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T cell epitope of arginine kinase with CpG co-encapsulated nanoparticles attenuates a shrimp allergen-induced Th2-bias food allergy

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ABSTRACT

T cell peptide-based immunotherapy (PIT) is an appealing therapeutic strategy for modulating allergic responses without IgE cross-linking. We propose a novel PIT that combines a T-cell epitope of the shrimp allergen arginine kinase (AKp) with TLR9 agonist CpG-ODN in nanoparticles (CpG-AKp NPs) to attenuate a shrimp allergen-induced food allergy. Treatment with CpG-AKp NPs demonstrated the attenuation of anaphylaxis responses such as the reduced incidence of diarrhea and hypothermia, lower levels of specific IgE and the induction of IgG2a in serum. Th2 cytokines were suppressed and higher Th1 cytokines were detected in the splenocyte culture supernatants. Treatment of CpG-AKp NPs also enhanced the protein expression of Foxp3 and IL-10 in small intestine but decreased the activation of STAT6 and GATA3 expression, which are related to differentiation of Th2. Our data indicated that CpG-AKp NPs may represent a promising PIT against shrimp allergy.

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KEYWORDS

Shrimp allergy; immunotherapy; arginine kinase; T epitopes; CpG-ODN

Food allergy (FA) continues to be a major public health problem that affects 4–8% children and 2–4% adults [1]. Recent studies indicate that people involved in seafood processing have a risk of food allergy [2,3]. Shrimp allergy is one of the most common types [4] and the prevalence of shrimp hypersensitivity is particularly high in the Asia-Pacific region [5,6]. Shrimp allergy, like most allergies, results from a Th2 polarization and IgE-mediated type I hypersensitivity rather than tolerance. The allergic symptoms of shrimp allergy include cutaneous, digestive and respiratory symptoms, as well as oral allergy syndrome and anaphylaxis [7]. Arginine kinase (AK) is known as a kinase involved in energy transformation. Amedee *et al.* [8] used AK and serum from shrimp allergic patients in two-dimensional immunoblotting, and AK was confirmed to be a novel and cross-reactive shrimp allergen [9]. Although the shrimp allergen AK has been characterized, clinical trials for shrimp allergy are still lacking. In addition to avoiding contact with allergens, active measures, especially allergen-specific immunotherapy (AIT) should be taken into consideration to treat shrimp allergy. AIT involves administering increasing doses of the allergenic proteins. However, this method has not been widely applied due to the potential risks of IgE-mediated side effects and relatively low effectiveness [10,11]. Therefore, the allergy treatment with low/no IgE reactivity is desirable.

Recently, the T cell epitopes of allergens-based immunotherapy (PIT) have been proposed as a safe and effective therapeutic strategy. These short peptides are designed to lack the conformation structure required to crosslink IgE on surface of mast cells and basophils [12] but maintain their immunological ability, which can modulate allergen-specific T cell responses and can be administered at higher doses [13]. However, T cell epitopes induce weak immune responses that need to be boosted by an adjuvant. In this regard, previous works have shown that the activation of Toll-like receptors (TLRs) can modulate the adaptive immune response and act as an immune adjuvant [14,15]. CpG-ODN, a known TLR9 ligand, has been shown to be effective in inducing Th1 immunity [16] as well as downregulating the established Th2 response [17]. Studies in animal models of peanut allergy have shown that CpG-coated peanut nanoparticles were significantly protected from anaphylaxis to peanut challenges with a significant decrease in peanut-specific IgE level and Th2 cytokines [18].

Previous studies have reported that the segment AK₈₁₋₁₀₀ showed the capacity to stimulate T cells from shrimp allergic patients [8]. Based on the data, a peptide of 20 amino acids was designed, spanning the amino acids 81–100 of AK. In the current study, we sought to delivery AK₈₁₋₁₀₀ by using CpG-coated poly (lactic-co-glycolic acid) nanoparticles (CpG-AKp NPs) and develop a novel method to improve the

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specific immunotherapy and the treatment of shrimp allergy.

Materials and methods

Materials

AK T epitope peptide (AKp) AK₈₁₋₁₀₀: LFDPIIEDYH VGFKQTDKHP was synthesized by ONTORES Biotechnologies Co., Ltd with purity $\geq 95\%$ and sequences were confirmed by HPLC and LC-MS spectrometry analysis (Supplementary data, Figure S1). The CpG-ODN was purchased from InvivoGen (San Diego, CA, USA). PLGA (lactide:glycolide 50:50, molecular weight 24,000–38,000) and polyvinyl alcohol (PVA) were obtained from Sigma-Aldrich (St Louis, MO, USA).

Preparation of recombinant shrimp AK

Synthetic sequences of AK (Gen Bank number: AFA45339.1) were ligated into a pMD19-T vector (Takara) and transformed into *E. coli* Top10. The target fragment was digested and ligated into PET-28a, then transformed into *E. coli* BL21(DE3) for expression. The positive clones were induced by isopropyl-D-thiogalactopyranoside (IPTG) for 4 h at 37°C. The bacteria were harvested in 50mM Tris-HCl, 100mM NaCl, pH 7.5 and then sonicated. The target protein was purified with Ni-NTA affinity column by rapid protein liquid chromatography at a flow rate of 2 mL/min. The nickel column was first balanced with liquid B (50 mmol/L Tris, 150 mmol/L NaCl), and then balanced with liquid B again after sample loading. The nonspecifically bound heteroproteins were washed with liquid C (50 mmol/L Tris, 150 mmol/L NaCl, 40 mmol/L imidazole, pH 8.0), and the target proteins were washed with liquid D (50 mmol/L Tris, 200 mmol/L NaCl, 300 mmol/L imidazole, pH 8.0). The endotoxin was replaced using an ion-exchange column and ToxinEraserTM Endotoxin Removal Kit (L00338, Genscript, China).

Immunogenicity identification of AKp

To verify the immunogenicity of recombinant AKp which was expressed and purified from *E. coli* BL21 (DE3). Briefly, 96-well plates were coated with AK or AKp (200 ng/well) and stored overnight at 4°C. The plates were washed with TBST and blocked with 5% nonfat milk for 2 h. Shrimp allergic mice serum and normal mice serum was respectively added to the plate and incubated at 37°C for 2 h. Plates were washed with TBST and then incubated with HRP-conjugated goat anti-mouse IgE or IgG (SouthernBiotech, Birmingham, AL) for 2 h. After washing, plates were incubated with TMB for 10 min at 37°C. The reaction was terminated by adding 2 M H₂SO₄ and the optical density was measured at 450 nm using a microplate reader.

Preparation of CpG-AKp NPs

CpG-AKp NPs were prepared by solvent evaporation double emulsion method as previously reported [18]. The final concentrations of CpG-AKp NPs contain 100 µg/mL CpG-ODN and 25 µg/mL AKp. The hydrodynamic diameter and PDI value of the particles were determined by dynamic light scattering (DLS) (Zetasizer Nano ZS, Malvern Instruments, UK). The obtained CpG-AKp NPs were 195.5 nm in diameter with monomodal size distribution: PDI 0.13 (Supplementary data, Figure S2).

Sensitization and challenge of mice

Animals

Female Balb/c mice (6 weeks) were acquired from the Experimental Animal Center (Guangzhou, China). The mice were maintained in an AK-free diet. The animal studies were carried out in accordance with the Institutional Guidelines for Animal Care and Use of Laboratory Animals at Shenzhen University and approved by the Animal Ethics Committee at Shenzhen University.

CpG-AKp NPs therapeutic treatment

The mice were randomly assigned to 4 groups (6 mice per group): (A) Naive group: Mice were treated with normal saline, (B) Model group: sensitized mice were treated with normal saline, (C) CpG-AKp NPs-treated group: sensitized mice were treated with CpG-AKp NPs (each mouse was received 25 µg AKp and 100 µg CpG-ODN each time), (D) blank NPs-treated group: sensitized mice were treated with blank NPs (blank carrier material PLGA and PVA without Akp and CpG-ODN). All the groups were received intraperitoneal (*i.p*) injection everyday for consecutive 6 days (Figure 1).

Sensitization and challenge of mice

The shrimp allergy model was established with the procedures reported previously with some modifications [19,20]. Briefly, mice were immunized with AK (100 µg per mouse) in 0.2 mL normal saline with 10 µg cholera toxin (CT) (Sigma-Aldrich, St Louis, USA) by intragastric (*i.g*) administration on day 0, 7 and 14. Naive mice received equal amounts of CT alone (10 µg per mouse). The mice were challenged 7 days after the last sensitization by receiving one dose of AK (600 µg pre mouse) via *i.g* administration. Diarrhea and core temperature were recorded within 30 min after AK challenge. Twenty-four hours after the final challenge, blood samples (500 µL) were taken from the mice eyes with local anesthesia before sacrifice and the serum was isolated. The jejunum section and spleen were immediately removed after death and processed in further experiments.

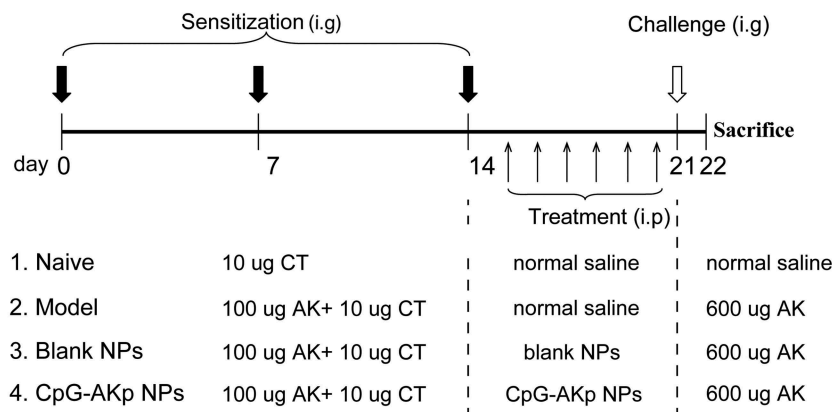


Figure 1. Experimental design.

Protocol for sensitization, treatment, and challenge: 6-week old female Balb/c mice were orally sensitized with arginine kinase (AK) and cholera toxin (CT) at day 0, 7, 14. Daily intraperitoneal treatments started at day 15 and continued through day 20 (black upward arrows). Mice underwent oral AK challenge at day 21 ($n = 6$).

Measurement of AK-specific IgE, IgG1, and IgG2a

The levels of AK-specific IgE, IgG1, and IgG2a in all experimental mice were determined by indirect ELISA method as demonstrated in section 3.3.

Histological examination

Harvest jejunum were fixed with 4% paraformaldehyde for 24 h and stained with Hematoxylin-Eosin stain after dehydration, embedding, and slicing. The structural and morphological changes were observed and analyzed under an OLYMPUS microscope.

Splenocyte cytokine level measurements

Spleens from different groups were removed and filtered through a 200-mesh screen. After erythrocyte lysis, spleen cells were resuspended in complete culture medium RPMI-1640 (containing 10% FBS, 100U/L penicillin, and 100 mg/L streptomycin). Then, the cells were cultured in 24-well plates at a density of 3×10^6 /well in the presence of AK (20 μ g/mL) and incubated at 37°C for 72 h. The culture medium was then collected and centrifuged at 1400 rpm for 5 min. Levels of cytokines IL-4, IL-13, IFN- γ , and IL-10 in the supernatants of the splenocyte culture medium were determined according to the instructions of the ELISA kit (Sino Biological, Beijing, China).

Flow cytometry analysis

Splenocytes from different groups were stained with anti-CD4-FITC (BD Biosciences Pharmingen) before fixed with BD Cytfix/CytoPerm Fixation/Permeabilization solution kit (BD Biosciences Pharmingen) and stained with anti-IL-4-PE, anti-IFN- γ -APC and anti-IL-10-PC5.5 (BD Biosciences Pharmingen). Isotype IgG was used as

control antibodies. Cells were analyzed using a FACS Calibur Flow cytometer (BD Biosciences). The data were analyzed using Flowjo software (7.6.1).

Western blot analysis

Small intestine tissues were lysed in RIPA buffer (Beijing Solarbio Science&Technology Co., Ltd) with proteinase inhibitor phenylmethanesulfonyl fluoride (PMSF) and homogenized using a tissue grinder (SCIENTZ-48, Ningbo Xingzhi Biotechnology Co, Ltd, China). Then, the mixture was centrifuged at 10,000 g for 5 min to collect the supernatant. The protein concentration of each sample was determined using BCA reagents (Thermo Scientific) and equivalent amounts of protein (20 μ g) were loaded in 12% SDS-PAGE gels and transferred by blotting to polyvinylidene fluoride membranes. Then, the membranes were incubated in blocking buffer (5% nonfat milk in TBST) for 2 h followed by overnight incubation in primary antibodies against mouse Foxp3, TGF- β , IL-10, STAT6, pSTAT6 or GATA3 (Cell Signaling Technology, Danvers, MA). After washing, the membranes were incubated with secondary antibodies. Finally, the membranes were developed using Pierce ECL Plus Substrate (Thermo Scientific). An antibody specific to GAPDH was used as a control. The immunoblotting densitometry of the targeted protein expression bands and GAPDH bands was quantified by Gel Imaging System (Bio-Rad).

Data statistics

The data (mean $\pm$ SD) were processed using GraphPad Prism5 (GraphPad Software, San Diego, CA, USA), and differences between groups were analyzed by Student's t-test or analysis of variance (ANOVA). The p value less than 0.05 was considered as statistically significant.

Results

Preparation of recombinant AK and AKp

In order to obtain AK protein for the subsequent experiments, we cloned AK cDNA into PET-28a (+), expressed and purified recombinant AK as described in the 'Materials and Methods' section. The result showed that His-tag-AK was successfully expressed by induction with IPTG and purified using His-Tag affinity column, and were detected by SDS-PAGE (Figure 2(a)). Figure 2(b) demonstrated that AKp reduced the AK-specific IgE (sIgE)-binding capacity but maintained the sIgG-binding ability, indicating Akp a potential hypoallergenic compared with AK (Figure 2(b)).

CpG-AKp NPs protect mice from anaphylactic symptoms to oral AK challenge

To test the effect of CpG-Akp NPs in the inhibition of shrimp allergy, we developed a food allergy mouse model by using the oral sensitization and challenge with AK. Mice were observed within 30 min after challenge. Model group and blank NPs-treated group showed observable signs of anaphylaxis, with 5/6 and 4/6 of the mice got a diarrhea at the end of the experiment, respectively (Figure 3(a)). In comparison, more than 2/3 of mice treated with CpG-AKp NPs were protected from diarrhea. Hypothermia is associated with anaphylaxis

symptoms in mice [21]. After AK challenge, model group and blank NPs-treated group had mean rectal temperature that were lower than those of mice in naive mice

(Figure 3(b)). While the rectal temperatures of CpG-AKp NPs-treated mice were significantly higher than model group ($P < 0.01$) and blank NPs-treated group ($P < 0.05$). Histological HE staining (Figure 3(c)) showed that the jejunum villi were fitly arranged and the tissue structure was clear and intact in naive mice. However, the jejunum villi in the model group were obviously damaged, shed, together with the significant decrease in the number. In the CpG-AKp NPs-treated group, the morphology of the intestinal mucosa and the jejunum villi were integral, with obvious differences with the model group. The above results indicated that CpG-AKp NPs treatment could protect mice from anaphylactic symptoms against oral AK challenge.

CpG-AKp NPs modified shrimp allergy-associated humoral immune responses

Serum IgE, IgG1 and IgG2a levels were measured by ELISA to evaluate the efficacy of CpG-AKp NPs. AK-sIgE levels in the model group were significantly higher than that in the naive mice ($P < 0.01$; Figure 4(a)). Much lower levels of AK-sIgE in CpG-AKp NPs-treated group were observed compared with

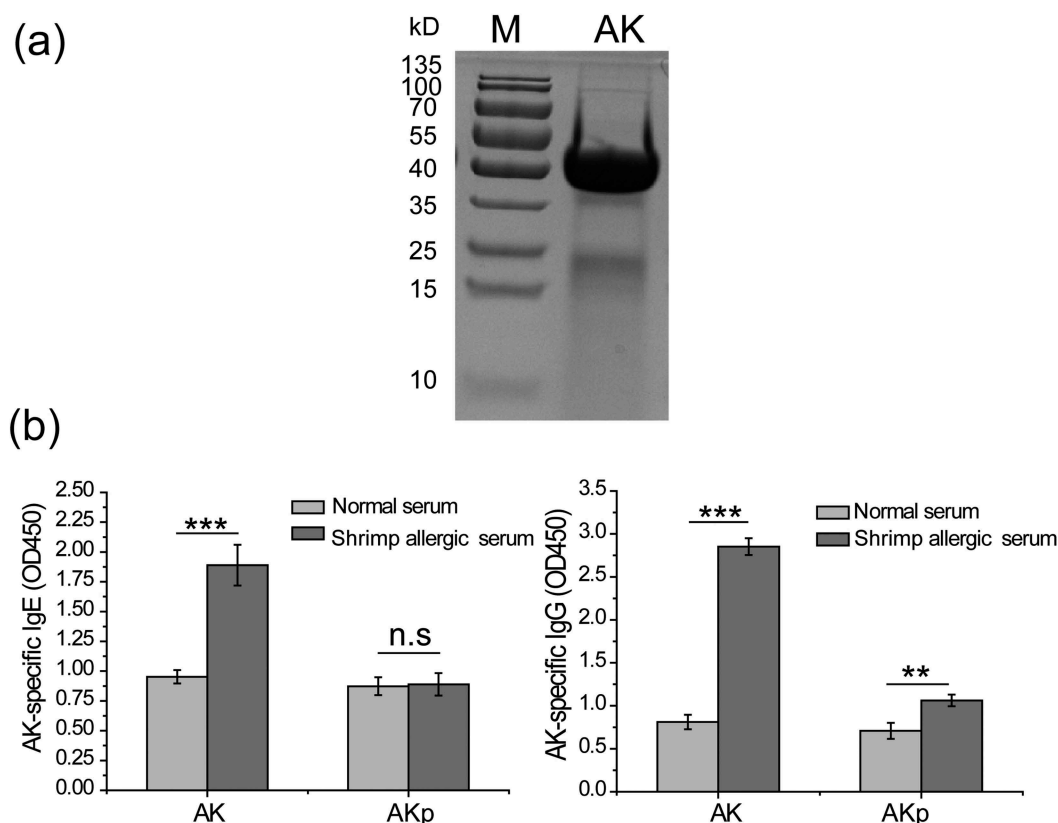


Figure 2. The preparation of recombinant AK and immunogenicity identification of AKp.

(a) SDS-PAGE analysis of the protein purified by 6His-PC-AK. The molecular weight is 40.1 kDa. (b) specific IgE and IgG levels induced by AK and AKp. The results demonstrated that, compared with AK, AKp would bind to AK-specific IgG (sIgG) but not sIgE, indicating the obtained Akp retained immunogenicity but reduced allergenicity. Results are expressed as mean $\pm$ SD (** $P < 0.01$, *** $P < 0.001$ vs normal serum, n.s no significant difference).

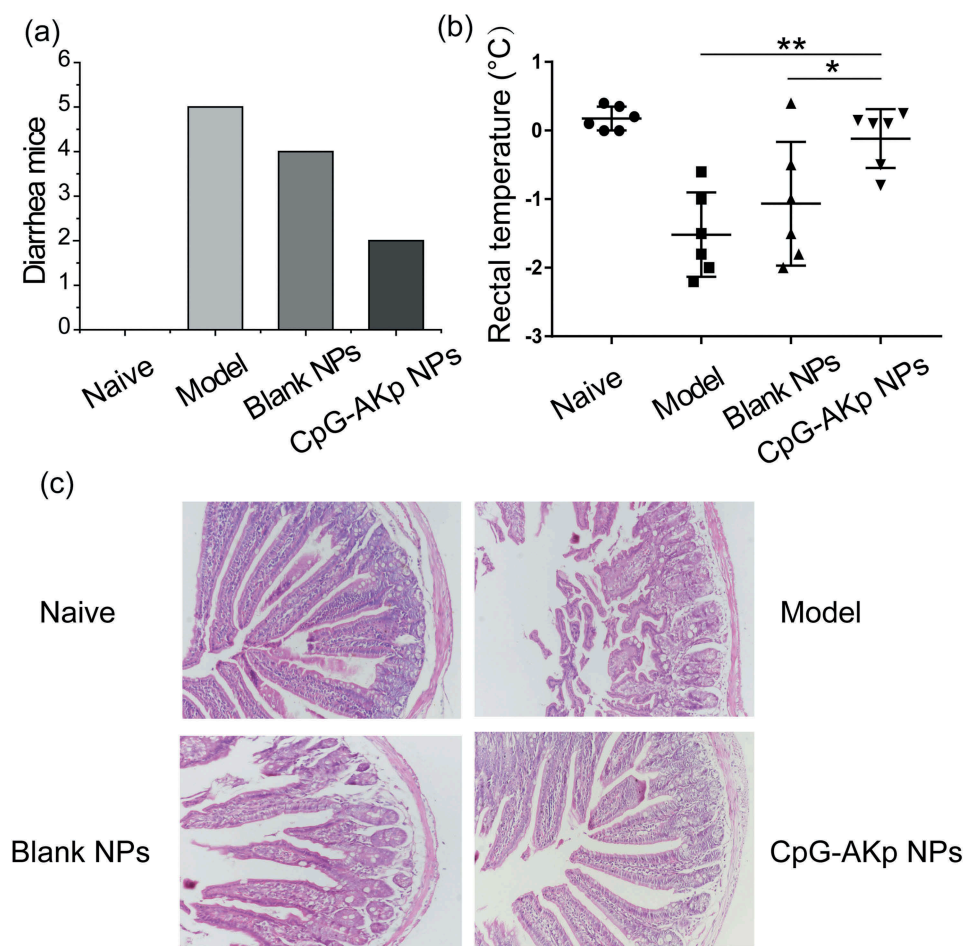


Figure 3. CpG-AKp NPs suppressed AK-induced anaphylactic reaction.

(a) Occurrence of diarrhea and (b) rectal temperatures were measured within 30 min after antigen challenge. (c) Histological examinations of jejunum from mice in naive, model, blank NPs and CpG-AKp NPs groups. The data are shown as mean $\pm$ SD from six individual mice (* P < 0.05, ** P < 0.01).

the model group (P < 0.01). Serum sIgE levels in the blank NPs and model groups did not differ significantly. A similar result was observed for AK-sIgG1, a Th2-associated immunoglobulin that has been implicated in anaphylactic reactions in mice (Figure 4(b)). Modulation of Th1/Th2 cytokine ratio has been shown to be important for IgG2a antibody production during immunotherapy [22]. We observed significantly higher sIgG2a levels in mice treated with CpG-AKp NPs compared with model group and blank NPs-treated group (Figure 4(c), P < 0.01). These findings indicated that mice received CpG-AKp NPs had a more balanced AK-specific antibody response than sensitized mice that did not receive CpG-AKp NPs.

CpG-AKp NPs suppresses allergy-associated Th2 cytokine responses

Further, to explore the T-cell responses, the cytokine secretion was determined after *in vitro* restimulation of splenocytes. Th2 cytokines (IL-4 and IL-13), Th1 cytokine (IFN- γ) and regulatory cytokines (IL-10 and TGF- β) were measured by ELISA (Figure 5). Therapeutic treatment with CpG-AKp NPs down-regulated the allergen-induced Th2 response, as indicated by the

significant decrease in IL-4 and IL-13 (P < 0.05) when compared with the model group. CpG-AKp NPs also induced a shift toward a prevalent Th1/Treg response, characterized by the increased level of IFN- γ (P < 0.05) and IL-10 (P < 0.05) in comparison with mice in the model group. No significant differences were detected in TGF- β levels of spleen cell culture supernatant of these groups (data not shown). The levels of splenocyte cytokine in the blank NPs group were almost identical to those in the model group. The Effects of CpG-AKp NPs on CD4⁺T cell phenotypes in the spleen were also analyzed by flow cytometric analysis. As shown in Figure 6, the frequency of CD4⁺IL-4⁺ T cells was significantly reduced while the frequency of CD4⁺IFN- γ ⁺ and CD4⁺IL-10⁺ was significantly increased in the spleen of mice treated with CpG-AKp NPs, which was consistent with the results of splenocyte supernatant.

CpG-AKp NPs enhanced the expression of Foxp3 and attenuated the expression of pSTAT6 and GATA-3 in small intestine

To understand how CpG-AKp NPs played a protective role against allergy, the protein expression of Foxp3 in small intestine was measured by Western blot. As

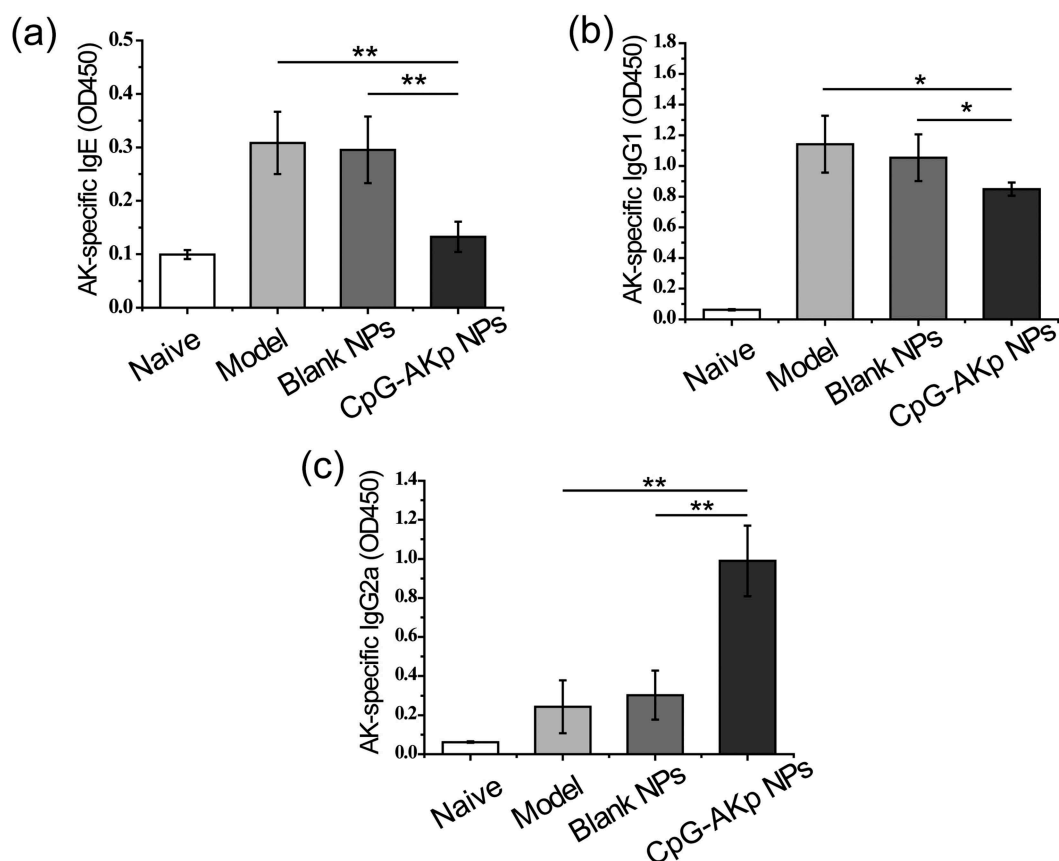


Figure 4. Allergen-specific antibodies in serum of experimental mice.

(a) AK-specific IgE. (b) AK-specific IgG1. (c) AK-specific IgG2a. Serum samples were taken from the mice 24 h after oral challenge. The data are shown as mean $\pm$ SD from six individual mice (* $P < 0.05$, ** $P < 0.01$).

shown in Figure 7(a), the level of Foxp3 and IL-10 protein increased significantly in CpG-AKp NPs-treated group compared with other groups. There was no significant difference among these groups in the expression of TGF- β , which were consistent with the cytokine secretion results. The transcription factors of STAT6 and GATA3, which are related to differentiation of Th2 immune response, were also explored. It was found that the phosphorylation of STAT6 and GATA3 expression all increased in the model group and blank NPs group compared with naive mice. However, the expression of pSTAT6 and GATA3 was inhibited in CpG-AKp NPs-treated group (Figure 7(b)).

Discussion

Food allergy represents a growing health problem that adversely affects the quality of life of children and adults [23]. AK is regarded as one of the major shrimp allergens, previous studies have shown that mice treated with AK produced specific IgE levels and allergic symptoms [9]. In the present study, a model of food allergy was generated by sensitization and challenge with recombinant AK, which has been purified and verified to ensure its validity in our previous study [24]. The use of recombinant AK to establish an

animal model of food allergy can overcome the uncertainties brought by the fluctuating allergen content in allergen extracts [25]. The AK-specific T cell epitope peptide AK₈₁₋₁₀₀ consists of 20 amino acids in length, which is smaller than the whole allergen and too short to cross-link IgE, offers advantages over traditional approaches in terms of safety and efficacy. CpG-ODN was reported to be a potential inhibitor of Th2 cell-driven inflammatory responses and has been applied in allergic disease [26]. In the present study, we prepared CpG-AKp NPs for co-delivery of allergenic epitope and adjuvant CpG-ODN to evaluate their therapeutic effects on AK-induced food allergy. We found administration of CpG-AKp NPs markedly reduced anaphylactic symptoms to oral AK challenge, serum specific IgE and Th2 cytokines (IL-4 and IL-13), with an increase of the production of IFN- γ and IL-10. These results are consistent with the previous studies in which the mice that undergoing immunotherapy with CpG-ODN in a food allergy model were induced a specific shift toward a Th1/Treg immune response [17,26,27].

T helper lymphocytes can be divided into different subsets of effector cells based on their functional capabilities and the profile of cytokines they produce. Under normal physiologic conditions, the ratio of Th1 to Th2 cells maintains a suitable level. The IgE-

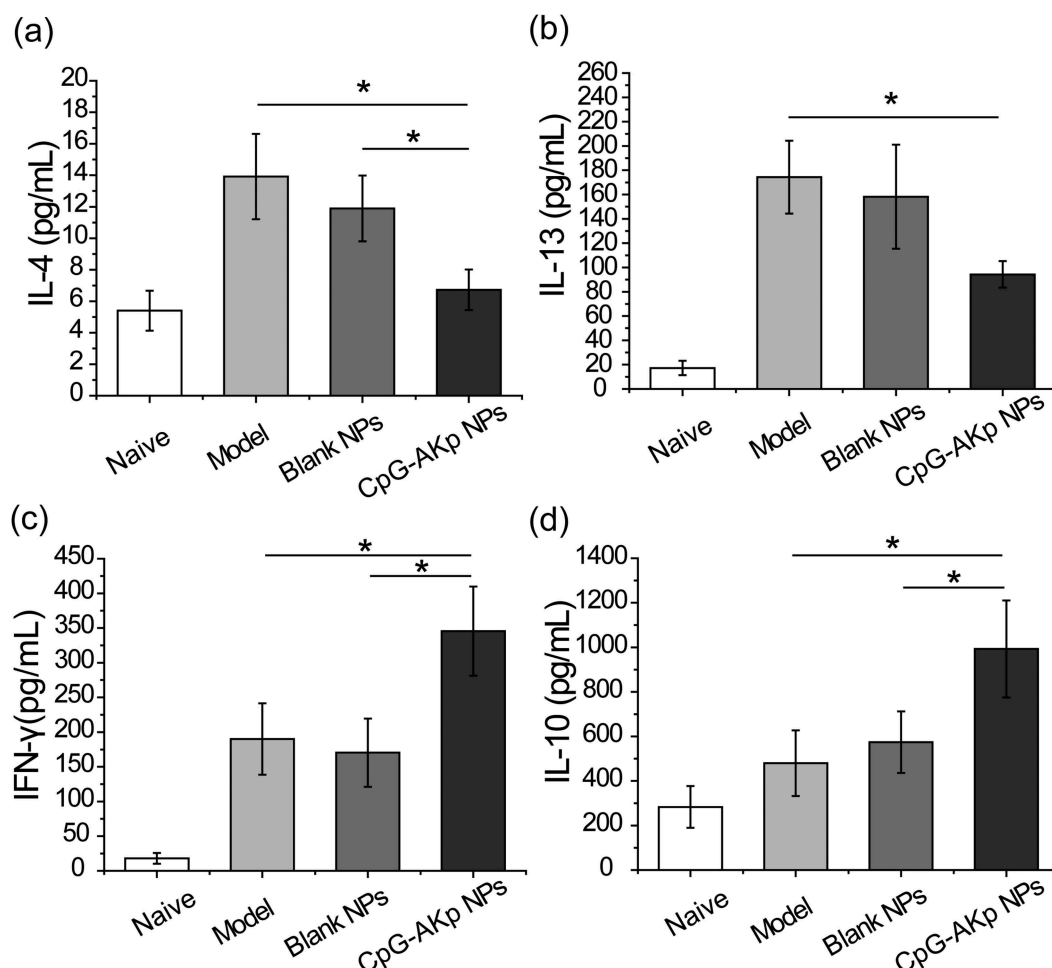


Figure 5. Cytokine production in spleen cell cultures from mice in different groups.

(a) IL-4 (b) IL-13 (c) IFN-γ (d) IL-10 levels (pg/mL) were measured in supernatants by ELISA and expressed as mean $\pm$ SD from six individual mice (*P < 0.05).

mediated FA is featured as Th2 polarization and over-production of IgE by B cells. It is well established that IL-4 can activate STAT6 and induce IgE production in mice [28,29]. GATA-3 has been shown to be responsible for the expression of the Th2 cytokines and depends on STAT6 activation, while suppressing the differentiation toward Th1 cells [30,31]. Here, we evaluated the effect of CpG-AKp NPs on STAT6 phosphorylation and GATA3 expression. It was observed that the lower levels of IL-4 and IL-13 in splenocyte supernatant in CpG-AKp NPs-treated group were correlated with downregulated the expression levels of pSTAT6 and GATA3. Therefore, the therapeutic effect of CpG-AKp NPs might be mediated by inhibition of pSTAT6 and GATA3.

In our study, a significant increase in Th1 cytokine IFN-γ was observed upon CpG-AKp NPs treatment. Increases in allergen-specific Th1 responses have been shown to be associated with favorable outcomes of immunotherapy [32]. However, the switch from Th2 to Th1 immune response needs to be well coordinated, since an uncontrolled bias toward Th1 response may

lead to other diseases [33]. Induction of Treg cells upon PIT may suppress excessive proliferation of both Th1 and Th2 cells due to its immune suppressive ability on both T cell subsets [34]. Although our study lacks a long-term follow-up after the treatment of CpG-AKp NPs, it is noteworthy that the expression of Treg cell linked proteins (IL-10 and Foxp3) was prominent in small intestine compared with other groups [35]. It is known that immune suppression for tolerance and maintenance of immune homeostasis depends on immunosuppressive cytokines such as IL-10 or TGF-β, which are produced by Treg cells [36,37]. In our model, the immune suppressive effect might be predominantly mediated by IL-10 since there were no differences in the level of TGF-β among groups. IL-10 was well established as key factor in the induction of tolerance, and modulating isotype response from IgE to an IgG4 dominated phenotype in the specific-immunotherapy [38]. Based on the above, it may be postulated that CpG-AKp NPs is promising to be a beneficial PIT for the treatment of AK-induced food allergy.

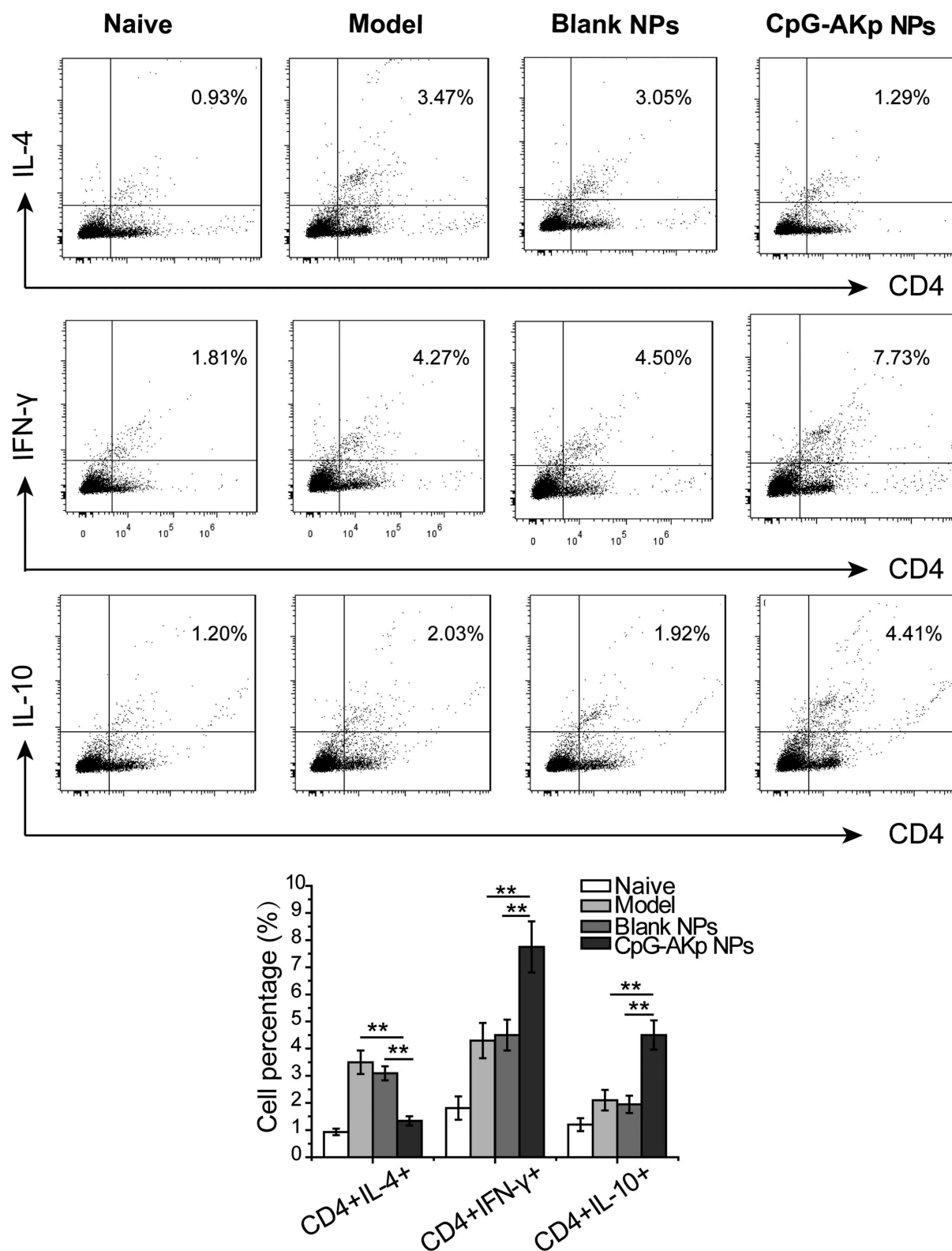


Figure 6. Isolated splenocytes CD4 + T-cell phenotypes from different groups.

Splenocytes were prepared and analyzed by using flow cytometric analysis. CD4⁺IL-4⁺, CD4⁺IFN-γ⁺ and CD4⁺IL-10⁺T cells were gated. The treatment was denoted above the panel. The % of each CD4 + T-cell phenotypes were shown. The results are presented as mean ± SD. **P < 0.01. Each group consists of 4–6 mice.

PIT has been widely employed in the treatment of food allergy. In the previous study, Yang et al. assessed the therapeutic potential of PIT based on the T cell epitope peptides of ovalbumin (OVA) [39]. Ueno et al.

have provided a practical approach for generating the T-cell epitope vaccines against major milk allergens [40]. Therefore, we assumed that the combination of these food allergen T-cell epitopes with CpG-ODN in the

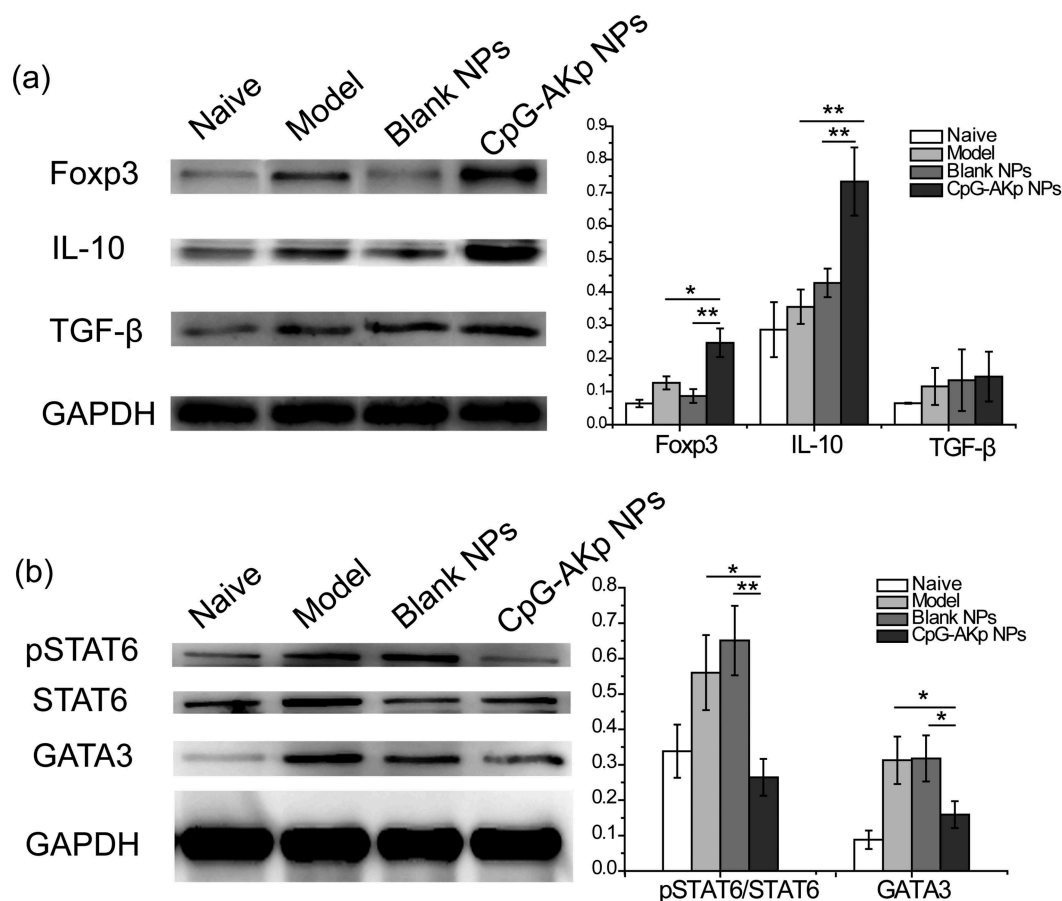


Figure 7. CpG-AKp NPs enhanced the expression of Foxp3 but attenuated the expression of pSTAT6 and GATA-3 in small intestine. (a) Western blotting for Treg linked proteins Foxp3, IL-10 and TGF-β. (b) Western blotting for Th2 transcription factors pSTAT6, STAT6, and GATA3. The data from at least three independent experiments are expressed as mean ± SD (*P < 0.05, **P < 0.01).

nanoparticle system might achieve better therapeutic efficacy in the treatments of different food allergy.

Conclusion

In summary, CpG-AKp NPs prepared in this study are potential as a safe and effective PIT approach. It can be prepared easily and prevent the *in vivo* allergic immune response in a shrimp allergy murine model through the suppression of Th2 cytokines (IL-4, IL-13) via inhibiting the activation of STAT6 and GATA3 expression. Meanwhile, CpG-AKp NPs could also induce a Th1/Treg response and promote the expression of Foxp3 in small intestine. These data suggested the potential of CpG-AKp NPs as a novel PIT against shrimp allergy in the future.

Author Contribution

JYH, QCG, XJX, HC, and RYY performed the experiments. ZGL and TQC organized the study and supervised experiments. JYH analyzed data and prepared the manuscript.

Disclosure statement

No potential conflict of interest was reported by the authors.

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Statement of Ethics

The animal studies were carried out in accordance with the Institutional Guidelines for Animal Care and Use of Laboratory Animals at Shenzhen University and approved by the Animal Ethics Committee at Shenzhen University.

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Supplementary Figures

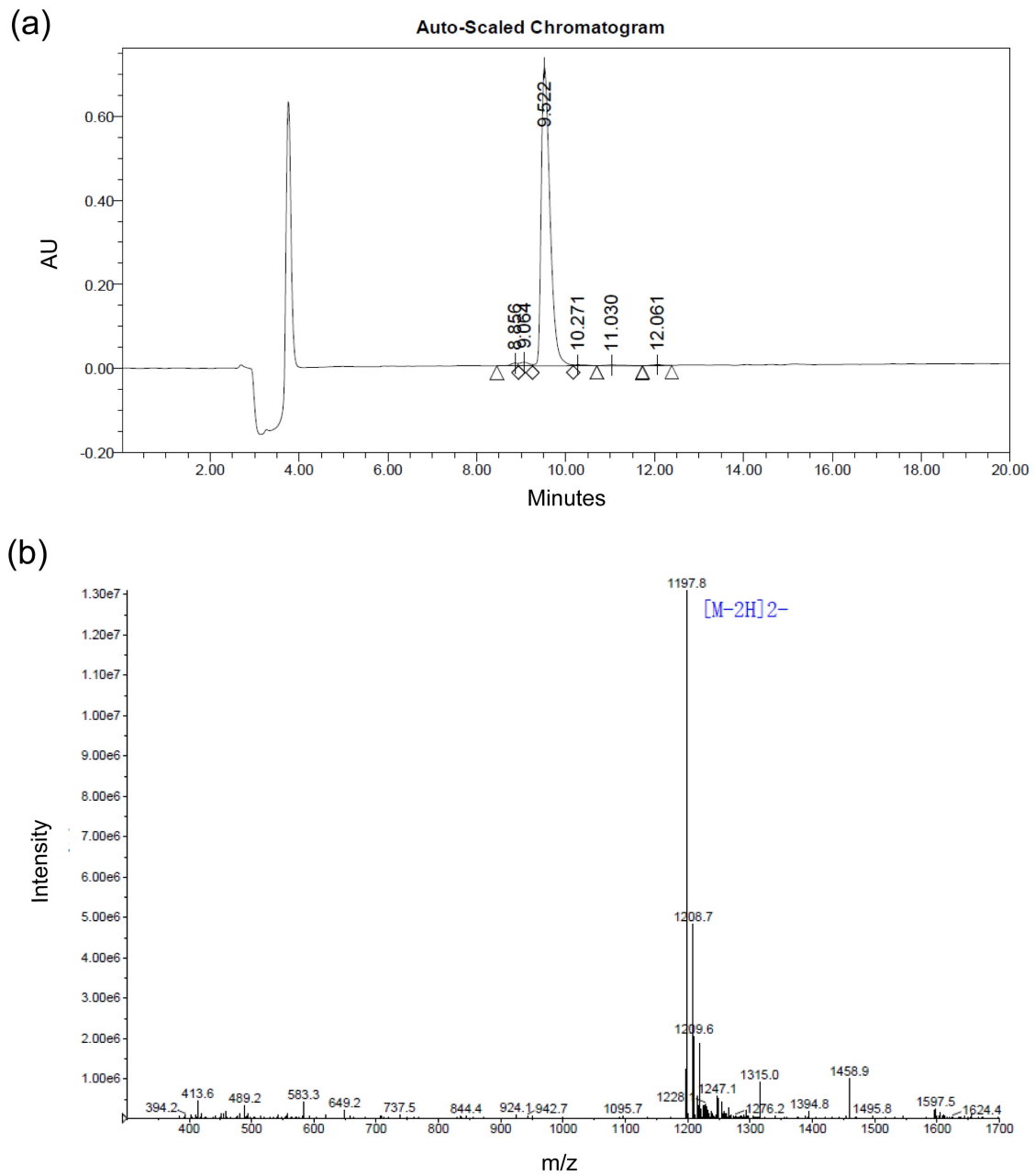


Figure S1. Characterization of AK₈₁₋₁₀₀ (LFDPIIEDYH VGFKQTDKHP). (a) HPLC Chromatograms of AK₈₁₋₁₀₀. (b) LC-MS spectrum of AK₈₁₋₁₀₀. The molecular weight of AK₈₁₋₁₀₀ is 2399.68 Da.

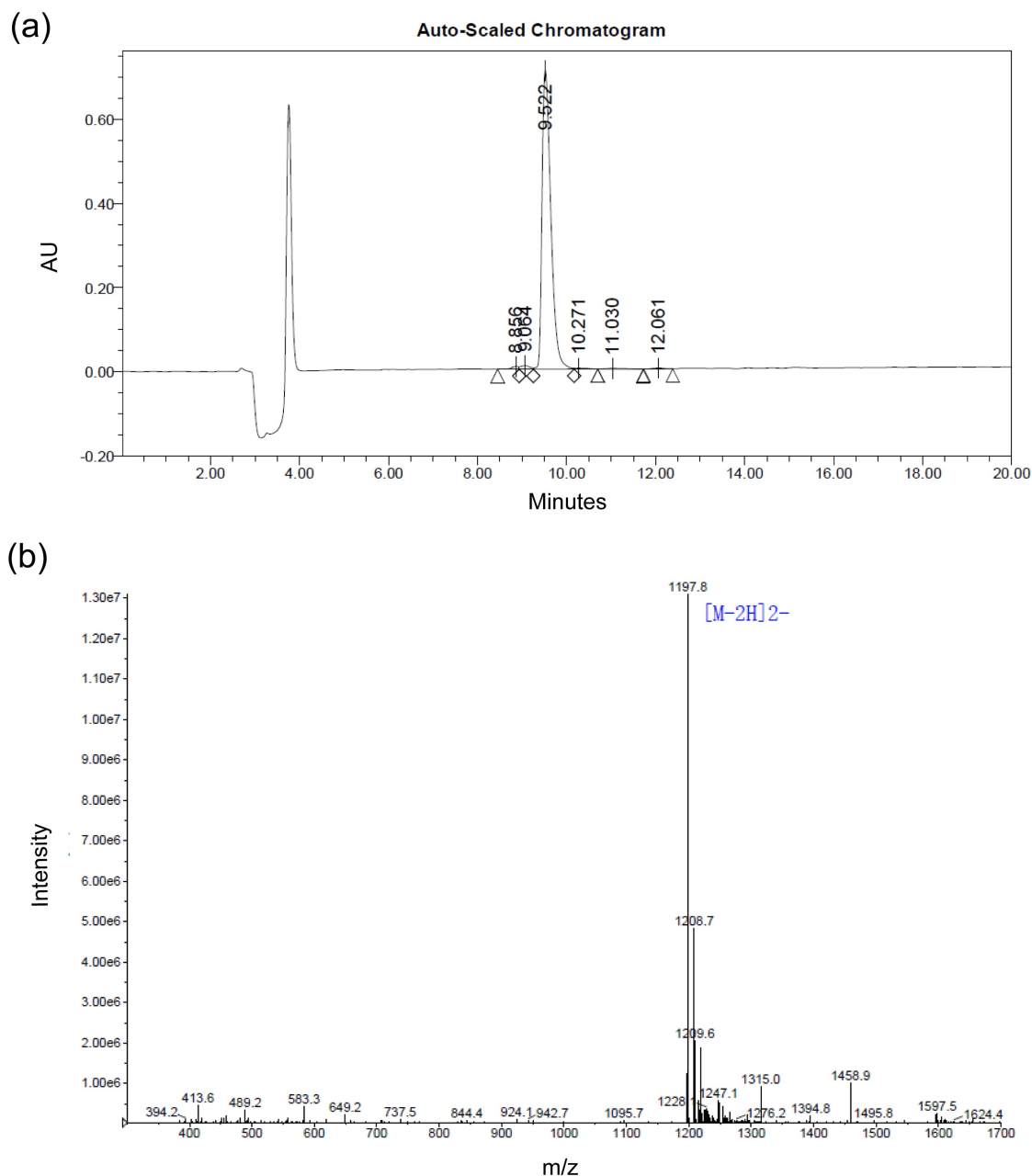


Figure S2. Characterization of the resultant CpG-AKp NPs. (a) The particle size distribution of CpG-AKp NPs were determined by DLS. Each sample was measured in triplicate (12 scans); (b) Transmission electron microscope of CpG-AKp NPs. One drop of the CpG-AKp NPs was placed on a 300-mesh copper grid, air-dried, and negatively stained with 2% (w/v) uranyl acetate for observation. The scale bar is 200 nm.