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Study on Immune Response to Antigens of *Francisella tularensis* using Humanized Mice Model

인간화마우스 모델을 활용한 *Francisella tularensis* 항원의 면역반응에 관한 연구

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ABSTRACT

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Francisella tularensis (*FT*), a highly infectious pathogen, is considered to be a potential biological weapon because of its low infectious dose, and the ability of aerosol transmission. Although an attenuated *FT* live vaccine strain (LVS) has been developed, this vaccine exhibits side effects and residual virulence. Therefore, subunit vaccines using parts of pathogens have been proposed as an alternative to live vaccines. Tul4 and FopA

are outer membrane proteins of *FT*, and have been reported to play an important role in the bacterium's immunogenicity, which support the potential of subunit vaccines as candidates against *FT*.

Although *in vitro* and mouse models are standard paradigms for vaccine development, these models have limitations including differences in susceptibility to *FT* between mice and humans. "Humanized mice" (hu-mice) bearing the human immune system have been developed to study human-specific diseases, and have been reported to produce human immunoglobulins against pathogens. Therefore, hu-mice have the potential to overcome the limitations of animal models used in clinical research. In this study, we reported that a subunit vaccine constructed based upon outer membrane epitopes, Tul4 and FopA, elicited the human-specific immunity in hu-mice model.

The study described in Chapter I reported the *in vitro* and *in vivo* immune response of a subunit vaccine comprising the recombinant peptides Tul4 and FopA generated from epitopes on *FT* outer membrane proteins. Dendritic cells (DCs) stimulated by recombinant peptides with adjuvant CpG oligodeoxynucleotide induced robust immunophenotypic changes in DC maturation and secretion of inflammatory cytokines (interleukin [IL]-6 and IL-12). In addition, the matured DCs enabled *ex vivo* proliferation of naive splenocytes in mixed lymphocyte reactions. Finally, I investigated *in vivo* immune responses by assessing antibody production in C57BL/6 mice. Total immunoglobulin (Ig) G was produced after immunization and levels peaked 6 weeks later. Moreover, Tul4-specific IgG was confirmed in mice receiving peptides with or without CpG. Based on these results, I revealed that the recombinant peptides Tul4 and FopA

have immunogenicity, and could be a safe subunit vaccine candidate against *FT*.

Chapter II described the immune response of mice—humanized with human CD34+ cells (hu-mice)—to a cocktail of recombinant Tul4 and FopA (rTul4 and rFopA), which were codon-optimized and expressed in *Escherichia coli*. Not only did the cocktail-immunized hu-mice produce a significant human immunoglobulin response, they also exhibited prolonged survival against an LVS, as well as human T cells in the spleen. These results suggest that a cocktail of rTul4 and rFopA had successfully induced an immune response in the hu-mice, demonstrating the potential of this mouse model for use in the evaluation of *FT* vaccine candidates.

In conclusion, the present study demonstrates the efficacy of recombinant Tul4 and FopA vaccine and the value of the hu-mice model for *FT* vaccine research. Overall, I suggest that such an approach might be widely applicable to vaccine studies of the *FT*.

Keywords : *Francisella tularensis*, Tul4, FopA, dendritic cells, humanized mice

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CHAPTER II

LIST OF ABBREVIATION

FT	*Francisella tularensis*
LVS	The live vaccine strain
HSC	Hematopoietic stem cells
DC	Dendritic cells
BMDC	Bone marrow derived dendritic cells
CDC	Centers for Disease Control and Prevention
OMP	Outer membrane protein
ODN	Oligodeoxynucleotides
FACS	Fluorescence-activated cell sorter
PE	Phycoerythrin
MMC	Mitomycin C
BSA	Bovine serum albumin
ANOVA	A one-way analysis of variance
Th1	T helper type 1
Th2	T helper type 2

NSG	NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ
DAPI	4,6-diamidino-2-phenylindole
MST	Median survival time

GENERAL INTRODUCTION

Tularemia, caused by *Francisella tularensis* (*FT*), is an acute febrile disease [1]. The severity of the disease in human depends on the route of infection and strain of *FT* [1]. The major subspecies (subsp.) of *FT,* which able to cause disease in humans, is tularensis (type A) and holarctica (type B) [2]. *FT* subsp. tularensis are more virulent than *FT* subsp. holarctica. Inhalation of aerosolized type A leads to respiratory tularemia, in which the mortality rate ranges from 30% to 60% without proper treatment [3]. For these reasons, *FT* has been considered a potential agent of biological terrorism. However, there is currently no vaccine available for public use anywhere in the world.

An attenuated type B strain—the so-called live vaccine strain (LVS)—was developed in the 1950s [4]. For reasons including unknown basis of attenuation, residual virulence, and the issue of mixed variant phenotype in the same vaccine lots, attempts to obtain a license for LVS have failed [5]. It is, therefore, important to develop new vaccines that can safely and effectively defend against *FT*.

Subunit vaccines, which consist of microbial antigens, are considered to be safe. As potential subunit vaccines for *FT*, various bacterial components have been studied. Recently, multi-antigen subunit vaccines using tobacco mosaic virus have been developed in combination with Tul4 (surface lipoprotein), DnaK (heat shock protein), SucB (dihydrolipoamide succinyltransferase) and OmpA (outer membrane proteins). Immunized mice were protected against a dose 10 times the LD100 dose for LVS infection [6]. In another study, DnaK and Tul4 isolated from *FT* induced protective

effects in mice against lethal respiratory challenge with LVS [7]. Replication-incompetent adenovirus with Tul4 demonstrated partial protection in mice against infection with LVS. Another component of *FT*, FopA (membrane protein A), incorporated into liposomes, demonstrated a specific antibody response and protection in mice against a lethal dose of LVS [8]. However, the optimal combination of protein subunits for the development of safe and effective vaccines is still ongoing globally. Moreover, the biological limitations of animal models for vaccine evaluation remain an obstacle.

Mouse and rat models have several advantages for use in biomedical research. These animals are cost effective, easy to handle, maintain and breed, and their genomes have high similarities to human working DNA [9]. In addition, several studies have reported that mice are suitable models to investigate infectious diseases [10, 11]. Nevertheless, the utility of mice as suitable models for the development of *FT* vaccines remains controversial. Although most preclinical experiments have been performed on mice, susceptibility and immune response to *FT* are different according to the strain of mice, and species differences between rodents and humans (e.g., C57BL/6 mice are significantly more sensitive to *FT* than BALB/c mice) [12, 13]. Mice are more susceptible to all subspecies of *FT* infection than humans [14]. Moreover, the systemic immune responses of non-human primates can better mimic the protection against high-virulence *FT* than mice [15, 16]. Thus, human responses based on *FT*-based mouse models cannot be accurately predicted, which has prompted the development of new animal models that can overcome these limitations.

A good example of an improved mouse model is “humanized mice” (hu-mice).

The basic concept of hu-mice is based on immunodeficient mice that are engrafted with human hematopoietic stem cells (HSC) and/or fetal tissue [17]. Engrafted human HSC can differentiate into several human immune cells in mice, such as granulocytes, monocytes, dendritic cells (DC), natural killer (NK), T and B cells, and even erythrocytes and platelets [18]. Options for the development of a human immune system in a mouse model are diverse, depending on the source of HSC, the strain of immunodeficient mice, and the technological methods for humanization [19-21]. Many previous studies, including mine, have described hu-mice with human-specific pathogens such as the Epstein-Barr virus (EBV) and HIV [22-25]. Furthermore, the antibodies produced in hu-mice against Dengue virus infection are functionally similar to antibodies isolated from human patients [26].

In the present study, in order to develop a safe and effective subunit combination vaccine, I evaluated the immune response to Tul4 and FopA of the *FT* outer membrane protein using mouse immune cells and C57BL/6 mice. Next, to develop an animal model capable of evaluating human-specific immune responses and confirm the efficacy of the Tul4 and FopA cocktail, a humoral response and survival challenging was confirmed in hu-mice. This study showed a noble approach that could be widely applied to further *FT* vaccine research.

LITERATURE REVIEW

Francisella tularensis

FT is an aerobic, gram-negative coccobacillus, able to survive for weeks at low temperatures and in harsh environments [27]. *FT* is divided into two major subspecies according to virulence and epidemiology features in humans: *FT* subspecies tularensis (type A); and subspecies holarctica (type B) [28]. Type A is known to be more virulent in humans and animals than type B [29, 30]. In particular, type A is considered to be a pathogen that could be continuously developed as a biological weapon because of its high virulence, low infectious dose, and potential for airborne dissemination [29]. Moreover, inhalation of <10 colony forming units of type A *FT* resulted in respiratory tularemia, which in humans can have a mortality rate of up to 30-60% without proper treatment [31].

Clinical manifestations of tularemia

The clinical presentation of tularemia according to the route of infection can include ulceroglandular, oculoglandular, oropharyngeal, respiratory, and typhoidal form. The most common form of tularemia is ulceroglandular, which develops by contact with blood-feeding arthropods or an infected animal [32]. Ulcers appears at the site of bacterial exposure with enlargement of the lymph nodes [32]. Oculoglandular form is a rare case of tularemia. *FT* is supposed to be transmitted by touching conjunctiva with contaminated fingers [33]. Oropharyngeal tularemia shows stomatitis and pharyngitis along with excessive regional lymphadenitis, which is caused by oral intake of contaminated water

or food [34]. Respiratory tularemia results primarily in flu-like symptoms such as high fever, chills, and cough [35]. These common symptoms may make it difficult to detect *FT* infection, and should be differentiated from other pathogenic agents. From the lung, this pathogen spreads to the lymph nodes, leading to systemic infection with severe inflammation and tissue damage, particularly in the spleen and liver [30, 36]. These common symptoms may make it difficult to detect early *FT* infection and should be differentiated from other pathogens that cause pneumonia.

Treatment of tularemia

The important factors in the treatment of tularemia are early diagnosis and appropriate antibiotic treatment. According to the WHO guidelines, patients with tularemia should receive aminoglycosides, such as streptomycin and gentamicin, for 7-14 days. Both antibiotics are effective treatments for Type A and B tularemia [37, 38]. Despite their successful treatment, aminoglycosides occasionally have side effects such as ototoxicity and nephrotoxicity. Alternatives to the aminoglycosides include ciprofloxacin, levofloxacin, and tetracyclines for clinical use. Ciprofloxacin and levofloxacin are considered first-line drugs to treat mild to moderate tularemia [37]. Children have the same risk for skeletal toxicity of quinolones as the adults [39]. Moreover, levofloxacin has reportedly been reported a successful treatment for human tularemia, without relapse [40]. Tetracyclines have much lower toxicity than aminoglycosides, but reportedly have a higher relapse rate than fluoroquinolones including ciprofloxacin and levofloxacin [41]. In the natural environment, *FT* has not been reported to be resistant to aminoglycosides,

tetracyclines, or fluoroquinolones. However, Georgi *et al* isolated *FT* with resistance to erythromycin [42]. In addition, Sutera *et al* experimentally obtained *FT* mutants resistant to fluoroquinolones [43]. These mutants indicated that the development of antibiotic resistance mutants in humans could be possible. Despite antibiotic therapy, several studies have reported that patients infected with *FT* who did not respond to antibiotics became more ill, experienced a relapse, or died [41, 44]. Whatever antibiotics are used, they have issues such as toxicity, relapses, and resistance. Thus, vaccine development could be a breakthrough beyond antibiotics.

Major virulence factors of *Francisella tularensis*

Major virulence factors of *FT* include lipopolysaccharide (LPS), capsule, and type IV pili. The capsule of *FT* is considered to be the structure resistant to environmental and immune responses of the host. The major component of the capsule is lipid, while the composition of other components remain unclear [45]. Capsule mutants exhibit a rough colony morphology and are sensitive to killing by serum [46]. In addition, several studies have reported that capsule mutants of *FT* lose virulence in animals [45, 46]. Although the capsule is a critical component for virulent expression in the host, further studies are needed to reveal the role of the capsule in developing a vaccine candidate.

LPS is a common structural component of gram-negative bacteria and an important antigen target for vaccines [47]. LPS from *FT* has a unique structure, including the lack of free phosphate moieties in lipid A, which exhibits low immunobiological activity compared with other bacteria [48]. Although less immunogenic than *FT* LPS,

atypical structural changes in LPS provide critical information of the blue-to-gray color variants of *FT* related to the loss of virulence, intracellular survival, and strategies for vaccine development [49, 50]. Moreover, O-antigen-deficient *FT* are controlled by autophagy in the macrophage [51].

Type IV pili are filamentous surface fibers associated with adhesion to host cells [52]. Type IV pili are critical for the virulence of the subspecies holarctica, in which these components may be related to dissemination of the pathogen in animals [53]. The type B strain, which lacks the *pilA* gene, exhibited a loss of virulence in mice [53]; however, the pathogenesis and function of type IV pili in the host have not been completely elucidated.

Aside from the major component, the *Francisella* pathogenicity island (FPI) is a gene cluster of related elements that are important for intracellular growth and virulence [54]. The function of individual genes in the FPI cluster has not been widely discussed, nor has their role in *FT* virulence been extensively investigated. Of the FPI genes identified to date, *IglA* and *IglB* are known to be involved in protein secretion as interacting cytoplasmic proteins [55]. Although the exact function of *iglC* remain unknown, *iglC* mutants exhibit a loss of intracellular growth ability [56]. FPI is considered to be a target cluster for vaccine development, and several ongoing studies are being conducted to determine the potential of FPI as a therapeutic target.

Vaccines for *Francisella tularensis*

Although the pathogenesis of *FT* and host immune response have not been fully elucidated, various vaccines have been developed based on the virulence factors and

pathogenesis that have been discovered to date.

Inactivated vaccine

FT LVS was inactivated in three ways: heat; exposure to paraformaldehyde; and ultraviolet radiation [57]. Baron *et al* reported that immunization with inactivated *FT* LVS, with IL-12 as an adjuvant, demonstrated 90 to 100% protection against lethal LVS [57]. This protective effect was correlated with reduced bacterial burden, lower expressions of cytokines including interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), and IL-6, and decreased inflammation in the lungs, liver, and spleen [57]. Similarly, immunization of inactivated *FT* LVS adjuvanted with immune-stimulating complexes (ISCOMS) and CpG oligonucleotides completely protected against type B strain HN63 [58]. However, these vaccine formulations failed to protect against SchuS4 (Type A strain) challenge. Inactivated *FT* LVS with other adjuvants such as immune complexes (ICs) and Cholera Toxin B subunit (CTB) showed successful protection against LVS and approximately 50% protection against SchuS4 [59, 60]. Better achievements in protection against SchuS4 challenge were also correlated with reduction of bacterial burden and decreased production of inflammatory cytokines [59, 60]. However, for more than a decade, attempts at producing a commercialized vaccine have failed, which suggests that although inactivated *FT* may confer complete defense in mice, it has a poor protective effect in humans due to different immune system.

Live attenuated vaccine

A live attenuated vaccine is developed by reducing virulence but still induces an immune response before the clearance by the host immune system. LVS, which was developed by isolating colonies of low-pathogenic wild type strains through subculture of the *FT* subspecies holarctica (type B), demonstrated protective effects according to inoculation routes and dose in humans [31, 61]. Although LVS based on the type B strain exhibits protective effects against the highly pathogenic type A, there are some problems in developing the *FT* vaccine, including unknown mechanism of attenuation; residual virulence of LVS; and the issue of mixed variant phenotypes in the same vaccine lots, which can exhibit different protective effects against *FT* [5, 62]. LVS appears blue and the variant as gray which is considered less virulent [63]. This variant showed increased nitric oxide production in macrophage, reduced intramacrophage survival, and altered the O-antigen structure of LPS [64, 65]. Because of these issues, the United States Food and Drug Administration has not yet approved it as a vaccine. Recently, to overcome these limitations, new live vaccine strains have been studied by eliminating or reducing the expression of genes related to *FT* virulence.

In the case of the type B strain, various mutations based on the LVS strain have been developed: chaperone protein (*clpB*); purine biosynthetic genes (*purMCD, guaA* and *B*); predicted lipoprotein gene (*Tul4*); superoxide dismutase gene (*sodB*); O-antigen polymerase gene (*wzy*) and LPS biosynthetic gene (*wbtA*) [66-73]. Major findings of LVS-based live attenuated vaccines is shown in Table 1. Despite various attempts, however, mice immunized with these vaccine candidates are not completely protected against *FT*.

Table 1. Major findings of LVS-based live attenuated vaccines

Mutation	Animal model	LVS challenge % protection (dose in CFU, route)	SchuS4 challenge % protection (dose in CFU, route)	Major findings	Ref.
SodB	C57BL/6	100% (1.2×10^6, IN)	40% (10^3, IN)	-Reduction of bacterial burden -Decreased tissue inflammation	[74]
ClpB	C57BL/6	100% (5×10^3, IN)	10% (30, IN)	-Increased IFN-γ+ CD4+ and CD8+ T cells -Proinflammatory cytokine production	[75]
dsbA	BALB/c	ND	50% (100, IN)	-Strong Th1-like antibody response	[76]
wbtA	BALB/c	100% (25 LD, IN)	25% (10, IN)	-Reduction of bacterial burden -Decreased tissue inflammation	[73]
Wzy	BALB/c	100% (1.2×10^5, IN)	84% (8, IN)	-Reduction of bacterial burden -Increased titers of antibody to LPS	[77]
purMCD	BALB/c	100% (5×10^3, IP)	ND	-Mutant failed to replicate in the cytosol or induce apoptotic and cytopathic responses in infected cells	[69]
guaA *guaB*	BALB/c	100%, (2.8×10^5, IP)	ND	-Mutants failed to replicate in macrophages -A robust proinflammatory cytokine response	[72]
Tul4	C3H/HeN	80% (1×10^5, ID)	ND	-Secretion of chemokines by human monocyte-derived macrophages	[67]

LVS, live vaccine strain; ND, not determined; ID, intradermal; IN, intranasal; IP, intraperitoneal; LD50, median lethal dose; LD100, absolute lethal dose.

Live vaccines based on the *FT* type A strain (SchuS4) have been considered to be strong vaccine candidates because of the partial protection of LVS mutants. These limitations are speculated to be differences in immune responses induced by each strain [78]. Only a few *FT* mutants have been developed as live vaccine candidates: purine biosynthesis gene (*purMCD*); pathogenicity island protein (*iglC*); heat shock protein gene (*clpB*), and the assembly of capsular poly- d-glutamic acid (*capB*) [70, 78-80]. Major findings of SchuS4-based live attenuated vaccines is shown in Table 2. However, these attempts have not reached clinical application levels due to the high infectivity of the type A strain. Developing a high-efficiency vaccine with safety and high immunogenicity, therefore, remains a challenge.

Table 2. Major findings of SchuS4-based live attenuated vaccines

Mutation	Animal model	LVS challenge % protection (dose in CFU, route)	SchuS4 challenge % protection (dose in CFU, route)	Major findings	Ref.
purMCD	BALB/c	ND	66% (100, ID) 14% (100, IN)	-Induction lung tissue damage -All mice died againt high dose Schu S4 challenge	[70]
iglC	BALB/c	11 days* (10, IN)	>35 days* (10, IN)	-Reduction of bacterial burden	[78]
clpB	C57BL/6	ND	0% (100, IN)	-Elevated levels of pulmonary IFNγ and IL-17	[79]
	BALB/c	ND	80% (100, IN)		
capB	BALB/c	ND	60% (10^3, SC)	-Increased serum IgG2a antibody -Elevated level of TNFα	[80]

* In this study, a protective effect in mice was evaluated by time to death against.

LVS, live vaccine strain; ND, not determined; ID, intradermal; IN, intranasal; SC, subcutaneous; LD50, median lethal dose; LD100, absolute lethal dose.

Subunit vaccines

To develop safe vaccines, several studies have been performed to investigate only some components, including protective surface-associated proteins, polysaccharides, and LPS. LPS can be a potential subunit vaccine because LPS immunization has been shown to induce antibody production and protection against *FT* [49, 81]. LPS-immunized mice depleted of either CD4+ or CD8+ T cells survived against LVS although the rate of bacterial burden was increased [81]. However, LPS failed to protect against aerosol challenge with *FT*.

Additional efforts to develop subunit vaccine against *FT*, it has been reported subunit vaccines using immunogenic proteins such as FopA, DnaK, and Tul4, but has not shown complete effectiveness against both *FT* strains [7, 8]. Ashtekar *et al* reported that immunization with DnaK or Tul4 together in C57BL/6 mice induced robust salivary IgA, and vaginal and bronchoalveolar IgA and IgG antigen-specific antibodies [7]. This immunization also stimulated splenic CD4+ T cells to produce IFN-γ, IL-10, and IL-17A in an antigen-specific manner, along with over 80% protection against a lethal respiratory challenge with *FT* LVS [7]. These components, derived from *FT* LVS and SchuS4, are extremely similar because the protein sequences encoding the DnaK and Tul4 T cell epitopes are conserved in *FT* LVS and Schu S4 [82, 83]. Thus, immune activation by DnaK and Tul4 is considered to be better components for vaccine development than the previously proposed candidates. However, the insufficient protection effect of 20% suggests that the multiple antigens strategy which can simultaneously stimulate cellular and humoral immunity should be recognized to confer effective protection against *FT*.

Recently, combinations of several vaccine candidates or use of a delivery system, such as tobacco mosaic virus, have been studied for improving of the efficiency of subunit vaccine candidates [84]. Moreover, new adjuvants, such as ISCOMS and CpGs are expected to improve the protective effect of subunit vaccine candidates [5, 58].

Other animal models for *Francisella tularensis*

In *FT* studies, not only mice but also various animals including rats, rabbits, and primates have been used. Representative animal model data against *FT* SchuS4 is shown in Table 3. Although small animals are natural reservoirs of *FT* and have been used in most studies on *FT,* the small animal model is considered to be insufficient for tularemia research. Their susceptibility to *FT* strains depends on the small animal species. Unlike humans, mice are highly susceptible to infection with LVS, but rabbit and Fisher 344 (F344) rat susceptibility is close to the human response to infection [85, 86]. Another difference between small animal models and humans is the immune response following infection. In humans, the V9γV2δ T-cell population has been reported in infection of *FT*, but these cells are absent from small animal models. Primates have this T-cell population and show similar symptoms of tularemia in humans such as skin ulcers and lymphadenopathy. Therefore, to date, the best model for development of the tularemia vaccine is primates. Despite the excellence of the primate model, due to the low accessibility of this model, the need of effective animal models to develop *FT* vaccine study is increasing.

Table 3. Summary of animal model data against *Francisella tularensis* SchuS4 [87]

	Rat	Rabbit	Marmoset	African Green Monkey	Rhesus Monkey	Grivet Monkey
Route of challenge	IT	Aerosol	Aerosol	Aerosol	Aerosol	IN
Time to symptoms (days)	NR	2-3	2.5	2	2-3	2-3
Initial symptoms	Weight loss, less active, less responsive	Accumulation of polymorphonuclear leukocytes in alveolar ducts (19 hrs), fever, weight loss, decreased food and water intake	Fever	Fever, elevated heart rate, high blood pressure	Fever	Fever, though blood chemistry differences were noted before fever
Culture data reported	NR	1/6 rabbits bacteremic	Blood, lung, liver, spleen, kidney	NR	NR	Positive in 100% nasal swabs
Organ involvement	NR	Lung, spleen, liver, kidney, intestines, lymph nodes	Lung, spleen, liver, lymph nodes	Lung, spleen, lymph nodes	Lung, spleen, lymph nodes, liver	Spleen, lynph nodes, liver
Lung pathology	NR	Lesions, parenchymal consolidation, loss of lung volume, hemorrhage, subpleural areas of nodular consolidation	Hemorrhage	Pleuritis, congestion, necrosis, granulomas, edema, hemorrhage	Nevrosis, abscess formation	Abscess formation, consolidation, pleuritis
Time to death (days)	10	4-7	4.5-7	7-11	6-10	5-7

IN, intranasal; IT, intratracheal; NR, not reported.

Humanized mice

Immunodeficient mice

"Hu-mice" generally refers to mice that have a human immune system. For human cells not be rejected by the mouse's immune system, the mouse must be severely immunodeficient. In this context, severe combined immunodeficiency (SCID) mice are an important starting point for modeling hu-mice [88]. SCID mice with a C.B-17 background carrying a mutation in *prkdc* (protein kinase, DNA activated, catalytic polypeptide gene) were first reported in 1988 [89]. *Prkdc*^{scid} mice exhibited failure of host T and B cell production, but still have a limitation, such as retention of NK cell activity, which hinders engraftment of human hematopoietic stem cells (HSC) into the mouse immune system [89]. Other efforts to eliminate the mouse immune system, including the generation of mice lacking recombination activating gene 1 or 2 (*Rag*1 or *Rag*2), have been developed [90]. Mice deficient in *Rag*1 or *Rag*2 are unable to initiate recombination of immunoglobulin, and lack functional T and B lymphocytes; however, this model also has the same limitations [90]. To develop an improved mice model, non-obese diabetic (NOD) strain have been used to generate new immunodeficient mice with mutation of these genes, which demonstrate higher engraftment of human HSC than existing models [90].

By additional mutation of the IL-2 receptor γ-chain (IL-2Rγ) locus, which is a key component of receptors with high affinity for IL2, IL4, and IL7 in SCID mice, these mice exhibit developmental impairments in T, B, and NK cells. Several research teams around the world have made efforts to develop a hu-mice model with a higher

engraftment rate targeting the IL-2Rγ mutation in several genetically modified mice. Representative NOG (NOD/shi-*Prkdc*^{scid} *IL2g*$^{tm1Sug/Jic}$), NSG (NOD/LtSz-*Prkdc*^{scid} *IL2g*$^{tm1Wjl/J}$), and BRG (BALB/c-*Rag2*^{null} *IL2rg*^{null}) mice have been developed by Ito et al, in Japan, Shultz et al. in the United States, and Kirberg et al. in the Netherlands, respectively [91-93]. Engraftment of human HSC into several immunodeficient mice resulted in human hematopoiesis, including lymphoid and myeloid lineages, at a high level compared with previous models [94].

Generation of humanized mice

Generation of a hu-mice model can be classified according to the types of human cells used for reconstitution and the methods of engraftment. The simplest method is intravenous injection of human peripheral blood, including lymphocytes, into adult immunodeficient mice. This transplantation method has resulted in human immune cells, such as T and B lymphocytes, lasting for several weeks in mice, and these human cells have functional roles including antibody production [95]. However, multilineage hematopoiesis does not occur, and graft rejection may occur due to transplanted T cells [96]. Moreover, lethal Epstein-Barr virus (EBV)-induced B cell lymphomas can naturally occur and complicate interpretation of study results using hu-mice with peripheral blood [21].

The second method is to transplant human organs, which interact with the blood lymphatic system, into immunodeficient mice. Fetal thymus, liver, bone, and spleen were suggested as hematopoietic sources in the construction of a hu-mice model. When

transplanted with thymus and liver, lymphopoiesis and differentiation into human T lymphocytes was observed in these organs [97]. SCID mice implanted subcutaneously with fetal bones demonstrated proliferation and differentiation of human HSC for 6 months [98].

The third method is to reconstitute human HSC using various routes, two of which are intravenous or intrafemoral injection of human HSC into adult immunodeficient mice; and intrahepatic injection in neonatal immunodeficient mice. Both methods can result in a human immune response to a variety of pathogens.

The fourth method is co-transplantation of fetal organs and human HSC. HSC-only transplantation into immunodeficient mice exhibit human leukocyte antigen restriction deficiency; however, this limitation can overcome by additional implantation of fetal immune tissue including liver and thymus [99]. These co-transplanted hu-mice models, using several immunodeficient mice such as NSG, NOG, and BRG, exhibit remarkable increases in T and B lymphocytes, macrophages, and dendritic cells, and stable maintenance of human hematopoiesis in mice [100]. However, these models encounter difficulties in acquiring fetal organs and increasingly challenging ethical issues [101].

Application of a humanized mice model

The most widespread use of hu-mice is in the study of infectious diseases. In particular, studies investigating pathogenesis, treatments, and preventive methods for human-specific pathogens have become possible with the design and development of hu-

mice models. An archetypal infectious disease using hu-mice models is AIDS. HIV type 1 (HIV-1), a causative pathogen of AIDS, infects human immune cells including CD4+ T lymphocytes, macrophages, and dendritic cells. Depletion of CD4+ T cells is caused by HIV-1, which in turn leads to the impairment of cellular immunity [102]. Thus, hu-mice reconstituted with human immune cells can be a good model for HIV-1 study. Several studies have reported similarities in the pathogenesis of HIV-1 in humans and hu-mice. Hu-mice infected with HIV-1 exhibit virus replication in the thymus, spleen, and lymph nodes, which are the same target organs in humans [103]. The hallmarks of HIV-1 infection include high levels of viremia, long-term HIV infection, and HIV latency via infection of resting CD4+ cells. These features were also observed in hu-mice infected with HIV [104, 105]. Hu-mice have also been actively used in research to develop AIDS therapies. Holt *et al* constructed hu-mice reconstituted with HSC that knockdown the chemokine receptor 5 gene (*CCR5*). Human *CCR5*, an essential factor for T lymphocyte infection, was down-regulated in T cells in hu-mice, which showed that CCR5 knockdown hu-mice are resistant to CD4+ T lymphocyte deficiency caused by HIV-1 [106]. Antiretroviral therapy using hu-mice has also been reported in the reduction of HIV infection, which suggests that hu-mice are a suitable model to develop new drugs and therapies [107].

Aside from HIV studies, hu-mice have been used to develop pathogenesis and infection models for several viruses. Infection of hu-mice with EBV results in the development of B cell lymphoproliferative disease and production of EBV-specific human IgM [108]. In the case of Ebola virus, infected hu-mice exhibit high levels

of viremia, hepatitis, and hypercytokinemia, similar to humans [109]. In other studies investigating hepatitis B virus (HBV) and hepatitis C virus (HCV), a persistent infection developed in hu-mice [110, 111]. Infected hu-mice also exhibit liver inflammation and fibrosis, and virus-specific immune responses [110, 111]. Hu-mice infected with the dengue virus (DENV) develop a decreased platelet count, fever, and erythema, which are major symptoms of dengue fever disease [112]. DENV-specific humoral immune response in hu-mice suggests that hu-mice are a realistic infection model for DENV [113]. Other virus studies using hu-mice are presented in Table 4.

Table 4. Humanized mice models for human-specific virus

Pathogen	Major findings	Humanized mice model	Ref.
Dengue virus	Dengue fever, skin rash, $CD8^+$ T-cell responses, virus neutralizing antibody	NSG, BRG, and NOD/*scid* engrafted with HSC	[114, 115]
EBV	Persistent infection, B-cell lymphoma formation, Protective $CD4^+$ and $CD8^+$ T-cell responses, IgM responses	NOG, BRG, NSG, and others engrafted with HSC or BLT	[116, 117]
Ebola virus	Viremia, liver steatosis, hemorrhage, high lethality, hypercytokinemia	NSG-A2 engrafted with HSCs, NSG engrafted with BLT	[109]
HBV	T cell immune responses, chronic hepatitis and fibrosis	NSG-A2 engrafted with hepatocytes and HSC	[118]
HCV	Hepatitis, and fibrosis T cell immune responses	BRG-AFC8 engrafted with HSC	[119]
HCMV	Viral latency and reactivation Arteriosclerosis	NSG engrafted with HSCs C57BL/6 *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ transplanted with HCMV-infected artery and engrafted with PBMCs	[120, 121]
HIV	Persistent infection, $CD4^+$ T-cell depletion, Latency, protective $CD8^+$ T-cell, antibody response	NOG, BRG, NSG, and others engrafted with HSCs or BLT	[24, 122]
HTLV-1	Infection of hematopoietic progenitor cells, $CD4^+$ T-cell lymphoma formation, ATL-like lymphoproliferation, Myelin disruption	NOG engrafted with HSC BRG engrafted with BLT	[123, 124]
HSV-2	Vaginal transmission, Mucosal innate and adaptive immune responses	BRG engrafted with HSC	[125]

NOG, NOD/Shi-*scid Il2rg*$^{-/-}$; BRG, Balb/c *Rag2*$^{-/-}$*Il2rg*$^{-/-}$; NSG, NOD/LtSz-*scid Il2rg*$^{-/-}$; NSG-A2, NSG with HLA-A2 transgene; BLT, immunodeficient mice with fetal liver, thymus, and bone marrow; BRG-AFC8, BRG with a transgene encoding a fusion protein of the FK506 binding protein and caspase 8; HSC, hematopoietic stem cells; PBMC, peripheral blood mononuclear cells; EBV, Epstein–Barr virus; HBV, Hepatitis B virus; HCV, hepatitis c virus; HCMV, human cytomegalovirus; HIV, human immunodeficiency virus; HTLV-1, human T-cell leukemia virus; HSV-2, herpes simplex virus type 2.

In the case of some bacteria and parasites, hu-mice are also useful in studies of infectious diseases because the infection and proliferation of pathogens are closely associated with human immune cells. *Plasmodium falciparum* causing malaria is the most lethal parasite in humans. The development of new drugs and therapies has been hampered by the parasite's tropism for human cells, especially hepatocytes and erythrocytes [126]. Several studies using engrafted NSG mice with human erythrocytes or hepatocytes have described hu-mice models for malaria, which are persistently infected with *P. falciparum* [127, 128]. *Salmonella enterica* serovar Typhi (*S. Typhi)* causing typhoid fever is a human-specific bacterial pathogen. Hu-mice models for *S. Typhi* infection demonstrate replication of the pathogen in the spleen and liver, with pathological changes similar to those in humans [129]. Moreover, innate and adaptive immune responses against *S. Typhi* have been observed in hu-mice [130]. Other studies using hu-mice models, including *Leishmania* parasite, *Borrelia* hermsii, *Neisseria meningitides*, and *Mycobacterium tuberculosis*, are reported in Table 5.

Table 5. Humanized mice models for human-specific bacteria and parasites

Pathogen	Major findings	Humanized mice model	Ref.
Mycobacterium tuberculosis	Granuloma formation supports bacterial replication $CD4^+$-dependent granuloma formation	NSG engrafted with HSC	[131, 132]
Salmonella typhi	Infection in spleen, liver, bone marrow, gall bladder, and blood, Cytokinemia, antibody responses	NSG engrafted with HSC	[129, 130]
Borrelia hernsii	Recurrent bacteremia, antibody response	NSG engrafted with HSC	[133]
Neisseria meningitidis	Bacterial proliferation in association with human vessel endothelium	*scid/beige* engrafted with skin tissue	[134]
Plasmodium falciparum	Replication in RBC and liver	TK-NOG engrafted with hepatocytes and infused with RBC repeatedly	[135, 136]
Leishmania major	Infection to macrophages, T cell immune responses	NSG engrafted with HSC	[137]

NOG, NOD/Shi-*scid Il2rg*$^{-/-}$; NSG, NOD/LtSz-*scid Il2rg*$^{-/-}$; HSC, hematopoietic stem cells; RBC, red blood cells; TK-NOG, NOG mice expressing a thymidine kinase transgene.

CHAPTER I

Synthetic Tul4 and FopA peptides cocktail of *Francisella tularensis* induced humoral and cell-mediated immunity in mice

1 INTRODUCTION

Tularemia, caused by *Francisella tularensis* (*FT*), is a highly infectious disease that has the various courses depended on the Francisella strain and the sites of infection [82]. Among the various route of inoculation, an aerosol release of *FT* induced acute fever beginning 3 to 5 days later, following 1 or more pharyngitis, bronchiolitis, hilar lymphadenitis, and pneumonitis [138]. The progression from respiratory symptoms to systemic illness may result in life-threatening pleuropneumonia and sepsis [138]. Without antibiotic treatment, the mortality rate of respiratory tularemia increased more than 30% [139]. Tularemia has been considered as a biothreat potential weapon in terror due to the ease of dissemination by aerosol, low dose of as few as 10 organisms to infection and the extreme virulence [82]. For these reasons, *FT* has been classified into a category A agent by the Centers for Disease Control and Prevention (CDC).

Thus far, *FT* live vaccine strain (LVS) has been the sole vaccine which is close to approval in humans. However, side effects and the incomplete protection as a general vaccine against *FT* remain major obstacles for approval of LVS in clinic [82, 140]. Since then, for developing a vaccine which completely protects against tularemia with the safety, there have been a lot of attempts including attenuated mutant Francisella, virus vector and subunit immunogens with carrier system [141]. Also a subunit vaccine using immunodominant antigens including outer membrane protein (OMP) has been considered as a potential alternative for LVS vaccination [7].

OMPs, surface-exposed domains, have been played a pathogenic role in adhesion to and invasion of host, virulence factors and activation both innate and adaptive host

immunity. These functions make them stand out as vaccines against bacterial infections [142]. Especially, OMPs of Francisella strains such as FopA and Tul4 induce an immunogenicity by the fact that FopA and Tul4 specific antibody. The recombinant FopA protein showed humoral immunity and protected naïve mice against LVS challenge [8]. Tul4 is also reported as a subunit vaccine that induced IFN-γ, IL-10 and IL-17A production in an antigen-specific manner and induces systemic antibody responses [7]. Therefore, FopA and Tul4 have been studied as strong candidates to develop a vaccine against tularemia.

In this study, I focused on a peptide vaccine using immunogenic epitopes of FopA and Tul4. Peptides based on epitopes are considered as activator of the cellular and humoral immunity and inducer of broad immune response against multiple serovars of pathogen by formulating various immunodominant epitopes. In addition, peptides are safe and economical owing to the small size as vaccines compared to proteins [143] and applicable as peptides cocktail vaccine that prompted a greater multi-target defensive response against antigens. For that reasons, peptides have gotten the limelight as attractive strategy for developing vaccines. Therefore, I demonstrate that a combination of FopA and Tul4 epitope elicited initial immune response related with dendritic cells *in vitro* and humoral immunity *in vivo*. These results demonstrate that a combination of FopA and Tul4 epitope could be a potential vaccine candidate with the safety and effectiveness improving the protection ability of individual FopA and Tul4

2 MATERIALS AND METHODS

2.1 Mice

7 weeks old C57BL/6 mice were purchased from Orient Bio (Gyeonggi, Korea) through a contract with the Institute of Laboratory Animal Resources of Seoul National University. The mice were housed at the laboratory animal facility, College of Veterinary Medicine, Seoul National University. All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the Seoul National University.

2.2 Synthetic peptides and adjuvant preparation

The Tul4 epitope consisted of 14 peptides (RLQWQAPEGSKCHD) with COOH-terminal amidation and NH_3-terminal acethylation. The FopA epitope consisted of 26 peptides (IGYNINKYFAVQYNQLVGRVFAGLGE) with COOH-terminal amidation and NH_3-terminal acethylation. All peptides used for the experiments were synthesized by Peptron, Inc. (Daejeon, Korea). CpG oligodeoxynucleotides (ODN) CpG ODN 1826 TCC ATG ACG TTC CTG ACG TT (GenoTech, Daejeon, Korea).

2.3 Generation of bone marrow derived dendritic cells (BMDCs)

Isolation of bone marrow derived hematopoietic precursors was performed as those previously described with minor modifications. Briefly, mouse bone marrow cells were harvested from the femurs and tibias of sacrificed mice. After removal of the red blood cells, the cells were resuspended at 1×10^6 cells/ml in RPMI-1640 medium (Gibco,

NY, USA) containing 10% (v/v) FBS (Gibco), 10 mM glutamine, and penicillin/streptomycin (Gibco). After culture for 3 h at 37◦C, the non-adherent cells were removed by two gentle washings, adherent cells were cultured in fresh RPMI 1640 medium containing 10 ng/ml recombinant granulocyte/macrophage colony-stimulating factor (GM-CSF) (Peprotech, NJ, USA) for 8 day. Adherent cells were harvested and purity was tested by FACS Aria II (BD Biosciences, CA, USA) equipped with FACS Diva Software. This procedure routinely yielded > 80% CD11c positive cells.

2.4 *In vitro* DC stimulation assay

The immature DCs were cultured in medium containing peptides (Tul4 40 μg/ml + FopA 20 μg/ml), CpG (20 μg/ml) and peptides+CpG. After culturing for 48 h, the medium was collected and cytokine contents were measured by enzyme-linked immunosorbent assay (ELISA kit, eBioscience, CA, USA) for IL-6 and IL-12p40. The absorbance was measured using a SpectraMax M2 Microplate Reader (Molecular Devices, CA, USA).

The stimulated DCs were harvested to examine their surface markers by fluorescence-activated cell sorter (FACS). Briefly, the DCs were washed twice with 0.01 M PBS, and treated with Fc-blocking reagent for 20 min on ice and then incubated with the following anti-mouse antibodies: phycoerythrin (PE)-conjugated monoclonal antibodies (mAbs) CD40, DC86 and MHC II (Biolegend, CA, USA), and FITC-conjugated mAb to CD11c (BD Biosciences) for 2 hours. Flow cytometry analysis was performed using a FACS Aria II (BD Biosciences) equipped with FACS Diva Software.

2.5 Mixed lymphocyte reaction (MLR)

Splenocytes from the spleens of C57BL/6 mice (7 weeks old) were isolated by using a mouse T cell enrichment column (R&D Systems, MN, USA). BMDCs were stimulated with peptides for 16 h. Cells were then harvested, washed, and incubated with 25 μg/ml mitomycin C (MMC) for 30 min at 37 ℃. Finally, the cells were washed and diluted with the prepared splenocytes in a ratio of 1:50 and 1:100 in U-bottomed 96-well culture plates for 5 days. Cell proliferation was determined by MTT assay. The absorbance of the dissolved solutions was detected by using a SpectraMax M2 Microplate Reader (Molecular Devices) at 570 nm.

2.6 Immunizations with peptides

Mice were immunized by the subcutaneous route with peptides (Tul4 40 μg/ml + FopA 20 μg/ml) CpG (20 μg/ml) or both on days 0, 14 and 28 (n=3 per each group). Control mice received PBS only. Serum samples were collected prior to immunization and at approximately 2-week intervals following the initial immunization. Blood samples were collected from the retroorbital plexus of mice anesthetized with mixture of xylazine (5mg/mg; Bayer Korea, Seoul, Korea) and alfaxan (60mg/kg; careside, Gyeonggi-do, Korea) using heparinized capillary tubes and the serum was obtained after centrifugation.

2.7 *Ex vivo* spleen re-stimulation

Mice were immunized by the subcutaneous route with peptides (Tul4 40 μg/ml +

FopA 20 μg/ml) CpG (20 μg/ml) or both on days 0, 14 and 28 (n=3 per each group). Control mice received PBS only. Serum samples were collected prior to immunization and at approximately 2-week intervals following the initial immunization. Blood samples were collected from the retroorbital plexus of mice anesthetized with mixture of xylazine (5mg/mg; Bayer Korea, Seoul, Korea) and alfaxan (60mg/kg; careside, Gyeonggi-do, Korea) using heparinized capillary tubes and the serum was obtained after centrifugation Splenocytes from peptides-immunized mice were suspended at 2×10^6/ml in RPMI 1640 medium supplemented with 10% FBS (complete medium). The cells were cultured in triplicate wells of U bottom 96-well plates at 37°C and 5%. Cells were restimulated with peptides, Tul4 (40 μg/ml), FopA (20 μg/ml) or both. Supernatants were removed after 72 h to test for IL-2 and IL-4 production. The concentrations of these cytokines were determined by specific ELISA kit (BD Biosciences) according to the manufacturer's instructions. The absorbance was measured using a SpectraMax M2 Microplate Reader (Molecular Devices).

2.8 Humoral and cellular immune response of the mice

Serum samples were assessed for total IgG antibody activity to Tul4 or FopA by ELISA. Briefly, microtiter plates (NUNC, Denmark) were coated with recombinant Tul4 (4 μg/ml) or FopA (4 μg/ml) with goat anti-mouse IgG antibodies (Southern Biotechnology Associates, AL, USA) in PBS. Blocking was done for 4 h at room temperature with PBS containing 1% bovine serum albumin (BSA). Serial twofold dilutions of the samples were added to wells in duplicate and the plates were incubated

overnight at 4°C. Samples were developed by the addition of the appropriate HRP-conjugated goat anti-mouse IgG antibody, followed by o-phenylenediamine substrate (Sigma, MO, USA). The absorbance was measured using a SpectraMax M2 Microplate Reader (Molecular Devices).

2.9 Statistical analysis

A one-way analysis of variance (ANOVA) followed by the Tukey HSD test was performed in SPSS Statistics (version 16). Experimental data were checked to determine if there was a significant difference. All of the data are expressed as the mean of samples ± standard error. *P*-values <0.05 were considered statistically significant.

3 RESULTS

3.1 Synthetic peptides-stimulated maturation of BMDCs

To confirm whether the peptides and CpG could stimulate the maturation of BMDCs, immune-phenotypes and cytokine secretion were analyzed after stimulation of naïve DCs. DCs were stimulated with peptides, CpG, or peptides + CpG; each concentrations of peptides or CpG were determined from dose-dependent test (data not shown). As shown in Figure 1A, the expression of CD86 and MHC class II were greatly upregulated on all stimulated DCs compared to unstimulated DCs. Also, the expression of CD40 was strongly enhanced on DCs stimulated with CpG or peptides + CpG, and mildly increased the expression was revealed on DCs stimulated with peptides only.

In cytokine analysis, the levels of IL-6 and IL-12p40 were significantly elevated in all experimental groups compared to that levels in control group (Figure 1B). Especially, the IL-6 and IL-12p40 levels in the group stimulated with peptides with/without CpG was higher than that in the group stimulated with CpG. Moreover, DCs co-stimulated with peptides + CpG released the highest amounts of IL-6 among the all experimental groups. These data indicate that peptides and CpG stimulation could induce the immune response *in vitro* through DCs activation with production of IL-6 and IL12p40.

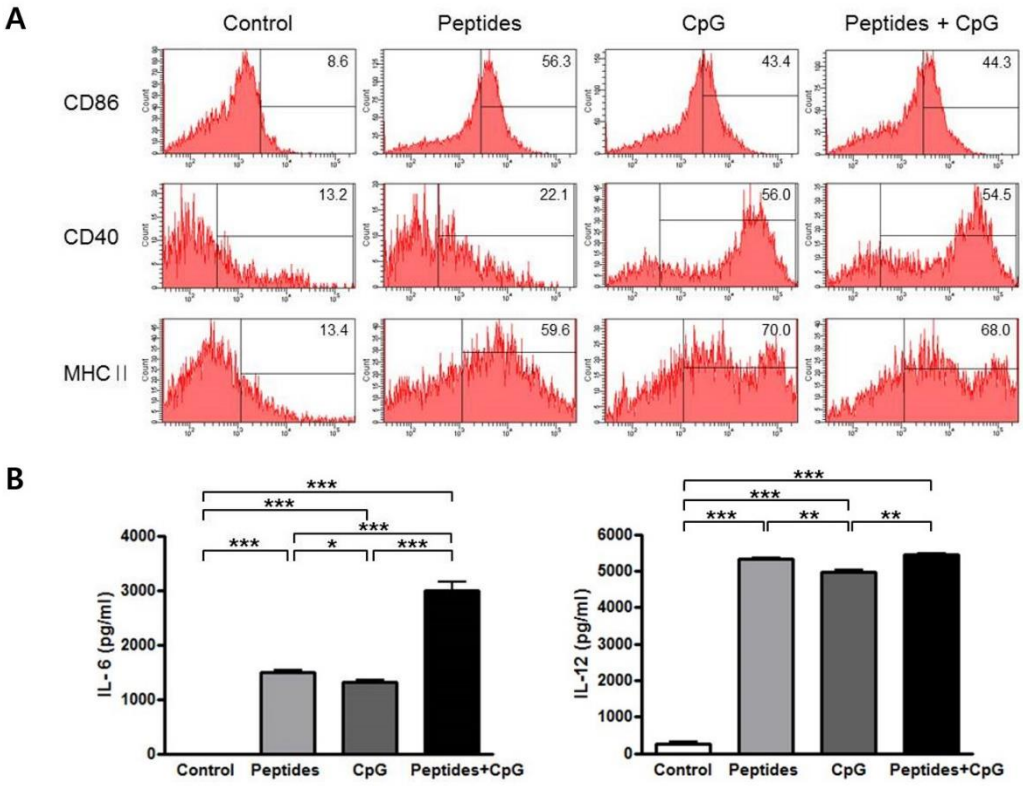

Figure 1. BMDCs maturation stimulated by peptides. (A) The expression of CD86, CD40, MHC class II on BMDCs following stimulation with peptides or peptides+CpG compared to those of control BMDCs. (B) Secretion of IL-6 and IL-12p40 by the BMDCs that were stimulated with peptides or peptides+CpG compared to control BMDCs. The data in bar graphs are presented as mean±SE. *p< 0.05, **p< 0.01, ***p< 0.001 between each group.

3.2 Allostimulatory function of BMDCs pulsed with synthetic peptides

Activated DCs promote lymphocyte proliferation, which contribute to initiate the adaptive immunity. Thus, I investigated whether the DCs stimulated with peptides, CpG, or both were capable of proliferation of naïve splenocytes. I pretreated MMC on activated DCs, and then co-cultured with allogeneic splenocytes. The proliferation of allogeneic splenocytes was measured by MTT assay. As a result, peptide-pulsed DCs elicited significant increase of splenocytes proliferation compared to control DCs, but rather decrease of that induced by CpG-pulsed DCs (Figure 2). Correlated with previous results of cytokine analysis, the highest induction of splenocytes proliferation was observed in DCs co-stimulated with peptides + CpG. These results showed activated DCs by peptides and CpG could mediate adaptive immunity through priming lymphocytes.

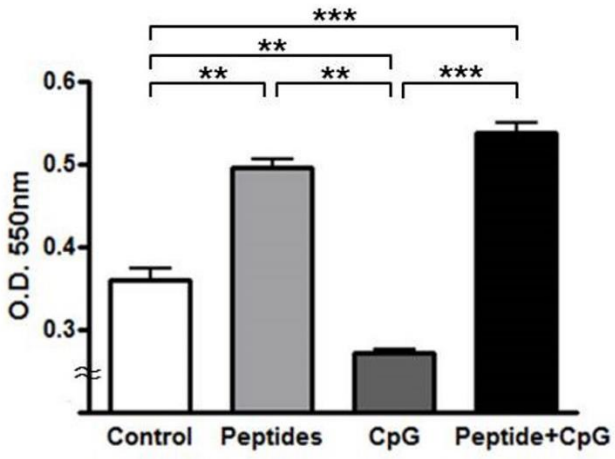

Figure 2. The proliferation of splenocytes co-cultured with peptides- or peptides+CpG- pulsed DCs. The data in bar graphs are presented as mean±SE. *p< 0.05, **p< 0.01, ***p< 0.001 between each group.

3.3 Immune response of splenocytes in mice immunized with synthetic peptides

I further examined the immune response of splenocytes that isolated from the mice immunized with peptides, CpG, or both. After restimulation of splenocytes with peptides, I measured each levels of T helper type 1 (Th1) related cytokine IL-2 and T helper type 2 (Th2) related cytokine IL-4 in the supernatant. As Figure 3, the levels of IL-2 were slightly increased with marginal significance (# = 0.059) in peptides and peptides+CpG groups compared to control group but not in CpG group. Moreover, the expressions of IL-2 in splenocytes primed with peptides+CpG were higher than those with peptides only groups. In Th2 related cytokine IL-4, the secretions were shown in all experimental groups with marginally detectable level, but there was no statistical difference among the groups. These *ex vivo* results supported that peptides could induce *in vivo* immune response by immunizing splenocytes.

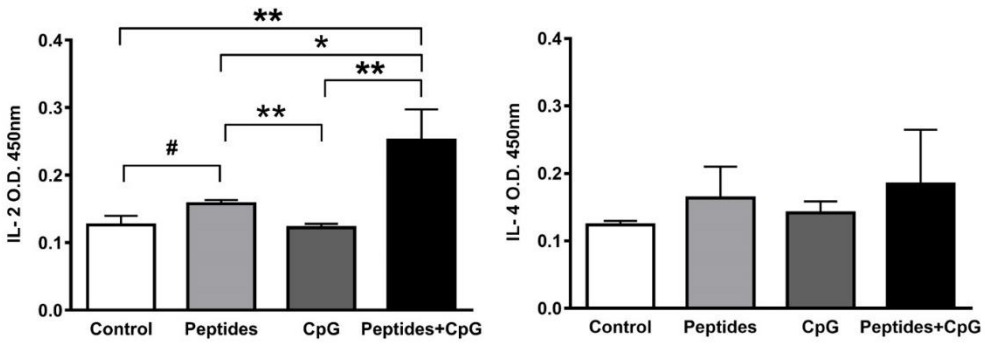

Figure 3. Secretion of IL-2 and IL-4 by the restimulated splenocytes with peptides. The data in bar graphs are presented as mean±SE. p *p< 0.05, **p< 0.01, between each group. Pound sign (#) indicate marginally significant differences in peptides group compared to control group.

3.4 Antibody response in mice immunized with synthetic peptides

In order to determine whether peptides with/without CpG was capable of inducing antibody after *in vivo* immunization, total IgG in the serum were collected from the mice immunized 3 times with 2 weeks intervals and were measured by ELISA. As presented in Figure 4A, the levels of total IgG in experimental groups were increased at each time point after the immunization. The total IgG in the group co-immunized with peptides and CpG was significantly higher than those in the peptides immunized groups at 4- and 8-week time point and, CpG immunized groups at 2-, 4- , 6- and 8-week time points ($p < 0.05$, each comparison). The peptides- and CpG- immunized groups do not reveal any significant difference. The levels of total IgG were peaked in 2 weeks after final boosting, and then declined at 8-week time point in all experimental groups.

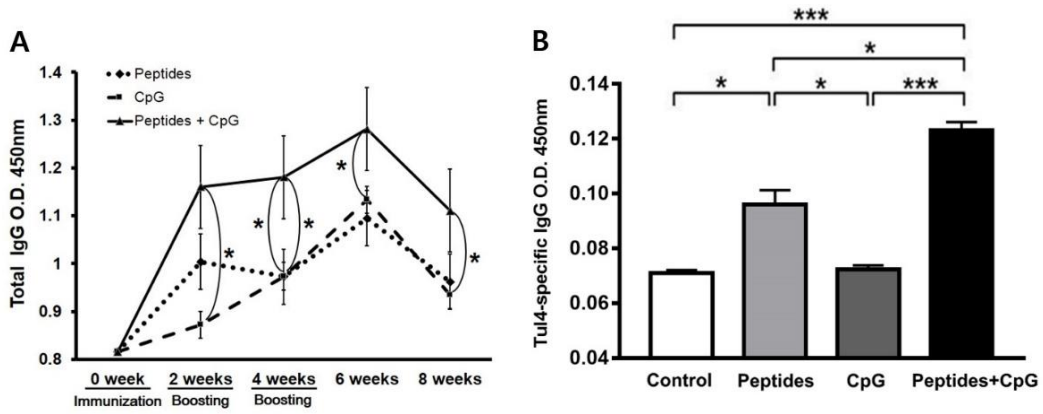

Figure 4. **The antibody responses in mice immunized with peptides (n=3 per each group).** (A) The level of total IgG in sera were collected from the mice immunized 3 times. (B) The level of Tul4 specific IgG in sera at 6-week time point. The data in bar graphs are presented as mean±SE. Statistics by one-way ANOVA. p *p< 0.05, ***p< 0.001, between each group.

I further examined specific IgG which functionally recognize and bind to Tul4 and FopA in the serum at 6-week time point when the total IgG level was the highest. As shown in the left panel of Figure 4B, in the mice immunized with peptides or peptides+CpG, the density of Tul4 specific IgG were significantly increased but not in the mice immunized with CpG without peptides. Interestingly, the density of Tul4 specific IgG in the mice immunized with peptides+CpG was statistically higher than that in the mice immunized with peptides alone, which indicates the synergic effects of peptides and CpG. On the contrary, FopA specific IgG was not detected in all immunized mice due to their insolubility of FopA peptides (Data not shown). On the basis of the results, I confirmed that immunization with peptides and/or CpG could elicit strong antibody response and pro-longed antibody production *in vivo*, and peptides could induce the generation of Tul4-specific antibody.

4 DISCUSSION

In the present study, I have developed the safe peptides vaccine candidates that originated from epitope of *FT* Tul4 and FopA conjugated with CpG, and evaluated the effectiveness as a new vaccine.

DCs are the initial antigen-presenting cell and particularly important in the initial stages of the immune response to antigen by expressing surface molecules. MHC II molecule plays a major role in signaling of antigen presenting cells and CD40 and CD86 have costimulatory functions. To investigate whether peptides and CpG could induce DC activation, I analyzed the phenotypic characteristics and cytokine secretion of activated DCs. As shown in Figure 1, peptides could stimulate the DCs to express CD40, and CpG strongly enhanced that expression. MHC class II and CD86 was also highly expressed in all groups. Based on these results, I could suppose that peptides and CpG treatments sufficiently induce the DCs activation for signaling to Th cells, but it was difficult to confirm the synergic effect with peptides and CpG in phenotype analysis, therefore, I conducted further cytokine analysis of immune response induced by peptides and CpG.

Cytokines secreted by stimulated DCs affect differentiation of T cells. IL- 12 plays important roles in the expansion of DCs and the development of IFN-γ producing cells for eliciting Th1 response [144]. IL-6 induces the immune responses by regulating the balance of immature and matured DCs [145]. In addition, IL-6 promotes Th2 differentiation and simultaneously inhibits Th1 polarization [146]. Previous studies reported that cytokines are produced from DCs through TLR 2 pathway by intracellular pathogens such as *FT* [147, 148] and TLR 9 pathway by CpG stimulation [149, 150]. For

these reasons, I supposed that synergistic effects of peptides and CpG are induced through TLR 2 and 9 pathways in DCs, then significantly upregulated the IL-6 and IL-12p40 level compared to other stimulated DCs. Moreover, as presented the MLR results, DCs stimulated with peptides+CpG induce the enhanced proliferation of allogeneic splenocytes through stimulatory signals. Contrary to expectations, CpG-pulsed DCs resulted a significant decrease of splenocytes proliferation compared to the control group, despite CpG's auxiliary function to immunity [151]. Interestingly, a previous study has reported that CpG application alone induces indoleamine 2,3-dioxygenase (IDO) expression and prohibits proliferation of T cells from splenocytes [152, 153]. Thus, I supposed that CpG-pulsed DCs suppressed proliferation of lymphocytes using a similar mechanism. Based on these results, I speculate that the increase of IL-12p40 and IL-6 secretion from stimulated DCs influence the differentiation of Th cells to Th1 or Th2 cells and control the cellular and humoral immunity. Simultaneously, stimulated DCs induce immune response by transferring signals to splenocytes including lymphocytes.

In vitro experiments with DCs to confirm the effectivity as a vaccine candidate have limitations for reflecting the multiple immune cell responses. Therefore, I examined cytokine response of splenocytes *ex vivo* and immunoglobulin levels in the serum from the mice immunized with peptides, CpG, or both. As presented Figure 3, when primed-splencocytes were restimulated with only Tul4 and FopA, Th1 cytokine IL-2 was strongly increased in peptides+CpG groups. However, a minimal level of IL-4 was induced in all experimental groups compared to IL-2. At such a low level, it is hard to detect the difference of IL-4 in each group. Although, the reason for Th1 cytokine biased induction

rather than Th2 cytokine biased induction remains unknown, previous studies partially explain that DNA/protein vaccination tends to induce a Th1-biased response [154], and Th1-type cytokines are hypothesized to suppress the production of Th2-type cytokines, such as IL-4 [155]. Of course, natural killer cells and macrophage in raw splenocytes could produce cytokines potentially [156], these cells may not affect antigen-specific cytokine reaction by repeated immunization. These results indicate that lymphocytes restimulated with Tul4 and FopA induced antigen specific immune response. Resultantly, peptides-immunizations could induce elicitation of adaptive immunity *in vivo*.

Lastly, I analyzed IgG in the serum from the mice to evaluate the humoral response induced by peptides stimulation. Figure 4 showed that the levels of total IgG were gradually increased and revealed the most activation state at 6 weeks after immunization. Total IgG levels were the highest in mice immunized with peptides+CpG, and similar patterns of Tul4 specific IgG levels were presented in Figure 4B. FopA specific IgG was not detected in all experimental groups, even previous studies have proven that FopA epitope could induces specific humoral immune response [8]. Taken together with total IgG and specific IgG, these results indicate that peptides immunization induces humoral immune responses following secretion of the peptides specific IgG by polarized B cells and CpG has synergistic effects with peptides to produce IgG *in vivo*.

Of course, challenging test is necessary for evaluating of vaccine efficacy. However, high-risk pathogens including *FT* and LVS are supervised by CDC, and required approval of experiments from CDC and Biosafety level 3 (BL3) facility. Further studies of challenging with *FT* in advanced BL3 facility could prove protective immunity

as an efficacious vaccine candidate.

The current study demonstrated that Tul4 and FopA epitope peptides conjugating with CpG could sufficiently enhance the immune response *in vitro* and *in vivo*. I have confirmed the phenotypic change, cytokine production and allostimulatory capacity of by peptides/CpG sensitized DCs in *in vitro* experiments. Also, *ex vivo* and *in vivo* experiments showed the induction of cellular immune response from splenocytes of immunized mice and an increase of humoral immune response through total IgG and antigen specific IgG production. Moreover, CpG could enhance the almost immune response of peptides in our experiments. These results support that Tul4 and FopA epitope peptides originated from *FT* have an effective immunogenicity, and could be an available candidates as safe and effective vaccine against *FT* infection.

CHAPTER II

Humanized mice for the evaluation of *Francisella tularensis* vaccine candidates

1 INTRODUCTION

Francisella tularensis (*FT*), the causative agent of tularemia, produces an acute infection in vertebrates, including humans, with clinical symptoms such as acute fever and inflammation of the respiratory system [157]. Moreover, because of its extreme virulence, ease of aerosol dissemination, and low infective dose (as few as 10 organisms), *FT* is considered as to be a potential biological weapon and bioterrorism agent [141]. The bacterium is classified as a Category A agent by the Centers for Disease Control and Prevention [158].

Several vaccine candidates, including live vaccines, that can impart effective protection against *FT* are under development [141]. However, no vaccine against tularemia is currently available for human use. An attenuated live vaccine strain (LVS) has been developed and has shown protection in animal studies, but it has not yet been licensed for public use by the US Food and Drug Administration owing to safety concerns [158]. Tul4, an outer membrane protein component of *FT*, is known to be an immunogen that stimulates lymphocyte and CD4+ T-cell proliferation, and has a partial protective effect against intranasal challenges with lethal dose of LVS [7, 159]. Another outer membrane protein component, FopA, when incorporated within liposomes and administered to mice, was shown to elicit FopA-specific antibodies and protection against intradermal and intranasal challenges with lethal dose of LVS [8, 159]. These studies have certainly contributed to a greater understanding of the immune mechanisms and the development of vaccine candidates; however, the relevance of the results obtained from experimental animal models to the immune responses in humans, is difficult to evaluate.

Although mouse models are most common in vaccine evaluations, this animal model has limitations because the immune response to and survival from *FT* infections are different for each mouse strain. In particular, C57BL/6 mice are known to be more susceptible than BALB/c mice to *FT* infection, and histopathological damage is more severe in former than in the latter [12, 13]. Therefore, an animal model is needed that can predict human immune responses to vaccines beyond differences between strains and species.

Humanized mice (hu-mice) have been used recently to study specific diseases of the human immune system, such as those caused by the human immunodeficiency virus and the Epstein-Barr virus [108, 160]. Moreover, it has been reported that the antibody produced by the humoral response to Dengue virus infection in hu-mice possesses functional similarity to the antibody isolated from human patients, and could neutralize the Dengue virus [26]. Therefore, hu-mice bearing the human immune system could potentially bridge the gap between animal models and human research, and this model could be optimized to assess vaccine efficacy with regard to the human immune system.

In the present study, I immunized hu-mice with recombinant Tul4 and FopA (rTul4 and rFopA), and then challenged the mice with LVS. In previous studies, I and other researchers had demonstrated rTul4 and rFopA to be excellent vaccine candidates for inducing cellular and humoral immune responses [161]. In this study, I used rTul4 and rFopA to evaluate the potential of hu-mice as a suitable animal model for vaccine development.

2 MATERIALS AND METHODS

2.1 Production of recombinant Tul4 and FopA proteins

The pET28b vector (Novagen®, Madison, WI, USA) was cloned respectively with the Tul4 and FopA genes, using both N-terminal and C-terminal six-histidine tags. Vector-Tul4 and vector-FopA were then respectively expressed in Rosetta™ 2(DE3)pLysS Competent Cells (Novagen®) and cultured in Luria Bertani medium. After sonication of the harvested cells, the inclusion bodies were collected from the lysates by centrifugation. These insoluble pellets were resolved in 6 M guanidine-HCl buffer, and purified by Ni-NTA affinity chromatography. The identity of the expressed rTul4 and rFopA was examined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with Coomassie Brilliant Blue staining. The concentration of each recombinant protein was estimated by the Bradford method.

2.2 Mice

NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (NSG) mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). The mice were bred and maintained under specific-pathogen-free condition at the animal facility in the College of Veterinary Medicine, Seoul National University. All experiments on animals were approved by the Seoul National University Institutional Animal Care and Use Committee (SNU-150202-1).

2.3 Establishment of humanized mice

Five-week-old NSG mice were intraperitoneally injected with busulfan (30 mg/kg body weight; Sigma Chemical Co., St. Louis, MO, USA). After 24 h, the busulfan-conditioned mice were inoculated with 1×10^5 human cord blood CD34+ cells (STEMCELL, Vancouver, BC, Canada) via the tail vein.

2.4 Analysis of human cell engraftment

At 6 weeks after transplantation of the human CD34+ cells, mice were anesthetized with a mixture of xylazine (5 mg/mg; Bayer Korea, Seoul, Korea) and alfaxan (60 mg/kg; Careside, Gyeonggi-do, Korea), following which blood samples from the retroorbital plexus were collected into heparinized capillary tubes. After removal of the red blood cells with ammonium-chloride-potassium lysis buffer (Lonza, Walkersville, MD, USA), the samples were stained with the following anti-human antibodies: hCD45-fluorescein isothiocyanate, hCD19-phycoerythrin, and hCD3-allophycocyanin (eBioscience, San Jose, CA, USA). Flow cytometry analysis was performed using a FACS Aria II device (BD Biosciences) equipped with FACS Diva Software, installed at the National Center for Inter-university Research Facilities at Seoul National University.

2.5 Vaccination of the humanized mice

The hu-mice were immunized subcutaneously with the rTul4 (40 μg/mL) and rFopA (20 μg/mL) combination or phosphate-buffered saline (PBS) on Days 0, 14, and 28. Blood samples were collected before the immunization and 2-week intervals

following the final injection, and the sera were separated by centrifugation and stored at -70 ℃. The hu-mice were anesthetized with a mixture of xylazine (5 mg/mg) and alfaxan (60 mg/kg) for the blood collections.

2.6 Humoral immune response

The concentrations of human IgM and IgG in the hu-mice were quantified through use of a commercial ELISA kit (Human IgM and IgG Ready-SET-Go!® ELISA; eBioscience), following the manufacturer's procedures. In brief, Corning Costar® 96-well plates were coated overnight at 4 ℃ with anti-human IgM and IgG monoclonal antibodies, respectively. After washing with PBS, the coated plates were blocked for 2 h with bovine serum albumin. Diluted serum samples were then added and the plates were left to stand for 2 h at room temperature. Known concentrations of human IgM and IgG were then applied, respectively, for use as standard curves. The plates were then incubated with horseradish peroxidase-conjugated anti-human-IgM and IgG monoclonal antibodies for 1 h at room temperature and thereafter developed with tetramethylbenzidine solution. After 15 min, the reactions of the substrate were stopped with 2N H_2SO_4 solution, and the plates were read at 450 nm using an Epoch microplate reader (BioTek, Winooski, VT, USA).

The concentrations of antigen-specific IgM and IgG were measured by the ELSIA method described above, but with slight modification, in that the 96-well plates were coated overnight at 4 ℃ with 4 μL/mL synthetic rTul4 and rFopA, respectively.

2.7 Challenge test

The *FT* LVS was cultured on cystine heart agar (Difco™, Becton Dickinson, Sparks, MD, USA) with 9% sheep blood at 37 ℃ for 48 h, and was suspended in PBS for the inoculations. Hu-mice (immunized three times at 2-week intervals) and control hu-mice were challenged intranasally with LVS (1×10^3 CFU), and were then monitored daily for 2 weeks for signs of mortality.

2.8 Histopathological analysis

After the challenge test, the spleens from the hu-mice were fixed in 10% neutral-buffered formalin and embedded in paraffin. The tissue paraffin blocks were sectioned into 3 μm slices, deparaffinized, rehydrated, and stained with hematoxylin and eosin (H&E). The sections were analyzed using a modified histopathological scoring system [74]. The criteria for histopathological scores are shown in Table 1. The inflammatory lesions in lungs were graded on a scale of 0-3 for peribronchiolar /bronchial infiltration, inflammation of the lung parenchyma, and given a numerical score ranging from 0 to 9. Spleen was assessed for degree of survived splenocytes.

Additional sections were deparaffinized and autoclaved for 20 min for antigen retrieval in a target retrieval solution (Dako, Carpinteria, CA, USA). The sections were washed with 0.025% PBST, blocked with normal horse serum for 1 h, and incubated overnight at 4 ℃ with monoclonal mouse anti-human CD3 antibody (diluted of 1:50; eBioscience). On the following day, after washing the slides, goat anti-mouse Alexa Fluor® 594 (diluted 1:1000; Invitrogen, Carlsbad, CA, USA) was applied to the sections

for 1 h. After washing again, the slides were mounted with VECTASHIELD® Antifade Mounting Medium with 4,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA, USA).

Table 1. The criteria of histopathological score for lung and spleen of hu-mice infected with *Francisella tularensis*

	Lung			Spleen
Score	peribronchiolar /bronchial infiltrates (% of sites)	Degree of peribronchial/bronchial inflammation	Parenchymal pneumonia	Survived splenocytes (% of sites)
0	None	None	None	<5%
1	Few (<25%)	Mild	Mild	5-15%
2	Many (25-75%)	Moderate	Heavy	15-30%
3	All (>75%)	Severe	Heavy and necrotizing	30-50%

2.9 Statistical Analyses

Statistical analyses were performed using Prism GraphPad version 6. Repeated measures analysis of variance followed by the Bonferroni post-hoc test, was used to analyze the data of total IgM and total IgG. The Mann-Whitney U test was applied for comparison of differences in the antigen-specific immunoglobulins. All data are expressed as the mean of samples ± standard error.

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3 RESULTS

3.1 Cloning, expression, and purification of the rTul4 and rFopA

In this study, I cloned the codon-optimized recombinant Tul4 and FopA genes of *FT*, individually into the pET-28b vector, expressed them in *E. coli* cells, and then purified the proteins using Ni-NTA agarose. The purity of the rTul4 (20.8 kDa) and rFopA (46 kDa) and their identity were monitored by SDS-PAGE, Coomassie Brilliant Blue staining (Figure 1A and C), and western blotting (Figure 1B), respectively.

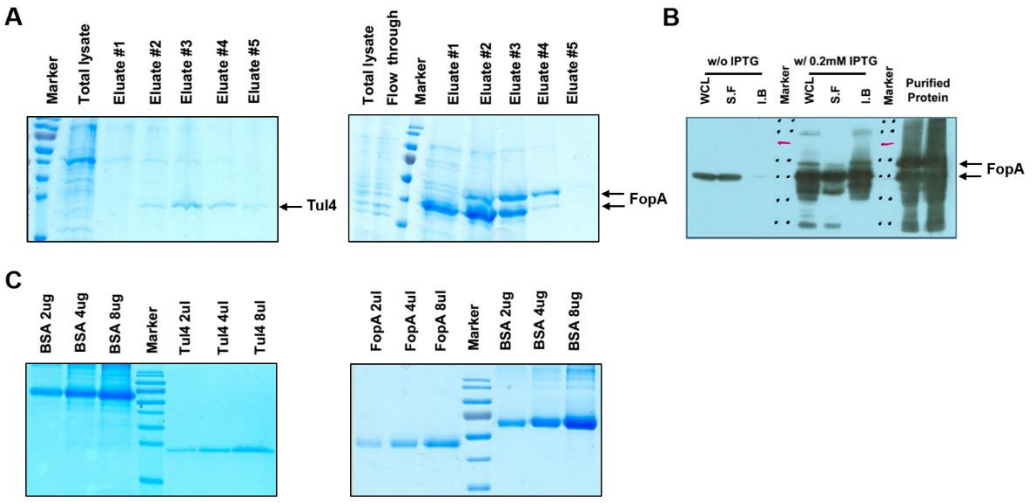

Figure 1. Cloning, expression, and purification of rTul4 and rFopA. (A) SDS-PAGE analysis of rTul4 and rFopA purification. SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis. (B) Western blot analysis of rFopA with anti-His antibody. WCL, whole cell lysates; S.F, soluble fraction; I.B, inclusion body. (C) Estimation of concentration of purified each protein. BSA, bovine serum albumin.

3.2 Human specific immune response after immunization in humanized mice

Human cord blood CD34+ cells were introduced into 5-week-old NSG mice via the tail vein. The establishment of a human immune system in the resulting hu-mice was confirmed by assessing the production of human T and B cells (Figure 2). Populations of human T and B cells were detected in the blood serum of the mice, and showed a continuous increase, reaching peak levels at 6 and 8 weeks after transplantation.

Serum samples from hu-mice immunized three times at 2-week intervals were collected at each sampling time point, and the levels of antibodies were measured using ELISA. As shown in Fig. 3A, levels of total human IgG were detectable at 4 weeks after the first immunization, and increased progressively until 6 weeks. On the contrary, no IgG was detected in the control hu-mice group at all sampling time points. In addition, the total human IgM levels showed a consistent increase with each subsequent boosting. A significant difference in the antibody levels was found between the two groups at 6 weeks after immunization. Unlike the total human IgG levels, total human IgM levels were detectable in the control group at all sampling time points.

To confirm the functional efficacy of the human antibodies produced in hu-mice, the levels of specific IgG and IgM against rTul4 and rFopA were measured in the blood serum at 6 weeks, when the antibody levels had reached a peak. Although it is difficult to quantify the amount of specific antibody, owing to the lack of a standard antibody against Tul4 and FopA, the production of specific IgG and IgM was confirmed by the significant differences in the absorbance values of serum samples between the two groups in the ELISA (Figure 3B). In the immunized mice, Tul4- and FopA-specific IgM

were dramatically increased relative to the control mice levels, whereas the Tul4-specific IgG was poorly produced even though the its absorbance was significantly higher than that in the control mice.

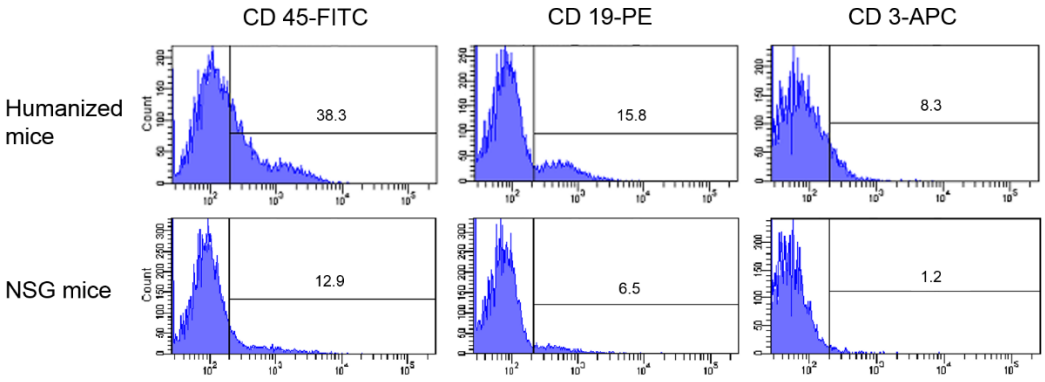

Figure 2. FACS analysis for confirmation of human CD34+ hematopoietic cell engraftment in NSG mice (resulting humanized mice). Cells in peripheral blood from humanized mice were labeled with fluorescent antibodies to a pan-leukocytes marker (FITC-conjugated anti-human CD45), a B cells marker (PE-conjugated anti-human CD19), and a T cells marker (APC- conjugated anti-human CD3). Non-humanized NSG mice were used as negative controls.

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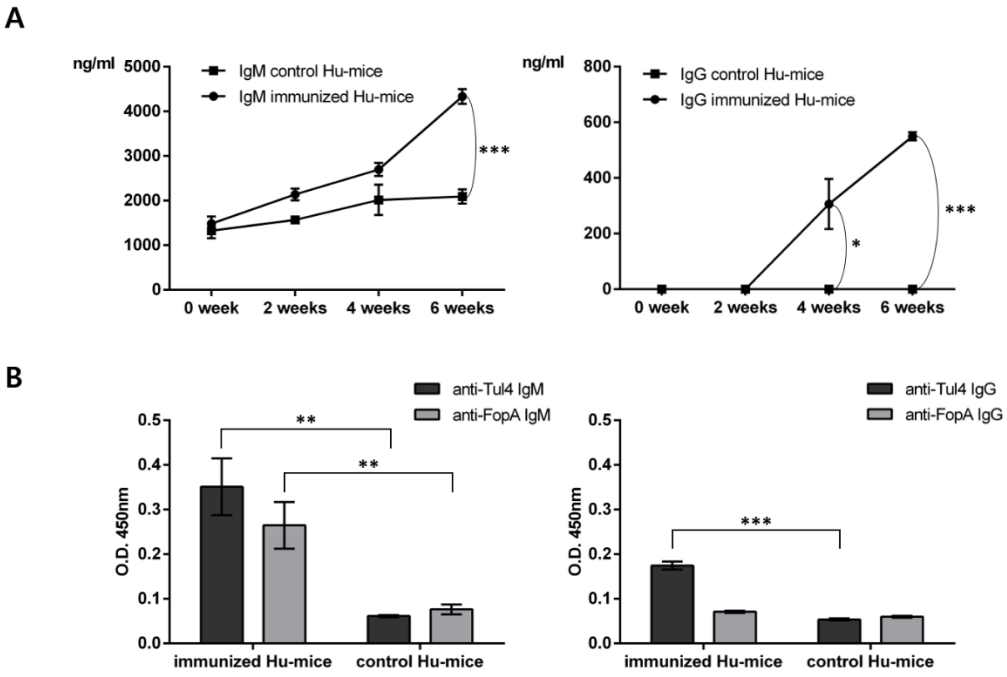

Figure 3. Antibody response in humanized mice after immunization. (A) The level of IgG and IgM in sera were collected from the hu-mice immunized 3 times. (B) The level of specific IgG and IgM against Tul4 and FopA in sera at 6-week time point. The data are presented as mean±SE. *** $p < 0.001$; * $p < 0.05$.

3.3 Partial protection against LVS in humanized mice immunized with rTul4 and rFopA

To determine whether the human antibodies induced in the blood could protect against a lethal dose of LVS (1×10^3 CFU), the hu-mice were challenged with an intranasal injection of LVS at 8 weeks after immunization. Owing to the potential virulence of the LVS, the challenge and survival experiments were performed in a Biosafety Level 3 facility. The survival curves and median survival time (MST) were measured after the LVS challenge (Figure 4). In this experiment, all animals in both the control and the rTul4- and rFopA-immunized groups showed a progressive loss of body weight with anorexia. Similar survival curves were observed for both these groups. However, the immunized group had a longer MST (9 days) than to the control group (7 days).

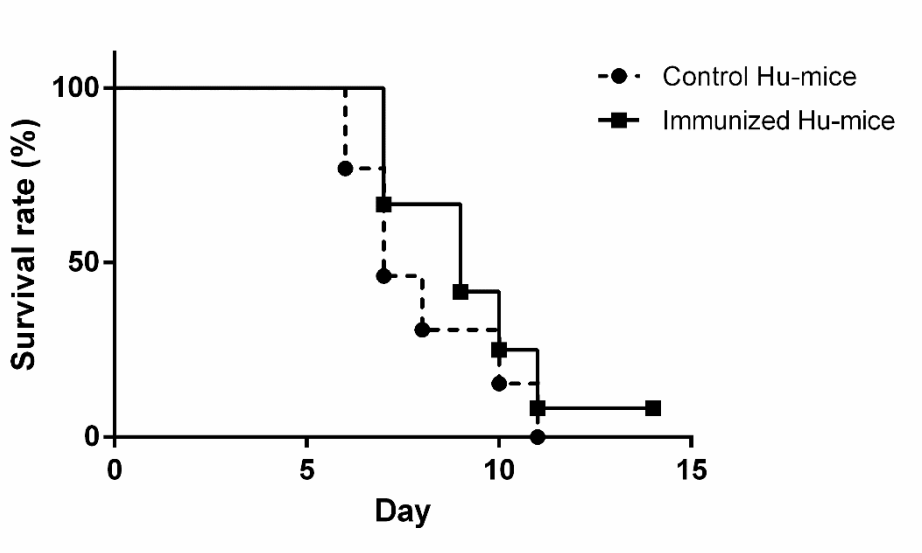

Figure 4. Survival of humanized and control mice following LVS (n= 11, n= 13, respectively). Humanized mice were immunized subcutaneously with the rTul4 (40 μg/mL) and rFopA (20 μg/mL) three times at 2-week intervals, and control humanized mice were injected subcutaneously with PBS. Survival curves and median survival time (MST) were measured after the LVS challenge (1×10^3 CFU). Survival of humanized mice was analyzed by Log-rank (*p* value = 0.1913) and Gehan-Breslow-Wilcoxon test (*p* value = 0.1480).

To confirm the effect of intranasal challenge in the immunized and control hu-mice, the lung tissues were analyzed. Although alveoli and parenchyma were filled with macrophages, lymphocytes, and neutrophils in both groups, alveolar structure remained in the immunized hu-mice compared to the controls (Figure 5). Moreover, in the control group, the alveolar septa were severely thickened, and the cell debris were observed together with severe necrosis. This result shows that the lungs were less damaged by intranasal LVS challenge in immunized hu-mice than in the control hu-mice.

I further confirmed the effect of rTul4 and rFopA on immune cells that were the main targets against *FT*, the spleen tissues from the immunized and control hu-mice, post LVS challenge, were prepared for histopathological analysis. Although rTul4 and rFopA immunization did not increase the survival rate of hu-mice from LVS challenge, there was higher splenocyte survival in the immunized hu-mice than in the controls (Figure 6). In addition, most of the splenocytes in the control group were undergoing apoptosis, and it was difficult to observe any normal ones. Relatively normal splenocytes that were resistant against LVS and retained a normal splenic structure including white pulp and red pulp, were observed in the immunized hu-mice only.

Next, to verify whether the splenocytes that had survived in the hu-mice were of human or mouse origin, the cells were stained with human CD3 antibody. As shown in Figure 7, CD3+ human T cells over DAPI-positive cells (staining live nuclei) were identified in the immunized hu-mice. Taken together with histopathological results, we conclude that rTul4 and rFopA immunization supports human T-cell survival in the spleens of hu-mice.

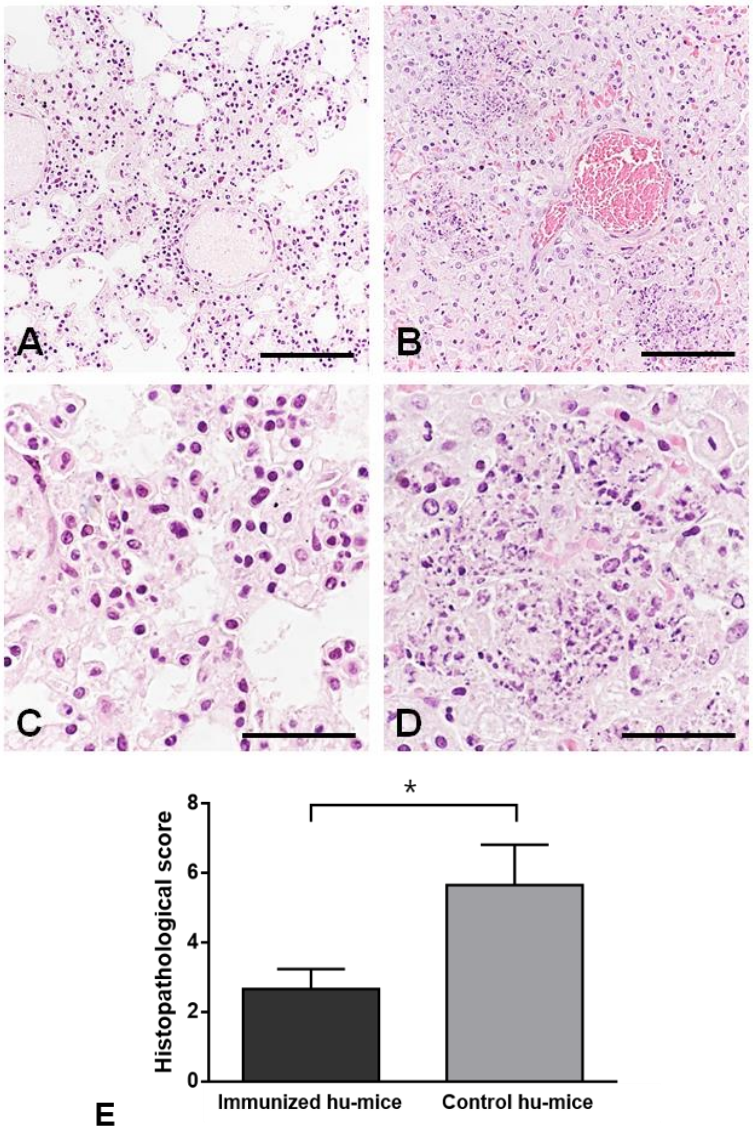

Figure 5. Histopathologic changes in lung from humanized mice (hu-mice) after challenge test. Two weeks after challenge, lungs were harvested, fixed, embed, and stained with H&E. (A and C) The lung of immunized hu-mice. (B and D) The lung of control hu-mice. (E) Histopathological score. Relatively normal lung structures were

observed in the immunized hu-mice compared to the controls. The alveolar space and septa showed increased alveolar macrophages and other mononuclear cells in both groups. (A and B) scale bar, 100 μm; (C and D) scale bar, 50 μm. The scoring data are presented as mean±SD. * p <0.05.

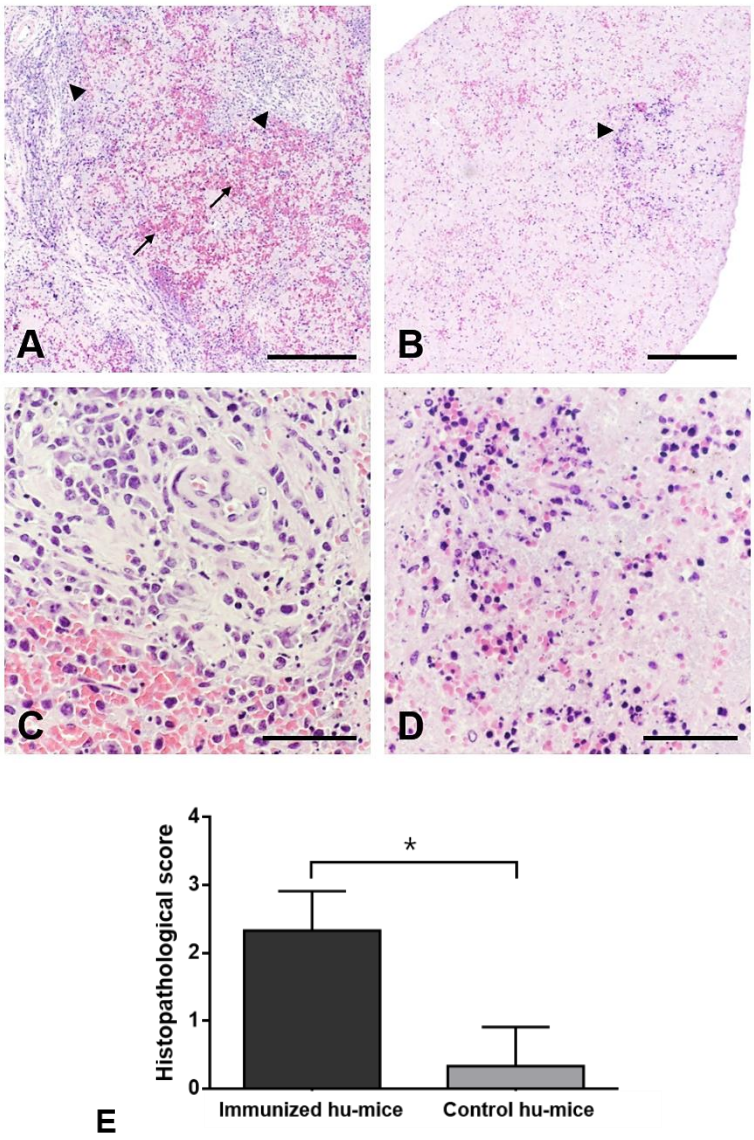

Figure 6. Histopathologic changes in spleen from humanized mice (hu-mice) after challenge test. Two weeks after challenge, spleens were harvested, fixed, embed, and stained with H&E. (A and C) The spleen of immunized hu-mice showed a histological structure close to normal, in which white pulp (arrow head) and red pulp (arrow) were

distinguished. (B and D) The spleen of control hu-mice showed apoptosis of the splenocytes and loss of histological structure. (A and B) scale bar, 250 μm; (C and D) scale bar, 50 μm. (E) Histopathological score. The scoring data are presented as mean±SD. * p <0.05.

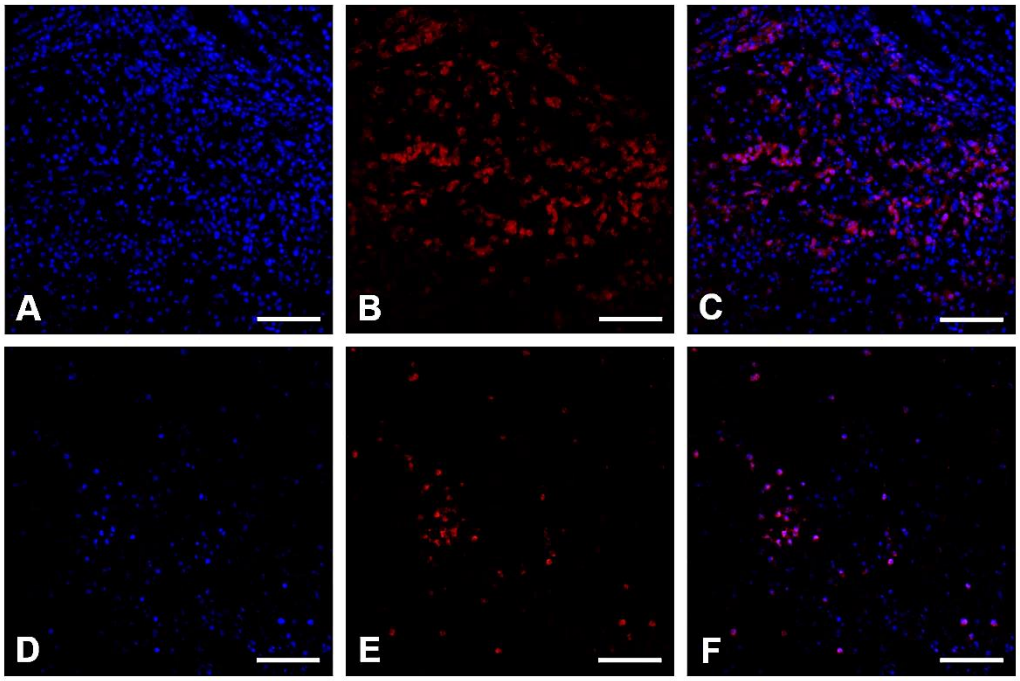

Figure 7. Immunohistochemistry in spleen from humanized mice after challenge test. The spleen slides were stained with anti-human CD3 antibody (B and E) and with DAPI for verification of nuclei (A and D). C and F showed a merged image of A and B, C and D, respectively. Scale bar, 50 μm.

4 DISCUSSION

Tul4 and FopA are representative outer membrane components of *FT*. The proteins are exposed to the surface and are most accessible to antibodies, indicating their high potential as candidates for a protective vaccine [8]. Therefore, I evaluated the possibility of a Tul4 and FopA combination as a vaccine candidate in mice with a human immune system.

In our study, I confirmed the elicitation of T and B cells between 6 and 8 weeks in mice bearing CD34+ cells indicating that a stable hu-mouse model had been established. In addition, the human total IgM and total IgG levels in the hu-mice were significantly increased after 2 weeks of final immunization. After 6 weeks, when humoral immunity was at its maximum, antigen-specific human IgM and IgG were detected. These results mean that the human immune system had been well developed by the human cord blood CD34+ cell graft in the hu-mice. In the human immune system, IgM responds most rapidly to exposure to external antigens, whereupon IgG increases rapidly to remove the antigen. I have shown that an increase in IgM levels preceded the increase in IgG levels in a manner similar to the human immune response. Interestingly, total IgG was not detected in the control hu-mice, and its increase in the immunized hu-mice was dependent on the immunizations. These results indicate that human IgG was induced in hu-mice by rTul4 and rFopA stimulation, suggesting that the immune system of hu-mice is almost similar to that of humans.

The production of antigen-specific human IgG in hu-mice was poor compared with antigen-specific human IgM, consistent with the results of previous studies [112,

162]. Several possibilities have been suggested for the insufficiency of specific IgG production in hu-mice, including the lack of interaction between B and T cells due to major histocompatibility complex mismatches [163] and the result of a series of processes involving the absence of interleukin-2 and the high susceptibility of T cells to apoptosis [164]. In contrast to a previous study in which FopA-specific IgG was identified, this study did not detect it in the hu-mice [8]. The reason for this is not clear, but it may be related to an insufficient induction of the humoral immune response resulting from transplantation of the human immune system into the mouse immune system, and the absence of an appropriate immune environment, such as human cytokines and secondary lymphoid organs for human immune cells [165-167]. Therefore, in future studies, it would be necessary to develop a hu-mouse capable of efficiently producing an antigen-specific antibody, including a neutralizing antibody.

FT is a highly contagious pathogen, and because of its risk, the challenge tests were conducted at an approved Biosafety Level 3 facility. There was no statistically significant difference in the survival rate between the immunized and control groups, but the MST showed an increase tendency in the immunized group. Several studies have shown that vaccine candidates made up of one or two subunits, such as DnaK, Tul4, and FopA, have a partial protective effect against the lethal dose of LVS and are less immunogenic than live attenuated vaccines [7, 8, 168]. In line with studies, our challenge results suggest a partial protective effect of the subunit vaccine candidate in the hu-mouse model against LVS.

The efficacy of these immunization candidates in hu-mice was further confirmed

by examining the histopathologic changes in the lungs and the immune responses in the spleen. It was found that immunized hu-mice had a partially normal alveoli structures in. In addition, the surviving splenocytes had retained a normal white pulp structure, and these immunized hu-mice had a greater number of surviving human CD3+ immune cells than the control mice. Therefore, although the efficacy of rTul4 and rFopA was insufficient to produce a 100% survival rate of the hu-mice, these vaccine candidates nevertheless were able to partially defend the CD3 cells against the LVS. In particular, the survival of the T cells in the spleen is presumed to be caused by rTul4 and rFopA, whereupon the immune cells could be protected from the LVS infection and avoided apoptosis. Cowley et al. [169] have reported that the T-cell subpopulation isolated from splenocytes inhibits and regulates the intracellular growth of the LVS. Thus, the support provided by rTul4 and rFopA toward T-cell survival suggests their potential for use as an adjuvant for the suppression and clearance of LVS infections. Above all, the survival of human T cells, not mouse T cells, was confirmed in the mice reconstituted with human immune cells, confirming that the experimental results obtained in the biological systems of these mice closely reflect the human immune system.

In conclusion, I confirmed that human CD34+ hematopoietic cells can be stably engrafted in mice, for construction of the human immune system. The immune responses elicited by the rTul4 and rFopA cocktail, were successfully evaluated in this system showing the feasibility of the hu-mouse model for use in the evaluation of tularemia vaccine candidates.

GENERAL CONCLUSION

FT is a high-risk pathogen that can be used for bioterrorism. However, LVS, which has been developed as an attenuated vaccine against *FT*, has limited applications in public use due to difficulties in predicting side effects and the efficacy of the vaccine. Therefore, the need for safe and effective vaccine development has increased and, in this regard, subunit vaccines have attracted attention. In Chapter I, I determined the immunogenicity of a cocktail of Tul4 and FopA peptides from the outer membrane protein of *FT* using BMDC from mice and a C57BL/6 mouse model. Tul4 and FopA elicited maturation of DC, production of IL6 and IL12p4 cytokines, and allostimulatory capacity. The cellular immune response was confirmed by restimulation of sensitized splenocytes, and the humoral immune response was confirmed by immunoglobulin evaluation in immunized mice.

Susceptibility to *FT* is different in mice and humans, which makes it more difficult to extrapolate vaccine evaluation results based on mouse models to humans. Hu-mice models with human immune systems are currently being used to study human-specific pathogens and produce human antibodies. Hu-mice were evaluated as suitable animal models for vaccine development using Tul4 and FopA, which confirmed the immunogenicity reported in Chapter I. Human CD34+ hematopoietic cells were engrafted and differentiated in mice. After immunization, human antibodies were produced in hu-mice. The hu-mice immunized with Tul4 and FopA were partially protected against LVS. In addition, the lungs and spleen of immunized hu-mice were less damaged than those of control mice.

These results confirmed the effective immunogenicity of Tul4 and FopA cocktails as candidates for an *FT* subunit vaccine and, moreover, that hu-mice could be a feasible model in vaccine evaluation.

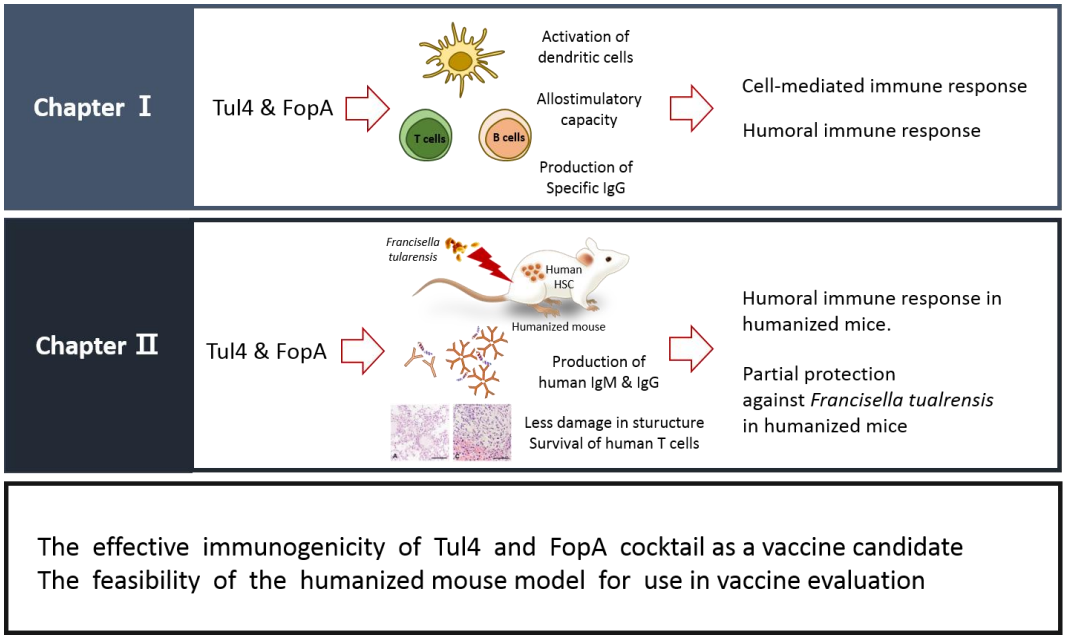

Figure 1. General conclusion of thesis.

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국문 초록

인간화마우스 모델을 활용한 *Francisella tularensis* 항원의 면역반응에 관한 연구

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고위험병원체인 *Francisella tularensis* (*FT*)은 낮은 감염용량 (infectious dose)과 에어로졸을 통한 전파력을 가지고 있어서, 잠재적인 생물학적 무기로

여겨진다. LVS (live vaccine strain)라고 불리는 약독화된 *FT*가 생백신으로 개발되었지만, 부작용과 병독성이 잔존하는 문제를 보여주었다. 따라서 최근 생백신의 대안으로, 병원체의 일부를 활용하는 subunit vaccine이 제시되고 있다. *FT*의 외막 단백질인 Tul4와 FopA는 박테리아의 면역원성에 중요한 역할을 하는 것으로 보고되었으며, *FT*에 대한 subunit vaccine 후보로 가능성이 있다.

In vitro 모델 및 마우스 모델은 백신 개발의 표준 평가 방법이다. 하지만 *FT*에 대한 감수성에 대해 마우스와 사람 간 차이를 나타냄으로써, 동물 모델의 한계점을 극복하지 못하고 있다. 사람의 면역체계가 구축된 인간화마우스 (humanized mice)는 사람 특이적인 질병을 연구하기 위해 개발되었으며, 병원체에 대한 사람의 면역글로불린의 생성이 보고되었다. 따라서 인간화마우스 모델은 동물 모델과 임상연구의 차이를 극복 할 수 있는 가능성을 제시한다. 이에, 본 연구에서는 *FT*의 외막 단백질 에피토프 (epitope)인 Tul4와 FopA를 기반으로 제작한 subunit vaccine이 인간화마우스 모델에서 사람-특이적인 면역반응을 야기함을 보고하고자 하였다.

제 1 장에서는 *FT*에 대한 subunit vaccine 후보물질로서, 외막 단백질의

Tul4와 FopA recombinant peptides의 면역반응을 *in vitro* 및 *in vivo* 상에서 관찰하였다. CpG 어쥬번트 (adjuvant)와 recombinant peptides에 의해 자극된 수지상세포 (dendritic cells)는 강력한 면역 표현형 변화가 유도되었다. 특히, 수지상세포의 성숙과 염증성 사이토 카인 (IL-6, IL-12)의 분비가 관찰되었다. 또한 성숙한 수지상세포는 혼합림프구반응시험 (mixed lymphocyte reaction)에서 미감작 비장세포의 *ex vivo* 증식이 관찰되었다. 마지막으로, C57BL/6 마우스에서 항체 형성 확인을 통해, 체액성 면역 반응을 평가했다. Tul4와 FopA를 마우스에 면역한 후, total IgG는 생성되기 시작하여, 6주에 최고점에 도달하였다. 더욱이, 마우스에서 Tul4에 대한 특이적 IgG (Tul4 specific IgG)가 확인되었다. 이러한 결과를 토대로, recombinant peptides Tul4와 FopA의 면역원성을 확인하였고, *FT*에 대한 안전한 subunit vaccine이 될 수 있을 것으로 사료된다.

제 2 장에서는 대장균에 최적화된 코돈으로 발현된 recombinant Tul4와 FopA (rTul4와 rFopA)의 칵테일의 면역 반응을, 사람의 CD34+ 조혈모세포를 생착한 인간화마우스 (humanized mice)를 활용하여 평가했다. rTul4와 rFopA

칵테일을 면역한 인간화마우스는 유의적으로 사람의 면역글로불린을 생성하였다. 뿐만 아니라, LVS 공격 시험에 대하여 생존 기간이 연장되는 경향을 보여주었고, 면역한 인간화마우스의 비장에서 사람의 T 세포가 상대적으로 생존한 것이 관찰되었다. 이러한 결과는 rTul4와 rFopA의 칵테일이 인간화마우스에서 성공적으로 면역 반응을 유도하였음을 나타낸다. 또한 *FT* 백신 후보 물질의 평가에 있어, 인간화마우스 모델의 잠재력을 보여주었다.

결론적으로, 본 연구는 재조합 Tul4 및 FopA 백신 후보물질의 효능 및 *FT* 백신 연구를 위한 인간화마우스 모델의 가치를 입증하였다. 종합적으로 인간화마우스를 활용한 본 연구의 시도가 *FT*의 백신 연구에 널리 적용할 수 있을 것으로 사료된다.

주요어: *Francisella tularensis*, Tul4, FopA, 수지상세포, 인간화마우스

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