

RESEARCH ARTICLE

A novel monoclonal antibody against the N-terminus of A β ₁₋₄₂ reduces plaques and improves cognition in a mouse model of Alzheimer's disease

Hai-Yan Xing¹, Bin Li¹, Dan Peng¹, Chun-Yan Wang², Guan-Ying Wang¹, Pan Li¹, Ying-Ying Le³, Ji-Ming Wang⁴, George Ye², Jian-Hong Chen^{1*}

1 Department of Pharmacy, Daping Hospital & Research Institute of Surgery, Third Military Medical University, Chongqing, China, **2** Anogen-Yes Biotech, Mississauga, Ontario, Canada, **3** Institute for Nutritional Sciences, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, University of Chinese Academy of Sciences, Shanghai, China, **4** Cancer and Inflammation Program, Center for Cancer Research, National Cancer Institute at Frederick, Frederick, Maryland, United States of America

* chenjh-110@263.net



OPEN ACCESS

Citation: Xing H-Y, Li B, Peng D, Wang C-Y, Wang G-Y, Li P, et al. (2017) A novel monoclonal antibody against the N-terminus of A β ₁₋₄₂ reduces plaques and improves cognition in a mouse model of Alzheimer's disease. PLoS ONE 12(6): e0180076. <https://doi.org/10.1371/journal.pone.0180076>

Editor: Stephen D Ginsberg, Nathan S Kline Institute, UNITED STATES

Received: December 21, 2016

Accepted: June 11, 2017

Published: June 29, 2017

Copyright: © 2017 Xing et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability Statement: All relevant data are within the paper.

Funding: This project was supported by the grants from the National Natural Science Foundation of China (Grant No. 81302867 and 81273608). We also gratefully acknowledge the financial support from the Chongqing Programs for Science and Technology Development (CSTC2015jcyjBX0018). Ji Ming Wang was funded by Federal funds from the National Cancer Institute, National Institutes of Health.

Abstract

Senile plaques consisting of Amyloid-beta (A β) peptides, in particular A β ₁₋₄₂, are the hallmark of Alzheimer's disease (AD) and have been the primary therapeutic targets. Passive immunotherapy with monoclonal antibodies (mAbs) has shown initial success in mouse models of AD. However, the existing A β -directed mAbs mostly were tested on animal models or patients with advanced disease. The effects and mechanisms of mAbs on animals or human trial participants in the prodromal phase of AD are not fully clarified. In the current study, a novel mAb (3F5) directed against the 1–11 amino acids of A β ₁₋₄₂ was generated by immunizing mice with an emulsion of full length human A β ₁₋₄₂. The mAb (3F5) showed the ability to disrupt A β ₁₋₄₂ aggregation and prevent A β -mediated neurotoxicity *in vitro*. In a mouse model of AD, administration with 3F5 for 3 months in 6 months-old mice demonstrated that the mAb specifically bound with A β ₁₋₄₂ to promote the depolymerization of A β fibrils, facilitated endocytosis of A β ₁₋₄₂ by microglia, and attenuated the death and apoptosis of neuronal cells, accompanied by neurite outgrowth. APP/PS1 double-transgenic mice treated with 3F5 mAb showed reduced memory loss, cognitive decline, and decreased levels of amyloid deposits in the brain. A β ₁₋₄₂ levels in cerebral tissues were also significantly reduced, whereas serum A β ₁₋₄₂ was markedly increased. Interestingly, the concentration of 3F5 in peripheral circulation is much higher than that in the brain. These results indicate that 3F5 is able to cross the blood-brain barrier (BBB) to bind A β and initiates the phagocytosis of antibody/A β complexes by microglia in the amyloid depositing mice. 3F5 also promotes A β efflux from the brain. As a consequence, the antibody reduces plaques in the AD mouse brain, in association with reduction in the pathology of AD.

Health, under contract No. HHSN 26/200800001E and supported in part by the Intramural Research Program of NCI, NIH, USA. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: We have declared that authors employed by Anogen-Yes Biotech participated in the study design, data collection and analysis. All authors have declared that no competing interests exist. The Anogen-Yes Biotech did not provide financial support for this project and will not alter our adherence to PLOS ONE policies on sharing data and materials. All data are available in the uploaded manuscript.

Introduction

Alzheimer's disease (AD), a progressive neurodegenerative disorder, is characterized by memory and cognition impairment. Strong genetic and biochemical evidence highlights β -amyloid deposition in the brain as a causative factor for this neurodegeneration cascade [1]. Thus, approaches to reducing Amyloid-beta (A β) production or remove A β _{1–42} deposits are actively explored as therapeutic modalities for AD [2]. Among these approaches, immunotherapy is considered as the first choice of disease-ameliorating treatment.

Passive immunization, treating patients with ex vivo produced monoclonal antibodies (mAbs) against A β peptides, has become potentially an effective strategy to prevent or treat AD [3]. Preclinical studies have shown that peripheral or intracerebroventricular injection of anti-A β antibodies reduces A β levels in the brain and improves cognitive function [4, 5]. Furthermore, antibodies against the N-terminus of A β _{1–42} may be superior to those against the middle and C-terminus A β _{1–42} segments to eliminate A β plaques [6]. However, global phase III trials of bapineuzumab, a mAb specific for the N-terminus of A β _{1–42} peptide, showed lack of efficacy on clinical endpoints in patients with mild to moderate AD [7]. A commonly held view is that the regimen of immunotherapy applied may be too late to halt or reverse the disease course. Consequently, there is a need to re-evaluate current immunotherapy strategies in terms of timing, safety and efficacy.

In the current study, a novel mAb (3F5) directing at the N-terminal 1–11 peptide region of A β _{1–42} was generated by immunizing mice with an emulsion of full length human A β _{1–42}. This mAb demonstrates the capacity to disrupt A β _{1–42} aggregation *in vitro* and reduce A β -mediated neurotoxicity. *In vivo*, in 6 month old APP/PS1 double-transgenic mice (Tg-mice), 3F5 crosses the blood-brain barrier (BBB) to bind A β to promote the phagocytosis of antibody/A β complex by microglia. 3F5 also initiates A β efflux from the brain. Thus, when given mice at an early stage of AD, 3F5 is able to markedly alleviate the progress of AD.

Materials and methods

Chemicals and reagents

Synthetic A β fragment peptides 1–11, 12–28, 25–35 and 33–42 were purchased from Bachem Biotechnology (Bachem, Switzerland). A β _{1–42} was purchased from American Peptide Company (California, USA). Non-specific IgG (#A7028), HRP-conjugated secondary antibody (#A0216) and Propidium Iodide (PI) (#ST512) were obtained from Beyotime Biotechnology (Shanghai, China). Goat serum (#SL038), β -mercaptoethanol (#M8210) and Fluorescein Isothiocyanate Isomer I (FITC) (#F8070) were purchased from Solarbio Life Sciences (Beijing, China). Thioflavin T (ThT) (#T3516), Methyl Thiazolyl Triumvirate (MTT) (#M2128), Dimethyl Sulfoxide (DMSO) (#472301), All-trans-retinoic Acid (RA) (#R2625), Paraformaldehyde (#16005) and Diaminobenzidine (DAB) (#DA1010) were purchased from Sigma—Aldrich (USA). All other reagents were from Sigma-Aldrich, unless otherwise indicated.

The development of mAb clone 3F5

The hybridoma of anti-A β _{1–42} mAb clone 3F5 was generated through intraperitoneal immunization of 6–7 week old Balb/C mice with an emulsion of full length human A β _{1–42} (Sequence: DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA) and Complete Freund's Adjuvant (Sigma, F5881). The immunization was boosted three times intraperitoneally with full length human A β _{1–42} in incomplete Freund' Adjuvant (Sigma, F5506). The spleen of the immunized mouse was isolated and the cells were dispersed with a 200 μ m mesh under sterile condition. The spleen cells were mixed with the myeloma cell line Sp2/0-Ag14 (ATCC, CRL-1581) at

a 10:1 ratio, while fusion reagent Polyethylene Glycol 1500 solution (Roch 783641) was added dropwise into the mixture. After plating the cell mixture to microwell plates, the hybridomas were selected by adding HAT Supplement (Gibco, 31062-011) to the medium. Anti-A β ₁₋₄₂ antibody secreting clones were screened and subcloned based on the reaction in full length human A β ₁₋₄₂ coated ELISA plates. The isotype of the clone 3F5, as determined using the Mouse Typer Isotyping Kit (Bio-Rad, 17-2055), is IgG2b Kappa. The antibody was purified using Gammabind Plus Sepharose (GE Healthcare, 17-0886-02). ELISA plates coated with different human A β peptides were used to analyze the reactivity of mAb clone 3F5.

Measurement of antigen-antibody affinity and specificity

Synthetic A β ₁₋₄₂ (100 μ L, American Peptide Company, California, USA) (100 ng/mL) was added to the wells in 96-well ELISA plates (Corning, USA). The samples were coated at 4°C overnight. The plates were washed and blocked using 5% bovine serum album (BSA) in 5% CO₂ atmosphere at 37°C for 2 h. Samples were then naturally dried for future use. Thereafter, synthetic A β ₁₋₄₂ (1 μ g/mL) without conjugation was diluted (1, 0.5, 0.25, 0.125, 0.0625, 0.03125 and 0 μ g/mL) and then pre-incubated with 3F5 (0.05 μ g/mL) at 4°C overnight. The mixtures were added in A β ₁₋₄₂ pre-coated ELISA plates for 90 min at 37°C to measure the competitive binding by 3F5 with free A β ₁₋₄₂ or coated A β ₁₋₄₂. The optical density (OD) was detected at 495 nm on a microplate reader (BIO-RAD Model 2550 EIA Reader, USA).

Classical peptide mapping using binding ELISA was performed to identify the epitope of A β ₁₋₄₂ recognized by 3F5. The 96-well ELISA plates were coated with different A β ₁₋₄₂ fragments (aa1-42, aa1-11, aa12-28, aa25-35, aa33-42), and 3F5 was then incubated with A β ₁₋₄₂ fragments for 72 h at 37°C. 3, 3', 5, 5' -Tetramethylbenzidine (TEM) was added as a substrate in each well and optical density (OD) was detected at 450 nm on a microplate reader (BIO-RAD).

Neurite outgrowth assay

Neurite outgrowth was examined according to previously described method [8]. Briefly, 2×10^3 SH-SY5Y cells/well were seeded in a 24-well plate and cultured for 24 h at 37°C in a 5% CO₂. 10 μ M all-trans-retinoic acid (RA) were co-incubated with SH-SY5Y cells for 5 days followed by incubation with 10 μ M A β ₁₋₄₂ fibrils. 3F5 antibody (10 μ g/mL and 20 μ g/mL) and IgG (10 μ g/mL) were then added into the plate for an additional 24 h. The supernatant was discarded and 200 μ L 4% paraformaldehyde was added to each well to fix the cells. Cell images were acquired by an inverted microscope (Olympus, Japan) and 10 fields (100 \times) were analyzed per group.

MTT assay

SH-SY5Y neuroblastoma cells were obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). Methyl thiazolyl triumvirate (MTT) assay was used to measure the ability of 3F5 to reduce the cytotoxicity of A β ₁₋₄₂ fibrils. Briefly, SH-SY5Y cells were grown on 96-well plates at a density of 4×10^5 for 24 h. After treatment with the different concentrations of 3F5 (40 μ g/mL, 20 μ g/mL, 10 μ g/mL and 5 μ g/mL) and 10 μ M A β ₁₋₄₂ for 48 h, the cells were incubated with 10 μ L MTT (Sigma, 5 mg/mL in PBS). After incubation at 37°C for 4 h, supernatants were removed and 150 μ L dimethylsulfoxide (DMSO) were added. The absorbance was monitored at 490 nm on a microplate reader (BIO-RAD).

Propidium iodide assay

SH-SY5Y cells were seeded at 2×10^4 per well in a 24-well plate in three replicates. A β ₁₋₄₂ fibrils at 10 μ M were added into the wells to co-incubate with 3F5, IgG, or PBS for 48 h. The samples then were mixed with propidium iodide (PI, 50 μ g/mL) and incubated for 15 min at room temperature in the dark. Cell images were visualized with blue filter in an inverted fluorescence microscope (IX70, Olympus, Japan).

Annexin V-FITC / PI assay

The number of apoptotic and necrotic cells was counted using an Annexin V-FITC/PI staining kit (KeyGEN BioTECH, Nanjing, China) according to the manufacturer's instructions. Briefly, SH-SY5Y cells were seeded in a 12-well plate at a density of 1×10^5 /well and then incubated in 5% CO₂ atmosphere at 37°C for 48 h. A β ₁₋₄₂ at 10 μ M was added in each well. Different concentrations of 3F5, IgG and PBS were added to the wells. IgG was considered as a negative control. SH-SY5Y cells were washed at 48 h later with cold PBS and collected by centrifugation at 92 g for 5 min. Subsequently, the cells were stained with a combination of fluorescein annexin V-FITC and PI for 10 min at room temperature. Apoptosis was examined with by flow cytometry (ACEA Biosciences, Santiago, USA).

Morris water maze

Tg-mice were obtained from the Jackson Lab of the University of Western Ontario in Canada, and the wild-type (WT) mice were derived from the Animal Experimental Center of Zheng Zhou University. They were housed in a temperature- and humidity-controlled environment under a 12-h light/12-h dark cycle (lights on at 8:00) and had ad libitum access to food and water. The health and welfare of the animals were monitored at least once a day. Experiments were approved by the Animal Care and Welfare Committee of the Third Military Medical University. All animal were performed in accordance with the standard guidelines for the care and use of laboratory animals and were approved by the Biological Research Ethics Committee of Third Military Medical University. It has been stated that during the experiment period, if an animal experienced symptoms of torture (self-injury behavior, abnormal posture, and crying etc.) and rapid weight loss (more than 20% in several days), it would be euthanized, with sodium pentobarbital (120mg/kg). No mice were observed having such a condition in this study.

Tg-mice (females and males, 6 months) and WT littermates (males, 6 months) were divided into four groups (n = 6): Tg control, Tg 3F5, Tg IgG and WT control. 3F5-treatment or IgG-treatment mouse group was injected intraperitoneally with 0.5 g/kg of 3F5 or IgG twice a week until 9 months of age. The Tg-control and WT mouse groups were injected intraperitoneally with normal saline (NS) at equal volume with the treatment groups.

Morris water maze (MWM) test was used to investigate spatial learning and memory of mice. The diameter of swimming pool for MWM is 1.2 meters, and the temperature was maintained at 18–22°C. Time of detection was limited to 60 seconds, and the record was terminated when mice stayed on the platform for over 3 seconds. Mice were examined in three quadrants except for aim quadrant each day. After 5 days, the platform was removed for probe trial. Indicators included time of water maze latency, speed of swimming, frequency of platform-crossing and the time spent in target quadrant in behavioral testing.

Thioflavin T assay

To measure the ability of 3F5 to disaggregate A β fibrils, synthetic A β ₁₋₄₂ was diluted to 10 μ M with PBS and then incubated in a 96-well brown plate at 37°C for 48 h to form fibrils. Thereafter,

100 μ L A β ₁₋₄₂ (10 μ M) was co-incubated with different concentrations of 3F5 at 37°C for 24 h, 48 h or 72 h, followed by 10 μ M thioflavin T (ThT) solution (50 mM phosphate buffer, pH 6.0) for 15 min in the dark at room temperature. Fluorescence was measured by Thermo Varioskan Flash (excitation at 450 nm and emission at 482 nm).

Cytotoxicity

Synthetic A β ₁₋₄₂ dissolved in DMSO was diluted by carbonate buffer (CBS, pH 9.6) to 2.5 mg/mL, and FITC was added to shake for 2 h in the dark. Murine microglial cell line N9 (a kind gift from Dr. P. Ricciardi-Castagnoli, Universita Degli Studi di Milano-Bicocca, Milan, Italy) was grown in Iscove's modified Dulbecco's medium supplemented with 5% heat-inactivated FCS, 2 mM glutamine, 100 units/mL penicillin, 100 μ g/mL streptomycin, and 50 μ M 2-mercaptoethanol. The cells were plated at a density of 1×10^5 /well in a 24-well plate and incubated overnight. A β ₁₋₄₂ labelled with FITC (1 μ g/mL) was mixed and co-incubated with 3F5 for 1 h at 37°C. The samples were added to the plates for co-incubation with N9 cells for 30 min in 5% CO₂ at 37°C. Fluorescence was monitored by NovoCyte Flow Cytometer (ACEA Biosciences, Santiago, USA).

Detection of A β ₁₋₄₂ and 3F5 in mouse cerebral tissues and peripheral blood

The concentration of A β ₁₋₄₂ and 3F5 in mouse cerebral tissues and peripheral blood was measured by ELISA (Elabscience BioTECH, Wuhan, China), according to the manufacturer's instructions. All mice were quickly decapitated. The procedure was performed without anesthesia to avoid the potential effect of anesthetic drugs. Whole blood was collected from mice. Then, the cerebral tissues were cut into fragments and suspended in PBS for sonication. Thereafter, the samples were centrifuged for 10 min at 9200 g at 4°C. The supernatants were collected for detecting A β ₁₋₄₂ and 3F5. The optical density (OD) was obtained on the plate reader at 450 nm (BIO-RAD).

Pathological staining and analysis for A β plaques and microhemorrhage

Immunohistochemistry was used to analyze the ability of 3F5 antibody to attenuate A β deposition in the brain of Tg-mice. 3 μ m brain sections were mounted onto microscope slides and dried for 2.5 h. The sections were then soaked in sodium citrate buffer solution for 20 min in a microwave oven at low temperature for antigen retrieval. Sections were blocked with goat serum for 30 min and then were incubated with 3F5 (1: 20000) overnight at 4°C. The sections were washed with PBS and incubated with HRP-conjugated secondary antibody (1: 500) for 30 min at room temperature. Staining was visualized with diaminobenzidine and images were monitored by Upright Metallurgical Microscope (BX53M, Olympus, Japan).

Prussian blue histological staining was performed to assess microhemorrhage in cerebral tissues by previously described method [9]. Briefly, 3 μ m brain sections attached to microscope slides were dewaxed and hydrated, then stained for hemosiderin in a solution containing 2% potassium ferrocyanide and 2% hydrochloric acid for 15 min. Thereafter, the sections were counterstained in a 1% Neutral Red solution for 10 min at room temperature. The sections were washed in distilled water, dehydrated and coverslipped with neutral balsam. The images of hemosiderin deposits were acquired by Upright Metallurgical Microscope (BX41, Olympus, Japan).

Statistical analysis

All experiments were performed at least three times, and the results were expressed as the means ± standard deviation (SD). All data for each variable were checked for normality and homogeneity of variance before performing statistical analyses. The results were analyzed by one-way analysis of variance (ANOVA) followed by a SNK-q test for multiple comparisons. Repeated measures ANOVA followed by the least significant difference (LSD) test was used to compare the escape latencies among mouse groups. *P* < 0.05 was considered statistically significant. Quantitative assessment of Aβ plaques area and the length of neurite were measured by ImageJ 1.48u.

Results

3F5 specially binds to the N-terminus of Aβ₁₋₄₂

As presented in Fig 1A, Aβ₁₋₄₂ (0.03–1 μg/mL) without FITC conjugation significantly reduced the binding of 3F5 to Aβ₁₋₄₂ conjugated with FITC. The optical density did not show significant change with the increasing dose of Aβ₁₋₄₂ not conjugated with FITC. On epitope mapping, the OD value in the presence of Aβ₁₋₁₁, Aβ₁₂₋₂₈, Aβ₂₅₋₃₅, Aβ₃₃₋₄₂ and Aβ₁₋₄₂ was markedly increased. Aβ₁₋₁₁ showed the greatest ability to bind 3F5 than other fragments of Aβ₁₋₄₂ (Fig 1B). These results indicate that 3F5 primarily binds the N-terminus of Aβ₁₋₄₂.

3F5 protects neuronal cells from the cytotoxicity of Aβ₁₋₄₂ fibrils

Aβ₁₋₄₂ fibrils are known to be toxic for neurites. We found that the viability of SH-SY5Y cell line and the neurite length were significantly decreased in the presence of Aβ₁₋₄₂ (Fig 2A and 2C). However, neurite length in cells treated with 3F5 (10 and 20 μg/mL) was significantly increased compared with the cells treated with Aβ₁₋₄₂ only. 3F5 improved the viability of SH-SY5Y cells with optional concentration of 20 μg/mL (Fig 2C). Moreover, as shown in Fig 3A and 3C, 3F5 significantly reduced the late apoptosis and necrosis of SH-SY5Y cells induced with Aβ₁₋₄₂ fibrils. 3F5 decreased the proportion of early, late and total apoptosis of SH-SY5Y

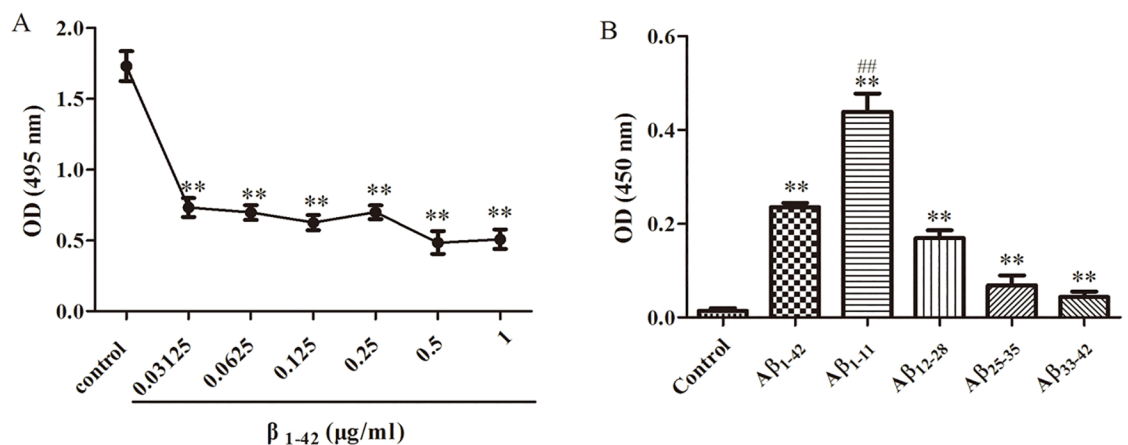


Fig 1. 3F5 specifically binds to the N-terminus of Aβ₁₋₄₂. (A) Aβ₁₋₄₂ (0–1 μg/mL) without FITC conjugation was pre-incubated with 3F5 overnight at 4°C. Thereafter, Aβ₁₋₄₂ (0–1 μg/mL) without FITC conjugation and Aβ₁₋₄₂ coated on ELISA plates competitively bound with 3F5 for 90 min at 37°C. The competitive binding of 3F5 with free or coated Aβ₁₋₄₂ was detected by ELISA. (B) On epitope mapping, 3F5 was incubated with different fragments of Aβ₁₋₄₂ coated on ELISA plates for 90 min at 37°C. The specificity of 3F5 for the epitopes of Aβ₁₋₄₂ was measured by ELISA. Experiments were performed in triplicate. Data are shown as mean±SD, and analyzed by One-way ANOVA. ***p* < 0.01 vs control, ##*p* < 0.01 vs Aβ₁₋₄₂ group.

<https://doi.org/10.1371/journal.pone.0180076.g001>

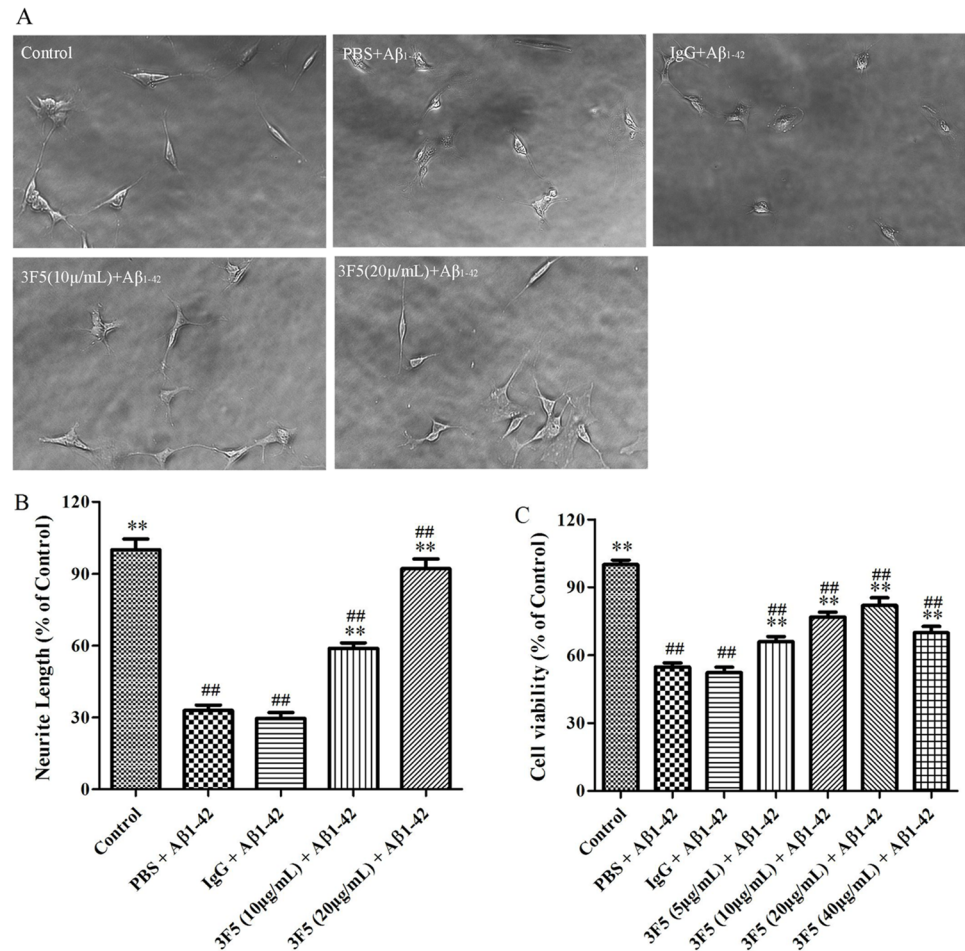


Fig 2. 3F5 reduces the neurotoxicity of Aβ₁₋₄₂ fibrils *in vitro*. SH-SY5Y cells were cultured with different concentrations of 3F5 and Aβ₁₋₄₂ fibrils for the indicated times. (A) SH-SY5Y cell images were shown by microscopy (100×). (B) Quantification of neurite length for the capacity of 3F5 to stabilize neurite cells. (C) The viability of SH-SY5Y cells treated with different concentrations of 3F5 measured by MTT. Experiments were performed in triplicate. Data are shown as the mean±SD and analyzed by One-way ANOVA. ***p* < 0.01 vs. PBS+ Aβ₁₋₄₂; ##*p* < 0.01 vs. Control.

<https://doi.org/10.1371/journal.pone.0180076.g002>

cells in the presence of Aβ₁₋₄₂ fibrils (Fig 3B and 3D). These findings confirm that 3F5 protects neurite cells from the cytotoxicity of Aβ₁₋₄₂ fibrils.

3F5 rescues cognition and memory defects in Tg-mice

Tg-mice exhibited excessive Aβ peptide deposits in the brain and defect spatial perception and memory [10]. Morris water maze test was used to assess the capacity of 3F5 to improve spatial cognition and memory of Tg-mice. Mauchly's sphericity test, tests of within-subjects effects and tests of between-subjects effects were performed. The results of Mauchly's sphericity test were *p* > 0.05. As shown in Fig 4A, the swimming speed of Tg- and WT-mice was not significantly different (Tests of Within-Subjects Effects, Time, Sphericity Assumed, *F* = 0.618, degree of freedom (df) = 3, *p* = 0.606), indicating that mice at early stage of AD exhibited no impaired motility. However, the latency time was significantly shortened in 3F5 treated Tg-mice and WT-mice after 5 days of training (Tests of Between-Subjects Effects, Groups, *F* = 25.797, df = 3, *p* = 0.000). There was no apparent changes in control Tg-mice and IgG-treated Tg-mice

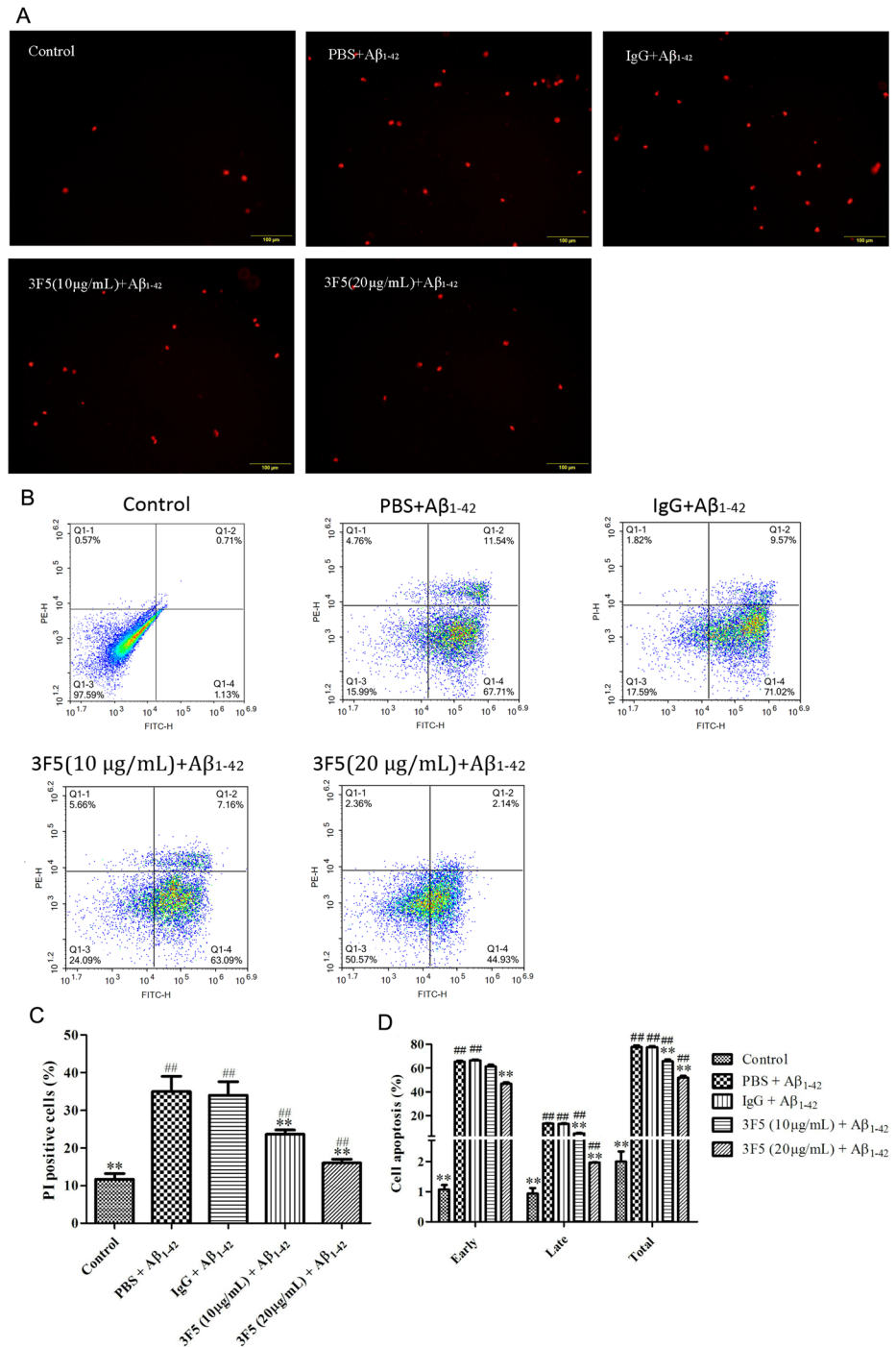


Fig 3. 3F5 inhibits the neuronal cell apoptosis induced by Aβ₁₋₄₂ fibrils. (A) Representative images of PI single staining to SH-SY5Y cells. SH-SY5Y cells were treated with 10 μM Aβ₁₋₄₂ fibrils and co-incubated with PBS, IgG or 3F5 for 48 h. The cells were then incubated with PI for 15 min in dark and analyzed with a fluorescence microscopy. (B) Representative images of SH-SY5Y cells stained with AnnexinV-FITC/PI. SH-SY5Y cells treated as above mentioned, then stained with a combination of fluorescein annexin V-FITC and PI for 10 min at room temperature. Cell apoptosis was examined with by flow cytometry. (C) The percentage of PI positive SH-SY5Y cells. (D) The percentage of cell apoptosis on SH-SY5Y cells stained with AnnexinV-FITC/PI. The experiments were repeated three times. Data are shown as the mean±SD and analyzed by One-way ANOVA. ***p* < 0.01 vs. PBS + Aβ₁₋₄₂; ###*p* < 0.01 vs. Control.

<https://doi.org/10.1371/journal.pone.0180076.g003>

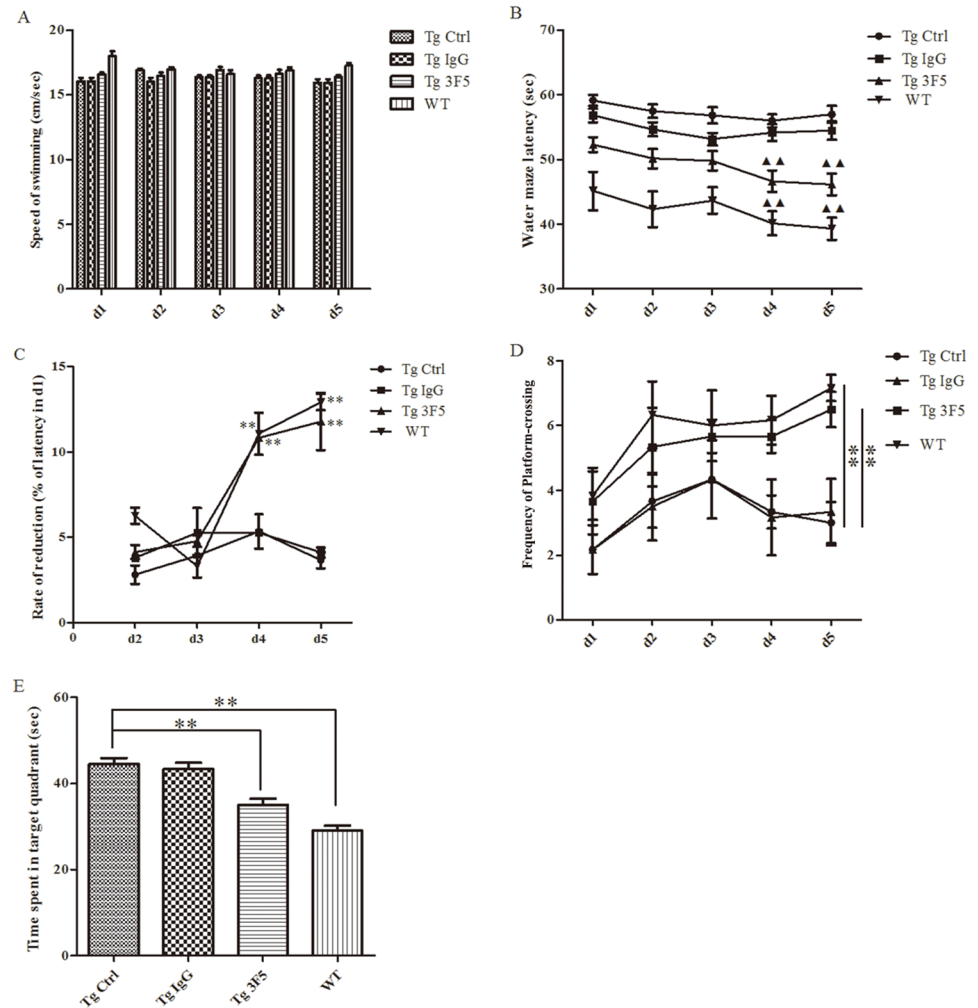


Fig 4. 3F5 improves the cognitive and memorial performance of Tg-mice. Mice were randomly divided into 4 groups (n = 6). Mice in the 3F5-treated and IgG-treated mouse groups were injected intraperitoneally with 0.5 g/kg 3F5 or IgG twice a week for 3 months. Tg-control and WT mice were injected intraperitoneally with normal saline (NS). Morris water maze (MWM) was used to evaluate the spatial learning abilities and the memory of mice. (A) Swim speed (cm/sec) was analyzed in MWM. (B) Escape latency (sec) of mice was recorded ($\blacktriangle p < 0.01$ vs. d1). (C) The percentage of reduction in escape latency relative to d1 was calculated ($\blacktriangle p < 0.01$ vs. Tg Ctrl). (D) The frequency of platform-crossing was recorded ($**p < 0.01$ vs. Tg Ctrl). (E) Time spent in quadrants of mice was recorded ($**p < 0.01$ vs. Tg Ctrl). Tg Ctrl denotes control Tg-mice; Tg IgG denotes Tg-mice treated with IgG; Tg 3F5 denotes Tg-mice treated with 3F5. Each bar represents the mean \pm SD from three independent experiments, and analyzed by repeated measures ANOVA (Fig. 4A-4D) and One-way ANOVA (Fig. 4E).

<https://doi.org/10.1371/journal.pone.0180076.g004>

in 5 days (Fig 4B). The reduction rate of escape latency in 3F5-treated Tg mice was similar to WT mice, and significantly prolonged than that in the Tg-control mice and the IgG-treated mice. In 3F5-treated Tg-mice, the reduction rate of escape latency reached $10.83 \pm 0.15\%$ and $11.78 \pm 1.68\%$ at d4 and d5 (Fig 4C, Between Groups, $F = 5.328$, $df = 3$, $p = 0.007$). WT- and 3F5-treated mice more frequently crossed the platform compared with control Tg-mice (Fig 4D, Tests of Between-Subjects Effects, Groups, $F = 59.715$, $df = 3$, $p = 0.000$). In comparison with control Tg-mice, WT- and 3F5-treated mice spent less time in the target quadrant. Under the same experimental conditions, IgG-treated Tg-mice showed no significant difference from

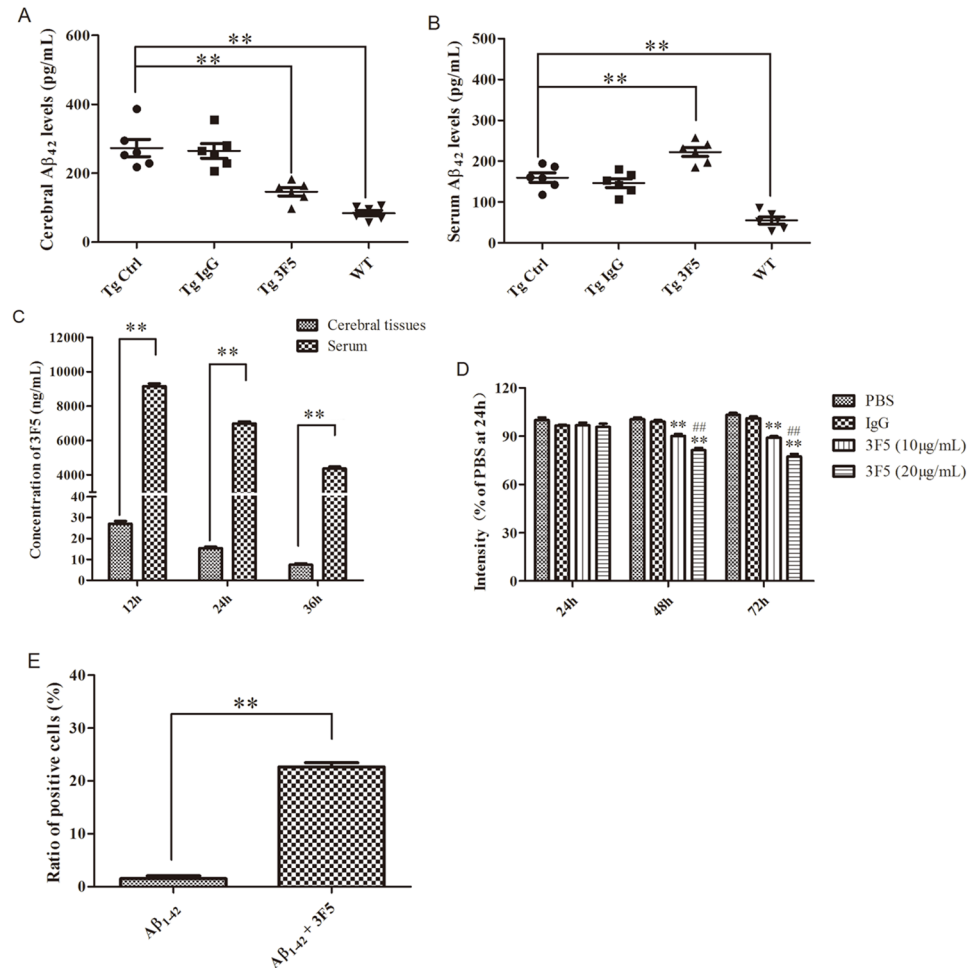


Fig 5. 3F5 promotes the clearance of Aβ₁₋₄₂ from the brain. Tg- and WT-mice were treated for 3-months with 3F5, IgG or NS. Aβ₁₋₄₂ levels in the cerebral tissues (A) and in the serum (B) of Tg-mice were detected by ELISA (***p* < 0.01 vs. Tg Ctrl). (C) The concentrations of 3F5 in the cerebral tissues and the serum of 3F5-treated Tg-mice were analyzed by ELISA (***p* < 0.01 vs. cerebral tissues). (D) 3F5 (10 μg/mL and 20 μg/mL) was co-incubated with Aβ₁₋₄₂ fibrils for the indicated times and the effect on Aβ aggregation was analyzed by ThT fluorescence (***p* < 0.01 vs. PBS, ##*p* < 0.01 vs. 10 μg/mL of 3F5). (E) Aβ₁₋₄₂ labelled with FITC (1 μg/mL) was co-incubated with 3F5 for 1 h followed by incubation with N9 microglial cells for 30 min at 37°C. The phagocytosis of Aβ₁₋₄₂ by N9 microglia cells was detected by NovoCyte Flow Cytometer (***p* < 0.01 vs. Aβ₁₋₄₂). Tg Ctrl denotes control Tg-mice; Tg IgG denotes Tg-mice treated with IgG; Tg 3F5 denotes Tg-mice treated with 3F5. Each experiment was repeated in triplicate. Data are shown as the mean ± SD and analyzed by One-way ANOVA.

<https://doi.org/10.1371/journal.pone.0180076.g005>

control Tg-mice (Fig 4E). These results suggest that 3F5 reduces cognition and memory deficits in Tg-mice.

3F5 promotes the clearance of Aβ₁₋₄₂ from the mouse brain

To examine the capacity of 3F5 to reduce Aβ burden in the brain of Tg-mice, the concentration of Aβ₁₋₄₂ and 3F5 in the cerebral tissues and peripheral blood were measured by ELISA. Treatment with 3F5 significantly decreased Aβ₁₋₄₂ levels in the cerebral tissues (Fig 5A) but a marked increase Aβ₁₋₄₂ in the serum (Fig 5B). As shown in Fig 5C, Aβ₁₋₄₂ levels in the serum of 3F5-treated Tg-mice reached 222.32 pg/mL, which was much higher than that in the cerebral tissues of 3F5-treated Tg-mice (Fig 5A and 5B). Interestingly, the concentrations of 3F5 in

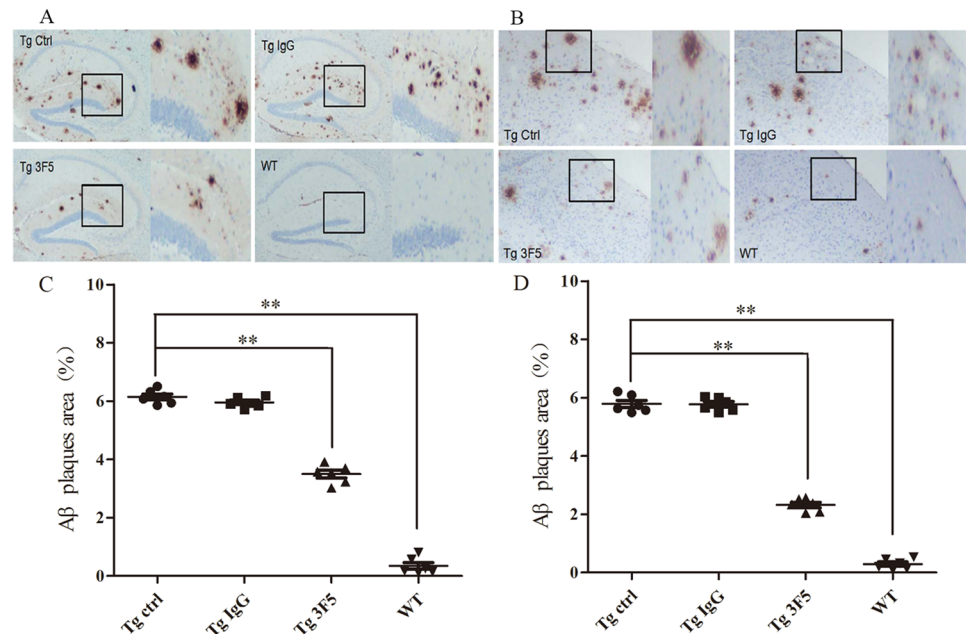


Fig 6. A β plaques in the hippocampus and cerebral cortex of mice. After 3-months treatment, the hippocampus and cerebral cortex of Tg- and WT-mice were collected for preparation of frozen sections. (A) Hippocampus sections of Tg- and WT- mice. High magnification of the deposited plaques from the boxed areas was shown. (B) Microhemorrhage associated with A β deposits mice in the cerebral cortex of Tg- and WT-mice (** $p < 0.01$ vs. the Tg-control group). (C) A β plaques in hippocampus area were quantitated. (D) Quantification of A β plaques area in the cerebral cortex (** $p < 0.01$ vs. the Tg-control group). Each experiment was in triplicate. Data are shown as the mean \pm SD and analyzed by One-way ANOVA.

<https://doi.org/10.1371/journal.pone.0180076.g006>

the serum of 3F5-treated Tg-mice were significantly higher than that in the cerebral tissues of 3F5-treated Tg-mice (Fig 5C). Moreover, 3F5 dose-dependently reduced A β fibril fluorescence intensity when co-incubated with preformed A β fibrils for 48 and 72 h (Fig 5D). As presented in Fig 5E, after 3F5 binding with A β ₁₋₄₂ for 1 h, the phagocytosis of A β ₁₋₄₂ by murine N9 microglial cells was significantly increased. Thus, A β burden in the cerebral tissues was reduced by 3F5 presumably by promoting the efflux of A β ₁₋₄₂ from the brain, deaggregation of A β fibrils and phagocytosis of A β ₁₋₄₂ by microglia.

3F5 decreases the number of plaques in the hippocampus and cerebral cortex of Tg-mice

Immunohistochemistry of hippocampus and cerebral cortex from Tg-mice after 3 months of antibody treatment showed (Fig 6A and 6C) A β plaque area in 3F5-treated mice decreased by 43% compared with the tissues from control mice which contained senior plaques mostly in the hippocampus. There were almost no senior plaques in WT mice until 9-month old (Fig 6C). IgG did not reduced A β plaques in Tg-mice (Fig 6A and 6C).

In agreement with Fig 6A and 6C, 3F5-treated Tg-mice exhibited 60% decrease in A β deposits in the cerebral cortex (Fig 6B and 6D). The number of A β plaques in the brain of Tg-mice and IgG-treated Tg-mice was not significantly different and there was no apparent A β deposition in the brains of WT mice (Fig 6D). These data demonstrate that 3F5 antibody is able to promote the clearance of senior plaques in the hippocampus and cerebral cortex of Tg-mice in association with improved cognition and memory.

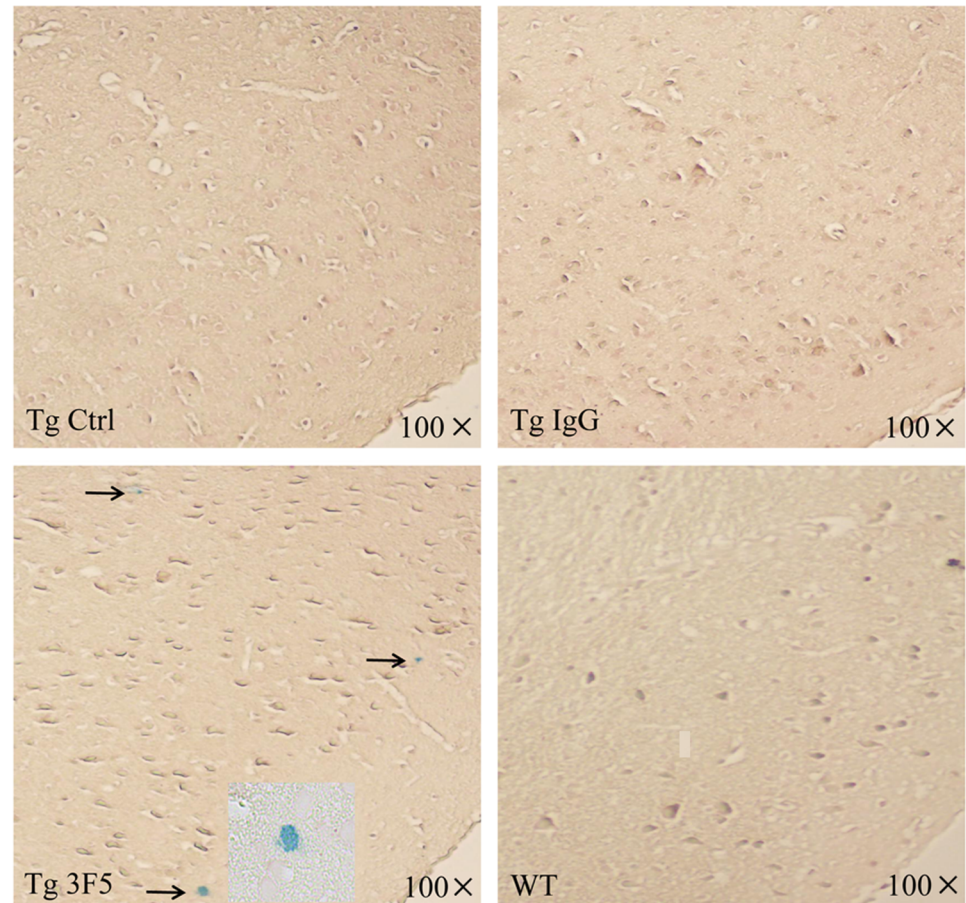


Fig 7. 3F5 antibody increases the frequency of microhemorrhages in the brain of Tg-mice. After 3-months treatment of 3F5, sections of the cerebral tissues in Tg- and WT-mice were stained with Prussian blue and counterstained with Neutral Red. The microhemorrhage lesions were detected by the presence of hemosiderin deposits in the cerebral tissues. Images per section were acquired by Upright Metallurgical Microscopy.

<https://doi.org/10.1371/journal.pone.0180076.g007>

3F5 causes minor microhemorrhage

Prussian blue staining was used to examine whether 3F5 antibody may increase the incidence of microhemorrhage in the brain of AD mice. As shown in Fig 7, lesions of prussian blue-positive microhemorrhage were not detected in the brain of WT-mice, control- and IgG-treated Tg-mice. However, tiny and scattered microhemorrhage lesions were detected in the cerebral tissues of 3F5-treated Tg-mice. Thus, 3F5 antibody increases lesions of microhemorrhage in the brain of 9-month-old Tg-mice, despite its beneficial effects in reverse the course of AD.

Discussion

AD is caused mainly by progressive accumulation of A β ₁₋₄₂ peptide in the brain to form fibrils and amyloid plaques, which lead to synaptic loss, neuronal dysfunction and death [11]. Therefore, A β ₁₋₄₂ has become a major therapeutic target for AD [12]. Although anti-A β mAbs including bapineuzumab and solanezumab failed to show beneficial effect in clinical trials [7, 13], passive immunotherapy still offers exciting preliminary results. In this study, we successfully developed an A β ₁₋₄₂ N-terminus targeting antibody 3F5, and confirmed that the mAb

specifically bound A β _{1–42} to promote depolymerization of A β fibrils, facilitate endocytosis of A β _{1–42} by microglia, and attenuate the death and apoptosis of neuronal cells, accompanied by neurite outgrowth. Moreover, therapy with 3F5 improved the behavioral abilities of Tg-mice. Further, 3F5 reduced A β burden and initiated A β efflux from the brain in Tg-mice.

Despite the development of potential therapies for AD, all double-blind, placebo-controlled Phase III studies of these therapies have failed to show clinical efficacy. Failure of all Phase III trials prompted the hypothesis that early intervention perhaps years before clinical symptoms of AD maybe crucial. [14]. Therefore, 6-month-old Tg-mice, which have not yet developed noticeable symptoms of memory loss were used in this study to investigate the therapeutic potential of 3F5. We found that 3F5 prevented memory and cognitive decline of Tg-mice, and decreased amyloid in the hippocampus and cortex. Although the accurate epitope remains "target" unclear, 3F5 may bind amino acids between 1 and 11 in the N-terminal of A β _{1–42} to induce depolymerization of A β fibrils therefore to facilitate endocytosis of A β _{1–42} by microglia. It is interesting to note that 3F5 preferentially bound A β _{1–11} vs A β _{1–42}, which contains amino acids 1–11, suggesting that conformational change in A β _{1–42} peptide structure aggregation process may interfere its binding by 3F5 [15, 16]. The exact mechanisms of the binding of 3F5 to a conformational epitope will be investigated by analyzing the crystal structures of 3F5 complexed with A β .

Mounting evidence confirms the efficacy of A β N-terminus targeting antibodies, but side-effects, notably microhemorrhage and vasogenic edema (VE) in the brain constitute risks for AD patients [17]. Our study showed that 3F5 causes minor microhemorrhage lesions in Tg-mice accompanied with clearance of A β _{1–42}. The finding is in agreement with a recent study showing that Bapineuzumab, a humanized IgG1, which recognizing the N terminus of A β (aa 4–10), clears plaques from the brains of AD patients in a phase II trial. However, it also causes cerebrovascular oedema and micro-haemorrhage in the cerebral vasculature [18, 19]. Preclinical studies suggest that Bapineuzumab may either decrease microglial response due to decreased plaque load, or enhance the cell response due to increased activation of Fc γ R upon phagocytosis of IgG-A β complexes [20, 21]. It have been reported that modifying the backbone of the antibody may reduce Fc γ R affinity and inflammatory responses therefore reduce the associated side effects [3]. However, the efficacy to remove plaques and the potential to induce side effects are difficult to predict due to use of different experimental models, epitope specificity of the antibody or antibody subclass [22]. Furthermore, studies in Tg mice have not translated into successful clinical trials in humans; therefore, the response to different anti-A β antibodies may differ in human AD patients [22]. Therefore, larger scale trials with longer durations should be crucial for future efficacy and safety analysis of 3F5. In addition, although A β N-terminus-directed antibodies are more effective in dissolving preexisting A β plaques in the brain [23], these antibodies may also promote the formation of A β oligomers, which are more neurotoxic. Therefore, combination of 3F5 with other neuroprotective therapy such as anti-tau agents, gene therapies, natural compounds, and stem cells may achieve more favorable outcomes [8].

There is an equilibrium for A β in the brain and in the circulation [24, 25]. Our study showed that after 3F5 treatment the level of A β _{1–42} in the brain tissues significantly decreased in Tg-mice while there is an increase of A β _{1–42} in the serums. Therefore, 3F5 facilitated the efflux of A β _{1–42} from the brain to the circulation [26]. It is likely that free A β peptides in the serum may be cleared by proteases including insulin-dependent enzyme, neprilysin, α -secretase, and other nonspecific proteases if A β do not form aggregates resistant to degradation [27]. Interestingly, the concentration of 3F5 in the circulation is much higher than that in the brain in 3F5-treated Tg-mice (Fig 5C). Thus, 3F5 may cross BBB to bind A β to initiate the phagocytosis of antibody/A β complex by microglia. 3F5 may also conjugate with A β in the

circulation to promote A β efflux from the brain and inhibit blood-to-brain influx of A β . This is consistent with previous study [6, 26], showing that anti-A β antibody may facilitate A β clearance through the interaction of antibody-amyloid plaque in the brain as “central clearance” and the interaction of antibody-A β in the circulation as “peripheral clearance” [8].

Further research is necessary to explore the efficacy, safety and functional mechanism of 3F5 in larger sizes of animals. Moreover, successful management of AD may require targeting not only A β but also A β -associated pathogenic mechanisms such as tau hyperphosphorylation, neuroinflammation, and oxidative stress [8]. Regardless, our novel anti-A β ₁₋₄₂ antibody 3F5 has shown potential as a basis for development of more effective therapeutic agents for AD.

Acknowledgments

This project was supported by the grants from the National Natural Science Foundation of China (Grant No. 81302867 and 81273608). We also gratefully acknowledge the financial support from the Chongqing Programs for Science and Technology Development (CSTC2015jcyjBX0018). Ji Ming Wang was funded by Federal funds from the National Cancer Institute, National Institutes of Health, under contract No. HHSN 26/200800001E and supported in part by the Intramural Research Program of NCI, NIH, USA.

Author Contributions

Conceptualization: George Ye, Jian-Hong Chen.

Data curation: Jian-Hong Chen.

Funding acquisition: Jian-Hong Chen.

Investigation: Jian-Hong Chen.

Methodology: Hai-Yan Xing, Bin Li, Dan Peng, Chun-Yan Wang, Guan-Ying Wang, Pan Li.

Project administration: Jian-Hong Chen.

Resources: Ying-Ying Le, Ji-Ming Wang, George Ye.

Supervision: Jian-Hong Chen.

Writing – original draft: Hai-Yan Xing, Bin Li.

Writing – review & editing: Hai-Yan Xing, Bin Li, Ji-Ming Wang.

References

1. Hardy J, Selkoe DJ. The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. *Science*. 2002, 297(5580):353–356. <https://doi.org/10.1126/science.1072994> PMID: 12130773
2. Bohrmann B, Baumann K, Benz J, Gerber F, Huber W, Knoflach F, et al. Gantenerumab: a novel human anti-Abeta antibody demonstrates sustained cerebral amyloid-beta binding and elicits cell-mediated removal of human amyloid-beta. *J Alzheimers Dis*. 2012, 28(1), 49–69. <https://doi.org/10.3233/JAD-2011-110977> PMID: 21955818
3. Adolfsson O, Pihlgren M, Toni N, Varisco Y, Buccarello AL, Antonello K, et al. An effector-reduced anti-beta-amyloid (Abeta) antibody with unique abeta binding properties promotes neuroprotection and glial engulfment of Abeta. *J Neurosci*. 2012, 32(28): 9677–9689. <https://doi.org/10.1523/JNEUROSCI.4742-11.2012> PMID: 22787053
4. Wang J, Li N, Ma J, Gu Z, Yu L, Fu X, et al. Effects of an amyloid-beta 1–42 oligomers antibody screened from a phage display library in APP/PS1 transgenic mice. *Brain Res*. 2016, 1635: 169–179. <https://doi.org/10.1016/j.brainres.2016.01.028> PMID: 26820640
5. Houacine J, Bolmont T, Aeschbach L, Oulad-Abdelghani M, Fraering PC. Selective neutralization of APP-C99 with monoclonal antibodies reduces the production of Alzheimer's Abeta peptides. *Neurobiol*

- Aging. 2012, 33(11): 2704–2714. <https://doi.org/10.1016/j.neurobiolaging.2011.12.033> PMID: 22317957
6. Bachmeier CJ, Beaulieu-Abdelahad D, Mullan MJ, Paris D. Epitope-dependent effects of Beta-amyloid antibodies on Beta-amyloid clearance in an in vitro model of the blood-brain barrier. *Microcirculation*. 2011, 18(5), 373–379. <https://doi.org/10.1111/j.1549-8719.2011.00096.x> PMID: 21418385
 7. Vandenberghe R, Rinne JO, Boada M, Katayama S, Scheltens P, Vellas B, et al. Bapineuzumab for mild to moderate Alzheimer's disease in two global, randomized, phase 3 trials. *Alzheimers Res Ther*. 2016, 8(1), 18. <https://doi.org/10.1186/s13195-016-0189-7> PMID: 27176461
 8. Ma L, Liu J, Zhang X, Qi J, Yu W, Gu Y. p38 MAPK-dependent Nrf2 induction enhances the resistance of glioma cells against TMZ. *Med Oncol* 2015, 32(3): 69. <https://doi.org/10.1007/s12032-015-0517-y> PMID: 25691294
 9. Jiao SS, Bu XL, Liu YH, Zhu C, Wang QH, Shen LL, et al. Sex Dimorphism Profile of Alzheimer's Disease-Type Pathologies in an APP/PS1 Mouse Model. *Neurotox Res* 2016, 29(2): 256–266. <https://doi.org/10.1007/s12640-015-9589-x> PMID: 26707129
 10. Webster SJ, Bachstetter AD, Van Eldik LJ. Comprehensive behavioral characterization of an APP/PS-1 double knock-in mouse model of Alzheimer's disease. *Alzheimers Res Ther*. 2013, 5(3), 28. <https://doi.org/10.1186/alzrt182> PMID: 23705774
 11. Association. As. 2016 Alzheimer's disease facts and figures. *Alzheimers Dement*. 2016, 12(4): 459–509. PMID: 27570871
 12. Underwood E. NEUROSCIENCE. Alzheimer's amyloid theory gets modest boost. *Science*. 2015, 349(6247), 464. <https://doi.org/10.1126/science.349.6247.464> PMID: 26228122
 13. Doody RS, Thomas RG, Farlow M, Iwatsubo T, Vellas B, Joffe S, et al. Phase 3 trials of solanezumab for mild-to-moderate Alzheimer's disease. *N Engl J Med*. 2014, 370(4): 311–321. <https://doi.org/10.1056/NEJMoa1312889> PMID: 24450890
 14. Amanatkar HR, Papagiannopoulos B, Grossberg GT. Analysis of recent failures of disease modifying therapies in Alzheimer's disease suggesting a new methodology for future studies. *Expert Rev Neurother* 2017, 17(1): 7–16. <https://doi.org/10.1080/14737175.2016.1194203> PMID: 27223100
 15. Enache TA, Chiorcea-Paquim AM, Oliveira-Brett AM. Amyloid-beta peptides time-dependent structural modifications: AFM and voltammetric characterization. *Anal Chim Acta* 2016, 926: 36–47. <https://doi.org/10.1016/j.aca.2016.04.015> PMID: 27216391
 16. Watt AD, Crespi GA, Down RA, Ascher DB, Gunn A, Perez KA, et al. Do current therapeutic anti-Abeta antibodies for Alzheimer's disease engage the target? *Acta Neuropathol* 2014, 127(6): 803–810. <https://doi.org/10.1007/s00401-014-1290-2> PMID: 24803227
 17. Yi K. Passive immunotherapy—a viable treatment for Alzheimer's disease. *Psychiatr Danub*. 2014, 26 Suppl 1, 256–265. PMID: 25413550
 18. Rinne JO, Brooks DJ, Rossor MN, Fox NC, Bullock R, Klunk WE, et al. 11C-PiB PET assessment of change in fibrillar amyloid-beta load in patients with Alzheimer's disease treated with bapineuzumab: a phase 2, double-blind, placebo-controlled, ascending-dose study. *Lancet Neurol* 2010, 9(4): 363–372. [https://doi.org/10.1016/S1474-4422\(10\)70043-0](https://doi.org/10.1016/S1474-4422(10)70043-0) PMID: 20189881
 19. Laskowitz DT, Kolls BJ. A phase 2 multiple ascending dose trial of bapineuzumab in mild to moderate Alzheimer disease. *Neurology* 2010, 74(24): 2026–2027. <https://doi.org/10.1212/WNL.0b013e3181e03844> PMID: 20548049
 20. McLaurin J, Cecal R, Kierstead ME, Tian X, Phinney AL, Manea M, et al. Therapeutically effective antibodies against amyloid-beta peptide target amyloid-beta residues 4–10 and inhibit cytotoxicity and fibrillogenesis. *Nat Med* 2002, 8(11): 1263–1269. PMID: 12379850
 21. Freeman GB, Brown TP, Wallace K, Bales KR. Chronic administration of an aglycosylated murine antibody of ponezumab does not worsen microhemorrhages in aged Tg2576 mice. *Curr Alzheimer Res* 2012, 9(9):1059–1068. PMID: 22631613
 22. Arai H, Umemura K, Ichimiya Y, Iseki E, Eto K, Miyakawa K, et al. Safety and pharmacokinetics of bapineuzumab in a single ascending-dose study in Japanese patients with mild to moderate Alzheimer's disease. *Geriatr Gerontol Int*. 2016, 16(5): 644–650. <https://doi.org/10.1111/ggi.12516> PMID: 26044070
 23. Dodel R, Balakrishnan K, Keyvani K, Deuster O, Neff F, Andrei-Selmer LC, et al. Naturally occurring autoantibodies against beta-amyloid: investigating their role in transgenic animal and in vitro models of Alzheimer's disease. *J Neurosci*. 2011(15), 31: 5847–5854. <https://doi.org/10.1523/JNEUROSCI.4401-10.2011> PMID: 21490226
 24. DeMattos RB, Bales KR, Cummins DJ, Dodart JC, Paul SM, Holtzman DM. Peripheral anti-A beta antibody alters CNS and plasma A beta clearance and decreases brain A beta burden in a mouse model of Alzheimer's disease. *Proc Natl Acad Sci USA*. 2001, 98:8850–8855. <https://doi.org/10.1073/pnas.151261398> PMID: 11438712

25. Zhang Y, Lee DH. Sink hypothesis and therapeutic strategies for attenuating Abeta levels. *Neuroscientist*. 2011, 17(2), 163–173. <https://doi.org/10.1177/1073858410381532> PMID: 21330304
26. DeMattos RB, Bales KR, Cummins DJ, Paul SM, Holtzman DM. Brain to plasma amyloid-beta efflux: a measure of brain amyloid burden in a mouse model of Alzheimer's disease. *Science*. 2002, 295(5563), 2264–2267. <https://doi.org/10.1126/science.1067568> PMID: 11910111
27. Selkoe DJ. Alzheimer disease: mechanistic understanding predicts novel therapies. *Ann Intern Med*. 2004, 140(8), 627–638. PMID: 15096334