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Nasal mucosal inhalation of amyloid-beta peptide 3–10 defective adenovirus attenuates cytotoxicity induced by beta-amyloid (1–42)

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Abstract

Three-month-old Alzheimer's disease model transgenic mice were immunized with $A\beta_{1–42}$, Plp-Adenovirus [Ad]-X-CMV-$(A\beta_{3–10})_{10}$-CpG [AdCpG-$(A\beta_{3–10})_{10}$] or AdCpG virus fluid *via* nasal mucosal inhalation, respectively. ELISA analysis of serum showed $A\beta_{42}$ antibody titers were significantly increased in mice immunized with $A\beta_{1–42}$ and AdCpG-$(A\beta_{3–10})_{10}$. Concanavalin A and AdCpG-$(A\beta_{3–10})_{10}$ stimulation significantly increased the number of proliferating spleen cells cultured from AdCpG$(A\beta_{3–10})_{10}$ and $A\beta_{42}$ groups compared with the control group. In the AdCpG$(A\beta_{3–10})_{10}$ group, levels of interleukin (IL)-4 and IL-10 were increased, while those of IL-2 and interferon-γ were decreased. In the $A\beta_{42}$ group, levels of IL-4, IL-10, IL-2 and interferon-γ were all increased. Experimental findings indicate that AdCpG-$(A\beta_{3–10})_{10}$ vaccine can produce strong T helper 2 (Th2) humoral immune responses in addition to the production of $A\beta_{42}$ antibody. The cellular immunologic response was weak and avoided $A\beta_{1–42}$-mediated cytotoxicity.

Key Words: *nerve regeneration; neurodegenerative disease; Alzheimer's disease; immunotherapy; amyloid-beta peptide vaccine; cytokines; humoral immunity; inflammation; NSFC grant; neural regeneration*

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Introduction

Current drug treatment for Alzheimer's disease includes cholinergic inhibitors for the improvement of cognitive function, and N-methyl-D-aspartate receptor antagonists for medium and severe patients. However, these drugs only alleviate the symptoms of Alzheimer's disease, and fail to affect irreversible cognitive dysfunction and effectively scavenge amyloid beta peptide (Aβ) in the brain. Aβ vaccines reduced and eliminated Aβ deposition in an Alzheimer's disease transgenic mouse model, and significantly improved behavioral and cognitive impairment (Arendash et al., 2001a; Dodart et al., 2002). In 1999, the application of vaccine AN1792 (ELAN Pharmaceuticals, Inc., San Diego, CA, USA) in a phase IIa clinical trial was terminated owing to the presence of aseptic meningitis in 6% subjects, but an autopsy report found it effectively scavanged brain Aβ plaques (Nicoll et al., 2003). AN1792-induced aseptic meningitis was caused by T cell-mediated autologous immune cellular responses (Town et al., 2002). T lymphocytes mediate cell-mediated immunity and immune regulatory effects. T helper cells can be classified into T helper (Th)1 cells and Th2 cells according to the type of cytokine produced. Th1 cells secrete interferon-γ (IFN-γ) and IFN-β, for cell immunity, while Th2 cells secrete interleukin (IL)-4 and IL-10 that function in humoral immunity. A safe and effective Aβ vaccine can reduce Th1 immune responses and increase Th2 immune responses, thus attenuating inflammation in the brain (Piras et al., 2014).

Numerous measures have been proposed to make the vaccine safe and effective, such as selecting different amino acid fragments of $A\beta_{1–42}$, using different adjuvants, and immune pathways (Serpente et al., 2014). A gene vaccine synthesized with an N-terminal amino acid fragment of $A\beta_{1–42}$ has attracted increasing attention, and $A\beta_{1–6}$ and $A\beta_{1–15}$ fragments have been investigated in animal experiments (Janus et al., 2000; Arendash et al., 2001b). However, it is important to avoid cellular immune responses caused by the Aβ vaccine. We constructed a recombinant defective adenovirus vaccine Plp-Adeno-X-CMV-$(A\beta_{3–10})_{10}$-CpG [AdCpG-$(A\beta_{3–10})_{10}$] using defective adenovirus carriers, by repeated synthesis of $A\beta_{3–10}$ that is bound to CpG (Guo et al., 2011). In this study, 3-month-old Alzheimer's disease transgenic mice were immunized with B6.Cg-Tg(APPswe, PSEN1dE9)85Dbo/J *via* nasal mucosal inhalation, to observe serum Aβ antibody production and effects on mouse

spleen cell inflammatory responses.

Materials and Methods

Vaccine and virus

Adenovirus AdCpG-$(A\beta_{3-10})_{10}$ was inserted into the target gene, and AdCpG virus without target gene adenovirus and adjuvant CpG were provided by our research group as previously described (Guo et al., 2011).

Immunization of transgenic mice

Eighteen double transgenic mice B6.Cg-Tg(APPswe, PSEN1dE9)85Dbo/J, aged 3 months, 9 males and 9 females, weighing 220–280 g, were provided by the Experimental Animals Center of China Medical University, China (license No. SCXK (Liao) 2008-0005). The experiment was performed under approval of the Experimental Animal Ethics Committee, the First Affiliated Hospital of China Medical University, China. The mice were randomly divided into three groups: $A\beta_{1-42}$ group, AdCpG group and $A\beta_{3-10}$ group. The mice of all three groups were nasally inhalated with 20 μL $A\beta_{1-42}$ (Sigma, St. Louis, MO, USA), AdCpG virus (containing 10^{10} vector particles, equivalent to 10^8 pfu virus) or AdCpG-$(A\beta_{3-10})_{10}$ (10^{10} vector particles, equivalent to 10^8 pfu virus) (Morgan et al., 2000). Nasal mucosal immunization was administered to mice every 3 weeks, for a total of eight immunizations.

Preparation of blood specimens

Tail vein blood (0.3 mL) was collected when mice were aged 3 months (1 week before immunization), 6 months (1 week after the fourth immunization), and 7.5 months (1 week after the sixth immunization). Cardiac blood (2 mL) was collected at the age of 10 months (4 weeks after the eighth immunization). The collected blood samples were placed at room temperature for 2 hours, and centrifuged at 4°C at 2,500 r/min, for 20 minutes. The serum was stored until further use.

Indirect enzyme-linked immunosorbent assay (ELISA) detection of serum anti-$A\beta_{42}$ antibody concentration

One hundred μL $A\beta_{1-42}$ (5 mL/L; AnaSpect, Fremont, CA, USA) was coated onto 96-well plates and incubated overnight at 4°C. The plate was then rinsed with PBS containing 0.05% Tween-20, three times, and incubated with 200 μL blocking buffer per well (PBS containing 0.5% fetal bovine serum and 0.05% Tween-20) at room temperature for 1 hour. Then, the buffer solution was discarded, the plate was rinsed with PBS (containing 0.05% Tween-20) three times, and incubated with mouse quantitive anti-$A\beta_{1-16}$ monoclonal antibody (100, 30, 10, 3, 1, 0 μg/L; Covance, Princeton, NJ, USA) at 4°C overnight. The serum and standard antibodies were removed, the plate was rinsed with PBS (containing 0.05% Tween-20) three times, and incubated with anti-mouse IgG (1:2,000; Thermo Fisher Biochemical Products Co., Ltd.,) at room temperature for 1 hour. The secondary antibody was removed, and the plate was rinsed five times and incubated with 3,3′,5,5′-tetramethylbenzidine (100 μL per well) at room temperature for 15 minutes, until the dye was visible. Terminating solution (100 μL) was added to each well, and the absorbance at 450 nm was calculated using a microplate reader (BioTex, Winooski, VT, USA).

In vitro culture of spleen cells after immunization

Ten-month-old mice were euthanized under anesthesia (10% chloral hydrate) and splenic tissue was harvested and placed in a petri dish containing RPMI-1640 medium (Thermo Fisher Biochemical Products Co., Ltd., Beijing, China). Spleen tissue was sheared and ground to obtain a cell suspension. This was centrifuged at 4°C at 1,000 r/min (centrifugal radius of 12.5 cm) for 10 minutes, and the supernatant was discarded. Erythrocyte lysis buffer (3 mL; Beijing Dingguo Changsheng Biotechnology Co., Ltd., Beijing, China) was added to the cells for 5 minutes and mixed with RPMI 1640 medium. The cells were re-suspended by centrifugation at 4°C at 1,000 r/min for 10 minutes, twice. The precipitates were added with RPMI 1640 medium containing 10% fetal bovine serum. The cell density was adjusted to 5×10^6/mL. The cells were then incubated into the 96-well plates containing 2 μg/μL concanavalin A (Beijing Dingguo Changsheng Biotechnology Co., Ltd.) and 20 μg/μL AdCpG-$(A\beta_{3-10})_{10}$, in a CO_2 incubator for 72 hours.

MTT assay for *in vitro* proliferation rate of spleen cells after immunization

Spleen cells were cultured *in vitro* and incubated with 20 μL MTT solution per well (5 mg/mL; Beijing Dingguo Changsheng Biotechnology Co., Ltd.) for an additional 4 hours, and centrifuged at 2,000 r/min (centrifugal radius of 12.5 cm) at 4°C for 10 minutes. The supernatant was removed and 150 μL DMSO was added to each well. The absorbance value at 492 nm was detected by ELISA (Corning, Steuben County, NY, USA).

ELISA detection of IFN-γ, IL-2, IL-4, and IL-10 levels in spleen cell culture medium

Different cytokines (IFN-γ, IL-2, IL-4, IL-10) were added to the microporous plate (Corning) and were incubated with 100 μL standard sample at different concentrations (GD Animal Health service, Deventer, Netherland) and 50 μL biotinylated antibody working solution (Thermo Fisher Biochemical Products Co., Ltd.) at 20–25°C for 120 minutes. All solutions in the wells were discarded and 100 μL enzyme conjugate working solution (Thermo Fisher Biochemical Products Co., Ltd.) was added to each well except for the blank, and incubated at 20–25°C for 30 minutes. Subseqently, 50 μL each of chromogenic agents A and B (Shanghai Baoman Bio-Technology Co., Ltd., Shanghai, China) were added and developed in the dark at 37°C for 10 minutes. Then the reaction was terminated. The absorbance value of each well at 450 nm was measured with a microplate reader. IFN-γ, IL-2, IL-4, and IL-10 content was obtained by comparison with the standard curve.

Statistical analysis

Data are represented as mean ± SD, and were analyzed using

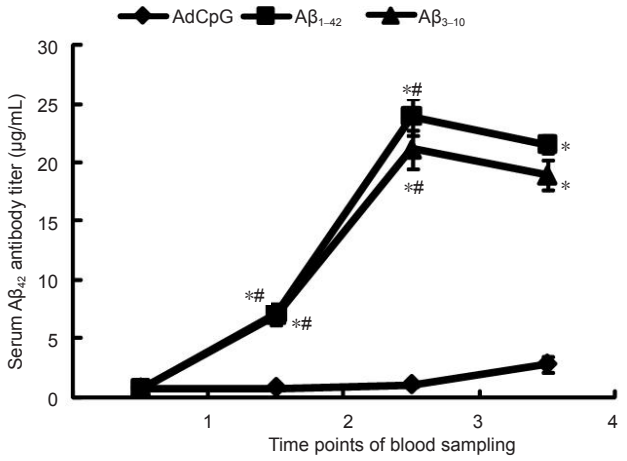

Figure 1 Changes in serum $A\beta_{42}$ antibody titers in transgenic mice after nasal mucosal immunization measured by enzyme-linked immunosorbent assay (ELISA).
Tail vein blood collected at an age of (1) 3 months (1 week before immunization); (2) 6 months (1 week after the fourth immunization); (3) 7.5 months (1 week after the sixth immunization); and (4) heart blood collected at 10 months of age (4 weeks after the eighth immunization). Data are expressed as mean ± SD of six mice per group. The difference between groups was compared using a paired *t*-test. $*P < 0.05$, *vs.* AdCpG group; $\#P < 0.05$, *vs.* previous time point.

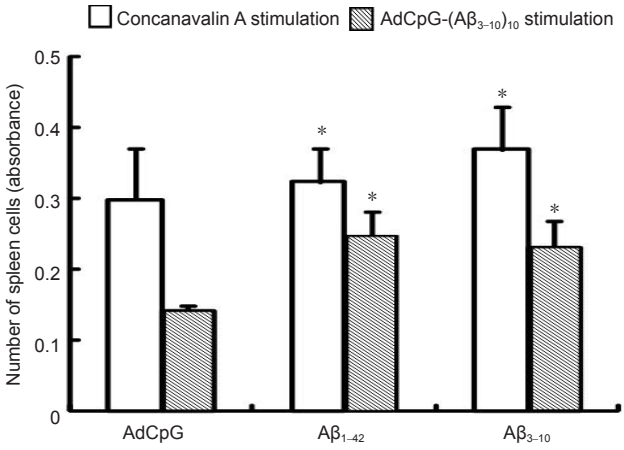

Figure 2 Proliferation of mouse spleen cells cultured *in vitro* after immunization (MTT assay).
Data are expressed as the mean ± SD of six mice per group. The difference between groups was compared by paired *t*-test. $*P < 0.05$, *vs.* AdCpG group.

SPSS 17.0 software (SPSS, Chicago, IL, USA). The difference between groups was compared using a paired *t*-test. A value of $P < 0.05$ was considered statistically significant.

Results

Serum anti-$A\beta_{42}$ antibody titers increased after immunization in transgenic mice

ELISA results showed that low serum $A\beta_{42}$ antibody levels could be measured before immunization. As the time after immunization increased, serum $A\beta_{42}$ antibody concentrations in the $A\beta_{1–42}$ group and $A\beta_{3–10}$ group gradually increased ($P < 0.05$). This increase was not statistically significant after the sixth and eighth immunizations ($P > 0.05$). In the AdCpG group, the serum $A\beta_{42}$ antibody concentration was unchanged during immunization. Compared with the AdCpG group, the $A\beta_{1–42}$ and $A\beta_{3–10}$ groups showed significantly higher serum $A\beta_{42}$ antibody concentrations ($P < 0.05$). There was no significant difference between the $A\beta_{1–42}$ and $A\beta_{3–10}$ groups ($P > 0.05$; Figure 1).

Nasal mucosal inhalation of AdCpG-$(A\beta_{3–10})_{10}$ promoted the *in vitro* proliferation of mouse spleen cells

After AdCpG-$(A\beta_{3–10})_{10}$ and $A\beta_{1–42}$ immunization, mouse spleen cells were cultured *in vitro*. MTT assay showed that stimulation with concanavalin A or AdCpG-$(A\beta_{3–10})_{10}$, significantly increased the number of spleen cells cultured in the $A\beta_{1–42}$ and $A\beta_{3–10}$ groups compared with the AdCpG group ($P < 0.05$), but there was no significant difference between the $A\beta_{1–42}$ and $A\beta_{3–10}$ groups ($P > 0.05$; Figure 2).

Effect of nasal mucosal inhalation of AdCpG-$(A\beta_{3–10})_{10}$ on IFN-γ, IL-2, IL-4 and IL-10 levels in spleen cell culture medium

ELISA results showed that after concanavalin A stimulation, IFN-γ and IL-2 levels in the culture medium of $A\beta_{42}$ immunized spleen cells ($A\beta_{1–42}$ group and $A\beta_{3–10}$ group) were significantly decreased compared with the AdCpG group ($P < 0.05$), whereas IL-4 and IL-10 levels showed no significant change. There was no significant difference in cytokine levels between the $A\beta_{1–42}$ and $A\beta_{3–10}$ groups ($P > 0.05$). After AdCpG-$(A\beta_{3–10})_{10}$ stimulation, IFN-γ, IL-2, IL-4 and IL-10 levels in the culture medium of $A\beta_{42}$ immunized spleen cells ($A\beta_{1–42}$ group and $A\beta_{3–10}$ group) were significantly increased compared with the AdCpG group ($P < 0.05$). IL-4 and IL-10 levels in the culture medium of AdCpG-$(A\beta_{3–10})_{10}$ immunized spleen cells were increased ($P < 0.05$), while IFN-γ and IL-2 levels were decreased ($P < 0.05$; Figure 3).

Discussion

Schenk et al. (2002) showed for the first time that $A\beta_{42}$ peptide vaccine reduced Aβ deposition and scavenged senile plaques in the brain of PDAPP mice, opening a new field for the immunotherapy for Alzheimer's disease. Subsequent studies have mostly supported the findings; for example Aβ immunization improved learning and memory functions in Alzheimer's disease transgenic mice (McLaurin et al., 2002). Furthermore, the presence of serum antibodies after immunization effectively inhibited Aβ fiber aggregation (McLaurin et al., 2002). Other studies have demonstrated the effect of immunotherapy against Alzheimer's disease. A phase I clinical trial using QS21 as an adjuvant for $A\beta_{42}$ peptide vaccine induced an immune response that generated Aβ antibodies that bound with amyloid-like plaques, thus significantly improving cognitive function in patients. Unfortunately, 6% of patients developed undersiable symptoms of cerebrospinal meningitis in a phase II clinical trial of the AN1792 vaccine (Hock et al., 2003), and the clinical trial was discontinued. A study by Dodart et al. (2002) confirmed aseptic meningitis occurred because of cellular immune responses mediated by T cells, which can be avoided.

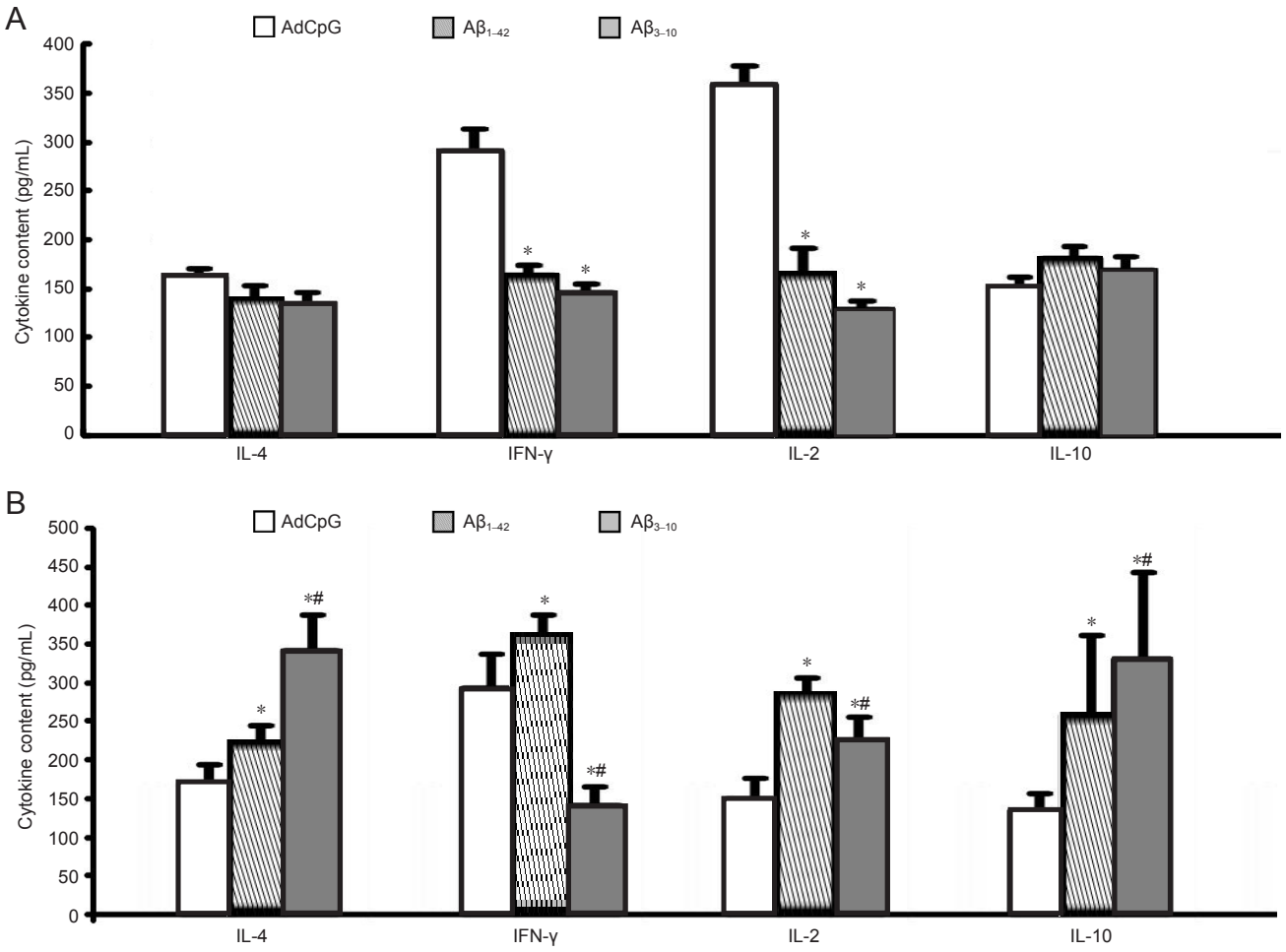

Figure 3 Differential cytokine expression in mouse spleen cells after stimulation with concanavalin A (A) and AdCpG-$(A\beta_{3-10})_{10}$ (B) by enzyme-linked immunosorbent assay.

Data are expressed as mean ± SD of six mice per group. The difference between groups was compared by paired *t*-test. ∗$P < 0.05$, *vs.* AdCpG group; #$P < 0.05$, *vs.* $A\beta_{1-42}$ group. IL-2: Interleukin-2; IL-4: interleukin-4; IL-10: interleukin-10; IFN-γ: interferon-γ.

Different types of cellular immune responses (Th1 and Th2) mediate either immune regulatory or inflammatory processes, and various types of T cell responses are important for developing Alzheimer's disease immunotherapeutic strategies. Extracellular pathogen infection (or antigen immunity) is mainly prevented by antibody and complement reactions as these antigens preferentially induce the differentiation of Th2 cells, whereas intracellular infections (or antigen) are dominated by Th1 responses. Infectious pathogen antigens are scavenged by activated macrophages or microglia in the central nervous system. The Th1 response mainly promotes the secretion of IL-2 and IFN-γ (Orgogozo et al., 2003), whereas Th2 responses produce IL-4 and IL-10, the expression of CD40, and the synthesis of IgG1, IgG3 and IgE antibodies from B cells. In addition, Th2 responses stimulate human B cells to produce IgG2, IgG4, IgA, IgE and other antibody isotypes that remove extracellular Aβ with no damage to surrounding tissues or cells. Therefore, a crucial strategy for treating Alzheimer's disease is to reduce Th1 immune responses and enhance Th2 immune responses.

A variety of methods have been proposed to develop a safe vaccine for Alzheimer's disease, including the synthesis of Aβ peptide chain amino acid sequences, adjuvants, vectors and immune system pathways. However, these methods affect the vaccine's efficacy. Kim et al. (2007) prepared a novel vaccine with $A\beta_{1-6}$ and *Pseudomonas* exotoxin A receptor delivered by adenoviral vector, and found that nasal mucosal immunization of this vaccine reduced brain Aβ plaques, induced Th2 responses, and inhibited Th1 responses in transgenic mice. Movsesyan et al. (2008) demonstrated that prophylactic immunization with DNA epitopes vaccine could induce strong Th2 immune responses and generate high concentrations of Aβ antibodies with cases of meningitis observed. Frenkel et al. (2001) elucidated that $A\beta_{3-6}$ is the epitope that prevents Aβ accumulation, and the lack of the 3rd amino acid significantly decreased the affinity of Aβ antibodies. It was also shown that the CpG funtion of activating the immune system in animals is mainly mediated by B lymphocyte proliferation and the secretion of cytokines from activated monocytes (Vellas et al., 2009). Accumulating evidence has demonstrated the application of adenoviral DNA as an immune adjuvant and therapeutic

drug (Ballas et al., 1996). Thus, it is feasible to use a defective adenovirus as the vector for nasal immunization (Lemere and Masliah, 2010). Furthermore, it is simple to use, is low cost, has a longer duration of expression, and causes less trauma.

Based on the above, we chose the N-terminal 3–10 amino acids of $A\beta_{1–42}$ as a macromolecular antigen, in an attempt to generate humoral immunity and reduce cell-mediated immunity. In addition, CpG functions as an adjuvant to increase the immune effects of $(A\beta_{3–10})_{10}$ antigen and overcome immune tolerance. The aim of this study was to induce Aβ antibodies and induce Th2 immune responses using the constructed AdCpG-$(A\beta_{3–10})_{10}$. Lemere et al. (2009) found that Aβ immunotherapy effectively prevented neurological damage in the brain of Alzheimer's disease patients, before Aβ aggregation. Therefore, we immunized 3-month-old transgenic mice with AdCpG-$(A\beta_{3–10})_{10}$ before senile plaques formed.

We examined the plasma of 6-month-old transgenic mice by ELISA and observed the successful induction of $A\beta_{42}$ antibodies at high concentrations. At 9 months of age (after the eighth immunization), plasma antibodies could still be detected, and the measured concentration was similar to that after the fourth immunization. This evidence indicated that a high concentration of antibodies was obtained after four immunizations, and were maintained at a high concentration after multiple immunizations. After $A\beta_{42}$ vaccine immunization, under the stimulation of antigen or concanavalin A, the number of proliferative spleen cells cultured *in vitro* was significantly increased, and the secretion of IL-2, IFN-γ, IL-4 and IL-10 was also increased. This evidence indicated that $A\beta_{42}$ peptides contain T cell and B cell epitopes, and induce both Th1 and Th2 responses. After mice were immunized with the AdCpG-$(A\beta_{3–10})_{10}$ vaccine, the number of proliferative spleen cells and IL-4 and IL-10 levels were increased, while IL-2 and IFN-γ levels were reduced. Therefore, the $A\beta_{3–10}$ subunit is B cell epitope antigen, that stimulates Th2 responses to activate cellular immune responses.

This study demonstrated that the constructed AdCpG-$(A\beta_{3–10})_{10}$ could induce high concentrations of Aβ antibodies in young transgenic mice after immunization, stimulate Th2 responses and reduce Th1 responses. When 3-month-old double transgenic mice B6.Cg-Tg(APPswe, PSEN1dE9) were nasal immunized with AdCpG-$(A\beta_{3–10})_{10}$ vaccine, IL-4 and IL-10 levels were significantly increased, indicating that the vaccine induced Th2 humoral immune responses, and no apparent cellular immune responses were observed. This evidence indicated the safety of the vaccine. We further detected serum Aβ antibody titers before, during and after immunization by ELISA, and found that high levels of serum antibody were present in AdCpG-$(A\beta_{3–10})_{10}$ immunized mice. AdCpG-$(A\beta_{3–10})_{10}$, as a second generation vaccine for Alzheimer's disease, could induce high levels of Aβ antibodies in young transgenic mice, produce Th2 responses, and reduce Th1 responses.

Author contributions: *Jiang TZ was responsible for the study design, collecting the data, and writing the manuscript. Guo WS, Sha S, Xing XN and Guo R implemented the study. Cao YP evaluated the study. Jiang TZ and Cao YP were responsible for the manuscript. All authors approved the final version of the manuscript.*
Conflicts of interest: *None declared.*

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