博士論文

Evaluation of *Francisella tularensis* Δ*pdpC* mutant as a candidate for live attenuated vaccine against tularemia using mouse and monkey models

（マウスおよびサルモデルを用いた野兎病菌 ***Francisella tularensis*** **Δ*pdpC*** 変異株の弱毒生ワクチンとしての有用性の評価）

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General introduction

Francisella tularensis is a gram-negative intracellular coccobacillus and the causative agent of the debilitating febrile illness, tularemia. Tularemia is zoonotic disease, and now sporadic and generally endemic. *F. tularensis* can cause severe disease with a low infectious dose and infect humans via multiple routes. This pathogenicity is taken alarm as potentials to be used as a biological weapon released intentionally into the environment to cause tularemia. On the other hand, recent studies on *F. tularensis* and closely related *Francisella* species have greatly increased our understanding about mechanisms by which these organisms infect and cause the disease to the host.

Depend on how easy the pathogen can be spread and the severity of illness, the pathogens for bioweapons are classified into three categories, from the highest risk category "A" to "C" in the United States of America (USA) (1). *F. tularensis* has been classified as a category A biological weapon (2-4). When *F. tularensis* is used as an airborne bioweapon, it causes acute, undifferentiated febrile illness with incipient pneumonia, pleurisy, and hilar lymphadenopathy with 3 to 5 days of incubation period (3).

The history of tularemia

Tularemia, also known as Francis' disease, Ohara's disease, rabbit fever or deer-fly fever, is a potentially fatal, multi-organ disease of humans and some animals and the causative bacterial pathogen, *F. tularensis* is also formerly called *Pasteurella tularensis*. The disease was named "tularemia" because the disease was first reported in 1911 as a disease of ground squirrels in Tulare County, California (5).

Tularemia in Japan was first described in 1837 by a surgeon as a poisonous disease, not an infectious disease (6). No one paid attention to this disease in Japan until 1924, when Dr. Hachiro Ohara

performed an extensive research on this disease (6). Therefore, tularemia used to be called as Ohara's disease in Japan.

The first description of tularemia in the paper was published in 1911 and named as "a plague-like disease of rodents" by George McCoy, a pathologist working in California who found this disease from ground squirrels (5). And in the following several years, McCoy and his team went on with this plague-like disease to elucidate the causative agent, and isolated the bacterium called *Bacterium tularense* (7). Human cases were first reported by Wherry and Lamp in 1914 (8).

In 1919, Edward Francis also reported the tularemia under the title of "Deer-fly fever, or Pahvant Valley plague, A disease of man of hitherto unknown etiology" (9). In this paper, he reproduced the same lesions as in human in guinea pigs and rabbits. He also cultured the causative bacterium on coagulated egg yolk, and proved that this bacterium was the same bacterium as McCoy reported in 1911 (5). In 1927, Francis reviewed the tularemia at that time, and reported the four types of pathophysiology, ulceroglandular type, oculoglandular type, glandular type and typhoid type (10). By his contribution to the disease, this pathogen was given its present name, *Francisella tularensis.*

Epidemiology of tularemia

In the beginning of the description of tularemia, because of a lack of the infectious disease knowledge, a person who ate hare meat and infected with this disease was considered to be suffered from poisoning (6). In 1911, from Dr. McCoy's paper "A plague-like disease of rodents", the research of tularemia started (5), and the etiological agent, *Bacterium tularense* was isolated and duplicated on other

laboratory animals (11).

Tularemia can be reproduced in squirrels, monkeys, guinea pigs, and rabbits by subcutaneous, nasal, and intraperitoneal inoculation (5). Tularemia can be transmitted to human by tick and fly bites, and exposure to contaminated water, food, and aerosols (12). The outbreaks are often related to water exposure. Some groups of people including hunters, hikers, foresters, persons in contact with meat and animals, farmers and their family, and laboratory workers have a high risk to tularemia infection (6, 13, 14). Until now, no person-to-person transmission has been reported. *F. tularensis* infection occurs in the northern hemisphere including North America, Europe, and Asia. Various species and subspecies of genus *Francisella* cause symptoms in humans and variety of animals including domestic animals, wild small mammals, and fish (12). Tularemia has recently been paid much attention as an important agent for a biological weapon, especially after anthrax attack in the United States in 2001 (15).

Francisella tularensis

Two species, *F. tularensis* and *F. philomiragia*, have been recognized in the genus *Francisella* based on the sequence of 16S ribosomal DNA (16), though seven new species are recently reported (List of prokaryotic names (LSPN) bacterio. net). There are four subspecies in *F. tularensis*, i.e. *tularensis* (Type A), *holarctica* (Type B), *novicida*, and *mediasiatica* (17). The pathogen of the first tularemia case found in Japan is considered to be a variant of *F. tularensis* subspecies *holarctica* (17). *F. tularensis* subspecies *tularensis* (Type A) is the most virulent subspecies, and distributes mainly in North America. Subspecies *holarctica*, also called Type B, has high virulence and distributes in the northern hemisphere.

Subspecies *mediasiatica* distributes in central Asia also has high virulence, while subspecies *novicida* shows low virulence and is rare (18).

Potential as a biological weapon

Contagious diseases and other biological weapons have been long recognized for their potential impact on both military and civilian (19). Especially after the anthrax attack in the United States of America (USA) in 2001, the USA has been investing in prevention, surveillance, and preparation for potential bioterrorism attacks (1). The ability of *F. tularensis* to cause severe disease with a low dose has long been considered as a potential biological weapon (20). During the Second World War, the former Soviet Union and other countries including Japan (21) developed and used bioweapons containing *F. tularensis,* and people were suffered from tularemia. After the War, tularemia became less frequent and lost attention until 2001, when the biosafety concerning tularemia drew attention again. The caution to this pathogen as a biological weapon was raised due to its high infectivity, easiness of dissemination, and the consequence of infection, although the morbidity is low. Among various ways to use *F. tularensis* as a bioweapon, aerosol release has been thought to cause the most serious consequences (3). Aerosol release causes a nonspecific febrile illness 3 to 5 days after exposure, and subsequently develops pulmonary symptoms, pneumonic tularemia (3). In order to prevent tularemia, a safe and highly efficacious vaccine is needed.

Vaccines against tularemia

In order to protect the host from tularemia, vaccine is thought to be an efficient method. Great efforts have been paid to develop successful vaccines against tularemia (22). The first vaccine of tularemia was a killed vaccine produced by Lee Foshay in 1930 (23). This vaccine provided protection against intracutaneous challenge of a virulent strain, SCHU S4 strain, of *F. tularensis* subspecies *tularensis* in human volunteers, but not against respiratory challenge (20, 24).

During the Second World War, live attenuated vaccines were developed and found to be protective (25). In 1956, ampoules of mixture of viable attenuated tularemia vaccine were transferred to the United States from the Institute of Epidemiology and Microbiology Imeni N. F. Gamaleia (Gamaleia Institute), the Soviet Union by Shope (25). From this mixture, a strain with low virulence was selected, tested for safety and designated as *F. tularensis* live vaccine strain, LVS (26). This strain has been the most widely used until now. In recent years, numerous mutant strains derived from *F. tularensis* LVS (such as Δ*sodB* (27) and Δ*dsbA* (28)), *F. tularensis* subsp. *novicida* (such as Δ*iglD* (29) and Δ*iglB* (30)), and *F. tularensis* SCHU S4 (such as Δ*clpB* (31) and Δ*ggt* (32)) have been demonstrated to have attenuated pathogenicity and been shown to have protective capacity against challenge with a virulent strain SCHU S4 in mice or rats. However until now, there is no licensed vaccine against tularemia for human use (33), thus development of a safe and efficacious tularemia vaccine is still needed.

Strain used in this study

The virulent strain of *F. tularensis*, SCHU, which was originally isolated by Foshay in 1941 from a human finger ulcer (34), was supplied to Ohara Research Laboratory, Ohara General Hospital,

Fukushima, Japan from Rocky Mountain Laboratory, National Institute of Allergy and Infectious Diseases, USA in 1958 (35). Then virulence of SCHU strain had been attenuated by extensive passages on pig liver glucose hemoglobin (LGH) agar medium for 30 years (35, 36). The attenuated SCHU strain was kindly provided by Dr. H. Fujita (Ohara Research Laboratory) to National Institute of Infectious Diseases (NIID) in 2002.

After NIID received the strain stored in sucrose phosphate glutamate (SPG) liquid medium, the bacterium was transferred to Eugon chocolate agar and incubated at 37°C for 3 days. The strain was then stored in skim milk glucose medium (3g skim milk and 5g glucose in 100 ml distilled water) at -80°C.

In 2010, Dr. Uda of NIID constructed a virulent strain from the attenuated SCHU strain by passages in mice for 9 generations and named SCHU P9. Based on the virulent SCHU P9 strain, the pathogenicity determinant protein C gene (*pdpC*) was disrupted by group II intron insertion using the TargeTron gene knockout system (Sigma-Aldrich), and Δ*pdpC* mutant was generated (37).

Objective of this study

A series of experiments using the virulent SCHU P9 strain and its mutant Δ*pdpC* constructed by Dr. Uda (37) were performed in mice and monkeys. A high pathogenicity of SCHU P9 strain and low pathogenicity of Δ*pdpC* mutant were confirmed in both mice and monkeys in this study.

Firstly, the stability of inserted intron sequence to disrupt *pdpC* gene and attenuated pathogenicity of the Δ*pdpC* mutant were confirmed after serial passages in mice.

Secondly, the median lethal dose (LD_{50}) of SCHU P9 strain in C57BL/6J mice were

determined via three different infection routes, intramuscular (i.m.), intranasal (i.n.), and subcutaneous (s.c.).

Then the protective effects of Δ*pdpC* mutant strain against a lethal challenge of SCHU P9 were analyzed using C57BL/6J mice (Chapter 1).

Finally, a potential of Δ*pdpC* mutant to protect the host was tested using nonhuman primate. In this experiment, six cynomolgus macaques were divided into 3 groups, two animals per group, and challenged with 1×10^6 CFU of either virulent SCHU P9 strain or Δ*pdpC* mutant with or without previous immunization by 1×10^6 CFU of Δ*pdpC*. The animals were observed for clinical symptoms such as fever, cough, and bad appetite and survival, for three weeks after challenge. Moribund and survived monkeys were euthanized and analyzed bacteriologically and histopathologically. Induction of antibodies against *F. tularensis* were also analyzed (Chapter 2).

Based on these data, both in mouse and monkey models, the Δ*pdpC* mutant was evaluated as a candidate of tularemia live attenuated vaccine.

Chapter 1

Evaluation of *Francisella tularensis* Δ*pdpC* mutant as a candidate for live attenuated vaccine against tularemia in C57BL/6J mouse model

1.1 Abstract

In this chapter, safety and stability of Δ*pdpC* mutant derived from a virulent strain SCHU P9 was tested first. The Δ*pdpC* strain was passaged serially in mice by intraperitoneal (i.p.) inoculation to confirm its stable attenuation. The ability to translocate to mouse spleen stayed low through the 1st to 10th passage. The bacterial loads in the spleen did not change significantly during *in vivo* passages of Δ*pdpC* in mice. The inserted intron sequence disrupting *pdpC* gene was completely maintained even after the 10 times passages in mice. Considering the stable attenuation and maintenance of the intron sequences in the *pdpC* gene, it was demonstrated that Δ*pdpC* showed good stability and safety in mouse model.

Then, the virulence of SCHU P9 via i.m., s.c., and i.n. inoculation was tested in C57BL/6J mice, and LD_{50} was calculated to be 3.2, 1.5 and 158 colony-forming units (CFU) upon i.m., s.c., and i.n. inoculation, respectively, using the Reed-Muench method. Because the virulence of SCHU P9 was comparable to that of SCHU S4, SCHU P9 strain was used as a virulent strain in the present study.

Finally, a potential of Δ*pdpC* as a vaccine candidate for tularemia was investigated. C57BL/6J mice immunized with 1×10^6 CFU of Δ*pdpC* via i.m., s.c., or i.n. were challenged with 100 LD_{50} of virulent SCHU P9 strain from the same route at 21 days after immunization. Mice were completely protected against challenge with 100 LD_{50} of the SCHU P9 strain when the immunization and challenge were given intramuscularly. On the other hand, 60% and 40% of the mice survived when they were immunized subcutaneously and intranasally, respectively. Thirty eight % of the mice survived after an intranasal challenge with 100 LD_{50} dose of SCHU P9 when they were immunized subcutaneously, though the survival rates were significantly higher in all these immunized groups compared with those in

unimmunized mice. These results suggest that the Δ*pdpC* mutant is a good candidate for live attenuated vaccine for tularemia.

1.2 Introduction

As has been described in “General introduction”, *Francisella tularensis* is a facultative intracellular bacterial pathogen capable of causing tularemia. The natural hosts of *F. tularensis* are rodents and lagomorphs, but the bacterium infects many other mammalian species including humans through ingestion of contaminated food or water, direct contact with infected animals, tick bites, or inhalation of aerosolized bacteria (12, 38). Although natural infection of this pathogen is less frequent these days, *F. tularensis* draw attention again because the virulent strains of the pathogen can be used as a biological weapon (3, 22, 39, 40).

Various approaches have been employed to develop vaccines for tularemia (41). A live attenuated vaccine derived from multiple passages of a virulent strain of *F. tularensis* subspecies *holarctica* LVS was once used as an investigational new drug in the USA (26). However, this vaccine did not offer a high level of protection against respiratory challenge with *F. tularensis*, and it has not been licensed (33, 42). In addition, cultivation of the LVS strain under certain conditions resulted in emergence of poorly immunogenic variants (43). So, various other variant strains with low pathogenicity have been developed as vaccine candidates against tularemia in recent years (44-46).

In tularemia research, the most applied virulent strain of *F. tularensis* subspecies *tularensis* is SCHU S4 strain (47). This highly virulent strain is a variant of virulent SCHU strain, which was originally isolated by Foshay in 1941 from a human finger ulcer (34). LD_{50} of SCHU strain for mice, guinea pigs, and rabbits were shown to be less than 10 organisms upon subcutaneous inoculation (48). The SCHU strain was supplied to Ohara Research Laboratory of Japan from Rocky Mountain Laboratory,

National Institute of Allergy and Infectious Diseases, the USA in 1958 (35). When SCHU strain was extensively passaged by culture on LGH agar in Ohara Research Laboratory, its virulence was attenuated (35). The SCHU strain kindly provided by Dr. H. Fujita to NIID was this attenuated strain. Uda *et al*. retrieved the virulence of the strain after serial 9 passages in mice and this virulent strain was referred to SCHU P9 (37). LD_{50} of SCHU P9 was comparable to SCHU S4. In the study, the authors compared the whole genome sequence of SCHU P9 with that of SCHU S4, both originated from the same SCHU strain, and found 35 differences including 29 single nucleotide mutations and 6 deletions/insertions (37). In Japan, it is difficult to obtain SCHU S4 strain from foreign laboratories because the transport of the bacterium is highly regulated by the Japanese infectious disease control law. In this regard, I used the SCHU P9 strain instead of SCHU S4 strain as a virulent strain in the present study.

Proteins encoded by genes on the *Francisella* pathogenicity island (FPI) have been demonstrated to play critical roles in the unique intracellular lifestyle of this bacterium (49-51). Pathogenicity determinant protein C gene (*pdpC*) is one of the genes within the FPI. Δ*pdpC* strain, in which *pdpC* was disrupted by insertion of intron sequence by TargeTron mutagenesis, exhibited a unique phenotype similar to the previous reports (52-54). In the previous study, Uda *et al*. reported that PdpC was crucial in determining the virulence of *F. tularensis* SCHU P9 because Δ*pdpC* displays significantly decreased intracellular growth in murine macrophage derived J774.1 cells and it did not induce severe disease in experimentally infected mice (37). Further, the virulence of Δ*pdpC* has been restored by complementation with a plasmid containing intact *pdpC* gene (37).

In this chapter, I investigated the stability of the attenuation of Δ*pdpC* after serial passages in

mice. Then I tested the virulence of SCHU P9 and Δ*pdpC* strain via i.m., s.c., and i.n. inoculation. Finally, the Δ*pdpC* strain was investigated whether it could be a candidate for live attenuated tularemia vaccine in a mouse model.

1.3 Materials and methods

1.3.1 Ethical statement

Experiments involving animals were performed in strict accordance with the Animal Experimentation Guidelines of the NIID. The protocol was approved by the Institutional Animal Care and Use Committee of NIID (Permission Numbers: 115024, 116005, 116046, and 215073).

1.3.2 Bacterial strains

F. tularensis subspecies *tularensis* strains SCHU P9 and Δ*pdpC* were established in the previous study (37). They were cultured in Chamberlain's defined medium (CDM) (37) at 37°C for 24 h, suspended in CDM containing 10% glycerol, and stored at −80°C until use. All experiments with live bacterial cultures were performed in a biosafety level 3 facility in accordance with regulations stipulated by the NIID.

1.3.3 Animal experiments

Seven-week-old female C57BL/6J mice purchased from Japan SLC, Inc. (Shizuoka, Japan) were kept in a biosafety level 3 animal room with suitable temperature and humidity and a 12-h/12-h light-dark cycle. Different groups of mice were housed in individual cages and provided *ad libitum* access to food and water. Mice were inoculated with bacteria via an intraperitoneal (i.p.) route under anesthesia with 3 μg of medetomidine hydrochloride (Domitor, Orion Diagnostica, Espoo, Finland), 40 μg of midazolam (Dormicum; Astellas Pharma, Tokyo, Japan), and 50 μg of butorphanol tartrate (Betorufaru; Meiji Seika Pharma Company, Tokyo, Japan). Euthanization of mice was conducted by inhalation of an

overdose of isoflurane (Pfizer Japan Inc., Tokyo, Japan).

Bacterial loads were quantified by inoculating 100 µl of diluted homogenates of lungs, spleens, and livers of infected mice prepared in RPMI 1640 as diluent onto Eugon chocolate agar plates, as previously reported (37, 55). After cultivation at 37°C for 4 days, colony-forming units (CFU) were calculated.

1.3.4 Stability of Δ*pdpC* strain through *in vivo* passages

Δ*pdpC* strain was serially passaged in mice to confirm its pathological and genetic stability (Fig 1.1). Three mice were inoculated via an i.p. route with 1×10^6 CFU of Δ*pdpC*. Three days after inoculation, spleens were collected from the euthanized mice. Serial dilutions of the splenic homogenates were inoculated onto chocolate Eugon agar plates and incubated at 37°C for 3 to 4 days. CFU were calculated from average number of colonies on the plate.

For the passage, one ml aliquot of the 10% splenic homogenates was spread onto Eugon agar plates and incubated at 37°C for 2 days. Bacterial cells were harvested and suspended in CDM. A bacterial suspension containing about 10^6 CFU was inoculated intraperitoneally into 3 C57BL/6J mice. Spleens were collected from the mice and passages of the bacteria were similarly performed until the 10th generation.

Genomic DNA was extracted from the bacterial suspension of each passage level using SepaGene (Sanko Junyaku, Tokyo, Japan) according to the manufacturer's instructions. DNA from Δ*pdpC* and SCHU P9 were used as controls for PCR. PCR was performed in a 50 µl reaction mixture

containing 1 U of KOD-Plus-Neo enzyme (Toyobo, Osaka, Japan), 1× PCR buffer, 0.02 mM dNTPs, 1.5 mM $MgSO_4$, 0.5 μM of the primers (pdpC-336s, 5'-ACT ATG GGG ATT AGT TGA TTT CGT-3'; and pdpC-725a, 5'-TGT TGT AAT CTT GGA GCT GCT T-3'), and 10 ng of bacterial genomic DNA in each reaction. The reaction conditions were as follows: initial denaturation at 94°C for 2 min; 30 cycles of denaturation at 94°C for 0.5 min, annealing at 58°C for 0.5 min, and extension at 68°C for 0.5 min; and a final extension at 72°C for 7 min. PCR products were confirmed by electrophoresis on a 1% agarose gel. Amplicons were treated with Illustra ExoProStar (GE Healthcare, Buckinghamshire, UK) to remove unincorporated primers and nucleotides according to the manufacturer's instructions. The nucleotide sequences of the amplicons were then determined by Eurofins Genomics Company (Tokyo, Japan).

1.3.5 Determination of the median lethal dose (LD_{50}) of the virulent strain, SCHU P9

Five C57BL/6J mice (7 week old females, SLC, INC. Shizuoka, Japan) in each group were inoculated via i.m., s.c., or i.n. route under anesthesia with 100μl (i.m. and s.c.) or 10μl (i.n.) of serially diluted SCHU P9 suspensions containing 10^{-1} to 10^{3} CFU in the inoculation volume. After inoculation of SCHU P9, health condition and body weight of the mice were observed daily. When mice showed severe clinical signs and/or >25% weight loss from the original body weight, they were humanely sacrificed by isoflurane inhalation and considered to be killed by SCHU P9 infection. Infection of SCHU P9 was confirmed by detection of the bacterium in the lungs, spleens, and livers of the mice. Reed-Muench method was used to calculate the LD_{50}.

1.3.6 Bacterial challenge from the route same as immunization

1.3.6.1 Immunization and challenge routes and doses

Ten mice in each group were immunized via an i.n., i.m., or s.c. injection with 1×10^6 CFU/10 µl, 1×10^6 CFU/100 µl, or 1×10^6 CFU/100 µl of Δ*pdpC*, respectively, whereas four or five mice of unimmunized group were administered the same amount of saline using the same routes. All immunized mice were observed daily for clinical signs and their body weights for 21 days after immunization. Five mice in each immunized group were sacrificed by isoflurane inhalation to detect bacterial burden in their organs after 21 days from immunization. Unimmunized mice and the remaining five immunized mice in each group were challenged with 100 LD_{50} of SCHU P9 from the same routes as for immunization at 21 days after immunization. The health conditions of the animals were observed daily and moribund animals were euthanized. Surviving mice were sacrificed at 21 days after SCHU P9 challenge by isoflurane inhalation.

1.3.6.2 IgG antibody titers determined by enzyme-linked immunosorbent assay (ELISA)

Blood samples were collected from five mice in each group from the tail vein before and 21 days after immunization. Sera were obtained by centrifugation at 3,500 × *g* at 4°C for 10 min, incubated at 56°C for 30 min, and then inactivated with UV irradiation at 254 nm for 10 min.

Antibody titers were measured by ELISA as previously reported (56). Briefly, SCHU P9 cultured on Eugon chocolate agar for 3 days was harvested, suspended in saline, adjusted to the

concentration corresponding to an optical density at 600 nm (OD_{600}) = 1.0, and inactivated at 100°C for 10 min. The antigen was stored at −80°C until use. Heat-killed SCHU P9 antigen was diluted 5 times with 50 mM carbonate-bicarbonate buffer, pH 9.0. Then 100 μl/well mixture was added onto the wells of a Nunc-Immuno plate (Thermo Scientific, Roskilde, Denmark), and the plates were left at 37°C overnight. After washing thrice with PBS containing 0.1% Tween 20 (PBST) to remove unbound antigen, all wells of the plates were blocked with 100 μl of PBST containing 3% skim milk at 37°C for 1 h. After washing thrice with PBST, fifty μl of the serum samples diluted to 1:2,000 in PBST containing 1% skim milk were added into antigen-coated wells in duplicate and incubated at 37°C for 1.5 h. After washing thrice with PBST, fifty μl of Peroxidase-Goat Anti-Mouse IgG (H+L) (Invitrogen, MD, USA) diluted to 1:10,000 was added into each well, and then the plates were incubated at 37°C for 1 h. The wells were washed thrice with PBST and developed with 100 μl of 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) ELISA HRP substrate (Roche, Germany) at 37°C for 30 min. Optical density at 405nm (OD_{405}) was measured in the each well using a BioRad 96-well plate reader (iMark, Bio-Rad Laboratories, CA, USA).

1.3.7 Bacterial challenge from the route different from immunization

1.3.7.1 Immunization and challenge routes and doses

Mice were then immunized from s.c. route, and challenged from i.n. route. The dose of immunization was 10^6 CFU/mouse, and the challenge dose was 100 LD_{50}. The mice were challenged 21 days after immunization, and body weight and health conditions were observed for 3 weeks after

challenge. When the mouse showed serious clinical symptoms and/or body weight loss reached to 25% of the original body weight, the mouse was euthanized.

1.3.7.2 IgG antibody titers determined by ELISA

Whole blood samples were collected from heart at 21 days after immunization. Blood samples were also collected from mice survived for 21 days after SCHU P9 challenge. Sera were obtained by centrifugation at 3,500 × g at 4°C for 10 min, incubated at 56°C for 30 min, and then inactivated via UV irradiation at 254 nm for 10 min. IgG antibody titers were measured by ELISA as described above, except that the serum samples were diluted to 1:100 in PBST containing 1% skim milk.

1.3.7.3 Cellular immunity

IFNγ concentration in sera from unimmunized and immunized mice at 0 day and 3 days post challenge (dpc) were measured using FlowCytomix mouse Th1/Th2 10plex bead kit (eBioscience, Vienna, Austria) in accordance with the instruction manual. Data were acquired by FACS Canto II (Becton–Dickinson, Franklin Lakes, NJ, USA) and analyzed by FlowCytomix Pro 2.2 Software (Bender MedSystems GmbH, Vienna, Austria).

Various tissues collected from the immunized and unimmunized mice at 3 dpc of SCHU P9 were immunohistochemically stained with the polyclonal antibodies against the serine protease granzyme B, which was known to be expressed specifically in cytotoxic T lymphocytes (CTL) and natural killer (NK) cells.

1.3.8 Statistical analysis

All statistical analyses were performed using GraphPad Prism v5 (GraphPad Software, CA, USA). Results were expressed as the mean ± standard deviation (SD). Between-group comparisons regarding IgG antibody titers were performed using Two-way analysis of variance with Tukey-Kramer's multiple comparisons post-test. Survival rates were analyzed using the log-rank test, and P-values were determined. Differences between the experimental groups were considered statistically significant at $P < 0.05$. CFU in the spleens of mice infected with Δ*pdpC* at 3 days post-inoculation (dpi) were statistically analyzed using linear regression analysis. IFNγ concentrations in sera were statistically analyzed by One-way ANOVA with Tukey's post-test (**$P<0.01$).

1.4 Results

1.4.1 Stability of Δ*pdpC* after serial passages in mice

The average bacterial burden in the spleen was constantly less than 1×10^6 CFU/10 mg during 10 passages of Δ*pdpC* in mice. Significant changes of bacterial loads in the spleens were not found through the 1st to 10th *in vivo* passages of Δ*pdpC* via linear regression analysis using Prism software (Fig 1.2).

The results of PCR amplification using the pdpC-336s/pdpC-725a primer pair are shown in Fig 1.3. The expected sizes of amplicons with and without intron sequences in *pdpC* were 1305 and 309 bp, respectively, and it is confirmed that Δ*pdpC* constantly harbored the intron during 10 passages (Fig 1.3). In addition, no nucleotide substitutions or deletions/insertions in the intron sequences were detected after the 10th passage (data not shown).

1.4.2 Determination of the LD_{50} of a virulent SCHU P9 strain in mice

All mice inoculated with 1×10^{-1} CFU survived, whereas mice inoculated with $> 1 \times 10^3$ CFU showed severe symptoms such as depression and bad appetite which humanely euthanized within 5 days after inoculation (Fig 1.4). Based on the survival rate for each inoculation dose, the LD_{50}s for SCHU P9 were calculated to be 3.2, 1.5 and 158 CFU after i.m., s.c. and i.n. inoculation, respectively. *F. tularensis* was detected in the liver, spleen and lung of dead or euthanized mice at about 10^8 CFU/g, while the bacterium was not found in these three organs of survived mice (Fig 1.5).

1.4.3 Immunization of mice from the route same as challenge

1.4.3.1 IgG antibody titers determined by ELISA

The IgG antibody responses against *F. tularensis* in the mice at 21 days after immunization were determined by IgG-ELISA. Compared with the naïve mice, the OD_{405} values in the IgG-ELISA were obviously higher in the immunized mice, regardless the mice immunized via i.m., s.c., or i.n. routes (Fig 1.6). However because the cut-off value was set at the average OD_{405} values of 15 naïve mice plus 3 × standard deviation (SD) (0.33), one of five mice in the s.c. immunization group was considered negative, though the antibody titer of this particular mouse was higher than those of all naïve mice (Fig 1.6).

1.4.3.2 Survival rate and body weight

No mice showed obvious symptoms during 21 days after immunization. Although body weight decreased after immunization, the body weight recovered to a normal range in 4 to 5 days. Live Δ*pdpC* strain was not recovered from the blood, lungs, spleens, and livers of mice at 21 dpi (data not shown).

All unimmunized mice were dead or sacrificed with severe symptoms within 6 days after challenge with SCHU P9 regardless of the challenge route (Fig 1.7). On the other hand, the mice immunized from different routes displayed different survival rates after the challenge with SCHU P9. In i.m. group, immunization with Δ*pdpC* elicited the highest level of protection, and all immunized mice

survived for 21 days (Fig 1.7A). Although the survival rates of mice in s.c. and i.n. groups were 60% (Fig 1.7B) and 40% (Fig 1.7C), respectively, the survival rate in each immunized group was significantly higher than those in unimmunized mice. The body weight decreased dramatically in the dead mice (Fig 1.8).

1.4.4 Protection of mice by Δ*pdpC* immunization from lethal tularemia via the challenge route different from immunization

1.4.4.1 Survival rate and body weight

All unimmunized mice challenged with SCHU P9 strain died or suffered from serious clinical symptoms and/or >25% weight loss and were humanely sacrificed within 6 days after challenge with SCHU P9 (Fig. 1.9). On the other hand, survival rate of mice in immunized group was 38% (Fig. 1.9) and the rate was significantly higher than that in unimmunized mice.

1.4.4.2 IgG antibody titers determined by ELISA

As the average OD_{405} value of 8 naïve mice in the ELISA was 0.158 and SD was 0.077, a cut-off OD_{405} value to judge as positive for antibody production was set to 0.389 (average + 3 × SD). In immunized mice, an average OD_{405} value and SD at 3 weeks after immunization were 0.945 and 0.247, respectively, indicating that immunization conferred humoral immune responses in mice. In the survived mice, an average OD_{405} value and SD in the ELISA were 1.903 and 0.119, respectively (Fig 1.10). These

data suggested that Δ*pdpC* could induce IgG antibody responses against *F. tularensis* upon s.c. immunization.

1.4.4.3 Cellular immune response in immunized mice

IFNγ concentration in sera was determined to evaluate Th1 cellular immune response in the immunized and unimmunized mice. Compared with 0 dpc, IFNγ were induced apparently in all mice at 3 dpc with virulent SCHU P9. However, the significant difference in serum IFNγ concentration was not observed between the immunized and unimmunized mice at 3 dpc (Fig. 1.11). Similarly, granzyme B, specifically expressed in functional cytotoxic T lymphocytes (CTL) and natural killer (NK) cells, were rarely detected in the immunized and the unimmunized mice at 3 dpc by immunohistochemistry (IHC) analysis (data not shown). Thus, it was suggested that prominent Th1 cellular immune response was not induced upon Δ*pdpC* immunization in mice.

1.5 Discussion

Because *F. tularensis* is a highly infectious bacterium that has the potential to be used as a biological weapon, the development of an effective vaccine is required (40, 57, 58). Several different strategies have been employed to develop an effective vaccine against tularemia. In general, live attenuated vaccines have been demonstrated to have the greater effects in protecting against viral and bacterial infection compared with killed or inactivated vaccines, although the risks of unintended side effects such as regain of virulence must be considered. *F. tularensis* subsp. *holarctica* live vaccine strain (LVS) was developed in the last century and used in human volunteers (20, 59). LVS was proved to provide partial protection against challenge with *F. tularensis* type A strain in humans (60). Although LVS remains in use in research (43, 61), this live vaccine strain is not licensed in the USA and other countries and precluded from public use, primarily due to uncertainty regarding the way of attenuation and instability in culture (26, 62, 63). Thus, the establishment of a novel vaccine against tularemia has remained a critical goal. The researchers aimed to develop a fully protective attenuated vaccine in which safety concerns are eliminated or at least minimized, and numerous live attenuated mutants derived from LVS or virulent SCHU S4 strain have been tried (33, 62).

In the previous study (37), a virulent strain SCHU P9 was derived from SCHU strain, the parent strain of SCHU S4 (35, 47). In mice, SCHU P9 was shown to be highly virulent, comparable to SCHU S4 (64, 65). In this study, I used SCHU P9 as a challenge strain, since it is difficult to obtain SCHU S4 strain from foreign laboratories because the transport of the bacterium is highly regulated by

the Japanese infectious disease control law.

SCHU P9 Δ*pdpC* mutant strain was generated by group II intron insertion using the TargeTron gene knockout system and the pKEK1140 plasmid (GenBank accession number: EU499313) (66) from the virulent SCHU P9 strain. Because this mutant does not induce any clinical symptoms in infected mice, I speculated that Δ*pdpC* had a potential to be used as a novel live vaccine strain.

Risks associated with reversion of live attenuated vaccine to cause tularemia should be considered because attenuated *F. tularensis* strains often regained virulence during several passages in mice (35, 37, 67). Safety and stability of a live attenuated strain should be confirmed before they are used as a vaccine. I demonstrated that attenuation of pathogenicity in Δ*pdpC* was stably maintained after 10 serial passages in mice in this study, and a low bacterial burden was detected in the spleens of mice infected with Δ*pdpC* after the 10th passage (Figs 1.1 and 1.2). In addition, I demonstrated the genetic stability of the intron sequences in Δ*pdpC* during these *in viv*o passages. Based on the results, Δ*pdpC* is stably and sufficiently attenuated to satisfy the requirements of a safe vaccine.

It is believed that the route of vaccination is important for the success of the immunization (68). The route of vaccination has also important influence on immune responses at the beginning of pathogen invaded to host (69). Because many bacterial and viral agents are thought to infect primarily from mucosal surfaces, the efficacy of vaccines against pathogens may be dependent on the effective induction of a mucosal immune response. Vaccines are generally administered through i.m., s.c., or i.n. routes. So in

this study, immunization through i.m., s.c., and i.n. routes were examined. The mice immunized via these three routes were challenged with SCHU P9 using the same routes as immunization, and effective protection against lethal infection was evaluated. It was observed that all mice in the i.m. immunization group were protected against a lethal challenge with the virulent SCHU P9 strain, while the survival rates of the mice immunized and challenged from s.c. and i.n. were inferior (Fig 1.7). Although inhalation and skin contact are the most common horizontal infection routes for human tularemia, aerosolized *F. tularensis* has long been considered as a prime candidate for use as a biological weapon (70). Therefore, protection with s.c. immunization against i.n. challenge was then evaluated and found to be 38% protection (Fig 1.9A). Statistical analysis indicated that the survival rates in all of the immunized groups were significantly (log-rank test analysis) higher than those in the respective unimmunized groups (Figs 1.7 and 1.9). These results indicate that Δ*pdpC* strain elicited protective immunity and that the strain is suitable as a candidate for live attenuated vaccine against *F. tularensis.*

It has been reported that antibodies against *F. tularensis* might offer a considerable survival advantage in patients with respiratory tularemia because the antibodies inhibited bacterial spread from the lungs to other organs (71, 72). In this study, antibody responses against *F. tularensis* were well established in mice immunized with Δ*pdpC* via three different routes (Figs 1.6 and 1.10). On the other hand, it is also reported that 100% protection against *F. tularensis* type A strains by vaccination was difficult to achieve (62). Recent reports illustrated that antibodies contributed to protection against *F. tularensis* LVS (type B)

challenge, while the importance of antibodies in *F. tularensis* SCHU S4 (type A) challenge was more controversial (62). Previous reports showed that it is difficult to demonstrate relationships between antibody responses and effective protection against virulent *F. tularensis* strains in mice (72-74).

It is well known that cellular immunity also plays an important role in protection against intracellular bacterial infections (75). Hence, we tried to observe the induction of functional cellular immunity in the unimmunized and immunized mice via s.c. route after virulent SCHU P9 challenge via i.n. route. IFNγ, which was indispensable for the differentiation of Th1 cells, were measured in the sera. In addition, the various tissues at 3 dpc were immunohistochemically stained with the polyclonal antibodies against granzyme B, which is known to be expressed specifically in CTL and NK cells (76). We could not detect significant differences in IFNγ concentration in sera from the immunized and unimmunized mice at 3 dpc with SCHU P9 (Fig. 1.11) and in the numbers of granzyme B positive cells in tissues such as liver and spleen (data not shown). Thus, the cellular immunity to protect the lethal challenge of virulent SCHU P9 in immunized mice could not be confirmed in this study. However, it appears clear that the synergy of humoral and cellular immunity is an important factor in protection against *F. tularensis* (77, 78). In this regard, i.m. immunization with Δ*pdpC* might have induced stronger cellular immunity. The survival rate in the immunized mice via s.c. and i.n. might be improved if the cellular immunity were induced forcefully more than in this study. Further study needed to be done for better understanding.

There are a lot of major factors influencing vaccine efficacy, which includes bacterial strain, growth conditions of attenuated vaccine and challenge strain, animal model and age of animal (62). Animal strain may also influence the results. C57BL/6 and BALB/c mice are often used as animal models of tularemia as well as for studies on *F. tularensis* vaccine development. The genetic background of the individual strain of mice often significantly affects the immune response and survival rates in murine models of infectious diseases and vaccination (31, 62, 79, 80). It is known that C57BL/6 mice are more susceptible to *F. tularensis* infection and that they are less easily protected by vaccination against challenge with the highly virulent type A *F. tularensis* than BALB/c mice (62). The survival rates obtained in this study might be different if BALB/c mice were used. I also cannot rule out the possibilities that the bacterial growth medium and sex-dependent host factors affected the efficacy of vaccination with Δ*pdpC*.

Virulent *F. tularensis* strains such as SCHU S4 can induce severe diseases and lethal infection at low dose in mice (20, 64). In this study, the LD_{50}s of SCHU P9 in the i.m. and s.c. challenge were 3.2 and 1.5 CFU, respectively. In the previous study, the LD_{50} in C57BL/6J mice inoculated intraperitoneally with SCHU P9 was 5 CFU (37). In other studies, the LD_{50}s in mice infected with type A strain via s.c. and i.d. inoculation routes were reported to be 1 and <10 CFU, respectively (81). There was no significant difference in the LD_{50}s of virulent *F. tularensis* strains for various inoculation routes in studies reported by different groups. In the s.c. and i.m. groups, 10 CFU of SCHU P9 caused lethal infection. Conversely,

mice inoculated intranasally with 10 CFU of the bacterium did not exhibit any clinical signs during the 21 day observation period (data not shown), and a 10-fold higher inoculum was necessary to induce lethal disease. The difference in LD_{50}s among various inoculation routes might be related to the local immune regulation of inflammation against pathogens (82).

Researchers are attempting to develop safe and effective immunizations using attenuated tularemia vaccines. Death following challenge with more than 100 CFU of SCHU S4 was prevented in mice immunized with attenuated strains derived from types A (Δ*clpB* (31), Δ*capB* (83), Δ*FTT_0918* (84), and Δ*ggt* (32)) and B strains (Δ*dsbA* (28) and Δ*sodB* (27)). In a recent report, Lindgren *et al*. illustrated that Δ*pdpC* could confer efficient protective immunity against intradermal (i.d.) challenge with SCHU S4, but it did not protect against i.n. challenge (52). The results of the present study indicated that Δ*pdpC* could be used as a vaccine candidate because the survival rates in the immunized groups were significantly higher than those in unimmunized mice (Figs 1.7 and 1.9). However, the survival rate in the i.n. group was lowest among the immunized groups. Although the precise reasons remain unclear, the earlier report pointed out that inflammation response delays during i.n. challenge, and respiratory challenge is hardest to protect (85). Taken together, induction of sterile immunity via vaccination against respiratory disease caused by virulent type A *F. tularensis* strains remains difficult if more than 100 CFU of the bacteria are introduced.

In conclusion, the inserted intron sequences used to disrupt *pdpC* gene using the TargeTron

system were stably maintained during serial passages in mice. The attenuated Δ*pdpC* strain elicited protective immunity against lethal infection of SCHU P9 in mice, but the survival rates were dependent on the routes of immunization and challenge. These data suggest that Δ*pdpC* is a safe and immunogenic tularemia vaccine candidate suitable for further clinical development.

Figure legends

Fig 1.1 The scheme of Δ*pdpC* mutant passage *in vivo*

Three C57BL/6J mice were inoculated i.p. with 1×10^6 CFU of Δ*pdpC* and were sacrificed 3 days after inoculation. The spleen samples from the 3 mice were homogenized with 10 times volume of tissue weight of RPMI 1640 medium. One milliliter of 10% spleen homogenates was plated onto Eugon chocolate agar plates and incubated at 37°C for 2 days. The bacterial growth on the plate was harvested, suspended in 1 × CDM, and adjusted the OD_{600}=1. Then 100 times dilution of the bacterial suspension was inoculated i.p. to three C57BL/6J mice. Subsequent passages were performed similarly until 10th generation. After 10 times passages in mice, *pdpC* gene region of the genomic sequences of the strain were amplified by PCR and its nucleotide sequences were determined.

Fig 1.2 Levels of bacterial burden of Δ*pdpC* in spleen during serial passages in mice.

One hundred μl of 10 fold serial diluted homogenates were inoculated on chocolate Eugon agar in 6-well plates. After an incubation period of 4 days, numbers of colonies were counted.

Fig 1.3 Genetic stability of the intron sequences of Δ*pdpC* during serial passages in mice.

Genomic DNAs extracted from SCHU P9 and passaged strains were amplified by PCR using pdpC336s and pdpC725a primer pair (upper panel). The amplicons and a molecular weight marker of 100 bp

Ladder (100-1000 bp; Nippongene, Tokyo, Japan) were electrophoresed on a 0.7% agarose gel. The predicted sizes of amplicons with and without intron were 1305bp and 390bp, respectively (lower panel).

Fig 1.4 Survival rates after SCHU P9 challenge

Five mice in each group were inoculated intramuscularly (i.m.), subcutaneously (s.c.) or intranasally (i.n.) with 1×10^{-1} to 1×10^{3} CFU of SCHU P9. Survival rates of mice were recorded until 21 days post-challenge (dpc). The mice were humanely euthanized when they showed severe clinical symptoms. (A) i.m. challenge. (B) s.c. challenge. (C) i.n. challenge.

Fig 1.5 Bacteria burden in liver, spleen, and lung after the challenge with SCHU P9 strain via i.m., s.c., and i.n. routes.

The amounts of bacteria were about 10^{8} CFU/g in three organs of all dead mice, while, no bacteria were detected from survived mice. (A) liver, (B) spleen, (C) lung.

Fig 1.6 Antibody (IgG) response in mice immunized with Δ*pdpC*

Blood samples were collected from C57BL/6J mice (n=5) through tail veins before (red square) and 21 days after immunization (blue circle). Levels of serum IgG antibody against *F. tularensis* were

determined by ELISA. Mean ± SD of IgG titers were shown. Asterisks indicate statistical significance between before and 21 days after immunization (**P<0.01 and ***P<0.001).

Fig 1.7 Protection by immunization with Δ*pdpC* via i.m., s.c., or i.n. route against SCHU P9 challenge from the same route.

Survival rates of mice challenged with 100 LD_{50} of SCHU P9 via i.m. (A), s.c. (B), and i.n. (C), with (blue) or without (red) immunization by 10^6 CFU of Δ*pdpC* from the identical route as immunization 21 days before challenge are shown. Mice were sacrificed when they showed the serious clinical symptoms and/or more than 25% weight loss during 21 days post-challenge (dpc). Statistical significance of survival rate between with and without immunization was determined using log-rank (Mantel-Cox) test (*P<0.05 and **P<0.01).

Fig 1.8 Body weights of mice challenged with 100 LD_{50} of SCHU P9 via i.m., s.c., and i.n. route with or without immunization.

Body weights of mice challenged with 100 LD_{50} of SCHU P9 via i.m. (A), s.c. (B), and i.n (C), with (blue) or without (red) immunization by 10^6 CFU of Δ*pdpC* from the identical route 21 days before challenge are shown. Mice were sacrificed when they showed more than 25% weight loss during 21 days post-challenge (dpc).

Fig 1.9 Protection from i.n. lethal challenge of SCHU P9 in mice immunized s.c. with Δ*pdpC*

C57BL/6J mice (n=8) were immunized via s.c. route with 10^6 CFU of Δ*pdpC* or saline, and challenged i.n. with 100 LD_{50} of SCHU P9. (A) Survival rates of mice with (solid line) and without (dashed line) immunization with Δ*pdpC* immunized. (B) Body weights of Δ*pdpC* immunized mice (black circle) and unimmunized mice (white circle).

Fig 1.10 Antibody (IgG) response in mice immunized with Δ*pdpC* and challenged with SCHU P9 from different route.

Blood samples were collected from C57BL/6J mouse tail veins of naïve (n=8) (circle), 21 days after immunization (n=8) (square) and survived for 21 days after SCHU P9 challenge (n=3) (triangle). Levels of serum IgG antibody against *F. tularensis* were determined by ELISA. Mean ± SD of IgG titers were shown. Statistical significance was determined by two-way ANOVA with Tukey's post-hoc tests (***P<0.001).

Fig 1.11 IFNγ concentration in sera in the immunized and unimmunized mice.

C57BL/6J mice were immunized via s.c. route with 10^6 CFUs of Δ*pdpC* or saline. At 21 days after immunization, the mice were challenged i.n. with 100 LD_{50} of SCHU P9. IFNγ concentration in sera

collected from the immunized and unimmunized mice at 0 and 3 dpc were measured. Mean ± SD of IFNγ concentration in sera are shown. Statistical significance was determined by One-way ANOVA with Tukey's post-test (** $p<0.01$).

Figures

Fig 1.1

Passage	1	2	3	4	5	6	7	8	9	10
Inoculation dose (CFU/100µl)	1×10^6	6×10^6	9×10^6	9×10^6	8×10^6	6×10^6	9×10^6	9×10^6	6×10^6	6×10^6
Average CFU /10mg in spleen at 3 dpi (n=3)	8×10^3	2×10^5	7×10^5	2×10^5	1×10^5	2×10^5	9×10^5	2×10^5	2×10^5	1×10^5

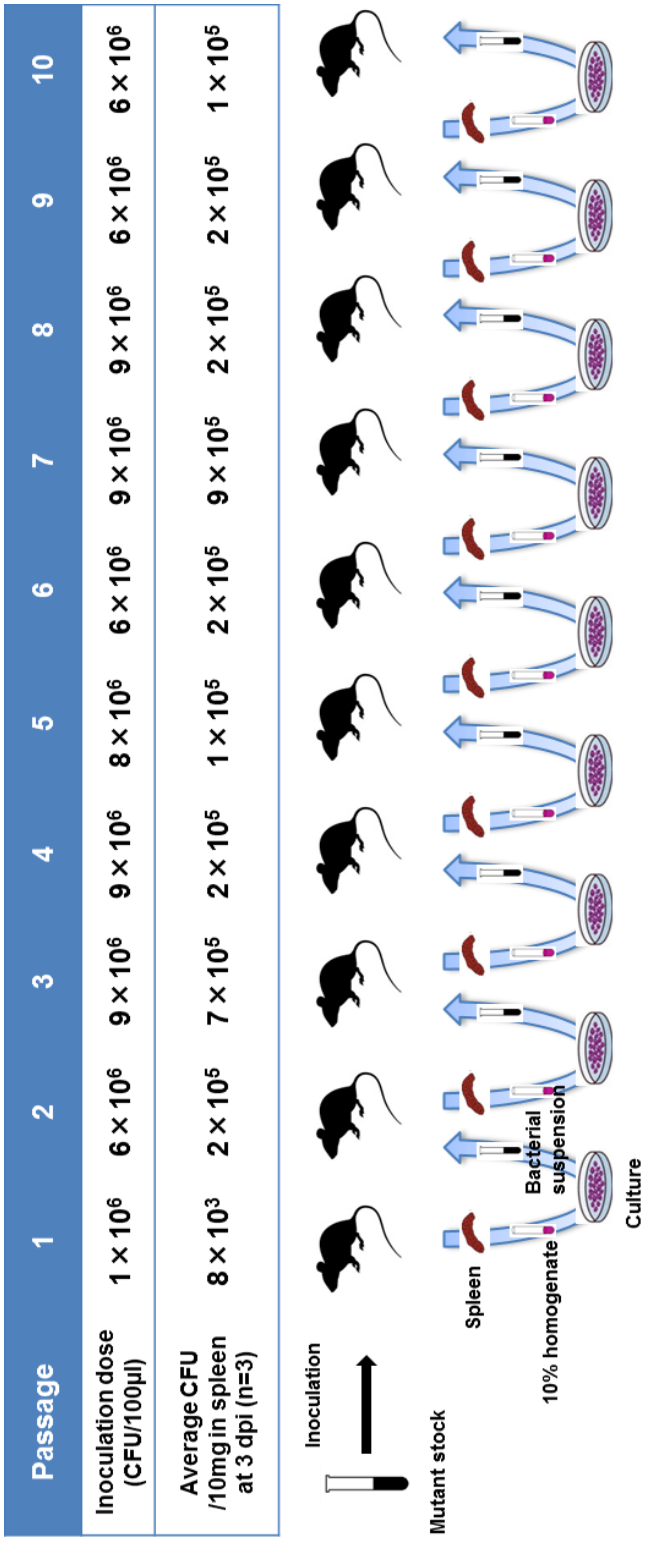


Fig 1.2

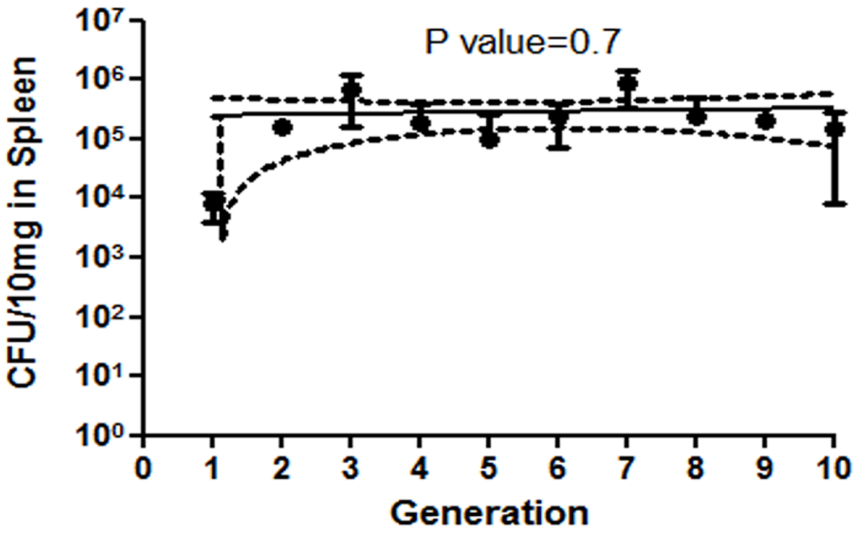

P value=0.7
CFU/10mg in Spleen
10⁷
10⁶
10⁵
10⁴
10³
10²
10¹
10⁰
0
1
2
3
4
5
6
7
8
9
10
Generation

Fig 1.3

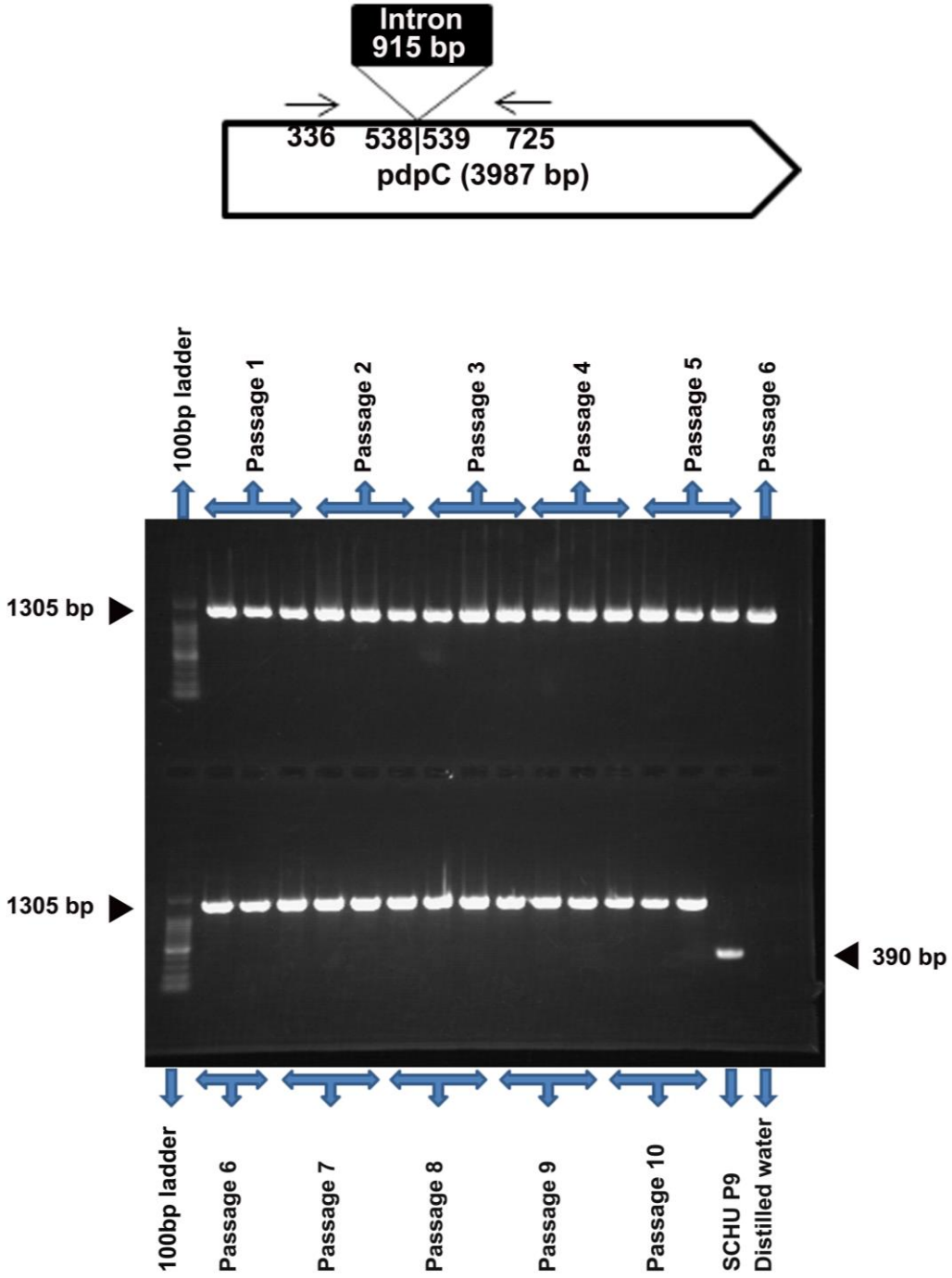

Intron
915 bp
336
538|539
725
pdpC (3987 bp)
100bp ladder
Passage 1
Passage 2
Passage 3
Passage 4
Passage 5
Passage 6
1305 bp
1305 bp
390 bp
100bp ladder
Passage 6
Passage 7
Passage 8
Passage 9
Passage 10
SCHU P9
Distilled water

Fig 1.4

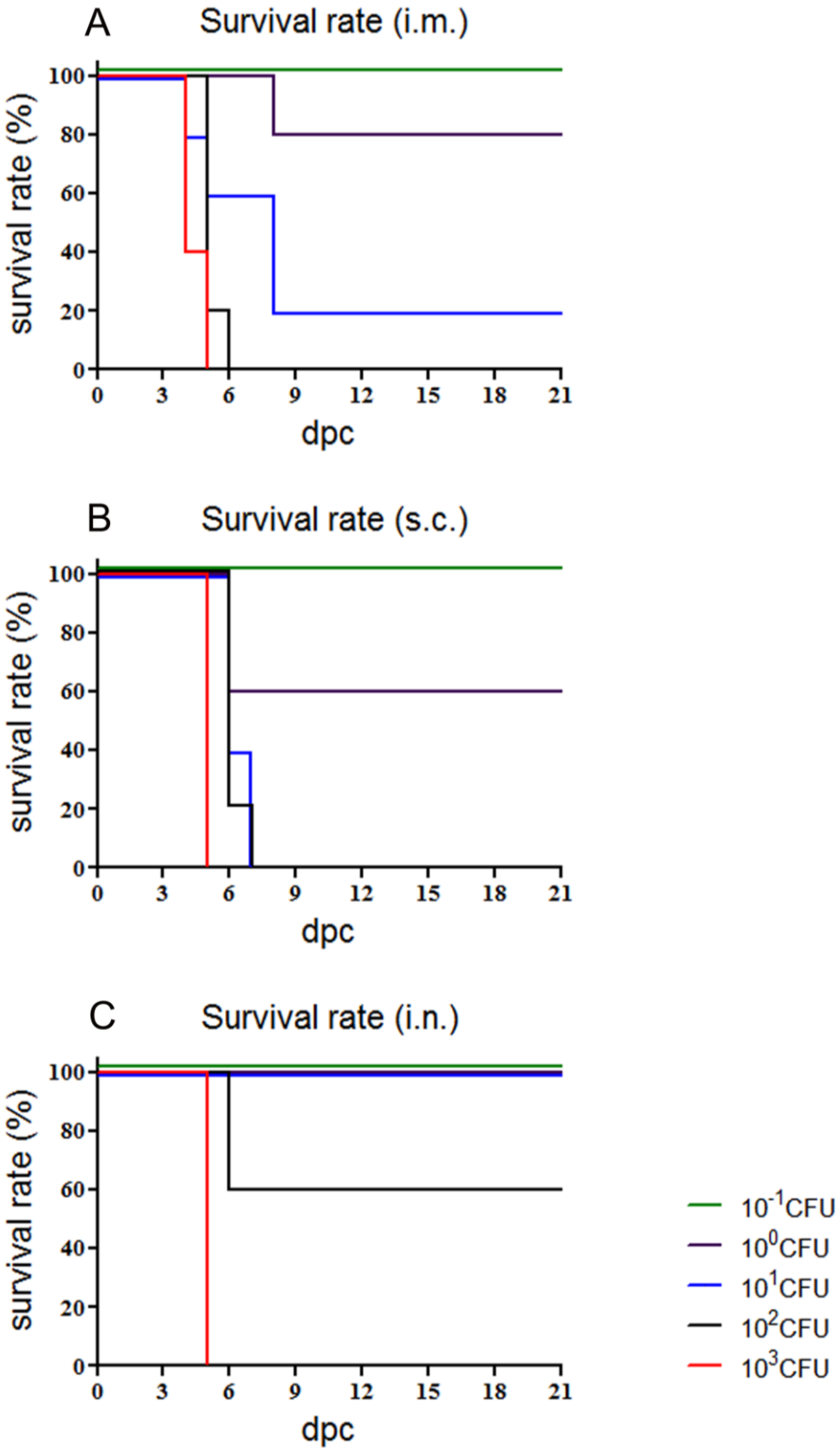

A
Survival rate (i.m.)
survival rate (%)
100
80
60
40
20
0
0
3
6
9
12
15
18
21
dpc
B
Survival rate (s.c.)
survival rate (%)
100
80
60
40
20
0
0
3
6
9
12
15
18
21
dpc
C
Survival rate (i.n.)
survival rate (%)
100
80
60
40
20
0
0
3
6
9
12
15
18
21
dpc
10-1CFU
100CFU
101CFU
102CFU
103CFU

Fig 1.5

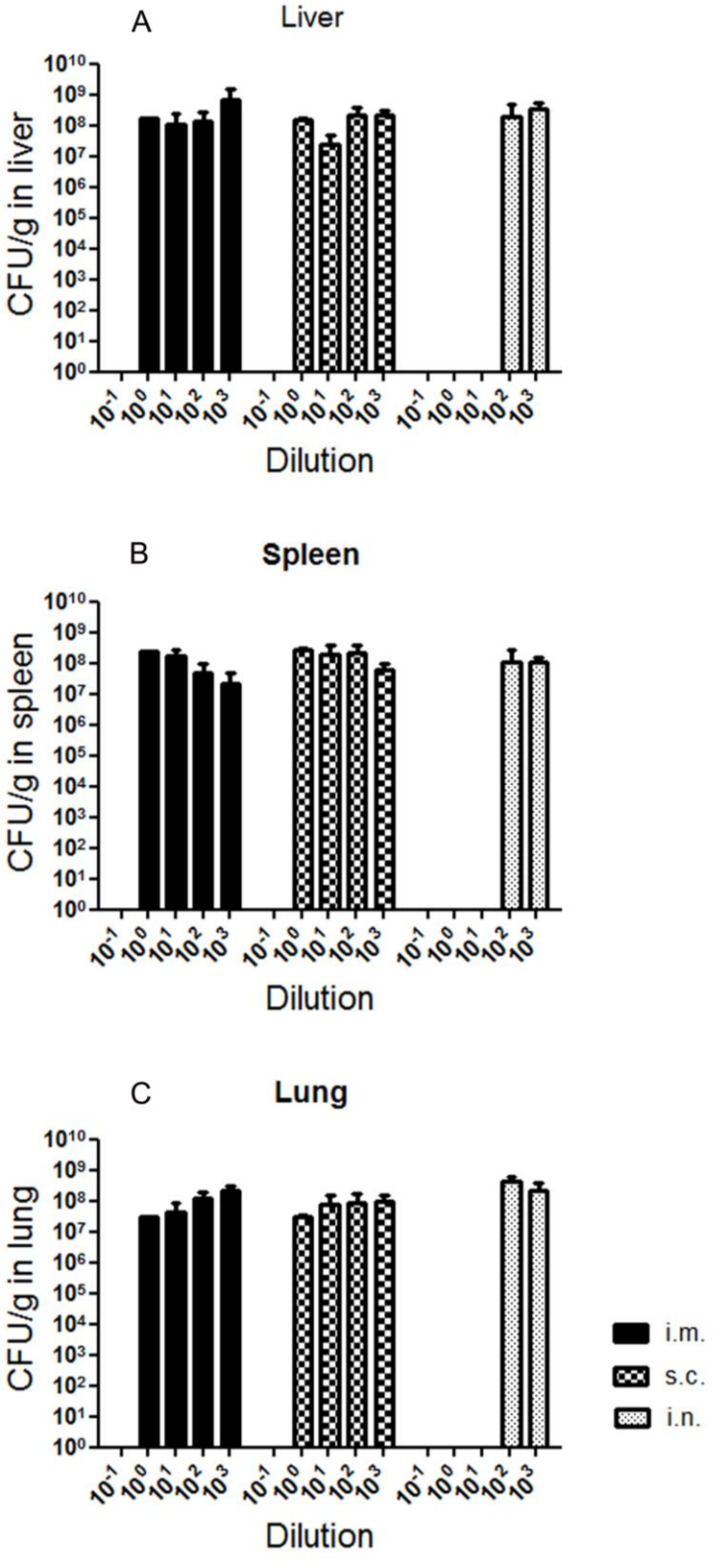

A
Liver
CFU/g in liver
Dilution
B
Spleen
CFU/g in spleen
Dilution
C
Lung
CFU/g in lung
Dilution
i.m.
s.c.
i.n.

Fig 1.6

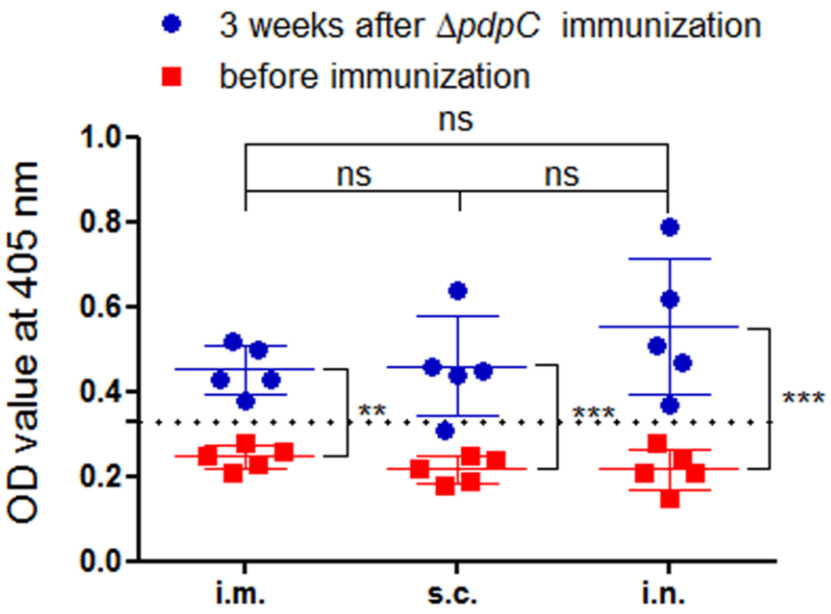

3 weeks after ΔpdpC immunization
before immunization
OD value at 405 nm
1.0
0.8
0.6
0.4
0.2
0.0
ns
ns
ns
**
***
***
i.m.
s.c.
i.n.

Fig 1.7

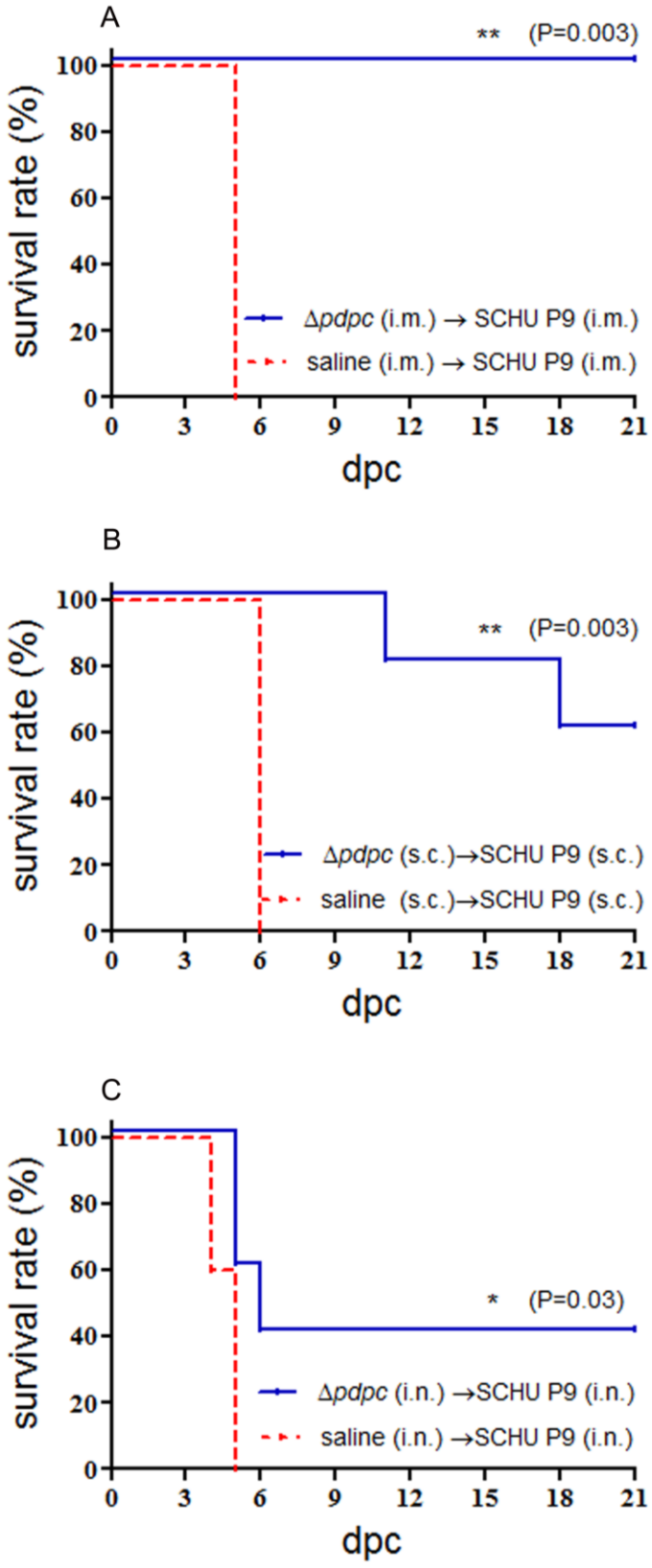

A
** (P=0.003)
survival rate (%)
100
80
60
40
20
0
0 3 6 9 12 15 18 21
dpc
Δpdpc (i.m.) → SCHU P9 (i.m.)
saline (i.m.) → SCHU P9 (i.m.)
B
** (P=0.003)
survival rate (%)
dpc
Δpdpc (s.c.)→SCHU P9 (s.c.)
saline (s.c.)→SCHU P9 (s.c.)
C
* (P=0.03)
survival rate (%)
dpc
Δpdpc (i.n.) →SCHU P9 (i.n.)
saline (i.n.) →SCHU P9 (i.n.)

Fig 1.8

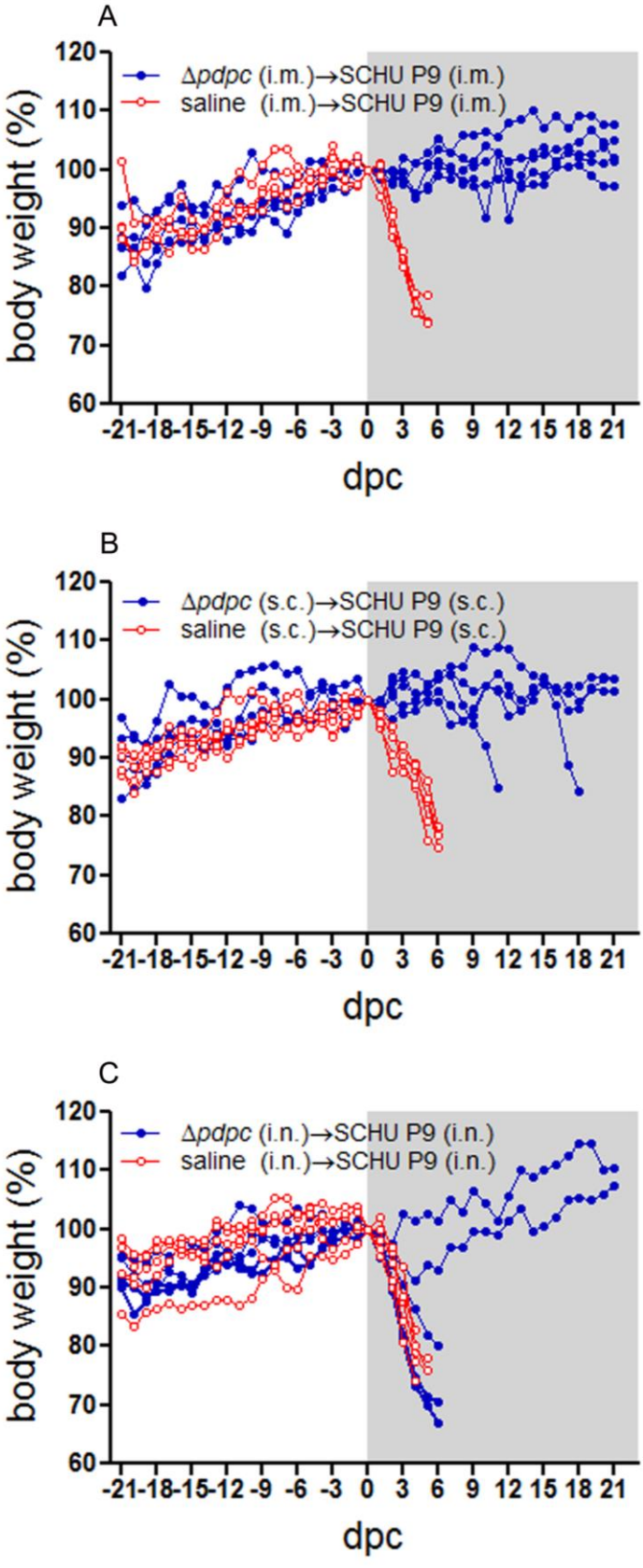

A
Δpdpc (i.m.)→SCHU P9 (i.m.)
saline (i.m.)→SCHU P9 (i.m.)
body weight (%)
120
110
100
90
80
70
60
-21 -18 -15 -12 -9 -6 -3 0 3 6 9 12 15 18 21
dpc
B
Δpdpc (s.c.)→SCHU P9 (s.c.)
saline (s.c.)→SCHU P9 (s.c.)
body weight (%)
120
110
100
90
80
70
60
-21 -18 -15 -12 -9 -6 -3 0 3 6 9 12 15 18 21
dpc
C
Δpdpc (i.n.)→SCHU P9 (i.n.)
saline (i.n.)→SCHU P9 (i.n.)
body weight (%)
120
110
100
90
80
70
60
-21 -18 -15 -12 -9 -6 -3 0 3 6 9 12 15 18 21
dpc

Fig 1.9

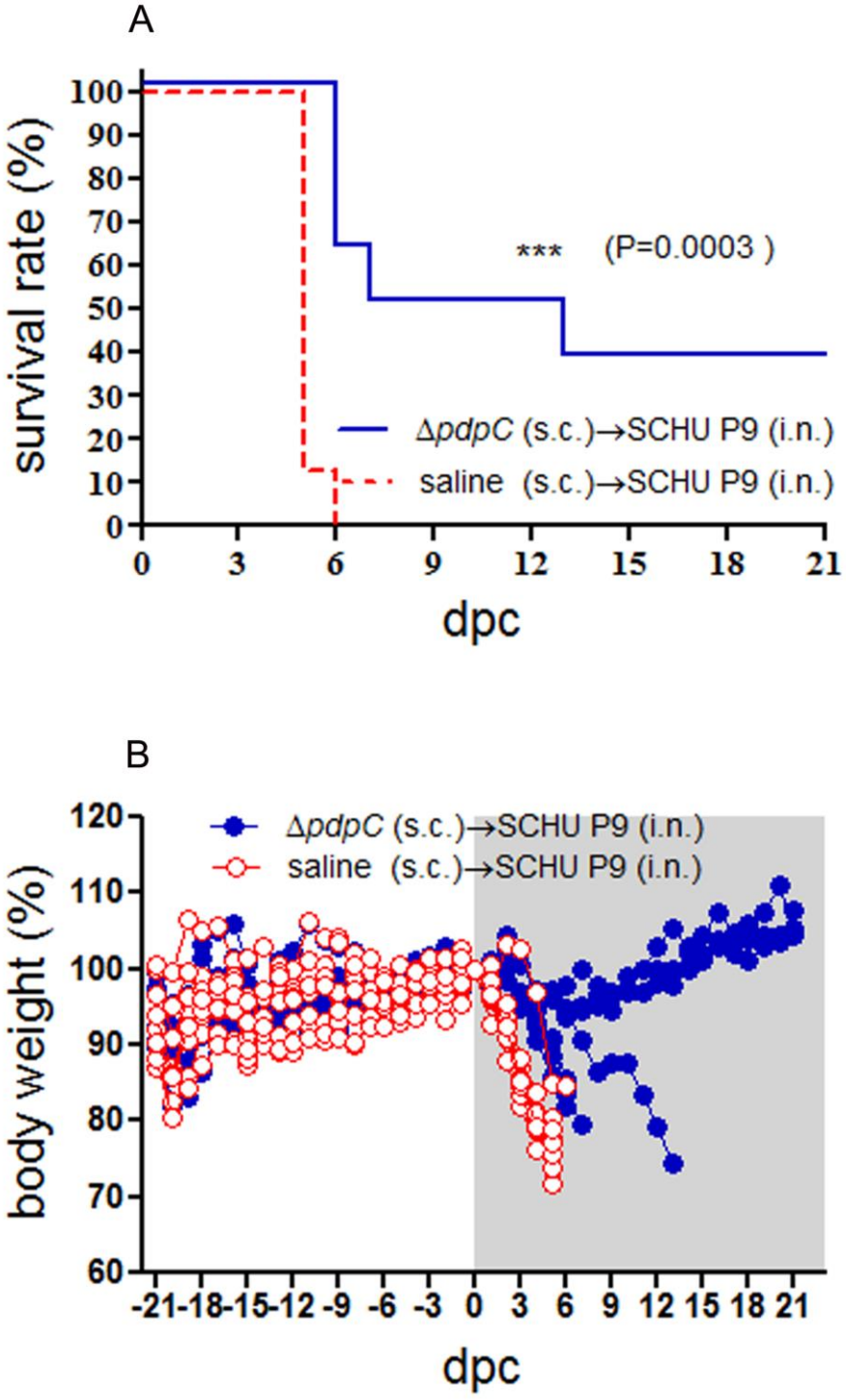

A
survival rate (%)
100
90
80
70
60
50
40
30
20
10
0
0
3
6
9
12
15
18
21
dpc
***
(P=0.0003)
ΔpdpC (s.c.)→SCHU P9 (i.n.)
saline (s.c.)→SCHU P9 (i.n.)
B
body weight (%)
120
110
100
90
80
70
60
-21
-18
-15
-12
-9
-6
-3
0
3
6
9
12
15
18
21
dpc
ΔpdpC (s.c.)→SCHU P9 (i.n.)
saline (s.c.)→SCHU P9 (i.n.)

Fig 1.10

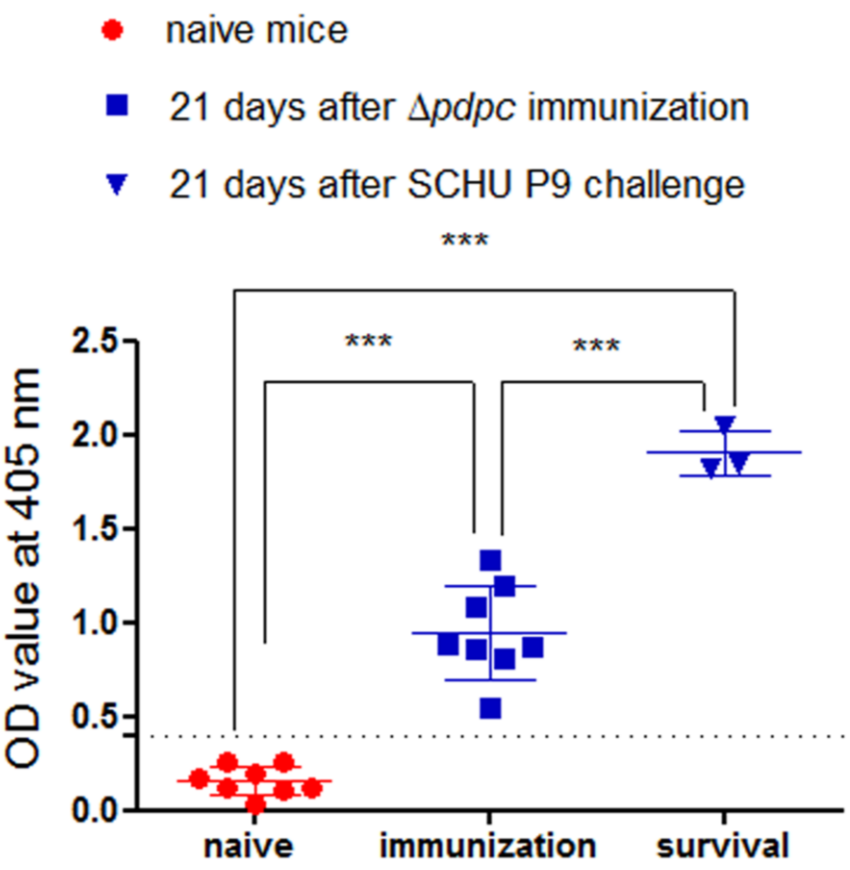
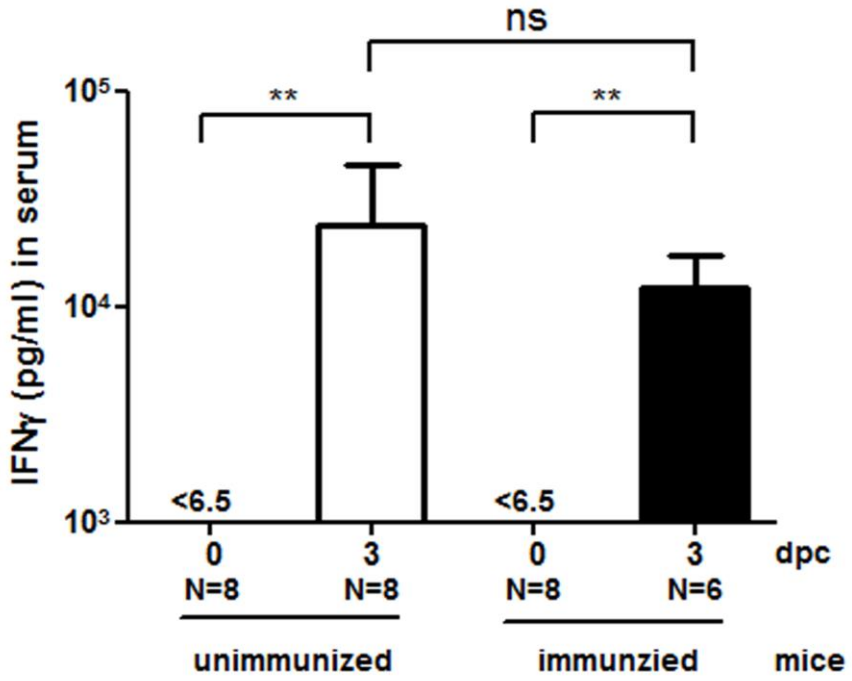

naive mice
21 days after Δpdpc immunization
21 days after SCHU P9 challenge
***
***
***
OD value at 405 nm
2.5
2.0
1.5
1.0
0.5
0.0
naive
immunization
survival

Figure 1.11


ns
**
**
IFNγ (pg/ml) in serum
10^5
10^4
10^3
<6.5
<6.5
0
3
0
3
dpc
N=8
N=8
N=8
N=6
unimmunized
immunzied
mice

Chapter 2

Protective effects of *Francisella tularensis* Δ*pdpC* mutant against its virulent parental strain SCHU P9 in cynomolgus macaques

2.1 Abstract

Tularemia caused by *Francisella tularensis*, is a severe infectious zoonotic disease. Although *F. tularensis* live vaccine strains attenuated by various methods have been attempted against tularemia, no vaccine has been licensed in the world until now. In the previous study, Uda *et al*. (37) have established knockout mutant strain with an attenuated phenotype by intron insertion into *pdpC* virulent gene of virulent strain SCHU P9 (Δ*pdpC*). In chapter 1, the mutant strain showed stability and good protection in a mouse model. In this chapter, I analyzed a protective effect of Δ*pdpC* strain against tularemia using a monkey lethal model. Six cynomolgus macaques were divided into 3 groups. Two monkeys were intratracheally challenged with 1×10^6 CFU of virulent SCHU P9 strain 3 weeks after subcutaneous inoculation with 1×10^6 CFU of Δ*pdpC* (Group-1). Two monkeys were intratracheally (i.t.) challenged with 1×10^6 CFU of virulent SCHU P9 strain 3 weeks after the inoculation of placebo (Group-2). Two monkeys were intratracheally challenged with 1×10^6 CFU of Δ*pdpC* 3 weeks after the inoculation of placebo (Group-3). The monkeys were observed for clinical symptoms and survival for 3 weeks after challenge. The 2 monkeys challenged with SCHU P9 strain without previous immunization with Δ*pdpC* strain (Group-2) were euthanized on 7 and 11 days after challenge when they showed severe clinical signs of tularemia such as fever, cough, bad appetite, and recumbent, while the 2 monkeys challenged with Δ*pdpC* strain (Group-3) showed no apparent clinical signs. On the other hand, the 2 monkeys with immunization by Δ*pdpC* strain (Group-1) survived for 3 weeks after SCHU P9 challenge, though one of the 2 monkeys developed mild symptoms of tularemia. Both of the 2 monkeys showed high levels of

antibodies against *F. tularensis*. Antibodies were confirmed by three methods, ELISA, micro-agglutination (MA) test, and western blotting. In this chapter, I also confirmed that *F. tularensis* invaded not only macrophages but also type II epithelial cells in the infected monkeys. These results suggest that Δ*pdpC* strain is a safe and promising candidate for tularemia vaccine.

2.2 Introduction

As described in general introduction, tularemia, caused by the Gram-negative intracellular bacterium *Francisella tularensis*, is a severe fatal zoonotic disease for both animals and human. Tularemia can be transmitted to humans in several ways; direct contact with infected animals, which is most frequent route of human infection, tick bites, and inhalation of aerosolized bacterium (12, 86). Although *F. tularensis* is naturally a pathogen for rodents and lagomorphs, humans are accidentally infected. In recent years, people pay much attention to *F. tularensis* because the bacterium can be used as a biological weapon (3, 40).

In order to prevent human tularemia, attempts to develop an effective vaccine have employed a lot of different strategies, and some vaccine candidates have been shown to have promising results in animal models (41).

Animal models including mouse, rat, rabbit, guinea pig, and monkey have been applied in the tularemia vaccine study (62). Among the animal models applied to test the efficacy of vaccine candidates, monkeys are suitable model since their close genetic background with humans, show clinical signs quite similar to human tularemia disease when focused on pulmonary infection (87, 88). The monkeys died on 5-7 days after virulent strain infection, and the target organs of the bacteria were the lung, liver, spleen and lymph nodes as observed in human tularemia (89). Thus, monkeys are suitable animals for studying the pathogenesis of tularemia and developing countermeasures, (20, 24, 90). In fact, from the last century, monkey models have been used to evaluate tularemia pathogenicity (89, 91-94) and its vaccine candidates

(95-98). Among the monkeys, cynomolgus macaque is a good model of inhalation tularemia compared with other nonhuman primate species (99), because the pathology of aerosolized *F. tularensis* virulent strain SCHU S4 in cynomolgus macaques (*Macaca fascicularis*) is similar to that in humans (100). For cynomolgus macaque, the LD_{50} of SCHU S4 is approximately less than 1 CFU via the aerosol (101). So in this chapter, six cynomolgus macaques were applied for assessing the pathogenicity of *F. tularensis* virulent strain and protective effects of its mutant strain as a vaccine candidate.

In chapter 1, I applied Δ*pdpC* mutant of *F. tularensis* SCHU P9 strain in mouse model and showed good protection against 100 LD_{50} virulent strain challenge. In this chapter, Δ*pdpC* mutant was further evaluated as a candidate for live attenuated vaccine against tularemia using a lethal animal model of cynomolgus macaque. The mortality and antibody titers in the monkey sera were investigated after the inoculation of Δ*pdpC* mutant followed by challenge of virulent SCHU P9 strain.

2.3 Materials and methods

2.3.1 Ethical statement

The experiments with animals were performed in strict accordance with the Animal Experimentation Guidelines of the NIID. The protocol was approved by the Institutional Animal Care and Use Committee of the NIID (Permission Number: 514005).

2.3.2 Bacterial strains

F. tularensis SCHU P9 and Δ*pdpC* used in this chapter were established in the previous report (37). These bacteria were cultured in Chamberlain's defined medium (CDM) at 37°C for 24 h, suspended in CDM containing 10% glycerol, and stored at -80°C until use. All work with live bacteria cultures were performed in a biosafety level 3 facility in accordance with the regulations stipulated by NIID.

2.3.3 Animals

Six healthy adult male cynomolgus macaques were obtained from animal center of NIID. The four monkeys (#4548, #4549, #4550, and #4552) were imported from Vietnam and used in a vaccine study of HIV infection. They were inoculated with recombinant BCG expressing SIV gag protein in 2001. The two monkeys (#4418, #4686) were laboratory-born animals in Tsukuba Primate Research Center, National Institutes of Biomedical Innovation Health and Nutrition and were used in vaccine studies of Hepatitis E virus (HEV) (#4418) and influenza virus (#4686) infection, infected with HEV and intranasally immunized with HA protein, respectively in 2000 to 2004.

When used in this experiment, the monkey's average weight was 5.6kg (range from 4.5 to 7.6 kg). Animals were in good physical condition and were free of clinical signs of any infection. During the period of the study, the animals were housed individually and monitored daily for signs of illness and distress.

2.3.4 Immunization and challenge

Six animals were randomly divided into three groups, 2 animals in each group. Two animals in Group-1 (monkeys number: #4550 and #4552) were subcutaneously immunized with 1 ml of Δ*pdpC* strain suspension containing 1×10^6 CFU, and four animals in Group-2 (monkey number: #4418 and #4548) and Group-3 (monkey number: #4549 and #4686) received an equal volume of saline via subcutaneous route as control. Three weeks later, the animals in Group-1 and Group-2 were challenged intratracheally with 1×10^6 CFU of SCHU P9 strain while the animals in Group-3 were challenged intratracheally with 1×10^6 CFU of Δ*pdpC* (Table 2.1).

2.3.5 Monitoring

Monkeys were monitored daily after the immunization, and their health conditions including appetite, water drinking, behavior, appearance, and feces were recorded. Body temperature and body weight were measured every two to three days as shown in a scheme (Fig 2.1). When monkeys showed serious clinical symptoms and became moribund, they were promptly euthanized by intramuscular injection with ketamine (5 mg/kg) and exsanguination.

2.3.6 Clinical observation

A clinical scoresheet was designed to record monkeys' health conditions. Animals were observed daily after the challenge with SCUH P9 and scored. The scoring parameters were as follows; i.e., activity (1: normal, 2: less active, 3: inactive), behavior (1: normal, 2: depressed, 3: ignoring everything),

stimulation response (1: normal, 2: entering room, 3: approaching cage), breathing (1: normal, 2: rapid, 3: abdominal), stool (1: normal, 2: loose stool, 3: liquid stool). When the monkey showed high clinical score, the other clinical symptoms were checked and the decision was made for termination of the experiment (Table 2.2).

2.3.7 Complete blood counts (CBCs) and serum collection

When the body temperature and body weight were measured, monkeys were anesthetized with ketamine (5 mg/kg) injected intramuscularly, and blood samples were collected from the femoral vein into 5 ml venoject II vacuum blood collection tubes containing citric acid (Terumo, Tokyo, Japan). CBCs were analyzed with auto analyzer (Vetscan HM2, Abaxis, Union City, CA, USA) according to the manufacturer's instructions. Then, sera were obtained by the centrifugation at 1,200 × g at 4°C for 30 min, incubated at 56°C for 30 min, and inactivated by UV irradiation at 254 nm for 10 min before use.

2.3.8 Necropsy and macroscopic pathology

Animals were euthanized when they became moribund or at the end of the study, and necropsies were performed in the BSL-3 facility. Macroscopic features of necropsied tissues of the monkeys were observed at dissection. For histopathology and immunohistochemistry analyses, brain, spinal cord, lymph nodes, lung, heart, liver, spleen, kidney, tonsil, testis, thymus, skin, and gastrointestinal tract tissue samples collected from each animal were immersion-fixed in 10%

neutral-buffered formalin for at least 21 days to kill all pathogens. Three different portions of lymph nodes (cervical lymph node, axillary lymph node, and inguinal lymph node), trachea, lung, liver, spleen, heart, and kidney were also submitted for bacterial culture at the time of postmortem examination.

2.3.9 Colony forming unit (CFU) measurement

The organs from the monkeys were homogenized in RPMI 1640, and the serial dilutions of the homogenates were cultured onto Eugon chocolate agar plates in duplicate and incubated at 37°C for 4 days. CFU were calculated from average number of colonies on the plate.

2.3.10 Histopathology and immunohistochemistry

Fixed tissues were embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Immunohistochemical detection of the *F. tularensis* antigen was performed on paraffin-embedded sections by using a polymer-based detection system, Dako EnVision+ System-horseradish peroxidase (HRP) Labeled Polymer Anti-Mouse (Dako Denmark A/S, Glostrup, Denmark). Without any antigen retrieval reaction, deparaffinized sections were treated with 0.3% hydrogen peroxide in methanol for 20 min to quench endogenous peroxidase activity. After washing with PBS, sections were incubated with 5% normal rabbit serum for 5 min, followed by incubation at 37°C for 30 min with mouse anti-*F. tularensis* LPS monoclonal antibody (clone T14, FB11; HyTest Ltd., Turku, Finland) as the primary antibody. Peroxidase activity was detected with 3, 3-diaminobenzidine (DAB; Sigma-Aldrich, St. Louis, MO) and

sections were counterstained by hematoxylin.

Double immunohistochemical staining of paraffin-embedded tissues was also performed with mouse anti-*F. tularensis* LPS monoclonal antibody (clone T14) and a mouse anti-human CD68 monoclonal antibody (clone KP1; Dako), specific to human macrophage, or a goat anti-human angiotensin converting enzyme 2 (ACE2) polyclonal antibody (R & D system, MN, USA). For double immunohistochemical staining of *F. tularensis* and CD68, sections were first incubated with the primary antibody against *F. tularensis* LPS. Dako EnVision™ G/2 System/AP, Rabbit/Mouse (Dako) was used to detect *F. tularensis* LPS according to manufacturer's instructions. Following heat inactivation of the antibody against *F. tularensis* LPS in 10 mM citrate buffer solution (pH 6.0) at 121℃ for 10 min, sections were incubated with antibody against human CD68 at 4℃ for overnight. Dako EnVision+ System-HRP Labeled Polymer Anti-Mouse (Dako) and DAB including 0.02% cobalt chloride were used to detect the antibody against human CD68. For visualization of the staining, sections were treated with fuchsin. Nuclei were counterstained by hematoxylin.

For double immunohistochemical staining of *F. tularensis* LPS and human ACE2, the *F. tularensis* LPS antigen was first detected by Dako EnVision+ System-HRP Labeled Polymer Anti-Mouse system. Following the heat inactivation of the antibody against *F. tularensis* LPS as described above, sections were incubated with the antibody against human ACE2 at 4℃ for overnight. Dako LSAB+ System–AP (Dako) was used according to manufacturer's instructions. For visualization of staining, the

sections were treated with fuchsin. Nuclei were counterstained by hematoxylin.

2.3.11 Detection of IgM and IgG antibody responses in the monkeys by ELISA

Total amount of IgM and IgG in the monkey sera were quantified by monkey IgG or IgM ELISA kits (Life Diagnostics, West Chester, PA, USA). All samples and reference standard provided in each kit were processed according to the manufacturer's instructions in duplicate and the absorbance values (A_{450}) were measured. Using a standard curve constructed from the mean absorbance value of reference standards, concentrations of IgM or IgG in the samples were calculated.

To detect *F. tularensis* specific antibody, IgM-ELISA and IgG-ELISA were performed as previously reported (56). Briefly, SCHU P9 cultured on Eugon chocolate agar for 3 days was harvested, suspended in saline, adjusted to the concentration corresponding to an optical density at 600 nm (OD_{600}) = 1.0, and inactivated at 100°C for 10 min. This SCHU P9 antigen was stored at −80°C until use. Heat-killed SCHU P9 antigen, centrifuged and diluted to 1:5 in 50 mM carbonate-bicarbonate buffer, pH 9.0 was added onto the wells (100 μl/well) of a Nunc-Immuno plate (Thermo Scientific, Roskilde, Denmark), and the plates were left at 37°C overnight. After washing thrice with PBS containing 0.1% Tween 20 (PBST) to remove unbound antigen, all wells were blocked with 100 μl of PBST containing 3% skim milk at 37°C for 1 h. After washing thrice with PBST, 50 μl of the serum samples diluted to 1:2,000 in PBST containing 1% skim milk were added into antigen-coated wells in duplicate and incubated at 37°C for 1.5 h. After washing thrice with PBST, 50 μl of HRP-conjugated goat anti-monkey IgM (μ

chain) antibody (Rockland, PA, USA) or HRP-conjugated rabbit anti-monkey IgG (whole molecule) antibody (Sigma-Aldrich, MO, USA) diluted to 1:10,000 was added into each well, and then the plates were incubated at 37°C for 1 h. The wells were washed thrice with PBST and developed with 100 µl of 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) ELISA HRP substrate (Roche, Germany) at 37°C for 30 min. Optical density at 405nm (OD_{405}) was measured in the each well using a BioRad 96-well plate reader (iMark, Bio-Rad Laboratories, CA, USA).

2.3.12 Micro-agglutination (MA) test

Antibody in serum samples from the monkeys was also detected by MA test (102, 103). Sera were diluted 10 times in the first well and the fowling wells in 2-fold serial dilutions, and 25 µl of dilution and an equal volume of formalin-inactivated *F. tularensis* SCHU P9 whole-cell suspension (OD_{560}=1.0) were mixed in a 96-well round bottom microtiter plate. The mixture was incubated at 37°C for 18 hours and checked for agglutination. *F. tularensis* immunized rabbit serum was used as positive control, and normal rabbit serum was used as negative control. The agglutination titer was expressed as the reciprocal of the highest serum dilution showing a positive response to the antigens. Agglutination at dilutions of 1:10 or higher were considered as MA positive.

2.3.13 Western blotting (WB)

Western blotting (WB) analysis for antibody in the serum was performed as described

previously (103). Suspension of SCHU P9 was adjusted to OD_{600} = 1.0, mixed with the same volume of laemmli sample buffer (Bio-Rad, Bio-Rad laboratories, USA), and inactivated at 100°C for 5 min. Whole cell lysate of *F. tularensis* SCHU P9 were subjected to SDS-PAGE using 5-20% precast polyacrylamide 2 well gel (e-PAGEL HR, EHR-MD520L, ATTO, Tokyo, Japan). Antigens were electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon, Millipore Corporation, Bedford, MA, USA). After incubating in PBST containing 3% skim milk for about one hour at room temperature, the membrane was washed with PBST for 5 min for 3 times, then partially dried and sliced into small strips. The monkey sera were diluted 1,000 times using PBST containing 1% skim milk and incubated with PVDF membrane strips at room temperature for 1 hour. After the reaction, the membrane strips were washed thrice in PBST for 5 minutes. Then the membrane strips were incubated with HRP-conjugated goat anti-monkey IgM (Rockland, Limerick, PA, USA) or HRP-conjugated rabbit anti-monkey IgG (Sigma-Aldrich, St. Louis, MO, USA) at a dilution of 4,000 times at room temperature for 1 hour. After the reaction, the membrane strips were washed thrice in PBST for 5 minutes. Finally, antibodies reacted with the bacterial antigens on the membrane were visualized by incubation with a substrate mixture of 0.02% DAB and 0.003% H_2O_2 in 0.05M Tris-HCL buffer (PH=7.6) for about 10 min, and washed twice in distilled water. The serum samples were considered to contain *F. tularensis*-specific antibodies when a typical ladder-pattern was observed (104).

2.3.14 Statistical analysis

All statistical analyses were performed using GraphPad Prism v5 (GraphPad Software, CA, USA). Complete blood counts and concentrations of IgM, IgG were analyzed using One-way ANOVA method. Difference between the experimental groups were considered statistically significant at a $P<0.01$ level.

2.4 Results

2.4.1 Protection from lethal challenge of *F. tularensis* in cynomolgus macaques immunized with Δ*pdpC*

Two monkeys challenged with virulent SCHU P9 strain without previous immunization with Δ*pdpC* strain (Group-2) showed fever and body temperature of monkey #4418 decreased sharply before death. They were suffered from serious clinical signs of tularemia including cough and bad appetite. After challenged with the virulent strain, they showed less active, depressed, recumbent position in the bottom of cage with very weak stimulation response and became moribund. Body weight of these monkeys decreased and euthanized at 7 (#4548) and 11 days (#4418) after challenge (Fig 2.2A). On the other hand, monkeys inoculated with Δ*pdpC* strain prior to SCHU P9 strain (Group-1) also showed fever after SCHU P9 challenge, but the body temperature decreased to normal after one week (Fig 2.2B). The body weight of one of these monkeys (#4550) decreased, but the general conditions including appetite was normal (Fig 2.2C) and survived until the end of the experiment. The monkeys inoculated with saline and challenged with Δ*pdpC* (Group-3) showed no clinical symptom and their body weight did not show any obvious change.

2.4.2 Complete blood counts (CBCs)

The CBC test was performed to observe the changes in the blood. White blood cell count (WBC), red blood cell count (RBC), platelet count (PLT), hemoglobin concentration (HGB), mean

corpuscular hemoglobin (MCH), and mean corpuscular volume (MCV) were observed during the experiment. All six parameters were basically in normal range during experiment. WBC of monkey #4548 obviously increased at 3 days after the challenge, while such increases in WBC were not observed in the other monkeys (Fig 2.3A). RBC of monkey #4550 increased significantly compared with other monkeys at 7 days after the challenge, then returned to the normal range (Fig 2.3B). PLT of monkey #4550 decreased at 7 days after the challenge, then increased to the normal range again at 10 days after the challenge, and was in the normal range thereafter. In monkey #4418 challenged with SCHU P9 without immunization, PLT decreased at 7 days after the challenge (Fig 2.3C). Decreases in HGB, MCH and MCV are related to anemia. From the data, HGB, MCH and MCV of these indexes were in the normal range for all animals, except for monkey #4550 which showed an increase in HGB at 7 days after the challenge (Figs 2.3D, E, F), indicating that they didn't have anemia.

2.4.3 Necropsy findings

Two monkeys challenged with virulent SCHU P9 strain without immunization (Group-2) showed obvious clinical signs of tularemia including ascites and hydrothorax (Tables 2.3 and 2.4). In the lung of these monkeys, severe pathology of tularemia, hemorrhage (asterisk) and white lesions (arrow) were observed (Fig 2.4B). The spleen of these two animals had blunt edge and one of these spleens had white lesions (Fig 2.4D). Two monkeys challenged with virulent SCHU P9 strain after immunization with Δ*pdpC* (Group-1) showed no gross abnormalities except that one of the monkeys (#4550) had white

lesions in the lung (Figs 2.4 and 2.5). Two monkeys challenged with Δ*pdpC* without immunization (Group-3) did not show any pathological observation (Figs 2.4 and 2.5).

2.4.4 Histopathology

Monkeys challenged with virulent strain SCHU P9 without immunization (Group-2) showed obvious histopathological lesions of tularemia, i.e. severe pneumonia and abscess accompanied with necrosis in whole body organs (Fig 2.6E). Lung structures were almost destroyed and neutrophils, macrophages and purulent necrotic substances filled the lung. Necrosises were also found in the liver and spleen (Fig 2.7). Monkey #4548 showed severe lung abscess, purulent lesion accompanied with necrosis extended from the end of bronchial to the alveoli (Fig 2.6G). Lymphocytopenia was observed in the spleen. Histopathological lesions in monkeys immunized with mutant Δ*pdpC* and challenged with SCHU P9 (Group-1) were found only in the lung, however, the lesions were less severe compared with those in the monkey of Group-2 (Fig 2.6A). Monkeys challenged with Δ*pdpC* (Group-3) did not show any histopathological lesions of tularemia (Fig 2.6I and 2.6K).

2.4.5 Immunohistochemistry

By immunohistochemistry, *F. tularensis* antigens were demonstrated in the lesions in the monkeys challenged with SCHU P9 without immunization (Group-2) (Fig 2.6F and 2.6H). *F. tularensis* antigens were also demonstrated in the necrosis region of the lung of the monkey #4550 (Group-1), which

was challenged with SCHU P9 after Δ*pdpC* immunization, while *F. tularensis* antigens were not detected in the area without lesion (Fig 2.6B).

Since *F. tularensis* is intracellular bacteria, it is of interest to determine the cells infected with bacteria. Thus, I performed double immunohistochemical staining of the lung lesion to demonstrate the bacterial antigens and cell markers (Fig 2.8A), and found that the bacteria invaded not only macrophage expressing CD68, but also other cells (arrows: bacteria, asterisks: macrophage) (Fig 2.8B). Double immunohistochemical staining of *F. tularensis* antigens and ACE2 showed that the cells with *F. tularensis* (arrows) were epithelial cells and considered morphologically to be alveolar type II epithelial cells (asterisks) (Figs 2.8C).

2.4.6 Bacteria burden

Three portions of lymph nodes (cervical lymph node, axillary lymph node, and inguinal lymph node), trachea, lung, liver, spleen, heart, and kidney were submitted for bacterial culture at the time of postmortem examination except for the lymph nodes of the monkey #4548 challenged with virulent strain without immunization (Group-2) because they were atrophied and could not take enough amounts for bacterial culture. In the monkey challenged with SCHU P9 without Δ*pdpC* strain immunization (Group-2), bacteria were detected in lymph nodes, trachea, lung, liver, spleen, and heart. *F. tularensis* was also detected in pleural effusion of the monkeys (data not shown). On the other hand, *F. tularensis* was detected only in the trachea and lung of monkey #4550 (Fig 2.9A-I) among 2 monkeys immunized with

mutant Δ*pdpC* and challenged with SCHU P9 (Group-1). All the organ and tissues collected from the monkeys challenged with Δ*pdpC* alone (Group-3) were *F. tularensis* culture negative.

2.4.7 IgG and IgM antibody titer determined by ELISA

Total amount of IgG and IgM in the sera were quantified using the monkey IgG or IgM ELISA kits (Life Diagnostics, West Chester, PA, USA). Total IgG and IgM concentration in Group-2 and Group-3 at -21 dpc, before the animals were treated with placebo, were considered as the normal level. Therefore, I thought that total IgG and IgM concentrations in all animals were remaining in the normal range during this experiment period (Fig 2.10A and 2.10B).

Francisella-specific IgG and IgM antibodies were also tested in ELISA as previously reported (56). Two monkeys immunized with Δ*pdpC* (Group-1) showed significant increases in serum IgG and IgM antibodies after immunization (Fig 2.10C and 2.10D). Two monkeys treated with saline and challenged with SCHU P9 (Group-2) did not show obvious increases in IgG and IgM antibodies, while monkey #4418 showed slight increases in IgG and IgM antibodies at 7 days after SCHU P9 challenge. Two monkeys injected with saline and challenged with Δ*pdpC* (Group-3) showed increases in IgG and IgM antibodies, but the increase was less than that of the monkeys in Group-1 (Fig 2.10C and 2.10D).

2.4.8 Micro-agglutination (MA) test

MA test proved that inoculation of Δ*pdpC* strain, either as immunization or as challenge,

induced the antibody against SCHU P9. The MA antibody titers of the monkeys immunized with Δ*pdpC* strain and challenged with SCHU P9 (Group-1) were higher than 1:10. The monkeys challenged with Δ*pdpC* strain (Group-3) also showed elevated MA antibody titers at 7 to 10 days after challenge, but the increase was less than those of the monkeys in Group-1. On the other hand, MA antibody was not detected in the monkeys challenged with SCHU P9 without immunization (Group-2) (Fig 2.10E).

2.4.9 Western blotting

The western blotting (WB) using whole bacterial lysates of *F. tularensis* SCHU P9 as antigen was performed to demonstrate *Francisella*-specific IgG and IgM antibody response. It was shown that *Francisella*-specific IgG and IgM antibodies were elicited in the sera of Group-1 monkeys at 7 days after immunization with Δ*pdpC* strain (Figs 2.11 and 2.12). The ladder-like bands were detected between 17 KD to 200 KD in IgG-specific WB (Fig 2.11), and those were detected between 15 KD to 100 KD in IgM-specific WB (Fig 2.12). The sera from Δ*pdpC* immunized monkeys showed ladder-like bands from 7 days after immunization (Figs 2.11 and 2.12). Of the sera from monkeys challenged with Δ*pdpC* (Group-3), ladder-like bands were only observed in monkey #4686 at 14 days after challenge and later (Figs 2.11 and 2.12). The sera without immunization with Δ*pdpC* (Group-2) showed no ladder-like bands after the SCHU P9 challenge except for monkey #4418 whose sera showed bands just before being moribund (Figs 2.11 and 2.12).

2.5 Discussion

F. tularensis subspecies *tularensis* is a highly infectious pathogen and causes lethal disease, tularemia. In addition to natural infection to humans and animals, its potential to be used as a biological weapon highlights the necessity to develop efficient vaccines to combat against this pathogen (70). Among many methods applied against this disease, attenuated live vaccine was often applied in these years (22, 62). The study in chapter 1 proved that Δ*pdpC* mutant had potential to protect the mice from lethal intranasal challenge of virulent strain via subcutaneous immunization. In chapter 1, I also confirmed the stability of the Δ*pdpC* through *in vivo* passages in mice until 10th generation. These data suggest that Δ*pdpC* is a promising candidate for tularemia vaccine. To further evaluate the possibility of Δ*pdpC* as a candidate for tularemia vaccine, a lethal monkey model was designed to analyze a protective potential of Δ*pdpC* in this chapter.

Because symptoms of tularemia in monkeys are more similar to humans compared with a murine model (90), monkeys have been used to study both the pathogenesis and efficacy of vaccine candidates of tularemia from the last century (29, 91, 93, 94). Cynomolgus macaque is suitable for an inhalation tularemia model compared to other nonhuman primate species utilized (99), since pathology of aerosolized *F. tularensis* virulent strain SCHU S4 in cynomolgus macaques is similar to that of humans (100). So in this chapter, I used 6 cynomolgus macaques to assess protective effects of Δ*pdpC* against an intranasal challenge of virulent SCHU P9 strain.

In chapter 1, I demonstrated that intranasal infection of tularemia was not completely protected

by subcutaneous immunization, the most common and convenient route for vaccination, in the mouse tularemia model. However, I employed subcutaneous immunization with Δ*pdpC*, and intratracheal challenge in the monkey model, because it is important to establish a vaccine effective even under tough conditions. For any strain applied as a live attenuated vaccine, the safety is one of the most important properties. Therefore, one monkey group was prepared for intratracheal inoculation of Δ*pdpC* as a challenge strain and investigated its pathogenicity.

In the present study, monkeys were challenged with 1×10^6 CFU dose of SCHU P9 via intratracheal route. I employed SCHU P9 strain, because it is difficult to obtain SCHU S4 strain from foreign laboratories due to the strict regulations for transportation of this bacterium by the Japanese infectious disease control law, as described in the chapter 1. LD_{50} of SCHU P9 strain in cynomolgus macaque is not determined because the number of monkeys was limited in the experiment. However, my previous study in mouse (chapter 1) showed that the LD_{50} in mouse of SCHU P9 strain was comparable to that of SCHU S4 strain, thus it is considered that the LD_{50} of SCHU P9 strain in cynomolgus macaque is at the same level of that of SCHU S4 strain. Earlier report showed that LD_{50} of SCHU S4 is between 14 and 4447 CFU based on aerosol particle size (91). For cynomolgus macaque, the LD_{50} of SCHU S4 is approximately 1CFU (101) or 294 CFU/animal (105) via the aerosol route. Based on my previous data in 10mice (chapter 1) and other person's data about virulence of strain SCHU S4 in monkey, I used the dose of 1×10^6 CFU in this study, because this dose is thought to be far above the LD_{50} to lead the monkeys to

death. Meantime for the consideration of bioterror attack, a great quantity of virulent *F. tularensis* strains inhaled by host during aerosol release. After the challenge with virulent SCHU P9 strain without immunization with Δ*pdpC* (Group-2), the monkeys were suffered from typical symptoms of tularemia and showed histopathological lesions essentially same as the previous reports with virulent *F. tularensis* strains (26, 45).

After Δ*pdpC* immunized monkeys before challenge, six hematological parameters were all in normal range (Fig 2.3), the results proved the safety of mutant as a vaccine. Tularemia can cause anemia and thrombocytopenia in squirrel monkey (106). In my experiment, PLT of monkey #4418 and monkey #4550 decreased 7 days after challenge and increased, indicating that they did not show thrombocytopenia. HGB, MCV and MCH, index for evaluation of anemia, were all in normal range during the experiment (Figs 2.3D, 2.3E, 2.3F) and the monkeys did not show anemia.

F. tularensis was isolated from the lesions and histopathologically the bacteria invaded not only into macrophages but also into other cells (Fig 2.7B). Double immunohistochemical staining of the lung with antibodies to *F. tularensis* and ACE2 revealed that SCHU P9 strain infects type II epithelial cells (Fig 2.8C) as previously reported with different *F. tularensis* strains (107-109)

It has been reported that fever is one of the common clinical signs of tularemia in humans (20, 24, 110), and monkeys (106). In this chapter, fever was observed in the monkey challenged with virulent SCHU P9 strain without immunization (Group-2). On the other hand, monkeys immunized with Δ*pdpC*

also had fever after the challenge with SCHU P9 strain, but the fever recovered to the normal range within two weeks after the challenge (Fig 2.2B). Two monkeys challenged with Δ*pdpC* strain without immunization (Group-3) showed no fever. These results clearly indicate that Δ*pdpC* strain is safe and effective against lethal tularemia in the cynomolgus macaques.

F. tularensis-specific antibodies were shown to play an important role to protect the host from *F. tularensis* infection (72, 111, 112). In the present study, IgG- and IgM-ELISA showed that serum antibodies, both IgG and IgM, were induced in the monkeys immunized with Δ*pdpC*. MA test and WB further proved the induction of *F. tularensis* specific antibodies. It has been reported that when the *F. tularensis* infected the host by inhalation, the serum antibodies contributed to protecting tularemia by preventing the spread of bacteria from the lung to liver and spleen, and to facilitating rapid bacterial clearance in the lung which offered considerable survival advantage (71, 72). Monkey #4552 showed higher antibody titer after immunization with Δ*pdpC* than monkey #4550. Monkey #4552 did not show obvious symptoms of tularemia, while monkey #4550 showed decrease in body weight, suggesting that stronger antibody response induced in monkey #4552 might contribute to protection against tularemia. The difference in protective immunity induced in the 2 monkeys may be due to the different genetic backgrounds of the two monkeys (113). In fact, the vaccine test in the human also reported different protection effects among individual volunteer from asymptomatic to mild or severe disease (20, 24, 60).

In monkey #4550, however, although the host could not clear all pathogens and the bacterium

was detected in the trachea and lung (Figs 2.9D and 2.9E), it seems that the bacterium was prevented from the lung to other organs.

The route of immunization is also important in influencing immune response when the pathogen invades into host (69). In this chapter, I demonstrated that *F. tularensis*-specific antibody responses after inoculation with Δ*pdpC* strain was different between monkeys inoculated subcutaneously (Group-1) and those inoculated intratracheally (Group-3). The monkeys in the two groups were inoculated with the same dose of Δ*pdpC* strain, while subcutaneous inoculation of Δ*pdpC* strain induced higher antibody response than intratracheal inoculation. Thus, an optimal route of administration, as well as suitable strain for vaccine, should be considered to induce better protection against tularemia.

In conclusion, I demonstrated that Δ*pdpC* strain was safe and efficious against the lethal challenge of virulent *F. tularensis* strain in the cynomolgus macaques. The data suggested that the Δ*pdpC* can be a good candidate for live attenuated vaccine against *F. tularensis*.

Figure legends

Figure 2.1 Time schedule of animal experiment.

Six monkeys were subcutaneously inoculated with Δ*pdpC* or saline at minus 21days before challenge, and the clinical manifestations were observed daily for 21 days. Serum samples, body weights and body temperatures were taken on 21, 19, 17, 14, 11, and 7 days before challenge. SCHU P9 or Δ*pdpC* was challenged via intratracheal route at a dose of 1×10^6 CFU, and 21 days of observation was applied. Serum samples, body weights and body temperatures were taken on 2, 4, 7, 10, 14, 17, 21 days after challenge. When monkey showed severe symptoms, it was euthanized with ketamine (5 mg/kg) intramuscularly and exsanguination. Time point showed was days of blood collection, body weight and temperature measurements.

Figure 2.2 Protection from lethal challenge of *Francisella* in cynomolgus macaques immunized with Δ*pdpC*.

Six healthy, adult male Cynomolgus macaques were obtained from animal center of NIID. The monkeys were randomly separated into three groups, Group-1 (G1, blue), Group-2 (G2, red), Group-3 (G3, black), two monkeys in each group. Two monkeys in G1 were subcutaneously immunized with 1×10^6 CFU of Δ*pdpC* mutant, and the other four monkeys in G2 and G3 were injected with saline via subcutaneous route. Three weeks later, four monkeys in G1 and G2 were challenged with 10^6 of virulent SCHU P9

strain via intratracheal route, and two monkeys in G3 were challenged with Δ*pdpC* via intratracheal route. All monkeys were monitored for 3 weeks.

(A)Two monkeys in G2 injected with saline and challenged with SCHU P9 showed serious clinical signs. Monkeys became moribund on day 7 and 11 after challenge and they were euthanized. The other four monkeys did not show serious symposium.

(B)The monkeys showed fever after challenge, and then the body temperature of monkey #4418 decreased sharply before death. Two monkeys immunized with mutant Δ*pdpC* also showed fever after challenge, but after one week, the temperature decreased to normal.

(C) Body weight of two monkeys challenged with Δ*pdpC* mutant did not show obvious change. For two monkeys challenged with virulent strain SCHU P9, the body weight decreased. One of the two monkeys immunized with mutant Δ*pdpC* decreased body weight.

Figure 2.3 Complete blood test results

(A) White blood cell count (B) Red blood cell count (C) Platelet count (D) Hemoglobin concentration (E) Mean corpuscular hemoglobin (F) Mean corpuscular volume.

Figure 2.4 Macroscopic pathologies

Necropsies were performed when the cynomolgus macaques developed server clinical symptoms nearly

to die or on the day of 21 after challenge. (A) Lung of the monkey challenged with Δ*pdpC* did not show pathological changes. (B) In the lung of monkeys challenged with SCHU P9 without immunization with Δ*pdpC*, severe pathology of tularemia, hemorrhage (asterisk) and white lesions (arrow) were detected. (C) Spleen of the monkey challenged with Δ*pdpC* did not show symptom. (D) Spleen of two animals challenged with SCHU P9 without immunization with Δ*pdpC* had blunt edge and spleen of monkey #4418 had white lesions.

Figure 2.5 Lung pathologies of monkeys

Lungs after dissection. (A) Monkey #4450, challenged with SCHU P9 after immunization with Δ*pdpC*, had white lesions. (B) Monkey #4552, challenged with SCHU P9 after immunization with Δ*pdpC*, did not have changes. (C and D) In the lung of two monkeys challenged with SCHU P9 without immunization with Δ*pdpC* showed white solid lesions. (E and F) Lungs of monkeys challenged with Δ*pdpC* were normal.

Figure 2.6 Histopathology and immunohistochemistry of the lung

Monkeys immunized with mutant Δ*pdpC* and challenged with SCHU P9 exhibited less severe histopathogical changes in the lung compared to the monkeys challenged with SCHU P9 without immunization with Δ*pdpC*. (A) In monkey #4550, abscess and neutrophil accumulation (asterisk) were

observed. (B) *F. tularensis* antigen was detected in the necrosis lesions of the lung of monkey # 4550. (C) Monkey #4552 did not show clinical symposiums. (D) Monkey #4552 showed *F. tularensis* antigen negative. (E) Monkey #4548 challenged with SCHU P9 without immunization with Δ*pdpC* and euthanized on 7 days after challenge showed severe lung abscess, purulent lesion accompanied with necrosis (asterisks) extended from the end of bronchial to the alveoli. Lung structures were destroyed and lumen of airways and adjacent alveolar spaces were filled with neutrophils, macrophage and purulent necrotic substances. (F) Lung of monkey #4548 showed *F. tularensis* antigen positive (arrows). (G) Monkey #4418, challenged with SCHU P9 without immunization with Δ*pdpC*, showed lung abscess (asterisks). Lung structures were almost destroyed, and neutrophils, macrophage and purulent necrotic substances filled the lung. (H) Lung of monkey #4418 showed *F. tularensis* antigen positive (arrows). (I) Monkey #4549, immunized with saline and challenged with mutant Δ*pdpC*, did not show clinical characteristics of tularemia. (J) Monkey #4549 was *F. tularensis* antigen negative. (K) Monkey #4686 immunized with saline and challenged with Δ*pdpC* did not show clinical characteristics of tularemia. (L) Monkey #4686 showed *F. tularensis* antigen negative.

Figure 2.7 Histopathology in the spleen and liver of monkey #4418

Monkey #4418, challenged with SCHU P9 without immunization with Δ*pdpC* showed severe pathologies in the spleen and liver. (A) Necrotic nests (asterisks) were detected and (B) *F. tularensis* antigen (arrow)

was positive in the spleen. (C) Hepatocyte necrosis (asterisk) was detected and (D) *F. tularensis* antigen (arrow) was positive in the liver.

Figure 2.8 Double immunohistochemical staining

Double immunohistochemical staining of the lung of monkey #4458 proved that *F. tularensis* is intracellular bacterium. Bacteria invaded not only macrophage (A, red (arrows): bacteria, black (asterisks): macrophage), but also other cells (B). Double immunohistochemical staining of *F. tularensis* and ACE2 showed that the invaded cells were type II epithelial cells (C, brown (arrows): bacteria, red (asterisks): type II epithelia).

Figure 2.9 Bacterial burden

From all organs of two monkeys challenged with SCHU P9 without immunization (monkey #4418 and monkey #4458), *F. tularensis* was detected. In two animals challenged with Δ*pdpC* (monkey #4549 and monkey #4686), *F. tularensis* was not detected. One of the two animals immunized with Δ*pdpC* and challenged with SCHU P9 showed *F. tularensis* in trachea and lung (monkey #4550), while bacterium was not detected in monkey #4552.

NA: Not applicable.

Figure 2.10 Results of serological tests

Total amount of IgG (A) and IgM (B) were quantified with ELISA method. IgG and IgM increased after Δ*pdpC* inoculation. *F. tularensis* specific IgG (C) and IgM (D) also increased 7-10 days after inoculation with Δ*pdpC*. MA test confirmed the ELISA result (E). Group 1: blue, group 2: red, group 3: black.

Figure 2.11 Western blotting for IgG

IgG reactive to SCHU P9 antigen in monkey sera from different time point were tested by western blotting method. The sera from Δ*pdpC* immunized monkeys showed ladder-like bands from 7 days after immunization. The sera form monkeys challenged with Δ*pdpC*, only monkey #4686 showed ladder-like bands from 14 days after challenge. In the sera without immunization with the mutant Δ*pdpC*, no ladder-like banding pattern was detected except that monkey #4418 showed bands on the day before euthanization. The bands for the IgG were detected between 17KD to 200KD.

Figure 2.12 Western blotting for IgM

IgM reactive to SCHU P9 antigen in monkey sera from different time point were tested by western blotting method. The sera from Δ*pdpC* immunized monkeys showed ladder-like bands from 7 days after immunization. The sera form monkeys challenged with Δ*pdpC*, only monkey #4686 showed ladder-like bands from 14 days after challenge. In the sera without immunization with the mutant Δ*pdpC*, no

ladder-like banding pattern was detected except that monkey #4418 showed bands on the day before euthanization. The bands for the IgM were detected between 15KD to 100KD.

Figures and Tables

Fig 2.1

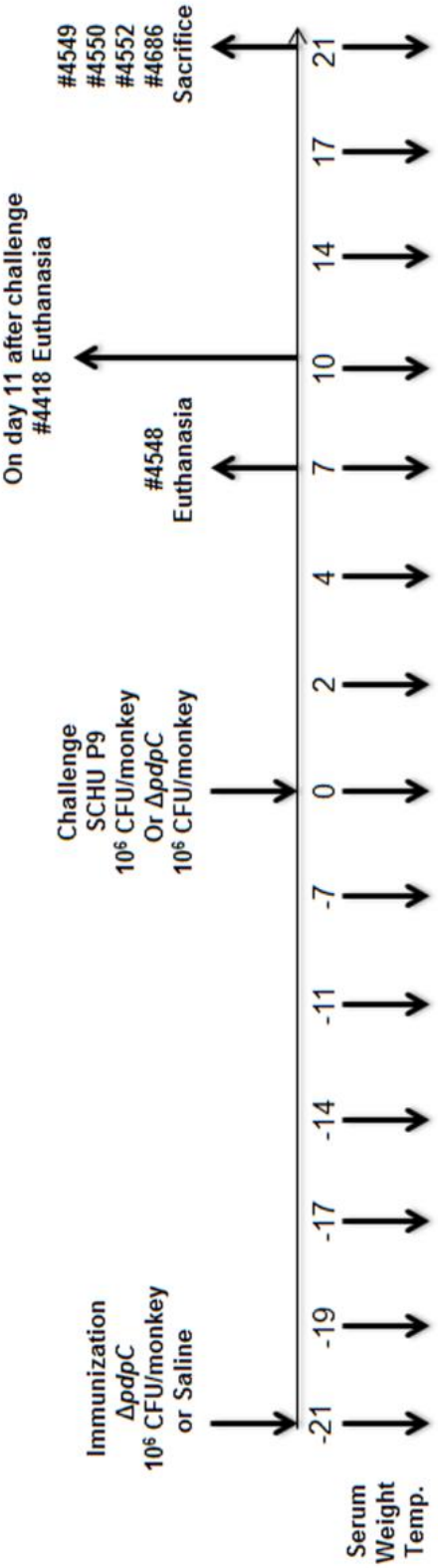


Fig 2.2

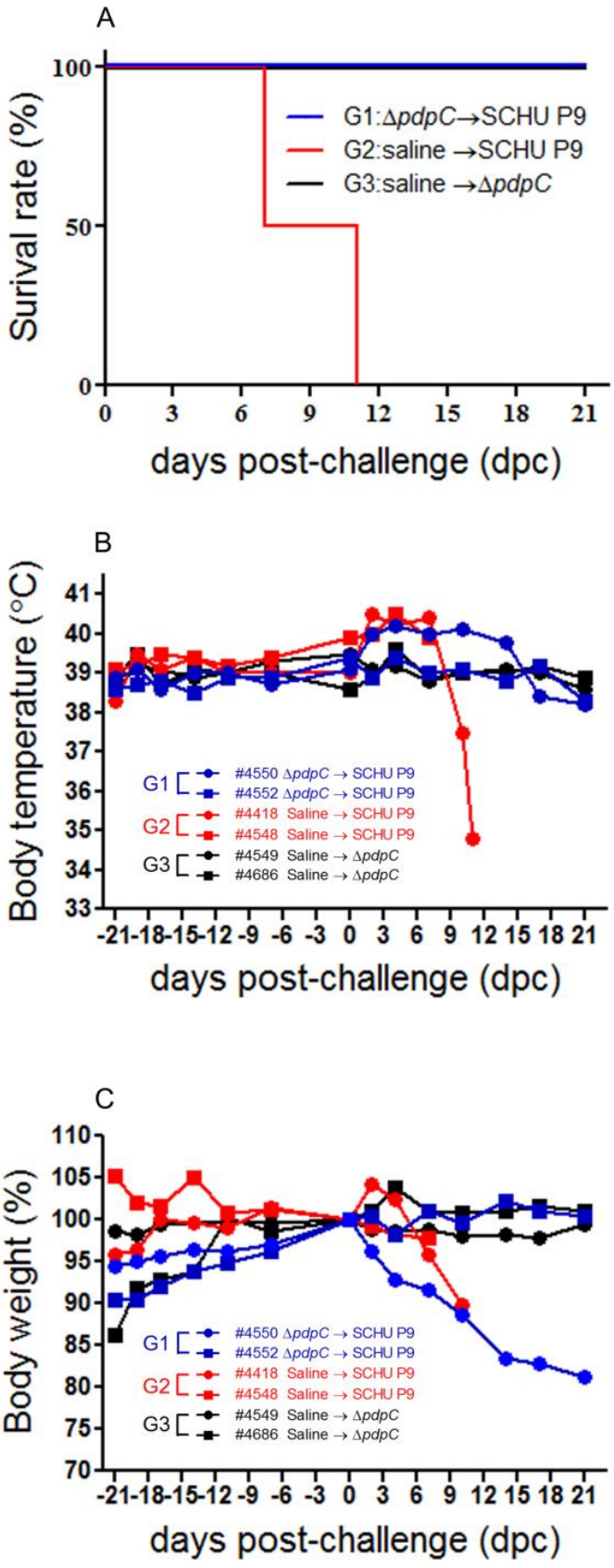

A
Surival rate (%)
100
50
0
0 3 6 9 12 15 18 21
days post-challenge (dpc)
G1:ΔpdpC→SCHU P9
G2:saline →SCHU P9
G3:saline →ΔpdpC
B
Body temperature (°C)
41 40 39 38 37 36 35 34 33
-21 -18 -15 -12 -9 -6 -3 0 3 6 9 12 15 18 21
days post-challenge (dpc)
G1
#4550 ΔpdpC → SCHU P9
#4552 ΔpdpC → SCHU P9
G2
#4418 Saline → SCHU P9
#4548 Saline → SCHU P9
G3
#4549 Saline → ΔpdpC
#4686 Saline → ΔpdpC
C
Body weight (%)
110 105 100 95 90 85 80 75 70
-21 -18 -15 -12 -9 -6 -3 0 3 6 9 12 15 18 21
days post-challenge (dpc)
G1
#4550 ΔpdpC → SCHU P9
#4552 ΔpdpC → SCHU P9
G2
#4418 Saline → SCHU P9
#4548 Saline → SCHU P9
G3
#4549 Saline → ΔpdpC
#4686 Saline → ΔpdpC

Fig 2.3

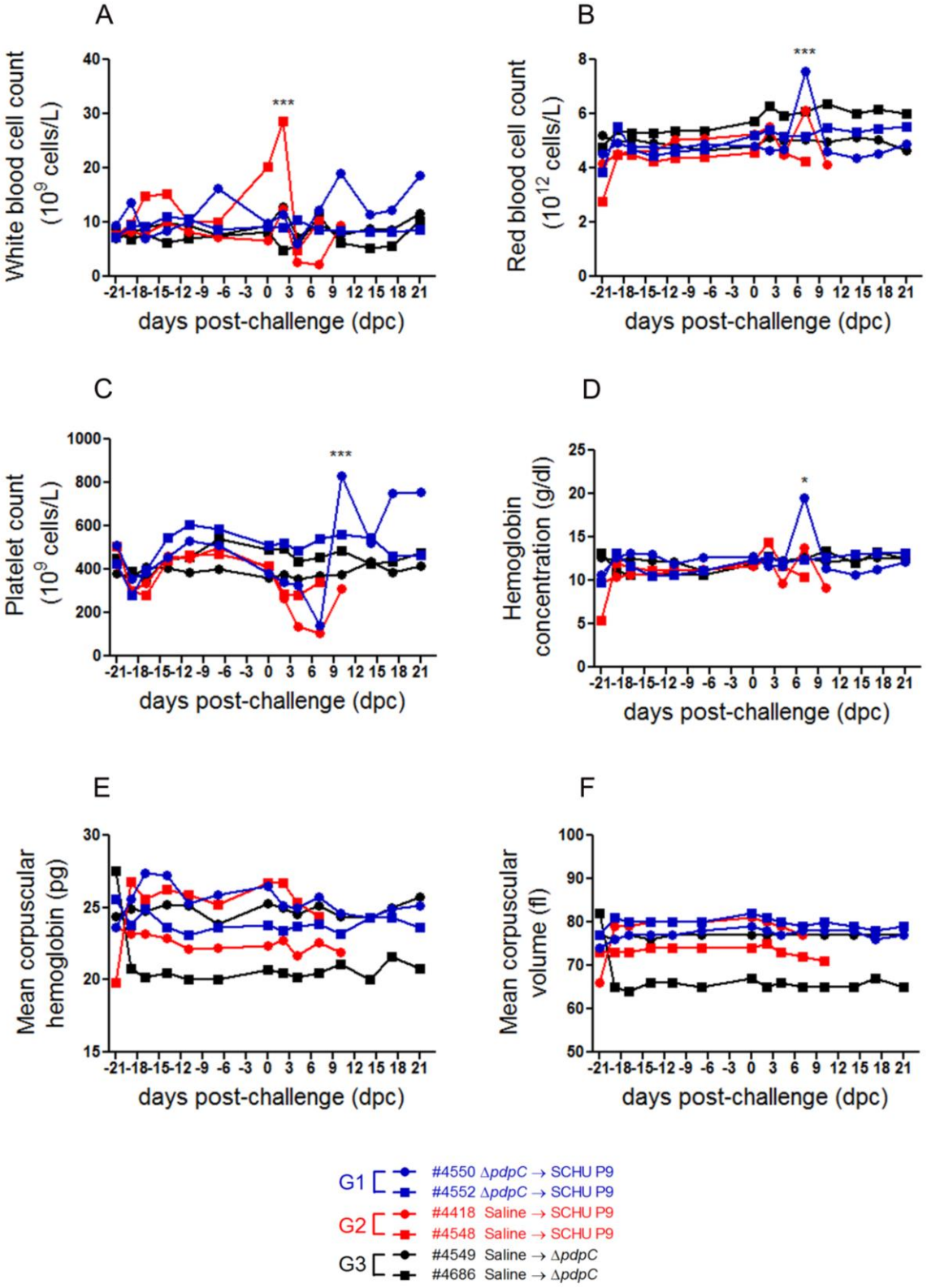
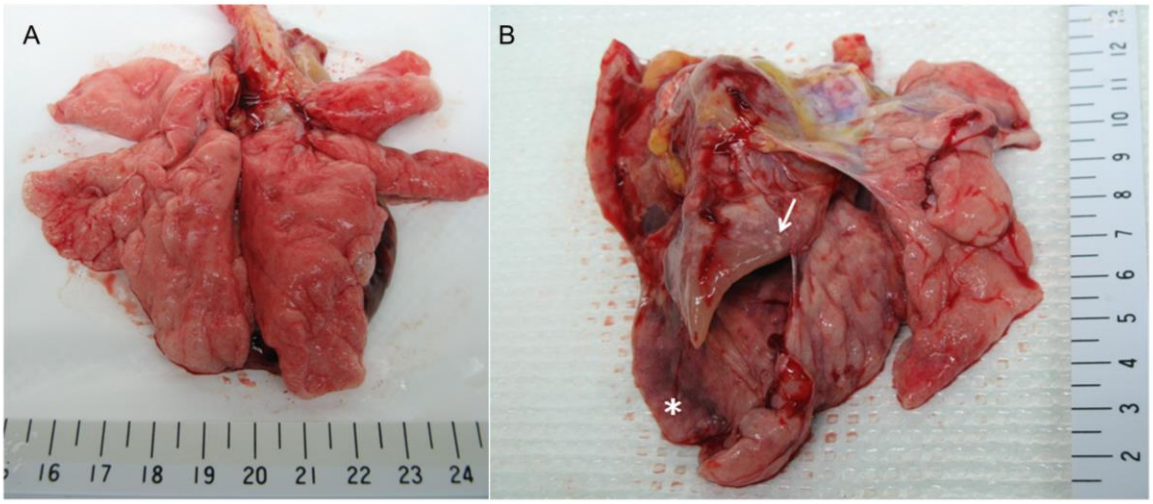
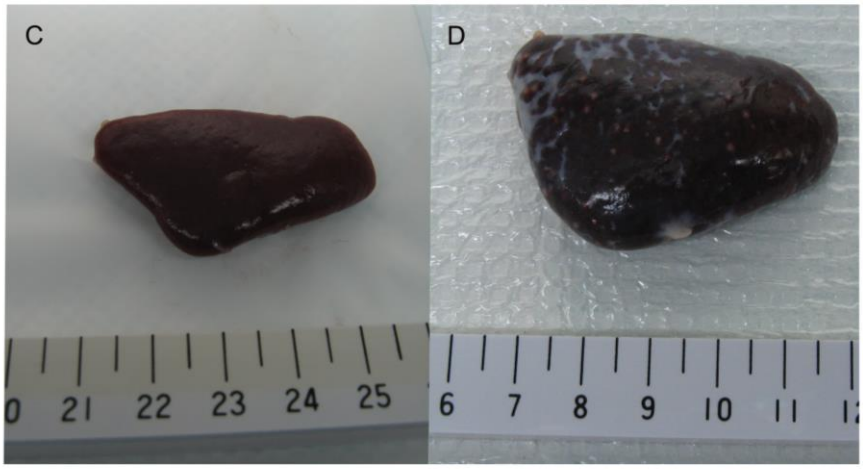

A
White blood cell count (10^9 cells/L)
***
days post-challenge (dpc)
B
Red blood cell count (10^{12} cells/L)
***
days post-challenge (dpc)
C
Platelet count (10^9 cells/L)
***
days post-challenge (dpc)
D
Hemoglobin concentration (g/dl)
*
days post-challenge (dpc)
E
Mean corpuscular hemoglobin (pg)
days post-challenge (dpc)
F
Mean corpuscular volume (fl)
days post-challenge (dpc)
G1 #4550 ΔpdpC → SCHU P9
#4552 ΔpdpC → SCHU P9
G2 #4418 Saline → SCHU P9
#4548 Saline → SCHU P9
G3 #4549 Saline → ΔpdpC
#4686 Saline → ΔpdpC

Fig 2.4



#4686 saline→Δ*pdpC* #4548 saline→SCHU P9



#4686 saline→Δ*pdpC* #4418 saline→SCHU P9

Fig 2.5

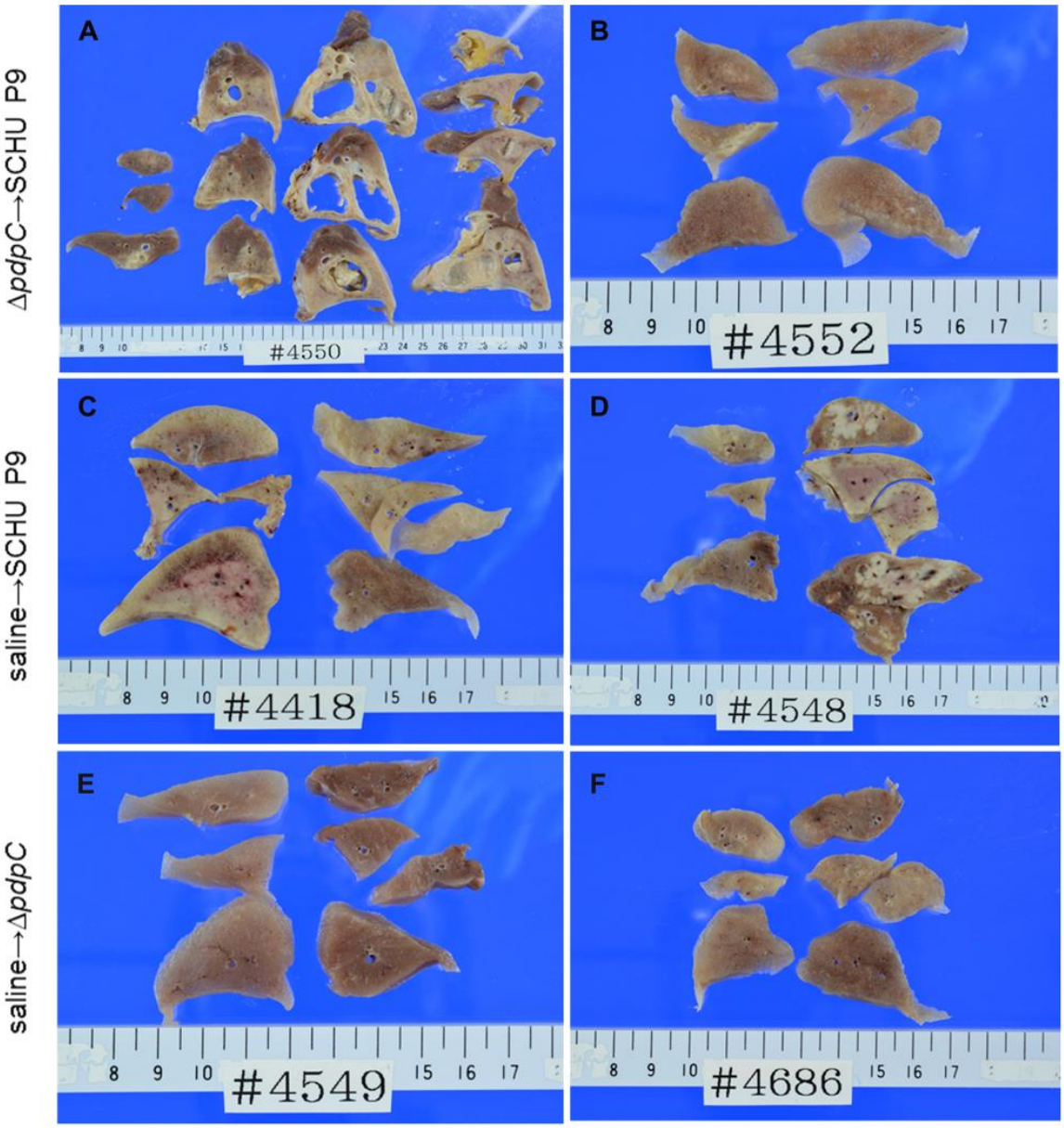

ΔpdpC→SCHU P9
saline→SCHU P9
saline→ΔpdpC
A
#4550
B
#4552
C
#4418
D
#4548
E
#4549
F
#4686

Fig 2.6

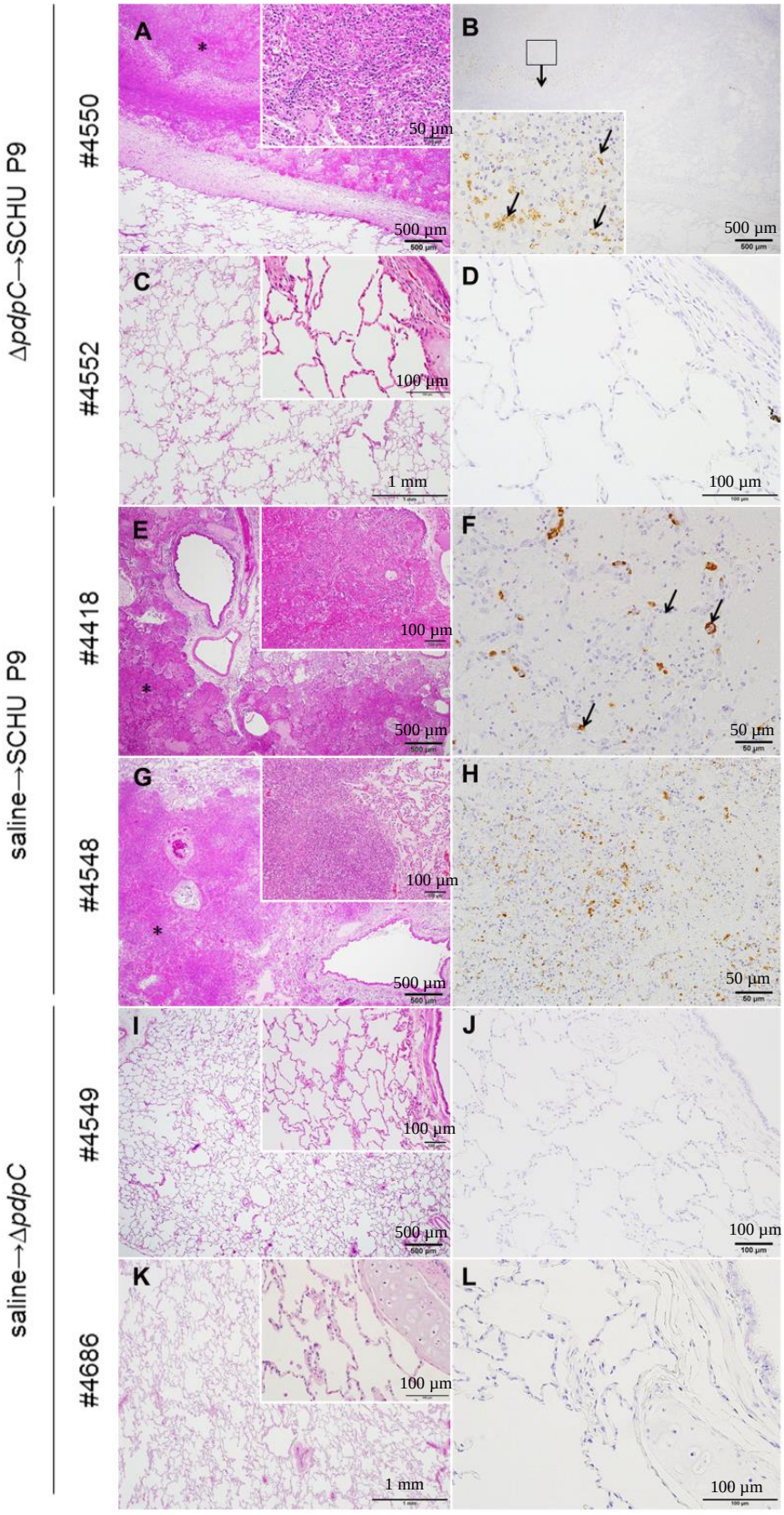
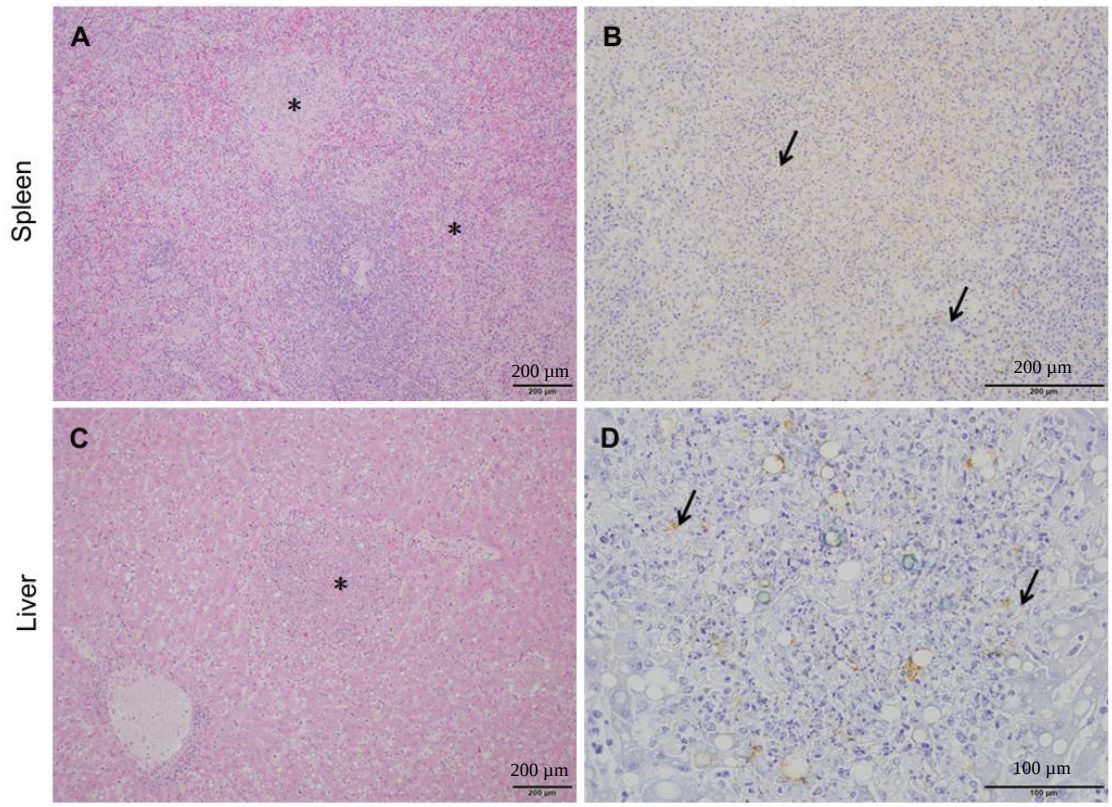

ΔpdpC→SCHU P9
saline→SCHU P9
saline→ΔpdpC
#4550
#4552
#4418
#4548
#4549
#4686
A
B
C
D
E
F
G
H
I
J
K
L
50 µm
500 µm
100 µm
1 mm

Fig 2.7



#4418 saline→SCHU P9

Fig 2.8

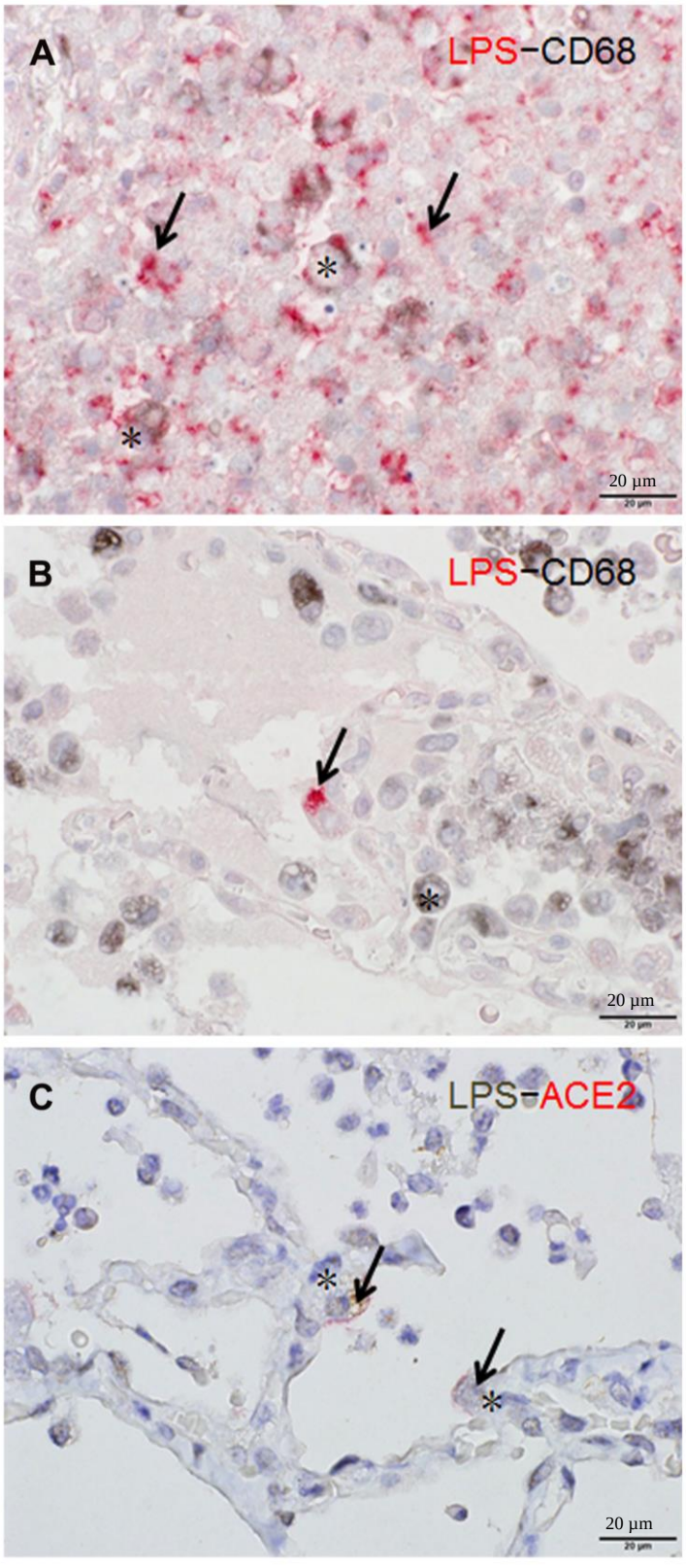


Fig 2.9

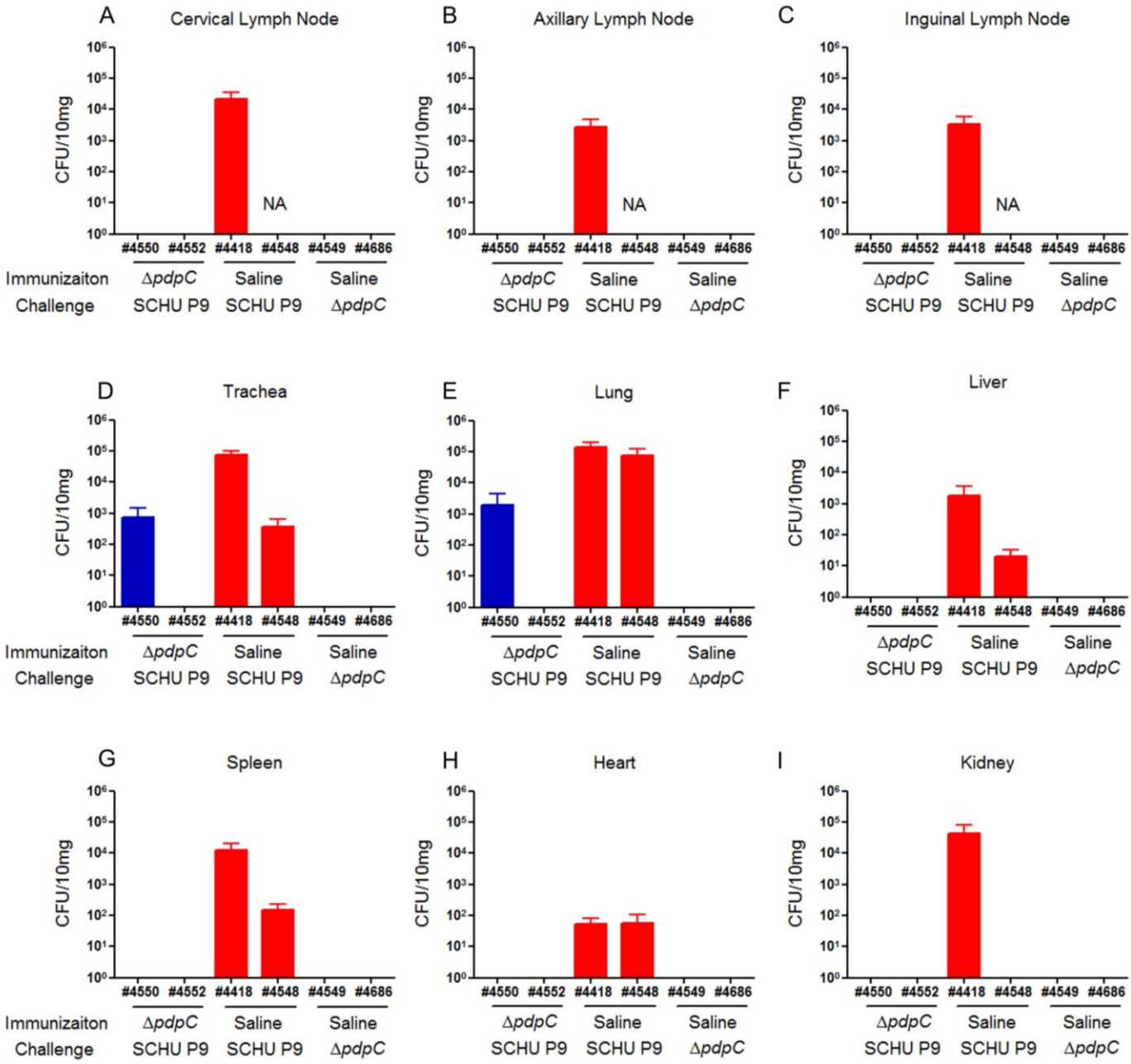

A
Cervical Lymph Node
B
Axillary Lymph Node
C
Inguinal Lymph Node
D
Trachea
E
Lung
F
Liver
G
Spleen
H
Heart
I
Kidney
CFU/10mg
NA
#4550 #4552 #4418 #4548 #4549 #4686
Immunizaiton
Challenge
ΔpdpC
SCHU P9
Saline
SCHU P9
Saline
ΔpdpC

Fig 2.10

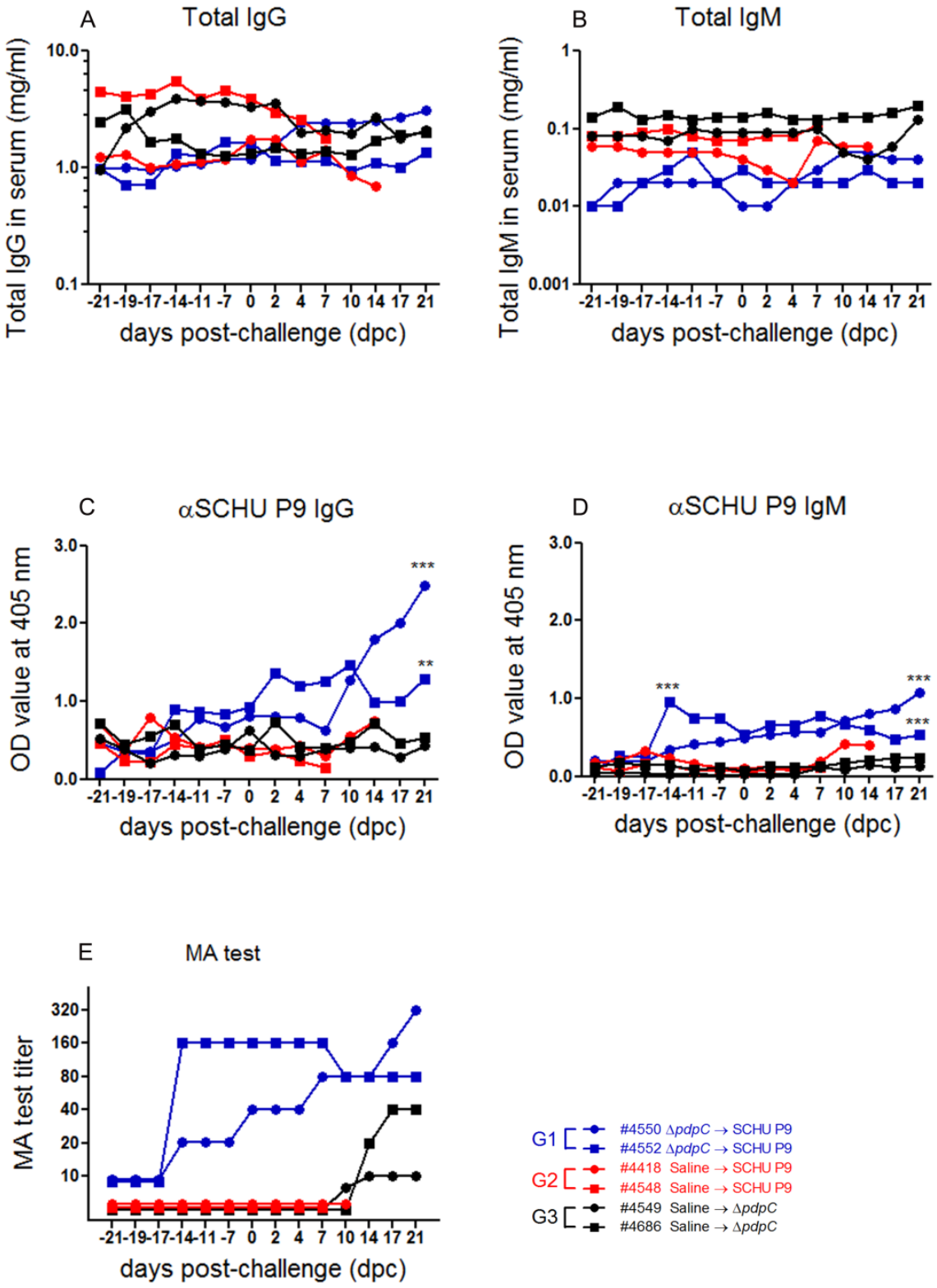

A
Total IgG
Total IgG in serum (mg/ml)
10.0
1.0
0.1
-21 -19 -17 -14 -11 -7 0 2 4 7 10 14 17 21
days post-challenge (dpc)
B
Total IgM
Total IgM in serum (mg/ml)
1
0.1
0.01
0.001
-21 -19 -17 -14 -11 -7 0 2 4 7 10 14 17 21
days post-challenge (dpc)
C
αSCHU P9 IgG
OD value at 405 nm
3.0
2.0
1.0
0.0
***
**
-21 -19 -17 -14 -11 -7 0 2 4 7 10 14 17 21
days post-challenge (dpc)
D
αSCHU P9 IgM
OD value at 405 nm
3.0
2.0
1.0
0.0
***
***
***
-21 -19 -17 -14 -11 -7 0 2 4 7 10 14 17 21
days post-challenge (dpc)
E
MA test
MA test titer
320
160
80
40
20
10
-21 -19 -17 -14 -11 -7 0 2 4 7 10 14 17 21
days post-challenge (dpc)
G1
#4550 ΔpdpC → SCHU P9
#4552 ΔpdpC → SCHU P9
G2
#4418 Saline → SCHU P9
#4548 Saline → SCHU P9
G3
#4549 Saline → ΔpdpC
#4686 Saline → ΔpdpC

Fig 2.11

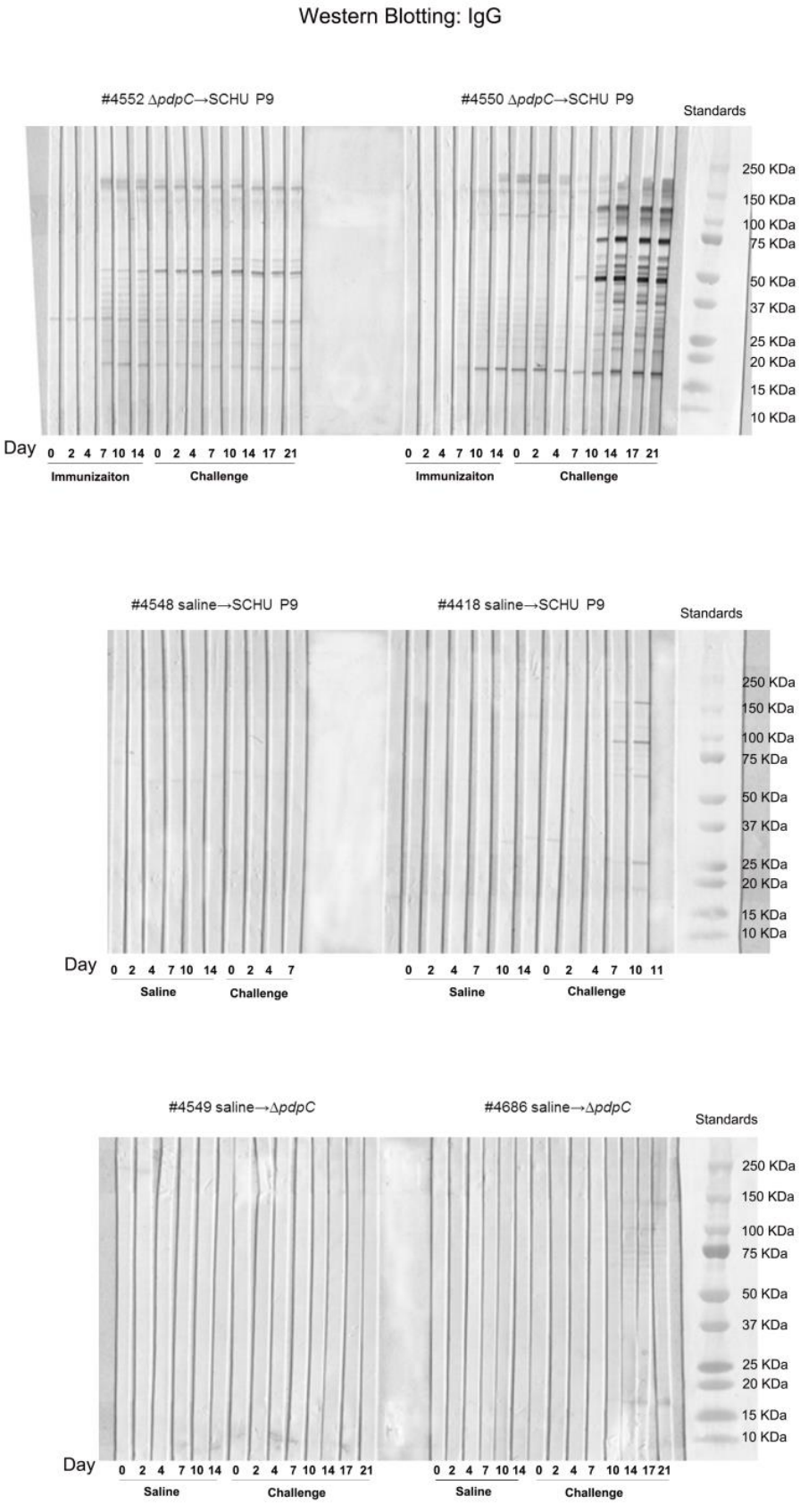
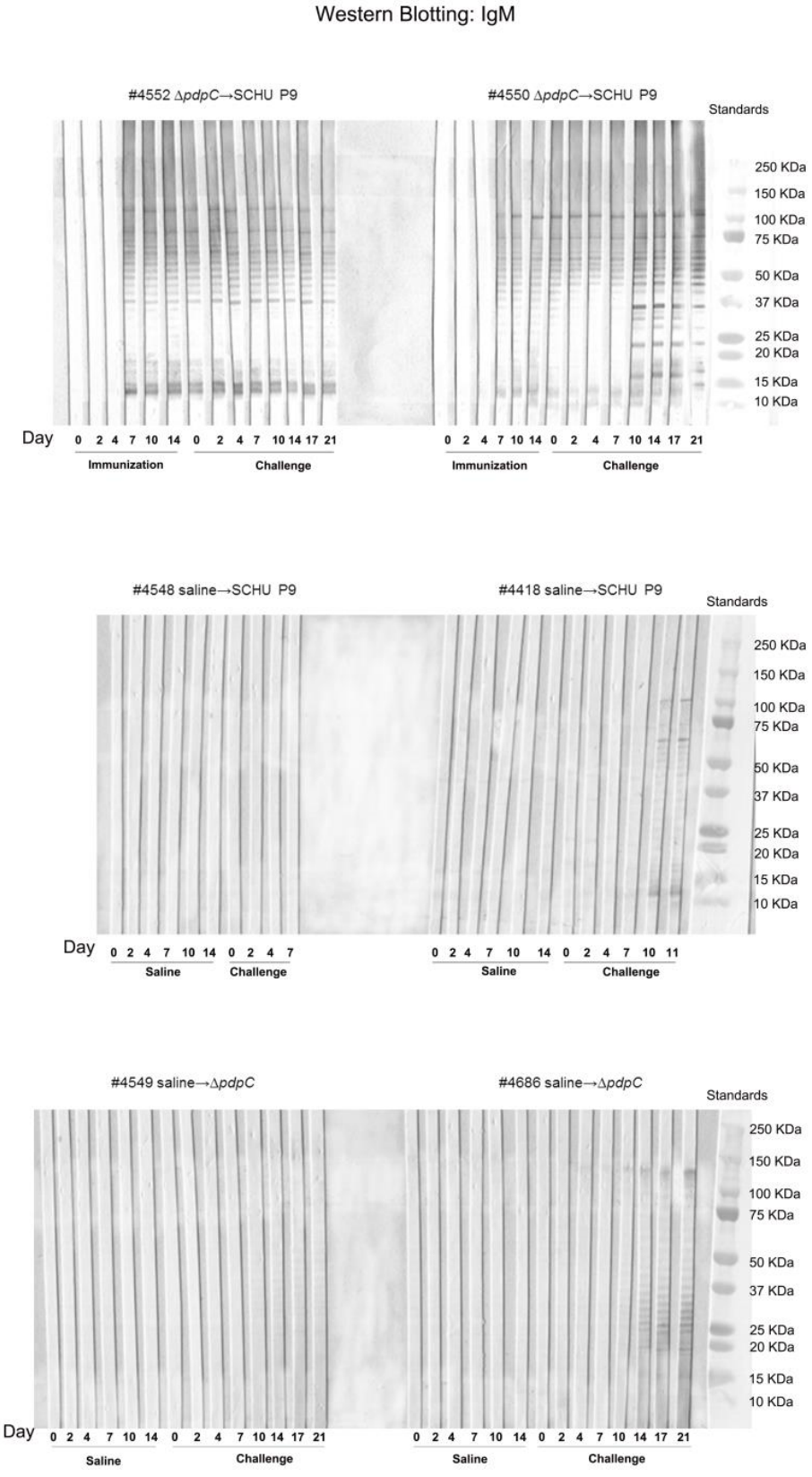

Western Blotting: IgG
#4552 ΔpdpC→SCHU P9
#4550 ΔpdpC→SCHU P9
Standards
250 KDa
150 KDa
100 KDa
75 KDa
50 KDa
37 KDa
25 KDa
20 KDa
15 KDa
10 KDa
Day
0 2 4 7 10 14 0 2 4 7 10 14 17 21
Immunizaiton
Challenge
0 2 4 7 10 14 0 2 4 7 10 14 17 21
Immunizaiton
Challenge
#4548 saline→SCHU P9
#4418 saline→SCHU P9
Standards
250 KDa
150 KDa
100 KDa
75 KDa
50 KDa
37 KDa
25 KDa
20 KDa
15 KDa
10 KDa
Day
0 2 4 7 10 14 0 2 4 7
Saline
Challenge
0 2 4 7 10 14 0 2 4 7 10 11
Saline
Challenge
#4549 saline→ΔpdpC
#4686 saline→ΔpdpC
Standards
250 KDa
150 KDa
100 KDa
75 KDa
50 KDa
37 KDa
25 KDa
20 KDa
15 KDa
10 KDa
Day
0 2 4 7 10 14 0 2 4 7 10 14 17 21
Saline
Challenge
0 2 4 7 10 14 0 2 4 7 10 14 17 21
Saline
Challenge

Fig 2.12


Western Blotting: IgM
#4552 ΔpdpC→SCHU P9
#4550 ΔpdpC→SCHU P9
Standards
250 KDa
150 KDa
100 KDa
75 KDa
50 KDa
37 KDa
25 KDa
20 KDa
15 KDa
10 KDa
Day
0 2 4 7 10 14 0 2 4 7 10 14 17 21
Immunization
Challenge
0 2 4 7 10 14 0 2 4 7 10 14 17 21
Immunization
Challenge
#4548 saline→SCHU P9
#4418 saline→SCHU P9
Standards
250 KDa
150 KDa
100 KDa
75 KDa
50 KDa
37 KDa
25 KDa
20 KDa
15 KDa
10 KDa
Day
0 2 4 7 10 14 0 2 4 7
Saline
Challenge
0 2 4 7 10 14 0 2 4 7 10 11
Saline
Challenge
#4549 saline→ΔpdpC
#4686 saline→ΔpdpC
Standards
250 KDa
150 KDa
100 KDa
75 KDa
50 KDa
37 KDa
25 KDa
20 KDa
15 KDa
10 KDa
Day
0 2 4 7 10 14 0 2 4 7 10 14 17 21
Saline
Challenge
0 2 4 7 10 14 0 2 4 7 10 14 17 21
Saline
Challenge

Tables

Table 2.1 Immunization and challenge strain, route and dose of *F. tularensis* to each monkey.

Groups	Animal No.	Immunization			Challenge			Time of Death (days)
		Strain	Route	Dose	Strain	Route	Dose	
Group-1	#4550	Δ*pdpC*	s.c.	10^6	SCHU P9	i.t.	10^6	NA
	#4552	Δ*pdpC*	s.c.	10^6	SCHU P9	i.t.	10^6	NA
Group-2	#4418	Saline	s.c.	10^6	SCHU P9	i.t.	10^6	11
	#4548	Saline	s.c.	10^6	SCHU P9	i.t.	10^6	7
Group-3	#4549	Saline	s.c.	10^6	Δ*pdpC*	i.t.	10^6	NA
	#4686	Saline	s.c.	10^6	Δ*pdpC*	i.t.	10^6	NA

Abbreviation: s.c., subcutaneous; i.t., intratracheal; NA, Not applicable.

Table 2.2 Clinical scores of monkeys.

Clinical Parameters		Score	Group-1		Group-2		Group-3	
			#4550	#4552	#4418	#4548	#4549	#4686
Activity	Normal	1		*			*	*
	Less active	2	*					
	Inactive	3			*	*		
Behavior	Normal	1	*	*			*	*
	Depressed	2			*			
	Ignoring everything	3				*		
Stimulation response	Normal	1	*	*			*	*
	Entering room	2						
	Approaching cage	3			*	*		
Breathing	Normal	1	*	*			*	*
	Rapid	2						
	Abdominal	3			*	*		
Stool	Normal	1	*	*			*	*
	Loose stool	2			*	*		
	Liquid stool	3						
Total			6	5	13	14	5	5

Table 2.3 Comparison of SCHU P9 infection features in monkeys.

	Group-1	Group-2	Group-3
General infection status			
Severity	Mild	Death	Mild
Major symptom(s)	Cough (#4550)	High fever, cough	No abnormal appearance
Clinical scores	Low	High	Low
Food and water intake	Normal	Little	Normal
Appearance	Normal	Abnormal	Normal
Histopathological findings in lesions			
Lymph node	Not obvious at last stage	Enlarge and hemorrhage (#4418) or Atrophy (#4548)	Little enlarge
Lung	Weak congestion (#4552)	White lesions	Not obvious at last stage
Spleen	Not obvious at last stage	White lesions	Not obvious at last stage
Liver	Not obvious at last stage	Not obvious at last stage	Not obvious at last stage
IgG expression	Positive	Negative	Weak positive
IgM expression	Positive	Negative	Weak positive

Table 2.4 Macroscopic lesions observed in the monkeys at necropsy.

Lesions	Group-1		Group-2		Group-3	
	#4550	#4552	#4418	#4548	#4549	#4686
Cervical lymph node	–	–	+(a,c)	+(b)	+(a)	+(a)
Axillary lymph node	–	–	+(a,c)	+(b)	+(a)	+(a)
Inguinal lymph node	–	–	+(a)	+(b)	+(a)	+(a)
Lung	–	+(c)	+(a,c)	+(a,c)	–	–
Liver	–	–	+(a)	–	–	–
Spleen	+(a)	–	+(a)	+(a)	–	–
Heart	–	–	–	–	–	–
Kidney	–	–	–	–	–	–
Ascites	–	–	+	+	–	–
Hydrothorax	–	–	+	+	–	–

+ or – indicated the presence or absence of the gross lesion

a, enlarged; b, atrophy; c, congestion and/or hemorrhage.

General Conclusions

Tularemia is zoonotic disease and the causative agent *Francisella tularensis* is a gram-negative intracellular coccobacillus. Although natural infections by *F. tularensis* are sporadic and generally localized now, the low infectious dose of this bacterium can cause severe disease and it has the ability to transmit to humans via multiple routes. These characteristics give this pathogen a potential to be used as a bioweapon and cause much attention. In order to protect the host from tularemia, a lot of studies had been applied against the disease and vaccine is thought to be an efficient method to prevent this disease. In this thesis, attenuated *F. tularensis* strain, Δ*pdpC*, in which the pathogenicity determinant protein C gene (*pdpC*) was disrupted by TargeTron mutagenesis, was investigated as a potential vaccine candidate for tularemia in mouse and monkey models. In order to identify contribution of vaccine-induced immunity against infection of virulent strain SCHU P9, I did a series of experiments to evaluate the safeness and efficacy of this mutant Δ*pdpC*.

In chapter 1, firstly Δ*pdpC* was passaged serially in mice to confirm its stable attenuation. Low bacterial loads in mouse spleens persisted during the 10 passages. Statistically significant changes in CFU were not observed during *in vivo* passages of Δ*pdpC*. Further, the inserted intron sequences for disrupting *pdpC* gene were completely maintained even after the 10th passage in mice. Considering the stable attenuation and maintenance of intron sequence, it was suggested that Δ*pdpC* is a promising tularemia vaccine candidate.

I estimated the LD_{50} of *F. tularensis* SCHU P9 strain via i.m., s.c., and i.n. routes, and showed

that quite small dose of the bacteria could lead mice to death within one week.

Further, I analyzed an efficacy of Δ*pdpC* as a live attenuated vaccine candidate of tularemia using mouse model. I immunized C57BL/6J mice with 1×10^6 CFU of Δ*pdpC* via three routes, i.m., s.c., and i.n. and challenged them with 100 LD_{50} of a virulent SCHU P9 strain at 21 days after immunization from the same route as immunization. All mice immunized intramuscularly were protected against challenge with 100 LD_{50} of the SCHU P9 strain. Conversely, the survival rates of the mice immunized subcutaneously and intranasally were 60% and 40%, respectively, when 100 LD_{50} of SCHU P9 was challenged via the same routes as immunization. Thirty-eight % of the mice survived after intranasal 100 LD_{50} dose of SCHU P9 when they were immunized subcutaneously.

After I confirmed the efficacy of the immunization with Δ*pdpC* strain in the mouse model, I tested the Δ*pdpC* strain in nonhuman primate model in chapter 2. In this chapter, six cynomolgus macaques were divided into 3 groups. Two animals were intratracheally challenged with 1×10^6 CFU of virulent SCHU P9 strain 3 weeks after cubcutaneous inoculation of 1×10^6 CFU of Δ*pdpC* (Group-1). Two animals were intratracheally challenged with 10^6 CFU of virulent SCHU P9 strain 3 weeks after the inoculation of placebo (Group-2). Two animals were intratracheally challenged with 1×10^6 CFU of Δ*pdpC* 3 weeks after placebo inoculation (Group-3). The animals were observed for clinical symptoms and survival for 3 weeks after challenge. The monkeys challenged with SCHU P9 strain without previous immunization with Δ*pdpC* strain (Group-2) were euthanized on 7 and 11 days after challenge when they

showed severe clinical signs of tularemia such as fever, cough, bad appetite, and recumbent. On the other hand, two monkeys immunized with Δ*pdpC* strain (Group-1) survived for 3 weeks after SCHU P9 challenge, though one of the 2 monkeys developed mild symptoms of tularemia. Both of the monkeys showed high level of antibodies against *F. tularensis*. Two monkeys challenged with Δ*pdpC* strain showed no apparent clinical signs. These results indicated that Δ*pdpC* strain is a safe and promising candidate for tularemia vaccine in monkey model.

Based on my data in both mouse and monkey models, it is suggested that Δ*pdpC* is a promising candidate of tularemia live attenuated vaccine.

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Abbreviations

Ag: antigen

BSL3: Biosafety level 3

CDM: Chamberlain's defined medium

CFU: Colony forming unit

DNA: Deoxyribonucleic acid

dpc: days post challenge

dpi: days post inoculation

DW: distilled water

ELISA: Enzyme-linked immunosorbent assay

F.: *Francisella*

FPI: *Francisella* Pathogenicity Island

G: Gram

H: Hour

i.d.: Intradermal

IFNγ: Interferon gamma

Ig: Immunoglobulin

IgG: Immunoglobulin G

IgM: Immunoglobulin M

IL: Interleukin

i.m.: Intramuscular

i.n.: Intranasal

i.p.: Intraperitoneal

i.t.: Intratracheal

kg: Kilogram

L: Liter

LD_{50}: median lethal dose

LPS: Lipopolysaccharide

LVS: Live vaccine strain

Mg: Milligram

Min: Minute

Ml: Milliliter

NCBI: National Center for biotechnology Information

NIID: National Institute of Infectious Disease

OD: Optical density

OD_{405}: optical density at 405 nm

OD_{600}: optical density at 600 nm

PBS: Phosphate buffered saline

PBST: PBS containing 0.1%Tween 20

PCR: Polymerase chain reaction

pdpC: pathogenicity determinant protein C

s.c.: subcutaneously

SD: standard deviation

TNF-α: Tumor necrosis factor alpha

type A: *Francisella tularensis* subspecies *tularensis*

type B: *Francisella tularensis* subspecies *holarctica*

µg: Microgram

µl: Microliter

µm: Micrometer

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