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# A new DNA vaccine fused with the C3d-p28 induces a Th2 immune response against amyloid-beta

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## Research Highlights

In this study, we successfully constructed a new plasmid DNA vaccine, p(A $\beta_{3-10}$ )10-C3d-p28.3, which not only boosted therapeutic levels of anti-amyloid-beta (A $\beta$ ) antibodies but also minimized the Th1 immune response. More importantly, the antibody bound to A $\beta$  plaques in brain tissue from a 12-month-old APP/PS1 transgenic mouse, demonstrating that p(A $\beta_{3-10}$ )10-C3d-p28.3 elicited anti-A $\beta$  antibodies are effective and have a strong affinity for A $\beta$  plaques.

## Abstract

To enhance anti-amyloid-beta (A $\beta$ ) antibody generation and induce a Th2 immune response, we constructed a new DNA vaccine p(A $\beta_{3-10}$ )10-C3d-p28.3 encoding ten repeats of A $\beta_{3-10}$  and three copies of C3d-p28 as a molecular adjuvant. In this study, we administered this adjuvant cyclically to female C57BL/6J mice at 8–10 weeks of age. Enzyme linked immunosorbent assay was used to detect the titer of serum anti-A $\beta$  antibody, isotypes, and cytokines in splenic T cells. A 3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay was used to detect the proliferation rate of splenic T cells. Brain sections from a 12-month-old APP/PS1 transgenic mouse were used for detecting the binding capacities of anti-A $\beta$  antibodies to A $\beta$  plaques. The p(A $\beta_{3-10}$ )10-C3d-p28.3 vaccine induced high titers of anti-amyloid- $\beta$  antibodies, which bound to A $\beta$  plaques in APP/PS1 transgenic mouse brain tissue, demonstrating that the vaccine is effective against plaques in a mouse model of Alzheimer's disease. Moreover, the vaccine elicited a predominantly IgG1 humoral response and low levels of interferon- $\gamma$  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(A $\beta_{3-10}$ )10-C3d-p28.3 vaccine is a promising immunotherapeutic option for A $\beta$  vaccination in Alzheimer's disease.

## Key Words

neural regeneration; Alzheimer's disease; amyloid- $\beta$ ; C57BL/6J mice; DNA vaccine; active immunotherapy; passive immunotherapy; C3d-p28 molecular adjuvant; Th2 immune response; grants-supported paper; neuroregeneration

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

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## INTRODUCTION

Alzheimer's disease is a neurodegenerative disease characterized pathologically by the presence of extracellular amyloid-beta ( $A\beta$ )-containing senile plaques and intracellular Tau protein neurofibrillary tangles<sup>[1]</sup>. The "amyloid cascade hypothesis" emphasizes that excessive production and insufficient clearance of  $A\beta$  protein precedes Alzheimer's disease-related dementia and neuronal loss<sup>[2]</sup>. Evidence supports a central role of aggregated forms of  $A\beta$  peptide in Alzheimer's disease<sup>[3-4]</sup>. Therefore, both augmenting clearance of  $A\beta$  and preventing accumulation of  $A\beta$  by  $A\beta$  active and passive immunotherapy are promising strategies for the treatment of Alzheimer's disease.

In 1999, Schenk *et al*<sup>[5]</sup> were the first to report that  $A\beta_{1-42}$  active immunization in PDAPP transgenic mice could increase the rate of clearance and prevent the aggregation of  $A\beta$ . Two additional reports demonstrated that  $A\beta$  vaccination in Tg CRND8<sup>[6]</sup> or APP/PS1tg<sup>[7]</sup> mice not only reduced levels of cerebral amyloid but also improved behavioral performance in learning and memory tasks. Recently, immunization targeting the  $A\beta$  peptide has been effective in halting progression of amyloid pathology in transgenic mice<sup>[8-9]</sup>. The method of the immunization of  $A\beta_{1-42}$  peptide (AN1792) in combination with the adjuvant QS-21 was translated to clinical trials by ELAN/Wyeth in 2000 and 2001. However, the trial was stopped because of the occurrence of meningoencephalitis in 18 of the 300 immunized Alzheimer's disease patients<sup>[10-11]</sup>. 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<sup>[12]</sup>, a Th1 type adjuvant and the presence of T and B cell self-epitopes of  $A\beta_{1-42}$  peptide. Holmes *et al*<sup>[13]</sup> found that  $A\beta_{1-42}$  peptide immunization did not improve survival or an improvement in the time to severe dementia, possibly because the immunization was administered too late in the course of the disease. Thus, early preventive immu-

nization prior to neuropathology and cognitive deficits may be essential for the effective treatment of Alzheimer's disease using a vaccine-based strategy.

Based on this rationale, in this study, we investigated the therapeutic potential of active  $A\beta$  immunotherapy. Current alternative immunotherapeutic strategies for boosting therapeutic levels of anti- $A\beta$  antibodies and minimizing the Th1 immune response after active  $A\beta$  vaccination have focused on  $A\beta$  DNA vaccine production, B cell  $A\beta$  epitope generation, adjuvant modifications, and alternative routes. DNA vaccination is a novel, safe, and effective vaccine that has the benefits of ease of production, the capacity 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<sup>[9]</sup>. Some studies have indicated that plasmid DNA encoding  $A\beta$  could generate specific antibodies against  $A\beta$  and elicit B cell immune responses without a significant T cell-mediated immune response in mice<sup>[9, 14-15]</sup>, rhesus monkeys<sup>[16]</sup> and rabbits<sup>[17]</sup>. The Th1 response reported in the AN1792 trial may relate to the presence of T and B cell self-epitopes of the  $A\beta_{42}$  peptide. The B cell epitopes are located in the N terminal of the peptide (11–15 amino acids)<sup>[18-19]</sup>, and are thought to be important for production of anti- $A\beta$  antibodies while avoiding a deleterious  $A\beta$ -specific T cell response. The T cell epitopes are derived from the C terminal of the peptide (16–42 amino acids), and are thought to activate the T cell response that triggers the meningoencephalitis<sup>[20]</sup>. It was reported that the  $A\beta_{4-10}$  fragment was the dominant peptide that anti- $A\beta_{42}$  IgGs specifically recognized with high affinity<sup>[21]</sup>. Moreover, the  $A\beta_{3-6}$  fragment (EFRH) affects the solubility and disaggregation of  $A\beta$  fibrils<sup>[22]</sup>.  $A\beta_3$  is also highly prone to oligomerization and is an important component of the  $A\beta$  deposited in senile plaques of the Alzheimer's disease brain<sup>[23]</sup>. To overcome its low immunogenicity of the short  $A\beta$  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<sup>[14-15, 17, 24]</sup>.

In this study, we constructed a new plasmid DNA vaccine, p(A $\beta_{3-10}$ )10-C3d-p28.3. We chose non-viral plasmid [pcDNA3.1(+)] as the vector. Here, we have chosen A $\beta_{3-10}$ , which is the minimal effective fragment, as the antigen of A $\beta_{1-15}$  has already been demonstrated to be a B cell epitope. To overcome the low immunogenicity of the A $\beta_{3-10}$  single peptide, we constructed a cDNA encoding ten tandem repeats of A $\beta_{3-10}$ . In addition, three copies of mouse C3d-p28 were added as an adjuvant to (A $\beta_{3-10}$ )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-A $\beta$  antibodies and induce a Th2 immune response. We hope that the p(A $\beta_{3-10}$ )10-C3d-p28.3 vaccine is a promising immunotherapeutic option for A $\beta$  vaccination in Alzheimer's disease.

## RESULTS

### Quantitative analysis of experimental animals

A total of 21 8–10-week-old female C57BL/6J mice were equally and randomly assigned to p(A $\beta_{3-10}$ )10-C3d-p28.3, pcDNA3.1(+), and A $\beta_{42}$  peptide groups. Mice were immunized intramuscularly (i.m.) in the left hind legs with p(A $\beta_{3-10}$ )10-C3d-p28.3, pcDNA3.1(+) or A $\beta_{42}$  peptide. All 21 mice were included in the final analysis.

### Construction of A $\beta$ plasmid DNA vaccine

Ten tandem repeats of cDNA encoding human A $\beta_{3-10}$  gene and three copies of cDNA encoding the mouse C3d-p28 gene were chemically synthesized and the DNA fragments were cloned into the eukaryotic expression vector pcDNA3.1(+). *Hind*III, *Bam*HI, *Eco*RI restriction sites were added to form the following DNA fragments: 5'—*Hind*III—kozak sequence—ATG (initiation codon)—10 × A $\beta_{3-10}$ —GGGS linker—*Bam*HI—3×C3d-p28—*Eco*RI—3'. The recombinant plasmid was amplified in DH5 $\alpha$  E.coli competent cells. The plasmids were purified and extracted in large quantities using plasmid preparation kits. 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 p(A $\beta_{3-10}$ )10-C3d-p28.3 plasmids was also confirmed in transiently transfected HEK293 cells by western blot assay.

### Generation of high titers of anti-A $\beta$ antibodies with a predominantly IgG1 isotype

All mice immunized with A $\beta_{42}$  peptide and p(A $\beta_{3-10}$ )10-C3d-p28.3 developed anti-A $\beta$  antibodies. Sera from A $\beta_{42}$  peptide and p(A $\beta_{3-10}$ )10-C3d-p28.3 vaccinated mice showed a steady increase in anti-A $\beta$  antibody after two immunizations. No anti-A $\beta$  antibodies were detected in the pcDNA3.1 (+) group (Figure 1A). The p(A $\beta_{3-10}$ )10-C3d-p28.3, similar to other gene vaccine, induced less anti-A $\beta$  antibodies than A $\beta_{42}$  peptide<sup>[25]</sup> ( $P > 0.05$ ).

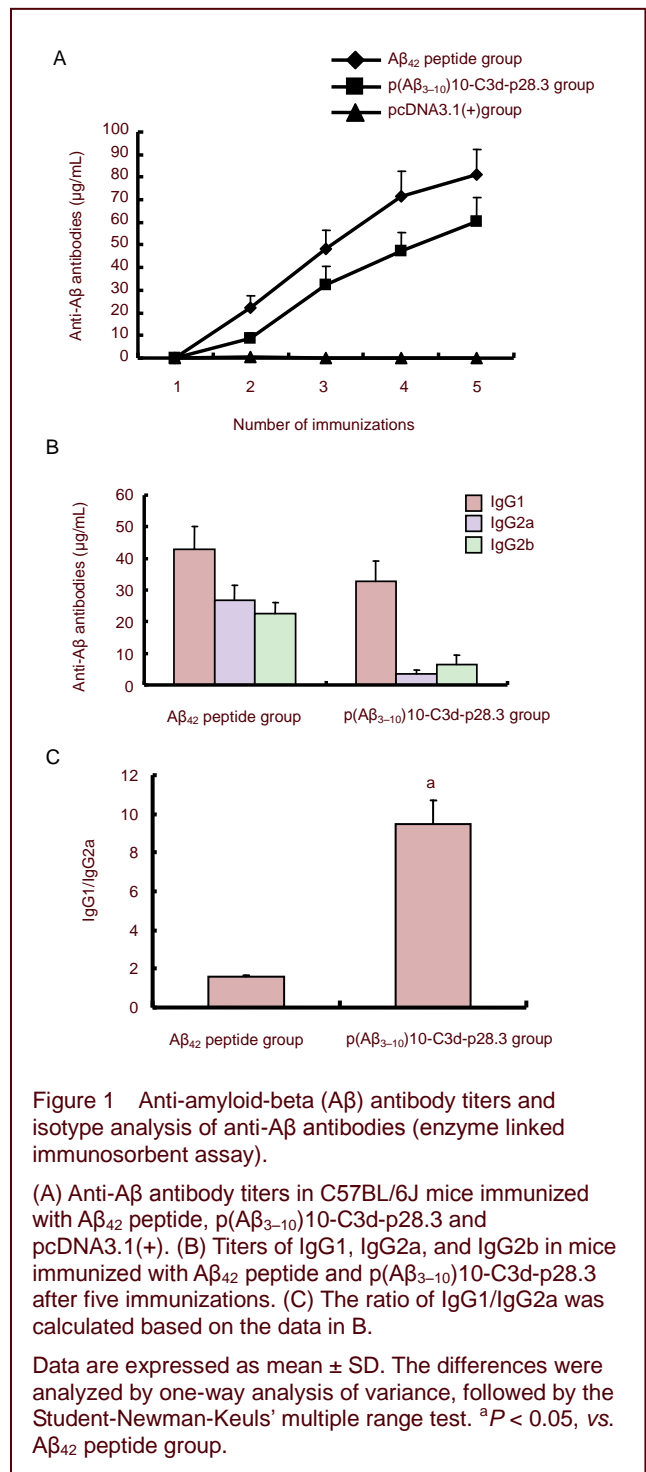


Figure 1 Anti-amyloid-beta (A $\beta$ ) antibody titers and isotype analysis of anti-A $\beta$  antibodies (enzyme linked immunosorbent assay).

(A) Anti-A $\beta$  antibody titers in C57BL/6J mice immunized with A $\beta_{42}$  peptide, p(A $\beta_{3-10}$ )10-C3d-p28.3 and pcDNA3.1(+). (B) Titers of IgG1, IgG2a, and IgG2b in mice immunized with A $\beta_{42}$  peptide and p(A $\beta_{3-10}$ )10-C3d-p28.3 after five immunizations. (C) The ratio of IgG1/IgG2a was calculated based on the data in B.

Data are expressed as mean  $\pm$  SD. The differences were analyzed by one-way analysis of variance, followed by the Student-Newman-Keuls' multiple range test. <sup>a</sup> $P < 0.05$ , vs. A $\beta_{42}$  peptide group.

Sera collected after the final immunization were used to quantify isotypes of immunoglobulin by enzyme linked immunosorbent assay. Immunization with p(A $\beta_{3-10}$ )10-C3d-p28.3 and A $\beta_{42}$  peptide induced a broad spectrum of anti-A $\beta$  antibody isotypes (IgG1, IgG2a, and IgG2b) (Figure 1B). The ratio of IgG1 to IgG2a was significantly higher in mice immunized with p(A $\beta_{3-10}$ )10-C3d-p28.3 versus A $\beta_{42}$  ( $P < 0.05$ ; Figure 1C). Immunization with p(A $\beta_{3-10}$ )10-C3d-p28.3 induced predominantly an IgG1 isotype with the IgG1/IgG2a ratio much greater than with the A $\beta_{42}$  peptide.

### Enhancement of T cell proliferation and induction of Th2-biased responses

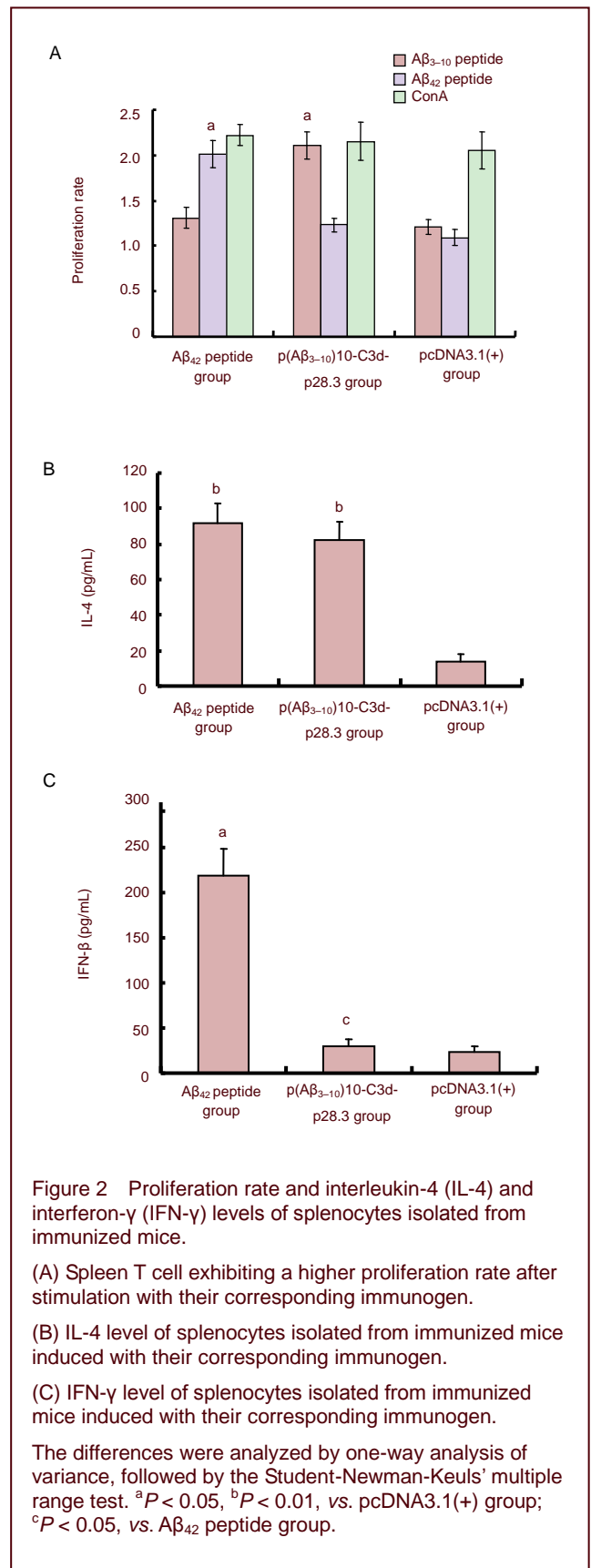
As shown in Figure 2A, spleen T-cells restimulated with concanavalin A exhibited the highest levels of proliferation in all groups. In the A $\beta_{42}$  peptide and p(A $\beta_{3-10}$ )10-C3d-p28.3 groups, a higher level of T cell proliferation was observed in splenocytes restimulated with their corresponding antigen, which was greater than restimulation with a different antigen and smaller than restimulation with concanavalin A, even though there was no significant difference between the two groups ( $P > 0.05$ ).

Enzyme linked immunosorbent assay was used to detect levels of interleukin-4 and interferon- $\gamma$  cytokines in splenocyte culture supernatants to determine the phenotype of the T-cell response. Higher levels of interleukin-4 were detected from the A $\beta_{42}$  peptide and p(A $\beta_{3-10}$ )10-C3d-p28.3 group as compared with the pcDNA3.1(+) group ( $P < 0.05$ ; Figure 2B), but there were no significant differences between two A $\beta$ -immunized groups ( $P > 0.05$ ). Splenocytes isolated from mice immunized with A $\beta_{42}$  peptide produced much higher interferon- $\gamma$  levels than those from p(A $\beta_{3-10}$ )10-C3d-p28.3 ( $P < 0.05$ ) and pcDNA3.1(+) ( $P < 0.05$ ; Figure 2C). However, approximately equal interferon- $\gamma$  levels were detected in the p(A $\beta_{3-10}$ )10-C3d-p28.3 and pcDNA3.1(+) groups ( $P > 0.05$ ).

### Induction of anti-A $\beta$ antibodies binding A $\beta$ plaques in the APP/PS1 transgenic mouse brain

Brain sections from a 12-month-old APP/PS1 transgenic mouse were used to detect the binding capacities of anti-A $\beta$  antibodies to A $\beta$  plaques. As shown in Figure 3A, sera of mice immunized with p(A $\beta_{3-10}$ )10-C3d-p28.3 bound to A $\beta$  plaques in right hemisphere sections from the APP/PS1 mouse. As a positive control, 6E10 anti-A $\beta$  monoclonal antibodies were used (Figure 3B). These two groups bound to A $\beta$  plaques in mouse right hemisphere brain sections. However, the sera before immunization and in pcDNA3.1(+) immunized mice did not bind to the

A $\beta$  plaques (Figure 3C, D). Immunization with pcDNA3.1(+) did not generate anti-A $\beta$  antibodies that could bind to A $\beta$  plaques in mouse right hemisphere brain sections.





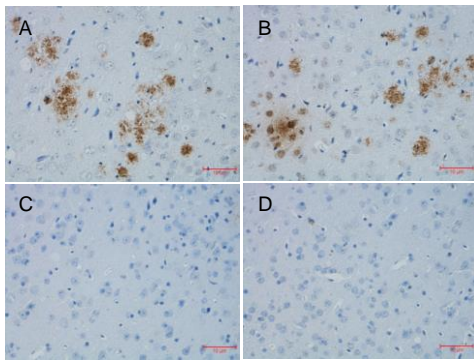


Figure 3 Induction of anti-amyloid-beta ( $A\beta$ ) antibodies binding  $A\beta$  plaques in the brain of a 12-month-old APP/PS1 transgenic mouse (immunohistochemistry).

(A) Serum from C57BL/6J mice immunized with  $p(A\beta_{3-10})10-C3d-p28.3$ . (B) Serum from C57BL/6J mice immunized with 6E10 anti- $A\beta$  monoclonal antibodies (positive control). (C) Pre-immune serum (negative control). (D) pcDNA3.1(+) group serum (negative control). (A) and (B) bound to  $A\beta$  plaques in brain sections. (C) and (D) did not bind to  $A\beta$  plaques in brain sections. Scale bars: 10 $\mu$ m.

## DISCUSSION

Development of a safe and effective  $A\beta$  immunotherapy for Alzheimer's disease requires the generation of therapeutic levels of anti- $A\beta$  antibodies as well as minimization of Th1 immune responses. We chose  $A\beta_{3-10}$  because  $A\beta$  immunogens minimize Th2-related T-cell epitopes present in full length  $A\beta_{1-42}$ , reducing the possibility of encephalitis as a complication. To overcome the low immunogenicity of the  $A\beta_{3-10}$  single peptide, we constructed cDNA encoding ten tandem repeats of  $A\beta_{3-10}$ . 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 or CD21, links innate and adaptive immunity<sup>[26]</sup>. Simultaneous conjugation of BCR and CD21/CD19 by antigen-C3d complex significantly reduces B lymphocytes activation thresholds, which induces much stronger B cell activation than antigens alone<sup>[27-28]</sup>. One early study showed that the complement receptor 2 binding site on C3d mapped to residues 1205–1214 (C3 sequence)<sup>[29]</sup>, 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<sup>[30]</sup>, but also is only

approximately 9% of the size of the entire C3d molecule<sup>[31]</sup>. Numerous studies have 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 increases after fusion of these immunogens with C3d-p28 molecular adjuvant<sup>[31-34]</sup>.

Enzyme-linked immunosorbent assay showed that all of the mice immunized with  $A\beta_{42}$  peptide and  $p(A\beta_{3-10})10-C3d-p28.3$  developed anti- $A\beta$  antibodies and serum anti- $A\beta$  antibody levels steadily increased after two immunizations. However, anti- $A\beta$  antibodies were not detected in the mice with pcDNA3.1(+). From the second immunization to the final immunization, the  $p(A\beta_{3-10})10-C3d-p28.3$  vaccine produced lower titers of anti- $A\beta$  antibodies than the  $A\beta_{42}$  peptide group, but showed no significant difference, suggesting that the  $A\beta_{3-10}$  repeat fragment plasmid exhibited similar immunogenicity with the  $A\beta_{42}$  peptide. However, the  $p(A\beta_{3-10})10-C3d-p28.3$  vaccine produced higher titers of anti- $A\beta$  antibodies than those observed in the pcDNA3.1 group, which is similar to what has been observed for other gene vaccines<sup>[25, 35]</sup>. Though the immune response induced by the DNA vaccine is gradual and of low intensity, our  $A\beta_{3-10}$  vaccine still elicited moderate titers of anti- $A\beta$  antibodies. DNA-based vaccines can induce prolonged, endogenous antigen synthesis because they are processed within the host's own immunized cells. Another important factor of choosing DNA-based vaccines is the available 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 the advantage that they can be mass-produced with a high purity and at a low price. We used moderate titers of anti- $A\beta$  antibodies so that the 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<sup>[25]</sup>, 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  $p(A\beta_{3-10})10-C3d-p28.3$  vaccine and  $A\beta_{42}$  peptide vaccine. After the final immunization, the  $p(A\beta_{3-10})10-C3d-p28.3$  vaccine predominantly elicited the IgG1 isotype, with low IgG2a and IgG2b isotypes.  $A\beta_{42}$  peptide immunized mice showed

high levels of the IgG1, IgG2a, and IgG2b isotypes. There was no difference in the levels of the IgG1 isotype between p(A $\beta_{3-10}$ )10-C3d-p28.3 immunized mice and A $\beta_{42}$  peptide immunized mice. However, the levels of IgG2a and IgG2b antibodies induced by p(A $\beta_{3-10}$ )10-C3d-p28.3 vaccine significantly was lower than in the A $\beta_{42}$  peptide group. Bard and colleagues<sup>[36]</sup> showed that antibodies against the N-terminus of A $\beta$  (3D6 against A $\beta_{1-5}$  or 10D5 against A $\beta_{3-7}$ ) were the most effective at reducing brain amyloid. It was also reported that IgG1 cleared cerebral A $\beta$  more efficiently than IgG2a and IgG2b<sup>[37]</sup>. Moreover, it has been suggested that Th1 type antibodies (IgG2a and IgG2b) contributed to inflammation in a clinical trial<sup>[11]</sup>. IgG2b, an Fc-receptor low affinity binder, was less protective against Alzheimer's disease<sup>[36]</sup>. Similar preferential IgG2b production was also observed when A $\beta$  peptide was used with monophosphoryl lipidA adjuvant<sup>[38]</sup>.

To distinguish the Th1 type and Th2 type immune responses, the ratio of the optical density values of IgG1 to IgG2a was calculated and the mean value was compared between the A $\beta$  vaccines after the final immunization. The ratio of IgG1 to IgG2a elicited by p(A $\beta_{3-10}$ )10-C3d-p28.3 vaccine was  $9.46 \pm 1.26$ , while A $\beta_{42}$  peptide immunized mice had an IgG1/IgG2a ratio of  $1.26 \pm 0.04$ . Evidence indicated that the ratio of IgG1 to IgG2a elicited by p(A $\beta_{3-10}$ )10-C3d-p28.3 vaccine was significantly higher than with the A $\beta_{42}$  peptide, which is consistent with results published from other groups<sup>[25, 39]</sup>. This means that the p(A $\beta_{3-10}$ )10-C3d-p28.3 group induced a more robust Th2 type immune response than the A $\beta_{42}$  peptide group. The antibody isotype generated by p(A $\beta_{3-10}$ )10-C3d-p28.3 vaccine was mainly IgG1, with a high ratio of IgG1/IgG2a, biased to Th2 type, which is more efficient at clearing cerebral amyloid plaques<sup>[37]</sup> 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. Zhang *et al*<sup>[40]</sup> found that immunization with A $\beta$  antibodies to APP mice carrying the APP mutation promoted primarily the clearance of diffused forms of A $\beta$  plaques whereas condensed plaque cores remained intact. Although high titers of anti-A $\beta$  antibodies were correlated with a reduction of A $\beta$  plaque pathology<sup>[14, 24, 41-42]</sup>, some cases demonstrated no profound plaque clearance regardless of A $\beta$  antibody production<sup>[43]</sup>, suggesting that either A $\beta$  antibody titers were not sufficient to clear the plaques or that plaques were resistant to antibody-mediated clearance in some patients. We also cannot exclude the possibility that in highly A $\beta$ -immunogenic patients clearance of antibody-resistant plaques was facili-

tated by infiltrating T cells and macrophages<sup>[43-44]</sup>.

Spleen T cell proliferation assay was used to detect whether immunization with p(A $\beta_{3-10}$ )10-C3d-p28.3 induced immune responses specific for A $\beta_{3-10}$  or full-length A $\beta_{42}$ . Spleen T cells restimulated with concanavalin A exhibited the highest levels of proliferation in all groups. In the pcDNA3.1(+) group, spleen T cell restimulated with concanavalin A exhibited much higher levels of proliferation than those stimulated with A $\beta_{42}$  and A $\beta_{3-10}$ . In the p(A $\beta_{3-10}$ )10-C3d-p28.3 and A $\beta_{42}$  peptide group, spleen T cell exhibits a higher proliferation rate after stimulation with their corresponding immunogen than in the pcDNA3.1(+) group, and there was no significant difference between the two groups. However, a lower proliferation rate was produced following restimulation with different antigens. Robust T cell proliferation was observed in splenocytes isolated from the p(A $\beta_{3-10}$ )10-C3d-p28.3 group after stimulation with A $\beta_{3-10}$ , but not with A $\beta_{42}$ . In contrast, because A $\beta_{42}$  itself possesses both B and T cell epitopes, immunization with A $\beta_{42}$  peptide induced A $\beta$  specific T cell proliferative responses after stimulation with A $\beta_{42}$ . This indicated that p(A $\beta_{3-10}$ )10-C3d-p28.3 immunization induced a specific cellular immunity targeted to A $\beta_{3-10}$ , other than full-length A $\beta_{42}$ . This could avoid the occurrence of auto-immune meningoencephalitis, which is consistent with results of a previously published study<sup>[45]</sup>.

Enzyme linked immunosorbent assay was used to detect levels of interleukin-4 and interferon- $\gamma$  cytokines in splenocyte culture supernatants to determine the phenotype of the Th immune response. The production of IgG1 antibodies is primarily induced by interleukin-4, which is a Th2 type cytokine, whereas production of IgG2 antibodies indicates the involvement of Th1 type cytokines such as interferon- $\gamma$ <sup>[25]</sup>. Consistent with the splenic T cell proliferation assay, high levels of interleukin-4 and interferon- $\gamma$  detected by enzyme linked immunosorbent assay were only observed in splenocytes that were restimulated with the corresponding immunogens, which is consistent with results published from another group<sup>[39]</sup>. Splenocytes isolated from mice immunized with either p(A $\beta_{3-10}$ )10-C3d-p28.3 or A $\beta_{42}$  peptide generated significantly higher levels of interleukin-4 than those from pcDNA3.1(+) immunized mice. There were no differences in the level of interleukin-4 between p(A $\beta_{3-10}$ )10-C3d-p28.3 immunized mice and A $\beta_{42}$  peptide immunized mice. However, higher levels of interferon- $\gamma$  were detected from the A $\beta_{42}$  peptide group as compared with the p(A $\beta_{3-10}$ )10-C3d-p28.3 group and the pcDNA3.1(+) group. Importantly, interferon- $\gamma$  levels were not signifi-

cantly different between the p(A $\beta_{3-10}$ )10-C3d-p28.3 group and the pcDNA3.1(+) group. These results indicate that A $\beta_{42}$  peptide immunized mice show a mixed Th1/Th2 immune response, whereas mice immunized with p(A $\beta_{3-10}$ )10-C3d-p28.3 develop a Th2-biased immune response that is less likely to induce detrimental inflammation. Cytokine analysis suggested that p(A $\beta_{3-10}$ )10-C3d-p28.3 immunization induced a considerable degree of Th2 immunity to the A $\beta_{42}$  peptide. The Th1 immune response reported in the AN1792 trial may have been caused by the presence of T and B cell self-epitopes of A $\beta_{42}$  peptide. Thus, choosing A $\beta$  epitope-specific vaccines could avoid a deleterious A $\beta$ -specific T cell response.

An A $\beta$  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 to detect the binding capability of anti-A $\beta$  antibodies to A $\beta$  plaques. The anti-A $\beta$  antibodies generated by the (A $\beta_{3-10}$ )10-C3d-p28.3 vaccine were able to bind to A $\beta$  plaques in brain tissue from this mouse. Binding of antibodies to the region of A $\beta_{42}$  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(A $\beta_{3-10}$ )10-C3d-p28.3 was immunoreactive to A $\beta$  deposits in the brain and therapeutically functional in a similar manner to the 6E10 anti-A $\beta$  monoclonal antibodies. Moreover, the anti-A $\beta$  antibodies elicited from the (A $\beta_{3-10}$ )10-C3d-p28.3 were effective and had strong affinity for A $\beta$  plaques. Both the pre-immune sera from the (A $\beta_{3-10}$ )10-C3d-p28.3 immunized mice and the sera of mice immunized with pcDNA3.1(+) did not bind to the A $\beta$  plaques. This demonstrated that immunized with pcDNA3.1(+) did not generate anti-A $\beta$  antibodies that could bind to A $\beta$  plaques in brain sections of Alzheimer's disease mice.

In summary, we report our findings with the novel DNA vaccine p(A $\beta_{3-10}$ )10-C3d-p28.3. This vaccine not only boosts anti-A $\beta$  antibody generation but also induces a Th2 immune response. The anti-A $\beta$  antibodies generated by the (A $\beta_{3-10}$ )10-C3d-p28.3 vaccine were able to bind to A $\beta$  plaques in brain tissue from a twelve-month-old APP/PS1 transgenic mouse. As such, a p(A $\beta_{3-10}$ )10-C3d-p28.3 vaccine may be a safe and effective candidate vaccine for A $\beta$  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 A $\beta$  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(A $\beta_{3-10}$ )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

8–10-week-old female C57BL/6J mice and a 12-month-old transgenic mouse (APP<sup>swe</sup>/PSEN1<sup>dE9</sup>) were purchased from the Center of Experimental Animals, China Medical University, China (license No. SYXK (Liao) 2008-0013). All mice were housed in a temperature- and light-cycle-controlled animal laboratory and allowed free access to food and water. 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<sup>[46]</sup>.

### Methods

#### Construction of plasmids

Ten tandem repeats of cDNA encoding the human A $\beta_{3-10}$  gene and three tandem repeats of cDNA encoding the 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 *Eco*RI (MBI Fermentas, Ottawa, Canada). The fusion expression protein contains (A $\beta_{3-10}$ )10 peptide and C3d-p28.3 oligonucleotide and there were seven amino acids, GlyGlyGlyGlySerGlySer (*Bam*HI) (MBI Fermentas), as the linker between them. This work was conducted at Yingrun Biotechnologies Inc. (Changsha, Hunan Province, China). The recombinant plasmid was amplified in DH5 $\alpha$  *E.coli* competent cells. The plasmids were purified and extracted in large quantities using plasmid preparation kits E.Z.N.A.<sup>TM</sup> Fast filter Endo-Free Plasmid Maxi Kit (OMEGA, Norcross, GA, USA). Plasmids were verified by agarose gel electrophoresis (KODAK, Rochester, NY, USA) and nucleotide sequence analysis (GenScript Inc, Nanjing, Jiangsu Province, China).

HEK293 cells (Stratagene, Santa Clara, CA, USA) were transiently transfected with 2 µg 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 blot assay using monoclonal anti-Aβ antibodies 6E10 (Signet, California, MA, USA).

### **Immunization**

All mice were immunized intramuscularly in the left hind legs for a total of five immunizations at 3-week intervals. The p(Aβ<sub>3-10</sub>)10-C3d-p28.3 and pcDNA3.1(+) groups were injected in the quadriceps femoris muscles with 100 µL of plasmid (100 µg in 100 µL of PBS) at each immunization and the mice were anesthetized by intraperitoneal injection of 10% chloral hydrate (0.03 mg/kg) before plasmid injection. 24 hours prior to plasmid injection, 50 µL (250 µg) of bupivacaine was injected into the left hind legs to induce mild muscle degeneration. The Aβ<sub>42</sub> peptide group was immunized with Aβ<sub>42</sub> peptide (50 µg per injection) (AnaSpec, San Jose, CA, USA) and Freund's adjuvant (Sigma, St. Louis, MA, USA). The protocol for the preparation of Synthetic Aβ<sub>42</sub> peptide and adjuvant was adapted from Schenk<sup>[6]</sup>.

Blood samples were collected from the orbital vein before immunization and 7 days after each immunization. The blood was incubated at room temperature for 2 hours and then centrifuged (Centrifugal radius = 13.5 cm) for 10 minutes at 5 000 r/min. Sera were then collected and stored at -70°C.

### **Detection of serum anti-Aβ antibody concentrations and isotypes**

Enzyme-linked immunosorbent assay was used to detect the titer of serum anti-Aβ antibodies. In brief, 96-micro well plates were coated with 5 µg/mL Aβ<sub>42</sub> and incubated overnight at 4°C. Micro well plates were treated with blocking buffer (5.0% goat serum, 1% bovine serum albumin, and 0.05% Tween-20 in PBS) and slightly shaken for 1 hour at room temperature. The serum samples were diluted to 1:1 000 with blocking buffer and the 6E10 monoclonal anti-Aβ antibody (Covance, Emeryville, CA, USA) was also diluted to 1:1 000. Then they were added to the plates in duplicate. After incubation (1.5 hours at 37°C) and washing, the goat anti-mouse IgG conjugated with horseradish peroxidase (Zymed, South San Francisco, CA, USA) was diluted to 1:1 000 and added to the plates and incubated (1 hour at 37°C). The reaction was developed by adding 3,3',5,5'-tetramethylbenzidine sub-

strate solution and stopped with 2 mol/L H<sub>2</sub>SO<sub>4</sub>. Plates were read in a spectrophotometer (Elx800; BioTek Instruments, Winooski, Vermont, USA) at 450 nm and antibody concentrations were calculated using a standard curve generated with known concentrations of 6E10 monoclonal antibody.

Horseshoe peroxidase-conjugated anti-mouse IgG1, IgG2a, and IgG2b (Zymed) were used to detect the isotypes of immunoglobulin and the results used to calculate the IgG1/IgG2a ratio. The method of detecting IgG1, IgG2a, and IgG2b was based on the manufacturer's instructions.

### **Detection of splenic T cell proliferation and cytokines**

At 2 weeks after the final immunization, the mice were sacrificed by cervical dislocation after intraperitoneal anesthesia and their spleens were excised. Single-cell suspensions were isolated and splenic T-cells were harvested using mouse splenocyte separation medium (Dakewe, Beijing, China). The splenic T-cells were plated in 96-well plates at 5 × 10<sup>6</sup> cells/mL in RPMI-1640 Medium (HyClone, Beijing, China). The cell suspension of each mouse was restimulated with Aβ<sub>42</sub> peptide (10 µg/mL), Aβ<sub>3-10</sub> peptide (10 µg/mL) (Shanghai Sangon Biological Engineering Technology & Services, Shanghai, China) or concanavalin A (2 µg/mL) (Sigma), respectively. At 48 and 72 hours, supernatants were collected and frozen at -20°C until analyzed for cytokine production by enzyme linked immunosorbent assay. Then, a 3-(4,5-dimethylthiazol-2-yl) 2, 5-diphenyl tetrazolium bromide assay was used to detect the proliferation rate of the cells. Absorbance at 490 nm was read using a micro plate reader (Elx800; BioTek Instruments, Winooski, VT, USA). The proliferation rate of cells was calculated as followings: (average absorbance of stimulating wells - average absorbance of blank wells) / (average absorbance of control wells - average absorbance of blank wells).

Mouse interferon-γ and interleukin-4 cytokines were detected in the culture media using a mouse cytokine enzyme linked immunosorbent assay 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 capacities of antisera to Aβ plaques in the APP/PS1 transgenic mouse brain. A 12-month-old-transgenic mouse (APP<sup>swe</sup>/PSEN1<sup>dE9</sup>) was sacrificed after



intraperitoneal anesthesia and the brain was removed. The right hemisphere was embedded in paraffin and cut into 4- $\mu$ m thick coronal sections. Sera from vaccinated mice were added to 4  $\mu$ m-thick brain sections of formalin-fixed cortical tissue. The titers of mice antisera were at a 1:500 dilution. We used the same dilutions of the preimmune sera and pcDNA3.1(+) group sera for the negative controls, and 6E10 monoclonal anti-A $\beta$  antibody (Covance, Emeryville, CA, USA) for the positive control experiments. The specimens were then incubated at 4°C overnight, followed by three washes with PBS. The corresponding biotinlabeled secondary rabbit anti-mouse IgG antibodies (1:500; Sigma) were added and incubated at 37°C for 30 minutes. After washing, horseradish peroxidase-labeled streptomycin avidin working solution was added and incubated at 37°C for 30 minutes. Binding of antibodies to the brain sections was visualized using the streptavidin biotin peroxidase complex Mouse IgG/DAB Substrate Biotin-Avidin System (both kits from Boster Biological Technology, Wuhan, Hubei Province, China) according to manufacturer's recommendations. A digital camera (Olympus, Osaka, Japan) was used to detect the plaques at 20  $\times$  image magnification.

### Statistical analysis

All data are expressed as mean  $\pm$  SD. The differences were analyzed by one-way analysis of variance, followed by the Student-Newman-Keuls' multiple range test. SPSS 17.0 (SPSS, Chicago, IL, USA) was used to perform all analyses. A value of  $P < 0.05$  was considered statistically significant.

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