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ORIGINAL PAPER

In Vivo Electroporation of a New Gene Vaccine Encoding Ten Repeats of Aβ3-10 Prevents Brain Aβ Deposition and Delays Cognitive Impairment in Young Tg-APPswe/PSEN1dE9 Mice

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Abstract Active immunization holds great promise for the treatment of Alzheimer's disease but the infiltration of T-lymphocytes and associated meningoencephalitis observed in clinical trials needs to be overcome. To avoid this toxicity, previous studies have used synthetic truncated derivatives of Aβ to promote humoral immunity. In this study, we developed a novel vaccine [p(Aβ3-10)10-MT] that expresses ten repeats of Aβ3-10 with melatonin (MT) as an adjuvant, and administered it intramuscularly in three-month-old Tg-APPswe/PSEN1dE9 (Tg) mice by in vivo electroporation. The p(Aβ3-10)10-MT vaccine induced high titers of anti-Aβ antibodies, which in turn reduced Aβ deposits in the mouse brains and decreased cognitive impairment. Immunoglobulin isotyping revealed a predominantly IgG1 response, indicating a Th2 anti-inflammatory response. Ex vivo cultured splenocytes exhibited a low IFN-γ and high IL-4 response. Immunohistochemical analysis revealed that glial cell activation was also attenuated. These results indicate that p(Aβ3-10)10-MT may potentially be an effective vaccine to reduce accumulated Aβ and attenuate cognitive deficits.

Keywords Alzheimer's disease · Gene vaccine · Amyloid β-protein · In vivo electroporation

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Introduction

Alzheimer's disease (AD) is the most common cause of dementia and currently there is no effective treatment to prevent its progression. According to the Amyloid Cascade Hypothesis adopted by most AD researchers, overproduction of Aβ is the primary pathological event, which precedes tau accumulation and leads to neurofibrillary degeneration [1, 2]. Therefore, preventing Aβ overproduction or accelerating its degradation has become an important therapeutic goal in the prevention and treatment of AD. Both active and passive Aβ immunotherapy has been reported to significantly decrease amyloid deposits, neuritic dystrophy and gliosis in the brain, as well as provide cognitive benefits in APP/Tg mice [3]. Based on previous studies, the AN-1792 clinical trial was initiated, but phase II of the trial was halted because of the occurrence of acute meningoencephalitis and cerebral amyloid angiopathy (CAA)-associated hemorrhage [4]. The inflammatory reaction in the central nervous system is presumably related to the induction of Th1-type responses and use of the conventional adjuvant QS21 [5].

Determination of the optimal treatment time point is also critical in the development of therapeutic approaches to AD. Interestingly, analysis of the AN-1792 clinical trial report suggests that "anti-amyloid immunization may be most effective, not as a therapeutic or mitigating measure, but as a prophylactic measure when Aβ deposition is still

minimal" [6]. Early protective immunization before substantial neuropathology, neuronal loss and cognitive deficits occur has become firmly established as potentially having the most benefit. Based on immunotherapy studies in young versus old Tg mice, a second potential benefit of early immunization may be a reduced incidence of adverse events in the cerebral vasculature [7]. Recently, a vaccine consisting of Aβ1–6 (CAD106) was developed in order to avoid T-cell activation and a Phase II clinical trial is ongoing. Results from pre-clinical trials in mice suggest that immunotherapy with CAD106 has a larger effect when immunization is started before amyloid deposition [8].

Establishing a safe vaccination against AD requires shifting the immune response to a Th2-type response, an outcome that can be achieved through genetic immunization and has been shown to have a long-term effect on Aβ clearance [9, 10]. The B-cell epitope is located within the Aβ1–15 region while the T-cell epitope has been mapped within Aβ15–42 [11]. The segregation of T and B-cell epitopes within the Aβ molecule offers the opportunity to use specific Aβ fragments that do not include potentially harmful T-cell epitopes [12, 13]. It has been reported that gene vaccines encoding alternative immunogens encompassing the N-terminal epitope of Aβ, but lacking the C-terminal T-cell reactive sites, such as Aβ1–15, Aβ4–10, and Aβ1–10, stimulate a robust humoral immune response without triggering meningoencephalitis or enhancing CAA when mutated or presented as multiple copies [14–16]. Previous studies have also shown that Aβ4–10 is the dominant peptide that anti-Aβ42 antibodies specifically recognize with a high affinity [15], that Aβ3–6(EFRH) affects the solubility and disaggregation of Aβ fibrils, and that its affinity for anti-Aβ antibodies is significantly decreased without the third amino acid [17]. To overcome the hurdle of the low iummunogenicity of Aβ, tandem repeats of a small self-peptide are constructed to permit self-tolerance and increase the molecular weight as well as reduce degradation of the peptide [18].

DNA-based vaccination uses direct injection of plasmid DNA encoding genes for peptide antigens. The facilitated uptake of the DNA by cells that then express the antigen provokes an immune response when the proteins are encountered by cells of the immune system [19]. Compared with conventional vaccines, DNA vaccines have several significant advantages 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 [20]. Furthermore, genetic immunization may simplify the vaccination protocol because the difficult steps of protein purification and combination with adjuvant, both routinely required for vaccine development, are eliminated [21]. Intramuscular administration of a gene vaccine allows for ready access to the bloodstream, thereby facilitating contact between the products of the gene expression and antigen-presenting cells [22]. In animal models, the application of in vivo electroporation has been shown to increase the transfection efficacy and the immunogenicity of plasmid vaccines by several fold [23]. With recent developments in electroporation systems for muscle delivery, the safety, tolerability, and clinically acceptable administration of DNA vaccines has advanced significantly [7].

In our current study, we chose pcDNA3.1 as a vector, used ten tandem repeats of the B-cell-activating epitope Aβ3–10 as an antigen to enhance immunogenicity and then added melatonin (MT), which is secreted by the pineal gland, as a novel adjuvant to enhance the humoral immune response. The vaccine was then injected intramuscularly into 3-month-old Tg-APPswe/PSEN1dE9 (Tg) mice using in vivo electroporation. The present study was designed to test the hypothesis that this vaccine should induce a robust humoral immune response, have prophylactic effects that prevent the deposition of Aβ, protect cognitive function in young mice, and may delay the pathogenesis of AD without an inflammatory response.

Materials and Methods

Plasmid Construction and Preparation

Ten tandem repeats of cDNA for the human Aβ3–10 gene were chemically synthesized with optimal codons for mammalian cell expression. The target genes were then subcloned into the eukaryotic expression vector pcDNA3.1(+) using the restriction sites HindIII and EcoRI by WanLei Life Sciences Corporation of Shen Yang, China. The recombinant plasmid was amplified in DH5α cells. The plasmids were purified and extracted in a large quantity using plasmid preparation kits (E.Z.N.A TM Fastfilter Endo-Free Plasmid Maxi Kit). The p(Aβ3–10)10 plasmids were then transfected into HEK293 cells (Strategene, USA) using lipofectamine 2000 (Invitrogen, USA). Cells transfected with the pcDNA3.1(+) vector alone were used as a negative control. Expression of the plasmids was analyzed in the supernatants of cells by Western blotting using monoclonal anti-Aβ 6E10 antibodies (Signet, MA, USA).

Immunization of Mice by In Vivo Electroporation

Twenty-one three-month-old APPswe/PSEN1dE9 mice were obtained from the Animal School of China Medical University. APPswe/PSEN1dE9 mice express a mouse/human amyloid precursor protein (APP) and mutant human presenilin 1 (PS1) and develop beta-amyloid deposits in the brain by 6–7 months of age. All mice were maintained in a

12-h light/dark cycle with food and water ad libitum. All animal experiments were performed in accordance with our institutional guidelines.

The mice were randomly assigned to three groups and injected with the p(Aβ3–10)10-MT vaccine (n = 7), an Aβ42 peptide (n = 7), or pcDNA3.1 (n = 7) respectively. The mice in the p(Aβ3–10)10-MT and pcDNA3.1 groups were injected with their plasmid (100 μg in 100 μl PBS) intramuscularly into the left hind leg for a total of 10 immunizations at three-week intervals. After the mice were anaesthetized, a pair of electrode needles (26-gauge) were inserted into the muscle 5 mm apart to cover the DNA injection sites and electrical pulses were delivered using an electric pulse generator (ECM830, BTX Harvard Apparatus company, USA). Six pulses of 75 V each were delivered at a rate of one pulse every 200 ms [24]. Aβ42 (50 μg per injection) was emulsified 1:1 (v/v) with complete Freund's adjuvant (Sigma, USA) for the first immunization, followed by a boost in incomplete Freund's adjuvant (Sigma, USA) at three-week intervals (total of 10 times). The protocol for the preparation of the synthetic Aβ42 peptide (AnaSpec, San Jose, CA, USA) and Freund's adjuvant was adapted from Schenk et al. [25]. Blood samples were drawn from each mouse via the orbital vein before immunization and 1 week after each immunization.

Morris Water Maze Test

Detailed methods for the Morris water maze test have been described previously [26]. The test was performed using a circular tank (1.25 m diameter and 0.4 m high), equipped with a digital pick-up camera to monitor animal behavior and a computer program for data analysis (ZH0065, Zhenghua Bioequipments, China). The Morris water maze test was performed at 2 weeks after the last immunization. The mice were trained for 2 days using a visible platform, followed by 2 days using a hidden platform, and then 1 day later a probe test was performed. Each mouse was lowered into the water from each quadrant and allowed 60 s to reach the platform. In the afternoon of the fifth day, the platform was removed from the pool and each mouse was allowed one 60 s swim probe trial. Finally, data for the escape latency, the number of times the platform location was crossed, and percentage of time spent in the target quadrant were analyzed.

Antibody Titers and Isotyping by ELISA

Enzyme-linked immunoabsorbent assay (ELISA) was used to monitor the humoral immune responses [10]. In brief, the Aβ peptide was detected in serum samples diluted with PBS at 1:1,000 by ELISA using 96-microwell plates coated with GST-Aβ proteins. Microtiter wells were treated with blocking buffer (5.0 % goat serum, 1 % BSA, and 0.05 % Tween-20 in PBS) for 2 h at room temperature. A standard curve was generated using serial dilutions of the standard 6E10 antibody (monoclonal anti-Aβ antibody, Covance). HRP-conjugated anti-mouse IgG1, IgG2a, and IgG2b (Zymed, CA) were used to determine the isotypes of immunoglobulin and the results used to calculate the IgG1/IgG2a ratio.

Splenic T Cell-Proliferation Assay and Detection of Cytokines

The mice were sacrificed 1 week after the morris water maze test. Following anesthesia, the spleen of each mouse was aseptically removed. Single cell suspensions were made by mincing and grinding the spleen through a 40-μm nylon cell strainer and T-cells harvested using mouse splenocyte separation medium (Dakewe, China). The suspension density of the T-cells was adjusted to 5×10^6 cells/ml and the cells plated on 96-well plates. The cell suspension from each mouse was restimulated, respectively, with Aβ1–42 peptide (10 μg/ml), Aβ3–10 peptide (10 μg/ml) (Shanghai Sangon Biological Engineering Technology & Services, Shanghai, China) and ConA (2 μg/ml) as positive control. For the blank well, 1640 medium containing 10 % FBS was added. Then, an MTT assay was used to detect the proliferation and stimulation index of the cells. Mouse Th1 (IFN-γ) and Th2 (IL-4) cytokines were detected in the culture media using a mouse cytokine ELISA kit according to the manufacturer's instructions (Dakewe Biotech Company, China).

ELISA Analysis of Aβ42 in Plasma and Brain Homogenate

The frontal lobe of the brain of each mouse was homogenized in 10 volumes of guanidine-tris buffer (5.0 M guanidine HCl/50 mM Tris–HCl, pH 8.0). The homogenates were mixed for 3 h at RT and stored at 20 °C until analyzed [35, 36]. The Aβ42 in heparinized plasma was also extracted with 10 volumes of quinidine–tris buffer. Plasma samples were diluted 1:100 in blocking buffer and Aβ levels were measured by sandwich ELISA. An Aβ42 ELISA kit (Invitrogen, Camarillo, CA, USA) was used to measure Aβ42 in the brain homogenates and plasma according to the manufacturer's instructions. The absorbance of the plates was read at 450 nm with a spectrophotometer.

Identification of Aβ Protein Expression in Muscles

To examine histologic changes within the injection site, muscle tissues were formalin-fixed, paraffin-embedded, or acetone-fixed then cryostat cut (5 μm thick), and incubated for 30 min with a monoclonal anti-Aβ 6E10 antibody (dilution 1:1,000) (Signet, MA, USA). Binding of antibodies to the muscle sections was tested using a SABC

Mouse IgG/DAB substrate biotin-avidin system (Boster Biological Technology, China) according to the manufacturer's recommendations.

Immunohistochemistry to Detect Aβ Burden and the Presence of Astrocytes

Aβ deposits in the mouse brains were detected with mouse anti-Aβ antibody (6E10, 1:500, Covance, USA) and astrocytes were detected with rabbit anti-GFAP (1:100, Boster Biological Technology, China). Sections were incubated overnight with primary antibodies at 4 °C, further developed with biotinylated secondary antibodies. The extent of the amyloid plaque burden was analyzed with Image J software (NIH) and the percentage of the plaque area compared to the total area observed was calculated in three representative images of the cortex and two of the hippocampus.

To detect the presence of hemorrhagic lesions in the mouse brain sections, Perl's Prussian blue stain was used to visualize ferric iron in hemosiderin present as a result of hemorrhaging. The lung, liver, heart, spleen and kidney sections were also routinely subjected to HE staining.

Statistical Analysis

Data are expressed as the mean ± standard deviation (SD) for each group of mice. The difference between groups was analyzed with a one-way ANOVA, followed by the Student–Newman–Keuls multiple range test. SPSS17.0 (Windows, release 10.0.5) was used to perform all analyses and $P < 0.05$ was considered statistically significant.

Results

Construction and Expression of Aβ Plasmid DNA Vaccines

The p(Aβ3–10)10-MT plasmid was successfully constructed as indicated by the digestion fragments resulting from the use of NotI and EcoRI restriction enzymes. The expression of p(Aβ3–10)10-MT plasmids was also confirmed in transiently-transfected HEK293 cells by Western blotting and by muscle tissue immunohistochemistry following administration of the vaccine.

High Titers of Anti-Aβ Antibodies were Induced in Immunized APPswe/PSEN1dE9 Mice and were Predominantly IgG1

Anti-Aβ antibodies were detectable in the Aβ42 and p(Aβ3–10)10-MT-vaccinated mice following the first immunization, showed a steady increase with each booster injection, reached the highest titers after the eighth immunization, and were then sustained at 92.43 ± 7.50 μg/ml and 55.05 ± 4.72 μg/ml, respectively. In contrast, the antibody titers in the mice vaccinated with the empty vector were at a background level (Fig. 1a). Immunization with p(Aβ3–10)10-MT or the Aβ42 peptide induced a broad spectrum of anti-Aβ antibody isotypes including IgG1, IgG2a, and IgG2b but the induced anti-Aβ antibodies were predominantly of the IgG1 isotype (Fig. 1b). The

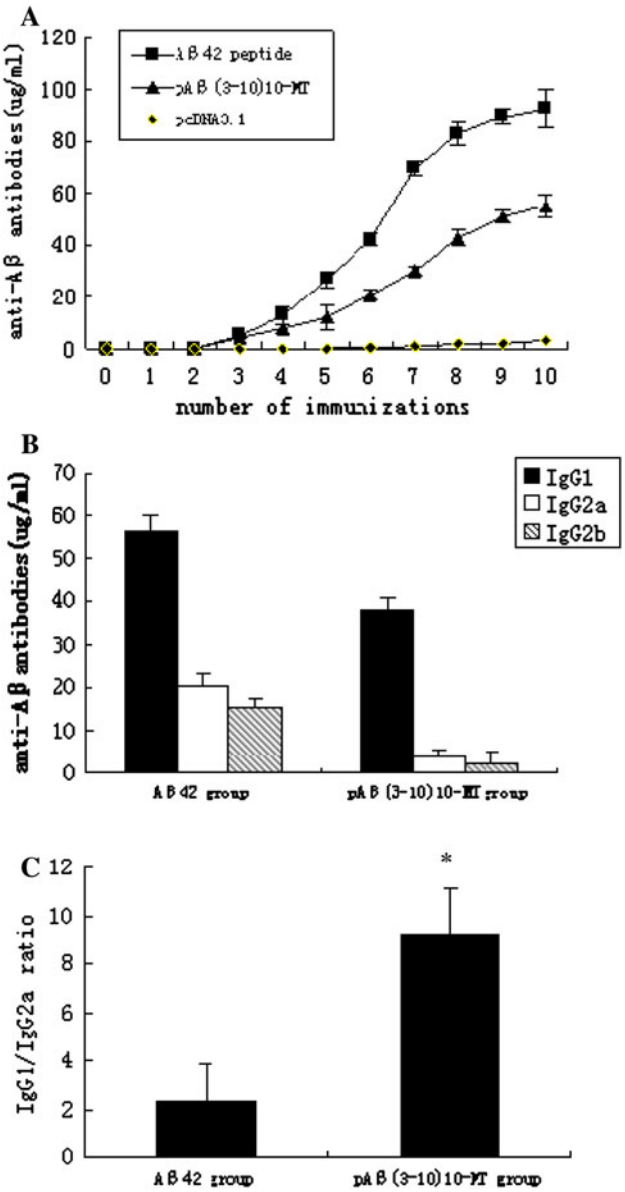


Fig. 1 Immune response against human Aβ42 in Tg mice immunized with our vaccine. **a** Anti-Aβ antibody titers were assayed by ELISA at the indicated immunization points. **b** Isotyping in sera from immunized mice after 10 immunizations. **c** IgG1/IgG2a ratios were calculated based on the data present in (**b**). *$P < 0.05$, compared with Aβ42 peptide group

IgG1/IgG2a ratio was 9.26 ± 1.86 for the mice vaccinated with p(Aβ3–10)10-MT, which was approximately four times greater than the IgG1/IgG2a ratio for the Aβ42 group (2.34 ± 1.49; $P < 0.05$) (Fig. 1c).

Cognitive Deficits were Attenuated in Immunized Mice According to the Morris Water Maze Test

Visible Platform Training

Two days of visible platform training showed that mice immunized with the Aβ42 peptide, p(Aβ3–10)10-MT, or the empty vector had a similar escape latency ($P > 0.05$), suggesting that immunization did not significantly affect mobility or vision in the mice.

Hidden Platform Training

The performance of mice in the hidden platform training significantly improved in all groups. The escape latency, an estimate of spatial learning and memory capacity, was significantly shorter for mice vaccinated with p(Aβ3–10)10-MT and those vaccinated with the Aβ42 peptide compared with those vaccinated with the empty vector (Fig. 2a) ($P < 0.01$). The Aβ42 peptide and p(Aβ3–10)10-MT-immunized mice showed no significant differences between them ($P > 0.05$).

Probe Test

Mice immunized with the Aβ42 peptide or p(Aβ3–10)10-MT spent significantly more time in the target quadrant and crossed the platform more often than mice vaccinated with the empty vector ($P < 0.05$), but there was no significant difference between the Aβ42 and p(Aβ3–10)10-MT groups ($P > 0.05$) (Fig. 2b, c). Analysis of swimming distance, swimming path, and average speed revealed no significant differences between the groups ($P > 0.05$).

Aβ-Specific T-cell and Cytokine Responses Could be Elicited in Splenocytes from Immunized Mice

To determine whether the cellular immune response was directed towards Aβ, splenocytes from immunized mice were restimulated with ConA, Aβ3–10, and Aβ42. As shown in Fig. 3a, splenic T-cells restimulated with ConA exhibited the highest levels of proliferation, but there was no significant difference between the groups ($P > 0.05$). Immunization with Aβ42 induced robust in vitro T-cell proliferation after restimulation with Aβ42. Importantly, splenocytes isolated from mice immunized with p(Aβ3–10)10-MT produced a nearly equally strong level of T-cell proliferation after stimulation with Aβ3–10, but not

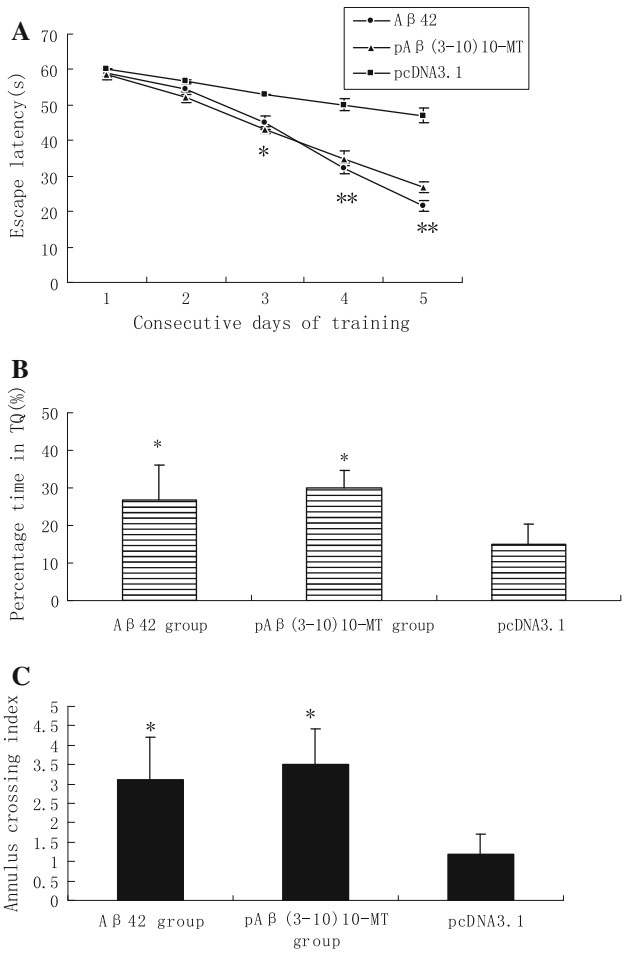


Fig. 2 Morris water maze assessment of APPswe/PSEN1dE9 mice after immunizations. **a** Learning curve showing the escape latency at days 1–5 in Aβ42 peptide and p(Aβ3-10)10-MT group immunized mice. $^{**}P < 0.01$ and $^{*}P < 0.05$ versus controls, respectively. **b** Percentage of time spent in the target quadrant (TQ) in probe trails in Aβ42 peptide and p(Aβ3-10)10-MT group. $^{*}P < 0.05$, compared with the empty vector group. **c** Annulus-crossing index during the probe trail after the last training trial on day 5. $^{*}P < 0.05$, compared with the empty vector group

with Aβ42 (P > 0.05). In the control mice, splenic T-cells stimulated with the Aβ3–10 and Aβ42 peptides exhibited much lower proliferation than those stimulated with ConA ($P < 0.05$).

Consistent with the splenic T-cell-proliferation assay, higher levels of IL-4 and IFN-γ detected by ELISA were only observed in splenocytes that were restimulated with the corresponding immunogens compared with the control group ($P < 0.05$), but the levels were lower than those observed following restimulation with ConA ($P < 0.05$). Mice immunized with p(Aβ3–10)10-MT or the Aβ42 peptide exhibited similar IL-4 levels in spleen cells restimulated with the corresponding immunogens ($P > 0.05$), but these levels were significantly greater compared with those observed in the mice immunized with the empty vector

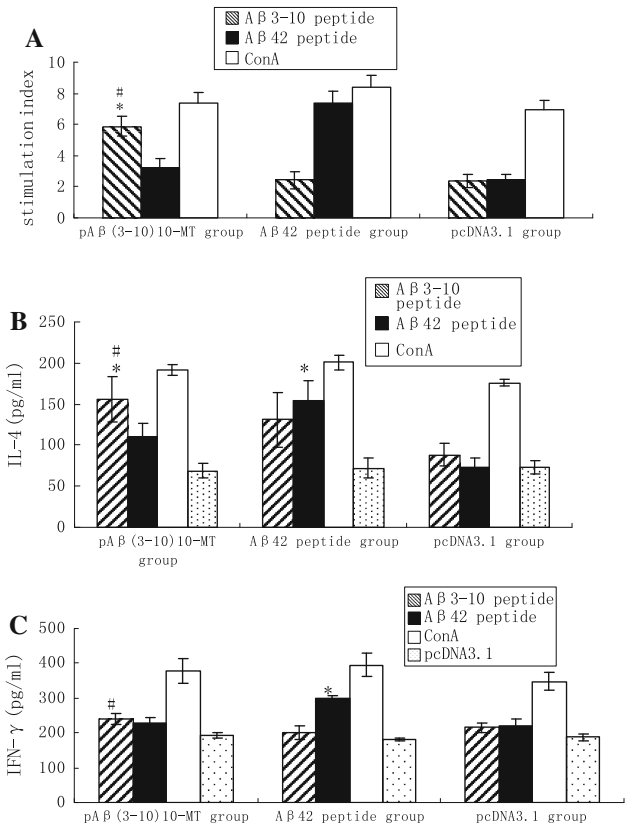


Fig. 3 Proliferation rate of spleen T lymphocytes in vitro induced with stimulating antigen, IL-4 and IFN-γ levels of splenocytes from immunized mice. **a** Spleen T lymphocytes exhibits a higher level of proliferation rate after stimulation with their corresponding immunogen. $^{*}P < 0.05$ compared with empty vector; $^{\#}P > 0.05$ compared with Aβ42 peptide group. **b** IL-4 levels in the spleen T lymphocytes are greater after stimulation with corresponding immunogen. $^{*}P < 0.05$, compared with empty vector; $^{\#}P > 0.05$ compared with Aβ42 peptide group. **c** IFN-γ levels in the spleen T lymphocytes of Aβ42 peptide group are greater after stimulation with corresponding immunogen. $^{*}P < 0.05$, compared with the p(Aβ3-10)10-MT group and the empty vector; no differences in the IFN-γ levels between the mice immunized with pAβ(3-10)10-MT and empty vector. $^{\#}P > 0.05$

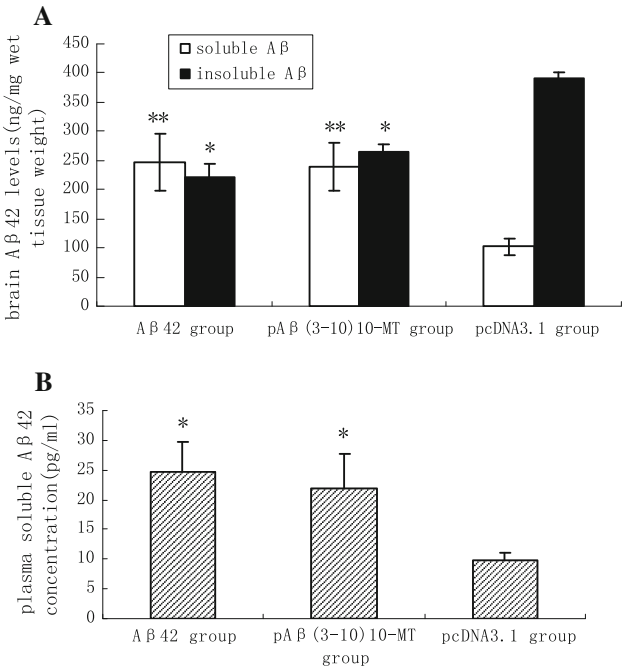


Fig. 4 Levels of Aβ42 in the brain (**a**) and in the blood (**b**) of Tg mice immunized with those vaccines. Insoluble Aβ42 levels in Aβ42 peptide and p(Aβ3-10)10-MT group were significantly reduced as compared to the control group ($^{*}P < 0.05$) while soluble Aβ42 levels significantly increased compared with empty vector group ($^{**}P < 0.01$). The Aβ42 levels in plasma increased significantly in mice immunized with Aβ42 peptide and p(Aβ3-10)10-MT compared with control group. ($^{*}P < 0.05$)

($P < 0.05$) (Fig. 3b). Spleen T-cells from mice immunized with the Aβ42 peptide exhibited greater IFN-γ levels when restimulated with the Aβ42 peptide than spleen cells restimulated with Aβ3–10 ($P < 0.05$) or spleen cells from mice immunized with the empty vector ($P < 0.05$). There were no difference in the IFN-γ levels produced by spleen T-cells from the mice immunized with p(Aβ3–10)10-MT and the empty vector ($P > 0.05$) (Fig. 3c).

Aβ42 Levels in Plasma and Brain Homogenates were Altered in Immunized Mice

Compared with soluble Aβ42, cerebral Aβ42 existed mainly in an insoluble form, whereas plasma Aβ42 was primarily soluble. As shown in Fig. 4a, levels of insoluble Aβ42 in the brain were significantly reduced by 32.5 and 43.3 % in the p(Aβ3–10)10-MT and Aβ42-immunized mice, respectively ($P < 0.05$). Compared with mice immunized with the empty vector, levels of soluble Aβ42 were significantly increased in the brains of mice immunized with p(Aβ3–10)10-MT or the Aβ42 peptide ($P < 0.01$), but there was no significant difference between the two groups ($P > 0.05$). As shown in Fig. 4b, Aβ42 levels in the plasma showed a similar pattern to the levels of soluble Aβ42 observed in the brain.

The Burden of Cerebral Aβ Plaques was Reduced in Immunized Mice

Immunohistochemistry and quantitative image analysis were performed to evaluate the Aβ burden in the brains of vaccinated and control mice. As shown in Fig. 5m, vaccination with p(Aβ3–10)10-MT reduced the Aβ burden in the cortex and hippocampus by 40.9 and 49.6 %, respectively, compared with the mice vaccinated with the empty vector ($P < 0.05$), while vaccination with the Aβ42 peptide reduced the Aβ burden in the cortex and hippocampus by 43.6 and 52.4 %, respectively ($P < 0.01$). Figure 5a–l shows representative images from the three groups of mice. The Aβ deposition in the hippocampus and cortex was

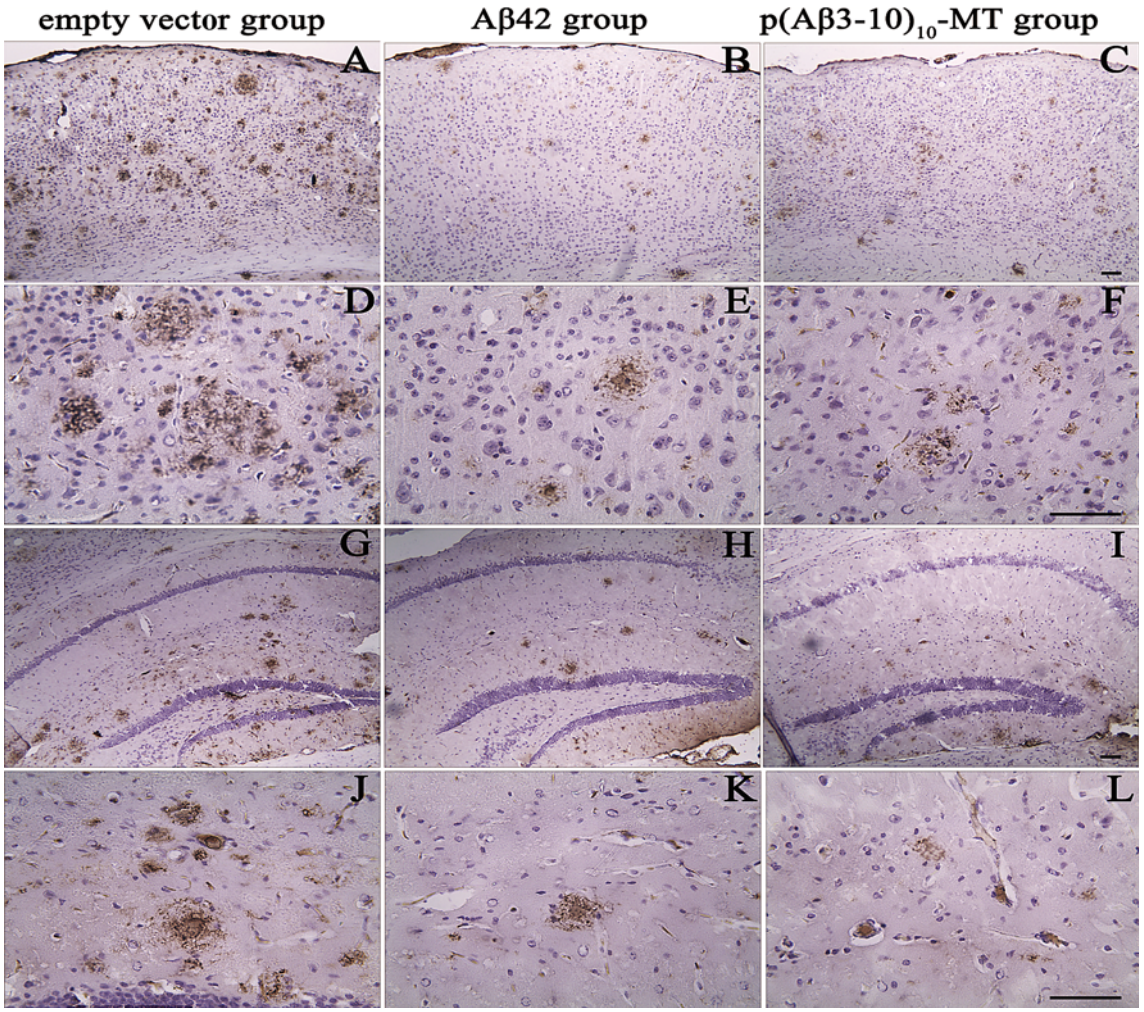


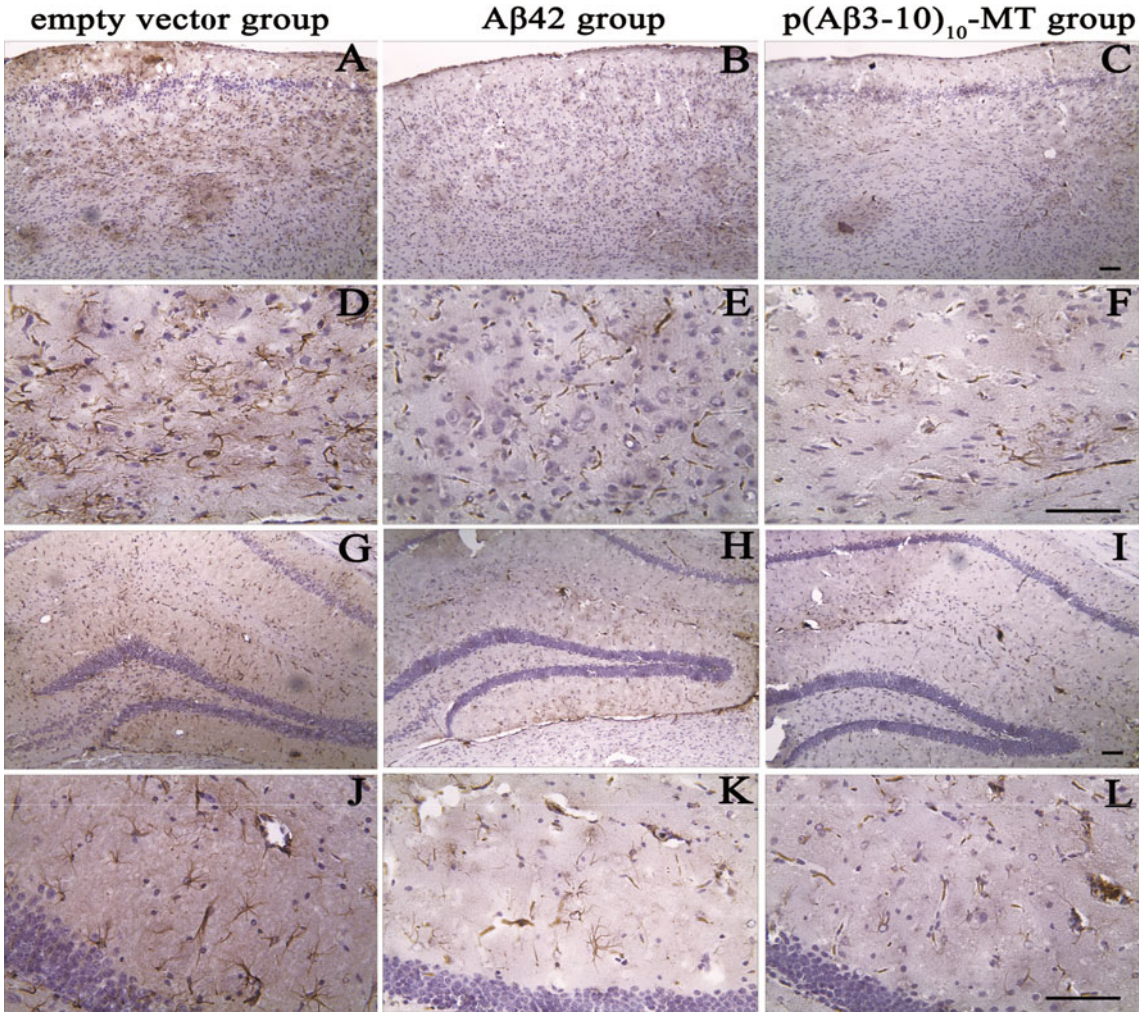
Fig. 5 Reduced Aβ deposition in the hippocampus and the parietal association cortex of vaccinated mice. Representative coronal sections were showed in **a–f**. **g–l** Are representative hippocampus sections. Brains sections are from mice immunized with p(Aβ3-10)10-MT (**a**, **d**, **g**, **j**), Aβ42 (**b**, **e**, **h**, **k**) and pcDNA3.1 group (**c**, **f**, **i**, **l**). Plaques of increased number and greater size were seen in control compared to the treated mice. **m** Show Aβ plaque quantification analysis of the hippocampus and parietal association cortex. $^{**}P < 0.01$, compared with the pcDNA3.1 group. $^{\#}P > 0.05$ compared with Aβ42 peptide group. *Scale bar* 50 μm

greatest in the control group and was observed as dark-brown and densely scattered, with coarse, granular senile plaques. In the brains of the mice immunized with p(Aβ3-10)10-MT or Aβ42 peptide the Aβ deposition was light-brown and sparsely scattered, with granular senile plaques.

Staining of Glial Fibrillary Acidic Protein was Reduced in Immunized mice

GFAP staining was used as a method of evaluating inflammation-related pathology in the brains of the mice.

The Aβ42 and p(Aβ3–10)10-MT vaccinations both reduced the number of GFAP-positive cells in the cortex and hippocampus compared with mice vaccinated with the empty vector ($P < 0.05$). Quantitative image analysis indicated that p(Aβ3–10)10-MT or Aβ42-immunized mice had a lesser degree of astrocytosis compared with the control group (Fig. 6m). In the control group, GFAP-positive cells were activated and exhibited increased



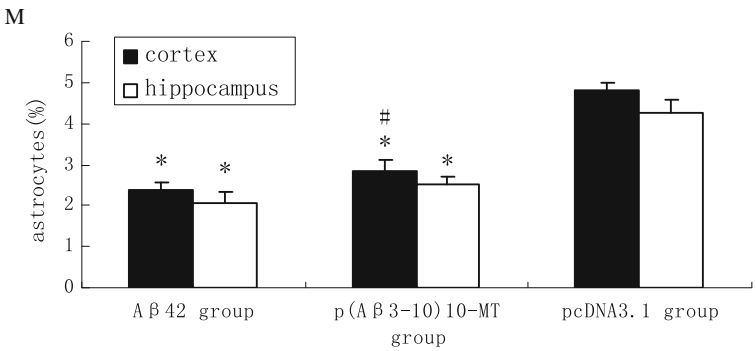


Fig. 6 Immunohistochemical analysis of glial fibrillary acidic protein in AD Tg mice. GFAP-positive cells significantly reduced in mice immunized with p(Aβ3-10)10-MT and Aβ42 peptide compared with empty vector group. **a–f** are the representative coronal sections and **g–l** are representative hippocampus sections. **a**, **d**, **g**, and **j**: the control group. **b**, **e**, **h**, and **k**: the Aβ42 treated group. **c**, **f**, **i**, **l**: the p(Aβ3-10)10-MT treated group. **m** Show the quantification analysis of GFAP-positive cells occupied areas in hippocampus and cortex. $^{*}P < 0.05$, compared with the empty vector group. $^{\#}P > 0.05$ compared with Aβ42 peptide group. *Scale bar* 50 μm

cytoplasm and longer synapses. GFAP-positive cells also showed an obvious tendency towards aggregation (Fig. 6a, d, g, j). In the mice immunized with p(Aβ3–10)10-MT (Fig. 6b, e, h, k) or Aβ42 (Fig. 6c, f, i, l), GFAP-positive cells were sparsely distributed with less cytoplasm and smaller, fewer, thinner, shorter synapses.

No Adverse Effects were Detected

Prussian blue staining was used to assess the potential adverse side effects of vaccination with the Aβ epitope. HE staining revealed normal structure and clear cell arrangement and shape in the brain and other organs (heart, liver, spleen, lung, and kidney) of all groups of mice. No blue stained hemosiderin was observed. Data is not shown.

Discussion

Holmes et al. suggested that plaque removal is not enough to halt progressive neurodegeneration in AD, prompting some intriguing challenges to the amyloid hypothesis [27]. It is plausible that Aβ vaccination was "too little, too late", and elevated Aβ levels/cerebral amyloidosis had already set into motion a pathogenic cascade such that removal of Aβ from the equation at a late stage would have little if any effect [28]. Prophylactic immunization against Aβ deposition in AD has been suggested to be the most beneficial approach to preventing the disease but the adverse response associated with clinical trials of Aβ42 vaccination needs to be overcome [3–7]. Thus, we developed a novel vaccine [p(Aβ3–10)10-MT] and then administered it to 3-month-old Tg-APPswe/PSEN1dE9 by intramuscular injection with in vivo electroporation and compared the effects against vaccination with the Aβ42 peptide or an empty vector.

MT, a indolamine hormone secreted by the pineal gland and lymphocytes, plays an important role in the immune system [29]. Indolamines have been shown to modulate the cytotoxicity of natural killer cells, modify antibody responses, and inhibit the proliferation of lymphocytes [30]. The present results suggest that MT acts on Th2-type cells, as shown by the predominant secretion of IL-4 and IgG1 antibodies but not IL-2, IFN-γ and IgG2a antibodies [31]. The solubility of MT in both lipids and water allows it to be easily distributed into cells. Secondly, its ability to cross the blood–brain barrier allows MT to enter the central nervous system [32]. Although there have been no reports on its use in AD-related clinical research, MT has been used extensively in clinical trials of cancer vaccines [33]. To our knowledge, our findings represent the first report that MT acts as an adjuvant to enhance humoral immune responses in prophylactic vaccination against Aβ42 deposition.

Intramuscular injection of p(Aβ3–10)10-MT into mice generates significantly higher titers of antibodies than those observed in the control group, but lower than the titers of antibodies produced in response to immunization with the Aβ42 peptide, which is similar to other gene vaccines [34]. The high titer of anti-Aβ antibodies, especially of the IgG1 type, was necessary to improve the behavioral abilities and to reduce the amyloid plaques and astrocytosis in the brains of the mice, although this effect plateaus off after the titers reach a certain level. The IgG1/IgG2a ratio in the mice vaccinated with p(Aβ3–10)10-MT is much greater than that in mice vaccinated with the Aβ42 peptide, suggesting the induction of Th2-biased responses. This response may be a key advantage of gene vaccination, as it could avoid cell-mediated encephalitis caused by immunization with the Aβ42 peptide.

In mice, the production of IgG1 antibodies is primarily induced by Th2 cytokines, whereas the production of IgG2a antibodies reflects the involvement of Th1 cytokines. IFN-γ and IL-4 cytokines are the two major primary cytokines produced by Th1 and Th2 cells, respectively [35]. The balance between IFN-γ and IL-4 produced by the splenocytes of immunized mice is associated with the ratio of IgG1/IgG2a. We found that mice immunized with p(Aβ3–10)10-MT or Aβ42 generate significant numbers of splenocytes that produce IL-4 and IFN-γ when stimulated with the corresponding antigen but the IFN-γ levels in mice immunized with p(Aβ3–10)10-MT are less than those observed in the Aβ42 peptide group. This result indicates that Aβ42-immunized mice show a mixed Th1/Th2 immune response, while the mice immunized with p(Aβ3–10)10-MT mount a more robust Th2 immune response.

The results of the splenocyte proliferation assay show that the highest stimulation indexes are observed in splenocytes restimulated only with their corresponding immunogen. Importantly, strong T-cell proliferation is observed in splenocytes isolated from the p(Aβ3–10)10-MT-vaccinated mice after stimulation with Aβ3–10, but not with Aβ42. In contrast, because Aβ42 itself possesses both B and T-cell epitopes, immunization with Aβ42 induces an Aβ-specific T-cell proliferative response after stimulation with Aβ42. These results demonstrate that immunization with p(Aβ3–10)10-MT induces a specific cellular immunity targeted to B-cell epitopes other than the full-length Aβ1–42 peptide.

Immunization of the mice significantly reduces the deposition of cerebral Aβ plaques and reverses behavioral impairment, as determined by immunohistochemistry and the Morris water maze test. There is a significant decrease in the amount of Aβ deposition and the concentration of

insoluble Aβ but no reduction in the soluble form of Aβ in the brain of immunized mice, as has been shown in other studies [36, 37]. FcR-mediated clearance within the brain by microglial cells [38], the peripheral sequestration and efflux of Aβ out of the CNS (the 'peripheral sink hypothesis') [39], and disruption of Aβ aggregation or promotion of its disaggregation by anti-Aβ antibodies [40] could account for the observed decreases in Aβ. Our findings are consistent with reports showing that Aβ-specific antibodies can improve cognitive performance [3], the mechanism of which could include promotion of humoral or cellular immune responses against soluble Aβ and the production of neurotrophic factors, which can promote synaptogenesis and normal synaptic functioning to improve memory and cognition.

Astrocytes may function as immunocompetent cells within the brain and astrocyte-produced inflammatory factors promote deposition and fibrotic transformation of Aβ [41]. In mice immunized with the Aβ42 peptide or p(Aβ3–10)10-MT the amount of plaque deposition and subsequent encapsulation by glial cells is decreased, indicating a reduction of activated astrocytes, which likely increases nutritional regulation for neurons and synapses. This result can be explained by two possible mechanisms. First, our vaccine induces a Th2 immune response that inhibits the inflammatory immune response involved in astroglial cell activation. And second, Aβ42 plaque deposition induces activation of glia cells while less deposition in vaccinated mice simultaneously reduces gliosis.

In summary, this study demonstrates that intramuscular administration of our novel vaccine p(Aβ3–10)10-MT by in vivo electroporation in young double transgenic mice can induce high levels of anti-Aβ antibodies that result in a more robust Th2-polarized humoral immune response, as well as decreased Aβ accumulation and deposition in the brain with a resultant decrease in plaque-associated astrocytosis and improved memory and cognition. Thus, immunotherapy with p(Aβ3–10)10-MT represents an effective strategy for ameliorating cognitive impairment and brings hope for the prevention of AD.

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