Molecular characterization of three major outer membrane proteins, TSA56, TSA47 and TSA22, in *Orientia tsutsugamushi*

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Abstract. *Orientia tsutsugamushi* (*O. tsutsugamushi*), the causative agent of scrub typhus, is an obligate intracellular pathogen. Recent studies have demonstrated the complete genome of *O. tsutsugamushi*. However, the route and detailed molecular mechanism for *O. tsutsugamushi* to get accessed into mammalian cells remains unclear. In this study, we demonstrated different adhesive properties of three major outer membrane proteins of *O. tsutsugamushi*, TSA56, TSA47 and TSA22. TSA56 showed higher antibody responses against patient serum samples compared with those of TSA47 and TSA22. In the adhesion assay, TSA56 exhibited a relative higher adhesion to host cells than TSA47 and TSA22, suggesting that TSA56 is the major outer membrane protein required for *O. tsutsugamushi* adhesion. Furthermore, the antigen domain (AD) I (residues 19-114) corresponding to the extracellular domain of TSA56 demonstrated a relative high antibody response against the patients' sera than the previously reported ADIII (residues 237-366), which has been suggested to facilitate the invasion of *O. tsutsugamushi* through interaction with fibronectin. Taken together, our results consistently showed that TSA56 of *O. tsutsugamushi* is important in the adhesion of *Escherichia coli* (*E. coli*) transformants to Vero cells. Moreover, in contrast to known ADIII-fibronectin interactions, TSA56-ADI may also play a role in the adhesion and/or invasion of *O. tsutsugamushi* to its host cells through unidentified receptors. A further study aimed at delineating the receptor of TSA56-ADI during *O. tsutsugamushi* infection is warranted.

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Introduction

Rickettsia are generally categorized into three groups: the spotted fever (SFG), the typhus (TG) and the scrub typhus (STG) group (1). The classification has been mainly based on the differences in antigenicity corresponding to their lipopolysaccharide and outer membrane proteins, as well as the disease symptoms (2). Scrub typhus or tsutsugamushi disease is an acute febrile disease caused by infection with *Orientia* (formerly *Rickettsia*) *tsutsugamushi* (3). Approximately 1 billion people in Asia are estimated to be at risk for the infection of scrub typhus and about 1 million new cases have been reported each year (4). Given the rapidly increase of clinical cases (5) and the newly reported outbreaks in the non-epidemic regions (6), scrub typhus infection has become a public health issue. Although the scrub typhus can be effectively controlled by treating with antibiotics, such as doxycycline and chloramphenicol, reinfections are common due to the fact that an antigenically distinct serotype scrub typhus can still infect the same individual (7,8). Even worse, a decreased efficacy of antibiotic therapy has been reported in several cases of scrub typhus infection (9,10). In spite of an increasing number of patients and recurrent outbreaks of scrub typhus reported in epidemic areas (5,10,11), an effective vaccine in controlling scrub typhus infection has yet to be developed (12).

O. tsutsugamushi is an obligate intracellular organism and the causative agent of scrub typhus (13). It belongs to the Gram-negative bacteria and shows great antigenic diversity. *O. tsutsugamushi*, first isolated in 1930, is a member of the family Rickettsiaceae and was classified into Gilliam, Karp, Kato, Boryong and other serotypes based on the antigenic variations of the '56-kDa' outer membrane major surface protein (14,15). Recently, the genomic sequences of the *O. tsutsugamushi* Boryong and Ikeda strains were decoded (16,17). The availability of genomic sequences allows conducting the sequence analysis for comparing any difference between these two strains (Boryong and Ikeda) (18) and between virulent and avirulent strains of *Rickettsia prowazekii* (19). The genome sequence of the *O. tsutsugamushi* strain Ikeda comprises a single chromosome of 2,008,987 bp (~2.1 Mb) and contains

1,967 protein coding sequences (CDS). The chromosome is much larger than those of other members of Rickettsiaceae (17). The genome of the strain Boryong, is comprised of 2.13 Mb and contains ~2,216 predicted genes (16). The genome of these *O. tsutsugamushi* strains has an extraordinary structure, with 37% identical repeats, which encode mobile genetic elements and accessory genes, putatively involved in host-parasite interactions. However, no genetic tools are available for determining whether these proteins are essential and sufficient for mediating host internalization of *O. tsutsugamushi*.

Bacterial invasion of host cells is mediated primarily by interactions between bacterial surface components and complementary host receptors. 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 nonphagocytic cells, such as endothelial cells and fibroblasts, as well as macrophages and polymorphonuclear leukocytes (PMN) (13,20-22). After entry into the host cells, the intracellular pathogens escape from vacuoles and move to the perinuclear region, where they replicate (23). Western blot analysis of *O. tsutsugamushi* cell lysates with patient sera has identified at least five protein antigens of molecular mass 110, 56, 47, 35 and 22 kDa (24). Among these antigens, the type-specific antigens (TSA) 56-kDa, 47-kDa and 22-kDa are the major outer membrane surface proteins of *O. tsutsugamushi* (25). The variable 56-kDa protein antigen is the most abundant and is recognized by almost all scrub typhus patients' sera (26). Moreover, the signal sequence (SP) is present in the N-terminal and the transmembrane domains (TMs) in the C-terminal end of the amino acid sequence of TSA56. In the middle of the sequence are three extracellular antigen domains (ADs) and four variable domains (VDs) (27). Previous studies reported that *O. tsutsugamushi* could bind to host fibronectin and utilize it for internalization via interactions with the outer membrane protein TSA56 (27,28). After adhering to the host cells, *O. tsutsugamushi* exploits integrin-mediated signaling and rearrangement of the actin cytoskeleton, which mediates phagocytosis in nonphagocytic host cells (29). However, the molecular basis of intracellular invasion by *O. tsutsugamushi* is poorly characterized.

In this study, we serologically determined the antibody responses of clinical *O. tsutsugamushi*-infected patients' sera against the TSA56, TSA47 and TSA22 major outer membrane proteins of *O. tsutsugamushi*. Then we further compared the adhesion ability of these surface antigens through a surrogate *Escherichia coli* (*E. coli)* expression system to investigate whether these type-specific proteins play a crucial role in facilitating bacterial adhesion and invasion. Our data showed that TSA56 and TSA56-ADI had strong antibody responses with patients' sera. Other than TSA56-ADIII, the TSA56-ADI may play a role in bacterial adhesion and/or invasion through an unspecified receptor.

Materials and methods

Bacterial strains and vectors. E. coli BL21 (DE3) (Novagen) was used as the host strain for prokaryotic expression vectors. The prokaryotic expression vector used was pET32a (Novagen). The GFP-tagged fusion protein expression vector used was pEGFP-C1 (Clontech Laboratories). Luria-Bertani (LB) medium was used for routine culture of *E. coli* strains. For all strains harboring the recombinant vectors, ampicillin (50 μg/ml) and kanamycin (50 μg/ml) was added to the culture medium. The culture was grown at 37˚C with vigorous shaking (200 rpm) until the mid-logarithmic phase was attained. When noted, isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma) was added to the culture of *E. coli* BL21 (DE3) harboring vectors to induce expression of the target genes.

Construction of recombinant expression vectors. To express the His-tagged surface proteins of *O. tsutsugamushi*, the full length type-specific antigen TSA56, TSA47, TSA22, TSA56-ADI, TSA56-ADIII and TSA56-ADI+ADII were amplified by a polymerase chain reaction (PCR) technique from the genomic DNA of the *O. tsutsugamushi* Taitung 6-like strain (a kind gift from Dr Li-Kuang Chen, Medical College, Tzu Chi University, Hualien, Taiwan). The PCR products were then directionally cloned into vector pET32a (Novagen) respectively via the restriction digestion as indicated in Table I to yield pET32a-TSA56FL, pET32a-TSA47FL, pET32a-TSA22FL, pET32a-TSA56-ADI, pET32a-TSA56-ADIII and pET32a-TSA56-ADI+ADII. Furthermore, in order to express the GFP-tagged surface proteins of *O. tsutsugamushi* through *E. coli* BL21 (DE3), the PCR products were also directionally cloned into vector pEGFP-C1 (Clontech Laboratories) respectively via the restriction digestion as indicated in Table I to yield pEGFP-TSA56FL, pEGFP-TSA47FL, pEGFP-TSA22FL, pEGFP-TSA56-ADI and pEGFP-TSA56-ADIII. We then used vector pEGFP-C1 and these five plasmids (pEGFP-TSA56FL, pEGFP-TSA47FL, pEGFP-TSA22FL, pEGFP-TSA56-ADI and pEGFP-TSA56-ADIII) as templates to amplify GFP, GFP-TSA56FL, GFP-TSA47FL, GFP-TSA22FL, GFP-TSA56-ADI and GFP-TSA56-ADIII DNA fragments by PCR. The PCR products were cloned into vector pET32a (Novagen) respectively via the restriction digestion as indicated in Table I to yield pET32a-GFP, pET32a-GFP-TSA56FL, pET32a-GFP-TSA47FL, pET32a-GFP-TSA22FL, pET32a-GFP-TSA56-ADI and pET32a-GFP-TSA56-ADIII. Each construct was amplified by PCR with the primers as listed in Table I.

Expression of TSA56, TSA47 and TSA22 genes of O. tsutsugamushi in *E.coli*. *E.coli* BL21 (DE3) (Novagen) transformed with the plasmid pET32a-TSA56FL, pET32a-TSA47FL, pET32a-TSA22FL, pET32a-TSA56-ADI, pET32a-TSA56-ADIII and pET32a-TSA56-ADI+ADII were propagated overnight in LB medium containing ampicillin (100 μg/ml) at 37˚C with gentle shaking. One milliliter of the overnight culture was inoculated into 100 ml of fresh medium containing the antibiotics and the culture was allowed to grow to an absorbance at 600 nm of 0.6. The culture was induced with 1 mM IPTG and grown for an additional 3 h at 37˚C. The cells were harvested by centrifugation at 2,000 x g for 10 min. The bacterial proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (5% stacking gel and 10% separation gel) and the recombinant proteins expressed in *E. coli* transformants were identified by immunoblotting assay.

Purification of recombination proteins. Protein expression was induced by adding IPTG to a final concentration of 1 mM

Table I. Constructs and primers used in this study.

Constructs	Restriction sites	Product size	Primer sequences (5'→3')
pET32a-TSA56FL	*Eco*RI/*Eco*RI	1608 bp	F: GGA ATT CAT GTT AAT TGCTAG TGC AAT GC R: GGA ATT CCC TAG AAG TTA TAG CGT ACA C
pET32a-TSA47FL	*Bam*HI/*Eco*RI	1401 bp	F: CGG GAT CCA TGA AAA AGG CAT TTT ATT CAC R: GGA ATT CTT ACT TAT TAA TGT TAG GTA AAG
pET32a-TSA22FL	*Bam*HI/*Eco*RI	606 bp	F: CGG GAT CCA TGA GTA AAG AAG CAA CAG AAC R: GGA ATT CTT ATC TTG CTA TAG AGT CTT TC
pET32a-TSA56-ADI	*Eco*RI/*Xho*I	288 bp	F: GGA ATT CTC TGC TAG TGC GAT AGA ATT G R: CCG CTC GAG ACC TCC AGA ATC TGC CTT AAC
pET32a-TSA56ADI+II	*Eco*RI/*Xho*I	588 bp	F: GGA ATT CTC TGC TAG TGC GAT AGA ATT G R: CCG CTC GAG AAC CAT AGG CCC ATT AGG
pET32a-TSA56-ADIII	*Bam*HI/*Eco*RI	390 bp	F: CAT GCC ATG GCA ATA CAT GAC CAT GAG CAA TGG R: GGA ATT CCC TAT GCT GCT GCT ACT GC
pET32a/GFP-alone	*Bam*HI/*Eco*RI	798 bp	F: CGG GAT CCA TGG TGA GCA AGG GCG AGG R: GAA TTC TTA TCT AGA TCC GGT GGA TC
pET32a/GFP-TSA56	*Eco*RI/*Eco*RI	2406 bp	F: GGA ATT CAT GGT GAC CAA GGG CGA G R: GGA ATT CCC TAG AAG TTA TAG CGT ACA C
pET32a/GFP-TSA47	*Bam*HI/*Bam*HI	2199 bp	F: CGG GAT CCA TGG TGA GCA AGG GCG AGG R: CGG GAT CCT TAC TTA TTA ATG TTA GGT AAA G
pET32a/GFP-TSA22	*Bam*HI/*Bam*HI	1404 bp	F: CGG GAT CCA TGG TGA GCA AGG GCG AGG R: CGG GAT CCT TAT CTT GCT ATA GAG TCT TTC
pET32a/GFP-TSA56 ADI	*Eco*RI/*Eco*RI	1083 bp	F: GGA ATT CAT GGT GAC CAA GGG CGA G R: GGA ATT CAC CTC CAG AAT CTG CCT TAA C
pET32a/GFP-TSA56 ADIII	*Bam*HI/*Bam*HI	1118 bp	F: CGG GAT CCA TGG TGA GCA AGG GCG AGG R: CGG GAT CCT GCT GCT GCT ACT GCT TCT TG
pEGFP-TSA56FL	*Eco*RI/*Eco*RI	1608 bp	F: GGA ATT CAT GTT AAT TGCTAG TGC AAT GC R: GGA ATT CCC TAG AAG TTA TAG CGT ACA C
pEGFP-TSA47FL	*Eco*RI/*Bam*HI	1401 bp	F: GGA ATT CCA TGA AAA AGG CAT TTT ATT CAC R: CGG GAT CCT TAC TTA TTA ATG TTA GGT AAA G
pEGFP-TSA22FL	*Eco*RI/*Bam*HI	606 bp	F: GGA ATT CCA TGA GTA AAG AAG CAA CAG AAC R: CGG GAT CCT TAT CTT GCT ATA GAG TCT TTC
pEGFP-TSA56-ADI	*Xho*I/*Eco*RI	288 bp	F: CCG CTC GAG CGT CTG CTA GTG CGA TAG AAT TG R: GGA ATT CAC CTC CAG AAT CTG CCT TAA C
pEGFP-TSA56-ADIII	*Eco*RI/*Bam*HI	390 bp	F: GGA ATT CCA TAC ATG ACC ATG AGC AAT GG R: CGG GAT CCT GCT GCT GCT ACT GCT TCT TG

for 4 h at 37˚C. Bacteria were pelleted by centrifugation and suspended in 5 ml binding buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0) containing 0.1% lysozyme and Roche protease inhibitor tablet, then sonicated until clear and finally centrifuged at 14,000 rpm for 30 min at 4˚C. Ni-charged resins (Novagen) were added to the supernatant, and the mixture was incubated on an end-over-end shaker for 16 h at 4˚C. The resins were washed three times with wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8.0). After washing, His-tagged proteins (His-TSA56FL His-TSA47FL His-TSA22FL, His-TSA56-ADI, His-TSA56-ADIII, His-TSA56-ADI+ADII) were released from the beads by elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0). Finally, the identity and purity of proteins were assessed by Coomassie blue staining and western blotting, respectively.

Immunoblotting assay. The 21 samples of clinical *O. tsutsugamushi*-infected patients' sera were prepared from 21 scrub typhus patients confirmed by the Centers for Disease Control (CDC) in Taiwan. Normal sera were collected from non-*O. tsutsugamushi*-infected controls. Both patient sera and normal sera were reacted against the purified recombinant TSA proteins. Horseradish peroxidase (HRP)-conjugated

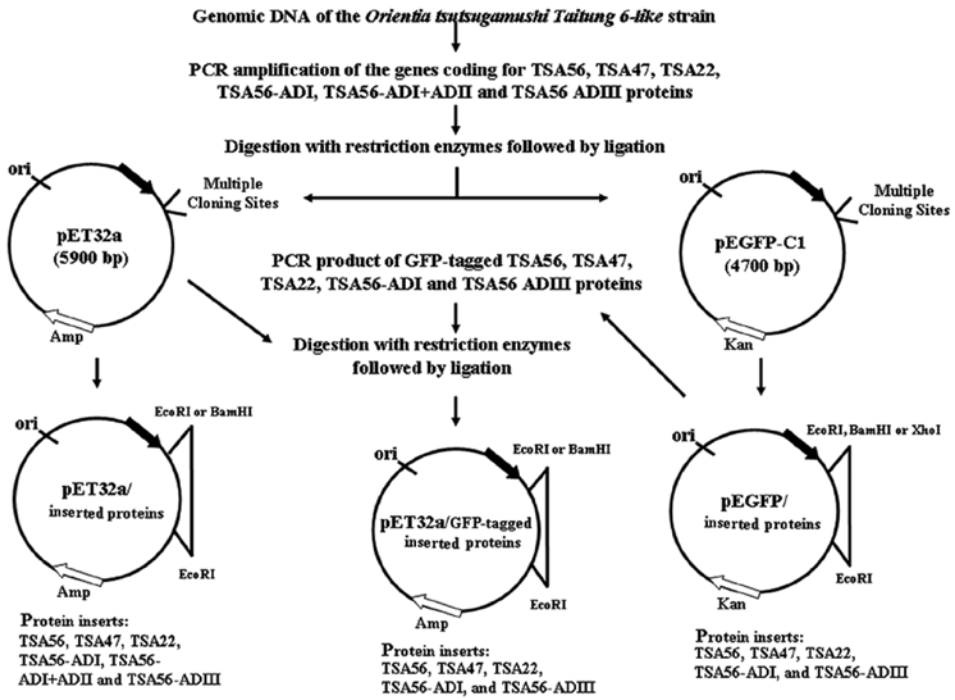

Figure 1. Strategies for cloning and construction of pET32a or pET32a-GFP tagged TSA56, TSA47, TSA22, TSA56-ADI, TSA56-ADI+ADII and TSA56 ADIII vectors that expressing either full-length or truncated recombinant proteins corresponding to *O. tsutsugamushi* Taitung 6-like strain.

goat-anti-human IgG (Invitrogen Life Technologies) was used as secondary antibody. Twenty micrograms of each recombinant protein (His-TSA56FL, His-TSA47FL, His-TSA22FL, His-TSA56-ADI, His-TSA56-ADIII, His-TSA56-ADI+ADII) was subjected and separated on 10% SDS-PAGE. Then the separated proteins were transferred to PVDF membranes using a semi-dry blot transferring apparatus. The membranes were further incubated for 1 h in blocking buffer (5% non-fat milk in 1X TBS with 0.1% Tween-20) at room temperature (or overnight at 4˚C). After being washed with TBS-T buffer (TBS with 0.1% Tween-20) for 5 times, the membrane was incubated with patients' sera in hybridization buffer (1% non-fat milk in 1X TBS-T; serum dilution 1:1,000) for 1 h at room temperature. After washing with TBS-T for another 5 times, the membrane was incubated with HRP-conjugated goat-anti-human IgG (1:10,000) as A secondary antibody in hybridization buffer for 45 min. The chemiluminescent signal was developed using the ECL plus kit (GE Healthcare) and the image was captured using the Kodak® BioMax X-ray film (Kodak). Densitometry was analyzed using ImageJ by which the signals of control serum samples were designated as the background to subtract the signals from the patents' sera firstly, and then the signal intensity was categorized into four orders as 'strong', 'moderate', 'weak' and 'no response' were indicated as '+++', '++', '+' and '-'. In general, the range of the signal intensity of the immnoblotting assay was defined and then further equally divided into four sub-ranges based on densitometry values. The antiserum of patient 8 was also used as a positive control across different batch experiments to normalize inter-batch variations.

Cell culture. Vero cells (kidney epithelial cells extracted from an African green monkey) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco-BRL) at 37˚C in incubator supplied with 5% CO_2.

Cell adhesion assay. Vero cells were cultured on 18-mm diameter glass coverslips in 12-well plates at a density of $1x10^5$ cells/well. Surrogate *E. coli* BL21 (DE3) were respectively transformed with pET32a-GFP, pET32a-GFP-TSA56FL, pET32a-GFP-TSA47FL, pET32a-GFP-TSA22FL, pET32a-GFP-TSA56-ADI and pET32a-GFP-TSA56-ADIII, and then induced with 1 mM IPTG at 37˚C for 3 h. After IPTG induction, *E. coli* BL21 (DE3) was added to a monolayer of Vero cells in serum-free media and incubated at 37˚C for 60 min. The contact between bacteria and the mammalian cells was synchronized by centrifugation at 200 x g. After being washed three times with PBS to remove non-adherent bacteria, the cells were fixed with 4% paraformaldehyde and were permeabilized in a 0.2% Triton X-100 solution for nuclear staining with DAPI (blue). The image was observed under a confocal microscope.

Results

Antibody responses against recombinant TSA56 are higher than those of TSA47 and TSA22 in infected-patient serum samples. To study the contribution of three major outer membrane proteins TSA56, TSA47 and TSA22 in *O. tsutsugamushi* invasion, an *E. coli*-based heterologous protein expression system was used. The PCR amplified fragments encompassing full-length *O. tsutsugamushi* TSA56, TSA47 and TSA22 genes were cloned into pET32a, an *E. coli* IPTG-inducible expression vector. The strategies for constructing TSA protein expressing vectors are shown in Fig. 1. The plasmids were transformed

Table II. Antibody responses against recombinant TSA56, TSA47 and TSA22 proteins in serum samples of patients with *O. tsutsugamushi* infection.

Patient serum	TSA56	TSA47	TSA22
Normal (n=4)			
Positive rate, %	0	0	0
Negative rate, %	100	100	100
O. tsutsugamushi-infected (n=21)			
+++	2/21	0/21	0/21
++	15/21	9/21	8/21
+	0/21	3/21	1/21
-	4/21	9/21	12/21
Positive rate (%)[a]	17/21 (81.0)	12/21 (57.1)	9/21 (42.9)

Serum dilution is equal to 1:1,000; symbols '+++', '++', '+' and '-' indicate strong, moderate, weak and no response, respectively. [a]The Fisher's exact test was used for discriminating the differences in the positive antibody response between the TSA56 and TSA47 groups (P=0.181) and the TSA56 vs. TSA22 groups (P=0.025).

Table III. Antibody responses against recombinant TSA56-ADI, TSA56-ADI+ADII and TSA56-ADIII in serum samples of patients with *O. tsutsugamushi* infection.

Patient serum	TSA56-ADI	TSA56-ADI+II	TSA56-ADIII
Normal (n=4)			
Positive rate, %	0	0	0
Negative rate, %	100	100	100
O. tsutsugamushi-infected (n=21)			
+++	0/21	2/21	0/21
++	13/21	15/21	7/21
+	2/21	0/21	2/21
-	6/21	4/21	12/21
Positive rate (%)[a]	15/21 (71.4)	17/21 (81.0)	9/21 (42.9)

Serum dilution is equal to 1:1,000; symbols '+++', '++', '+' and '-' indicate strong, moderate, weak and no response, respectively. [a]The Fisher's exact test was used for discriminating the difference in the positive antibody response between the TSA56ADI and TSA56ADI+II groups (P=0.520) and the TSA56ADI vs. TSA56ADIII groups (P=0.181).

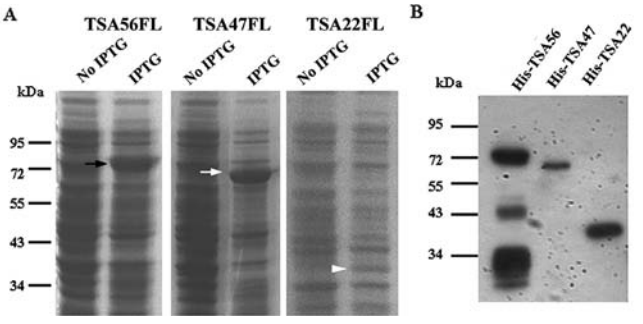

Figure 2. Expression and purification of recombinant His-TSA56, TSA47, TSA22 proteins in *E. coli*. (A) Bacterial lysates of *E. coli* expressing recombinant proteins were separated by SDS-PAGE and stained with Coomassie blue. Black arrow, white arrow and white arrowhead indicate TSA56, TSA47 and TSA22, respectively. (B) Purified full-length His-TSA56, TSA47 and TSA22 proteins were separated by SDS-PAGE and were detected with anti-His antibody by immunoblotting.

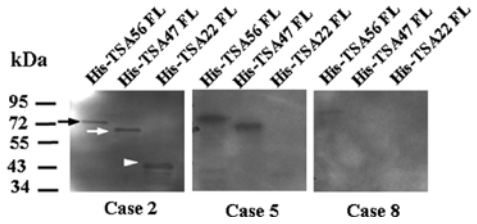

Figure 3. Representative responses of sera from scrub typhus patients against recombinant His-TSA56, TSA47 and TSA22 by immunoblotting. Black arrow, white arrow and white arrowhead indicate TSA56, TSA47 and TSA22, respectively.

into the *E. coli* strain, BL21 (DE3) and further induced for TSA protein expression (Fig. 2A). All recombinant TSA56, TSA47 and TSA22 proteins were purified and were detected by immunoblotting assay with anti-6xHis antibody (Fig. 2B). To further examine the antibody responses of patient sera against these recombinant TSA proteins, TSA56FL, TSA47FL and TSA22FL proteins were resolved in SDS-PAGE and further incubated with either 21 samples of clinical *O. tsutsugamushi*-infected patient sera or with normal serum from controls. Of the 21 *O. tsutsugamushi*-infected patients' sera, 81% (17/21) were found to have antibody responses in against TSA56, 57.1% (12/21) for TSA47 and 42.9% (9/21) for TSA22, respectively. No antibody responses were found for normal serum from controls (Table II). Representative results from patient sera against three different TSA proteins are shown in Fig. 3. Of these serum samples, most can recognize the recombinant TSA56 protein rather than the TSA47 and TSA22 as shown in Table II. Besides, the positive rate of the antibody response against the TSA56 group was significantly higher than that of the TSA22 group (P=0.025), but not the TSA47 group (P=0.181).

TSA56-ADI is more highly recognized by the patients' sera than TSA56-ADIII. The TSA56 protein of *O. tsutsugamushi* is recognized by most of the patient serum samples during the convalescence phase of the scrub typhus life cycle (Table II). Further molecular mapping for the TSA56 antigenic domain that is responsible for inducing the antibody responses of patients' sera, was performed by functionally expressing the *O. tsutsugamushi* TSA56-ADI, -ADI+ADII and -ADIII truncated proteins. The cloning strategies for preparing the protein expression constructs are shown in Fig. 1. All recombinant TSA56-ADI, TSA56-ADI+ADII and TSA56-ADIII proteins were purified from an *E. coli* host and were detected in the patients' sera using immunoblotting (Fig. 4). We found that the positive rates of anti-TSA56-ADI, -ADI+ADII and -ADIII were 71.4% (15/21), 81.0% (17/21) and 42.9% (9/21), respectively (Table III). No antibody response was detected for the

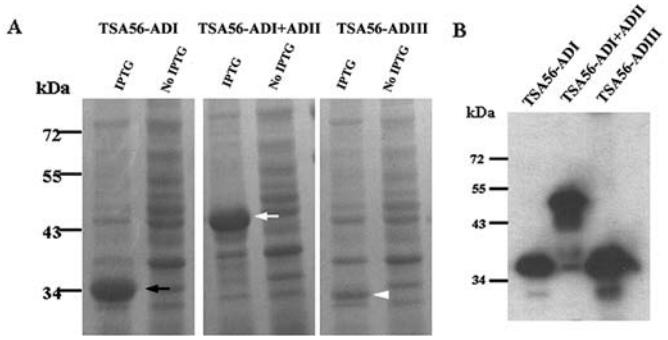

Figure 4. Expression and purification of His-TSA56-ADI, TSA-ADI+ADII, and TSA56-ADIII recombinant proteins. (A) Bacteria lysates of *E. coli* expressing recombinant proteins were separated by SDS-PAGE and stained with Coomassie blue. Black arrow, white arrow and white arrowhead indicate TSA56-ADI, TSA-ADI+ADII and TSA56-ADIII, respectively. (B) Purified proteins were separated by SDS-PAGE and were detected with an anti-His antibody by immunoblotting.

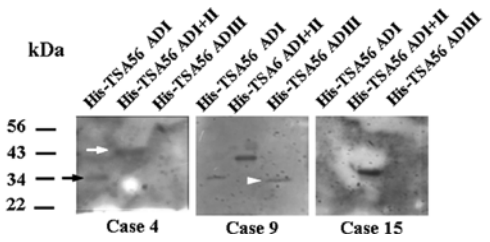

Figure 5. Representative results in serum samples of scrub typhus patients against recombinant His-TSA56-ADI, TSA56-ADI+II and TSA56-ADIII using immunoblotting assay. Black arrow, white arrow and white arrowhead indicate TSA56-ADI, TSA56-ADI+II and TSA56-ADIII, respectively.

antiserum from normal controls. The representative results in respect to the anti-TSA56-ADI, -ADI+ADII, and -ADIII responses in serum samples of *O. tsutsugamushi*-infected patients are shown in Fig. 5. These results, are inconsistent with some findings from a previous study showing that ADIII and the adjacent C-terminal region of TSA56 is a putative Fn-binding domain and may facilitate Fn-mediated infection with *O. tsutsugamushi* (27). However, our data showed that the TSA56-ADI-truncated proteins had a higher antibody response than TSA56-ADIII in patient sera (71.4% vs. 47.6%) even though the difference was not significant (P=0.719). Thus, our findings suggest that TSA56-ADI may have a role in disease trajectory of *O. tsutsugamushi* infection. Moreover, the TSA56-ADI may be potential molecular target for developing a peptide-based vaccine against *O. tsutsugamushi*.

Adhesion ability of TSA56 is higher than that of TSA47 and TSA22, and TSA56-ADI rather than TSA56-ADIII is associated with adhesion to host cells. As known, bacterial invasion of host cells is mediated primarily by interactions between bacterial surface components and their complementary host receptors. To further investigate and compare the adhesion ability of all three major outer membrane proteins of *O. tsutsugamushi*, cell adhesion assays were performed by which green fluorescent protein (GFP)-fused TSA proteins were expressed in heterologous *E. coli* protein expression system and the adhesion ability of these *E. coli* transformants to Vero cells among different TSA protein groups was examined. pET32a-GFP, GFP-TSA56, GFP-TSA47 and GFP-TSA22 were constructed (the strategies for preparing these vectors are shown in Fig. 1). All GFP-fused proteins or GFP were purified and confirmed for successfully expressing in *E. coli* host by immunoblotting assay with an anti-GFP antibody (Fig. 6A). The *E. coli* transformants with pET32a-GFP or with pET32a-GFP-TSA56, GFP-TSA47 and GFP-TSA22 were further induced with IPTG and incubated with Vero cells for the adhesion assay. The adhered GFP-expressing *E. coli* transformants on the Vero cells was observed and counted by fluorescence microscopy. Among different GFP-fused TSA groups, the GFP-TSA56 group showed a significantly higher number of adhered *E. coli* transformants on Vero cells even after extensive washing with PBS buffer (Fig. 6B). On the contrary, a limited number of adhered *E. coli* transformants was found for the GFP-TSA47 group and almost no adhered *E. coli* transformants were observed for the GFP-alone and the GFP-TSA22 groups (Fig. 6B). The ratio of adhered GFP-expressing *E. coli* transformants to the Vero cells in each group was also estimated as shown in Fig. 6C. The GFP-TSA56 group demonstrated a significantly higher adhering ability compared to the GFP-TSA47 and GFP-TSA22 groups.

It has been documented that *O. tsutsugamushi* can internalize into the host cells via interaction between its TSA56-ADIII and host fibronectin (25,26). Our data also showed that TSA56 is the most important component among the three outer membrane proteins of *O. tsutsugamushi*, in terms of the responses for the adhesion to the host cells. Thus, we further analyzed and compared the adhesion ability of TSA56-ADI and TSA56-ADIII to the host cells. Both GFP-fused truncated TSA proteins were expressed, purified from *E. coli* transformants and further confirmed by immunoblotting assay with an anti-GFP antibody (Fig. 7A). The GFP-expressing and GFP-TSA56-expressing *E. coli* strains were used as negative and positive controls, respectively. Fluorescence microscopy showed the *E. coli* transformants expressing GFP-TSA56, GFP-TSA56-ADI and GFP-TSA56-ADIII adhered to the host cells (Fig. 7B). However, the GFP-TSA56 and GFP-TSA56-ADI group was detected with an increased number of adhered *E. coli* compared to the GFP-TSA56-ADIII group (Fig. 7B and C). This result was consistent with the finding from immunoblotting assay using patient serum samples (Table III), indicating that TSA56-ADI may induce a higher antibody response than TSA56-ADIII and as an important component responds for adhesion. Taken together, our data suggest that the TSA56 revealed a higher adhesion ability than TSA47 and TSA22; in addition, TSA56-ADI may facilitate adhesion and mediate *O. tsutsugamushi* invasion to the host cells.

Discussion

O. tsutsugamushi is an obligate intracellular organism and the causative agent of scrub typhus (13). The ability of *O. tsutsugamushi* to bind to and invade target mammalian cells is a critical initial event during pathogenesis. Bacterial adhesions have become recognized as attractive targets for therapeutics and vaccine development (30,31). Previous studies have shown that the strong immune responses of humans to the outer membrane protein type-specific antigen (TSA) of *O. tsutsugamushi*

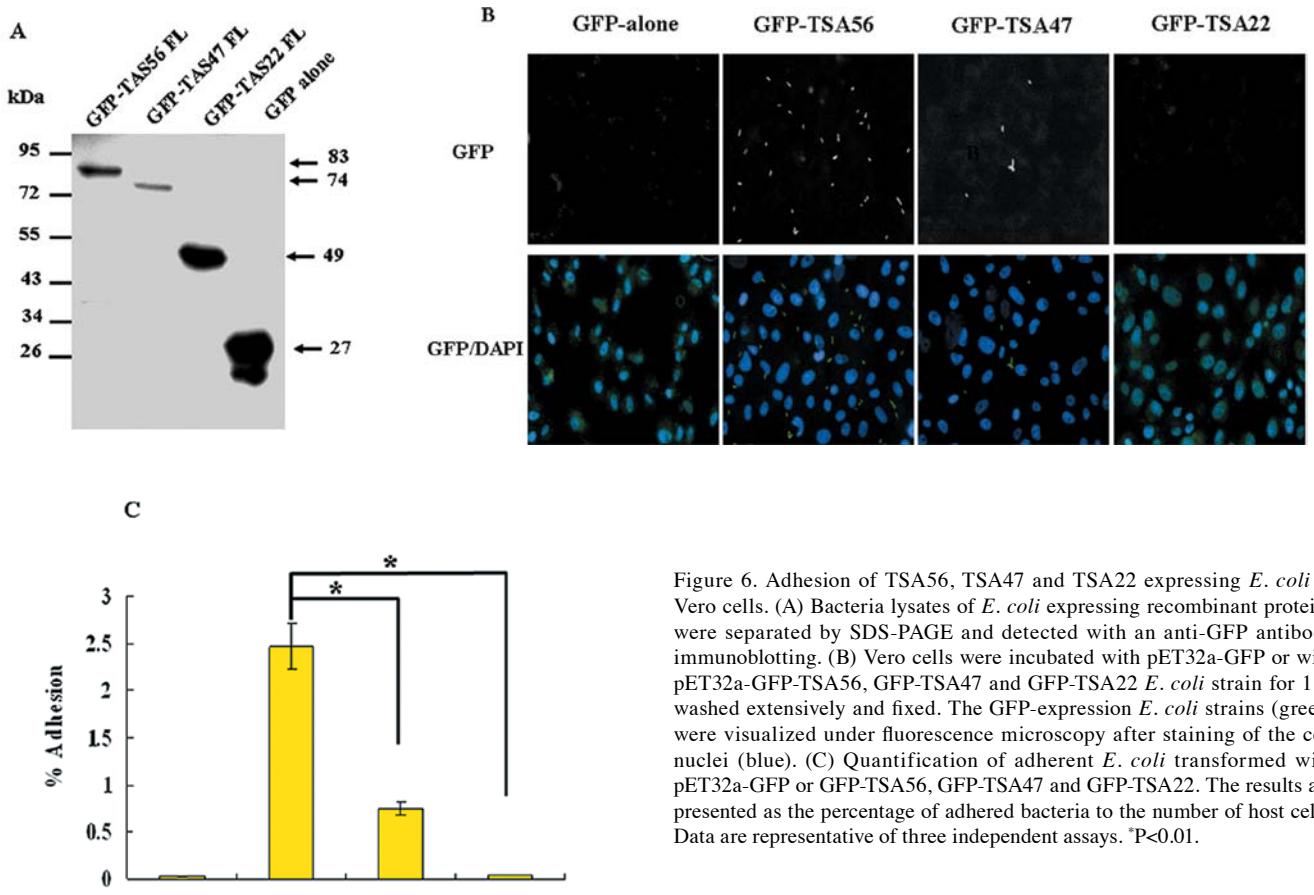

Figure 6. Adhesion of TSA56, TSA47 and TSA22 expressing *E. coli* to Vero cells. (A) Bacteria lysates of *E. coli* expressing recombinant proteins were separated by SDS-PAGE and detected with an anti-GFP antibody immunoblotting. (B) Vero cells were incubated with pET32a-GFP or with pET32a-GFP-TSA56, GFP-TSA47 and GFP-TSA22 *E. coli* strain for 1 h, washed extensively and fixed. The GFP-expression *E. coli* strains (green) were visualized under fluorescence microscopy after staining of the cell nuclei (blue). (C) Quantification of adherent *E. coli* transformed with pET32a-GFP or GFP-TSA56, GFP-TSA47 and GFP-TSA22. The results are presented as the percentage of adhered bacteria to the number of host cells. Data are representative of three independent assays. *P<0.01.

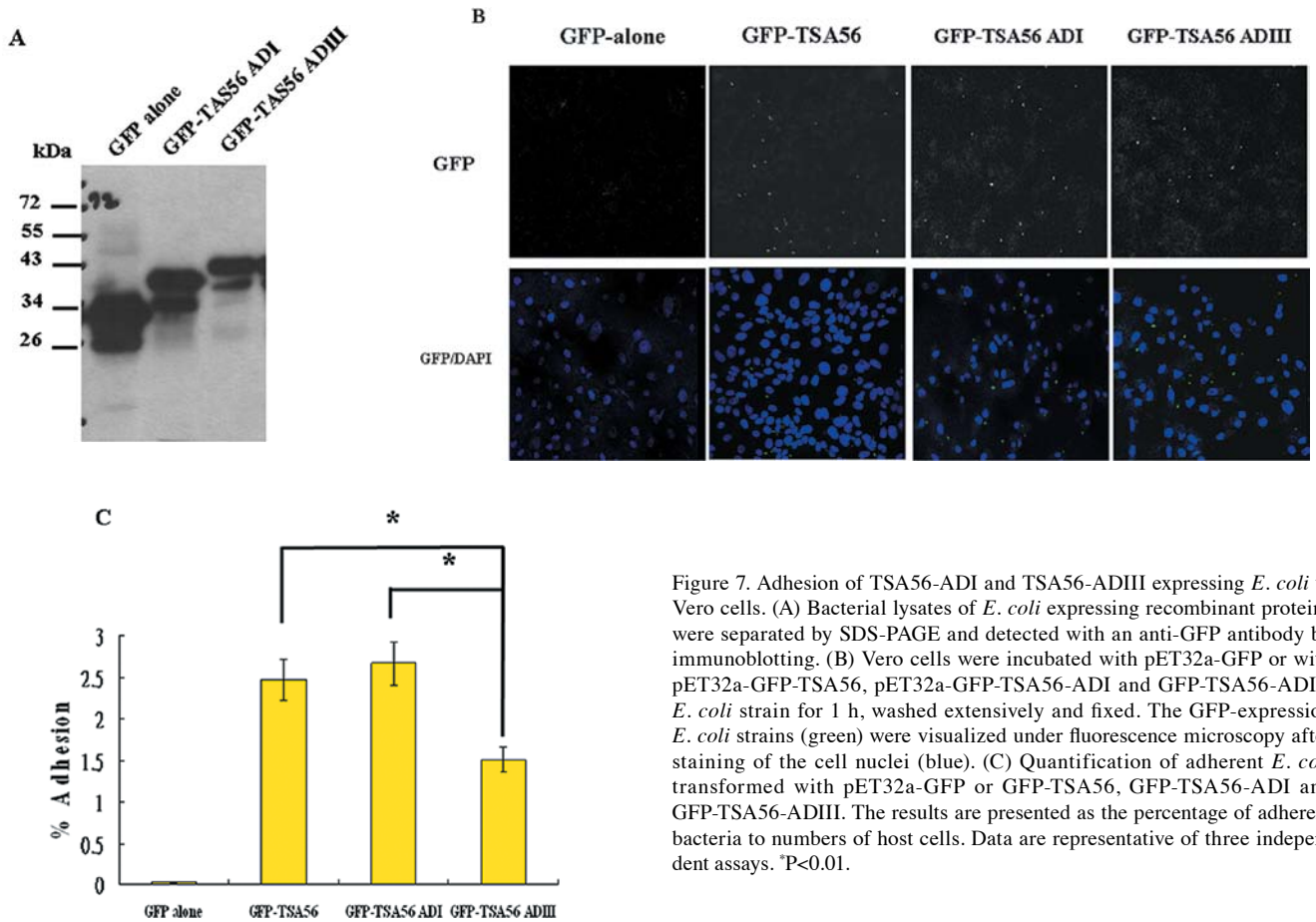

Figure 7. Adhesion of TSA56-ADI and TSA56-ADIII expressing *E. coli* to Vero cells. (A) Bacterial lysates of *E. coli* expressing recombinant proteins were separated by SDS-PAGE and detected with an anti-GFP antibody by immunoblotting. (B) Vero cells were incubated with pET32a-GFP or with pET32a-GFP-TSA56, pET32a-GFP-TSA56-ADI and GFP-TSA56-ADIII *E. coli* strain for 1 h, washed extensively and fixed. The GFP-expression *E. coli* strains (green) were visualized under fluorescence microscopy after staining of the cell nuclei (blue). (C) Quantification of adherent *E. coli* transformed with pET32a-GFP or GFP-TSA56, GFP-TSA56-ADI and GFP-TSA56-ADIII. The results are presented as the percentage of adherent bacteria to numbers of host cells. Data are representative of three independent assays. *P<0.01.

reflect their abundance on the cell surface and their potent immunogenicity (32-36). The outer membrane proteins of *O. tsutsugamushi* have been reported to play an important role during its infection and induction of the host immune response (37). At least four protein antigens of *Orientia* with molecular weights of 22, 47, 56 and 110 kDa have been identified. Almost all clinically diagnosed patient serum samples recognize the 56-kDa antigen, but not every patient's serum reacts with the 22-kDa, 47-kDa, or 110-kDa antigens (38,39). Unfortunately, there are still no successful vaccines against *O. tsutsugamushi*. Thus, the development of inhibitor candidates based on recombinant proteins will require the identification of specific defined antigens that allow discrimination of *O. tsutsugamushi*. In this study, we clearly demonstrated that the *O. tsutsugamushi* outer membrane protein TSA56 had a strong response compared with TSA47 and TSA22 in Taiwanese patients. Moreover, our data also showed that the antibody responses and adhesion ability of TSA56-ADI to host cells was significantly higher than those of TSA56-ADIII. Our results support TSA56-ADI as a novel candidate for developing subunit vaccine against scrub typhus.

In this study, we observed that the TSA56 protein of *O. tsutsugamushi* was recognized by 17/21 (81%) of the patient serum samples (serum dilution 1:1,000) in the convalescence phase of scrub typhus in Taiwan (Table II). However, 4/21 patient serum samples were not reactive with the TSA56 protein. We firstly rationalized that this result may be due to the over dilution of the patients' sera since the original titer of the TSA56 antibody in the antiserum may be low during the immunoblotting assay. However, 19/21 (90.4%) serum samples of patients reacted with the TSA56 protein in the titer of 1:100, but the positive antibody responses to the TSA47 and TSA22 proteins was 13/21 (61.9%) and 10/21 (47.6%), respectively (data not shown). These data indicate that the TSA56 protein has a strong antibody response in scrub typhus patients in Taiwan in spite of the dilution factors of the patients' sera, which is consistent with other reports (26,38-41).

Furthermore, cell adhesion assays using a heterologous *E. coli* expression system showed that the expression of GFP-TSA56 fusion protein enhanced the antibody responses (Fig. 6). Previous studies reported that *O. tsutsugamushi* could bind to host fibronectin and utilize it for internalization via interactions with its outer membrane protein TSA56 (27,28). Our data also confirmed the TSA56 adhesion ability to host cells. Alternatively, when the adhesion ability of TSA56 is compared with that of TSA47 and TSA22, the TSA22 group displayed almost no bacterial adhesion to host cells and the TSA47 group showed very limited bacterial adhesion (Fig. 6). It may be presumed that TSA47 mediates, but to a low degree, *O. tsutsugamushi* adhesion to host cells. Yu *et al* (42) demonstrated that the scrub typhus antigen 56-47 (Sta56-47) fusion protein containing the antigenic properties of the 56- and 47-kDa major surface antigen of *O. tsutsugamushi* is an efficient recombinant antigen for eliciting humoral and cellular immunity against scrub typhus. Converging this finding with our results, it is possible that TSA47 may synergistically act with TSA56 to facilitate *O. tsutsugamushi* adhesion to host cells. Taken together, the data suggest that *O. tsutsugamushi* exploits the abundant TSA56 to elicit antibody responses of the host and as a major outer membrane protein, TSA56 participates in the adhesion of *O. tsutsugamushi* to the host cells.

The amino acid sequence of TSA56 contains the signal sequence (SP) in the N-terminal and the transmembrane domains (TMs) in the C-terminal, and in the middle of the sequence there are 3 extracellular antigen domains (ADs) and 4 variable domains (VDs) (27,37,43). In this study, we found that TSA56-ADI may elicit higher antibody responses against patients serum samples than TSA56-ADIII, as well as a higher adhesion ability. This finding is in agreement with results from previous studies: i) Seong *et al* (43) demonstrated that human immunoglobulin M (IgM) antibodies are predominantly bound to ADI (residues 19-113) and ADIII (residues 243-328); and ii) human IgG preferentially binds to ADI. In accordance with our data, the positive antibody responses were higher in the ADI (residues 19-114) or the ADI+II (residues 19-214) groups than in the ADIII (residues 237-366) group (Table III). Previously, Choi *et al* (37) demonstrated that the amino acid region encompassing residues 131-201 (containing ADII and VDII) from Karp, Kato and Boryong strains were reactive with both homotypic and heterotypic antibodies. Lacking of the antibody specificity, and thus the ADII region was suggested to be dispensed and unsuitable for vaccine development (37).

Recent studies have shown that the scrub typhus pathogen, *O. tsutsugamushi*, can bind to fibronectin (Fn) via a major antigenic membrane protein, TSA56 (27,28). The ADIII and adjacent C-terminal region of TSA56 was sufficient for Fn interaction and prohibiting Fn-enhanced invasion. However, GST-TSA56-F1 and GST-TSA56-F2, which contain ADI and ADII respectively, did not interact with Fn and had no effect on the inhibition of Fn-enhanced invasion by bacteria (27). In our case, the ADI+II region (residues 19-214) contains the variable domain I and II (VDI and II) and shows high reactivity against serum samples of scrub typhus patients (Table III). It could be that ADII and VDII enhance the antibody responses (37). In addition, our results also showed that the expression of GFP-TSA56-ADI fusion protein enhanced the adhesion ability of *E. coli* to Vero cells compared to GFP-TSA56-ADIII (Fig. 7). The results suggest that the ADI region of TSA56 reflects an adhesion site other than ADIII, to invade eukaryotic host cells. Recently, in addition to TSA56-ADIII, fibronectin was identified as a potential receptor for ScaC, an autoreceptor of *O. tsutsugamushi* (44). Using microbeads conjugated to recombinant ScaC or a surrogate *E. coli* expression system which partly consistent with our approach, Ha *et al* (44) showed that ScaC was sufficient to mediate attachment to, but not invasion of, nonphagocytic mammalian cells. The interactions between recombinant *O. tsutsugamushi* ScaC and host fibronectin were also confirmed by a yeast two-hybrid system and GST pull-down assay. Thus, we further determined whether blocking host cell receptors by neutralizing the fibronectin receptor would lead to an inhibition of the bacteria-host cell interaction (in our study, we firstly hypothesized that TSA56-ADI also interacts with fibronectin similarly to TSA56-ADIII). Unexpectedly, we found that pre-conditioned Vero cells with a fibronectin antibody showed no significant inhibition of bacterial-host cell interactions (data not shown). We suspected that negative results of interrupting bacteria-host cell interactions by fibronectin neutralization may be due to antibody recognition sites on fibronectin which are different from the binding sites of TSA56-ADI or -ADIII. Another possibility is

that TSA56-ADI may interact with unknown components or complementary host receptors, such as the Toll-like receptor (TLR) family to invade host cells.

In conclusion, our results suggested that the TSA56 protein is a more efficient recombinant antigen and that TSA56-ADI shows higher reactivity against serum samples of scrub typhus patients in Taiwan. Moreover, expression of GFP-TSA56 and GFP-TSA56-ADI in *E. coli* enhances bacterial adherence to nonphagocytic host cells. Taken together, our results suggest that the TSA56-ADI truncated protein may be a novel potential target for vaccine development against scrub typhus. Further identifying the regions within the fibronectin molecule that are associated with TSA56-ADIII binding or other complementary host receptors like Toll-like receptor (TLR) family that is responsible for binding to each of the bacterial ligands may provide valuable clues for their contribution to the initial stages of infection.

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