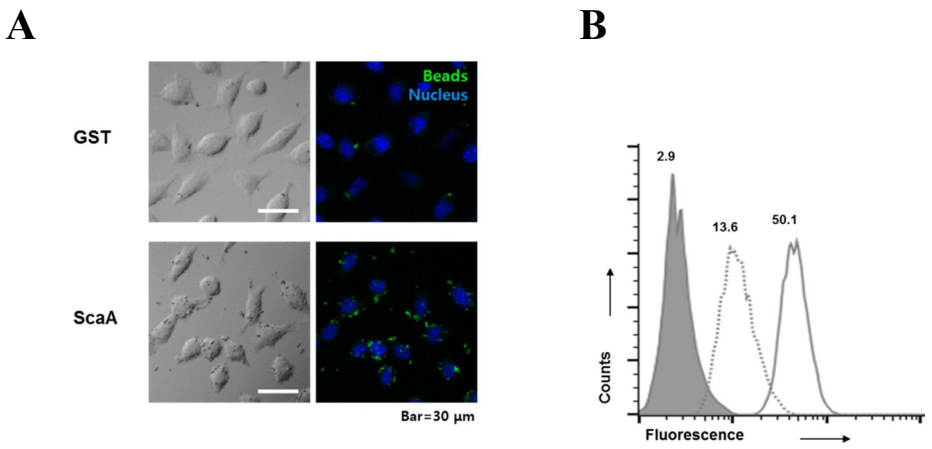


Figure 7. Adhesion of ScaA-coated microbeads to HeLa cells.

(A) Cells were incubated with fluorescent microbeads coated with GST or GST-ScaA (ScaA) for 1 h, washed extensively, and fixed. Cell-bound microbeads (green) were visualized by fluorescence microscopy after staining of cell nuclei (blue). Scale bars, 10 μm.

(B) Relative binding of the microbeads coated with GST (dotted line) or GST-ScaA (thick line) to HeLa cells was quantified directly using flow cytometric data analysis. The gray histogram represents unbound cells (cells not incubated with microbeads).

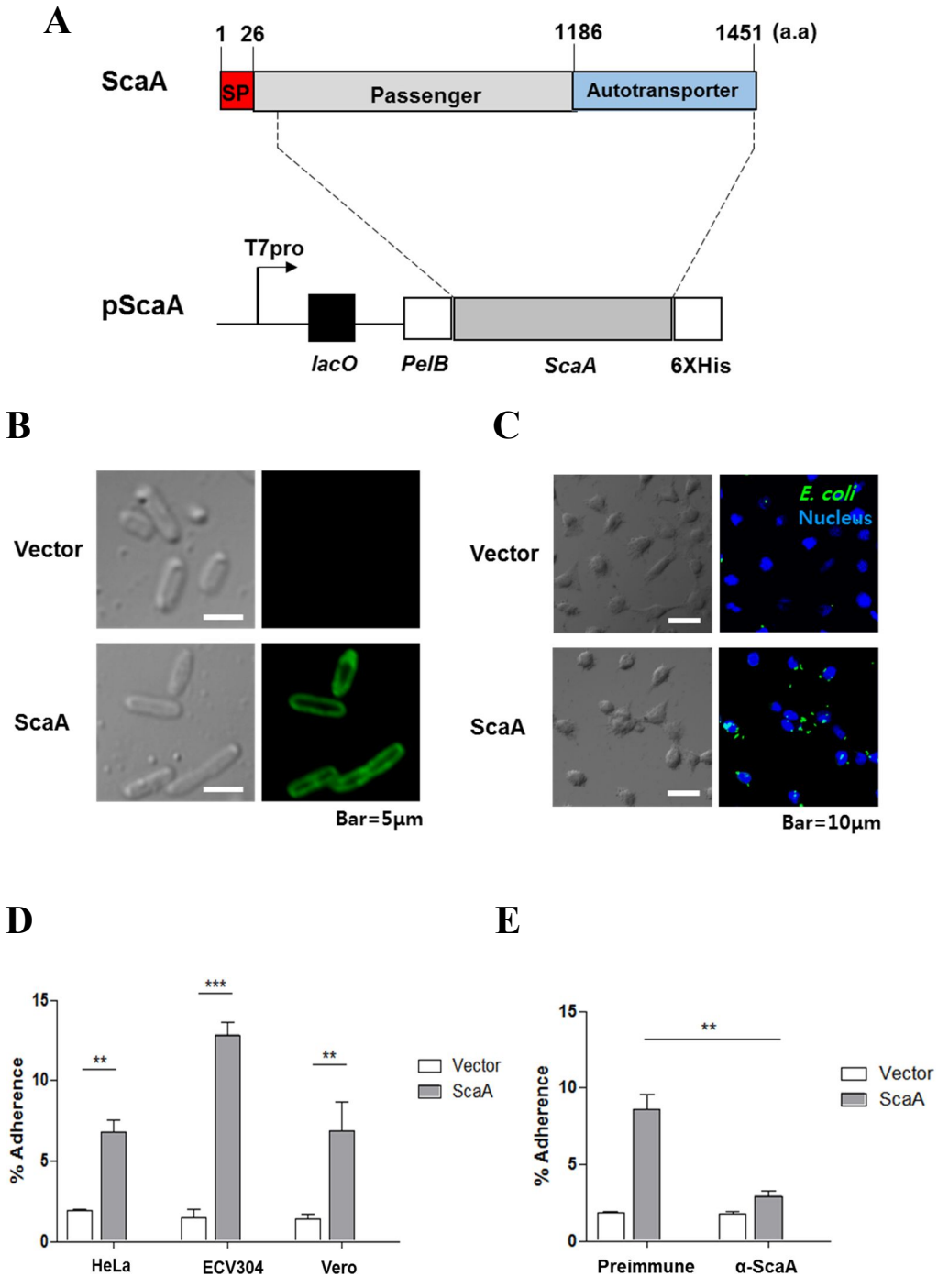


Figure 8. Adhesion function of ScaA.

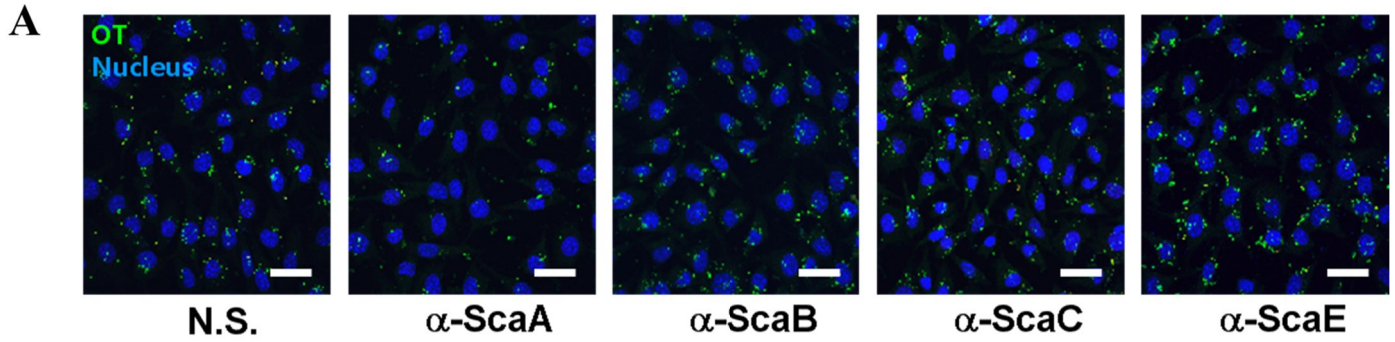
(A) Schematic diagram of the *scaA*-containing pET28a plasmid (pScaA). This vector encodes a recombinant protein fusion containing an N-terminal *E. coli* PelB signal sequence, *O. tsutsugamushi* ScaA, and a C-terminal 6 X His tag.

(B) Immunofluorescence microscopy using an anti-ScaA antibody revealed the presence of ScaA on the surface of the recombinant *E. coli* (lower panels). Preimmune serum did not detect the recombinant protein (upper panels). Scale bars, 5 μm

(C) *E. coli* transformed with the pET28a vector or with pScaA was induced with IPTG and incubated with HeLa cells. After being washed to remove adherent bacteria, the cells were fixed, permeabilized, and stained with an anti-E. coli antibody (green) and ToPro-3 for nuclear staining (blue). Scale bars, 10 μm

(D) CFU-based quantification of adherent *E. coli* transformed with the vector or pScaA was performed. The results are presented as percentages of adherent bacteria relative to the total bacterial input. Data are representative of three independent assays for each of the host cells. **, $p < 0.01$, ***, $p < 0.001$.

(E) Inclusion of anti-ScaA serum in the medium (α-ScaA) significantly inhibited adhesion of *E. coli* expressing ScaA into host cells. After addition of anti-ScaA or preimmune serum into infection media, CFU-based quantification of adherent *E. coli* transformed with the vector or pScaA was performed. **, $p < 0.01$.


A
OT
Nucleus
N.S.
α-ScaA
α-ScaB
α-ScaC
α-ScaE

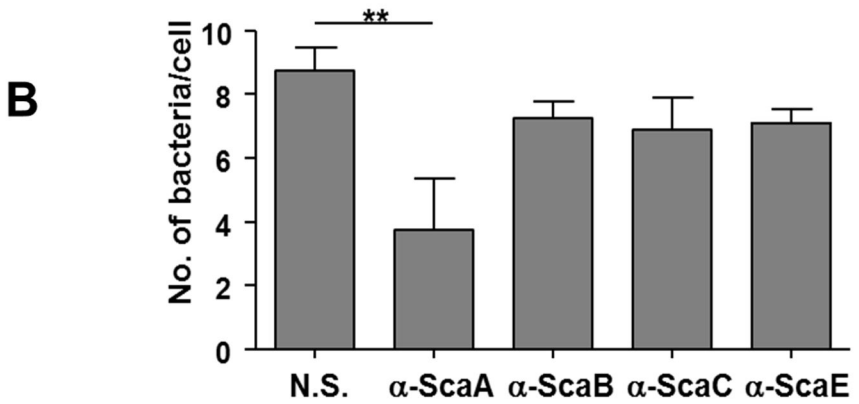

B
**
No. of bacteria/cell
0
2
4
6
8
10
N.S.
α-ScaA
α-ScaB
α-ScaC
α-ScaE

Figure 9. Protective role of anti-ScaA immunity.

(A) Anti-ScaA antibody inhibited *O. tsutsugamushi* infection into host cells. HeLa cells were infected with the pathogen in the presence of the indicated anti-Sca antibodies or nonimmune serum. At 4 h after infection, bacterial infection was examined using confocal microscopy after differential immunoflourescent stain.

(B) The *O. tsutsugamushi* per host cell ratio was determined from three independent experiments in (A). **, $p < 0.01$.

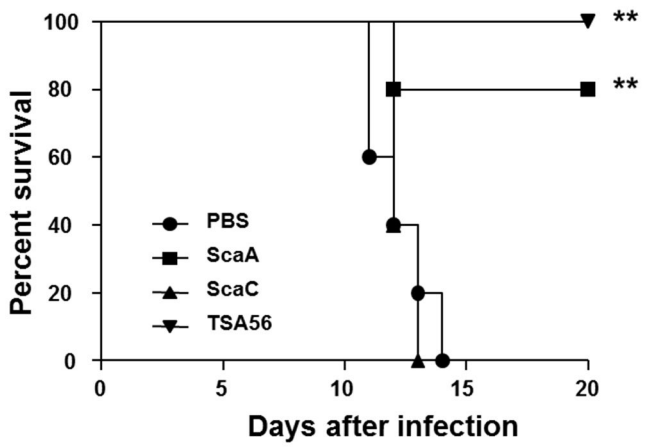


Figure 10. Protective role of immunization against *O. tsutsugamushi* infection.

Survival curves of immunized mice following lethal challenge with *O. tsutsugamushi*. Mice (n = 5/group) were immunized with the indicated antigen from the Boryong strain and challenged intraperitoneally with 100 x LD50 of *O. tsutsugamushi* Boryong strain. Their survival was monitored until all the surviving mice recovered from the disease. This graph is a representative survival curve of two experiments. **, $p < 0.01$ when compared with non-immunized group (PBS).

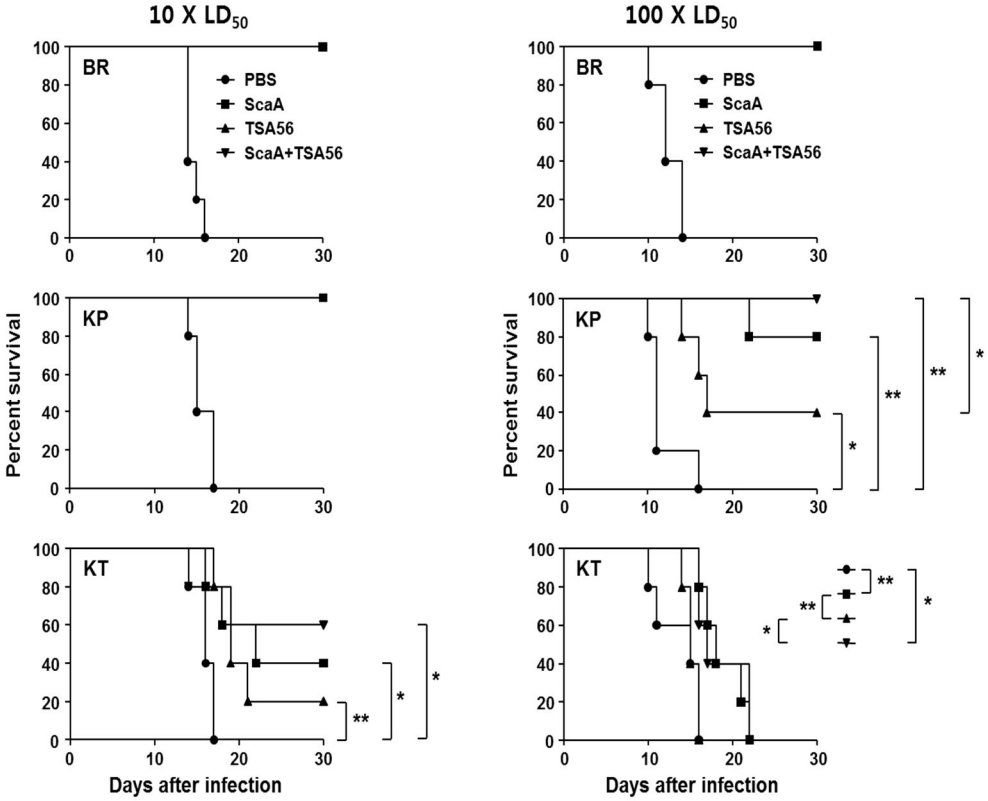


Figure 11. Protective role of ScaA or combined immunization against heterologous strain infection.

Mice (*n*=5/group) were immunized with the indicated antigens and challenged intraperitoneally with 10 x LD_{50} (A) or 100 x LD_{50} (B) of *O. tsutsugamushi*. Mice were immunized with antigens from the Boryong strain and challenged with the indicated strains (BR: Boryong, KP: Karp, KT: Kato). *p* value and median survival are summarized in Table 5. *, $p < 0.05$; **, $p < 0.01$.

100 X LD50 / KP	PBS	ScaA	TSA56	Median survival
PBS				11
ScaA	0.0020			U.D.*
TSA56	0.0169	0.1492		17
ScaA+TSA56	0.0020	0.3179	0.0494	U.D.

10 X LD50 / KT	PBS	ScaA	TSA56	Median survival
PBS				16
ScaA	0.0203			22
TSA56	0.0084	0.6045		19
ScaA+TSA56	0.0353	0.6819	0.4453	U.D.

100 X LD50 / KT	PBS	ScaA	TSA56	Median survival
PBS				15
ScaA	0.0082			18
TSA56	0.1277	0.0084		15
ScaA+TSA56	0.0174	0.8327	0.0181	17

Table 4. Statistical analysis on survival rates.

Statistical analysis were performed using the Mantel-Cox Log Rank test. A *p*-value of < 0.05 was considered statistically significant (red). *:undefined

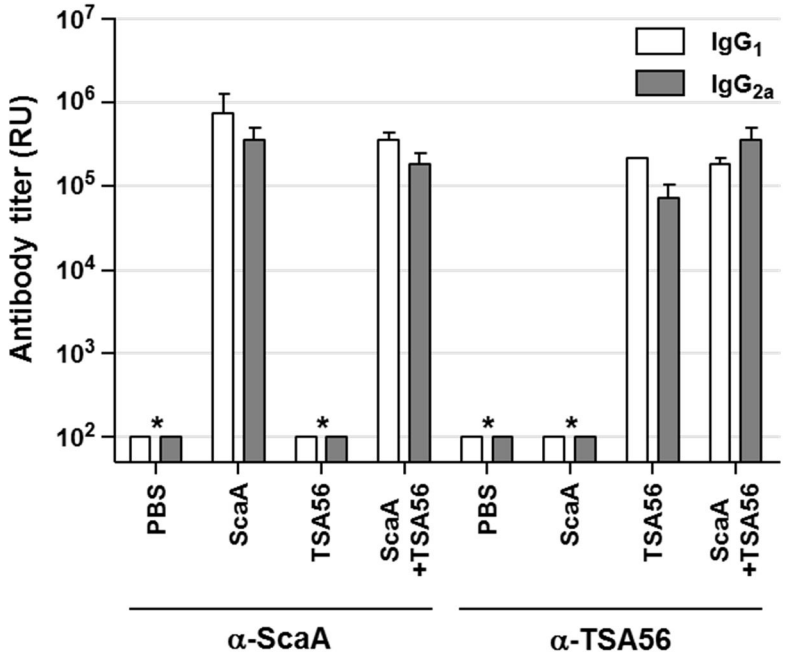


Figure 12. Antibody responses of different isotypes in immunized mice. Antibody titers in mice (n = 3) at one week after third immunization presented in relative units (RU) as serial dilution of serum relative to antibody end-point titers. *: titer < 100

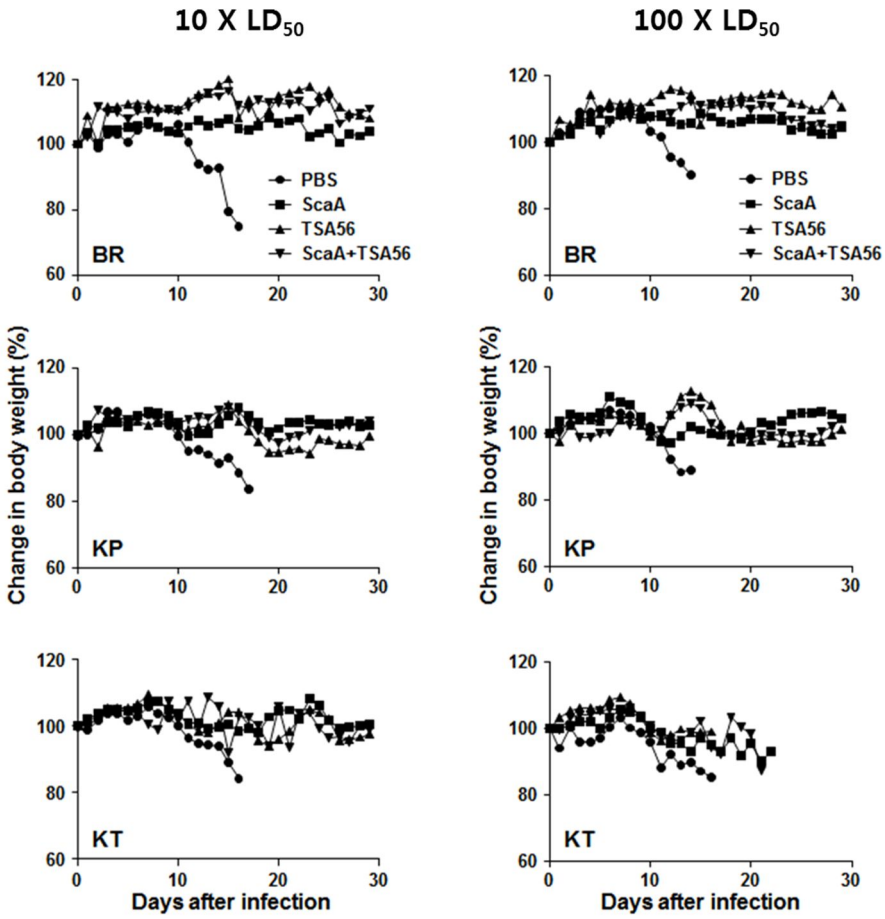


Figure 13. Body weight change of mice challenged with diverse *O. tsutsugamushi* strains.

Mice (*n*=5/group) were immunized with the indicated antigens and challenged intraperitoneally with 10 x LD_{50} (left panel) or 100 x LD_{50} (right panel) of *O. tsutsugamushi* (the same sets in Fig. 10). Mice monitored and weighed daily for a month after inoculation of the pathogen and the average body weight of the surviving mice of each group is presented.

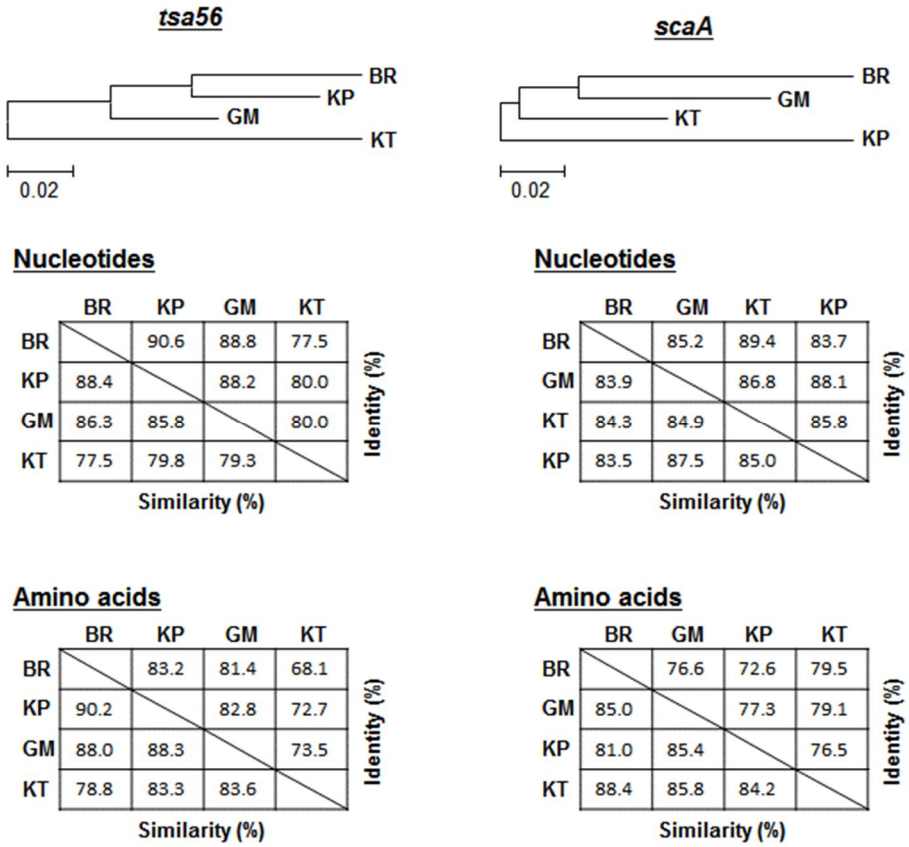


Figure 14. Identity and similarity of *tsa56* and *scaA* sequences among different strains.

Nucleotides and amino acids sequences from the indicated strains of *O. tsutsugamushi* were compared. Nucleotide sequence alignments for constructing phylogenetic trees were processed by Clustal W with the maximum likelihood method. The similarity and identity of those nucleotides and amino acids were calculated through Matrix Global Alignment Tool (MatGAT) (see Methods section). BR: Boryong, KP: Karp, GM: Gilliam, KT: Kato.

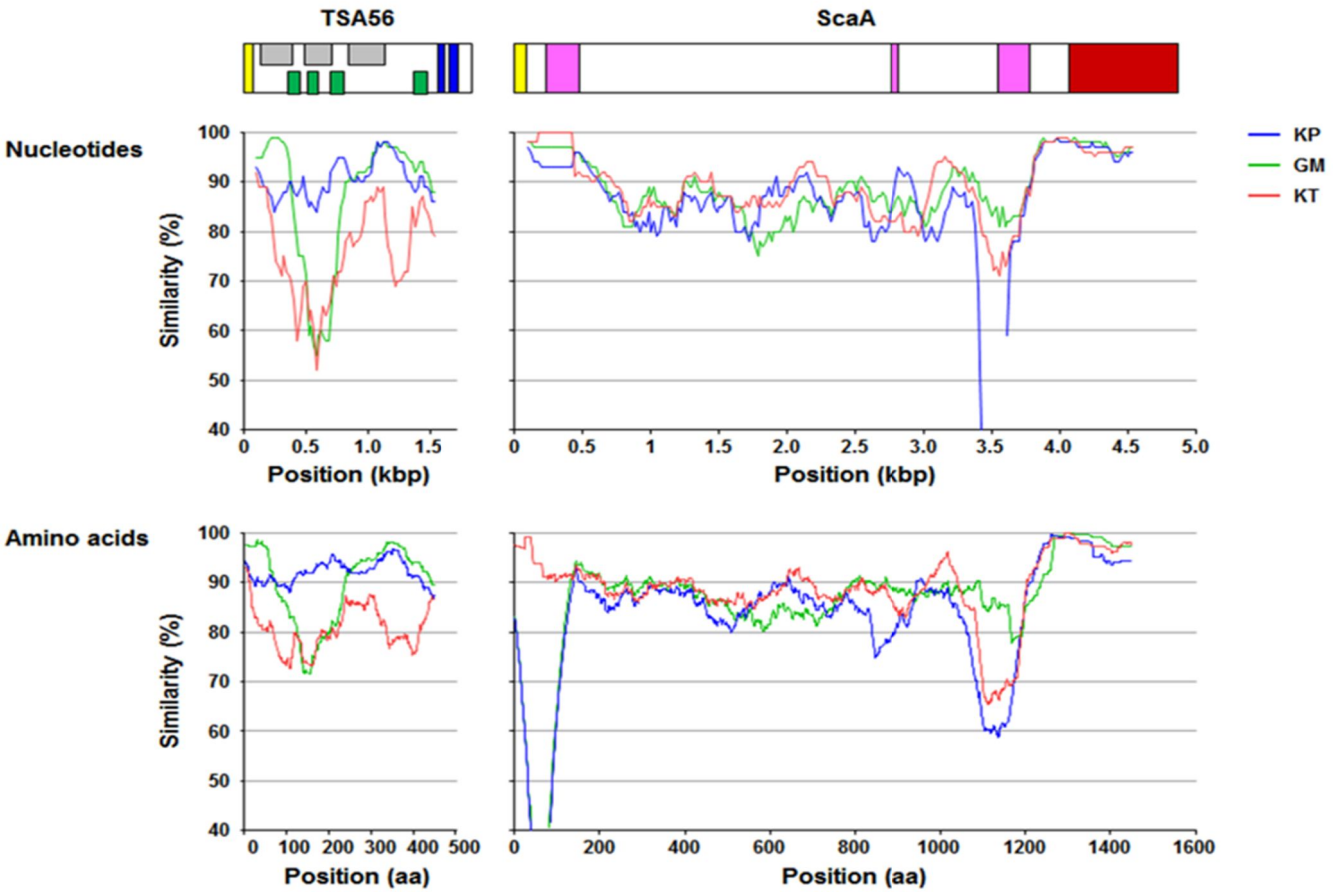

TSA56
ScaA
Nucleotides
Amino acids
Similarity (%)
Position (kbp)
Position (aa)
KP
GM
KT

Figure 15. Similarity plots of a set of *tsa56* and *scaA* sequences from the indicated strains compared to sequences from the Boryong strain.

Each plotted point is the percent identity within a sliding window of 100 bp or 100 amino acids wide centered on the position plotted, with a step size between points of 10 bp or amino acids. Diagrams above the graphs show the relative sizes of TSA56 and ScaA proteins and their sequence motifs. Yellow box: signal peptide, gray box: antigenic domain, green box: variable domain, blue box: transmembrane domain, pink box: repeated sequences, brown box: autotransporter domain.

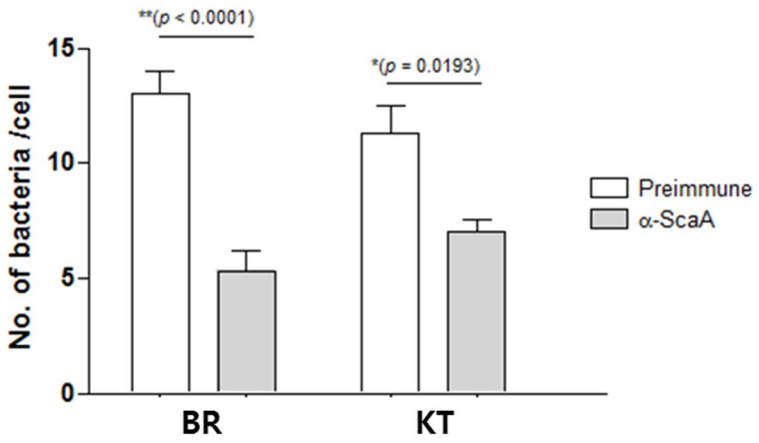


Figure 16. Differential inhibition of anti-ScaA antibody on heterologous bacterial infection.

Anti-ScaA antibody generated by immunization with ScaA antigen from Boryong strain differentially inhibited *O. tsutsugamushi* Boryong (BR) or Kato (KT) strain infection into host cells. ECV304 cells were infected with the indicated strain in the presence of anti-ScaA antibody or nonimmune serum. At 4 h after infection, bacterial infection was examined using confocal microscopy after differential immunoflourescent staining and the *O. tsutsugamushi* per host cell ratio was determined from three independent experiments.

DISCUSSION

Despite continuous efforts to develop a vaccine for scrub typhus since World War II, an effective vaccine is not yet available. Earlier human studies using inactivated whole bacteria failed to show evidence of protection [25, 26]. A more recent study using a formalin-inactivated antigen prepared from chicken egg-adapted *O. tsutsugamushi* showed protection in mice against the same strains used for immunization, but failed to protect from infections with other strains [69]. The requirement of a biosafety level 3 facility for the cultivation of the pathogen is an additional barrier for the mass production of a cost-effective vaccine using the whole bacterial antigen. Therefore, whole cell vaccine products may not be practical and economically feasible, and the majority of the recent studies of potential scrub typhus vaccines mainly focus on selecting subunit antigens as vaccine candidates [24, 70]. Before the genomic era, most of the vaccine studies were performed using antigens recognized by sera obtained from immunized animals and infected humans, such as 22-, 47-, 56-, 58-, and 110 kDa proteins [24, 70]. Among them, the type-specific antigen (TSA), a 56 kDa protein, has long been tested as a vaccine candidate since it is highly immunogenic and plays an important role in *O. tsutsugamushi* attachment to and invasion into host cells [39, 40, 71]. Thus far, TSA56 has been the best antigen to provide protective immunity in mouse infection models, but only to homologous strain infection due to its antigenic diversity [29]. Another conserved major antigen, a 47 kDa protein, has been tested as a vaccine antigen [72]. However,

this bacterial antigen failed to provide significant protection against homologous strain challenge and did not improve vaccine efficacy even when combined with TSA56 [72]. In addition, the 47 kDa antigen may induce cross-reactivity against human serine proteases due to sequence homology, and thus potentially contributes to autoimmune responses or enhanced pathology in some scrub typhus patients [73]. Immunogenic 22- and 110-kDa proteins have also been considered as vaccine antigens, but their efficacy have never been proven in *in vivo* infection models [70].

Based on the genomic information of two *O. tsutsugamushi* strains [49, 50], there are 161 *O. tsutsugamushi*-specific genes that are absent in all other sequenced *Rickettsia* species [50]. Among them, we identified 58 genes that are present in both the Boryong and Ikeda strains but are absent in all other bacteria species in the NCBI database (cutoff e-value < 10^{-20}) (Table 1). In the whole bacterial proteome analysis, 17 *O. tsutsugamushi*-specific genes including *tsa56* and *scaA* were identified to be translated [51].

Diagnosis of scrub typhus is an important issue that determines the administration of rapid and proper antibiotic therapy in local clinics of endemic region [74]. However, all currently available serological tests and PCR-based detection methods have limitations in terms of sensitivity, costs, and convenience. Moreover, evaluation of the diagnostic methods has been hampered because the current gold standard, IFA, is imperfect [74]. Therefore, there is an urgent need for alternative diagnostic methods that use rapid and accurate point-of-care technologies that are readily available, especially for patients in the acute phase of scrub typhus. Recently, combinatorial detection

methods consisting of rapid immunochromatographic techniques (ICT) and bacterial DNA amplification have been proposed [75, 76]. Combinatorial diagnosis can achieve 50-67% sensitivity when applied to a robust reference comparator set, scrub typhus infection criteria (STIC), which is comprised of four parameters: positive cell culture isolation, an admission IgM titer ≥ 1:12,800 using the gold standard IFA, a four-fold increase of IFA IgM titer, and/or a positive result in at least two out of three PCR assays. The combination of DNA- and antibody-based detection methods has increased sensitivity with a minimal reduction of specificity, and has expanded the timeframe of adequate diagnostic coverage to extend throughout the acute phase of scrub typhus when compared to conventional single detection techniques and methods [75].

Although the combinatorial detection method can improve the sensitivity of diagnosis during the acute phase, several limitations still remain. First, sensitivity is still less than 70% when applying the STIC to define scrub typhus with a high level of confidence. Second, PCR detection and rapid ICT are largely dependent on a single gene, *tsa56*, encoding a 56 kD major outer membrane protein, and antibody responses against TSA56. Since *tsa56* is highly variable, it may affect PCR sensitivity [77] and, more critically, multiple antigenic variants should be used to cover the diverse serotypes for serological detection of specific antibodies [78]. Third, the method cannot differentiate between genetic variations of *O. tsutsugamushi* strains if restriction fragment-length polymorphism (RFLP) mapping or sequencing is

not performed. To date, more than 20 antigenically distinct strains have been reported [3]. In addition to antigenic variation, multiple studies showed great interstrain variability in virulence in humans and rodents [3]. Therefore, differentiation of genetic variation is an important issue in scrub typhus diagnosis and epidemiology.

Here, I report that Sca proteins of *O. tsutsugamushi* can induce antibody responses in scrub typhus patients. Specific antibody responses against ScaA and ScaC were observed mainly in scrub typhus patients with high IFA titers, thereby the serological test using the Sca antigens may not be efficient if solely used, especially during the early phase of infection. However, it is notable that ELISA using ScaA or ScaC showed 100% of sensitivity (Figure 4) and the use of these antigens with TSA56 may synergistically enhance the sensitivity of serological diagnosis such as ICT. Previously, it was suggested that 47 kD protein, a HtrA homolog of *O. tsutsugamushi*, might be a useful antigen if combined with TSA56 for serological diagnosis [74, 79]. However, the specific antibody titers against the 47 kD antigen in the human patients are relatively low during the acute phase of infection [79], which might be due to antibody cross-reaction with human serine protease or HtrAs of other bacteria [73]. Consistently, I also observed relatively low levels of specific antibody responses against 47 kD antigen in our human patients (Figure 4). Given that the amino acid sequences of Sca passenger domains are highly specific to *Orientia* and show low levels of sequence identity (less than 30%) with other Rickettsial Sca proteins [52], these bacterial antigens can be effective targets

for scrub typhus diagnosis. Although I may need to further confirm the antigenic variation of the diverse strains and antibody responses against Sca antigens of different strains in human scrub typhus patients, the sequence conservation observed in this study support the potential benefits of using Sca antigens as novel diagnostic targets when combined with TSA56.

In addition to their use in serological methods, the *sca* genes could also be useful targets for molecular diagnosis such as PCR-based detection. Currently, PCR-based methods targeting *tsa56*, 47 kD gene, and *groEL* (Citrate Synthase gene) have been used for species-specific amplification [74, 80]. The sensitivity of these DNA detection methods maximizes at around 40 % [74, 75]. Although targeting 16S rRNA gene yields better sensitivity (37.5 – 52.3%) in real world conditions [74], all of these DNA-based techniques require sequencing after PCR amplification in order to identify the specific strain that has infected the patient. In this report, I found that two of the *sca* genes, *scaB* and *scaE*, were amplified differentially in different strains, suggesting a differential presence of the genes in the genomes of different strains. In fact, the *scaB* gene is present in the Boryong strain but absent in the Ikeda strain when their complete genome sequences were examined [52]. Therefore, the molecular detection of *sca* genes could provide direct clues about *O. tsutsugamushi* strains as well as the species, without sequencing. Furthermore, the size of the passenger domain of the *scaD* gene is highly variable among the different strains due to the different numbers of internal repeat sequences (Figure 2 and Figure 3). This characteristic of the gene can provide valuable

information on the genotypes of *O. tsutsugamushi* without any sequencing. Currently, I examined three prototype strains, Boryong, and Ikeda strain for sequence comparison. Considering the geographic distribution of various genotypic variants in the real world, further studies on the genetic variation of s*ca* genes in a wide geographic area may facilitate the usage of this gene family as molecular targets for scrub typhus diagnosis. I propose for the first time that a panel of serological and molecular detection methods using the *sca* gene family as a novel target can provide rapid and cost-effective technologies for scrub typhus diagnosis as well as for epidemiologic studies, when combined with classical assay methods.

In this study, I also showed that ScaA, an autotransporter protein of *O. tsutsugamushi*, is expressed on the bacterial periphery and functions as a bacterial adhesion factor. Autotransporter proteins of gram-negative bacteria share a common sequence organization: a signal peptide followed by an N-terminal passenger domain and a C-terminal translocator domain [81]. The sequences and functions of the passenger domains can be quite diverse and are frequently associated with various virulent phenotypes, including bacterial adhesion, invasion, biofilm formation, and cytotoxicity [81]. The apparent role of autotransporter proteins in virulence and host cell interactions naturally make them potential targets for the design of novel vaccines directed against human pathogens [52, 82]. For example, a major virulence factor of *Bordetella pertussis*, pertactin that mediates bacterial adhesion to the lung epithelium [83] and resistance to neutrophil-mediated clearance [84], has been

successfully used to provide the acellular components of a pertussis vaccine [85]. The passenger domain of the *Haemophilus influenzae* autotransporter protein, Hap, which mediates attachment and entry into epithelial cells as well as attachment to extracellular matrix proteins [86], elicits significant antibody responses and protects preimmunized mice from nasopharyngeal colonization [87].

In this study, I examined the neutralizing activity of antibodies against four Sca proteins (ScaA, B, C, and E) encoded in the *O. tsutsugamushi* genome and found that only the antibody against ScaA inhibited bacterial infection in a cell culture model, whereas antibodies against other Sca proteins of *O. tsutsugamushi* had marginal effects (Figure 9). In addition, immunization with ScaA provided protective immunity against *O. tsutsugamushi* infection in mice as efficiently as TSA56, whereas ScaC failed to induce protection, indicating that ScaA could provide specific and protective immunity against *O. tsutsugamushi,* at least against the homologous strain. When combined with TSA56, ScaA immunization significantly enhanced protective immunity against infection with heterologous strains, resulting in better survival or extended half-life of infected mice. To my knowledge, this is the most promising result of scrub typhus vaccination against infection of heterologous strains in a mouse model. When we compared the sequences of *scaA* from the four different strains, the overall level of sequence similarity of *scaA* nucleotides (83.5 ~ 87.5%) and amino acids (81.0 ~ 88.4%) is similar to those of *tsa56* (nucleotides: 77.5 ~ 88.4%, amino acids: 78.8 ~ 90.2%) (Figure 14). However, a similarity plot shows that *tsa56* has more local variation among

the four strains than *scaA* (Figure 15). Sequence variation observed in *scaA* is mainly due to the differential presence of repeated sequences found in the 5'-region (nucleotides 203 ~ 241 in Boryong strain) and 3'-end of the passenger domain (nucleotides 3,243 ~ 3,314 in Boryong strain) of each strain. When I examined the neutralizing activity of anti-ScaA antibody generated by immunizing ScaA protein from Boryong strain, it showed less inhibitory effect on the cellular invasion of Kato strain than Boryong strain (Figure 16), suggesting that the sequence variation of ScaA may also affect *in vitro* neutralizing activity of anti-ScaA antibody. It remains to be determined whether the variable regions and their repeated sequences affect the antigenicity or neutralizing activity of antibodies against ScaA protein. Since protective immunity against *O. tsutsugamushi* infection is provided by antigen-specific IFN-γ-producing T cells [88, 89] as well as humoral immunity [29, 90], the protective role of ScaA-specific Th1 cells also needs to be investigated. Nevertheless, the passenger domains of ScaA proteins from different strains are relatively well conserved and those conserved areas could make it a better antigen for scrub typhus vaccine for targeting multiple strains of *O. tsutsugamushi*.

Recently, several infection models using mice have been proposed to study pathologic changes and vaccine development for scrub typhus [91-94]. Intradermal or intravenous inoculation of the pathogen partially represented the specific pathology of human scrub typhus [92, 93]. An infection model using *O. tsutsugamushi*-infected mites to mimic the natural transmission was also shown that the species of infected chigger and their *O. tsutsugamushi*

genotypes produced different clinical presentations in ICR mice [91]. Previously, diverse strains of mice showed differential morbidity and mortality to the infection with specific strains of *O. tsutsugamushi* [95, 96]. Therefore, various factors such as infection routes and genetic backgrounds of host and the pathogen may affect the susceptibility and disease severity of scrub typhus. In the current study, I used C57BL/6 inbred mice model after intraperitoneal injection of *O. tsutsugamushi* strains, which resulted in 100% mortality when unimmunized. Valid models in C57BL/6 mice also open the opportunity to study genes involved in the mechanisms of immunity and pathogenesis by the use of gene knockout mice [21]. The development of animal models that accurately portray human scrub typhus is an important step toward understanding and managing disease [93, 94]. Although there are differences in target cells of *O. tsutsugamushi* infection and the disease progression depending on the route of infection and the genotypes, these models closely parallels the clinical course and pathological legions described from lethal scrub typhus in human and, therefore, may provide valuable tools to characterize the molecular and cellular factors responsible for immunological pathogenesis of scrub typhus [93]. Further studies on the bacterial virulence mechanisms [97-99] and the underlying mechanisms of immunological pathogenesis in human scrub typhus patients [100] should also be followed to improve our understanding for the weak and transient immunity against the bacterial infection in human and to facilitate the development of effective vaccine for scrub typhus.

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국문 초록

서론: 쯔쯔가무시균은 리켓치아과의 그람 음성 절대 세포내 기생세균이며, 털진드기를 매개로 사람에게 쯔쯔가무시병을 일으키는 원인균이다. 임상적인 특징으로는 가피, 발진, 발열, 근육통을 동반하며, 독시싸이클린이나 클로람페니콜 등의 항생제 처방이 적절히 이루어지지 않으면, 다발성 장기부전으로 인하여 35-50%의 높은 치사율을 보인다. 쯔쯔가무시병은 우리나라 및 아시아-태평양 지역, 러시아 동부, 그리고 서남아시아 지역에서 주로 발생하고 있으며, 이들 지역 안에 20 가지 이상의 항원형이 다른 균주가 있다고 알려져 있다. 최근에는 항균제 내성 균의 출현과 쯔쯔가무시병 발생환자 수가 점차 증가 함에 따라 백신 개발의 필요성이 점차 대두 되고 있지만, 현재까지 개발된 효과적인 백신은 없다. 이는 사람을 포함한 영장류에서 백신에 의한 면역기억이 1 년 이상 지속되지 못하는 점과 주요 백신 항원 물질로 연구가 되어온 막항원인 TSA56 단백항원의 유전적 변이 차이가 크기 때문 일 것으로 생각된다. 따라서 효과적인 백신 개발을 위해서는 다양한 항원형의 균주들이 공통적으로 발현하는 단백항원 중에 유전적 변이 차가 적은 새로운 백신 후보 물질의 선발이 우선적으로 시행 되어야 한다.

연구방법 및 결과: 본 연구에서는 쯔쯔가무시균의 전체유전체 정보를 바탕으로, 자가수송단백을 암호화하는 *sca* 유전자들을 선발하였으며, 중합효소연쇄반응(Polymerase chain reaction) 및 염기서열 분석을 통해 다양한 균주들에서 검지된 *sca* 유전자가 비교적 높은 상동성을 보이는 것을 확인하였다. 각각의 Sca 단백질들을 쯔쯔가무시병 환자 혈청과 반응시켜 항체반응이 일어나는지 효소결합면역흡착 분석법(Enzyme-linked absorbant assay)을 통해 확인하였으며 이 중 ScaA, ScaC 단백이 쯔쯔가무시병 환자 혈청에서 특이항체가 유의하게 증가되어 있는 것을 하였다. 면역원성이 상대적으로 높은 ScaA 를 선정하여 대장균에서 발현시켰을 때, 대조군에 비해 *scaA* 유전자를 발현하는 대장균이 숙주세포로의 부착능이 증가해 있는 것을 확인하였으며, ScaA 특이항체를 쯔쯔가무시균과 반응시킨 후, 숙주 세포에 감염시켜 중화반응 효과를 관찰한 결과, ScaA 특이항체와 반응시킨 쯔쯔가무시균은 숙주세포로의 부착능이 저해되는 것을 확인하였다. 그리고 동물실험을 통해 ScaA 단백항원 단독 혹은 TSA56 단백항원과 혼합하여 면역한 마우스에서 동종 또는 이종 균주의 감염에 대해서도 효과적인 보호면역을 유도 하는지 관찰 하였다. ScaA 단독으로 면역한 경우, TSA56 단독으로 면역한 실험군에 비해 마우스의 생존률이 증가해 있는 것을 관찰 하였으며, ScaA 와 TSA56 혼합항원을 면역 한 실험군에서는 단독항원을 면역한 실험군보다 마우스의 생존률이 유의하게 증가해 있는 것을 관찰하였다.

결론: 항원형이 다른 쯔쯔가무시 균주들에서 다양한 *sca* 유전자들이 존재 하는 것을 확인하였으며, 이 유전자들의 존재 유무와 변이정도 및 항체 반응을 활용하면 쯔쯔가무시병 진단기술의 민감도와 특이도를 향상시킬 수 있을 것으로 사료된다. 또한 ScaA 백신항원으로 사용할 경우, 기존에 사용되어 온 TSA56 단백 보다 유사하거나 더 향상된 보호면역 반응을 유도할 수 있는 것이 확인되었으며, 특히 이종 균주에 대한 보호면역을 유의하게 향상시키는 결과를 확인하였다. 따라서 ScaA 단백은 쯔쯔가무시병의 새로운 백신항원 후보물질로 될 수 있을 것으로 판단되며, 기존에 주로 사용되어 온 TSA56 단백과 병용 사용할 경우 백신의 효능을 증가시키는데 기여할 수 있을 것으로 판단된다

주요어 : 쯔쯔가무시병, 자가수송 단백, 백신 개발

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