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A Synthetic Tul4 and FopA Peptide Cocktail of *Francisella tularensis* Induces Humoral and Cell-Mediated Immune Responses in Mice

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Francisella tularensis is a highly virulent pathogen of humans and other mammals. Moreover, *F. tularensis* has been designated a category A biothreat agent, and there is growing interest in the development of a protective vaccine. In the present study, we determine the in vitro and in vivo immune responses of a subunit vaccine composed of recombinant peptides Tul4 and FopA from epitopes of the *F. tularensis* outer membrane proteins. The recombinant peptides with adjuvant CpG induced robust immunophenotypic change of dendritic cell (DC) maturation and secretion of inflammatory cytokines (IL-6, IL-12). In addition, the matured DCs enabled ex vivo proliferation of naive splenocytes in a mixed lymphocyte reaction. Lastly, we determined the in vivo immune response by assessment of antibody production in C57BL/6 mice. Total IgG levels were produced after immunization and peaked in 6 weeks, and moreover, Tul4-specific IgG was confirmed in the mice receiving peptides with or without CpG. Based on these results, we concluded that the recombinant peptides Tul4 and FopA have immunogenicity and could be a safe subunit vaccine candidate approach against *F. tularensis*.

Keywords: *Francisella tularensis*, Tul4, FopA, subunit vaccine, immune response

Introduction

Tularemia, caused by *Francisella tularensis*, is a highly infectious disease that has various courses depending on the *Francisella* strain and the site of infection [19]. Among the various routes of inoculation, an aerosol release of *F. tularensis* induced acute fever beginning 3 to 5 days later, followed by one or more of pharyngitis, bronchiolitis, hilar lymphadenitis, and pneumonitis [4]. The progression from respiratory symptoms to systemic illness may result in life-threatening pleuropneumonia and sepsis [4]. Without antibiotic treatment, the mortality rate of respiratory tularemia increased by more than 30% [10]. Tularemia has been considered as a potential biothreat weapon in terror due to the ease of dissemination by aerosol, low dose of as few as 10 organisms for infection, and the extreme virulence [19]. For these reasons, *F. tularensis* has been classified as a category A agent by the Centers for Disease Control and Prevention (CDC).

Thus far, the *F. tularensis* live vaccine strain (LVS) has been the sole vaccine that is close to approval in humans. However, side effects and the incomplete protection as a general vaccine against *F. tularensis* remain major obstacles for approval of LVS in clinic [2, 19]. Since then, for developing a safe vaccine that completely protects against tularemia, there have been a lot of attempts, including attenuated mutant *Francisella*, virus vector, and subunit immunogens with carrier system [6]. Moreover, a subunit vaccine using immunodominant antigens, including outer membrane proteins (OMPs), has been considered as a potential alternative for LVS vaccination [1].

OMPs, surface-exposed domains, have played a pathogenic role in the adhesion to and invasion of host, virulence factors, and activation of both innate immunity and adaptive host immunity. These functions make them stand out as vaccines against bacterial infections [8]. Specifically, OMPs of *Francisella* strains such as FopA and Tul4 induce immunogenicity by eliciting FopA and Tul4 specific antibodies. The recombinant FopA protein showed humoral immunity and protected naïve mice against LVS challenge

[7]. Tul4 is also reported as a subunit vaccine that induced IFN-γ, IL-10, and IL-17A production in an antigen-specific manner and induced systemic antibody responses [1]. Therefore, FopA and Tul4 have been studied as strong candidates to develop a vaccine against tularemia.

In this study, we focused on a peptide vaccine using immunogenic epitopes of FopA and Tul4. Peptides based on epitopes are considered as activators of cellular immunity and humoral immunity and inducers of the 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 with proteins [12] and are applicable as a peptide cocktail vaccine to prompt a greater multitarget defensive response against antigens. For those reasons, peptides have gotten the limelight as an attractive strategy for developing vaccines. Therefore, we demonstrate that a combination of FopA and Tul4 epitopes elicited an initial immune response related with dendritic cells in vitro and humoral immunity in vivo. These results demonstrate that a combination of FopA and Tul4 epitopes could be a potential vaccine candidate with the safety and effectiveness, improving the protection ability of individual FopA and Tul4.

Materials and Methods

Animals

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

Synthetic Peptides and Adjuvant Preparation

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

Generation of Bone Marrow-Derived Dendritic Cells (BMDCs)

Isolation of bone marrow-derived hematopoietic precursors was performed as 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, 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, and adherent cells were cultured in fresh RPMI 1640 medium containing 10 ng/ml recombinant granulocyte/macrophage colony-stimulating factor (Peprotech, USA) for 8 day. Adherent cells were harvested and the purity was tested by FACS Aria II (BD Biosciences, USA) equipped with FACS Diva software. This procedure routinely yielded >80% CD11c-positive cells.

In Vitro Dendritic Cell 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 the cytokine contents were measured by enzyme-linked immunosorbent assay (ELISA kit; eBioscience, USA) for IL-6 and IL-12p40. The absorbance was measured using a SpectraMax M2 Microplate Reader (Molecular Devices, USA).

The stimulated DCs were harvested to examine their surface markers by fluorescence-activated cell sorting. 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-conjugated monoclonal antibodies (mAbs) to CD40, DC86, and MHC II (Biolegend, USA), and FITC-conjugated mAb to CD11c (BD Biosciences) for 2 h. Flow cytometry analysis was performed using the FACS Aria II equipped with FACS Diva software.

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, 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°C. 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.

Immunizations with Peptides

Mice were immunized by the subcutaneous route with the peptides (Tul4 40 μg/ml + FopA 20 μg/ml) or CpG (20 μg/ml) or both on days 0, 14, and 28. 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 a mixture of xylazine (5 mg/mg; Bayer Korea, Korea) and alfaxan (60 mg/kg; Careside, Korea), using heparinized capillary tubes and the serum was obtained after centrifugation.

Ex Vivo Spleen Restimulation

Splenocytes from peptide-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. The supernatants were removed after 72 h to test for IL-2 and IL-4 production. The concentrations of these cytokines were determined by a specific ELISA kit (BD Biosciences) according to the manufacturer's instructions. The absorbance was measured using a SpectraMax M2 Microplate Reader.

Humoral and Cellular Immune Responses 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, USA) in PBS. Blocking was done for 4 h at room temperature with PBS containing 1% bovine serum albumin. Serial 2-fold 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, USA). The absorbance was measured using a SpectraMax M2 Microplate Reader.

Statistical Analysis

A one-way analysis of variance followed by the Tukey HSD test was performed in SPSS Statistics (ver. 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.

Results

Synthetic Peptide-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 concentration of peptides or CpG was determined from a dose-dependent test (data not shown). As shown in Fig. 1A, the expression of CD86 and MHC class II were greatly upregulated on all stimulated DCs compared with unstimulated DCs. Moreover, the expression of CD40 was strongly enhanced on DCs stimulated with CpG or peptides + CpG, and only mildly increased expression was revealed on DCs stimulated with the peptides only.

In the cytokine analysis, the levels of IL-6 and IL-12p40 were significantly elevated in all experimental groups compared with that in the control group (Fig. 1B). In particular, the IL-6 and IL-12p40 levels in the group stimulated with peptides with/without CpG were higher than those 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 DC activation, with production of IL-6 and IL12p4.

Allostimulatory Function of BMDCs Pulsed with Synthetic Peptides

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

Immune Response of Splenocytes in Mice Immunized with Synthetic Peptides

We further examined the immune response of splenocytes that were isolated from mice immunized with peptides, CpG, or both. After restimulation of the splenocytes with peptides, we measured each level of T helper type 1 (Th1)-related cytokine IL-2 and T helper type 2 (Th2)-related cytokine IL-4 in the supernatant. As shown in Fig. 3, the levels of IL-2 were slightly increased with marginal significance ($p = 0.059$) in the peptides and peptides+CpG groups compared with the control group but not in the CpG group. Moreover, the expression levels of IL-2 in splenocytes primed with peptides+CpG were higher than those with peptides only groups. For Th2-related cytokine IL-4, its secretion was 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 an in vivo immune response by immunizing splenocytes.

Antibody Response in Mice Immunized with Synthetic Peptides

In order to determine whether peptides with/without

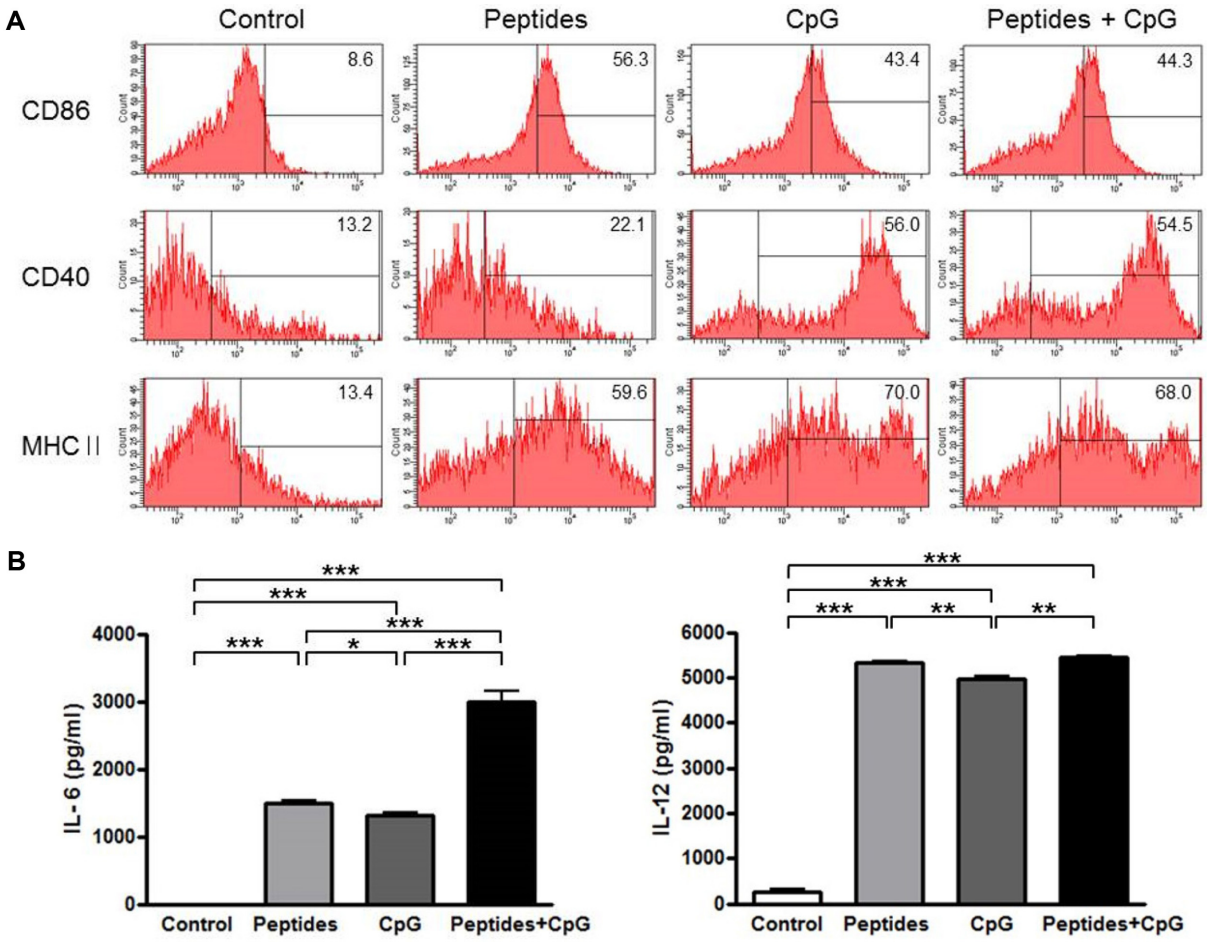

Fig. 1. Bone marrow-derived dendritic Cell (BMDC) maturation stimulated by peptides.
(**A**) The expression levels of CD86, CD40, and MHC class II in BMDCs following stimulation with peptides or peptides+CpG compared with those of control BMDCs. (**B**) Secretion of IL-6 and IL-12p40 by the BMDCs that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

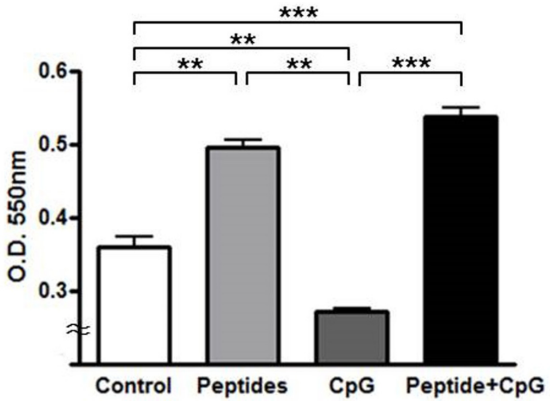

Fig. 2. The proliferation of splenocytes co-cultured with peptides- or peptides+CpG-pulsed DCs.
The data in bar graphs are presented as the mean ± SE. **$p < 0.01$, ***$p < 0.001$ between each group.

CpG are capable of inducing antibody after in vivo immunization, total IgG in the serum was collected from the mice immunized 3 times with 2 weeks intervals and were measured by ELISA. As presented in Fig. 4A, the levels of total IgG in the 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 peptide-immunized groups at the 4- and 8-week time points and CpG-immunized groups at the 2-, 4- , 6-, and 8-week time points ($p < 0.05$, each comparison). The peptide- and CpG-immunized groups did not reveal any significant difference. The levels of total IgG were peaked in 2 weeks after final boosting, and then declined at the 8-week time point in all experimental groups.

We further examined for specific IgG that functionally

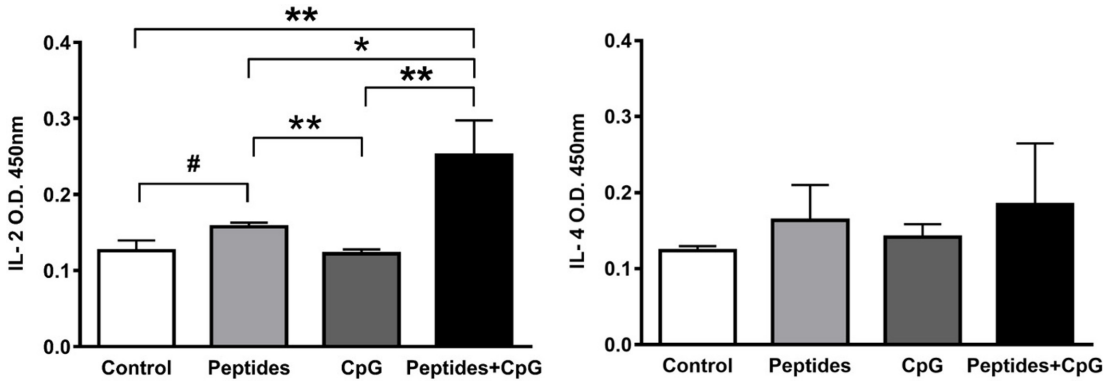

Fig. 3. Secretion of IL-2 and IL-4 by the restimulated splenocytes with peptides.
The data in the bar graphs are presented as the mean ± SE. *$p < 0.05$, **$p < 0.01$, between each group. #indicates marginally significant differences in the peptide group compared with the control group.

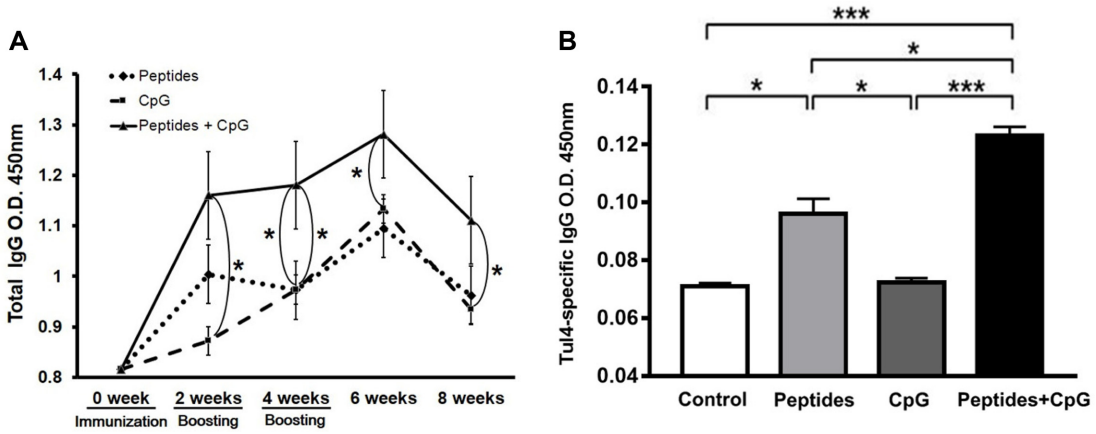

Fig. 4. The antibody responses in mice immunized with peptides.
(**A**) The total IgG in sera were collected from the mice immunized 3 times. (**B**) The level of Tul4-specific IgG in sera at the 6-week time point. The data in the bar graphs are presented as the mean ± SE. *$p < 0.05$, ***$p < 0.001$,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

recognize and bind to Tul4 and FopA in the serum at the 6-week time point when the total IgG level was the highest. As shown in Fig. 4B, in the mice immunized with peptides or peptides+CpG, the density of Tul4-specific IgG was 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 the peptides and CpG. On the contrary, FopA-specific IgG was not detected in all immunized mice owing to the insolubility of FopA peptides (data not shown). On the basis of the results, we confirmed that immunization with the peptides and/or CpG could elicit a strong antibody response and prolong antibody production in vivo, and the peptides could induce the generation of Tul4-specific antibody.

Discussion

In the present study, we have developed safe peptide vaccine candidates that originated from epitopes of *F. tularensis* Tul4 and FopA conjugated with CpG, and evaluated the effectiveness as a new vaccine.

DCs are the initial antigen-presenting cells and are particularly important in the initial stages of the immune response to antigens by expressing surface molecules. The 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, we analyzed the phenotypic characteristics and cytokine secretion of activated DCs. As shown in Fig. 1, peptides could stimulate the DCs to express CD40, and CpG strongly enhanced that expression. MHC class II and CD86 were also highly expressed in all

groups. Based on these results, we could conclude that the peptide and CpG treatments sufficiently induce DC activation for signaling to Th cells, but it was difficult to confirm the synergic effect of the peptides and CpG in phenotype analysis, and therefore, we conducted further cytokine analysis of the immune response induced by the peptides and CpG.

Cytokines secreted by stimulated DCs affect the differentiation of T cells. IL-12 plays important roles in the expansion of DCs and the development of IFN-γ-producing cells for eliciting the Th1 response [13]. IL-6 induces the immune responses by regulating the balance of immature and matured DCs [16]. In addition, IL-6 promotes Th2 differentiation and simultaneously inhibits Th1 polarization [5]. Previous studies reported that cytokines are produced from DCs through the TLR2 pathway by intracellular pathogens such as *F. tularensis* [17, 18] and the TLR9 pathway by CpG stimulation [9, 22]. For these reasons, we supposed that the synergistic effects of peptides and CpG are induced through the TLR2 and the 9 pathways in DCs, and then significantly upregulated the IL-6 and IL-12p40 levels compared with other stimulated DCs. Moreover, as presented in 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 in a significant decrease of splenocyte proliferation compared with the control group, despite CpG's auxiliary function to immunity [15]. Interestingly, a previous study has reported that CpG application alone induces indoleamine 2,3-dioxygenase expression and prohibits proliferation of T cells from splenocytes [14, 21]. Thus, we supposed that CpG-pulsed DCs suppressed the proliferation of lymphocytes using a similar mechanism. Based on these results, we speculate that the increase of IL-12p40 and IL-6 secretion from stimulated DCs influences the differentiation of Th cells to Th1 or Th2 cells and controls the cellular immunity and humoral immunity. Simultaneously, stimulated DCs induce immune responses by transferring signals to splenocytes, including lymphocytes.

In vitro experiments with DCs to confirm the effectivity of a vaccine candidate have limitations for reflecting the multiple immune cell responses. Therefore, we examined the cytokine response of splenocytes ex vivo and immunoglobulin levels in the serum from the mice immunized with peptides, CpG, or both. As presented in Fig. 3, when primed-splenocytes were restimulated with only Tul4 and FopA, Th1 cytokine IL-2 was strongly increased in the peptides+CpG groups. However, a minimal level of IL-4 was induced in all experimental groups compared with IL-2. At such a low level, it is hard to detect the difference of IL-4 in each group. Although the reason for the 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 [3], and Th1-type cytokines are hypothesized to suppress the production of Th2-type cytokines such as IL-4 [11]. Of course, natural killer cells and macrophages in raw splenocytes could potentially produce cytokines [20], and these cells may not effect the antigen-specific cytokine reaction by repeated immunization. These results indicate that lymphocytes restimulated with Tul4 and FopA induced antigen-specific immune responses. Resultantly, peptide immunizations could induce elicitation of the adaptive immunity in vivo.

Lastly, we analyzed IgG in the serum from the mice to evaluate the humoral response induced by peptide stimulation. Fig. 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 Fig. 4B. FopA-specific IgG was not detected in all experimental groups, and even previous studies have proven that FopA epitope could induce a specific humoral immune response [7]. Taken together with the total IgG and specific IgG, these results indicate that peptide immunization induces a humoral immune response following secretion of the peptide-specific IgG by polarized B cells and CpG has synergistic effects with peptides to produce IgG in vivo.

Of course, a challenge test is necessary for evaluating the vaccine efficacy. However, high-risk pathogens including *F. tularensis* and LVS are supervised by the CDC, and require approval of experiments from the CDC and Biosafety Level 3 (BL3) facility. Further studies of challenging with *F. tularensis* in an 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. We have confirmed the phenotypic change, cytokine production, and allostimulatory capacity of peptides/CpG-sensitized DCs in in vitro experiments. Additionally, ex vivo and in vivo experiments showed the induction of the cellular immune response from splenocytes of immunized mice and an increase of the humoral immune response through total IgG and antigen-specific IgG production. Moreover,

CpG could enhance the almost immune response towards peptides in our experiments. These results support that Tul4 and FopA epitope peptides originated from *F. tularensis* have effective immunogenicity, and could be used as a safe and effective vaccine against *F. tularensis* infection.

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