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Co-immunization with DNA and protein mixture: A safe and efficacious immunotherapeutic strategy for Alzheimer's disease in PDAPP mice

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Active immunotherapy targeting β-amyloid (Aβ) is the most promising strategy to prevent or treat Alzheimer's disease (AD). Based on pre-clinical studies and clinical trials, a safe and effective AD vaccine requires a delicate balance between providing therapeutically adequate anti-Aβ antibodies and eliminating or suppressing unwanted adverse T cell-mediated inflammatory reactions. We describe here the immunological characterization and protective efficacy of co-immunization with a 6Aβ15-T DNA and protein mixture without adjuvant as an AD immunotherapeutic strategy. Impressively, this co-immunization induced robust Th2-polarized Aβ-specific antibodies while simultaneously suppressed unwanted inflammatory T cell reactions and avoiding Aβ42-specific T cell-mediated autoimmune responses in immunized mice. Co-immunization with the DNA + protein vaccine could overcome Aβ42-associated hypo-responsiveness and elicit long-term Aβ-specific antibody responses, which helped to maintain antibody-mediated clearance of amyloid and accordingly alleviated AD symptoms in co-immunized PDAPP mice. Our DNA and protein combined vaccine, which could induce an anti-inflammatory Th2 immune response with high level Aβ-specific antibodies and low level IFN-γ production, also demonstrated the capacity to inhibit amyloid accumulation and prevent cognitive dysfunction. Hence, co-immunization with antigen-matched DNA and protein may represent a novel and efficacious strategy for AD immunotherapy to eliminate T cell inflammatory reactions while retaining high level antibody responses.

Alzheimer's disease (AD) is the most common form of dementia in the elderly. As a neurodegenerative disease, it is a debilitating disorder that can lead to significant cognitive deficits and ultimately lead to complete dependency and death[1–4]. Based on the amyloid cascade hypothesis, production and accumulation of excessive β-amyloid (Aβ) in the brain may be the main cause of AD[1,3,5–7]. Many studies have supported the central role of immunotherapy targeting β-amyloid for AD[1,7–9]. In many reports anti-Aβ42 antibodies were thought to be key for eliminating Aβ load and improving learning and memory performances[10–12]. Fibrillar Aβ42 was formulated with a strong Th1-type adjuvant QS21 as the AN-1792 vaccine and tested in a phase IIa clinical trial. However, this trial was halted when 6% of treated patients developed meningoencephalitis[7,13,14]. The self T cell epitopes in the C-terminal portion of Aβ42 may have induced Aβ42-specific T cell immune responses resulting in meningoencephalitis. Although the AN1792 clinical trial failed, subsequent examination of the patient brains showed a reduction in Aβ load after immunotherapy[5,15].

Due to the potential for autoreactive T cell inflammation causing side effects such as meningoencephalitis, Aβ42 epitope-based vaccines with deleted T epitopes are preferred. Prior analyses of peptide- and DNA-based epitope vaccines suggest that this active immunotherapy strategy for AD would be efficacious and safe[5,7,8,16,17]. Recently, two 6Aβ15-T DNA- and protein-based epitope vaccines derived from the Aβ1-15 B-cell epitope attached to the promiscuous foreign T helper epitope pan HLA DR-binding peptide (PADRE)[18–20] were prepared and assessed as an active immunotherapy strategy for AD in our lab[21,22]. Immunizations with DNA vaccines typically induce low antibody titers, and adjuvants such as QS-21 in peptide/protein vaccines may result in unforeseen adverse side effects[7,13,23,24]. In a heterologous prime-boost immunization regimen for AD immunotherapy[25,26], the first immunization initiates the priming of the immune response and subsequent immunizations would trigger further expansion of antigen-specific immune responses. However, this type of prime-boost

strategy would not avoid the side effects associated with adjuvants such as alum or Quil A accompanying the peptide/protein vaccines, and T cell responses potentially can be auto-reactivated following multiple immunizations.

Here we implemented a novel strategy by immunizing mice with a mixture of DNA and protein without any adjuvants, which could overcome the above mentioned disadvantages and elicit strong antibody responses. Furthermore, this co-immunization approach with DNA and protein vaccines based on the same antigen could suppress T cell-mediated immune responses[27–30], which would be beneficial for AD immunotherapy[31]. Importantly, this suppression had no effect on antibody production. The results showed that immunization with the mixture of 6Aβ15-T DNA and protein-based vaccines could induce high anti-Aβ antibody titers with mild non-self T cell-mediated responses in mice. Moreover, prophylactic active immunization with this DNA and protein vaccine mixture could effectively reduce the amyloid accumulation and prevent the development of behavioral deficits in AD mice without unwanted side effects.

Results

Preparation of DNA, protein and DNA + protein vaccines. Our lab previously constructed a plasmid pVAX1-6Aβ15-T encoding a chimeric *6Aβ15-T* minigene, which contains six copies of Aβ1-15 (6Aβ15) fused with PADRE (T), as a DNA epitope vaccine for AD[21]. A recombinant chimeric antigen 6Aβ15-T expressed and purified in *Escherichia coli* (BL21) was also prepared as a subunit protein vaccine for AD in our lab[22]. In the current study, a novel immunization regimen consisting of a mixture of the pVAX1-6Aβ15-T DNA and 6Aβ15-T recombinant protein antigen was used to immunize mice without adjuvant as a candidate vaccine for AD.

Co-immunization with DNA and protein induces robust Th2-polarized Aβ-specific antibodies and suppresses T cell-mediated inflammatory responses in Balb/c mice. As described in the Methods (Figure 1A), three groups of Balb/c mice were injected with three different vaccine regimens separately. Significantly stronger Aβ42-specific antibody responses were induced in DNA + protein-immunized mice compared with DNA or protein-immunized ones ($p < 0.001$ or $p < 0.01$, respectively). Mice in the co-immunization group (DNA + protein) clearly produced high titers of Aβ42-specific antibodies after the third injection. Antibody titers of mice in the co-immunization group after the third injection were even higher than those of mice in the protein-immunized group after the fourth immunization ($p < 0.05$), while no obvious antibody responses were detected in the DNA-immunized mice (Figure 2A). The detection of antibody subtypes showed that the co-immunization of DNA + protein induced strong Th2-polarized Aβ-specific humoral responses in mice, as was observed with the protein vaccine (Figure 2B).

As expected, these vaccines induced PADRE-specific, but not Aβ-specific T cell immunity, in the immunized mice. Of note, the PADRE-specific T cell immune response was mild in DNA + protein-immunized mice (Figure 2C), with no statistically significant difference between the co-immunization and control vector groups ($p > 0.05$). The level of IL-4 (Th2) was much higher, while the level of IFN-γ (Th1) was much lower, in the co-immunization group compared to the DNA or protein group (Figure 2D, E). Specifically, the highest IL-4 cytokine levels corresponded with the strongest Th2-polarized antibody responses in the co-immunization group. Furthermore, the combined vaccine elicited a low IFN-γ cytokine level, comparable to that in the control group, which was consistent

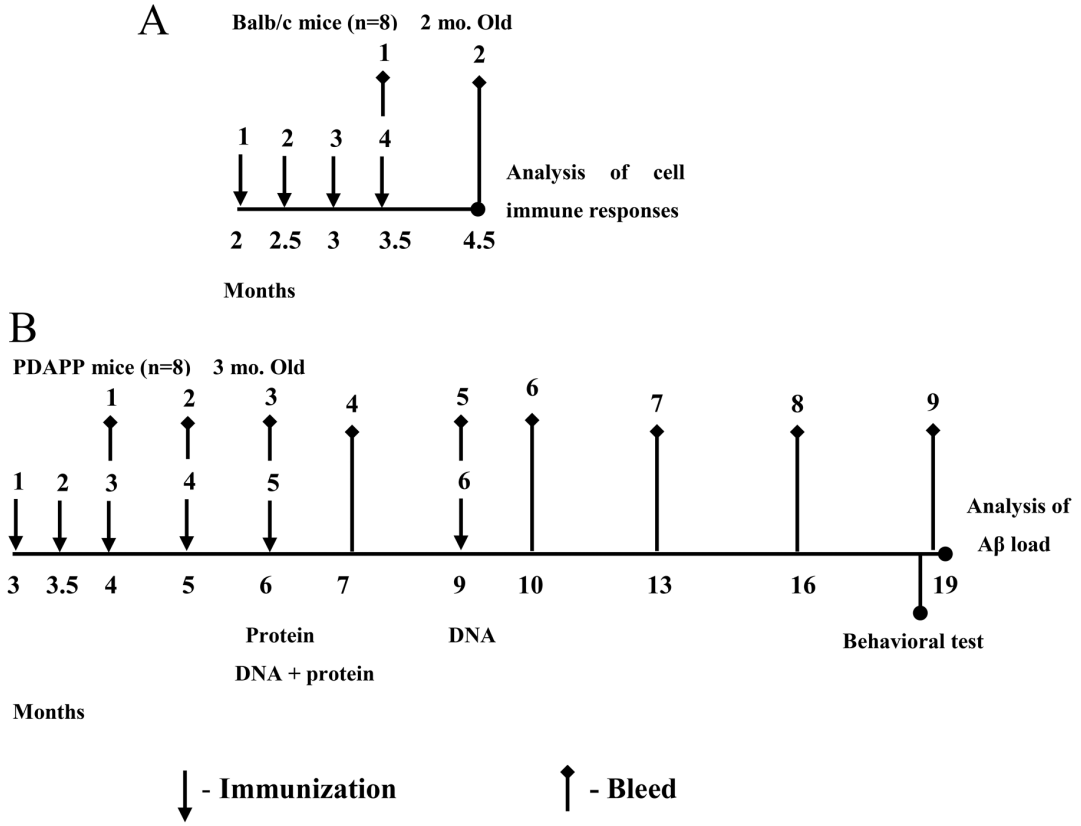

Figure 1 | Immunization protocols for Balb/c and PDAPP mice. (A) Balb/c mice were immunized four times biweekly. Blood was collected after three and four immunizations for analysis of humoral immune responses. Two weeks after the last immunization, mice were sacrificed and cellular immune responses were analyzed in splenocytes. (B) PDAPP mice were immunized five or six times as indicated. Blood was collected, and humoral immune responses were analyzed. Upon completion of the Morris water maze testing of 19-month-old PDAPP mice of experimental and control groups, they were sacrificed and analyzed for neuropathological changes in the brain.

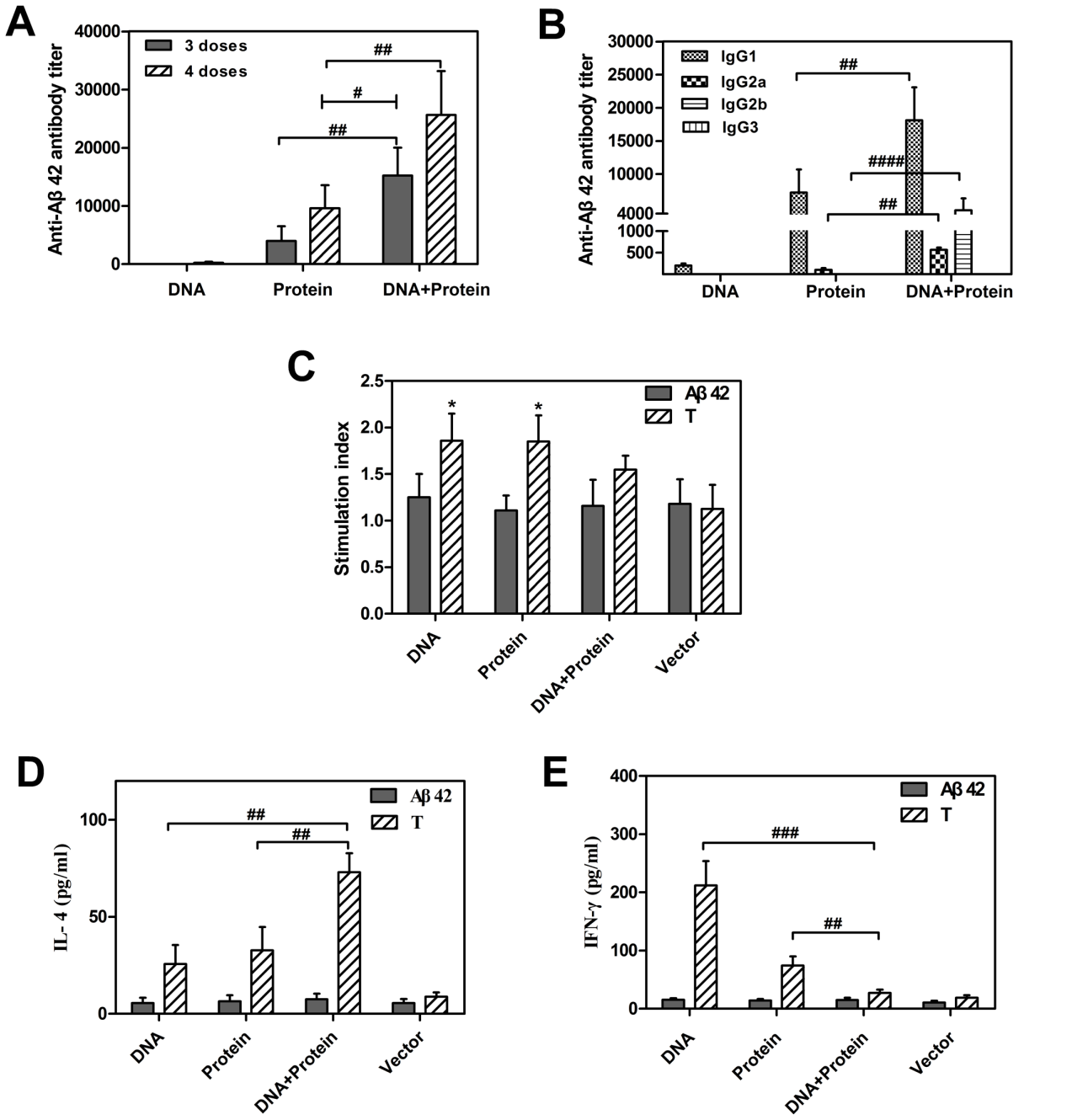

Figure 2 | Co-immunization with DNA and protein mixture induces robust Th2-polarized Aβ-specific antibodies and suppresses T cell-mediated responses in Balb/c mice. (A) Anti-Aβ42 IgG titers in Balb/c mice immunized with three or four doses of pVAX1-6Aβ15-T (DNA), 6Aβ15-T (protein) or pVAX1-6Aβ15-T + 6Aβ15-T (DNA + protein). (B) Anti-Aβ42 IgG isotype (IgG1, IgG2a, IgG2b, and IgG3) titers in mice immunized with DNA, protein or DNA + protein after the fourth injection. (C) T cell proliferation in immunized mice. Splenocytes from mice were restimulated *in vitro* with Aβ42 or PADRE (T) peptide. (C, D) IL-4 and IFN-γ cytokine levels produced by splenocytes in immunized mice. Statistical analysis was performed by ANOVA, and t-tests were used to compare two groups. $^{*}p < 0.05$, compared with control vector group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, compared with other vaccination groups (DNA or protein).

with the induction of a robust Th2-polarized antibody response. The observed low IFN-γ cytokine levels also indicated that co-immunization could suppress T-cell mediated inflammatory responses. Altogether, these results confirmed that the DNA and protein immunogens together could induce robust Th2-polarized Aβ-specific antibodies and suppress T cell-mediated inflammatory responses in mice.

Co-immunization with DNA and protein promotes and prolongs anti-Aβ42 antibody responses in PDAPP mice. The DNA + protein combined vaccine showed good immunogenicity in Balb/c mice, supporting its use as an appropriate candidate for AD immunotherapy in PDAPP model mice (Figure 1B). As shown in Figure 3, very strong and significantly higher anti-Aβ42 antibody responses were detected in the DNA + protein-immunized mice than that in the other two immunization groups ($p < 0.01$). Impressively, the combined vaccine group showed a trend towards relatively stable and adequate antibody levels over a one-year period after the last vaccination. Therefore, co-immunization with the DNA and protein could promote and prolong anti-Aβ42 antibody

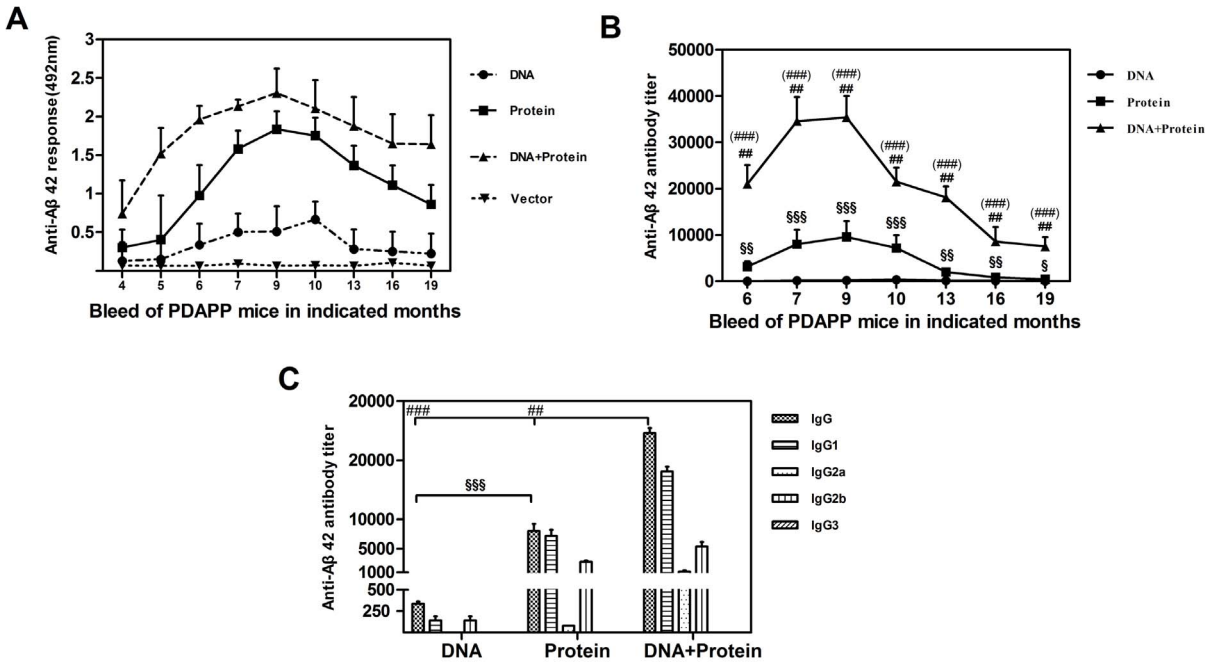

Figure 3 | Duration of DNA + protein-induced immunity in PDAPP AD model mice and IgG isotype profiling after final immunization. (A) Serum samples from PDAPP mice immunized with pVAX1-6Aβ15-T (DNA), 6Aβ15-T (protein), pVAX1-6Aβ15-T + 6Aβ15-T (DNA + protein) or pVAX1 (vector) were collected at various time points, and specific anti-Aβ42 antibody responses were analyzed by a standard single dilution (1 : 100) ELISA. Data are represented as the mean ± SD of n = 8 samples. (B) Anti-Aβ42 IgG titers of sera from immunized PDAPP mice at indicated time points were analyzed by ELISA. (C) Serum antibodies of IgG1, IgG2a, IgG 2b and IgG3 subtypes from immunized PDAPP mice after the final injection were tested, and the GMT was calculated for each group (n = 8). Statistical analysis was performed by ANOVA. $^{\#\#}p < 0.01$, compared with protein vaccination group; $^{\#\#\#}p < 0.001$, compared with DNA vaccination group. $^{\S}p < 0.05$, $^{\S\S}p < 0.01$, $^{\S\S\S}p < 0.001$, compared with DNA vaccination group.

responses in PDAPP mice. Of note, an anti-inflammatory Th2-polarized antibody response characterized by predominant anti-Aβ42 IgG1 was also revealed in the co-immunized PDAPP mice by subtype profiling (Figure 3C), similar to that observed in Balb/c mice.

Co-immunization with DNA and protein significantly prevents cognitive dysfunction of AD model mice. In the behavioral test, the latency to reaching the platform for PDAPP mice immunized with the protein or DNA + protein vaccine significantly decreased on day 3 or 4 of training ($p < 0.05$ or $p < 0.01$, respectively), similar to results with a group of age-matched non-transgenic C57/BL6 mice (non-Tg), when compared to the negative control vector-vaccinated group (vector) or unvaccinated PDAPP mice (PDAPP), but not DNA-immunized PDAPP mice ($p > 0.05$) (Figure 4A). In the probe trials for spatial memory ability, a significantly shorter initial latency to reach the platform was observed in protein or DNA + protein-immunized PDAPP mice when compared to the control PDAPP mice ($p < 0.05$ or $p < 0.01$, respectively, Figure 4B). Notably, the significantly increased number of correct quadrant crosses was only observed in the co-immunized PDAPP mice compared to the control PDAPP mice (Figure 4C, $p < 0.05$). The DNA + protein-immunized PDAPP mice displayed the best behavioral performances, consistent with their production of the highest anti-Aβ42 antibody levels among all groups. Together, the results indicated that co-immunization with the DNA and protein significantly prevented cognitive deficits in PDAPP mice.

Co-immunization with DNA and protein significantly reduces cerebral Aβ burben of AD model mice. To evaluate the Aβ burden in the immunized and control PDAPP mouse brain, immunohistochemistry and quantitative image analysis were performed. As shown in Figure 5, immunization with the protein or DNA + protein vaccine significantly reduced plaque load, as assessed by plaque counts and percent area in the hippocampus and cortex of the brains occupied by plaques of PDAPP mice versus control PDAPP mice ($p < 0.01$ or $p < 0.001$, respectively), while it was non-significantly reduced by DNA immunization alone ($p > 0.05$). Importantly, the hippocampus and cortex Aβ load levels in the co-immunized PDAPP mice were significantly reduced by 44.3% and 50.6%, respectively, compared with that of the control group. Meanwhile, no microhemorrhage was observed in the brain sections from the co-immunized PDAPP mice by hematoxylin and eosin (HE) staining (data not shown).

Moreover, insoluble or soluble Aβ42 and Aβ40 levels of PDAPP mouse brain were assessed by ELISA to investigate whether cerebral Aβ levels were affected by co-immunization. As demonstrated in Figure 6, immunization with protein or DNA + protein vaccine significantly reduced insoluble or soluble Aβ42 and Aβ40 peptides in the brains of PDAPP mice versus control PDAPP mice ($p < 0.01$ or $p < 0.001$, respectively), while it was non-significantly reduced in that of the immunized-DNA mice ($p > 0.05$). Notably, the co-immunization significantly reduced the insoluble or soluble Aβ42 and Aβ40 peptides in the brains of PDAPP mice versus the immunization with DNA or protein alone ($p < 0.01$ or $p < 0.05$, respectively). Therefore, the combined vaccine group had the lowest content of insoluble or soluble Aβ42 and Aβ40 peptides. The same trend was observed with the Aβ plaque load.

Discussion

After the AN-1792 trial was halted, the safety of AD vaccines needed to be addressed, and adverse T cell-mediated autoimmune responses were thought to be induced by T cell epitopes found in the C-terminal of the Aβ42 peptide. Thus, a second generation of AD vaccine mainly targeting N-terminal B-cell epitopes of Aβ42 was developed. This type of vaccine in recent clinical trials, such as

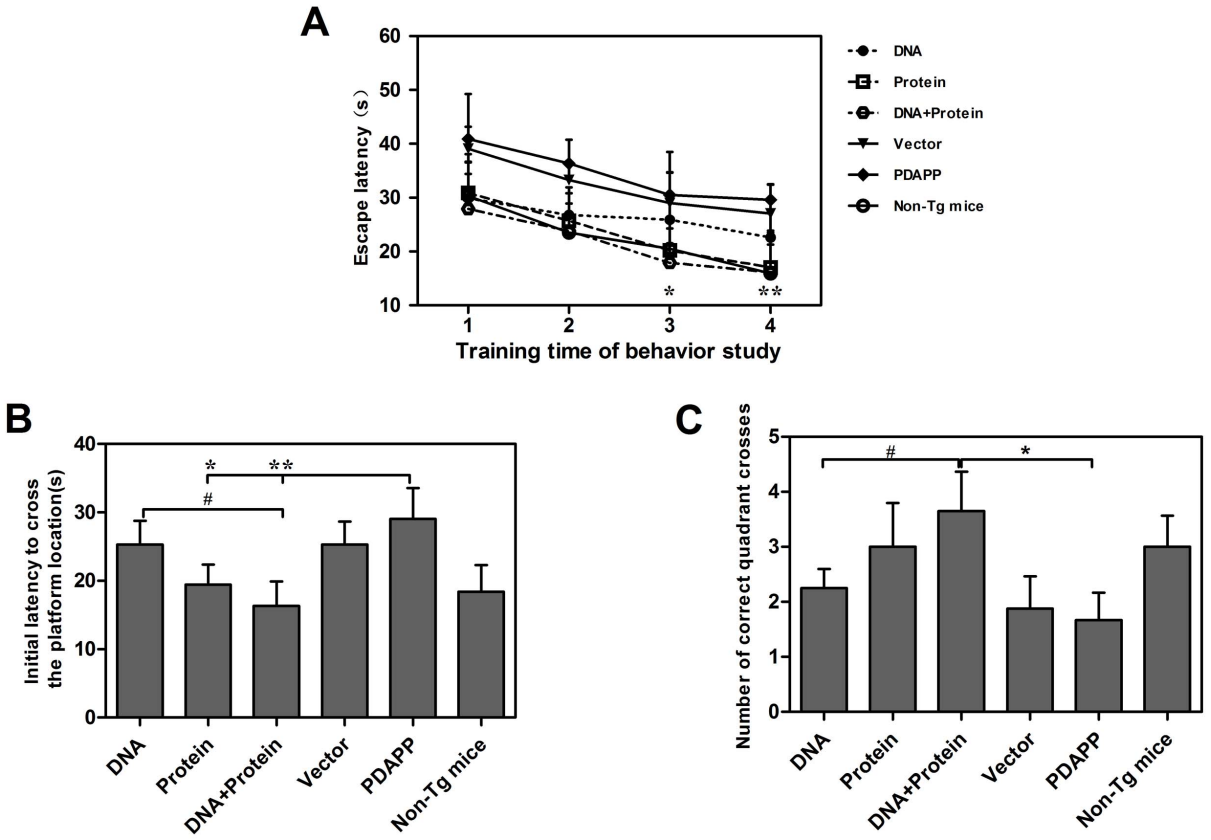

Figure 4 | Co-immunization with DNA and protein mixture significantly improves acquisition and retention of spatial memory in PDAPP mice. (A) Escape latency to reach the platform on days 1–4. (B) Initial latency to reach the platform location in probe trials after the last training trial. (C) Numbers of correct quadrant crosses during the probe trial after the last training trial. Data are represented as the mean ± SD for each group. Statistical analysis was performed by ANOVA, and t-tests were used to compare two groups. $^{*}p < 0.05$, $^{**}p < 0.01$, compared with control groups (vector or PDAPP). $^{\#}p < 0.05$, compared with other vaccination groups (DNA or protein).

ACC-001, CAD106, V950, UB311 and ACI-24, has shown good potential[3,5,16], not only by inducing antibody response but also avoiding adverse T cell-mediated autoimmune responses. Therefore, a safe and effective AD vaccine must adequately provide therapeutic anti-Aβ antibodies but also eliminate or suppress unwanted adverse T cell-mediated immune responses.

Recently, we prepared two 6Aβ15-T DNA- and protein-based epitope chimeric vaccines using six copies of Aβ1-15 fused with PADRE. Immunization with the pVAX1-6Aβ15-T DNA by electrical pulses induced a very low anti-Aβ42 antibody response following six immunizations and did not confer a protective effect in AD model mice, while the Alhydrogel-formulated 6Aβ15-T subunit vaccine produced an adequate anti-Aβ42 antibody response and protective effect in AD model mice. Several studies reported that co-immunization with antigen-matched DNA and protein vaccines could eliminate or suppress inflammatory T cell reactions, but yet retain a high level of antibody production. Therefore, we hypothesized that co-immunization may be an effective strategy for AD immunotherapy to suppress unwanted T-cell mediated immune responses and simultaneously boost beneficial antibody responses. In the current study, a mixture of 6Aβ15-T DNA and protein without alum adjuvant was evaluated as a candidate AD vaccine by assessing its immunological characterization and protective effects. Two strains of mice were used to confirm the effects of this combined vaccine. Our results showed that immunization with the combined vaccine could induce very high Aβ42-specific antibody titers in both Balb/c and PDAPP mice. Thus, it could overcome the immune tolerance and induce long persistence of strong Aβ42-specific antibodies in PDAPP mice. Significantly higher levels of antibody titers were observed in the combined vaccine group than the DNA or protein-immunized groups. Tests of cellular immune responses showed that co-immunization was a safe strategy for AD therapy. Impressively, the combined vaccine group showed suppression of PADRE-specific foreign helper T cell responses and also exhibited no adverse Aβ42-specific T cell-mediated autoimmune responses. Therefore, our results clearly showed that this DNA + protein combined vaccine could induce high antibody titers and simultaneously suppress T cell-mediated responses.

The effectiveness of the combined vaccine in preserving cognitive function also was demonstrated in PDAPP mice. The DNA + protein vaccine significantly reduced Aβ accumulation and prevented the development of behavioral deficits in PDAPP mice without obvious Aβ pathology before immunization (3-month-old mice) compared to the effect of DNA or vector vaccine. Whereas, there is not statistical difference in the number of correct quadrant crosses between the protein vaccine alone and vector control mice ($p > 0.05$). Furthermore, the co-immunization significantly reduced the insoluble or soluble Aβ42 and Aβ40 peptides in the brains of PDAPP mice versus the protein alone immunization. Altogether, the co-immunization displayed the best behavioral performances and the lowest content of insoluble or soluble Aβ42 and Aβ40 peptides, consistent with their production of the highest anti-Aβ42 antibody levels among all groups. The neurotoxic soluble Aβ peptides are thought to be responsible for the disruption of cognitive functions in the onset and progression of AD[3,32,33]. Therefore, the combined vaccine in our study appeared to be beneficial by reducing both plaques and insoluble or soluble Aβ42/40 in 19-month-old PDAPP mice. The results suggested that the therapeutically Aβ-

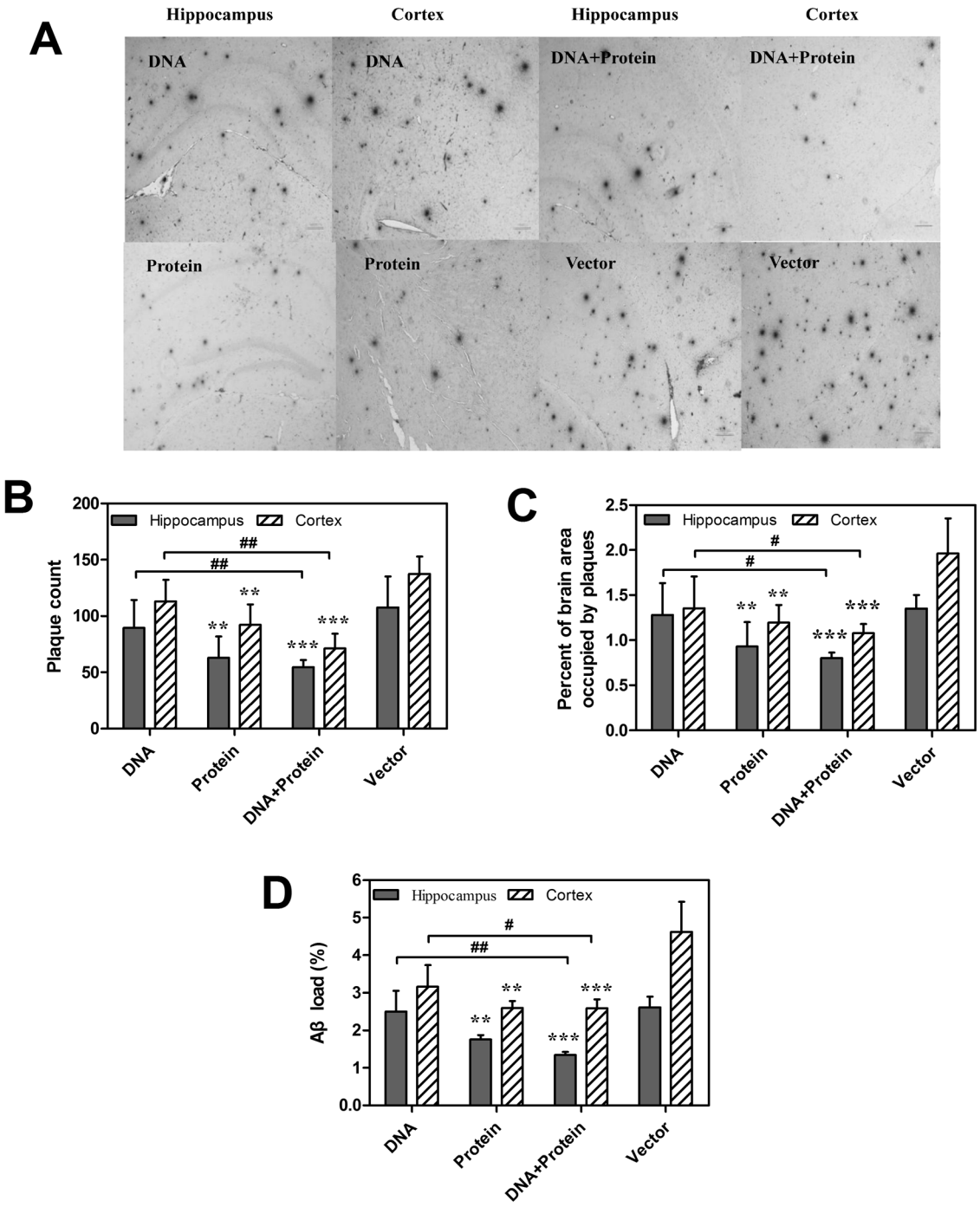

Figure 5 | Plaque count, load and percent of brain area occupied by plaques are significantly decreased in combined vaccine-immunized PDAPP mice. (A) Representative images of distribution of Aβ plaques stained by 6E10 antibody in the hippocampus and cortex. Scale bar, 300 μm. (B–D) Quantification of Aβ plaques in the hippocampus and cortex of brain. Data are represented as the mean ± SD for each group. Statistical analysis was performed by ANOVA, and t-tests were used to compare two groups. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, compared with control vector group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, compared with other vaccination groups (DNA or protein).

specific antibodies produced by co-immunization could prevent or block aggregation of Aβ and further neutralize or clear the soluble Aβ over the immunization period[21,22]. The best behavioral performances by the DNA + protein-immunized PDAPP mice among the vaccinated groups may be attributed to the induction of high anti-Aβ42 antibody levels and resulting low Aβ load in the brain.

The low anti-Aβ42 antibody titers observed in the AN-1792 trial may be due to immune tolerance induced by the Aβ42 self-antigen[1,34–36]. However, the low dose of DNA + protein vaccine with three immunizations effectively elicited high anti-Aβ42 antibody titers (210 ± 50 μg/ml) in PDAPP mice in our study. More importantly, a striking feature of the combined vaccine was the maintenance of high antibody titers which lasted at least 12 months after the last immunization. Therefore, our data clearly indicated that co-immunization with DNA and protein could overcome Aβ42-associated hypo-responsiveness in AD mice. Consistent with the results of pre-clinical and clinical trials[37–39], the presence of high titers of Aβ42-specific antibody in the sera of DNA + protein–immunized PDAPP mice correlated with the reduction of Aβ pathology and was linked to improved cognitive function. Therefore, the long persist-

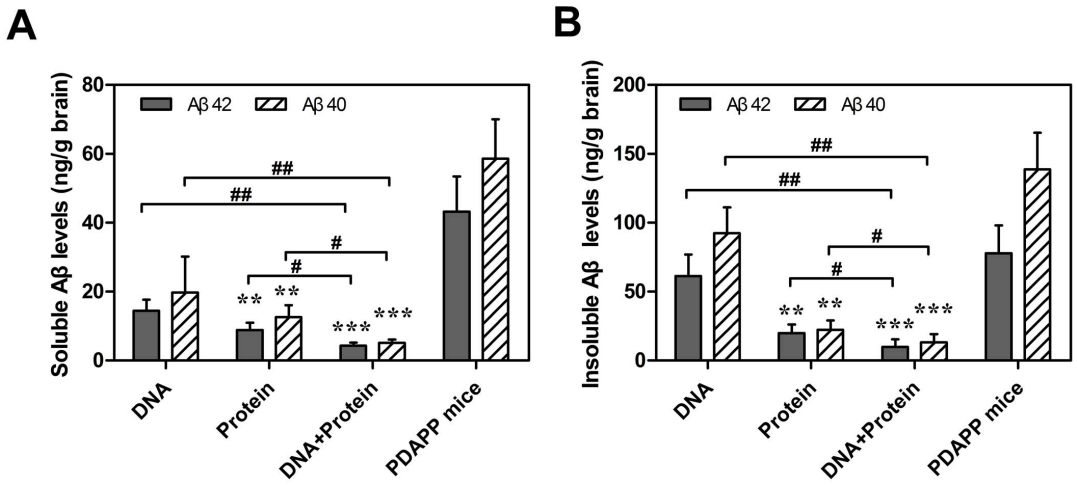

Figure 6 | Combined vaccine significantly reduces soluble and insoluble Aβ levels in brain homogenates of PDAPP mice. (A) Soluble Aβ42 and Aβ40 levels were measured by sandwich ELISA. (B) Insoluble Aβ42 and Aβ40 levels were measured by sandwich ELISA. Data are represented as the mean ± SD for each group (n = 8). Statistical analysis was performed by ANOVA, and t-tests were used to compare two groups. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, compared with control group (PDAPP). $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, compared with other vaccination groups (DNA or protein).

ence of strong Aβ42-specific antibodies helped to maintain antibody-mediated clearance of Aβ42 and accordingly alleviated symptoms of AD in co-immunized PDAPP mice.

The meningoencephalitis occurring in the AN-1792 clinical trial may have resulted from the strong autoimmune T cell responses and inflammation elicited by immunization with Aβ42 formulated in a strong Th1-type QS21 adjuvant. Another AD vaccine trial (ACC-001) was temporarily suspended due to vasculitis (inflammation of the blood vessels) detected in one patient, although it was not clear if the skin lesions were related to the antigen, the alternative adjuvant QS-21 or an entirely unrelated factor[7]. The Th1-type QS21 or ISCOMATRIX adjuvant has been used in other clinical trials such as CAD106, V950, UB311 and ACI-24. While these adjuvants boosted antibody responses, they also elicited strong T-cell responses and induced cytokines such as IFN-γ, which could cause inflammatory side effects. Thus, some safety concerns still remain regarding the use of the strong inflammatory QS21 or other adjuvant.

Previous studies have shown that abnormally high IFN-γ production increases neuronal death in response to amyloid[40,41], and IFN-γ production by Aβ42-specific Th1 cells promotes microglial activation and increases plaque load in AD model mice[42]. Therefore, suppression of Aβ42-specific or non-specific IFN-γ production during AD immunotherapy should be beneficial by reducing amyloid load and improving behavioral performances without pro-inflammatory side effects. In the current study, a DNA + protein combined vaccine without any adjuvant as a novel strategy was tested in Balb/c and PDAPP mice. Our results clearly indicated that the combined vaccine could induce robust Th2-polarized Aβ-specific antibodies, while suppressing or eliminating unwanted adverse T cell-mediated immune responses without Aβ42-specific T-cell mediated autoimmune responses. At the same time, co-immunization reduced the production of inflammatory cytokine IFN-γ and increased the production of IL-4 in $CD4^+$ T cells, suggesting that an anti-inflammatory effect was induced in the co-immunized mice. Likewise, Wang et al. recently reported that co-immunization with Aβ42 peptide and Aβ42-coding DNA ameliorated AD pathology while inhibiting Aβ42-specific T-cell-mediated inflammatory reactions in mice[43], further supporting the feasibility of this approach. Moreover, the lack of adjuvant use with our combined vaccine is another advantage which can reduce potential side effects associated with strong adjuvants as reported in the AN-1792 and ACC-001 trials.

In summary, we have provided proof-of-principle for the feasibility of a second generation AD vaccine by co-immunization with antigen-matched DNA and protein. Our combined vaccine, by inducing an anti-inflammatory Th2-type immune response with high level Aβ-specific antibody and low level IFN-γ production, demonstrated the capacity to inhibit amyloid accumulation and prevent cognitive dysfunction. Indeed, co-immunization with DNA encoding 6Aβ15-T plus 6Aβ15-T protein may serve to effectively provide immunotherapy for AD while eliminating adverse Th1 type autoimmune responses or suppressing inflammatory immune responses. Our study demonstrated that co-immunization without any adjuvant is a safe and efficacious strategy for AD immunotherapy to avoid T cell inflammation while retaining a high level of antigen-specific antibody. Therefore, this combined immunization strategy warrants further assessment in 3xTg-AD mice or large animals with fewer immunizations, and the results of those trials will provide important perspectives to support the development of a DNA and protein co-immunization approach for AD.

Methods

Immunization. Eight-week-old specific pathogen-free female Balb/c mice were purchased from the Beijing Laboratory Animal Center (Beijing, China) and randomly assigned to different treatment groups. Each group of eight mice were intramuscularly (i.m.) injected with 30 μg of plasmid pVAX1-6Aβ15-T[21] (designated "DNA" vaccine group) along with electrical pulses as previously described[44], 5 μg of purified recombinant 6Aβ15-T antigen[22] (designated "protein" vaccine group) diluted in 10% (v/v) Alhydrogel (aluminum hydroxide, Sigma), or a mixture of 30 μg pVAX1-6Aβ15-T and 5 μg 6Aβ15-T without adjuvant (designated "DNA + protein" vaccine group). Injections were administered four times at biweekly intervals (200 μl per injection) as indicated in Figure 1A.

$PDAPP^{V717I}$ transgenic AD model mice (derived from parental C57/BL6 mice)[45] were obtained from the Institute of Experimental Animals, Chinese Academy of Medical Sciences & Peking Union Medical College (Beijing, China). Groups of eight PDAPP mice (3 months old, 28 ± 2 g, female and male in half) were immunized with the same doses of pVAX1-6Aβ15-T (DNA), 6Aβ15-T (protein) or pVAX1-6Aβ15-T + 6Aβ15-T (DNA + protein) as above in Balb/c mice. Three vaccinations were performed at biweekly intervals and two boosters were given with one month intervals for all three vaccine groups. Another booster only for the DNA vaccine group was given three months later as indicated in Fig. 1B. For negative control (vector), mice were injected with 30 μg of pVAX1 vector as above. All animal procedures were conducted with the approval of the Beijing Institute of Biotechnology Institutional Animal Care and Use Committee and were in full compliance with the Committee's guidelines.

Detection of antibody responses. ELISAs were used to test anti-Aβ42 antibodies in sera from mice in the different treatment groups as described previously[21,22]. Briefly, the sera were diluted proportionally before testing, starting at the 100-fold dilution. After incubation with the secondary antibody, reactivity was visualized by adding 100 μl of citrate buffer (pH 5.0) containing 0.04% (w/v) o-phenylenediamine and 0.02% (v/v) hydrogen peroxide for 10 min at room temperature. The reaction was stopped with 50 μl of 2 M H_2SO4, and the absorbance was read at 492 nm using a

Thermo Labsystems microplate reader (Franklin, MA). Serum samples from individual mice were assayed, and the geometric mean titer (GMT) was calculated for each group (n = 8).

Lymphocyte proliferative responses and evaluation of cytokine levels. Spleens were removed from immunized mice two weeks after the last immunization, and splenocytes were resuspended in RPMI 1640 at 2×10^6 cells/ml and plated in 96-well flat-bottom plates in 100 μl per well. Subsequently, 100 μl per well of medium with or without 10 μg/ml Aβ42 and PADRE (T) was added and mixed. After 72 h of incubation, supernatants were harvested, and proliferative responses were measured by using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI) according to the manufacturer's protocol. The stimulation index (SI) was calculated as the ratio of average optical density (OD) value of wells containing antigen-stimulated cells to that of wells containing cells only with medium. Levels of mouse IL-4 and IFN-γ were assayed using commercial ELISA kits according to the manufacturer's protocol (Bender Medsystems, Vienna, Austria). Data are presented as means ± SD of n = 8 samples.

Behavioral test. The Morris water maze test was performed with 19-month-old PDAPP and non-transgenic C57/BL6 mice to assess their spatial learning and memory abilities as described previously[21,22]. Briefly, old mice that had received five or six vaccinations were trained to swim in a water maze apparatus, a circular tank (1.0 m in diameter and 0.5 m high) subdivided into four parts. Each part had a unique marker inside of the tank top edge at designated start locations. The movements of the animal in the tank were recorded by a video camera and analyzed by a computer. Prior to training, the tank was filled with opaque water maintained at 23–25°C, and each mouse was allowed to swim freely for 60 s in the tank without the hidden platform. On each trial, the mouse was released into the tank at one of four designated start locations and allowed to find and climb onto the hidden platform, which was fixed in one part of the tank throughout the learning period. If a mouse failed to find the platform within 60 s, it was manually guided to the platform and allowed to remain there for 15 s. During the learning period, each mouse was subjected to a daily four-trial session for four consecutive days. The escape latency was recorded as the time from being placed into the water to climbing the escape platform during training. Retention (probe trial) of spatial learning was assessed 24 h after the last training trial. The platform was removed from the pool, and each mouse received one 60 s swim probe trial. Mice were monitored with the camera mounted on the ceiling directly over the pool and recorded on videotape for subsequent analysis. The parameters measured during the probe trial were the initial latency to reach the platform location and number of correct quadrant crosses.

Immunohistochemistry and semi-quantitative image analysis. Brains were removed from PDAPP mice after the Morris water maze test and divided sagittally along the interhemispheric fissure. Formalin-fixed, paraffin-embedded hemi-brains were cut into 4-μm sections sagitally across the hemisphere. The dewaxed sections were incubated with the anti-human Aβ monoclonal antibody 6E10 (Covance, Emeryville, CA, 1 : 500). After the incubation and washing, horseradish peroxidase (HRP)-conjugated anti-mouse antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were used to identify 6E10. Bound antibodies on the sections were detected with 0.005% diaminobenzidine (DAB).

Quantitative image analysis of Aβ plaques was performed as previously described[21,22]. Six 4-μm coronal sections at 40-μm intervals for each mouse were immunostained using the monoclonal anti-Aβ antibody 6E10 to assess plaque load. The total number of plaques (across all sections) and the percent of brain area occupied by plaques were determined for each group. Images were captured using a digital camera mounted to a Nikon ECLIPSE Ti-U inverted microscope (Tokyo, Japan). Images were analyzed using NIS-Elements BR3.1 imaging software (Nikon, Instruments Inc., Mellville, NY). For each mouse, quantitative analysis was performed on an area of about 3 × 2.5 mm for each image at 40× magnification by a single examiner blinded to sample identities.

Detection of soluble and insoluble Aβ peptide. Each of the other half brains collected from PDAPP mice were homogenized in 2% SDS solution with protease inhibitor. After ultrasonication, these samples were centrifuged at 100,000 × *g* for 2 h at 4°C. The supernatant was collected to detect soluble Aβ40 or Aβ42. The sediment was treated with 5 M guanidine buffer in order to solubilize the Aβ peptide. Protein concentrations were determined using the BCA Protein Assay Kit (Pierce, Rockford, IL). Aβ42 and Aβ40 ELISA kits (Invitrogen) were utilized according to the manufacturer's instructions to measure Aβ levels in brain homogenates. Aβ levels were calculated as ng/g wet brain.

Statistical analysis. Statistical analyses for all parameters used in immunological, neuropathological and behavioral studies were performed with SPSS software (version 16.0, Chicago, IL). Statistically significant differences between groups were determined using a t-test, or one-way analysis of variance (ANOVA) and Tukey's multiple comparisons post-test. For all tests only data resulting in P values < 0.05 were regarded as statistically significant.

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Author contributions

Y.Z.Y. and Q.X. conceived and designed the experiments. S.L., D.Y.S., H.C.W and Y.Z.Y. performed the experiments. S.L. and Y.Z.Y. analyzed the data. H.C.W. and S.Z.W. contributed reagents/materials/analysis tools. S.L. and Y.Z.Y. wrote the paper.

Additional information

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