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Evaluation of a Recombinant Vaccine Candidate r56Lc-1 in a Chigger Challenge Mouse Model

Wei-Mei Ching[1,3*], Woradee Lurchachaiwong[2], Zhiwen Zhang[1,3] Temitayo Awoyomi[1,3], Chien-Chung Chao[1,3] and Anthony Schuster[2]

[1]*Viral and Rickettsial Diseases Department, Infectious Diseases Directorate, Naval Medical Research Center, Silver Spring, USA*

[2]*Entomology Department, Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand*

[3]*Uniformed Services University of the Health Sciences, Bethesda, USA*

*Corresponding author: Wei-Mei Ching, PhD, Viral and Rickettsial Diseases Department, Infectious Diseases Directorate, Naval Medical Research Center, 503 Robert Grant Ave, RM3N71, Silver Spring, MD 20910, USA, Tel: 301 319 7438; Fax: 301 319 7451; E-mail: WeiMei.Ching@med.navy.mil

Abstract

Scrub typhus, an acute, febrile disease is transmitted by the bite of an *Orientia* infected chigger. We evaluated the protective potential of a recombinant 56 kDa antigen in a chigger challenge mouse model which mimics the natural transmission of *Orientia*. Chiggers from an *L. chiangraiensis* line 1 (Lc-1) was chosen for this challenge because the line produces chiggers with stable infectivity of 90-100% in several generations. The 56kD antigen gene of *O. tsutsugamushi* was cloned into an expression vector, expressed, purified, and refolded. All ICR mice were immunized 3 times at 4 weeks intervals and challenged by placing an individual chigger in the inner ear of each mouse 4 weeks after the last immunization. Mice were immunized with adjuvant Montanide+CpG or 25 g of r56Lc-1 emulsion with Montanide+CpG. Both groups were challenged by an individual Lc-1 chigger (un-infected or infected). There was no death in the groups of mice challenged by an uninfected chigger, with or without the vaccine candidate r56Lc-1. There was no survival in the groups of mice immunized with adjuvant only and challenged by an infected chigger. Our data demonstrated that r56Lc-1 provided 20-30% of protection consistently upon challenge by an infected chigger which carries the same *Orientia* strain based on the sequence of 56 kDa antigen. Time to mortality was delayed in mice immunized with the vaccine candidate then challenged with infected chiggers as compared to their adjuvant counterparts Since *Orientia* is an intracellular pathogen, the clearance of this pathogen may heavily depend on T-cell immune responses rather than B-cell immune responses and may require a vaccine candidate that mainly promotes T-cell immunity.

Introduction

Scrub typhus is caused by the infection of *Orientia tsutsugamushi*, an obligate intracellular Gram-negative bacterium [1]. The disease is characterized by fever, rash, eschar, pneumonitis, meningitis, and in some cases, disseminated intravascular coagulation that may lead to circulatory failure [2]. It accounts for up to 23% of all febrile episodes in endemic areas of the Asia-Pacific, while recent estimations in Thailand are about 20% [3]. Scrub typhus can be fatal if left untreated [2,4] and reports from India have documented 12-17% fatality rates [5,6]. Geographic distribution of the disease occurs within an area of about 13 million square kilometers and includes Pakistan, India and Nepal in the West to Japan in the East, and from South eastern Siberia, China, and Korea in the North to Indonesia, Philippines, Northern Australia and the intervening Pacific islands in the South [7]. The disease has been re-emerging and occurs in new areas in many countries located in the Eastern Hemisphere [8-10]. Recently, two publications have documented the presence of scrub typhus in the Middle East (United Arab) and South America (Chile) [11,12]. The presence of scrub typhus outside the traditional geographic distribution suggests an increased risk for US military personnel and travellers entering into these newly emerged regions. At this present time, no vaccine is available for protection against scrub typhus

[13-16]. In addition, evidence of antibiotic resistance cases further emphasizes the need for a scrub typhus vaccine [17,18]. Due to the fact that *O. tsutsugamushi* exhibits considerable strain variations, previous vaccine development has been difficult.

In the past, effective vaccination in mice has been achieved with subcutaneous biovaccines in which live organisms are combined with tetracycline or with gamma-irradiated live organisms [19,20]. Also, immunization of volunteers with live vaccine in combination with chloramphenicol prophylaxis elicited immunity comparable to that of natural infection [21]. However, mass production of purified *O. tsutsugamushi* and retaining its stability upon storage are extremely difficult [22-24]. Therefore, whole cell vaccine products are unlikely to be economically feasible or suitable for manufacturing with current Good Manufacturing Practices Act standards of purity, potency, and lot-to-lot consistency. It is therefore, essential to develop a vaccine composed of genetically engineered materials which are easy to produce and capable of inducing protective immunity in human subjects after testing in relevant mouse models.

The 56 kDa protein is the most immunodominant antigen of *O. tsutsugamushi* which accounts for 10-15% of the total cell protein [25-27]. This protein has four variable domains as well as five conserved domains and is responsible for the antigenic divergence of *Orientia* [7]. Almost every clinically diagnosed patient serum reacts with 56 kDa antigen. Recombinant 56 kDa protein and DNA plasmid carrying this protein gene have been shown to be protective in mice against homologous challenge by intraperitoneal injection (IP) or in

cynomolgus monkey by intradermal injection [25-29]. The observed protective immune responses include antibody production, T cell proliferation, and production of IL-2 and IFN-γ [28-32].

Since the scrub typhus disease is transmitted via the bite of an infected chigger and rodent is the natural reservoir of chigger, the best way to evaluate a vaccine candidate is using the chigger-mouse feeding model, which has been established at The Armed Forces Research Institute of Medical Sciences, Thailand (AFRIMS) [33-36]. A total of 12 *Orientia* infected and 3 uninfected chigger lines derived from three *Leptotrombidium* species are maintained [21,33,34] at AFRIMS [33,34]. Among these *Orientia* infected chigger lines; Lc-1 exhibited a 90% or higher infection rate [34]. This chigger line was identified as the most suitable line mimicking the natural challenge to evaluate vaccine candidates, i.e. infecting the mice by feeding a chigger in the mouse ear [34]. It was established previously that protein antigen based on the 56 kDa protein could provide very good protection in mouse challenge by injection of live *Orientia tsutsugamushi* intraperitoneally [31,32]. However, the protective effect of the recombinant protein antigen in mouse directly infected by the bite of chigger, the natural vector of *O. tsutsugamushi* has not been evaluated. In order to evaluate whether recombinant protein antigen-based vaccine could provide protection in mouse challenged by an *O. tsutsugamushi*-infected chigger, we have produced the recombinant protein for 56 kDa antigen based on the sequence of the *Orientia* strain carried by the chigger line Lc-1 (r56Lc-1).

This study described the production of r56Lc-1, the immunization regimen of out bred mice, and the observations of immunized mice after challenge in a chigger-mouse feeding model. The results have suggested that the protective efficacy ranged from 20-30%, in addition to the effect of prolonged survival days in non survivors. This is the first report for the evaluation of a vaccine candidate in a natural challenge mouse model.

Materials and Methods

Cloning and expression of 56 kDa protein genes from Lc-1

Primers with built-in BamH1 and NdeI restriction sites for the 56 kDa protein gene from amino acid 80-456 (according to the sequence of Karp strain) of the open reading frame were designed based on the available DNA sequences of Karp strain in the NCBI database. Genomic DNA extracted from the liver of mouse, which was infected by Lc-1 chiggers, was extracted as previously described using QiAamp DNA mini kit [35] and used in a polymerase chain reaction (PCR). The amplicons were cloned into a pET24a vector (Novagen, Wisconsin, USA). Colonies were selected and sequences of the amplicons were verified.

The plasmid was transformed into BL21 (DE3) cells (Invitrogen, Carlsbad, CA) for protein expression. BL21 (DE3) transformants containing correct amplicons were selected, grown in LB in the presence of 50 μg/mL kanamycin (Invitrogen) in a 37°C shaker and agitated at 200 rpm. The cells were induced with 1mM Isopropyl β-D-1-thiogalactopyranoside (IPTG, Sigma, St. Louis, MO) when OD600 reached 0.8-1.0. After induction for 19 hours, the cell culture was centrifuged at 4,000 rpm for 30 minutes in a SS34 Sorvall rotor to separate cells from LB medium. The cell pellet was stored in -20°C until use.

Extraction and solubilization of inclusion bodies containing the recombinant 56 kDa (r56Lc-1) proteins

The inclusion bodies were extracted as described previously [36]. Briefly, the cell pellet was thoroughly resuspended in 2% Deoxycholate (DOC, Sigma) in 20 mM Tris-HCl, pH 7.5 (buffer A) and sonicated (Sonicator Ultrsonic Liquid Processor Model XL2020 with a standard tapered microtip) on ice. Following centrifugation at 8,000xg for 30 minutes at 4°C, the resulting pellet was resuspended with 2 M urea (Arcos Organics USA, Morris Plains, NJ) in buffer A, incubated with gentle rocking for 30 min and centrifuged again as described. The entire process was repeated with 4 M, 6 M and 8 M urea in buffer A. The extraction supernatants were analyzed by SDS-PAGE. The supernatant from 4 M urea wash (approximately 10 ml) contained the majority of r56Lc-1 proteins.

Chromatographic purification of recombinant r56Lc-1 in the presence of 6M urea

The 4 M urea supernatant containing r56Lc-1 protein was purified by DEAE anion-exchange chromatography with a 50 minute linear gradient of 0.30-0.70 M NaCl (Sigma-Aldrich, Missouri, USA) in the presence of 6 M urea in buffer A (Buffer B). The purity of protein in each fraction was accessed by SDS-PAGE and fractions >95% pure were pooled together. If the purity of protein was not satisfactory, a second run of DEAE chromatography was carried out after dialysis of the pooled fractions to remove NaCl. The procedure for the second DEAE was similar to the first DEAE purification but the linear gradient was done in 0.30-0.60 M NaCl for 50 minutes. The final fractions containing r56Lc-1 were evaluated for protein purity by SDS-PAGE.

Refolding of purified recombinant 56 kDa proteins by dialysis

The purified r56Lc-1 in 6 M urea was refolded by sequential dialysis in decreasing concentrations of urea as described by Chao et al. [36]. The purified polypeptides at approximately 0.5 mg/mL in Buffer B were transferred into a dialysis bag (24 mm, molecular weight cutoff at 12,000 Daltons) and dialyzed sequentially against 4 M, 2 M, and 0 M urea in Buffer A for 30 min twice at each concentration of urea with gentle stirring. All dialysis procedures except the last step without urea were done at room temperature. The dialysis was continued overnight without urea in large excess of Buffer A at 4°C to remove traces of urea.

Chigger line, animals and immunization

Chiggers from the *Leptotrombidium chiangraiensis* line 1 (Lc-1) were selected for these challenge experiments because this line of chiggers exhibit stable infectivity of 90-100% in several generations [34]. *Mus musculus*, ICR strain female mice ordered from a Charles River Technology colony (BioLASCO, Taiwan) were maintained by the Department of Veterinary Medicine, AFRIMS, and used for this study as previously described in Lurchachaiwong et al. [34]. Each experimental group consisted of 40 mice each in 3 independent experiments, bringing the total number to 120 mice. Each experiment used 40 mice and the experiment was performed 3 times at 3 separate times. All animal procedures were approved by the AFRIMS Institutional Animal Care and Use Committee (IACUC). The refolded r56Lc-1 protein was dialyzed in 0.2X PBS and mixed with CpG 1826. Subsequently, equal volume of the protein-CpG solution and

Montanide were emulsified and 200 μL of emulsified solution (containing 25 μg of r56Lc-1 and 10 μg CpG) was administered subcutaneously at 2 different sites (100 μL/ site) on the back for each immunization. For the groups receiving only the adjuvant, the same preparation was used except r56Lc-1 was replaced by 0.2X PBS. Mice were randomly selected from the colony for each experimental design group. Each mouse was immunized 3 times at 4 week intervals before challenge via chigger feeding.

Chigger challenge

Immunized mice were challenged by chigger bite 4 weeks after the last immunization. As previously described by Lurchachaiwong et al. [34], an individual chigger were placed into the inner ear of anesthetized females. Mice were placed in special holding cages during chigger feeding to restrict their movement and prevent chigger removal via scratching or grooming. A pan of water was placed under each cage to catch any chiggers fall from mice. The ear notching method was used to permanently identify each mouse. In general, the chiggers were fully engorged by day 2-3 and dropped off from the mouse (Figure 1). Forceps were used to remove chiggers still attached at day 3. Control mice were fed on by uninfected chiggers of *L. chiangraiensis* species. Dates of survival and symptoms onsets, such as the appearance of ruffled hair and the mobility of the mice, were recorded for 30 days after the challenge. Mice with limited mobility after moderate stimulation or those unable to move and had moderately or very rough hair were euthanized [37].

Figure 1: Chigger size and feeding status. Feeding status of chigger mite are shown over time, comparing an unfed mite (0 hour.) to a fully fed mite (>48 hours). Pictures were taken at 60X magnification by Dr. Kriangkrai Lerdthusnee, Dept. of Entomology, AFRIMS.

Antibody titer determination by ELISA

The ELISA experiments were performed as previously described [36]. The concentration of r56Lc-1 protein antigen was adjusted to 0.3 μg/100 μL by 0.2X PBS based on previous experiments. The plate was coated at 4°C overnight. On the day of experiment, plates were first washed 3 times with 1X PBS containing 0.1% Tween 20 (1X PBST). The plates were then blocked with 200 μL/well of 10% milk in 1X PBST and the plates were then incubated for 1 hour at room temperature. Mouse sera were diluted in 5% milk in 1X PBST. For titration purposes, primary antibody was prepared for a serial dilution differed by a factor of 4. Plates were incubated for 1 hour at room temperature. After incubation, plates were washed 3 times with 1X PBST. HRP conjugated goat anti-mouse IgG (1:5,000) or IgM (1:2,000) (Santa Cruz) was used as the secondary antibody. The secondary antibody was diluted in 5% milk in 1X PBST and 100 μL/well of diluted secondary antibody was applied and plates were incubated for 1 hour at room temperature. At the end of incubation, plates were washed 3 times with 1X PBST. A 1:1 ratio of ABTS® Peroxidase Substrate Solution A and Peroxidase Substrate Solution B (Kirkegaard & Perry Laboratories, Gaithersburg, MD) was prepared and added at 100 μL/well. Plates were incubated for 15-30 minutes in a dark drawer. Plates were read after incubation for 30 minutes at 405 nm-650 nm on a UVmax kinetic microplate reader (Molecular Devices, Sunnyvale, CA).

Data analysis

Days for onset of symptoms, the survival dates after onset of symptoms, and overall survival rates for control group and vaccinated group were calculated as the means of 3 independent experiments comprising of 40 mice per group using GraphPad Prism version 5.03 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com. Error bars represent standard error or the mean (+SEM).

Results

r56Lc-1 vaccine formulation

In order to evaluate whether recombinant protein antigen-based vaccine could provide protection in mouse challenged by an *O.*

tsutsugamushi-infected chigger, we prepared a recombinant protein antigen r56Lc-1. The gene was amplified from the *Orientia* strain infected chigger line 1 (and was named *Orientia* strain Lc-1). Our goal was to establish a homologous challenge model to exclude the consideration of factors due to heterogeneity in the 56 kDa antigen. The r56Lc-1 protein, similar to previously expressed and purified 56 kDa proteins, was mainly present in inclusion bodies [38]. A similar purification procedure was applied and shown to be effective in generating a highly pure fraction of r56Lc-1 (Figure 2A). Once purified, the r56Lc-1 protein was evaluated for its reactivity with serum samples collected from mice infected by Lc-1 chigger or naïve chigger as shown on the western blot (Figure 2B). The purified recombinant protein was also evaluated via ELISA for detection of antibody responses elicited in mice challenged with live Lc-1 chiggers. Results confirmed that the protein was in a conformation recognized by the naturally-produced anti-56Lc-1 antibody (data not shown). Furthermore, purifying with the HPLC DEAE column effectively eliminates bacterial LPS which may lead to unrelated immune activation that can interfere with the results. We also observed earlier that recombinant 56 kDa proteins derived from Karp, Kato and Gilliam strains of *O. tsutsugamushi* precipitated in 1X PBS (Ching et al., unpublished results). Therefore, all the vaccine formulation was prepared by dialyzing the purified r56Lc-1 in 0.2 x PBS before making the emulsion with Montanide.
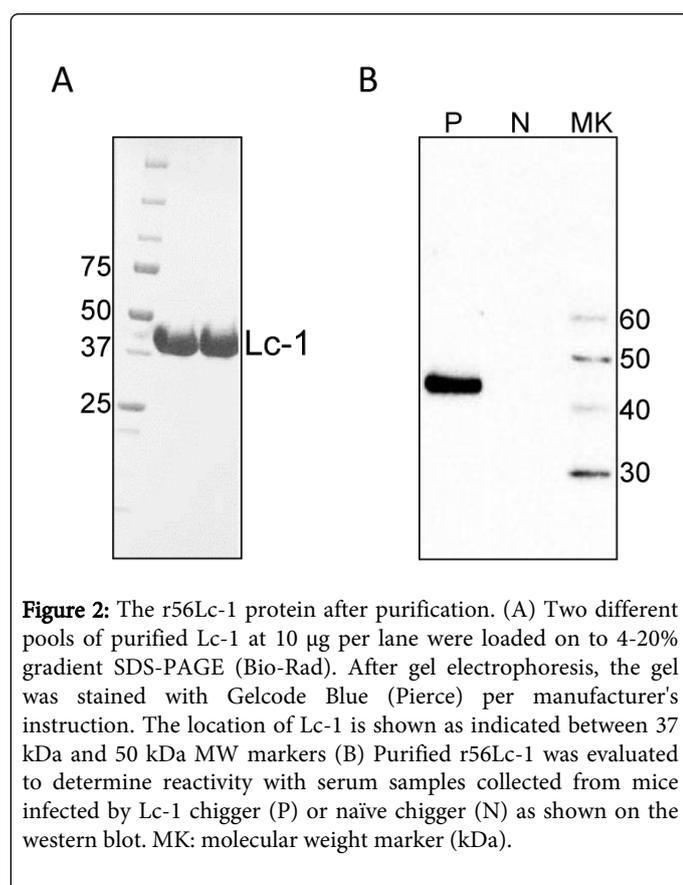

Figure 2: The r56Lc-1 protein after purification. (A) Two different pools of purified Lc-1 at 10 µg per lane were loaded on to 4-20% gradient SDS-PAGE (Bio-Rad). After gel electrophoresis, the gel was stained with Gelcode Blue (Pierce) per manufacturer's instruction. The location of Lc-1 is shown as indicated between 37 kDa and 50 kDa MW markers (B) Purified r56Lc-1 was evaluated to determine reactivity with serum samples collected from mice infected by Lc-1 chigger (P) or naïve chigger (N) as shown on the western blot. MK: molecular weight marker (kDa).

Evaluation of r56Lc-1 in chigger challenge model

ICR mice were immunized with newly prepared emulsion (Montanide ISA-51) which contained 25 µg of r56Lc-1 and 10 µg of CpG subcutaneously. The mice were immunized 3 times at 4 week

intervals. The immunized mice were then challenged 4 weeks after the last immunization by an individual chigger from Lc-1 line of mite placed in the inner ear. After the mice were fed on by infected chiggers, all mice were observed daily and a record of their mobility and the roughness of their hair were taken until the end of study (day 30 after initiating chigger challenge). Delay of death and survival rate were also recorded and analyzed. Montanide emulsified in CpG 1826 was used as the adjuvant in the concentrations described above. Table 1 shows results for 3 independent challenge experiments. In each experiment, Group A comprised of ICR mice immunized with the adjuvant alone, Group B mice were immunized with the vaccine candidate. Within each group, there were 2 sub-groups: one group was challenged with uninfected chiggers (as controls) and the other challenged with infected chiggers. The survival rate for each group at the end of 30 days is shown in Table 1. In each group, challenge by infected chiggers served as the mimic of the natural infection route. Experiments 1 and 2 presented 30% survival rates after immunization with the r56Lc-1 vaccine candidate plus Montanide, while only 20% survived after the challenge in experiment 3.

		Immunogen	Chigger Challenge	Survival rate
Expt. 1	Group A	Montanide	Uninfected chigger	10/10
		Montanide	Lc-1 chigger	0/10
	Group B	r56Lc-1+Montanide	Uninfected chigger	10/10
		r56Lc-1+Montanide	Lc-1 chigger	3/10
Expt. 2	Group A	Montanide	Uninfected chigger	10/10
		Montanide	Lc-1 chigger	0/10
	Group B	r56Lc-1+Montanide	Uninfected chigger	10/10
		r56Lc-1+Montanide	Lc-1 chigger	3/10
Expt. 3	Group A	Montanide	Uninfected chigger	10/10
		Montanide	Lc-1 chigger	0/10
	Group B	r56Lc-1+Montanide	Uninfected chigger	10/10
		r56Lc-1+Montanide	Lc-1 chigger	2/10

Table 1: Evaluation of r56Lc-1 in chigger challenge model. ICR mice were immunized with either 25µg r56Lc-1 emulsion (Montanide) in the presence of CpG or adjuvant only before being fed on by either control chiggers or Orientia infected Lc-1 chiggers. Survival rates from 3 independent experiments are shown.

Representative data for the survival proportion analysis for experiment 2 (40 mice total) is shown in Figure 3A. No death was observed in the groups of mice challenged by uninfected chiggers, with or without the r56Lc-1 vaccine candidate. None of the mice immunized with adjuvants only survived the challenge by infected chiggers. Although the rate of survival is low, mice immunized with the vaccine fared better than those immunized with the adjuvant only. We measured the IgM and IgG responses of mice after three immunizations. The IgM titer was 1:6400 and IgG titer was greater than 1: 12800. In previous studies, we monitored the antibody responses after each immunization of r56Kp, which has a 96% protein homology to r56Lc-1. The IgM titers were 1:1600 after one or two immunization and increased to 1:3200 after the third immunization.

The IgG titers were all greater than 1:12800 regardless of the number of immunizations (Lerdthusnee et al., unpublished data). Since r56Kp and r56Lc-1 exhibit high protein sequence homology, we expect that similar antibody responses would be observed after one or two immunizations with r56Lc-1 as we observed for r56Kp immunization.
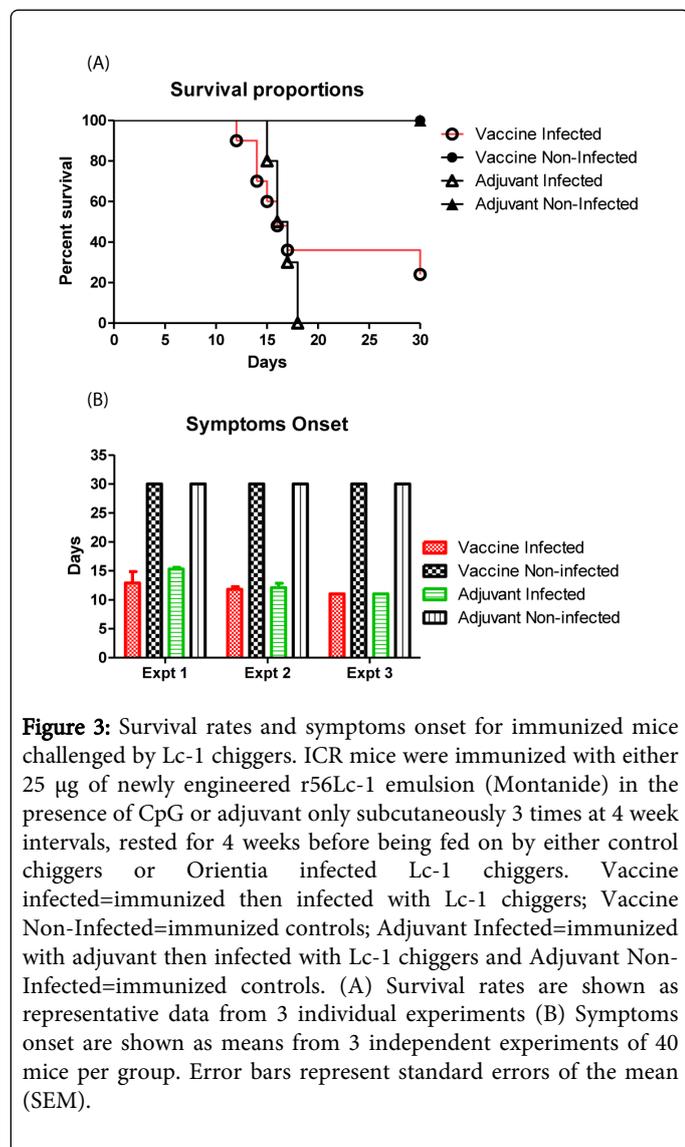

Figure 3: Survival rates and symptoms onset for immunized mice challenged by Lc-1 chiggers. ICR mice were immunized with either 25 μg of newly engineered r56Lc-1 emulsion (Montanide) in the presence of CpG or adjuvant only subcutaneously 3 times at 4 week intervals, rested for 4 weeks before being fed on by either control chiggers or *Orientia* infected Lc-1 chiggers. Vaccine infected=immunized then infected with Lc-1 chiggers; Vaccine Non-Infected=immunized controls; Adjuvant Infected=immunized with adjuvant then infected with Lc-1 chiggers and Adjuvant Non-Infected=immunized controls. (A) Survival rates are shown as representative data from 3 individual experiments (B) Symptoms onset are shown as means from 3 independent experiments of 40 mice per group. Error bars represent standard errors of the mean (SEM).

Symptoms onsets, as characterized by the roughness of their hair and degree of mobility, were recorded for mice challenged with either uninfected or infected chiggers for 30 days, after which the surviving mice were euthanized. As shown in Figure 3B, no symptoms were observed in all non-infected sub-groups, while the vaccinated mice challenged with infected chiggers began to exhibit symptoms at around day 11. All mice fed on by uninfected chiggers did not show any symptoms and no death occurred until the end of study. While those fed on by an infected chigger after immunization with the vaccine candidate, death occurred as early as day 8. Thus, the data presented here demonstrates that r56Lc-1 provides 20-30% of homologous protection consistently upon challenge by an infected chigger. To further understand the intricacies of the protection provided by the vaccine candidate, we analysed the time between the initial observation of symptoms and death in all groups of mice. Since

the non-infected groups showed no symptoms and all survived, the time between symptoms onset and death, are analysed for those mice challenged by infected chiggers. As shown in Figure 4, we can clearly see that the vaccine infected group survived 2-3 days longer than their adjuvant infected counterparts in experiments 2 and 3. The r56Lc-1 - vaccinated mice were alive for an average of 7-8 days after the chigger challenge while those vaccinated with the adjuvant were alive for an average of 4-6 days. This r56Lc-1 vaccine candidate was able to keep the mice alive longer than the adjuvant groups, a positive result that signifies that further studies are warranted to decipher the efficacy of r56Lc-1 as an effective vaccine candidate.
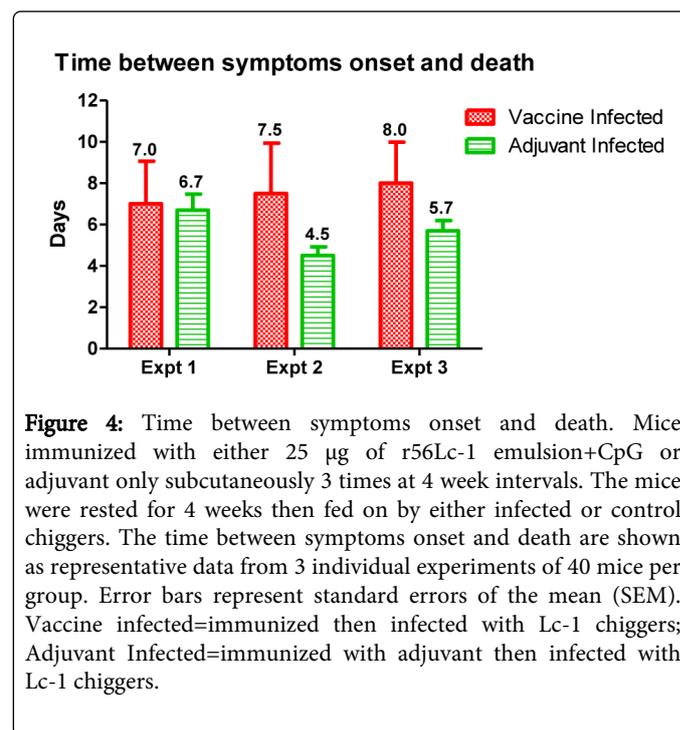

Figure 4: Time between symptoms onset and death. Mice immunized with either 25 μg of r56Lc-1 emulsion+CpG or adjuvant only subcutaneously 3 times at 4 week intervals. The mice were rested for 4 weeks then fed on by either infected or control chiggers. The time between symptoms onset and death are shown as representative data from 3 individual experiments of 40 mice per group. Error bars represent standard errors of the mean (SEM). Vaccine infected=immunized then infected with Lc-1 chiggers; Adjuvant Infected=immunized with adjuvant then infected with Lc-1 chiggers.

Discussion

We have produced and evaluated a promising vaccine candidate in a chigger challenge mouse model. This candidate, r56Lc-1 is composed of the variable 56 kDa outer membrane protein, the most immunodominant antigen of *O. tsutsugamushi*, which accounts for 10-15% of the total cell protein. Almost every clinically diagnosed patient serum reacts with 56 kDa antigen [25,26,28,29]. *O. tsutsugamushi* exhibits considerable strain variation [13-16]. Homologous protection developed from natural infection persists for at least one year, but heterologous protection may remain for less than six months [21,39]. The 56 kDa protein contains five conserved domains as well as four variable domains and is responsible for the antigenic divergence of *Orientia*. The 56 kDa protein has been shown to be protective in mice against homologous challenge [25,26,28,29]. Both humoral and cell-mediated immune responses are important in protective immunity against scrub typhus [40-44]. Strong humoral and cellular immune responses were also induced in cynomolgus monkeys by r56Kp, with antigen-specific IgM and IgG produced to almost maximum levels within one week of a single immunization [31]. In addition, peripheral blood mononuclear cells from vaccinated animals showed an induction of antigen-specific proliferation and gamma interferon production [31]. All these observations have

strongly suggested that r56 has the potential to be an effective vaccine candidate.

To investigate the protective capabilities of r56Lc-1 against *Orientia* infection in a chigger challenge model, we produced the vaccine and combined it with CpG and Montanide as the adjuvants. Montanide has been shown to elicit required cellular and humoral immune responses when used alone or in emulsions with other components [45-47]. It is also safe and well-tolerated in humans [47,48]. CpG has been shown to induce strong Th1 responses and to enhance vaccine-induced humoral immune responses [49]. CpG is also safe when used as an adjuvant in human vaccines [50,51]. ICR mice, like CD-1s, are outbred mice [52] and our choice of the ICR mouse strain was based on the premise that as outbred mice, the efficacy of this vaccine will provide valuable data that may have more similarities with what is expected in humans. Also, the lethal nature of the *O. tsutsugamushi* Lc-1 strain in ICR mice meant that we could better characterize the responses observed. Some of the effects of ICR infection after chigger feeding include reduction in food intake, lowered body temperatures, reduced mobility and loss of weight [34]. We observed reduced mobility and roughness in the fur in mice fed on by infected chiggers, irrespective of whether they were immunized by the adjuvant only or by the emulsified vaccine candidate.

Previously, we observed that r56 proteins were able to provide very good homologous protection but relatively poor heterologous protection in needle challenge mouse model. We anticipated a better survival rate by using the homologous immunogen in this chigger challenge model. However, our results indicated that although 3/10 (30%) of mice immunized with the vaccine survived the chigger challenge in experiments 1 and 2, only 20% survived in the third experiment. This observation is in contrast to our previous experiments, where mice were immunized 3 times with r56Kp in a similar regimen as described in this study and 70-100% of the mice survived an intraperitoneal needle challenge (Ching et al. unpublished data). There are many different factors, such as the site of inoculation, the duration of infection upon chigger feeding, the components in chigger saliva, and interactions between the chigger vector and the host could have contributed to the discrepancies we observed. Also, during a natural chigger infection, there is the possibility of various strains of *Orientia* inhabiting the feeding mite, passing on various strains to the host. However, this is not the case with Lc-1, as only 1 sequence (94.2% similar to Karp) was found in the analysed chiggers [35]. Even though the intraperitoneal route has been used extensively and has facilitated the elucidation of the mechanism of action of diseases [15,28,29,53], alongside in the testing of vaccine candidates [20,54,55], it only takes a couple of seconds to introduce the infectious organisms into mice. The chigger bite on the other hand, goes far beyond the initial break in the skin. The various components of the chigger saliva may play a role in the transmission of *Orientia* to the host. As an example of this concept, the sand fly (*Leishmania infantum*), which regurgitates infectious parasites and saliva into host skin during the blood meal, possesses saliva components that have been found to impair the classical pathway of the complement system in dogs, guinea pigs and rats [56]. It is therefore reasonable to hypothesize that the chigger may have similar effects on the host as it feeds on the epithelium. Another example is the tick saliva protein complex, 1xscS-1E1. After injected into the host during the blood meal, it inhibits platelet aggregation and clotting by interfering with thrombin [57]. This allows the tick to feed longer on the host. Another key point is the composition of the chigger saliva may depend on the stage of feeding as can be speculated from the huge size difference of

the chigger at difference stage. Such effects were demonstrated during a study in cattle ticks. A difference in the amount of transporter proteins was observed between the saliva of fully or partially engorged cattle ticks [58]. Partially engorged ticks produced more of these transport proteins thought to transport active pharmacological agents from the tick to the host [58]. Although chiggers take the body fluid, from its host, this may also be the case for chigger mites. Another key point may be the composition of the chigger saliva depending on the stage of feeding. Such effects were demonstrated during a study in cattle ticks. A difference in the amount of transporter proteins was observed between the saliva of fully or partially engorged cattle ticks [58]. Partially engorged ticks produced more of these transport proteins thought to transport active pharmacological agents from the tick to the host [58]. This may also be the case for chigger mites considering the changes in the size of chigger from unfed to fully fed (Figure 1). The salivary components and the long-time feeding, which fosters interactions between vector-pathogen-host, may explain why the protective efficacy in chigger challenge model is not as good as those in needle challenge model.

In conclusion, our data demonstrated that r56Lc-1 provided 20-30% of protection consistently upon homologous challenge by an infected chigger. Since *Orientia* is an intracellular pathogen, the clearance of this pathogen may heavily depend on T-cell immune responses rather than B-cell immune responses. Vaccine candidate and adjuvants in formats which can promote mainly T-cell immunity may be able to improve the protective efficacy of the vaccine.

Acknowledgements

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