

# Oral Immunization with Soybean Storage Protein Containing Amyloid- $\beta$ 4–10 Prevents Spatial Learning Decline

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**Abstract.** Amyloid- $\beta$  (A $\beta$ ) plays a central role in the pathogenesis of Alzheimer's disease (AD). Because AD pathologies begin two decades before the onset of dementia, prevention of A $\beta$  amyloidosis has been proposed as a mean to block the pathological cascade. Here, we generate a transgenic plant-based vaccine, a soybean storage protein containing A $\beta$ 4–10, named A $\beta$ +, for oral A $\beta$  immunization. One mg of A $\beta$ + or control protein (A $\beta$ -) was administered to TgCRND8 mice once a week from 9 weeks up to 58 weeks. A $\beta$ + immunization raised both anti-A $\beta$  antibodies and cellular immune responses. Spatial learning decline was prevented in the A $\beta$ + immunized group in an extended reference memory version of Morris water maze test from 21 to 57 weeks. In Tris-buffered saline (TBS), sodium dodecyl sulfate (SDS), and formic acid (FA) serial extractions, all sets of A $\beta$  species from A $\beta$  monomer, low to high molecular weight A $\beta$  oligomers, and A $\beta$  smears had different solubility in TgCRND8 brains. A $\beta$  oligomers decreased in TBS fractions, corresponding to an increase in high molecular weight A $\beta$  oligomers in SDS extracts and A $\beta$  smears in FA fraction of the A $\beta$ + treated group. There was significant

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inhibition of histological A $\beta$  burden, especially in diffuse plaques, and suppression of microglial inflammation. Processing of amyloid- $\beta$  protein precursor was not different between A $\beta$ <sup>+</sup> and A $\beta$ <sup>-</sup> groups. No evidence of amyloid-related inflammatory angiopathy was observed. Thus, A $\beta$ <sup>+</sup> oral immunization could be a promising, cheap, and long-term safe disease-modifying therapy to prevent the pathological process in AD.

Keywords: Alzheimer's disease, Alzheimer vaccines, amyloid- $\beta$  oligomers, plant, prevention, soybean, spatial memory

## INTRODUCTION

Based on the amyloid cascade hypothesis in Alzheimer's disease (AD) [1, 2], many disease-modifying therapies (DMTs) are now being developed. However, none have succeeded in phase III clinical trials. Although the first clinical trial with an amyloid- $\beta$  (A $\beta$ ) vaccine, AN1792, was stopped because of meningoencephalitis [3], subsequent studies revealed that AN1792 induced anti-A $\beta$  antibodies, removed A $\beta$  accumulations, and slowed the progression rate of cognitive dysfunction [4, 5]. Since then, many trials have attempted to improve the safety of A $\beta$  immunotherapies by avoiding T-cell autoimmune responses [6–8]. In a phase Ib randomized trial, aducanumab, an antibody against aggregated forms of A $\beta$ , reduced A $\beta$  burden accompanied by a slowing of cognitive impairment in prodromal and mild AD patients [9]. A phase II study of A $\beta$  vaccine CAD106 against A $\beta$ <sub>1–6</sub> evoked a strong serological response and demonstrated acceptable safety and tolerability [10]. A phase IIa trial of A $\beta$  vaccine UB-311 against A $\beta$ <sub>1–14</sub> has been started based on favorable phase I trial results [11]. Case studies, the Alzheimer Disease Neuroimaging Initiative (ADNI), and the Dominantly Inherited Alzheimer's Network (DIAN) have shown that AD pathology begins more than 20 years before the onset of dementia [12–14]. For this reason, DMT trials aimed at preventing the onset of AD, such as the DIAN-Trials Unit [15] and Alzheimer's Prevention Initiative (API) [16], are now ongoing.

Mucosal vaccination is the ideal immunization for good accessibility, needle-free delivery, and protective immune responses in both mucosal and systemic immune compartments [17]. This method induces regulatory T cells, leading to a decrease in the systemic T-cell response and increased secretion of immune-inhibitory cytokines. Plants are advantageous platforms for recombinant vaccines because of their low cost, industrial scale production, and the absence of contamination from toxins and pathogens that are produced in bacterial and yeast systems [18]. Although plant-based A $\beta$  vaccines using potatoes, tomatoes, green pepper leaves, rice, and tobacco

have been reported [19–21], their effect on amyloid deposition and learning was examined only in the study with transgenic rice [21]. Booster injections of A $\beta$  peptide were necessary in their procedure, and most transgenic plants did not produce sufficient amounts of A $\beta$ . We have developed an approach based upon an innovative transgenic soybean that produces 870 mg/g of transgenic soybean seed storage protein containing A $\beta$ <sub>4–10</sub> (A $\beta$ <sup>+</sup>), which is sufficient sequence of Th2 epitope [22] for safe oral immunization without T-cell responses and additional A $\beta$  peptide booster injections [23]. Here, we validate the efficacy of A $\beta$ <sup>+</sup> oral vaccines using an AD mouse model, TgCRND8 [24, 25].

## MATERIALS AND METHODS

### *Preparation of transgenic soybean protein A $\beta$ <sup>+</sup>*

Three tandem repeats of the Th2 epitope portion of A $\beta$ <sub>4–10</sub> (FRHDSGY) [22] were inserted into three portions of the flexible disordered regions II–IV of soybean glycinin A1aB1b, a carrier subunit protein of 11S globulins [26] (Fig. 1A). The seed specific glycinin promoter, cDNA for A1aB1b containing A $\beta$ <sub>4–10</sub> sequences or a wild type A1aB1b cassette for controls, and glycinin 1 terminator constructs to plasmids were transformed into soybean immature embryos. A1aB1b with A $\beta$ <sub>4–10</sub> and wild type A1aB1b were expressed in protein storage vacuoles [23]. Purified A1aB1b containing A $\beta$ <sub>4–10</sub>, referred to as A $\beta$ <sup>+</sup>, and wild type control A1aB1b, referred to as A $\beta$ <sup>-</sup> were used.

### *Oral immunization*

TgCRND8 expresses a mutant (K670N/M671L and V717F) human amyloid- $\beta$  protein precursor (A $\beta$ PP) 695 transgene under the regulation of the Syrian hamster prion promoter on a C3H/B6 strain background [24, 25]. TgCRND8 mice show spatial learning deterioration at 3 months of age that are accompanied by both increasing levels of A $\beta$  and increasing numbers of amyloid plaques in the

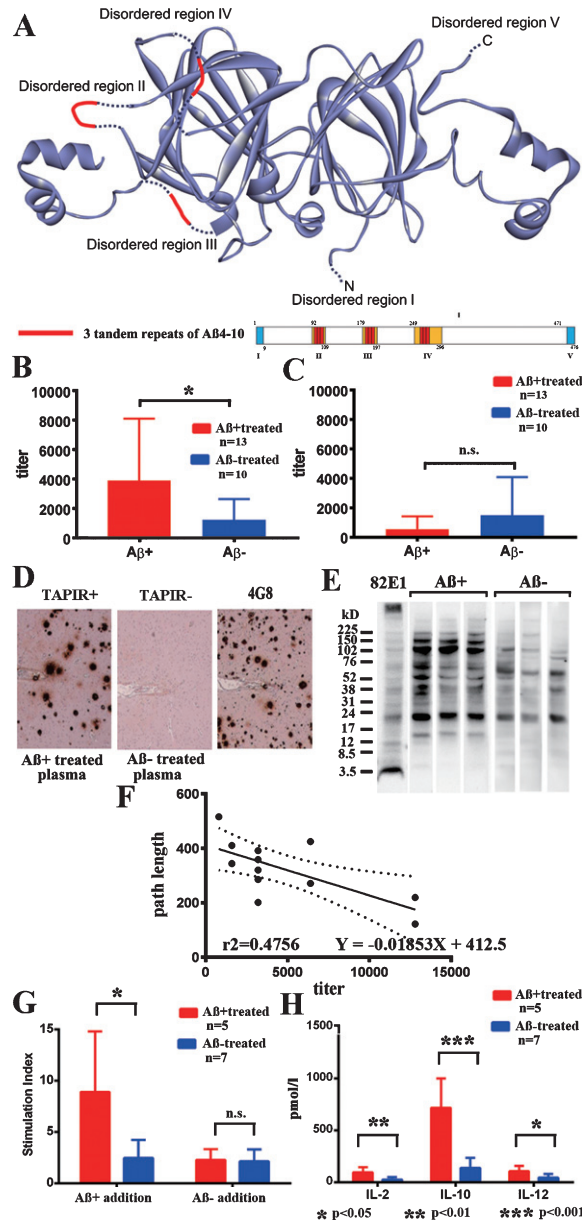


Fig. 1. Transgenic plant protein A1aB1b and immune responses. A) The structure of transgenic plant protein A1aB1b containing A $\beta_{4-10}$  (A $\beta$ +). Three tandem repeats of A $\beta_{4-10}$  were inserted into the three disordered regions of soybean 11S globulin, A1aB1b, that are marked in red. B) IgG antibody titer against A $\beta$ + in plasma from A $\beta$ + immunized mice (red,  $n = 12$ ) significantly increased compared with A $\beta$ - immunized mice (blue,  $n = 10$ ,  $p < 0.05$ ). C) IgG antibody titer against A $\beta$ - in plasma was much less than that against A $\beta$ + and did not differ between A $\beta$ + (red,  $n = 12$ ) and A $\beta$ - (blue,  $n = 10$ ) immunized mice. D) A $\beta$ + immunized mouse plasma diluted with blocking solution (1:2,000) labeled senile plaque A $\beta$  amyloid in an AD brain (TAPIR+; left). Control stain of the AD brain using A $\beta$ - treated mouse plasma (1:2,000, TAPIR-; middle) and anti-A $\beta$  antibody (4G8; right). The figures show representative staining from A $\beta$ + and A $\beta$ - treated mice. E) The SDS fraction of non-oral vaccine treated TgCRND8 mice was stained with plasma (1:400) from A $\beta$ + and A $\beta$ - treated mice. Plasma from A $\beta$ + treated mice showed more staining compared with that from A $\beta$ - treated mice in bands at 16, 40, 56, 102, 150, and 200 kD. F) The antibody titer against A $\beta$ + and average path length to reach the hidden platform during 1–10 days of the last Morris water maze test showed a significant linear regression correlation. Determination coefficients ( $r^2 = 0.4756$ ) and regression equations ( $Y = -0.01853X + 412.5$ ) are shown ( $n = 12$ ;  $n = 6$  for 43 weeks, and  $n = 6$  for 59 weeks). G) Thymidine uptake by stimulation of A $\beta$ + was significantly increased in A $\beta$ + treated mouse splenocytes (red,  $n = 5$ ) compared with those in the A $\beta$ - treated group (blue,  $n = 7$ ,  $p < 0.05$ ). No increase in thymidine uptake by stimulation of A $\beta$ - was shown in both A $\beta$ + and A $\beta$ - treated groups ( $p = 0.628$ ). H) Stimulation of A $\beta$ + significantly increased the amounts of released cytokines, IL-2 ( $p < 0.01$ ), IL-10 ( $p < 0.001$ ), and IL-12 ( $p < 0.05$ ) in A $\beta$ + treated mouse splenocytes (red,  $n = 5$ ) compared with those in A $\beta$ - treated mice (blue,  $n = 7$ ).

brain [24]. One mg of purified A $\beta$ + or control A $\beta$ - with 10  $\mu$ g cholera toxin subunit B (Crucell, Leiden, Netherlands) was administered into the guts via a catheter every week from 9 weeks old until 22~58 weeks old. All animal experiments followed the ARRIVE guidelines, and were approved by the Ethics Committee of Hirosaki University (approval number M13007-1).

#### *Morris water maze (MWM) test*

Memory was evaluated by a spatial reference memory version of the MWM test every 4 weeks, as previously described [24, 25, 27]. Tests began on the first day of 13 weeks old and continued for 9 more consecutive days just 4 weeks after the first oral administration at 9 weeks old. These consecutive 10-day tests were repeated every 4 weeks until 21, 41, and 57 weeks old. The swim path of a mouse during each trial was recorded by a video camera connected to a video tracking system (Noldus EthoVision XT, Noldus Information Technology, Wageningen, Netherlands). The mouse was given 4 consecutive 60 s training trials for 10 days. The location of a hidden escape platform was in the center of one of the pool's quadrants and was left in the same position during 10 consecutive days. Probe trial was administered 24 h after the 10th day of training. During the probe trial, the escape platform was removed from the pool, and the mice were allowed to search the pool uninterrupted for 60 s [24, 27].

#### *Brain preparation*

Under anesthesia with halothane, brains and cerebrospinal fluid (CSF) were collected at 23 weeks (A $\beta$ +  $n$ =9, male 5, female 4, A $\beta$ -  $n$ =11, male 4, female 7), 43 weeks (A $\beta$ +  $n$ =7, male 4, female 3, A $\beta$ -  $n$ =6, male 4, female 2), and 59 weeks (A $\beta$ +  $n$ =6, male 4, female 2, A $\beta$ -  $n$ =7, male 5, female 2) after the last MWM test. Brains were removed and cut into sagittal sections along the midline. One hemisphere was fixed in 4% paraformaldehyde with 0.1 M phosphate-buffered saline (PBS, pH 7.6) for 8 h, and embedded in paraffin. The other half of the brain was fractionated by three sequential extraction steps using Tris-buffered saline (TBS) with protease inhibitors (Complete<sup>®</sup>, Roche Diagnostics, Basel, Switzerland), 2% sodium dodecyl sulfate (SDS) in water with the same protease inhibitors, and then 70% formic acid (FA) in water for biochemical analysis of A $\beta$  species [28, 29].

#### *Immune response to oral administration of A $\beta$ + and A $\beta$ -*

Microplates (MICROLON, Greiner bio-one, Austria) were coated overnight at 4°C with A $\beta$ +, A $\beta$ -, or A $\beta$ 1-42 peptides (0.5  $\mu$ g/well) with 0.01 M PBS (pH 7.4), washed with PBS, and blocked with Blocker Casein in PBS (Thermo Fisher, Waltham, MA). After incubating with plasma samples in each well for 45 min at room temperature and washing, samples were reacted with anti-mouse IgG- or IgA-conjugated horseradish peroxidase (Thermo Fisher) in Blocker Casein PBS at 37°C for 30 min, and color development using 100  $\mu$ l of tetramethylbenzidine for 15 min was performed. H<sub>2</sub>SO<sub>4</sub> was added to stop the reaction, and signals were measured at 450 nm using an ELISA reader.

Splenocytes from 59-week-old mice were isolated, cultured, and restimulated, as previously described [30]. A $\beta$ +, A $\beta$ -, or A $\beta$ 1-42 was added to splenocytes at final concentrations of 100  $\mu$ g/ml in triplicated wells, and 1  $\mu$ Ci of [<sup>3</sup>H]-thymidine was added to cells at 72 h. Cells were harvested after 18 h and thymidine incorporation was measured using a 1450 Microbeta liquid scintillation counter (Perkin Elmer, Waltham, MA). The stimulation index (SI) was calculated using the following formula: counts per minute (CPM) of the well with antigen per CPM with no antigen. An SI index >3 indicates a proliferative cellular immune response of the splenocytes. Supernatants were collected just before the addition of [<sup>3</sup>H]-thymidine and stored at -80°C for cytokine assays. Released cytokines were measured using Mouse Pro-Inflammatory TH1/TH2 9-plex (MesoScale Discovery, Rockville, MD) according to the manufacturer's protocol. The Multi-Spot ELISA plates were precoated with antibodies specific for the following cytokines: interferon- $\gamma$ , Interleukin (IL)-1 $\beta$ , IL-10, IL-12 total, IL-2, IL-4, IL-5, keratinocyte chemoattractant/human growth-regulated oncogene (KC/GRO), and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), and detected with SULFO-TAG detection antibodies. Light emitted upon electrochemical stimulation was read using a SECTOR Imager 2400A (Meso Scale Discovery).

#### *Antibodies for western blot and immunostaining*

The following antibodies to A $\beta$  were used for western blots and immunostaining. Monoclonal antibodies: 82E1 (anti-A $\beta$ 1-16, IBL, Fujioka, Gunma, Japan), BA-27 (anti-A $\beta$ 1-40) [31], BC-05

(anti-A $\beta_{35-43}$ ) [31], 4G8 (anti-A $\beta_{18-22}$ , Signet Lab), and 6E10 (anti-A $\beta_{3-8}$ , Covance Research Products Inc); Polyclonal antibodies: A $\beta$ -N (anti-A $\beta_{1-5}$ , IBL), Ab9204 [32], anti-A $\beta_{40}$  (Cat# 44-348, Thermo Fisher), and anti-A $\beta_{42}$  (Cat#44-344, Thermo Fisher). A monoclonal antibody against A $\beta_{4-10}$ , named PEP3, was newly produced. Other antibodies included Iba1 for microglial markers (Wako Cat# 019-19471), anti-GFAP (Dako Cat# N1506), anti-CD5 (Cat# 550522 Clone 53-7.3, BD Pharmingen, Franklin Lakes, NJ), anti-A $\beta$ PP antibody Saeko (anti-C-terminal 30 amino acids of A $\beta$ PP [29]), anti-mouse tau antibody, TAU-5 (Thermo Fisher Cat# MA1-26600), and PHF-1 against phosphorylated tau (pTau) at serine 396/serine 404 (gift from Davies P).

#### ELISA for levels of A $\beta_{40}$ , A $\beta_{42}$ , A $\beta$ Os, and sA $\beta$ PP

Human  $\beta$  Amyloid ELISA Kits for A $\beta_{x-40}$  and A $\beta_{x-42}$  (294-64701 for A $\beta_{x-40}$ ; 290-62601 for A $\beta_{x-42}$ ; Wako), Human Amyloid  $\beta$  oligomers (82E1-specific) Assay Kit-IBL (#27725, IBL [33]), a Human sAPP $\alpha$  (highly sensitive) Assay Kit (#27734, IBL), and a Human sAPP $\beta$ -sw (highly sensitive) Assay Kit (#27733, IBL).

#### Western blot analysis

All prepared samples were boiled at 70°C for 10 min in SDS sample buffer, separated on a 4–12% NuPAGE Bis-Tris Gel (Cat# NP0321, Thermo Fisher), and electrotransferred to an Immobilon P (MerckMillipore, Burlington, MA) membrane at 100 V for 1.5 h. The signal intensities of proteins labeled using Supersignal (Cat#34076, Thermo Fisher) were quantified using a luminoimage analyzer (LAS 1000-mini, Fuji Film, Tokyo, Japan). A $\beta_{42}$  peptides (Cat#A9810, Sigma-Aldrich, St. Louis, MO) were used as control A $\beta$  peptides.

#### Pathological analysis

Five- $\mu$ m-thick sections were immersed in 0.5% periodic acid to block intrinsic peroxidase, and then treated with 99% FA for A $\beta$  and tau staining for 3 min. After blocking with 5% normal goat or horse serum in 50 mM PBS (pH 7.4) containing 0.05% Tween 20 and 4% Block Ace (Cat# UK-B80, DS Pharma Biomedical, Suita, Osaka, Japan), sections were incubated overnight with the primary antibodies. Specific labeling was visualized using a Vectastain Elite ABC

kit (Vector, Burlingame, CA). Tissue sections were counterstained with hematoxylin. Immunostaining areas of A $\beta$  or Iba1 in 10 randomly selected ROIs (872  $\mu$ m  $\times$  671  $\mu$ m) in the frontal, temporal, and parietal cortex of 3 serial slides were measured in total using Image Pro Plus ver.4.5 (Media Cybernetics, Rockville, MD) after adjustment for artifact staining. The presence of hemorrhage was examined using Berlin blue staining. For the tissue amyloid plaque immunoreactivity (TAPIR) to identify antibody raised against A $\beta$ +, paraffin sections of brains from AD patients and controls were stained with plasma from A $\beta$ + and A $\beta$ - treated mice diluted with blocking solution (1:2,000). Congo red stain was used to stain core plaques.

#### Statistical analysis

Data were expressed as the mean  $\pm$  standard deviation (SD) except for Fig. 2. Two-way analysis of variance (ANOVA), with *post hoc* tests (Bonferroni's multiple comparisons test), was used for analyzing longitudinal alteration. In Fig. 2, data are expressed as the mean  $\pm$  standard error (SE), and two-way repeated ANOVA was used. Mann-Whitney *U* test was applied for comparison between two groups. Prism 7 software (GraphPad, La Jolla, CA) and IBM SPSS Statistics 25 (IBM, Armonk, NY) were used for statistical analyses. A value of  $p < 0.05$  was considered to be significant for all statistical tests.

## RESULTS

#### Oral immunization raised adapted immune responses

Significant positive rates of IgG antibodies against A $\beta$ + were found in the A $\beta$ + treated group (Fig. 1B;  $p < 0.05$ ). IgG antibodies against A $\beta$ - were much lower than those against A $\beta$ +, and there was no difference between the A $\beta$ + and A $\beta$ - treated groups (Fig. 1C). IgG antibodies against A $\beta_{1-42}$  peptides, and IgA antibodies against A $\beta$ +, A $\beta$ -, and A $\beta_{1-42}$  were not detected. Plasma (1:2,000) from 59-week-old A $\beta$ + immunized mice labeled A $\beta$  amyloid plaques in the human AD brain (TAPIR+; Fig. 1D). The SDS fraction of non-oral vaccine treated TgCRND8 mice was stained with plasma (1:400) from A $\beta$ + and A $\beta$ - treated mice. Plasma from A $\beta$ + treated mice showed more staining compared with that from A $\beta$ - treated mice in bands at 16, 40, 56, 102, 150, and 200 kD (Fig. 1E). The

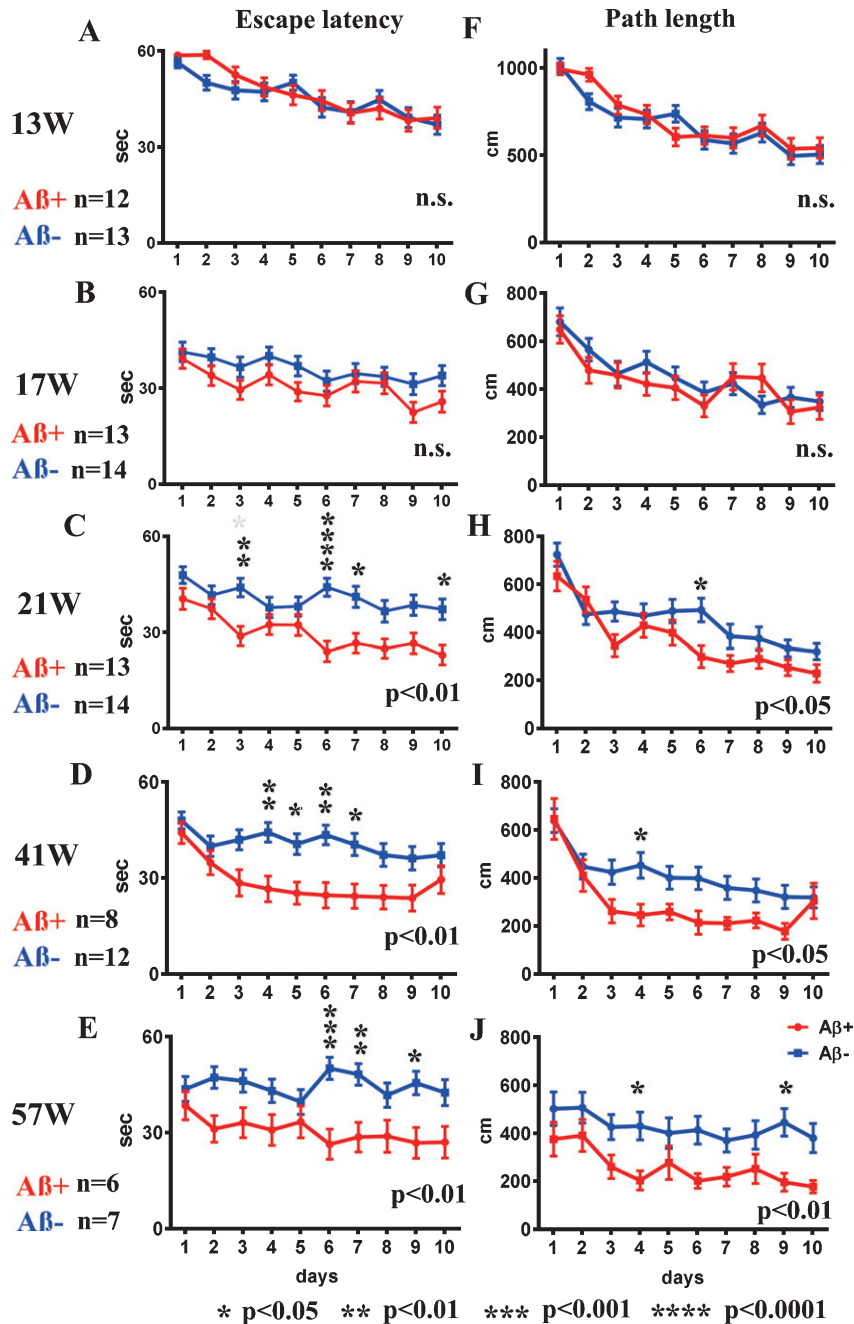


Fig. 2. Spatial reference memory version of the MWM test from 13 to 57 weeks old. A-E) escape latency, and F-J) path-length at 13, 17, 21, 41, and 57 weeks. Escape latency and path-length analyses showed significant improvement in A $\beta$ + treated mice (red line) from 21 weeks old (C, H) compared with A $\beta$ - treated mice (blue line) (escape latency  $p < 0.01$ , path-length  $p < 0.05$ ). A $\beta$ + treated mice continued to show significantly better performances in escape latency and path-length than those of A $\beta$ - treated mice until 57 weeks old ( $p < 0.05$  to 0.01; D, E, I, J). Statistical significance by two-way repeated ANOVA was shown in lower right of each line graph. The asterisk shows the result of *post hoc* analysis at each day. Mann-Whitney U test was applied for comparison between two groups. Analyzed mice numbers are  $n = 12$  for A $\beta$ +,  $n = 13$  for A $\beta$ - at 13 weeks;  $n = 13$  for A $\beta$ +,  $n = 14$  for A $\beta$ - at 17, 21 weeks;  $n = 8$  for A $\beta$ +,  $n = 12$  for A $\beta$ - at 41 weeks; and  $n = 6$  for A $\beta$ +,  $n = 7$  for A $\beta$ - at 57 weeks.

antibody titer against A $\beta$ + and average path length to reach the hidden platform during 1–10 days of the last Morris water maze test showed a significant linear regression correlation (Fig. 1F;  $p < 0.05$ ). This finding suggested a close correlation between evoked anti-A $\beta$  oligomers antibody titers and preservation of special learning ability, as shown in the Fig. 2.

Splenocytes from the A $\beta$ + treated group showed significant proliferation against A $\beta$ + addition compared with those of the A $\beta$ - treated group (Fig. 1G;  $p < 0.05$ ). A $\beta$ - treated splenocytes did not react with both A $\beta$ + and A $\beta$ - addition. Significantly increased levels of IL-2, IL-10, and IL-12 were revealed in the media from the A $\beta$ + treated group compared with the A $\beta$ - treated group ( $p < 0.01$  for IL-2,  $p < 0.001$  for IL-10, and  $p < 0.05$  for IL-12). The levels of interferon- $\gamma$ , IL-1 $\beta$ , IL-4, IL-5, KC/GRO, and TNF- $\alpha$  were not different between the groups. IL-10 levels were markedly increased compared with those of IL-12, suggesting inhibition of proinflammatory cytokines and the predominance of a Th2 response (Fig. 1H).

#### *A $\beta$ + prevented spatial learning decline*

In the first trial at 13 weeks, there were no differences between A $\beta$ + and A $\beta$ - treated mice in escape latency or path-length to reach the hidden platform (Fig. 2A and F). For escape latency and path-length analysis, improvements due to learning effects were recognized in the second trial in both groups (Fig. 2B, G). The escape latency and path-length in A $\beta$ + treated mice were significant shorter than those of the A $\beta$ - treated group at 21 weeks old (escape latency Fig. 2C;  $p < 0.01$ , path-length Fig. 2H;  $p < 0.05$ ) suggesting improved learning in A $\beta$ + treated mice. A $\beta$ + treated mice continued to show significantly better performances than A $\beta$ - treated mice until 57 weeks old (Fig. 2D, E, I, and J;  $p < 0.05$ – $0.01$ ). There were no significant differences in the probe trials.

#### *Decreased soluble A $\beta$ O<sub>s</sub> by ELISA*

Almost all A $\beta$ <sub>40</sub> and A $\beta$ <sub>42</sub> monomers accumulated in the SDS and FA fractions (Fig. 3B, C, E, F). In the TBS, SDS, and FA soluble fractions, the amount of A $\beta$ <sub>40</sub> and A $\beta$ <sub>42</sub> did not differ between the A $\beta$ + and A $\beta$ - groups at any of the ages tested (Fig. 3A, B, D–F), except for increased A $\beta$ <sub>40</sub> in the FA fraction of A $\beta$ + group (Fig. 3C;  $p < 0.05$ ). A $\beta$ O<sub>s</sub> measured by 82E1/82E1 ELISA longitudinally showed that the amount of A $\beta$ O<sub>s</sub> in the TBS fraction was significantly

decreased in the A $\beta$ + treated group compared with the A $\beta$ - treated group (Fig. 3G;  $p < 0.0001$ ). A $\beta$ O<sub>s</sub> ELISA showed trace amounts of soluble A $\beta$ O<sub>s</sub> in both the SDS and FA extracted fractions compared with the amounts of A $\beta$ <sub>40</sub> and A $\beta$ <sub>42</sub> in the A $\beta$ + and A $\beta$ - groups, and no difference between the A $\beta$ + and A $\beta$ - treated groups (Fig. 3H, I).

#### *Longitudinal appearance of A $\beta$ O<sub>s</sub> species in TgCRND8 without oral immunization trial*

Since 82E1/82E1 ELISA could only measure some A $\beta$ O<sub>s</sub> species, the basic age-dependent presence of all A $\beta$  species were directly examined by western blots in 3-step brain extracts from TgCRND8 at 13, 23, 43, and 59 weeks of age. In TBS fractions, A $\beta$  monomers were labeled by 82E1 from 13 weeks, and the amount increased with age (Fig. 4A, D). A $\beta$ O<sub>s</sub> except high molecular weight (HMW) A $\beta$ O<sub>s</sub> larger than 200 kDa were difficult to detect in this fraction because of the existence of mouse IgGs, as shown in lane 59N from a non-transgenic TgCRND8 littermate at 59 weeks old (Fig. 4A, G). In SDS fractions, however, there were marked A $\beta$  monomers, low molecular weight (LMW) A $\beta$ O<sub>s</sub> (di-, tri-, tetra-, and A $\beta$ \*56 [34], and HMW A $\beta$ O<sub>s</sub> larger than 200 kDa. Respective A $\beta$ O<sub>s</sub> species increased with age (Fig. 4B, E, H). In FA fractions, A $\beta$  monomers, A $\beta$  dimers and diffuse smear patterns were observed (Fig. 4C, F, I). Many A $\beta$  species with different solubility and aggregation properties were accumulated from the early period in TgCRND8 brains, suggesting that 82E1/82E1 ELISA detected only some of the accumulated A $\beta$ O<sub>s</sub>. A $\beta$  monomers increased with age in the TBS, SDS, and FA fractions, HMW oligomers increased with age in the TBS and SDS fractions, and A $\beta$  smear increased in the FA fractions, although not significantly with the small sample size.

4G8, anti-A $\beta$ <sub>40</sub>, and anti-A $\beta$ <sub>42</sub> weakly detected LMW A $\beta$ O<sub>s</sub>, but HMW A $\beta$ O<sub>s</sub> could not be detected (Fig. 4J–L). These findings suggest that the C-terminal site of A $\beta$  caused conformational changes in A $\beta$  when incorporated into HMW large assemblies, leading to C-terminal epitope blockade of A $\beta$  species including A $\beta$ <sub>40</sub> and A $\beta$ <sub>42</sub>. In support of this, western blots of aggregated synthetic A $\beta$ <sub>42</sub> showed that N-terminus antibodies (Ab9204, A $\beta$ -N, and PEP3) clearly detected HMW A $\beta$ O<sub>s</sub>; however, antibodies against the mid portion (6E10 and 4G8) and C-terminus 42 (anti-A $\beta$ <sub>42</sub> and BC-05) of A $\beta$  could not detect HMW A $\beta$ O<sub>s</sub> (Fig. 4M). Antibodies against C-terminus 40 (anti-A $\beta$ <sub>40</sub>, BA-27) did not detect

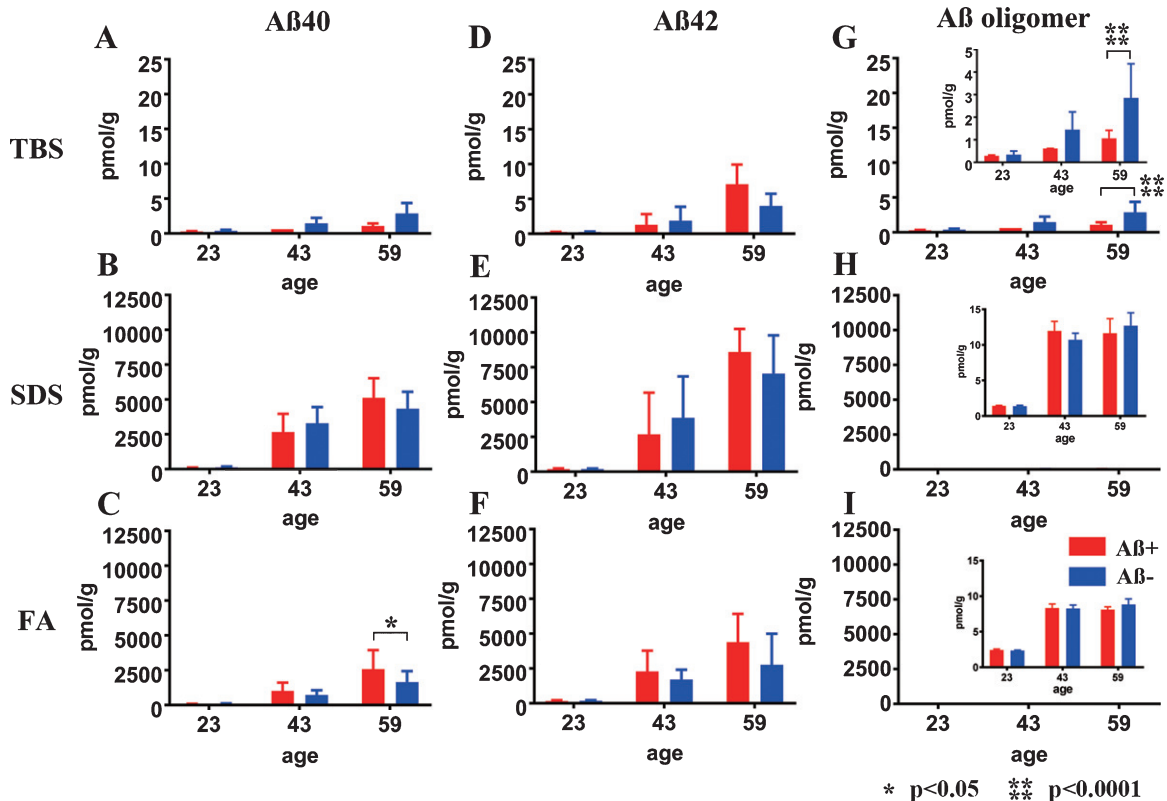


Fig. 3. Longitudinal change in A $\beta_{40}$ , A $\beta_{42}$ , and A $\beta$ O in TBS, SDS, and FA fractions from A $\beta^+$  or A $\beta^-$  treated mouse brains. Amounts of A $\beta_{x-40}$  (A–C), A $\beta_{x-42}$  (D–F), and A $\beta$ O (G–I) in TBS, SDS, and FA fractions of A $\beta^+$  (red) and A $\beta^-$  (blue) immunized mouse brains measured using ELISA at 23, 43, and 59 weeks old. There were no significant differences in the levels of A $\beta_{x-40}$  (A, B) and A $\beta_{x-42}$  (D–F) between A $\beta^+$  and A $\beta^-$  treated groups at any time points or in any fractions, except for the A $\beta_{x-40}$  in the FA fraction at 59 weeks old (C: \* $p < 0.05$ ). Significant suppression of A $\beta$ O in TBS soluble fractions was revealed in A $\beta^+$  treated mice compared with A $\beta^-$  treated mice (G and enlarged illustration, \*\*\* $p < 0.0001$ ). Amounts of A $\beta$ O in SDS and FA fractions were very low compared with A $\beta_{40}$  and A $\beta_{42}$  in the same fraction (H, I). Mice at 23 weeks ( $n = 9$  for A $\beta^+$ ,  $n = 11$  for A $\beta^-$ ), 43 weeks ( $n = 7$  for A $\beta^+$ ,  $n = 6$  for A $\beta^-$ ), and 59 weeks ( $n = 6$  for A $\beta^+$ ,  $n = 7$  for A $\beta^-$ ) were measured by ELISA.

synthetic A $\beta_{42}$ . The C-terminal epitopes of HMW A $\beta$ O were blocked resulting no staining by C-terminus-specific antibodies. Immunostaining of TgCRND8 brains showed decreased immunostaining by anti-A $\beta_{40}$  or anti-A $\beta_{42}$  compared with that by A $\beta$ -N (Fig. 4N). C-terminal epitope blockade of HMW A $\beta$ O is one reason for the decrease of A $\beta$  burden labeled by anti-C terminal antibodies.

#### Western blotting of A $\beta^+$ treated brains

Based on these analyses, A $\beta$  species in three fractions of both A $\beta^+$  and A $\beta^-$  groups were analyzed by western blots using 82E1 at 23, 43, and 59 weeks of age. In TBS fractions, soluble A $\beta$  monomers in the A $\beta^+$  treated group were decreased at 23, 43, and 59 weeks. Trace amounts of HMW A $\beta$ O were detected at 59 weeks; however, there was no differ-

ence between the A $\beta^+$  and A $\beta^-$  groups (Fig. 5A–C). In SDS fractions, A $\beta$  monomers and LMW A $\beta$ O were detected equally at 23, 43, and 59 weeks. However, accumulation of HMW A $\beta$ O was slightly increased in the A $\beta^+$  treated group at 23, 43, and 59 weeks (Fig. 5D–F). In FA fractions, monomers and dimers of A $\beta$  increased with age equally in both groups (Fig. 5G–I). Smear patterns of A $\beta$  were markedly observed in the A $\beta^+$  group at 59 weeks of age (Fig. 5I). Using quantification of the bands, TBS soluble A $\beta$  monomer decreased significantly in A $\beta^+$  treated groups by two-way ANOVA ( $p < 0.001$ , Fig. 5J). Using *post-hoc* analysis, significance was detected at 59 weeks ( $p < 0.001$ , Fig. 5J). SDS soluble HMW oligomers and A $\beta$  smear in the FA fraction increased significantly in A $\beta^+$  treated groups by two-way ANOVA (Fig. 5N,  $p < 0.01$  for SDS soluble HMW oligomers, and Fig. 5O,  $p < 0.0001$  for



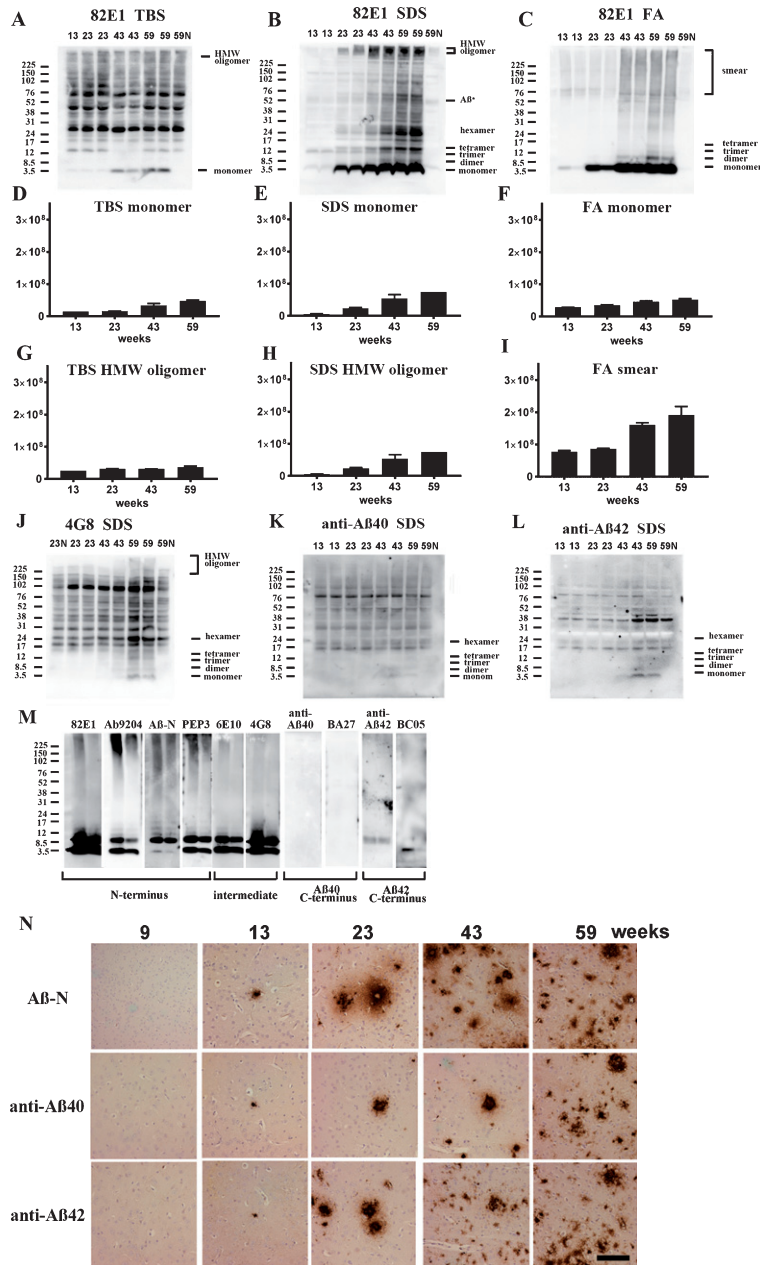


Fig. 4. Age-dependent increase of A $\beta$  monomer and A $\beta$  oligomers in brains of TgCRND8 without an oral immunization (A–L), antibody epitope mapping of aggregated synthetic A $\beta$ <sub>1–42</sub> (M), and immunostaining of TgCRND8 brains without an oral immunization trial (N). D–I are quantification of A $\beta$  monomer in each fraction (D–F), HMW oligomers in TBS and SDS fraction (G, H), and A $\beta$  smear in the FA fraction (I). 59N indicates nontransgenic littermates at 59 weeks. A) In TBS fractions, A $\beta$  monomers were detected by 82E1 from 13 weeks and the amount increased with age (D). Other molecular weight oligomers except HMW oligomers (G) were difficult to detect because of the existence of mouse IgGs. B) In SDS fractions, A $\beta$  monomers and A $\beta$ O<sub>s</sub>, including di-, tri-, tetra-, and A $\beta$ \*56, and HMW A $\beta$ O<sub>s</sub>, were visualized from 13 weeks. Respective species increased with age (E, H). C) In FA fractions, A $\beta$  monomers from 13 weeks, A $\beta$  dimers from 43 weeks, and diffuse smear patterns from 43 weeks were found (F, I). J–L) 4G8, anti-A $\beta$ <sub>40</sub>, and anti-A $\beta$ <sub>42</sub> weakly detected LMW A $\beta$ O<sub>s</sub>, but could not detect HMW A $\beta$ O<sub>s</sub>. M) HMW A $\beta$ O<sub>s</sub> were detected by antibodies against the N-terminus (82E1, Ab9204, A $\beta$ -N, and PEP3), and were weakly detected by antibodies against the mid portion of A $\beta$  (6E10 and 4G8), but were not detected by anti-A $\beta$ <sub>42</sub> and BC-05. Anti-C-terminus to A $\beta$ <sub>40</sub> (anti-A $\beta$ <sub>40</sub> and BA-27) did not detect A $\beta$ <sub>1–42</sub>. N) Immunostaining of TgCRND8 brains using A $\beta$ -N (1:1000), anti-A $\beta$ <sub>40</sub> (1:1000), and anti-A $\beta$ <sub>42</sub> (1:400) showed age-dependent A $\beta$  deposition. A $\beta$  burden labeled by anti-A $\beta$ <sub>40</sub> and anti-A $\beta$ <sub>42</sub> were weaker than those by A $\beta$ -N. Bar represents 100  $\mu$ m. One mouse at 13 weeks, 2 mice at 23 weeks, 43 weeks, and 59 weeks were used for analysis.

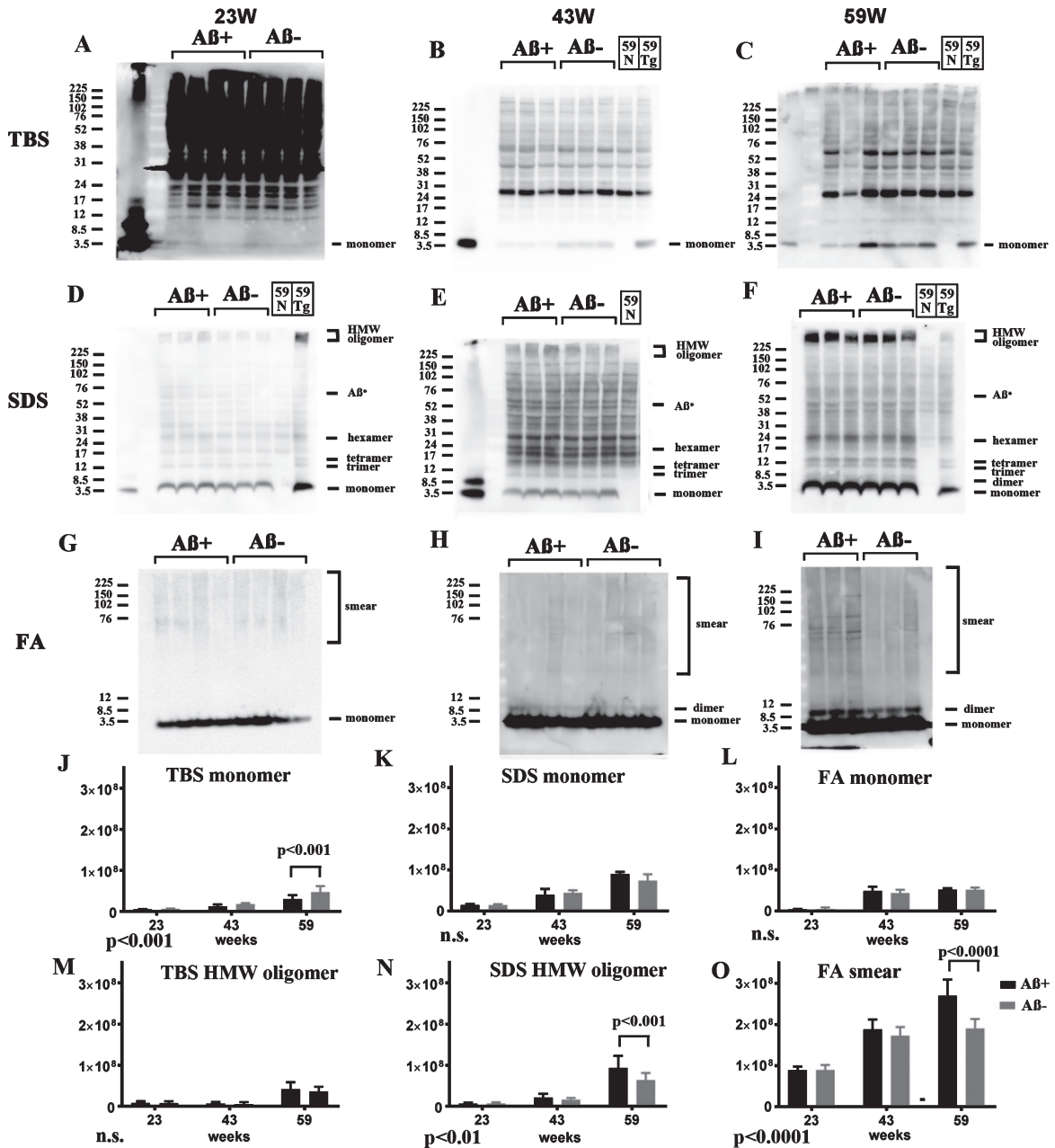


Fig. 5. Longitudinal comparison of A $\beta$  species (23, 43, and 59 weeks) in TBS (A–C), SDS (D–F), and FA (G–I) extracts from A $\beta$ + and A $\beta$ - treated mice in western blots using 82E1. For controls, a nontransgenic mouse at 59 weeks (59N) and a TgCRND8 mouse at 59 weeks without treatment (59Tg) were used. A $\beta$  monomer in each fraction (J–L), HMW oligomers in the TBS fraction (M), and SDS fraction (N), and A $\beta$  smear in the FA fraction (O) were quantified. The results of two-way ANOVA are shown in the lower left of each figure (J–O). In TBS fractions, soluble A $\beta$  monomers in A $\beta$ + treated mice were decreased compared with those in A $\beta$ - treated mice at 23, 43, and 59 weeks (A–C, J). To detect A $\beta$  monomers clearly at 23 weeks, the membrane was exposed for longer than the other blots (A). Small amounts of HMW A $\beta$ O were detected only at 59 weeks, but they did not differ between the A $\beta$ + and A $\beta$ - groups (A–C, M). In SDS fractions, A $\beta$  monomers and LMW A $\beta$ O were detected equally at 23, 43, and 59 weeks (D–F, K). Accumulation of HMW A $\beta$ O was inversely increased in the A $\beta$ + treated group at 23, 43, and 59 weeks (D–F, N). In FA fractions, monomers and dimers of A $\beta$  increased with age equally in both groups (G–I, L). However, smear patterns of A $\beta$  were markedly visualized in the A $\beta$ + group at 59 weeks (G–I, O). Mice at 23 weeks ( $n = 9$  for A $\beta$ +,  $n = 11$  for A $\beta$ -), 43 weeks ( $n = 7$  for A $\beta$ +,  $n = 6$  for A $\beta$ -), and 59 weeks ( $n = 6$  for A $\beta$ +,  $n = 7$  for A $\beta$ -) were used.

A $\beta$  smear). By *post-hoc* analysis significance was detected at 59 weeks ( $p < 0.001$  for SDS soluble HMW oligomers, Fig. 5N, and  $p < 0.0001$  for A $\beta$  smear, Fig. 5O). HMW oligomers in the TBS fraction and A $\beta$  monomer in the SDS and FA fractions did not differ between the A $\beta$ + and A $\beta$ - groups (Fig. 5M,K,L). Since TBS soluble A $\beta$ Os detected by 82E1/82E1 ELISA seem to be recovered as A $\beta$  monomers in western blots using SDS sample buffer, these results correspond to A $\beta$ Os ELISA result that A $\beta$ + immunization suppressed soluble A $\beta$ Os. Furthermore, it was suggested that A $\beta$ + immunization accelerated the conversion of LMW A $\beta$ Os to HMW A $\beta$ Os in SDS fractions, and finally increased smear A $\beta$  accumulation in most insoluble FA fractions.

#### *Suppressed A $\beta$ -immunoreactive load and adverse reaction*

Age-related A $\beta$ -immunoreactive load measured using the total area of A $\beta$ -N immunostaining was significantly suppressed in the A $\beta$ + treated group compared with that of the A $\beta$ - group (Fig. 6A, C;  $p < 0.0001$ ). Congo red staining showed that most A $\beta$  deposition was Congo red-negative diffuse plaques (Fig. 6A). Separate evaluation of core plaques (Fig. 6D;  $p < 0.0001$ ) and diffuse plaques (Fig. 6E;  $p < 0.0001$ ) revealed that a large part of the age-related increase in the area of amyloid burden and also decrease by immunization consisted of diffuse plaques. Immunostaining by anti-A $\beta$ <sub>40</sub> or anti-A $\beta$ <sub>42</sub> also showed significant suppression of amyloid burden in the A $\beta$ + group compared with the A $\beta$ - group (Fig. 6F;  $p < 0.001$ , 6G  $p < 0.0001$ ).

Microglial burden based on Iba1 labeling was significantly decreased in the A $\beta$ + treated group compared with the A $\beta$ - treated group (Fig. 6B, H;  $p < 0.0001$ ). Astrocytosis detected using an anti-GFAP antibody was not different between both groups of mice at any age. Both infiltration of CD5-positive lymphocytes or macrophages, and microhemorrhage by Berlin blue staining, were not detected in both A $\beta$ + and A $\beta$ - treated mice in any examined brain sections (data not shown).

#### *No alteration in A $\beta$ PP processing and tau*

Full length A $\beta$ PP, C-terminal fragments of A $\beta$ PP (CTFs) showed no differences between the A $\beta$ + and A $\beta$ - groups from 23 to 59 weeks (Fig. 6I). The amount of  $\alpha$ - and  $\beta$ -cleaved soluble A $\beta$ PP (sA $\beta$ PP $\alpha$ , sA $\beta$ PP $\beta$ ) detected by ELISA also showed no differ-

ences in both groups during 23–59 weeks (Fig. 6J, K). Levels of the total tau and pTau amount (Fig. 6L, M) did not differ between the A $\beta$ + and A $\beta$ - treated groups at all ages. Additionally, there were almost no bands of pTau in western blots of FA extracts of TgCRND8 brains, corresponding with the lack of neurofibrillary tangles in TgCRND8 brains by Gallyas silver staining (data not shown).

#### *CSF A $\beta$ <sub>40</sub> and A $\beta$ <sub>42</sub>*

A $\beta$ <sub>40</sub> and A $\beta$ <sub>42</sub> in CSF decreased with aging. They were decreased in A $\beta$ + treated mice compared with A $\beta$ - treated mice, although there was no significant difference between A $\beta$ + and A $\beta$ - mice with the small sample size (A $\beta$ +  $n = 7$  for 23 weeks,  $n = 4$  for 43 weeks and  $n = 6$  for 59 weeks; A $\beta$ -  $n = 9$  for 23 weeks,  $n = 2$  for 43 weeks, and  $n = 4$  for 59 weeks) because of the difficulty in drawing CSF from mice (Fig. 6N,O).

## DISCUSSION

To evaluate the potential utility of A $\beta$ + oral immunization to prevent cognitive decline in TgCRND8, we adopted an extended reference memory version of the MWM test, requiring hippocampus-dependent spatial working memory, with a longitudinal design that mimicked human clinical trials [24]. At 21 weeks, escape latencies and path-lengths became worse in the A $\beta$ - group, whereas the A $\beta$ + group remained in the same levels. The performances of the A $\beta$ + group were significantly better during 21–59 weeks than those in the A $\beta$ - group. Our extended MWM test study showed the preventative efficacy of A $\beta$ + oral immunization on A $\beta$ -related learning impairment. As previously indicated [35], probe tests were inadequate for extended MWM test which consisted of extensive long-time and repeated behavioral evaluation. The same repeated trials in every 4 weeks in A $\beta$  immunization trial of TgCRND8 mice also failed to show significant difference in probe trials [24]. Extensive overtraining by trials for a long duration may achieve the saturated level of memory, and may make it difficult to detect subtle changes in probe trials even among hippocampal damaged animals [36, 37]. Recent cohort observation studies have confirmed that A $\beta$  amyloidosis caused impairment of episodic memory in the preclinical stage, and executive and global functions in the symptomatic stage before onset of dementia [38–40]. Rigorous and detailed cognitive assessments to repeatedly evaluate subtle cognitive impairments over a long duration are necessary for preclinical prevention trials such

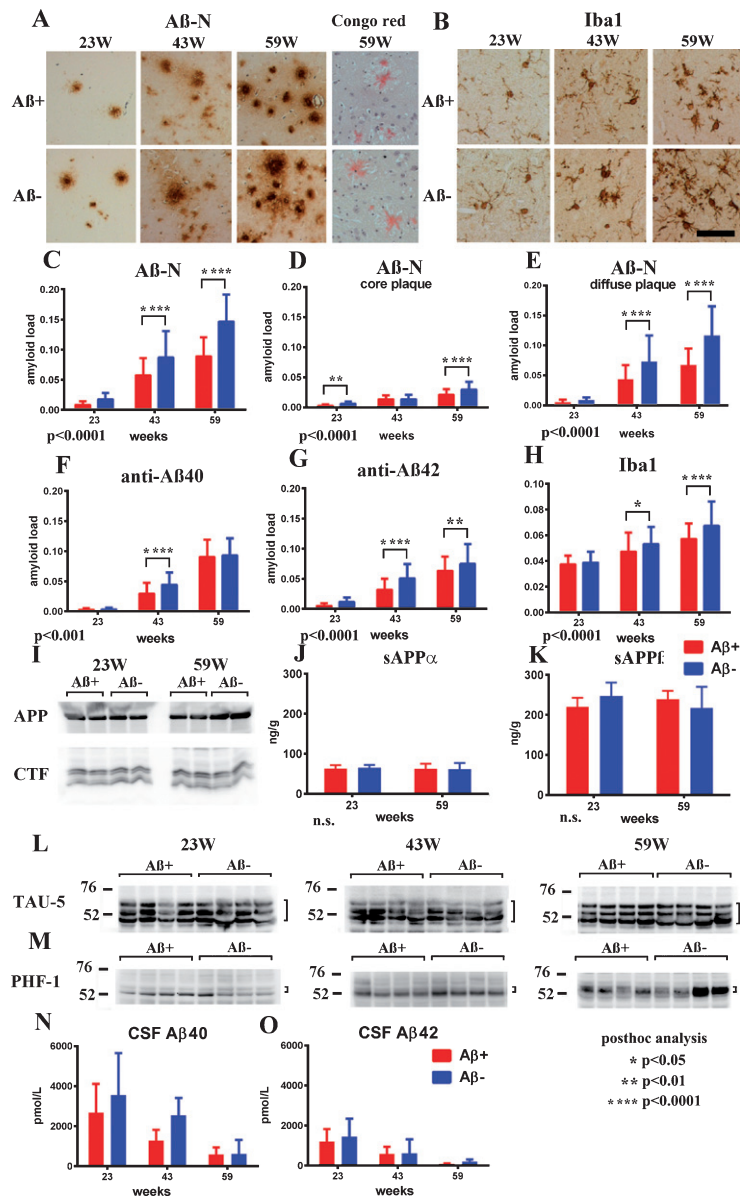


Fig. 6. Staining of A $\beta$ + or A $\beta$ - treated brains with A $\beta$ -N and Congo red (A), microglial marker Iba1 (B) in A $\beta$ + or A $\beta$ - treated mouse brains, quantification of A $\beta$  burden (C-G), Iba1 (H), western blot of A $\beta$ PP and CTFs (I), ELISA of sA $\beta$ PP $\alpha$  (J), sA $\beta$ PP $\beta$  (K), western blotting of total tau (L), phosphorylated tau (M), CSF A $\beta$ <sub>40</sub> (N), and A $\beta$ <sub>42</sub> (O) measured by ELISA. A) A $\beta$ -immunoreactive load using A $\beta$ -N was suppressed in the A $\beta$ + treated group compared with that of the A $\beta$ - group. Most A $\beta$  staining showed diffuse plaques that are not stained with Congo red. B) Microgliosis was weaker in the A $\beta$ + treated group than that in the A $\beta$ - treated group. Bar represents 100  $\mu$ m in A and 200  $\mu$ m in B. C) The area labeled by A $\beta$ -N was significantly suppressed in the A $\beta$ + treated group compared with that in the A $\beta$ - treated group ( $p < 0.0001$ ). D, E) The area occupied by diffuse plaques was more than 3-fold of that by core plaques. Both plaques were suppressed by A $\beta$ + treatment (D,  $p < 0.0001$ ; E,  $p < 0.0001$ ). Large part of the A $\beta$  decrease by immunization consisted of diffuse plaques. A $\beta$  burdens detected by anti-A $\beta$ <sub>40</sub> (F) and anti-A $\beta$ <sub>42</sub> (G) were significantly decreased in the A $\beta$ + treated group (F,  $p < 0.001$ ; G,  $p < 0.0001$ ). H) The area of Iba1 staining in A $\beta$ + treated mice was smaller than that in A $\beta$ - treated mice ( $p < 0.0001$ ). The numbers of mice analyzed: 23 weeks ( $n = 9$  for A $\beta$ +,  $n = 11$  for A $\beta$ -), 43 weeks ( $n = 7$  for A $\beta$ +,  $n = 6$  for A $\beta$ -), and 59 weeks ( $n = 6$  for A $\beta$ +,  $n = 7$  for A $\beta$ -). I) A $\beta$ PP, CTF $\beta$ , and CTF $\alpha$ , and other CTFs in SDS fractions did not differ between the A $\beta$ + and A $\beta$ - treated groups from 23 to 59 weeks. (J, K) The amount of sA $\beta$ PP $\alpha$  and sA $\beta$ PP $\beta$  in the TBS fractions detected by ELISA did not differ between A $\beta$ + (Red) and A $\beta$ - (Blue) treated groups from 23 to 59 weeks. L, M) The amount of total tau (TAU-5) and phosphorylated tau (pTau) (PHF-1) did not differ between A $\beta$ + and A $\beta$ - treated mice from 23 to 59 weeks. N, O) A $\beta$ <sub>40</sub> and A $\beta$ <sub>42</sub> in CSF decreased with aging. They were decreased in A $\beta$ + treated mice compared to A $\beta$ - treated mice, although there was no significance difference between A $\beta$ + and A $\beta$ - mice (A $\beta$ +  $n = 7$  for 23 weeks,  $n = 4$  for 43 weeks and  $n = 6$  for 59 weeks; A $\beta$ -  $n = 9$  for 23 weeks,  $n = 2$  for 43 weeks, and  $n = 4$  for 59 weeks).

as DIAN, API, or A4 studies [15, 16, 41]. Based on these findings, our extended reference memory version of the MWM test is a competent way to develop preventive DMTs in basic level of model animal experiments.

A previous A $\beta_{42}$  immunization study reported a reduction in behavior impairment and plaques in TgCRND8 mice; however, A $\beta$  amounts measured by ELISA did not decrease [24]. Administration of anti-A $\beta$  antibody m266 to PDAPP mice and BAM-10 to Tg2576 mice rapidly reversed the memory deficits without altering brain A $\beta$  burden [42, 43]. The same findings were observed in our experiments, which revealed reduced cognitive impairment and A $\beta$  burden, but no differences in the total amounts of A $\beta_{40}$  and A $\beta_{42}$  measured by ELISA between A $\beta^+$  and A $\beta^-$  groups except for increased A $\beta_{40}$  in the FA fraction. Decreased TBS-soluble A $\beta$ Os were the significantly suppressed A $\beta$  species. This observation supported the notion that these soluble A $\beta$ Os species contribute to neuronal/synaptic injury and cognitive impairment.

Because the ELISA-based A $\beta$  assay could not recover the whole amount of A $\beta$  species, we next intensely surveyed A $\beta$  species using western blotting of the same 3-step extracts to avoid overlooking other cardinal A $\beta$  molecules. Analyses of untreated TgCRND8 mice revealed the age-related accumulation of all A $\beta$  species: A $\beta$  monomers in the TBS fraction; a large part of all sets of A $\beta$ Os with LMW to HMW in the SDS fraction [28]; and age-related A $\beta$  monomers, dimers, and smears in the FA fraction. These different LMW to HMW A $\beta$ Os species were not measured by A $\beta$ Os ELISA, and the C-terminus of HMW A $\beta$ Os species were blocked [44]. Histological examination also confirmed increased plaque immunoreactivity by N-terminal antibodies compared to those by anti-A $\beta_{40}$  and A $\beta_{42}$ . Thus, diverse A $\beta$  species with conformational changes, modifications, and different lengths and solubilities were accumulated in the brains. For this reason, we carefully analyzed A $\beta$  accumulation using not only ELISA, but also conventional western blots to decide which is the cardinal A $\beta$  molecule that is toxic for the nervous system and causes cognitive dysfunction.

In a comparison study between A $\beta^+$  and A $\beta^-$  treated groups, visualized TBS soluble A $\beta$  monomers were decreased by A $\beta^+$  treatment during weeks 23–59. Because A $\beta$ Os in the TBS fraction were easily degraded into monomers using SDS sample buffer in western blotting experiments, these findings may partially correspond with the decreased amount of

A $\beta$ Os detected by ELISA in the A $\beta^+$  treated group. Consistent with ELISA findings, almost all insoluble aggregated A $\beta$  species in the SDS and FA fractions did not show obvious differences between the A $\beta^+$  and A $\beta^-$  treated groups. Conversely, HMW A $\beta$ Os in the SDS fraction and smear A $\beta$  in the FA fraction were increased in the A $\beta^+$  treated group compared with the A $\beta^-$  treated group. These findings suggested that toxic soluble A $\beta$ Os in A $\beta^+$  treated brains may be sequestered into highly insoluble and aggregated A $\beta$ Os and A $\beta$  fibrils. The same findings have been suggested previously, because the amyloid fibril is a protective structure in AD pathology [45–47]. CSF A $\beta_{40}$  and A $\beta_{42}$  levels were decreased in A $\beta^+$  treated mice compared with A $\beta^-$  treated mice, although there was no significant difference between A $\beta^+$  and A $\beta^-$  mice because of the small sample size. Previous immunization studies showed increased A $\beta$  in CSF by increased clearance of A $\beta$  [48]. Decreased amounts of CSF A $\beta$  are thought to be due to the deposition of A $\beta$  in the brain, because CSF A $\beta$  decreases correlate with A $\beta$  plaques [29, 49]. Decreased amounts of A $\beta$  in A $\beta^+$  treated mice suggested that the effect of immunization is not by enhanced clearance of A $\beta$  but sequestration of A $\beta$  in an insoluble fraction in the brain.

Histological evaluation showed that reduction of diffuse plaques was more prominent than the reduction of core plaques by A $\beta^+$  immunization. Diffuse plaques were composed of scant A $\beta$  amyloid fibrils. No difference of A $\beta$  levels by ELISA may imply a lower decrease in core plaques. Together with biochemical analyses, A $\beta^+$  immunization may facilitate the sequestration of toxic soluble A $\beta$ Os into insoluble and HMW A $\beta$ Os within the compact aggregated A $\beta$  amyloid core. Liu et al. separated A $\beta$ Os into neurotoxic type 1 A $\beta$ Os and non-neurotoxic type 2 A $\beta$ Os. Type 2 A $\beta$ Os, which occupy the majority of A $\beta$ Os sequestered around dense core plaques (~95% in 21-month-old Tg2576 mice), accounted for less than 15% of the cortex [46]. Hong et al. showed that diffusible, highly bioactive oligomers represent a critical minority of soluble A $\beta$  in AD brain [50]. It is still unclear what types of A $\beta$ Os exert the main neurotoxicity [33, 42–54]. We have previously shown that accumulation of A $\beta$  dimers in lipid rafts is the earliest event corresponding to behavioral deficits in Tg2576 mice [55]. A $\beta$  dimers are also shown to be cardinal molecules for synaptic dysfunction in A $\beta$  amyloid cascades in AD pathogenesis [56, 57]. Injection of LMW oligomers into the brain induced rapid and persistent impairment of memory, associated with

decreased hippocampal synaptophysin [58]. A $\beta$ O neurotoxicity and their induction of tauopathy are canceled by A $\beta$ O-specific monoclonal antibodies [59]. The recent success of a phase Ib randomized trial of aducanumab also showed that antibodies against aggregated forms of A $\beta$  are clinically useful [9]. Together with these findings, our study suggested that soluble A $\beta$ O are the cardinal A $\beta$  species responsible for neurotoxicity and are valid targets for DMTs.

Inverse increases in HMW A $\beta$  oligomers and A $\beta$  smear have not been reported in previous A $\beta$  immunization studies. There are some studies on decreased A $\beta$  neurotoxicity through the accelerated conversion of A $\beta$  oligomers into A $\beta$  fibrils. Using chaperone protein HspB1, A $\beta$  oligomers were converted into large nontoxic aggregates, and their toxicity was sequestered [60]. Peptides that enhance the formation of amorphous aggregates of A $\beta$  attenuated the paralysis of transgenic *Caenorhabditis elegans* [61]. Meanwhile, activated microglia are shown to take up A $\beta$ , cluster A $\beta$  inside, and release aggregated A $\beta$ , which contributed to plaque growth [62]. Based on these reports, we speculated the presence of sequestration mechanisms that convert toxic A $\beta$  oligomers into HMW A $\beta$  aggregates for reduction of A $\beta$  toxicity.

Both ELISA and TAPIR showed the presence of specific IgG antibodies against A $\beta$ <sub>4–10</sub> structural epitopes within A1aB1b in A $\beta$ + immunized mice. In western blotting, antibodies were shown to detect several A $\beta$  oligomers. Splenocytic proliferation and cytokine release against A $\beta$ + stimulation also implied the presence of adaptive cellular immune responses. We found no histological meningoencephalitis or bleeding in mouse brains. Our mucosal A $\beta$ + immunization significantly decreased microgliosis, indicating that it suppressed glial-mediated inflammatory responses. These findings suggested that our A $\beta$ + oral immunization could safely raise moderate and continuous innate and humoral immune responses.

Our A $\beta$ + immunization did not alter basic A $\beta$ PP processing or the signaling process of the A $\beta$ PP intracellular C-terminal domain [63]. Accumulation of pTau was only detected in dystrophic neurites associated with core plaques. Pathological conversion and spreading of aggregated tau was facilitated in the dystrophic neurites around core plaques [64]. Because numbers of core plaques were not so different, the amount of pTau was not significantly different.

Preclinical initiation and long-term maintenance of DMT against A $\beta$  amyloidosis have been proposed

to halt the progression of cognitive impairment and the onset of dementia. This strategy, which will likely necessitate weekly or monthly injections of antibodies, carries two risks. First, it may enhance risks of side effects. Second, the cost of long-term treatment with antibodies is likely to be prohibitively high when applied on national scales to large numbers of people. The use of oral plant vaccines offers advantages of extremely low cost and better safety compared with synthetic compounds or antibodies. For these reasons, our plant-based soybean A $\beta$ + oral vaccine strategy is cheap, safe and as effective as others.

In conclusion, our results revealed that A $\beta$ + oral immunization suppressed soluble A $\beta$ O production and prevented cognitive impairment without obvious adverse reactions. Oral immunization by A $\beta$ + could be a promising DMT for prevention of the pathological processes of AD.

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