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For t 2 DNA Vaccine Prevents *Forcipomyia taiwana* (Biting Midge)

Allergy in a Mouse Model

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Short title: DNA vaccine prevents biting midge allergy in a mouse model

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ABSTRACT

Background: *Forcipomyia taiwana* (biting midge) is the most prevalent allergenic biting insect in Taiwan and 60% of the exposed subjects develop allergic reactions. Subjects with insect allergy frequently limit their outdoor activities to avoid the annoyingly intense itchy allergic reactions, leading to significant worsening of their quality of life. Allergen specific immunotherapy is the only known therapy that provides long-term host immune tolerance to the allergen, but is time-consuming and cumbersome. This study tested if the For t 2 DNA vaccine can prevent allergic symptoms in For t 2 sensitized mice.

Materials and Methods: Two consecutive shots of For t 2 DNA vaccine were given to mice with a 7-day interval before sensitization with recombinant For t 2 proteins, using the two-step sensitization protocol reported previously.

Results: The For t 2 DNA vaccine at 50 µg prevented the production of For t 2-specific IgE ($p<0.05$), as well as midge-allergen challenge induced scratch bouts, midge-allergen induced IL-13 and IL-4 production from splenocytes, and inflammatory cell infiltrations in the lesions 48 hours after intra-dermal challenge.

Conclusions: This study is the first to demonstrate that DNA vaccine encoding midge allergen is effective in preventing allergic skin inflammation induced by biting midge. Immunotherapy using For t 2 DNA vaccine can protect mice from being sensitized by midge allergen and may be a promising treatment for biting midge allergy in the future.

INTRODUCTION

Forcipomyia taiwana (biting midge) is the most prevalent allergenic biting insect in Taiwan, with 60% of the exposed subjects developing allergic reactions to its bites (1). The midge is a very small (around 1-1.5 mm) blood-sucking insect that attacks in groups in the daytime. It is widely found in Taiwan and in southern China (2, 3). People with insect allergy frequently limit their outdoor activities to avoid the annoyingly intense itchy allergic reactions, leading to significant worsening of their quality of life (4).

Previous studies have shown that immediate reactions to midge bites are IgE-mediated, with at least 11 allergens involved. For t 1, For t 2, and For t 3 are the major *F. taiwana* allergens (1). In contrast, delayed reactions involve inflammatory cell proliferations and increased production of multiple cytokines, including IFN-gamma, IL-10, IL-6, and TNF-alpha (5).

Symptoms of IgE-mediated allergy can be ameliorated by temporary suppression of mediators and immune cells by antihistamines and corticosteroids. However, the only long-term curative treatment is allergen-specific immunotherapy by the administration of gradually-escalating doses of allergen-specific vaccines (6-9). Allergen-specific immunotherapy has been proven to induce allergen-specific immune tolerance, probably through the up-regulation of allergen-specific IL-10 and TGF-beta producing regulatory T cells and B cells (7, 10). Aside from the mounting evidence of therapeutic effects among

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patients with asthma, allergic rhinitis, and venom allergies (11, 12), patients with severe mosquito allergy also show improved symptoms after immunotherapy with mosquito extract (13-15).

However, conventional immunotherapy using protein-based vaccine is time-consuming and cumbersome. Recent studies have tried many strategies to minimize allergenicity or enhance immunogenicity to improve the efficiency (6, 7, 16, 17). One strategy is DNA vaccines. In previous studies, DNA vaccines induce balanced immune responses to infectious, cancerous, and allergic diseases, and are relatively inexpensive. Such advantages make DNA vaccine promising for both human and veterinary applications (18-23).

For t 2 is the most predominant allergen for *F. taiwana*, with 75% of the midge-allergic patients showing specific IgE to For t 2 (24). Recombinant For t 2 does not only bind to serum IgE of the sensitized patients, it also induces the production of pro-inflammatory cytokines IL-6 and IL-8 from skin fibroblasts, the first immune barrier cells, in a concentration-dependent manner (25). This study therefore aimed to determine if a For t 2 DNA vaccine can prevent the development of allergic symptoms on For t 2 sensitized mice using a two-step sensitization protocol (26).

MATERIALS AND METHODS

Preparation of For t 2 recombinant protein

E. coli-expressed For t 2 recombinant protein (rFor t 2) was purified by rapid affinity column chromatography (Novagen, Darmstadt, Germany). The rFor t 2 was further purified by Endotoxin Detoxi-Gel (Pierce, Rockford, IL, USA) and sterilized with a 0.22- μ m syringe filter (Millipore, Billerica, MA, USA). Lastly, protein concentration was determined by the Coomassie brilliant G-250 protein-dye binding method of Bradford, with bovine serum albumin as a standard according to manufacturer's procedures (Bio-Rad, Hercules, CA, USA).

Preparation of For t 2 recombinant plasmid DNA vaccine

The For t 2-encoding fragment (GenBank accession number EU678971) was amplified from *F. taiwana* cDNA by PCR. The sequence of the amplified For t 2 were verified by DNA sequencing. The For t 2-encoding fragment was subcloned into the pCI-neo expression vector. The protein expression of the pCI-neo-For t 2 construct was verified in mammalian cells by transfecting human lung epithelial cell line A549 (ATCC, Manassas, VA, USA) with 2.5 μ g of the plasmid using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA).

Two days after culture in Opti-MEM reduced serum medium (Gibco, Grand Island, NY, USA), culture supernatants from transfected A549 cells were analyzed by Western blotting, with rabbit anti-For t 2 antibodies. Large-scale purification of the pCI-neo-For t 2

recombinant plasmid (For t 2 DNA vaccine) was then performed with Endo Free Plasmid Maxi kits (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions.

Animals

Female BALB/c mice (5-6 weeks old) were purchased from the Taiwan National Laboratory Animal Center and raised under specific pathogen-free conditions. All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee of Taichung Veterans General Hospital.

Experimental design of prophylactic DNA vaccination

The grouping of experimental mice and protocols were summarized in **Figure 1**. There were 6 mice per group initially, however, as one mouse at V25 group died incidentally during IP sensitization, resulting in 5-6 mice per group for the final measurement of scratching and cytokines. To evaluate the prophylactic effect of DNA vaccination, mice were first given two intra-muscular (IM) injections (days 0 and 7) of For t 2 DNA vaccine with doses of 25, 50, and 100 µg for the three experimental groups. On days 14 and 21, the mice were sensitized intra-peritoneally (IP) with 10 µg rFor t 2. Within days 35-40, the mice were sensitized intra-dermally (ID) with 1µg of rFor t 2 for three consecutive days. Blood samples were collected from the retro-orbital venous plexus at days 0, 14, 35, and 42. The scratching behaviors were videotaped on days 0 and 40, and the mice were sacrificed on day 42.

Measurement of specific antibodies by ELISA

The specific IgE, total IgG, IgG1, and IgG2a were determined by ELISA, with the required antibodies purchased from BD Pharmingen (San Jose, Calif). Micro-titer plates were coated with rFor t 2 for 2 hours at 37°C. After washing with PBST, plates were blocked with 5% skimmed milk (for IgE) or 2% BSA (for all IgGs) for 2 h at room temperature. Sera were diluted (1:10 for IgE or 1:100 for all IgGs) in PBST and incubated at 4°C overnight (for IgE) or at room temperature for 2 h (for all IgGs).

For IgE measurement, the plates were incubated with biotin-conjugated rat anti-mouse IgE (1:4000) for 2 h at room temperature. Subsequently, the plates were reacted with horseradish peroxidase-conjugated streptavidin (1:10,000) for 1 h, developed by adding TMB (Sigma), and stopped with 1 M H₃PO₄.

For IgG measurement, the plates were incubated with horseradish peroxidase-conjugated rabbit anti-mouse IgG1 (1:5,000) or IgG2a (1:1000) for 2 h at room temperature and developed by adding ABTS solution (Sigma). The optical density was then analyzed on a Sunrise Absorbance Reader (TECAN, Austria) at 450 nm and 415 nm, respectively.

Evaluation of scratching behavior of immediate type reaction

On days 0 and 40, the scratching behaviors were videotaped for 1 h starting immediately after the intra-dermal challenge with 5 µg rFor t 2. Counts of scratching were made using video playback. The observation of scratching behavior was performed as described previously (26).

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Scratches of the challenged site by the fore paws were counted and that of other sites was disregarded. The first scratch after rFor t 2 challenge was observed within 5 min after intradermal allergen challenge among all mice almost completely subsided by 30 min after challenge. Data showed the scratch bouts from 0-20 min after challenge.

Histologic examination of delayed type reaction

On day 40, the mice were intra-dermally challenged with rFor t 2 and videotaped their scratch bouts immediately. After 48 h, the mice were sacrificed and the abdominal skins from the challenge sites were removed and placed in 10% formalin overnight at room temperature. Briefly, tissues were embedded in paraffin, cut into 5- μ m sections, de-paraffinized, dehydrated, and stained with hematoxylin and eosin (H and E). Sections were further stained with rabbit anti-CD4 (800-fold dilution) or anti-CD8 (400-fold dilution) polyclonal antibodies (Bioss, MA, USA).

To detect CD marker positive cells, the sections were incubated with peroxidase-conjugated goat anti-rabbit IgG and stained in a DAB solution in Bond automatic system (Leica, Newcastle, UK). Inflammatory cell infiltrates were examined by light microscopy and corresponding images were shot by the Olympus BX51 microscopic/DP71 Digital Camera System (Nagano, Japan)

Measurement of cytokine production

Splenocytes were cultured in 24-well flat-bottomed plates at a concentration of $1 \times 10^6/\text{ml}$ and stimulated with various doses of rFor t 2 at 37°C for 3-5 days. The culture supernatants were collected at each time interval and stored at -20°C until the cytokine assay. The levels of IL-4, IL-6, IL-10, IL-13, interferon (IFN)- γ , and TNF- α in the culture supernatants were measured with murine ELISA development kits (PeproTech, Rocky Hill, NJ, USA) according to the instructions of the manufacturer. Cytokine concentrations were determined by interpolation from a standard curve for each cytokine derived from the cytokine standards. The results were represented as stimulation index to correct the baseline differences between individuals. The stimulation index of cytokine was calculated as a ratio of the cytokine levels after rFor t 2-stimulation divided by the basal levels from the unstimulated cells.

RNA preparation and quantitative RT-PCR

Total RNA from splenocytes after stimulation were purified using the RNeasy mini kit (Qiagen) according to the manufacturer's manual. Pre-designed primer sequences were used as described previously (26). Briefly, cDNA were prepared from 1 μg total RNA using a SuperScript III kit (Invitrogen, Carlsbad, CA). A total volume of 20 μl PCR mixture, which included 10 μl Real-Time SYBR Green/ROX PCR master mix from Applied Biosystems (Life technologies, Carlsbad, CA), 9 μl double-distilled H_2O , and 1 μl template cDNA, were added in each well of the PCR array.

Amplification by PCR was conducted with an initial 10-min step at 95°C , followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. The fluorescent signal from SYBR Green

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was detected immediately after the extension step of each cycle, and the cycle at which the product was first detectable was recorded as the cycle threshold. Data were imported into an Excel database and analyzed using the comparative cycle threshold method with normalization of the raw data to β -actin.

Statistical analysis

Analyses were performed using the Statistical Package for the Social Science (IBM SPSS version 22.0; International Business Machines Corp, New York, USA) with appropriate methods. Statistical significance was set at $p < 0.05$.

RESULTS

Construction and expression of For t 2 DNA vaccine

The 987-bp For t 2-encoding fragment amplified from PCR was cloned into the EcoRI and NotI sites of the pCI-neo vector, with agarose analysis (**Fig. 2A**). High-quality and endotoxin-free plasmid DNA were purified from the host strain DH5 α . The expression level of the For t 2 vaccine construct was then tested in A549 cells. The expressed 36-kDa protein of For t 2 was shown in **Figure 2B**. No For t 2 proteins were detected in the control sample transfected with the empty vector.

Effects of For t 2 DNA vaccine on rFor-t 2-induced specific antibodies

The levels of For t 2-specific IgE and IgG1 were high in the not vaccinated and vector only (VO) groups (**Fig. 3**). In contrast, the For t 2 vaccine 50 µg (V50) group prevented the induction of For t 2-specific IgE ($p<0.05$) and significantly induced levels of For t 2-specific IgG2a ($p<0.001$). The levels of For t 2-specific IgE in group V50 were significantly lower than that of the not vaccinated group as well as VO group and maintained at almost the same level as the sentinel control group.

Effects of For t 2 DNA vaccine on scratch behavior to rFor t 2 challenge

On final ID challenge (day 40), all groups, including the not vaccinated group, VO, V25, and V100 groups, showed significantly more scratch bouts on the challenge sites than on the starting day (day 0). Only the For t 2 vaccine 50 (V50) group showed a decrease in scratch bouts to the rFor t 2 ID challenge (**Fig. 4**).

Effects of For t 2 DNA vaccine on cytokine production of splenocytes

The cytokine mRNA expression on day 3 and protein levels on day 5 in response to rFor t 2 were determined by real-time PCR and ELISA, respectively (**Fig. 5**). There was a marked down-regulation of mRNA expression and protein secretions of IL-13 in the V25, V50, and V100 groups (**Fig. 5A**). Levels of mRNA and protein of IL-4 significantly decreased only at the V50 group as compared to both the not vaccinated and VO groups (**Fig. 5B**). Protein levels of IL-10 were elevated in the V50 group compared to the not vaccinated group but not

to the VO group (**Fig. 5C**). There were no differences in the expression of IFN- γ mRNA as compared to not vaccinated and VO groups as well as protein level as compared to not vaccinated group but an increased in protein levels at V25 and V50 groups as compared to VO group. (**Fig. 5D**) Levels of mRNA and proteins of TNF- α and IL-6 showed no significant changes (**Figs. 5E and 5F**) among all of the groups.

Effects of For t 2 DNA vaccine on delayed skin reactions

Skin biopsy from the not vaccinated and VO groups 48 h after the rFor t 2 challenges showed marked leukocyte infiltration similar to that observed in the murine model of midge allergy (**Fig. 6A**). Immuno-histologic staining revealed higher numbers of murine CD4⁺ and CD8⁺ T cells of both the epidermis and dermis compared to the PBS in the sham and VO groups. However, histologic analysis revealed that skin from the V50 group had significantly reduced in total inflammatory cell infiltration, especially the eosinophils (**Fig. 6B**)

DISCUSSION

This study used For t 2 DNA vaccine from the major allergen of biting midge, *Forcipomyia taiwana*, to pre-treat BALB/c mice, using two injections in seven days apart, before being sensitized with recombinant For t 2 allergen. The For t 2 DNA vaccine at 50 μ g (V50 group) was able to prevent the development of skin itching symptoms represented by midge allergen-challenge induced scratch bouts. The vaccine also prevented the development

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of midge allergen-induced allergic inflammation, including the production of For t 2-specific IgE, midge-allergen induced IL-13 and IL-4 productions from splenocytes, as well as inflammatory cell infiltrations, in the lesions 48 h after intra-dermal challenge. For t 2-specific IgG2a, one of the surrogate makers of successful allergen specific immunotherapy in mice, elevated at all treatment groups and most significantly at V50 group. Proinflammatory cytokines related with bacterial CpG contamination, including TNF-alpha and IL-6 (27) showed no significantly elevate among all study groups in current study.

The protective effect of DNA vaccine to allergic airway inflammation has been reported in those encoding the cockroach major allergen Bla g 1, house dust mite allergen Der p 1, and birch pollen allergen Bet v 1 in mouse models (28-30) This study demonstrates for the first time that the DNA vaccine encoding allergen is also effective in preventing allergic skin inflammation induced by biting midge.

However, this study has its limitations. Whether the DNA vaccine is as effective in mouse already sensitized, reflecting the treatment effects, remains unanswered. A DNA vaccine encoding the human calcium-activated chloride channels (hCLCA1) induced antibodies lasted a few weeks in asthmatic mice (31). The durability of the immuno-deficiency virus (HIV)-1 DNA vaccine was evaluated recently, revealing specific immune responses that lasted at least 6 months in a phase 2a trial among HIV-uninfected human adults (23). However, the duration of the prophylactic effect of the For t 2 DNA

vaccine in mice or in humans warrants further investigation.

Furthermore, previous studies show that even though the effects of DNA vaccines in mice, horses, and pigs appear promising (32), DNA-based vaccines are typically weaker immunogens in humans compared to the traditional protein-based vaccines (33-35). Thus, several strategies have been reported to enhance the DNA vaccine immunogenicity in humans and primates. For example, adding traditional (36-38) or plasmid-encoding adjuvants (39, 40), designing a strong promoter to increase transcription and translation (41, 42), using a different DNA vaccine delivery methods (43), and various prime-boost protocols (44, 45). Whether the prophylactic effects of For t 2 DNA vaccine in the mouse model can translate into human subjects require further studies. Strategies to improve the For t 2 DNA vaccine potency in humans will be necessary. In addition, though For t 2 allergen accounts for 64-75% of the midge-allergic patients from our previous studies (24, 25), it may be necessary to add other major midge allergens in the future for a more comprehensive coverage.

In conclusion, immunotherapy using For t 2 DNA vaccine can protect mice from midge allergen sensitization, as shown by multiple clinical, immunologic, and histologic evidences. The For t 2 DNA vaccine is a promising treatment for this annoying allergy in the future.

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LEGEND

Figure 1. Treatment schedule of For t 2 DNA vaccinations on the rFor t 2-sensitized mice.

VO denoted vector only.

Figure 2. Construction and expression of For t 2 DNA vaccine. 100 ng of rFor t 2 protein was used as a positive control. Numbers on the left lane indicate sizes of DNA (lane M1) and protein markers (lane M2) (A) Agarose gel electrophoresis of empty vector or pCL-neo-For t 2 plasmid digested with enzymes *EcoRI* and *NotI*. (B) Western blot analysis of culture supernatant from A549 cells transfected with empty vector or pCI-neo-For t 2 using rabbit anti-For t 2 antibody. Recombinant For t 2 proteins were seen at 36kDa as detected by rabbit anti-For t 2 antibody. Faint bands between 40-70 kDa at lanes pCL-neo and pCL-neo-For t 2

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of panel B are non-specific bands due to the high sensitivity of chemiluminescent exposure.

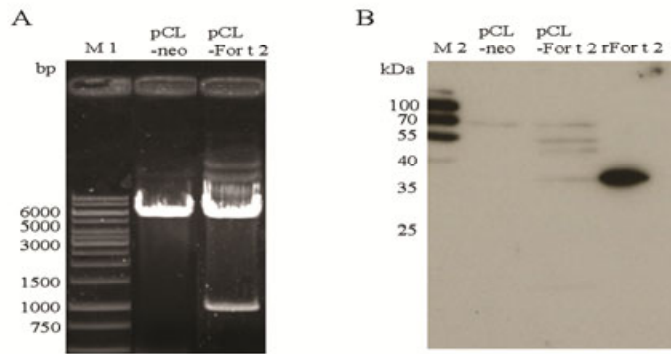
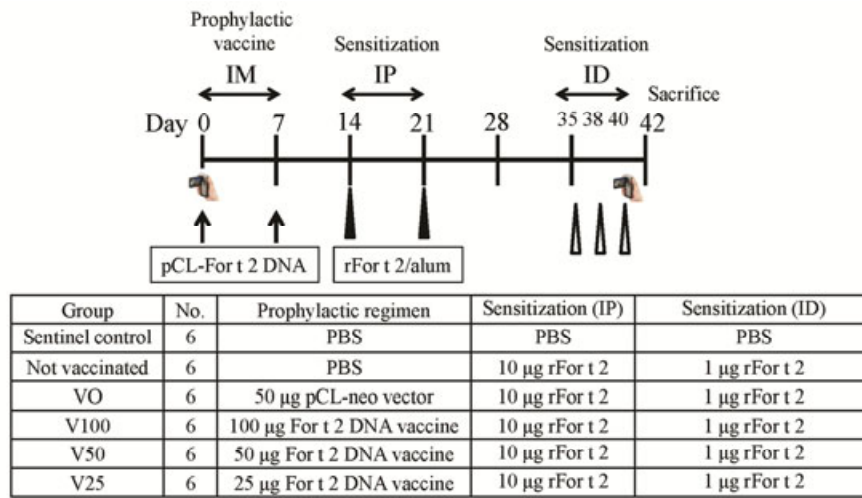
Figure 3. Serum levels of For t 2-specific IgE, IgG1 and IgG2a determined by ELISA. The data represent the mean $\pm$ SEM from 5-6 mice/group. * denoted $p < 0.05$, ***denoted $p < 0.001$, NS denoted not statistically significant, VO denoted vector only. The statistics show comparisons between not vaccinated group and DNA vaccine groups by Wilcoxon signed-rank test.

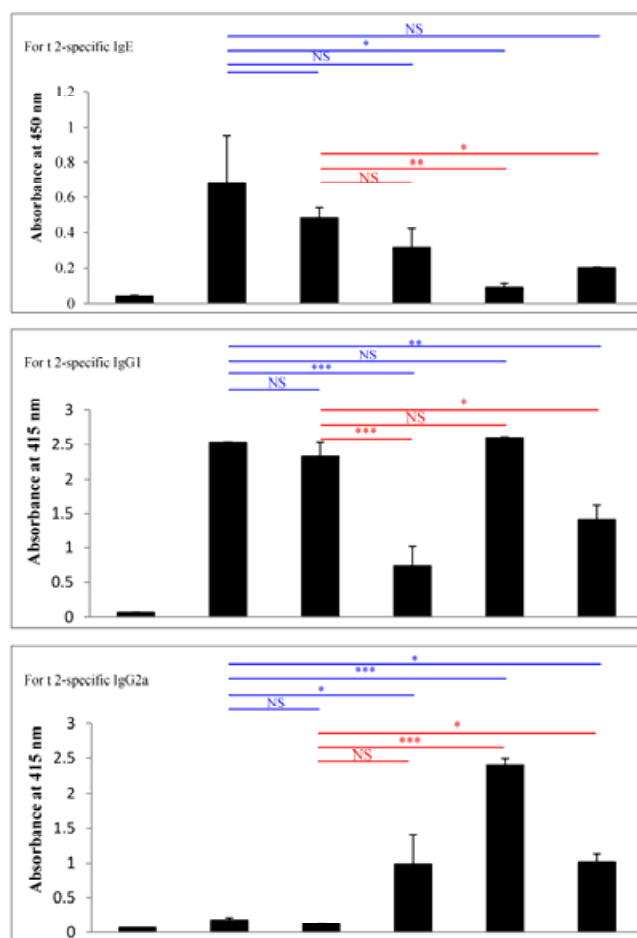
Figure 4. Counts of scratching bouts from each group mice induced by intra-dermal injection of rFor t 2. The scratching behavior was videotaped and data represent the number of scratches within 20 min after intra-dermal allergen challenge. Results were mean $\pm$ SEM of 5-6 mice from each group. The statistics show comparisons between day 0 (W0) and day 40 (W6) from each group by Wilcoxon signed-rank test. VO denoted vector only. * denoted $p < 0.05$.

Figure 5. Cytokine mRNA and protein expression levels of splenocytes from each group of mice (n=5-6 per group) by real-time PCR and ELISA. (A) IL-13, (B) IL-4, (C) IL-10, (D) Interferon γ (IFN- γ), (E) TNF- α and (F) IL-6. Real-time PCR data are expressed as mean of increasing fold $\pm$ SEM. Cytokine releasing data are showed as mean of stimulation index $\pm$ SEM. The statistical significance of differences between not vaccinated group and vaccine groups was assessed by Wilcoxon signed-rank test. VO denoted vector only.

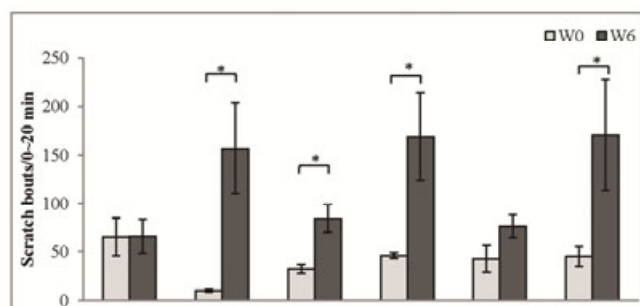
Figure 6. (A) Effects of For t 2 vaccine on histopathology of abdominal skins by H&E and

immunostaining. Figures showed the representative skin sections obtained 48 hours after intra-dermal challenge test and stained with H&E or with anti-mouse CD4 (mCD4) and CD8 (mCD8) antibodies from sentinel control, not vaccinated control, vector only control (VO) and V50 vaccinated mice under 400x light microscope. (B) The infiltrating inflammatory cells were quantified in H&E-stained skin sections under 400-fold view from each mouse (n=5-6 per group). The results are expressed as mean±SEM of the cell numbers per square millimeter of biopsy specimen. The statistical significance of differences among groups was assessed by the Wilcoxon signed-rank test. VO denoted vector only. * denoted $p < 0.05$.



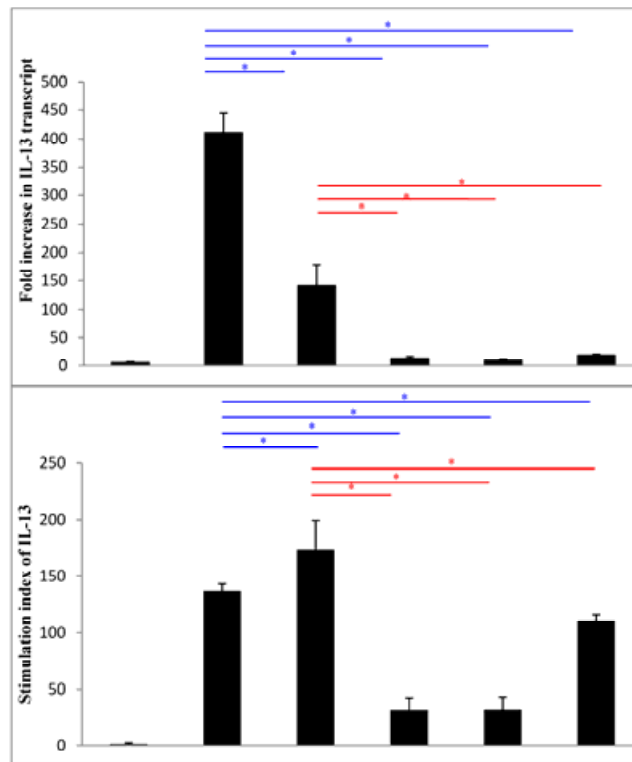


Sensitized with PBS	+	-	-	-	-	-
Sensitized with rFor t 2	-	+	+	+	+	+
DNA vaccine (μg)	PBS	PBS	pCL 50μg	For t 2/pCL 25μg	For t 2/pCL 50μg	For t 2/pCL 100μg
Group	Control	Not vaccinated	VO	V25	V50	V100



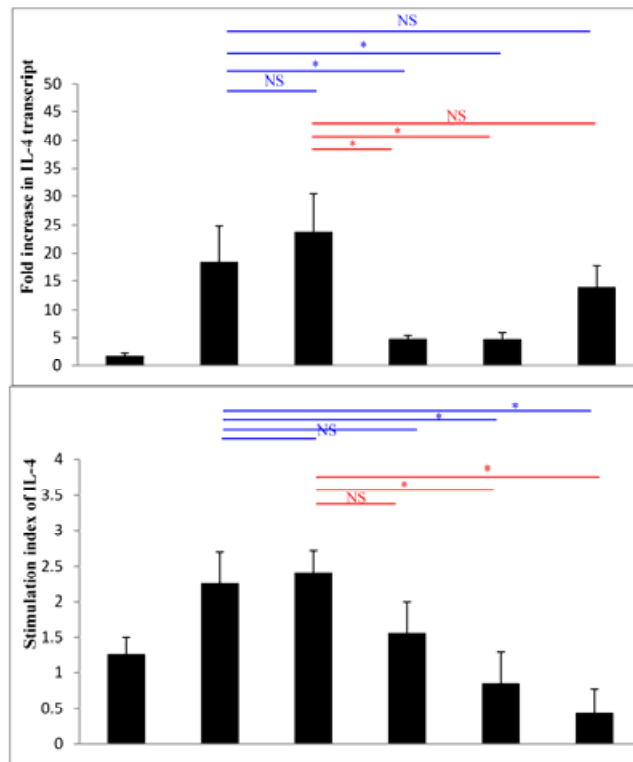
Sensitized with PBS	+	-	-	-	-	-
Sensitized with rFor t 2	-	+	+	+	+	+
DNA vaccine (µg)	PBS	PBS	pCL 50µg	For t 2/pCL 25µg	For t 2/pCL 50µg	For t 2/pCL 100µg
Group	Control	Not vaccinated	VO	V25	V50	V100

(A) IL-13



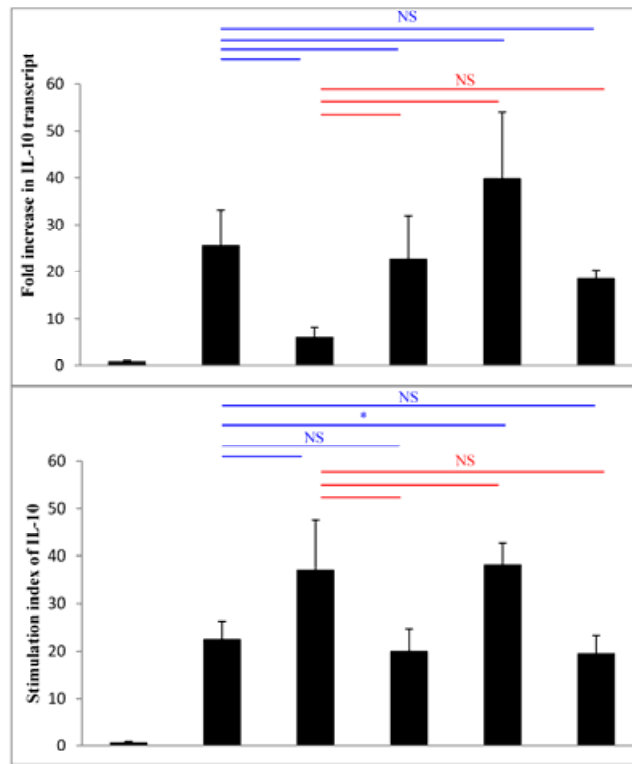
Sensitized with PBS	+	-	-	-	-	-
Sensitized with rFor t 2	-	+	+	+	+	+
DNA vaccine (µg)	PBS	PBS	pCl. 50µg	For t 2/pCl. 25µg	For t 2/pCl. 50µg	For t 2/pCl. 100µg
Group	Control	Not vaccinated	VO	V25	V50	V100

(B) IL-4

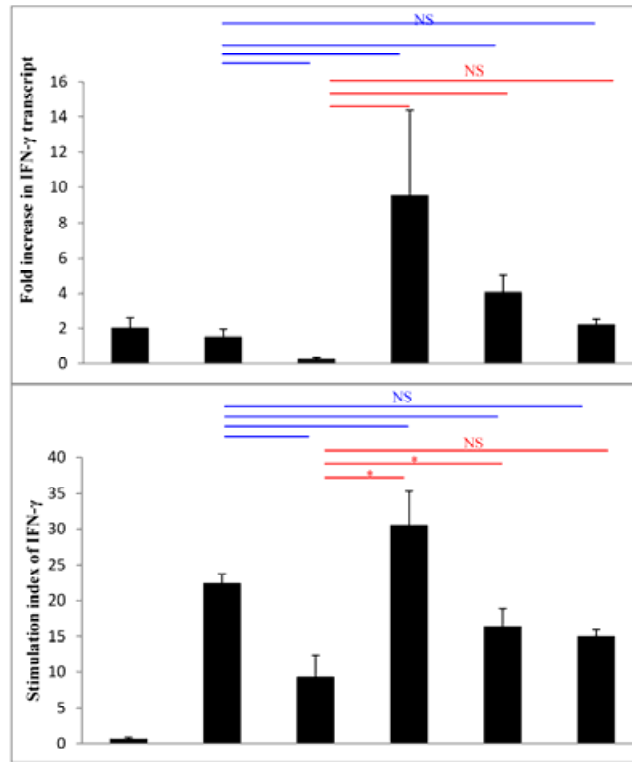


Sensitized with PBS	+	-	-	-	-	-
Sensitized with rFor t 2	-	+	+	+	+	+
DNA vaccine (µg)	PBS	PBS	pCL 50µg	For t 2/pCL 25µg	For t 2/pCL 50µg	For t 2/pCL 100µg
Group	Control	Not vaccinated	VO	V25	V50	V100

(C) IL-10

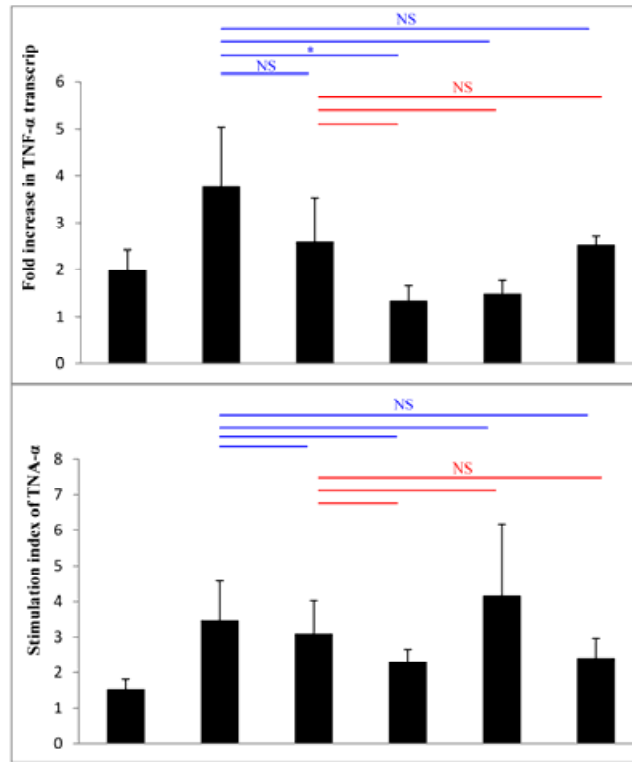


Sensitized with PBS	+	-	-	-	-	-
Sensitized with rFor t 2	-	+	+	+	+	+
DNA vaccine (µg)	PBS	PBS	pCL 50µg	For t 2/pCL 25µg	For t 2/pCL 50µg	For t 2/pCL 100µg
Group	Control	Not vaccinated	VO	V25	V50	V100

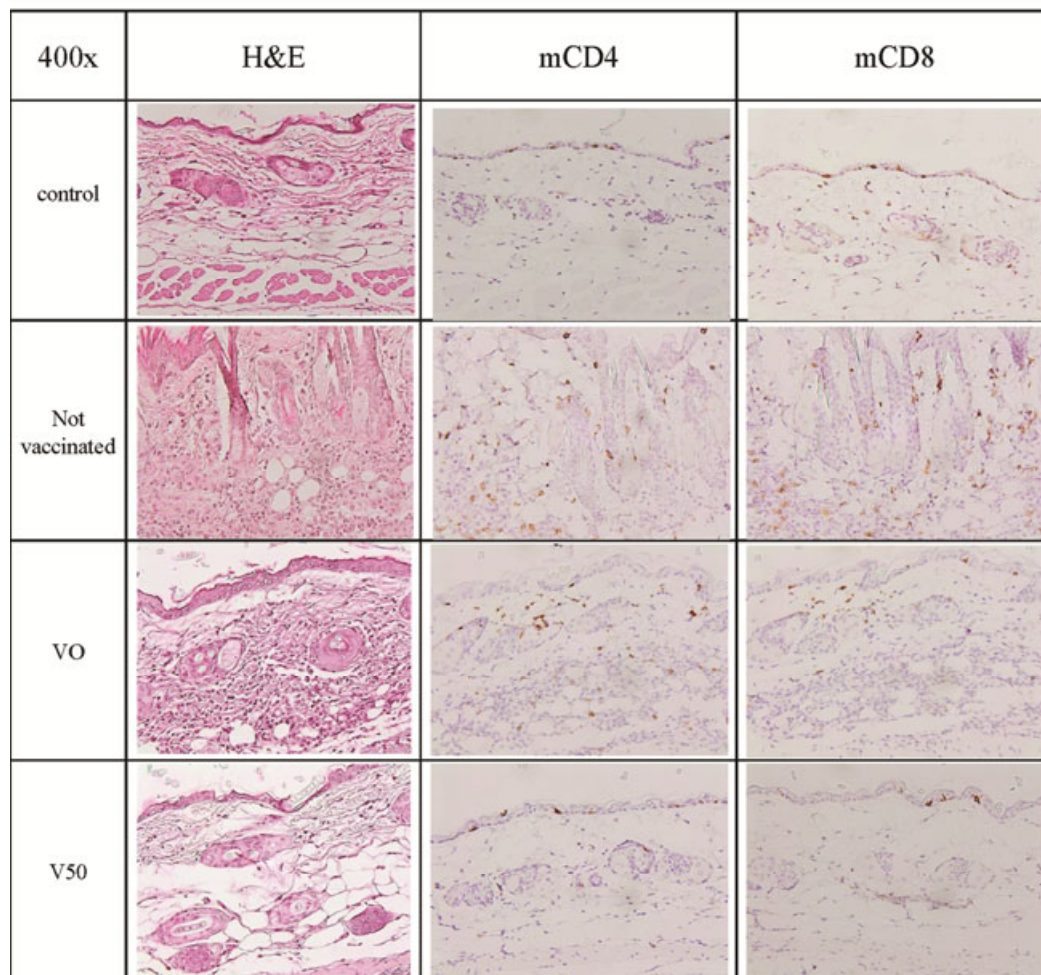
(D) IFN- γ 

Sensitized with PBS	+	-	-	-	-	-
Sensitized with rFor t 2	-	+	+	+	+	+
DNA vaccine (μ g)	PBS	PBS	pCL 50 μ g	For t 2/pCL 25 μ g	For t 2/pCL 50 μ g	For t 2/pCL 100 μ g
Group	Control	Not vaccinated	VO	V25	V50	V100

(E) TNF- α



Sensitized with PBS	+	-	-	-	-	-
Sensitized with rFor t 2	-	+	+	+	+	+
DNA vaccine (μ g)	PBS	PBS	pCl. 50 μ g	For t 2/pCl. 25 μ g	For t 2/pCl. 50 μ g	For t 2/pCl. 100 μ g
Group	Control	Not vaccinated	VO	V25	V50	V100



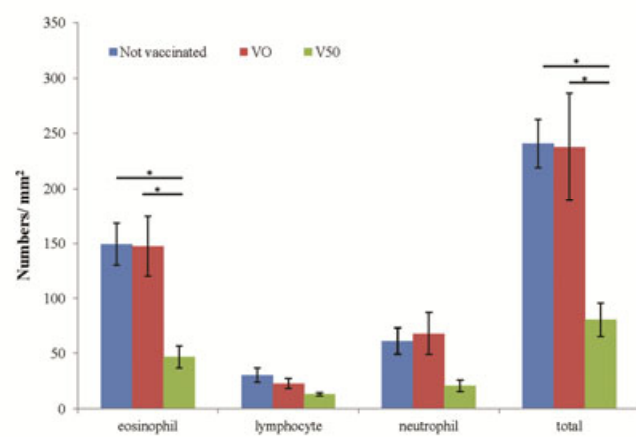


Table 1. The sequences of gene-specific primers used in real-time PCR

Gene (mouse)		Sequences	Produce size (bp)
TNF-α	F	5' CAT CTT CTC AAA ATT CGA GTG ACA A 3'	175
	R	5' TGG GAG TAG ACA AGG TAC AAC CC 3'	
IL-6	F	5' CCA TCC AGT TGC CTT CTT G 3'	223
	R	5' AAG TGC ATC ATC GTT GTT CAT AC 3'	
IL-4	F	5' AGC CAT ATC CAC GGA TGC GAC AAA 3'	176
	R	5' AAT ATG CGA AGC ACC TTG GAA GCC 3'	
IL-13	F	5' AGA CCA GAC TCC CCT GTG CA 3'	123
	R	5' TGG GTC CTG TAG ATG GCA TTG 3'	
IL-10	F	5' CCA AGC CTT ATC GGA AAT GA 3'	155
	R	5' AGG GGA GAA ATC GAT GAC AG 3'	
IFN-γ	F	5' GGC CAT CAG CAA CAA CAT AAG CGT 3'	118
	R	5' TGG GTT GTT GAC CTC AAA CTT GGC 3'	
β-actin	F	5' GGCCAACCGTGAAAAGATGA 3'	251
	R	5' CACGCTCGGTCAGGATCTTC 3'	