A new DNA vaccine fused with the C3d-p28 molecular adjuvant enhances anti-amyloid-β antibody generation and induces a Th2 immune response in young C57BL/6J mice

Wanshu Guo¹, Sha Sha¹, Xiaona Xing¹, Tongzi Jiang², Yunpeng Cao¹

¹ Department of Neurology, First Affiliated Hospital of China Medical University, Shenyang 110001, Liao Ning Province, China
² Department of Neurology, The First People’s Hospital of Shenyang City, Shenyang 110041, Liao Ning Province, China

Abstract

Both active and passive Aβ immunotherapy could increase the clearance of Aβ and decrease the progressive cognitive decline. To enhance anti-amyloid-β (Aβ) antibody generation and induce a Th2 immune response, we constructed a new DNA vaccine [p(Aβ3-10)10- C3d-p28.3] encoding ten repeats of Aβ3-10 and three copies of C3d-p28 as a molecular adjuvant and administered it intramuscularly in eight to ten-week-old female C57BL/6J mice. ELISA was used to detect the titers of serum anti-Aβ antibody, isotypes and splenic T cells cytokines. MTT assay was used to detect the proliferation rate of the splenic T cells. Brain sections from a twelve-month-old APP/PS1 transgenic mouse were used for detecting the binding capability of anti-Aβ antibodies to Aβ plaques. The p(Aβ3-10)10- C3d-p28.3 vaccine induced high titers of anti-amyloid-β antibodies which were able to bind to Aβ plaques in APP/PS1 transgenic mouse brain tissue, demonstrating that the vaccine is effective against plaques in a mouse model of AD. Moreover, the vaccine elicited a predominantly IgG1 humoral response and low levels of IFN-γ in ex vivo cultured splenocytes, indicating that the vaccine could shift the cellular immune response towards a Th2 phenotype. This indicated that the vaccine did not elicit a detrimental immune response and had a favorable safety profile. Our results indicate that the p(Aβ3-10)10- C3d-p28.3 vaccine is a promising immunotherapeutic option for Aβ vaccination in Alzheimer’s disease.

Key Words

Alzheimer’s disease; Amyloid-β; C57BL/6J mice; DNA vaccine; Active immunotherapy; Passive immunotherapy; C3d-p28 molecular adjuvant; Th2 immune response

Research Highlights

(1) We have chosen the Aβ3-10 fragment, as the antigen as Aβ1-15 has already been demonstrated to be a B cell epitope, which could avoid a Th1 cell mediated meningoencephalitis in Alzheimer’s disease immunotherapy.

(2) C3d-p28 molecular adjuvant is an attractive adjuvant. Three copies of mouse C3d-p28 could not only enhance B cell response to a vaccine antigen but also shift the cellular immune response towards a Th2 phenotype. Thus, in this study, three copies of mouse C3d-p28 were added as an adjuvant to (Aβ3-10)10 to further enhance the immunogenicity.

(3) We successfully constructed a new plasmid DNA vaccine, p(Aβ3-10)10- C3d-p28.3, which could not only boost therapeutic levels of anti-Aβ antibodies generation but also minimize the Th1 immune response, more importantly, the antibody were able to bind to Aβ plaques in brain tissue from a twelve-month-old APP/PS1 transgenic mouse. It demonstrated that the anti-Aβ antibodies elicited from the (Aβ3-10)10- C3d-p28.3 were effective and had strong affinity for Aβ plaques.
INTRODUCTION

Alzheimer’s disease (AD) is a neurodegenerative disease characterized pathologically by the presence of extracellular amyloid-β (Aβ)-containing senile plaques and intracellular Tau protein neurofibrillary tangles. At present, there are 36 million people globally with dementia and it is predicted that there will be 115 million by 2050 [1]. It is one of the most important health care, social, and economic challenges of the 21st century. However, there are no effective treatments for Alzheimer’s disease. The “amyloid cascade hypothesis” emphasized that excessive production and insufficient clearance of Aβ protein precedes Alzheimer’s disease-related dementia and neuronal loss [2]. Evidences supported that a central role of aggregated forms of amyloid-β (Aβ) peptide might cause Alzheimer’s disease [3, 4]. Therefore, both augment clearance of Aβ and prevent accumulation of Aβ by Aβ active and passive immunotherapy have been thought of as a promising strategy for the treatment of Alzheimer’s disease.

In 1999, Schenk [5] and colleagues were the first to report that Aβ1-42 active immunization in PDAPP transgenic mice could increase the rate of clearance and prevent the aggregation of Aβ. Two additional reports demonstrated that Aβ vaccination in Tg CRND8 [6] or APP/PS1tg [7] mice not only reduce levels of cerebral amyloid but also improved behavioral performance in learning and memory tasks. Recently, immunization targeting the Aβ peptide is effective in halting progression of amyloid pathology in transgenic mice [8, 9]. The method of the immunization of Aβ1-42 peptide (AN1792) in combination with the adjuvant QS-21 was translated to clinical trials by ELAN/Wyeth in 2000 and 2001. But the trial was stopped due to the occurrence of meningoencephalitis in 18 of the 300 immunized Alzheimer’s disease patients [10, 11]. It is important to note that meningoencephalitis is a serious side effect of active immunotherapy and accumulating evidence suggests that the occurrence of meningoencephalitis may be related to QS21, a Th1 type adjuvant and the presence of T and B cell self-epitopes of Aβ1-42 peptide. Initially, for generating a robust antibody response in AN1792 vaccinated elderly, researchers chose QS21 as an adjuvant to induce a strong cell-mediated immune response [12]. But just QS21, may result in the occurrence of meningoencephalitis. Subsequent report showed that Aβ1-42 peptide immunization could indeed lower Aβ plaques in patients compared to the placebo control patients, which provided a proof that it is possible to remove brain amyloid by immunotherapy. However, Holmes also found that Aβ1-42 peptide immunization did not improve survival or an improvement in the time to severe dementia [13], possibly because the immunization was administered too late in the course of the disease. Thus, early preventive immunization prior to neuropathology and cognitive deficits may be essential for the effective treatment of Alzheimer’s disease using a vaccine based strategy.

Bengt Winblad and colleagues [14] had constructed a novel second-generation Aβ vaccine (CAD106), a small Aβ peptide fragment (Aβ1–6) that is a B-cell epitope avoiding an Aβ-specific T-cell response. In order to elicit an immune response, the peptide was used coupled to an adjuvant carrier formed by multiple copies of the coat protein of bacteriophage Q8. Immunization with CAD106 could prevent the aggregation of Aβ in two transgenic mouse models of Alzheimer’s disease, with reductions of up to 80% in the plaque area compared with controls. Subsequently, researchers tested the safety, tolerability, and Aβ-specific antibody response of CAD106 in patients with mild-to-moderate Alzheimer’s disease. Winblad and colleagues [15] enrolled patients, both men and women, 50-80 years of age, with Alzheimer’s disease who had a mini-mental state examination score of 16–26. Patients were randomly allocated either to three injections of 50 mg CAD106 (24 patients in cohort one) or 150 mg CAD106 (22 patients in cohort two) or to placebo (seven patients in cohort one and five in cohort two). The study-period was 52 weeks, with a 2-year follow-up period. Although nine patients reported serious adverse reactions, none of the serious adverse events seen in the 52-week study or the 2-year follow-up was thought to be related to the study drug. No cases of meningitis, meningoencephalitis, or vasogenic oedema were detected clinically or by use of MRI during the trial period. However, longer studies with larger study populations are needed to confirm the safety and establish the efficacy of CAD106 in Alzheimer’s disease. In addition to CAD106, three other active immunization approaches are under clinical testing: one in phase 1 (V950) and two in phase 2 (ACC-001 and ACI-24), all of which target the amino-terminal part of Aβ but with differing peptide conjugates. Full results from these trials are not yet available.

Passive immunization studies using Aβ antibodies against the N-terminus, mid-domain, and C-terminus of Aβ have been used in transgenic mice. Bard [16] showed that the Aβ antibodies were able to enter the central nervous system (CNS), bind plaques and induce
clearance of pre-existing amyloid. Later, the same authors performed that antibodies against the N-terminus of Aβ (3D6 against Aβ1-5 or 10D5 against Aβ3-7) were the most effective at reducing brain amyloid [17]. Passive immunization of PDAPP tg mice with the 10D5 antibody led to improved cognitive performance [18]. DeMattos [19] used an antibody that binds to the mid-domain of Aβ. Treatment of transgenic mice for 5 months using such an antibody (m266.2) reduced plaque burden [19]. Cattepoel [20] showed that intranasal treatment with an anti-Aβ30-42 scFV antibody ameliorates amyloid pathology in APPswe/PS1dE9 mice. Bapineuzumab, a monoclonal antibody (3D6) binding to the N-terminal portion of Aβ, is in clinical development. Although meningoencephalitis has not been reported with bapineuzumab in a phase 2 study, using magnetic resonance imaging (MRI), vasogenic edema was seen in approximately 10% of patients [21]. Subsequent report showed that reduction of amyloid burden following treatment with bapineuzumab was demonstrated in a phase 2 amyloid imaging 11C-PIB positron emission tomography (PET) study [22].

125I-labeled 3D6, the mouse parent antibody of the phase 3 clinical candidate bapineuzumab, was explored the pharmacokinetic profile of this antibody in the brain and serum. Meanwhile, researchers tested the correlation between plaque accumulation and antibody blood levels [23]. Solanezumab (LY2062430) is the humanized analog of the murine antibody m266.2. In a preclinical study [24], m266.2 did not produce microhemorrhage in transgenic mice. In a single-dose clinical study, solanezumab was generally well tolerated when administered to 16 mild to moderate Alzheimer’s disease patients [25]. However, repeated injections of monoclonal anti-Aβ antibody in passive Aβ immunotherapy may result in micro-hemorrhage and vasogenic edema [21, 24]. Furthermore, passive immunization is not useful to prevent the diseases with insidious onset such as Alzheimer’s disease. And more importantly, the passive approach is the potential for patients to eventually develop neutralizing antibodies against the passive therapy.

Based on this rational, in our study, we investigated the potential of active Aβ immunotherapy. Current alternative immunotherapeutic strategies for boosting therapeutic levels of anti-Aβ antibodies generation as well as minimizing the Th1 immune response after active Aβ vaccination are focused on: Aβ DNA vaccines production, B cell Aβ epitope generation, adjuvant modifications, and alternative routes. DNA vaccine is a new safe, effective vaccine including their ease of production, the ability to modify genes coding for desired antigens, the stability of episomal DNA, and more importantly, the ability to induce the desired type of immune response [9]. Some studies indicated that plasmid DNA encoding Aβ could generate specific antibodies against Aβ and elicit B cell immune responses without a significant T cell-mediated immune response in mice [9, 26, 27], rhesus monkeys [28] and rabbits [29]. The Th1 response reported in the AN1792 trial may relate to the presence of T and B cell self-epitopes of Aβ42 peptide. The B cell epitopes are located in the N terminal of the peptide (11-15 amino acids) [30, 31], and are thought to be important for production of anti-Aβ antibodies while avoiding a deleterious Aβ-specific T cell response. The T cell epitopes are derived from the C terminal of the peptide (16-42amino acids), and are thought to activate the T cell response that triggers the meningoencephalitis [32]. It was reported that the Aβ4-10 fragment was the dominant peptide that anti-Aβ42 IgGs specifically recognized with high affinity [33]. Moreover, the Aβ3-6 fragment (EFRH) affects the solubility and disaggregation of Aβ fibrils [34]. Aβ3 is also highly prone to oligomerization and is an important component of the Aβ deposited in senile plaques of the Alzheimer’s disease brain [35]. To overcome its low immunogenicity of the short Aβ peptide, tandem repeats of a small self-peptide are constructed to increase the molecular weight as well as to reduce the degradation of the peptide [26, 27, 29, 36].

In this study, we constructed a new plasmid DNA vaccine, p(Aβ3-10)10-C3d-p28.3. We chose non-viral plasmid [pcDNA3.1(+)] as a vector. Here, we have chosen Aβ3-10, which is the minimal effective fragment, as the antigen as Aβ1–15 has already been demonstrated to be a B cell epitope. To overcome the low immunogenicity of the Aβ3-10 single peptide, we constructed a cDNA encoding ten tandem repeats of Aβ3-10. In addition, three copies of mouse C3d-p28 were added as an adjuvant to (Aβ3-10)10 to further enhance the immunogenicity. The vaccine was then injected intramuscularly into eight to ten week-old female C57BL/6J mice. The current study was designed to test whether the vaccine could boost the production of high titers of anti-amyloid-β antibodies and induce a Th2 immune response. We hope that the p(Aβ3-10)10-C3d-p28.3 vaccine could be a promising immunotherapeutic option for Aβ vaccination in Alzheimer’s disease.

RESULTS

Quantitative analysis of experimental animals
Construction of Aβ plasmid DNA vaccine

Ten tandem repeats of cDNA encoding human Aβ3-10 gene and three copies of cDNA encoding mouse C3d-p28 gene were chemically synthesized and the DNA fragments were cloned into the eukaryotic expression vector pcDNA3.1(+) (+). HindIII, BamHI, EcoRI restriction sites were added to form the following DNA fragments: 5′—HindIII—kozak sequence—ATG (initiation codon)—10×Aβ3-10—GGGGS linker — BamHI—3×C3d-p28—EcoRI—3′. The recombinant plasmid was amplified in DH5α E.coli competent cells. The plasmids were purified and extracted in large quantities using plasmid preparation kits (E.Z.N.A TM Fast filter Endo-Free Plasmid Maxi Kit). The sequence of the target genes was confirmed by restriction enzyme digestion and agarose gel electrophoresis. Plasmid sequences were confirmed using nucleotide sequence analysis. The in vitro expression of pcDNA3.1 (+) -C3d-p28.3 plasmids was also confirmed in transiently transfected HEK293 cells by Western blotting.

Generation of high titers of anti-Aβ antibodies of predominantly IgG1 isotype

All of the mice immunized with Aβ42 peptide and p(Aβ3-10)10-C3d-p28.3 developed anti-Aβ antibodies. Sera from Aβ42 peptide and p(Aβ3-10)10-C3d-p28.3 vaccinated mice showed a steady increase in anti-Aβ antibody after two immunizations, initiating an average of (22.07±5.35μg/ml) and (8.46±2.42μg/ml), reaching an average of (81.03±11.38 μg/ml) and (60.53±10.55 μg/ml) after the final immunization, respectively. No anti-Aβ antibodies were detected in the pcDNA3.1 (+) group. Antibody titers from pcDNA3.1 (+) vaccinated mice remained at background levels (Fig. 1A). The p(Aβ3-10)10-C3d-p28.3, similar to other gene vaccine, induced less anti-Aβ antibodies than Aβ42 peptide [38].

Sera collected after the final immunization was used to quantify isotypes of immunoglobulin by ELISA. Immunization with p(Aβ3-10)-C3d-p28.3 and Aβ42 peptide induced a broad spectrum of anti-Aβ antibody isotypes (IgG1, IgG2a, and IgG2b). The IgG1 antibody response (32.65 ± 6.55 μg /ml), IgG2a antibody response (3.57 ± 1.13 μg /ml) and IgG2b antibody response (6.45 ± 2.9 μg /ml) were detected in the p(Aβ3-10)10-C3d-p28.3 group (Fig. 1B). And in the Aβ42 peptide group, the IgG1 antibody response (42.86 ± 7.28 μg /ml), IgG2a antibody response (26.76 ± 4.73 μg /ml) and IgG2b antibody response (22.62 ± 3.46 μg /ml) were detected (Fig. 1B). The isotypes of IgG can be used as an indirect measure of cytokine production during immune response. In mice, IgG1 is mainly induced by Th2 cytokines, whereas IgG2a reflects the involvement of Th1 cytokine [38]. The ratio of IgG1 to IgG2a was significantly higher (p<0.05) in mice immunized with p(Aβ3-10)10-C3d-p28.3 (9.46 ± 1.26) versus Aβ 42 (1.26 ± 0.04) (Fig. 1C). Immunization with p(Aβ3-10)10-C3d-p28.3 induced predominant IgG1 with IgG1/IgG2a ratio much greater than Aβ42 peptide. This means that the p(Aβ3-10)10-C3d-p28.3 group induced a more robust Th2 immune response than the Aβ42 group.

Enhancement of T cell proliferation and inducement of Th2-biased response

Spleen T cell proliferation assay was used to detect whether immunization with p(Aβ3-10)10-C3d-p28.3 induced immune response specific for Aβ3-10 or
full-length Aβ42. As shown in Fig.2A, spleen T-cells restimulated with ConA exhibited the highest levels of proliferation in all groups. In the Aβ42 peptide group, a higher level of T cell proliferation was observed in splenocytes restimulated with Aβ42 peptide, which was greater than restimulated with Aβ3-10 and smaller than restimulated with ConA. In the Aβ3-10 peptide group, a higher level of T cell proliferation was observed in splenocytes restimulated with Aβ3-10 peptide, which was greater than restimulated with Aβ42 and smaller than restimulated with ConA. In other words, in the Aβ42 peptide and p(Aβ3-10)10- C3d-p28.3 group, a higher level of T cell proliferation was observed in splenocytes restimulated with their corresponding antigen, which was greater than restimulated with a non-corresponding antigen and smaller than restimulated with ConA, although there was no significant difference between the two groups (p>0.05). In the pcDNA3.1(+) group, spleen T-cells restimulated with ConA exhibited much higher levels of proliferation than those stimulated with Aβ42 and Aβ3-10. The results indicated that p(Aβ3-10)- C3d-p28.3 immunization induced a specific cellular immunity targeted to Aβ3-10 (B-cell epitopes) other than full-length Aβ42.

ELISA was used to detect levels of IL-4 and IFN-γ cytokines in splenocyte culture supernatants to determine the phenotype of the T-cell response. Higher levels of IL-4 were detected from the Aβ42 peptide (91.63 ±11.02 pg/ml) and p(Aβ3-10)10- C3d-p28.3 group (82.26 ± 9.94 pg/ml) as compared with the pcDNA3.1(+) group (13.74 ± 4.25 pg/ml) (p<0.05) (Fig.2B), but there were no significantly differences between two Aβ-immunized groups(p>0.05). Splenocytes isolated from mice immunized with Aβ42 peptide produced much higher IFN-γ levels (218.89 ±29.35 pg/ml) than those from p(Aβ3-10)10- C3d-p28.3 ( 29.77 ±7.76 pg/ml) (p<0.05) and pcDNA3.1(+) (23.13 ±7.16pg/ml) (p<0.05) (Fig.2C). However, approximately equal IFN-γ levels were detected in the p(Aβ3-10)10- C3d-p28.3 and pcDNA3.1(+) group (p>0.05). The p(Aβ3-10)10- C3d-p28.3 vaccine elicited a high levels of IL-4 and low levels of IFN-γ in vitro cultured splenocytes. Therefore, the p(Aβ3-10)10- C3d-p28.3 vaccine biased cellular immunity towards an anti-inflammatory Th2-type response.

**Figure 2** Proliferation rate and IL-4, IFN-γ levels of splenocytes isolated from immunized mice. (A) Spleen T cell exhibits a higher proliferation rate after stimulation with their corresponding immunogen. *p < 0.05 compared with pcDNA3.1(+) group; #p > 0.05 compared with Aβ42 peptide group. (B) IL-4 level of splenocytes isolated from immunized mice with their corresponding immunogen. *p < 0.01 compared with pcDNA3.1(+) group; #p > 0.05 compared with Aβ42 peptide group. (C) IFN-γ level of splenocytes isolated from immunized mice with their corresponding immunogen. *p < 0.01 compared with p(Aβ3-10)10-C3d-p28.3 and pcDNA3.1(+) group; #p > 0.05 compared with pcDNA3.1(+) group.

**Induction of anti-Aβ antibodies binding Aβ plaques in an APP/PS1 transgenic mouse brain**

Brain sections from a 12-month-old APP/PS1 transgenic mouse were used for detecting the binding capability of anti-Aβ antibodies to Aβ plaques. As shown in Fig.3A, sera of mice immunized with p(Aβ3-10)10- C3d-p28.3 bound to Aβ plaques in APP/PS1 mouse right hemisphere sections. As a positive control, 6E10 anti-Aβ monoclonal antibodies were used (Fig.3B). These two groups were able to bind to Aβ plaques in mouse AD brain sections. This demonstrated that the anti-sera of mice immunized with p(Aβ3-10)10- C3d-p28.3 was immunoreactive to Aβ deposits in the brain and therapeutically functional in a similar manner to the 6E10 anti-Aβ monoclonal antibodies. However, no plaques were detected by sera from pre-immune sera and pcDNA3.1(+) immunized mice. This demonstrated that the pre-immune sera from these mice did not bind to the Aβ plaques (Fig.3C). And the sera of mice immunized with
pcDNA3.1(+) also did not bind to the Aβ plaques (Fig.3D). Immunized with pcDNA3.1(+) did not generate anti-Aβ antibodies that could bind to Aβ plaques in mouse AD brain sections.

![Image](67x486 to 165x582)

Figure 3 Sera from C57BL/6J mice react with Aβ plaques in brain of twelve-month-old APP/PS1 transgenic mouse. (A) serum from C57BL/6J mice immunized with p(Aβ3-10)10-C3d.p28.3. (B) serum from C57BL/6J mice immunized with 6E10 anti-Aβ monoclonal antibodies (positive control). (C) pre-immune serum (negative control). (D) pcDNA3.1(+) group serum (negative control). (A) and (B) bound to Aβ plaques in brain sections. (C) and (D) did not bind to Aβ plaques in brain sections.

**DISCUSSION**

Development a safe and effective Aβ immunotherapy for Alzheimer’s disease requires therapeutic levels of anti-Aβ antibodies generation as well as minimizing the Th1 immune response. In this study, we successfully constructed a DNA vaccine encoding ten tandem repeats of Aβ3-10 fused with three copies of mouse C3d-p28 to elicit anti-Aβ antibodies and enhance Th2 type of immune responses. We have chosen Aβ3-10, such an Aβ immunogen avoided the Th2-related T-cell epitopes present in full length Aβ1-42, reducing the possibility of encephalitis as a complication. To overcome the low immunogenicity of the Aβ3-10 single peptide, we constructed a cDNA encoding ten tandem repeats of Aβ3-10. Meanwhile, we used three copies of C3d-p28 as a molecular adjuvant to modulate the DNA vaccine elicited immune response and to enhance its intensity.

C3d- p28 is an attractive adjuvant for eliciting enhanced B cell responses to a vaccine antigen. C3d, a terminal degradation fragment of the complement C3 component, binding to the complement receptor 2 (CR2) or CD21, links innate and adaptive immunity [39]. Simultaneous conjugation of BCR and CD21/CD19 by antigen-C3d complex significantly reduced B lymphocytes activation threshold, which induces much stronger B cell activation than antigen alone [40]. Movsesyan [41] showed that immunization of mice with 3Aβ 1-11-PADRE epitope vaccine alone generated only moderate levels of anti-Aβ antibodies and a pro-inflammatory T helper (Th1 phenotype) cellular immune response. However, the addition of 3C3d to the vaccine construct significantly augmented the anti-Aβ humoral immune response and, importantly, shifted the cellular immune response towards the potentially safer anti-inflammatory Th2 phenotype. One early study showed that the CR2 binding site on C3d mapped to residues 1205-1214 (C3 sequence) [42], and that this minimum binding domain was contained within a 28-amino acid peptide (p28). The p28 not only has similar adjuvant properties as the entire C3d molecule [43], but also is only ~9% the size of the entire C3d molecule [44]. Researches has shown that the efficacy of DNA vaccines encoding antigens from different pathogens (West Nile virus, HBV, porcine reproductive and respiratory syndrome virus, circumsporozoite protein of Plasmodium berghei) dramatically increased after fusion of these immunogens with C3d-p28 molecular adjuvant [44-46, 28].

Results from plasmid restriction enzyme digestion and sequencing analysis demonstrated that ten repeats Aβ3-10 and three copies of pC3d-p28 were successfully combined into the pcDNA3.1(+) virus. Enzyme-linked immunosorbent assay results showed that all of the mice immunized with Aβ42 peptide and p(Aβ3-10)10-C3d-p28.3 developed anti-Aβ antibodies and serum anti-Aβ antibody levels steady increased after two immunizations. However, the mice with pcDNA3.1(+) could not detect any anti-Aβ antibodies. From the second immunization to the final immunization, the p(Aβ3-10)10-C3d-p28.3 vaccine produced lower titers of anti-Aβ antibodies than the Aβ42 peptide group, but showed no significant difference, suggesting that the Aβ3-10 repeat fragment plasmid exhibited similar immunogenicity with the Aβ42 peptide. However, the p(Aβ3-10)10-C3d-p28.3 vaccine produced higher titers of anti-Aβ antibodies than observed in the pcDNA3.1 group, which is similar to what has been observed for other gene vaccines [38, 47]. Though the immune response induced by the DNA vaccine is gradual and of low intensity, our Aβ3-10 vaccine still elicited moderate titers of anti-Aβ antibodies. Be-
cause DNA-based vaccines can induce prolonged, endogenous antigen synthesis as they are processed within the host’s own immunized cells. Another important factor of choosing DNA-based vaccines is related to technology. When DNA vaccines are in clinical use, large amounts of vaccines are necessary for treatment of a large number of patients who would be treated for a long period. DNA vaccines have an advantage because they can be mass-produced with a high purity at a low price. The reasons of moderate titers of anti-Aβ antibodies were related to the use of C3d-p28 molecular adjuvant and bupivacaine pretreatment 1 day prior to immunization could enhance antigen immune response.

Antibody isotypes have been used as an indirect measure of the contribution of Th1 type cytokines and Th2 type cytokines to the immune response [38], as the production of IgG1 antibodies is primarily induced by Th2 cytokines and the production of IgG2 antibodies is promoted by Th1 cytokines. Based on this we investigated the Th phenotype induced by p(Aβ3-10)-C3d-p28.3 vaccine and Aβ42 peptide vaccine. After the final immunization, immunization with p(Aβ3-10)-C3d-p28.3 vaccine predominantly elicited the IgG1 isotype, low IgG2a and IgG2b isotype. Aβ42 peptide immunized mice show high levels of IgG1 isotype, IgG2a and IgG2b isotype. There is no difference in the IgG1 isotype between p(Aβ3-10)-C3d-p28.3 immunized mice and Aβ42 peptide immunized mice. However, the level of IgG2a and IgG2b antibodies induced by p(Aβ3-10)-C3d-p28.3 vaccine significantly lower than Aβ42 peptide group. Bard and colleges [17] showed that antibodies against the N-terminus of Aβ (3D6 against Aβ1-5 or 10D5 against Aβ3-7) were the most effective at reducing brain amyloid. It was also reported that IgG1 cleared cerebral Aβ more efficiently than IgG2a and IgG2b [48]. Moreover, it is also believed that Th1 type antibodies (IgG2a and IgG2b) contributed to the inflammation in the clinical trial [11]. IgG2b, an Fc-receptor low affinity binder, was less effective in the protection from AD [17]. Similar preferential IgG2b production was also observed when Aβ peptide was used with monophosphoryl lipidA adjuvant [49].

In order to distinguish the Th1 type and Th2 type immune response, the ratio of the optical density values of IgG1 to IgG2a was calculated and the mean value was compared between the Aβ vaccines after the final immunization. The ratio of IgG1 to IgG2a elicited by p(Aβ3-10)-C3d-p28.3 vaccine was (9.46 ±1.26) while Aβ42 peptide immunized mice had an IgG1/IgG2a ratio of (1.26 ±0.04). Evidence indicated that the ratio of IgG1 to IgG2a elicited by p(Aβ3-10)-C3d-p28.3 vaccine was significantly higher than with the Aβ42 peptide, which is consistent with results published from other groups [38, 50]. This means that the p(Aβ3-10)-C3d-p28.3 group induced a more robust Th2 type immune response than the Aβ42 peptide group. The antibody isotype generated by p(Aβ3-10)-C3d-p28.3 vaccine was mainly IgG1 and high ratio of IgG1/IgG2a, biased to Th2 type, which is more efficient at clearing cerebral amyloid plaques [48] and avoids excessive neuroinflammation. Therefore, for effective Alzheimer’s disease immunotherapy, achieving a predominantly Th2 type response may be more important than increasing antibody titers alone. Wang and colleges [51] found that immunization with Aβ antibodies to APP mice carrying the APP mutation promoted primarily the clearance of diffused forms of Aβ plaques while condensed plaque cores remained intact. Although high titers of anti- Aβ antibodies were correlated with a reduction of Aβ plaque pathology[26, 36, 52, 53], some cases demonstrated no profound plaque clearance regardless of Aβ antibody production[54], suggesting that either Aβ antibody titers were not sufficient to clear the plaques or that plaques were resistant to antibody-mediated clearance in some of the patients. We also cannot exclude the possibility that in highly Aβ-immunogenic patients clearance of antibody-resistant plaques was facilitated by infiltrating T cells and macrophages [54, 55].

Spleen T cell proliferation assay was used to detect whether immunization with p(Aβ3-10)-C3d-p28.3 induced immune response specific for Aβ3-10 or full-length Aβ42. Spleen T cell restimulated with ConA exhibited the highest levels of proliferation in all groups. In the pcDNA3.1(+) group, spleen T cell restimulated with ConA exhibited much higher levels of proliferation than those stimulated with Aβ42 and Aβ3-10. In the p(Aβ3-10)-C3d-p28.3 and Aβ42 peptide group, spleen T cell exhibits a higher proliferation rate after stimulation with their corresponding immunogen than pcDNA3.1(+) group. And there was no significant difference between the two groups. However, a lower proliferation rate was produced when restimulated with non-corresponding antigens. A robust T cell proliferation was observed in splenocytes isolated from the p(Aβ3-10)-C3d-p28.3 group after stimulation with Aβ3-10, but not with Aβ42. In contrast, because Aβ42 itself possesses both B and T cell epitopes, immunizing with Aβ42 peptide induced Aβ specific T cell proliferative response after stimulation with Aβ42. This indicated that p(Aβ3-10)-C3d-p28.3 immunization induced a specific cellular immunity targeted to Aβ3-10 other than full-
length Aβ42. It demonstrated that this could avoid the occurrence of auto-immune meningoencephalitis, which is consistent with result published from the other group [56].

ELISA was used to detect levels of IL-4 and IFN-γ cytokines in splenocyte culture supernatants to determine the phenotype of the Th immune response. The production of IgG1 antibodies is primarily induced by IL-4, which is a Th2 type cytokine, whereas production of IgG2 antibodies indicates the involvement of Th1 type cytokines such as IFN-γ [38]. Consistent with the splenic T cell proliferation assay, high levels of IL-4 and IFN-γ detected by ELISA assay were only observed in splenocytes that were restimulated with the corresponding immunogens, which is consistent with result published from the other group [50]. Splenocytes isolated from mice immunized with either p(Aβ3-10)10- C3d-p28.3 or Aβ42 peptide generated significantly higher levels of IL-4 than those from pcDNA3.1(+) immunized mice. And there were no differences in the level of IL-4 between p(Aβ3-10)-C3d-p28.3 immunized mice and Aβ42 peptide immunized mice. However, higher levels of IFN-γ were detected from the Aβ42 peptide group as compared with p(Aβ3-10)-C3d-p28.3 and the pcDNA3.1(+) group. Importantly, IFN-γ levels were not significantly different between the p(Aβ3-10)-C3d-p28.3 group and the pcDNA3.1(+) group. These results indicate that Aβ42 peptide immunized mice show a mixed Th1/Th2 immune response, while mice immunized with p(Aβ3-10)-C3d-p28.3 develop a Th2-biased immune response that is less likely to induce detrimental inflammation. Cytokine analysis suggested that p(Aβ3-10)-C3d-p28.3 immunization induced a considerable degree of Th2 immunity to the Aβ42 peptide. Previous study also showed that Aβ42 DNA trimer immunization has a high probability to be effective and safe to treat patients with early AD as it diminishes Th1 and Th17 cell proliferation [57]. The Th1 immune response reported in the AN1792 trial may have been caused by the presence of T and B cell self-epitopes of Aβ42 peptide. Thus, choosing Aβ epitope-specific vaccines could avoid a deleterious Aβ-specific T cell response.

An Aβ plaque load in the brain is the most definitive marker of Alzheimer’s disease diagnosis, and its clearance has been used to evaluate the efficacies of therapeutic means in Alzheimer’s disease mouse models. Brain sections from a twelve-month-old APP/PS1 transgenic mouse were used for detecting the binding capability of anti-Aβ antibodies to Aβ plaques. The anti-Aβ antibodies generated by the (Aβ3-10)10- C3d-p28.3 vaccine were able to bind to Aβ plaques in brain tissue from a twelve-month-old APP/PS1 transgenic mouse. Binding of antibodies to the region of Aβ42 peptide coincides with the ability of antibodies to bind native plaques in brain tissue. This demonstrated that the anti-sera of mice immunized with p(Aβ3-10)-C3d-p28.3 was immunoreactive to Aβ deposits in the brain and therapeutically functional in a similar manner to the 6E10 anti-Aβ monoclonal antibodies. And the anti-Aβ antibodies elicited from the (Aβ3-10)10- C3d-p28.3 were effective and had strong affinity for Aβ plaques. Both the pre-immune sera from the (Aβ3-10)10- C3d-p28.3 immunized mice and the sera of mice immunized with pcDNA3.1(+) did not bind to the Aβ plaques. This demonstrated that immunized with pcDNA3.1(+) did not generate anti-Aβ antibodies that could bind to Aβ plaques in mouse AD brain sections.

There are three factors to think over for selecting better vaccination path: transfection efficiency of gene vaccine to organism cell; expression level of antigen gene; presentation capability of antigen to immunity system. Currently, there are several vaccination paths for Alzheimer’s disease gene vaccine such as intramuscularly immunization, gene gun utilizes, oral immunity, hypodermic injection, intraperitoneal injection, intranasal administration, vivo electroporation and so on. Intramuscularly immunization was still most commonly method in the gene vaccination at recently. Both skeletal muscle and cardiac muscle could high efficiency intake and express exogenous gene. Moreover skeletal muscle had many advantages such as large of immunization capacity, convenient injection and security and so on. Yoriko Tokita [58] demonstrated that newly developed nonviral amyloid-β (Aβ) DNA vaccines are safe and effective in reducing Aβ burdens in the brain of rhesus monkeys. Six months after the first vaccination, it was demonstrated that anti Aβ antibodies in plasma of vaccinated monkeys were significantly elevated than that of control monkeys. Immunohistochemical examinations revealed that DNA vaccination reduced the Aβ burden to approximately 50% of that found in control monkeys. There was neither inflammation nor microhemorrhage in the brain and no significant changes in cytokine and chemokine levels in the blood throughout the observation period. However, another strategy to increase the generation of Aβ antibodies and the safety of active immunization is to optimize administration routes of the vaccine delivery. We found that intranasal administration of Aβ 3-10 vaccine [59] led to a predominantly Th2-biased immune response and a lowering of cerebral Aβ in the absence of any adverse effects. Transcutaneous im-
munication such as vivo electroporation and gene gun utilizes [60] antigen presentation by Langerhans cells in the skin. Town and colleagues showed that transcutaneous immunization with Aβ1-42 with cholera toxin adjuvant resulted in robust anti-Aβ antibody titers, reduced cerebral Aβ levels, and increased Aβ in blood, while avoiding T cell infiltration into brain and cerebral microhemorrhage[61]. An oral DNA vaccine consisting of an adenovirus-associated viral vector carrying Aβ cDNA (AAV/Aβ) without adjuvant induced the expression and secretion of Aβ1-43 or Aβ1-21 in Tg2576 mice, leading to the generation of long-lasting anti-Aβ antibodies [62].

In summary, this study shows that a novel DNA vaccine, p(Aβ3-10)10- C3d-p28.3. This vaccine not only boosts anti-Aβ antibody generation but also induces a Th2 immune response. The anti-Aβ antibodies generated by the (Aβ3-10)10- C3d-p28.3 vaccine were able to bind to Aβ plaques in brain tissue from a twelve-month-old APP/PS1 transgenic mouse. The p(Aβ3-10)10- C3d-p28.3 vaccine may be a safe and effective candidate vaccine for Aβ immunotherapy in Alzheimer’s disease. As the average lifespan increases worldwide, the number of Alzheimer’s disease patients who suffer from this devastating neurodegenerative disease grows as well. Although there is much work to be done, we remain hopeful that Aβ immunotherapy, either alone or in combination with other therapies, will succeed in preventing or treating Alzheimer’s disease. We are currently testing the therapeutic potency of the p(Aβ3-10)10- C3d-p28.3 vaccine construct in the APP/PS1 mouse model of Alzheimer’s disease.

**MATERIALS AND METHODS**

**Design**
A randomized, controlled animal experiment.

**Time and setting**
This experiment was performed at the Central Laboratory, the First Affiliated Hospital of China Medical University, China between December 2011 and August 2012.

**Materials**

**Animals**
Eight to ten week-old female C57BL/6J mice and twelve-month-old transgenic mouse (APPSwe/ PSEN1dE9) were bred in the Center of Experimental Animals, China Medical University, China (license No.SYXK (Liao) 2008-0013). Experimental protocols were in accordance with the Guidance Suggestions for the Care and Use of Laboratory Animals, formulated by the Ministry of Science and Technology of China [37].

**Methods**

**Construction of plasmids**
Ten tandem repeats of cDNA encoding human Aβ3-10 gene and three tandem repeats of cDNA encoding mouse C3d-p28 gene were chemically synthesized and the DNA fragments were cloned into the eukaryotic expression vector pcDNA3.1(+) using the restriction sites Hind III and EcoRI (FERMENTAS (MBI), Ottawa, Canada). The fusion expression protein contains (Aβ3-10)10 peptide and C3d-p28.3 oligonucleotide and there were seven amino acids, GlyGlyGlyGlySerGlySer (BamHI) (FERMENTAS (MBI), Ottawa, Canada), as the linker between them. This work was conducted at Yingrun Biotechnology Co. Ltd. Changsha, Hunan, China). The recombinant plasmid was amplified in DH5α E.coli competent cells. The plasmids were purified and extracted in large quantities using plasmid preparation kits (E.Z.N.A TM Fast filter Endo-Free Plasmid Maxi Kit, OMEGA, Norcross, GA, USA). Plasmids were verified by agarose gel electrophoresis (KODAK, USA) and nucleotide sequence analysis (GenScript Inc. Nanjing, Jiangsu, China).

HEK293 cells (Strategene, Santa Clara, CA, USA) were transiently transfected with 2μg plasmid DNA by Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s recommendation and cells transfected with pcDNA3.1(+) vector were used as a negative control. Expression of these plasmids was analyzed in the supernatants of cells by Western blotting using monoclonal anti-Aβ antibodies 6E10 (Signet, California, MA, USA).

**Immunization**
21 eight to ten week-old female C57BL/6J mice were randomly assigned to three groups (seven mice per group), p(Aβ3-10)10- C3d-p28.3 group, pcDNA3.1(+) group, Aβ42 peptide group. And all mice were immunized intramuscularly (i.m.) in the left hind legs for a total of five immunizations at three-week intervals. The p(Aβ3-10)10- C3d-p28.3 and pcDNA3.1(+) groups were injected in the quadriceps femoris muscles with 100μl of plasmids (100μg in 100μl PBS) at each immunization and the mice were anesthetized by intraperitoneal injection of 10% chloral hydrate (0.03 mg/kg) before plasmid injection. 24h prior to plasmid injection, 50μl (250μg) bupivacaine was injected into the left hind legs to induce mild muscle degeneration. The Aβ42 peptide group was immunized with Aβ 42 peptide (50μg per injection)
Technology & Services, Shanghai, China) or ConA (10μg/ml) (Shanghai Sangon Biological Engineering Technology & Services, Shanghai, China) or ConA (10μg/ml), Aβ3–10 peptide (10μg/ml) (Shanghai Sangon Biological Engineering Technology & Services, Shanghai, China) or ConA (2μg/ml) (Sigma, St. Louis, MA, USA), respectively. At 48 h and 72 h, supernatants were collected and frozen at −20°C until analyzed for cytokine production by ELISA. Then, a MTT assay was used to detect the proliferation rate of the cells. The OD at 490nm was read using a micro plate reader (Elx800, BioTek Instruments, Winoski, Vermont, USA). The proliferation rate of cells was calculated as followings: (average OD of stimulating wells – average OD of blank wells)/ (average OD of control wells – average OD of blank wells). Mouse IFN-γ and IL-4 cytokines were detected in the culture media using a mouse cytokine ELISA kit (R&D Systems China Co.Ltd., Shanghai, China) according to the manufacturer’s instructions.

**Detection of Aβ plaques in an APP/PS1 transgenic mouse brain**

Immunohistochemistry was used to detect the binding capability of antisera to Aβ plaques in an APP/PS1 transgenic mouse brain. A 12-month-old-transgenic mouse (APPswe/PSEN1dE9) was sacrificed by cervical after intraperitoneal anesthesia and the brain was removed. Right hemisphere was paraffin-embedded and cut into 4-μm thick coronal sections. Sera from vaccinated mice were added to 4μm-thick brain sections of formalin-fixed cortical tissue. The titer of mice antisera were 1:500 dilution. We used the same dilutions of the preimmune sera and pcDNA3.1(+) group sera for the negative controls, and 6E10 monoclonal antibody (monoclonal anti-Aβ antibody, Covance, Emeryville, California, USA) for the positive control, respectively. The specimens were then incubated at 4 °C overnight, followed by three washes with PBS. The corresponding biotin labeled secondary antibodies (rabbit anti-mouse IgG, 1:500, Sigma, St. Louis, MA, USA) were added and incubated at 37 °C for 30 min. After washing, horseradish peroxidase-labeled streptomyacin avidin working solution was added and incubated at 37°C for 30 min. Binding of antibodies to the brain sections was visualized using the SABC Mouse IgG /DAB Substrate Biotin-Avidin System (both kits from Boster Biological Technology, LTD, Wuhan, Hubei, China) according to manufacturer recommendations. A digital camera (Olympus, Osaka, Japan) was used to detect the plaques at 20×image magnification.

**Statistical analysis**

Data are expressed as the mean ± standard deviation (SD). The differences were analyzed by one-way ANOVA, followed by the Student–Newman–Keuls multiple range test. SPSS17.0 (SPSS, Chicago, IL, USA) was used to perform all analyses and p < 0.05 was consid-
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Author contributions: Wanshu Guo had full access to the experimental design, experimental implementation, statistical analysis and wrote the manuscript. Sha Sha and Xiaona Xing assisted in accomplishing the experiments Tongzi Jiang was responsible for statistical analysis. Yunpeng Cao directed the research and supervised the manuscript writing. All authors read and approved the final version of the manuscript.

Conflict of interest: None of the authors have any conflicts of interest to declare.

Ethical approval: This study was approved by the Animal Research Committee, China Medical University, China.

Author statements: The manuscript is original, has not been previously published in any language or any form, including electronic, and contains no disclosure of confidential information.

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