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**RESEARCH ARTICLE**

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**APOE Genotype Differentially Modulates Effects of Anti-Aβ, Passive Immunization in APP Transgenic Mice**

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**Abstract**

**Background:** APOE genotype is the foremost genetic factor modulating β-amyloid (Aβ) deposition and risk of sporadic Alzheimer’s disease (AD). Here we investigated how APOE genotype influences response to anti-Aβ immunotherapy.

**Methods:** APP⁺/PS1⁺E9 (APP) transgenic mice with targeted replacement of the murine Apoe gene for human APOE alleles received 10D5 anti-Aβ or TY11-15 isotype control antibodies between the ages of 12 and 15 months.

**Results:** Anti-Aβ immunization decreased both the load of fibrillar plaques and the load of Aβ immunopositive plaques in mice of all APOE backgrounds. Although the relative reduction in parenchymal Aβ plaque load was comparable across all APOE genotypes, APP/ε4 mice showed the greatest reduction in the absolute Aβ plaque load values, given their highest baseline. The immunization stimulated phagocytic activation of microglia, which magnitude adjusted for the post-treatment plaque load was the greatest in APP/ε4 mice implying association between the ε4 allele and impaired Aβ phagocytosis. Perivascular hemosiderin deposits reflecting ensued microhemorrhages were associated with vascular Aβ (VAβ) and ubiquitously present in control mice of all APOE genotypes, although in APP/ε3 mice their incidence was the lowest. Anti-Aβ immunization significantly reduced VAβ burden but increased the number of hemosiderin deposits across all APOE genotypes with the strongest and the weakest effect in APP/ε2 and APP/ε3 mice, respectively.

**Conclusions:** Our studies indicate that APOE genotype differentially modulates microglia activation and Aβ plaque load reduction during anti-Aβ immunotherapy. The APOE ε3 allele shows strong protective effect against immunotherapy associated microhemorrhages; while, conversely, the APOE ε2 allele increases risk thereof.

**Keywords:** Alzheimer’s disease, Apolipoprotein E, β-amyloid, Brain hemorrhages, Microglia, Neurodegeneration, Therapy, Vasculopathy

**Background**

Accumulation of β-amyloid (Aβ) in the brain is a culprit early in Alzheimer’s disease (AD) pathogenesis, and triggers down-stream neurodegenerative cascades including inflammatory responses, intraneuronal neurofibrillary pathology, and synaptic and neuronal loss (reviewed in [1–3]). Susceptibility to sporadic AD is foremost modulated by APOE genotype, which also influences the load of Aβ parenchymal plaques and vascular Aβ (VAβ) deposits.

A single copy of the APOE ε4 allele endows a ~3 fold increase in AD risk, and 2 APOE ε4 copies result in a ~15 fold risk increase, while an APOE ε2 allele halves AD risk relative to 2 copies of ε3 (reviewed in [4]). Autopsy series and more recently positron emission tomography (PET) imaging studies of fibrillar Aβ plaque load in AD patients have shown ε4 > ε3 > ε2 allele gradation effect on Aβ deposition [1–3]. To counteract down-stream neurodegenerative effects triggered by Aβ accumulation, development of anti-Aβ therapeutic strategies including anti-Aβ immunotherapy have been proposed and pursued. Several anti-Aβ monoclonal antibodies (mAbs) have been tested in clinical trials in AD patients and were found to significantly reduce load of fibrillar Aβ as demonstrated using...
PET Aβ imaging [5–8]. Whether APOE genotype also differentially modulates degree of Aβ plaque load reduction in response to anti-Aβ passive immunization remains unknown due to limited clinical data and because preclinical testing of anti-Aβ mAbs has been exclusively conducted in AD transgenic (Tg) mice models expressing wild type, murine apoE [9–12]. The main adverse effects associated with administration of certain anti-Aβ mAbs during clinical trials were amyloid related imaging abnormalities (ARIA) identified on magnetic resonance imaging (MRI) scans. These included vasogenic edema (ARIA-E) and cerebral microhemorrhages (ARIA-H) that in about 20% of cases are associated with clinical symptoms and signs [13, 14]. Frequency of ARIA events and in particular ARIA-E was significantly higher among APOE ε4 allele carriers compared to non-carriers, making the APOE ε4 allele a risk factor for vascular complications of anti-Aβ immunotherapy [5, 8, 14, 15]. In view of these considerations, we sought to re-examine the effects of passive immunization in APPSWE/PS1ΔE9 Tg mice with targeted replacement of the murine Apoe gene for various human APOE alleles, which expression remains controlled by the native murine Apoe promoter [16, 17]. These Tg mouse lines, hereafter designated as APP/ε2, APP/ε3, and APP/ε4, reflect the differential effect of APOE alleles on the load of Aβ parenchymal plaques and VAB known from AD patients [18]. The vaccination experiment was started in 12 months old mice with advanced load of Aβ deposits [19, 20], to emulate the stage of human disease in regard to Aβ deposition in which anti-Aβ immunotherapy is currently occurring. We used mAb 10D5 direct against Aβ3−7 epitope [10], which is known to penetrate the blood–brain-barrier (BBB) and directly binds to deposited Aβ triggering microglial cells to clear Aβ plaques through Fc receptor-mediated phagocytosis [9]. Our study in APOE humanized APPSWE/PS1ΔE9 mice provides evidence for differential effect of APOE alleles on response to anti-Aβ immunotherapy and occurrence of vascular complications associated with thereof.

Methods
All reagents and antibodies, unless stated otherwise, were purchased from Sigma-Aldrich (St. Louis, MO).

Animals and antibody treatment
All mouse care and experimental procedures were approved by Institutional Animal Care and Use Committees of the New York University School of Medicine and the Washington University School of Medicine. Generation of APP/ε2, APP/ε3, and APP/ε4 mice by cross-breeding of APOE ε2, APOE ε3, or APOE ε4 targeted replacement mice with APPSWE/PS1ΔE9 mice and detailed genotyping procedures have been previously described [19, 20]. This study was performed using non-breeder mice of both sexes with each sex contributing approximately half to the total animal number in each experimental group. Animals separated by sex were aged in a barrier facility with a 12/12 h. light/dark cycle and ad libitum food and water access. The antibody treatment in animals of all APOE backgrounds was commenced at the age of 12 months and was carried out continuously for three months till the animals were killed at the age of 15 months. Anti-Aβ 10D5 mAb or TY11-15 an isotopic control IgG2a antibody [9, 10, 21] were administered once a week (10 mg/kg) through intraperitoneal injections. The dose of 10D5 mAb used in this study was previously shown to be effective in removing Aβ plaques in PDAPP AD Tg mice expressing wild type murine apoE [9, 10, 22]. Prior to each injection mice were weighted and the accurate dose of administered antibodies was quantified. Antibodies were injected diluted in 0.25 ml of sterile phosphate buffered saline (PBS), pH 7.4 using a 29 gauge needle. Both 10D5 mAb and TY11-15 control IgG were produced, purified and provided by Janssen Alzheimer Immunotherapy. In addition to 10D5 mAb treatment and TY11-15 control groups, for each APOE genotype we analyzed a group of age-matched mice receiving no treatment or sham injections. During the treatment all animals were closely monitored for any signs of toxicity by the veterinary staff. 10D5 mAb and TY11-15 control IgG2a injections were well tolerated by the animals, which showed no abnormalities in the following monitored parameters: body weight, physical appearance, occurrence of unprovoked behavior, and blunted or exaggerated responses to external stimuli. At the age of 15 months all animals were killed with an overdose of sodium pentobarbital (150 mg/kg) and transcardially perfused with heparinized (1,000 units/L) 0.01 M PBS at pH 7.4 and temperature 37°C.

Histological processing and immunohistochemistry
Brains were extracted from skulls and fixed by immersion in 4% paraformaldehyde in 0.1 M phosphate buffer at pH 7.4 for 72 h. and then dehydrated in a mixture of 20% Dimethyl sulfoxide and 20% glycerol in 0.01 PBS at pH 7.4 and temperature 4°C. Brains were cut serially along the entire rostro-caudal axis using a freezing microtome (Leica Microsystems, Wetzlar, Germany), into coronal, 40-µm thick sections, which were alternately collected into 10 series. Randomly selected series of sections were used for the following histological and immunohistochemical procedures: Thioflavin-S (Th-S) stain to visualize fibrillar component of Aβ parenchymal plaques and cerebral amyloid angiopathy (CAA); anti-Aβ immunohistochemistry using HJ3.4 mAb (1:250) [19, 23]; Perls’ Prussian blue stain to detect perivascular ferric iron deposition [24–26]; and a combination of anti-Iba1 immunostaining (anti-Iba1 rabbit polyclonal antibody 1:1,000; Wako Chemicals USA Inc., Richmond VA) or anti-CD68 immunostaining (anti-CD68 rabbit polyclonal antibody 1:250; Abcam Inc, Cambridge,
MA) and Th-S to characterize microglia response in association with fibrillar Aβ parenchymal plaques. The intensity of HJ3.4 immunostaining was enhanced by formic acid (FA) pretreatment and the immunohistochemistry protocol was concluded using M.O.M. 3,3’-diaminobenzidine (DAB) kit (Vector Laboratories; Burlingame, CA) as per manufacturer manual. The anti-CD68 and anti-Iba1 immunostaining protocol was concluded using mouse anti-rabbit IgG biotinylated antibody (1:500) followed by streptavidin conjugated Cy3 fluorochrome (1:200), as previously described [19]. Additional series from selected brains were double-stained with Perls’ Prussian blue stain and HJ3.4 anti-Aβ mAb or Th-S stain to visualize association of VΑβ with hemosiderin deposits. One series of sections from five randomly selected APP/ɛ4 mice treated with 10D mAb and TY11-15 IgG controls were immunostained with 4G8 mAb (1:2,000) (Covance; Princeton, NJ) directed against Aβ residues 17–24 [20, 27]. In this case, sections were also FA pretreated to enhance Aβ immunodetection and the staining procedure was concluded using M.O.M. DAB kit. This was done to verify that 10D5 mAb does not obscure binding of HJ3.4 mAb to deposited Aβ since both mAbs are directed against the N-terminus of Aβ while 4G8 mAb binds a central Aβ epitope.

**Analysis of fibrillar parenchymal plaque load and cerebral amyloid angiopathy (CAA) load**

These metrics were determined using an unbiased, whole-section quantification approach. The entire cross-sectional profile of the right hemisphere stained with Th-S, was photographed under ×4 objective magnification using a high-sensitivity, cooled, monochrome DS-Qi1Mc camera (Nikon Corp. Tokyo, Japan). Adjacent frames were combined into a single image using the photo-stitching function of the NIS Elements Imaging Software, and automatically thresholded and filtered by size according to the preset algorithm to discriminate nonspecific staining and Aβ positive capillaries using NIH Image J v 1.47 (Bethesda, MD). The load of immunopositive Aβ plaques per brain was calculated as the average of load values obtained from all three analyzed brain sections.

**Assessment of cerebral capillary Aβ (CCA)**

Prevalence of HJ3.4-immunoreactive capillaries was analyzed on three coronal cross-sectional profiles through the brain cortex at the aforementioned anatomical levels and on three to four subsequent coronal cross-sectional profiles through the thalamus. Aβ immunopositive capillaries (defined as vessels with diameter <8 μm [28, 29]) were manually counted at 40× objective magnification and their number was expressed as n/mm².

**Microglia analysis**

The analysis was performed on sections immunostained against Iba1 or CD68 antigen and counterstained with Th-S. Microphotographs of the brain cortex were taken at ×20 objective magnification under Tetramethylrhodamine (for Iba1/Cy3 and CD68/Cy3) and Fluorescein isothiocyanate (for Th-S) channels of 80i Nikon fluorescent microscope (Nikon Corp. Tokyo, Japan) using DS-Qi1Mc monochrome camera (Nikon Corp. Tokyo, Japan). The load of Iba1 and CD68 positive cells (defined as the percentage of a microphotograph area occupied by anti-Iba1 or anti-CD68 positive immunostaining) was automatically thresholded and filtered according to the preset algorithm to discriminate nonspecific staining using NIH Image J v 1.47 (Bethesda, MD). To control for variable Aβ parenchymal plaque load...
across APOE genotypes the Iba1 load and the CD68 load was divided by the load of Th-S positive Aβ parenchymal plaques determined on the same microphotograph as described above.

Analysis of perivascular hemosiderin deposits
A complete series of brain coronal cross-sections evenly spaced 400 μm apart from each animal was analyzed. Such series typically includes 13–15 sections, which
were stained with Perl’s Prussian blue method without counterstain to maximize sensitivity of detection. Manual count of perivascular hemosiderin deposits in all brain structures (Additional file 2: Figure S2 A-C) was performed under × 20 objective magnifications. Deposits ≥ 15 μm in diameter were classified as large.

Measurement of the serum cholesterol level
At the conclusion of the experiment blood samples were collected and centrifuged to separate serum. The total serum cholesterol concentration was measured using a standard enzymatic assay based on the Cholesterol E kit (Wako Diagnostics, Richmond, VA) as previously described [19, 24].

Statistical analysis
Distribution of data within all data sets was analyzed with Kolmogorov-Smirnov and Shapiro-Wilk tests. Multiple data sets conforming to the normal distribution were first compared using Kruskal-Wallis one-way analysis of variance between selected data sets. All statistical analyses were performed using GraphPad Prism v 6.05 (GraphPad Software, Inc.). All data were reported as the mean and the standard error of the mean (SEM) per APOE genotype and treatment arm for each outcome measure.

Results
APOE genotype modulates reduction in parenchymal plaque load by Aβ immunotherapy
APOE targeted replacement APPSwPS1ΔE9 mice faithfully reflect the differential effect of APOE alleles on Aβ brain deposition. At the age of 15 months, the load of Th-S positive, fibrillar plaques in the brain cortex of TY11-15 control APP/e4 mice was 3.1 and 2.6 fold higher than that in TY11-15 control APP/e2 and APP/e3 mice, respectively, while in the hippocampus it was 2.7 and 2.1 fold higher, respectively (p < 0.0001) (Fig. 1a-c). The load of immunopositive Aβ plaques in the brain cortex of TY11-15 control APP/e4 mice was 1.3 and 1.6 fold higher, than that in TY11-15 control APP/e2 and APP/e3 mice, respectively, while in the hippocampus it was 1.5 and 1.7 fold higher, respectively (p < 0.001) (Fig. 2a-d). The plaque load in the brain cortex for matching APOE genotypes was higher than that in the hippocampus and the fold difference ranged from 1.4 (APP/e3) to 1.8 (APP/e4) for fibrillar plaque load (p < 0.01) and from 1.1 (APP/e4) to 1.3 (APP/e2) for immunopositive Aβ plaque load (p < 0.05 for APP/e2 only). As we found no statistically significant differences in the load of fibrillar and immunopositive Aβ plaques between male and female mice of the same APOE genotype, thus animals of both sexes were combined for all analyses.

10D5 mAb immunization decreased both fibrillar and immunopositive Aβ parenchymal plaque loads. The degree of reduction relative to TY11-15 controls was comparable across all three APOE genotypes. Thus, the fibrillar plaque load in the brain cortex in 10D5 mAb treated APP/ε2, APP/ε3, APP/e4 mice was reduced by 27.7% (p < 0.01), 24.4% (p < 0.01), and 22.7% (p < 0.0001) of the TY11-15 control mean value for matching APOE genotype (Fig. 1a, b), respectively; while the load of Aβ immunopositive plaques was reduced by 43.7% (p < 0.0001), 36.0% (p < 0.01), and 44.3% (p < 0.0001) (Fig. 2a, b), respectively. Similarly, the load of fibrillar plaques in the hippocampus of 10D5 mAb treated APP/ε2, APP/ε3, APP/e4 mice was decreased by 26.7% (p < 0.05), 34.0% (p < 0.01), and 42.5% (p < 0.0001) of the TY11-15 control mean value for matching APOE genotype (Fig. 1a, c), respectively; while the load of Aβ immunopositive plaques was lowered by 38.7% (p < 0.001), 30.1% (p < 0.05), and 38.3% (p < 0.0001) (Fig. 2c, d), respectively. However, given significantly higher plaque load baseline in APP/e4 mice, the absolute plaque load reduction effected by same dose of 10D5 mAb was incomparably higher in APP/e4 mice than in other genotypes. Thus, the fibrillar plaque load in the brain cortex was reduced from 1.93 ± 0.05% of the cortical cross-sectional area in TY11-15 APP/e4 controls to 1.49 ± 0.06% in 10D5 mAb treated APP/e4 mice (absolute reduction = 44%), while the load of immunopositive Aβ plaques was reduced from 2.67 ± 0.07% to 1.49 ± 0.09% between TY11-15 and 10D5 mAb groups, respectively (absolute reduction = 1.18%). For comparison in APP/ε2 and APP/ε3 mice the absolute reduction in the fibrillar plaque load in the brain cortex was 0.17% and 0.18%, respectively; while the absolute reduction in the load of Aβ immunopositive plaques was 0.89% and 0.61%, respectively. Analogously, the fibrillar plaque load in the hippocampus of APP/e4 mice was reduced from 1.08 ± 0.10% of the hippocampal cross-sectional area in TY11-15 controls to 0.62 ± 0.05% in 10D5 mAb treated mice (absolute reduction = 0.46%), while the load of immunopositive Aβ plaques was reduced from 2.43 ± 0.15% to 1.50 ± 0.14% between TY11-15 and 10D5 mAb groups, respectively (absolute reduction = 0.93%). In APP/ε2 and APP/ε3 mice the absolute reduction in fibrillar plaque load in the hippocampus was 0.11% and 0.18%, respectively; while the absolute reduction in the load of Aβ immunopositive plaques was 0.62% and 0.42%, respectively. Despite greater treatment effect seen in APP/e4 mice, post-treatment load of both fibrillar and immunopositive Aβ parenchymal plaques in APP/e4 mice remained significantly higher than these in 10D5 mAb treated APP/ε2 and APP/ε3 mice (Fig. 1b, c and Fig. 2b, d).

We found no statistically significant differences between values of fibrillar plaque loads in the brain cortex and in
the hippocampus between TY11-15 isotype control IgG2a antibody treated mice and untreated age-matched mice for corresponding APOE genotypes (Fig. 1a-c). Likewise the loads of immunopositive Aβ plaques in TY11-15 control and untreated age-matched mice for corresponding APOE genotypes were similar (Fig. 2a-e).

Since 10D5 mAb used for immunotherapy and HJ3.4 mAb used for immunohistochemistry are both directed against the N-terminus of Aβ we immunostained brain section from TY11-15 control and 10D5 mAb treated APP/ε4 mice with 4G8 mAb directed against midportion of Aβ [27] and compared Aβ plaque load on immunostaining with both antibodies. This analysis was done to eliminate unlikely possibility that 10D5 mAb hinders the HJ3.4 epitope resulting in reduced ability to detect plaques. Average values of parenchymal Aβ plaque loads revealed by 4G8 mAb immunostaining were 2.57% ± 0.10% and 1.49% ± 0.09 in TY11-15 control
and 10D5 mAb treated APP/ε4 groups; respectively; and they were not significantly different from values obtained with HJ3.4 mAb for the same animal groups (Additional file 1: Fig. S1 A, B).

10D5 mAb exerts enhanced effector response on microglia in the setting of the APOE ε4 allele

Expression of two microglia markers, Iba1 and CD68 were compared across APOE genotypes and treatment arms. Iba1 is a calcium-binding protein specifically expressed by microglia and macrophages and its expression increases with microglia activation [30]. CD68 antigen is a glycoprotein localized to the lysosomal membrane in microglia and blood derived macrophages and is up regulated in actively phagocytic cells [31]. Activated microglia expressing both markers were found in the vicinity of Th-S positive plaques in control animals of all APOE genotypes but their loads were significantly higher in APP/ε4 mice compared to APP/ε2 and APP/ε3 mice. The load of Iba1 positive cells in the brain cortex of TY11-15 APP/ε4 control mice was 2.9 and 2.6 fold greater than in TY11-15 APP/ε2 and APP/ε3 control mice (p < 0.0001) (Fig. 3a, b), respectively; while the CD68 load was approximately 2.9 greater (p < 0.0001) (Fig. 3d, e). The isotype control...
antibody TY11-15 treatment had no significant effect on Iba1 and CD68 loads as their values were similar in TY11-15 control mice and untreated age-matched mice of corresponding APOE genotypes.

Passive immunization with 10D5 mAb effected significant microglia response across all APOE genotypes. The Iba1 load in 10D5 mAb treated APP/ε2, APP/ε3, and APP/ε4 mice was increased by 23.8% (p < 0.05), 39.0% (p < 0.05), and 39.4% (p < 0.0001) relative to the mean Iba1 load in TY11-15 controls of corresponding APOE genotypes, respectively (Fig. 3a, b); while the CD68 load was increased by 29.5% (p < 0.05), 32.8% (p < 0.05), and 23.3% (p < 0.0001), respectively (Fig. 3d, e).

Since the load of Iba1 and CD68 positive microglial was distinguishably the greatest in TY11-15 APP/ε4 mice, which also show the highest Aβ plaque load, we additionally analyzed the ratio of Iba1 and CD68 loads to the load of Th-S-positive parenchymal plaques for the same test area (Fig. 3c, f). This analysis enabled us to compare the level of microglia activation across APOE genotypes. We found no statistically significant differences in the Iba1/Th-S ratio and in the CD68/Th-S ratio between TY11-15 control and untreated age-matched control mice across all APOE genotypes suggesting that both Iba1 load and CD68 load correlate with fibrillar plaque load. 10D5 mAb immunization effected significant increase in both the Iba1/Th-S ratio and the CD68/Th-S ratio. The magnitude of increase was comparable between APP/ε2 and APP/ε3 genotypes while in APP/ε4 mice it was substantially higher. In 10D5 mAb treated APP/ε4 mice the Iba1/Th-S ratio became 1.4 fold higher than in 10D5 mAb treated APP/ε2 and APP/ε3 mice (p < 0.0001), while the CD68/Th-S ratio was 1.33 (p < 0.0001) and 1.21 (p < 0.01) fold higher, respectively (Fig. 3c, f).

Using the combination of anti-Iba1 immunostaining and Th-S counterstaining enabled us also to analyze morphology of microglia across APOE genotypes and treatment arms. Microglia associated with fibrillar plaques displaying features of activated microglia/macrophage ie. enlargement and rounding of the perikaryon and shortening of processes to their nearly disappearance, were observed in untreated age-matched and TY11-15 controls of all APOE backgrounds (Fig. 3a). To clarify, Iba1 positive activated microglia/macrophages was significantly more pronounced in 10D5 treated APP/ε4 mice. Peri-plaque recruitment of Iba1 positive activated microglia/macrophages and accompanying upsurge of CD68 expression in 10D5 mAb treated mice was associated with reduced plaque diameter as demonstrated by double anti-Iba1/Th-S and anti-CD68/Th-S stainings (Fig. 3a, d). We also observed Iba1 positive activated microglia and CD68 positive puncta, which were dissociated from Th-S positive plaque. They likely define activated microglia engaged with diffuse, immunopositive Aβ plaques lacking fibrillar component. Their load also became markedly increased with 10D5 mAb treatment.

**APOE genotype has strong differential effect on VAβ burden and its reduction with Aβ immunotherapy**

Two forms of Aβ angiopathy were observed in brains of APOE targeted replacement APPSWI/PS1ΔE9 mice and they were analyzed separately. The first type was CAA defined as Th-S positive deposits of fibrillar Aβ in walls of meningeal arteries and arteries penetrating the brain cortex (Fig. 4a). CAA load was quantified in the brain cortex on Th-S stained sections and defined as the percent of the cross-sectional area of the cortex occupied by CAA laden vessels. The second type of Aβ angiopathy concerned immunoreactive Aβ deposits along walls of capillary vessels, which were Th-S negative (CCA) (Fig. 5a). CCA incidence was analyzed by calculating a number of Aβ immunopositive capillary profiles per mm² in the brain cortex where they appeared in abundance across all APOE genotypes. In addition, CAA was analyzed in the thalamus as in APP/ε2 mice it was associated with outstanding number of perivascular hemosiderin deposits.

There was no significant difference in the CAA load between untreated age-matched and TY11-15 control mice for each of APOE genotypes analyzed. However across the TY11-15 control groups, APP/ε4 mice showed two fold higher CAA load than APP/ε3 mice (p < 0.01) (Fig. 4a, b). In contrast to APP/ε3 and APP/ε4 lines, in APP/ε2 mice CAA laden arteries were rarely occurring and were present in only some of the mice which precluded reliable quantification of the CAA load in this genotype (Fig. 4a). CAA laden vessels were rarely and inconsistently observed in the hippocampus and in subcortical structures including the thalamus in mice of any APOE genotype. 10D5 mAb treatment reduced the CAA load in the brain cortex in APP/ε3 and APP/ε4 mice by 56% (p < 0.05) and 71% (p < 0.01) relative to the mean CAA load value in TY11-15 controls of corresponding genotypes. However, given significantly higher CAA load baseline in APP/ε4 mice the absolute CAA load reduction in APP/ε4 mice was 2.5 fold higher than that in APP/ε3 mice. Thus, the CAA load in APP/ε4 animals was reduced from 0.41 ± 0.03% in TY11-15 controls to 0.11 ± 0.02% in 10D5 mAb treated group.
(absolute CAA load reduction = 0.30%) while in APP/e3 mice the absolute reduction in the CAA load was 0.12%. Unlike CAA, which was the most prevalent in APP/e4 mice, the greatest abundance of CCA was associated with the APOE e2 allele (Fig. 5a-c). The density of CCA associated capillary profiles per mm² in the brain cortex of APP/e2 TY11-15 control mice was 1.9 and 1.3 fold higher than these in the brain cortex of APP/e3 (p < 0.0001) and APP/e4 (p < 0.05) TY11-15 controls, respectively; while in the thalamus of APP/e2 TY11-15 controls, the average density of CCA associated capillary profiles per group was 13.1 and 5.3 fold higher than in the thalamus of APP/e3 (p < 0.0001) and APP/e4 (p < 0.001) TY11-15 control mice, respectively. There was no statistically significant difference in the CCA incidence between untreated age-matched and TY11-15 control mice in the brain cortex and in the thalamus for any of matching APOE genotype. One needs to mention that CCA was absent in the thalamus of two thirds of control APP/e3 mice and of one half of control APP/e4 mice. Besides the thalamus, the incidence of CCA in other subcortical structures was scanty especially in APP/e3 and APP/e4 mice, thus no other structure specific quantification was performed.

10D5 mAb treatment effected significant CCA reduction across all three APOE genotypes. In the brain cortex of APP/e2 and APP/e3 mice the density of Aβ immunopositive capillary profiles was reduced approximately two-fold (p < 0.001 and p < 0.05; respectively), while in the brain cortex of APP/e4 mice it was reduced by 1.6 fold (p < 0.05).

10D5 mAb treatment reduced CCA in the thalamus of APP/e2 mice 8.5 fold (p < 0.0001). Marked reduction also was observed in the thalamus of APP/e3 and APP/e4 mice, but it did not reach statistical significance as the data did not conform to normal distribution hence weaker non-parametric statistical analysis was used.

APOE e2 and e3 alleles show opposing effect on the risk of cerebral microhemorrhages

Perivascular hemosiderin deposits, reflecting ensued microhemorrhages, were found associated both with CAA and CCA positive vessels (Fig. 6a, b). They frequently occurred in untreated age-matched mice of all APOE genotypes and their number did not increase in TY11-15 treated control animals (Fig. 7a-d). There were no statistically significant differences in the number of the total brain hemosiderin deposits, the number of deposits subclassified as small and large (with cut off ≥ 15 μm in diameter), and the fraction of deposits localized to the thalamus between TY11-15 treated APP/e2 and APP/e4 mice (Fig. 7a-d). In contrast, untreated age-matched and TY11-15 APP/e3 control mice had approximately two-fold less hemosiderin deposits for each listed category compared to APP/e2 and APP/e4 TY11-15 controls.

10D5 mAb treatment resulted in a significant increase in the number of hemosiderin deposits yet the magnitude of this effect was differentially modulated by the APOE genotype. In APP/e3, and APP/e4 mice, an increase by 12.4 and 15.6 deposits per brain on average was observed,
respective; bringing their number in APP/e3 mice from 20.5 ± 1.9 per brain in TY11-15 controls to 32.9 ± 3.8 per brain in 10D5 mAb treatment group (p < 0.05), while in APP/e4 mice, from 41.1 ± 4.6 per brain in TY11-15 controls to 56.7 ± 4.1 per brain in the 10D5 mAb treatment group (p < 0.01) (Fig. 7a). The increase only concerned small deposits (Fig. 7b), while the number of large deposits did not change in either APP/e3 or APP/e4 mice (Fig. 7b, c). Differences in the number of all hemosiderin deposits and small hemosiderin deposits between 10D5 mAb treated APP/e3 and APP/e4 mice were statistically significant (p < 0.0001 and p < 0.01, respectively) (Fig. 7a, b). In APP/e2 mice 10D5 mAb treatment caused a nearly two-fold higher upsurge in the number of hemosiderin deposits compared to APP/e3 and APP/e4 mice. The count of all hemosiderin deposits was increased on average by 27.0 per brain bringing it from 42.5 ± 3.8 per brain in TY11-15 controls to 69.5 ± 4.4 per brain in 10D5 mAb treated group (p < 0.0001) (Fig. 7a, b). Unlike other APOE genotypes, APP/e2 mice experienced an increase both in the number of small and large hemosiderin deposits. The count of large hemosiderin deposits was increased on average by 7.3 per brain from 3.7 ± 0.9 in TY11-15 controls to 11.0 ± 1.4 in 10D5 treated mice (a nearly three-fold increase) (p < 0.0001) (Fig. 7c).

The bulk of perivascular hemosiderin deposits was found in the brain cortex (Additional file 2: Figure S2 A-C). In Age control and TY11-15 control APP/e3 and APP/e4 mice they constituted between 65 to 70% of the brain total hemosiderin deposits, while in Age control and TY11-15 control APP/e2 mice 53% and 58%, respectively. As compared to other APOE genotypes APP/e2 mice showed greater number of hemosiderin deposits to the thalamus. In APP/e2 TY11-15 control mice 14.9 ± 2.8% of all brain hemosiderin deposits was found in the thalamus compared to 5.1 ± 1.9% in TY11-15 APP/e3 controls (p < 0.05) and 12.3 ± 3.1% in TY11-15 APP/e4 controls (non-significant) (Fig. 7d). While in APP/e3 and APP/e4 mice 10D5 mAb treatment did not affect the proportion of thalamic hemosiderin deposits to all brain deposits, in 10D5 mAb treated APP/e2 mice this proportion nearly doubled and it was 27.7 ± 3.6% on average (p < 0.0001) (Fig. 7d; Additional file 2: Figure S2 A). Except for minor increase in the number of hemosiderin deposits in the hippocampus in APP/e2 mice, no other brain structure but the brain cortex in animals of all APOE genotypes and the thalamus in APP/e2 mice only, showed significant regional increase in the number of hemosiderin deposits with anti-Ab passive immunization (Additional file 2: Figure S2 A-C).

APOE alleles have differential effect on cerebrovascular disease risk factors in APPWt/PS1dE9 mice

APOE e2 targeted replacement mice are established model of type III hyperlipidemia. APP/e2 TY11-15 control mice used in this study showed average total serum
cholesterol level of 477.3 ± 49.5 mg/dl at the age of 15 months. For comparison total serum cholesterol level in TY11-15 control APP/ε3 and APP/ε4 mice was 83.8 ± 4.7 mg/dL and 97.9 ± 7.7 mg/dL, respectively (p < 0.0001 vs. APP/ε2) (Fig. 8a). APP/ε2 mice were also obese. The average weight of APP/ε2 TY11-15 control mice was 46.6 ± 0.8 g, while average weight of TY11-15 control APP/ε3 and APP/ε4 mice was 33.6 ± 1.6 g and 35.9 ± 1.5 g, respectively (p < 0.0001 vs. APP/ε2) (Fig. 8b). Neither serum cholesterol level nor animal weight was significantly changed with 10D5 mAb treatment. No significant differences in respect to total serum cholesterol level and animal body weight between untreated age-control and TY11-15 control mice for matching APOE genotypes were observed.

Discussion

APOE genotype critically affects the burden of Aβ pathology in sporadic AD. ApoE isoforms encoded by various APOE alleles differentially influence the rate of soluble Aβ clearance from the brain interstitial space and formation of Aβ parenchymal plaques and VAβ [32, 33]. Our study provides novel evidence that APOE genotype also differentially affects multiple aspects of response to Aβ immunotherapy. Firstly, we observed that APOE genotype is associated with variable reduction in the load of Aβ par enchymal plaques and APP/ε4 mice had greater reduction in absolute Aβ plaque load values than APP/ε2 and APP/ε3 mice. The reduction concerned both Th-S positive, (fibrillar) and immunopositive Aβ (total) plaque loads with the later metric showing greater change than the former across all APOE genotypes. As the immunopositive Aβ plaque load is inclusive of Th-S positive plaques this suggests that stimulated by 10D5 mAb microglia clear diffuse component of Aβ plaques more effectively than they do fibrillar Aβ aggregates. However, despite enhanced treatment effect in APP/ε4 mice the post-treatment Aβ plaque load in these mice and especially the fibrillar Aβ plaque load, remained significantly higher than in APP/ε2 and APP/ε3 mice, what is a result of distinguishably greater Aβ plaque load, associated with the ε4 allele. This observation may inform design of future passive immunization experiments in APP Tg model mice of AD and clinical trials suggesting specific tailoring of the anti-Aβ passive immunization protocol in APOE ε4 allele.

Fig. 6 Demonstration of perivascular hemosiderin deposits reflecting ensued microhemorrhages. a Shown is an example of hemosiderin deposition associated with CAA laden vessel in the cerebral cortex of 10D5 mAb treated APP/ε4 animal. The histological section was double-stained with Thioflavin-S and Pearls’ Prussian blue stain for ferric iron and photographed under fluorescence and bright-field microscopy, respectively. Positive perivascular hemosiderin stain (arrowhead) was digitally converted to red color and superimposed on green Thioflavin-S stain of CAA (Merge). An arrow indicates a vessel affected by CAA, without evidence of bleeding. b Representative microphotograph of CAA related hemosiderin deposits (arrowheads) in the brain cortex of 10D5 mAb-treated APP/ε2 mouse. A coronal section of the cerebral cortex was immunostained with anti-Aβ HJ3.4 mAb and counterstained with Pearls’ Prussian blue stain. c Examples of large hemosiderin deposits in the thalamus typical of APP/ε2 mice; Pearl’s iron stain without counterstain. An arrow on the left panel indicates an isolated thalamic hemosiderin deposit in TY11-15 control APP/ε2 animal, while arrowheads on the right panel point at the cluster of large hemosiderin deposits (≥15 μm in diameter) identified in 10D5 mAb treated APP/ε2 mouse. Abbreviations: h-hippocampus, t-thalamus. Scale bars: 75 μm (a), 25 μm (b), 500 μm (c)
carriers to achieve end-point plaque load reduction level compared to that expected among non-ε4 allele carriers. Potential modifications of the immunization protocol may include increasing antibody dose, earlier commencement of the treatment and extension of treatment duration. However one needs to be mindful that escalation of anti-Aβ mAbs dose in APOE ε4 allele carriers may trigger increased rate of vasculotropic adverse events associated with immunization [5, 8, 14]. With the advancement of certain newer anti-Aβ immunotherapies such as Gantenerumab and Aducanumab that appear to lower Aβ plaque load in humans to a greater extent than seen previously [6, 8], it will be interesting to see if the effects of APOE genotype noted here in animal models are also present in humans.

The primary mechanism of action of 10D5 mAb is based on its direct binding to deposited Aβ and stimulating Fc receptor-mediated Aβ plaque removal by activated microglia and possibly blood derived macrophages [9, 34]. To compare microglial response across APOE genotypes we immunostained brain sections against Iba1 and CD68 antigens and counterstained them with Th-S for fibrillar Aβ. Iba1 is a microglia specific marker, which expression increases with microglia activation and macrophage...
transformation [30], while CD68 is a lysosomal/endosomal membrane glycoprotein up regulated in actively phagocytic cells [31]. The load of Iba1 and CD68 positive cells was significantly higher in Age control and TY11-15 control APP/e4 mice compared to APP/e2 and APP/e3 controls. Both Iba1 and CD68 loads correlated with the load of Th-S positive fibrillar deposits and when adjusted for it, they were comparable across all APOE genotypes in control animals. Following 10D5 mAb treatment both Iba1 and CD68 loads increased across all APOE genotypes, however when adjusted for the post-treatment fibrillar plaque load, they were significantly higher in APP/e4 mice than in APP/e2 and APP/e3 animals. This finding illustrates increased effector function of 10D5 mAb exerted on microglia in the setting of the APOE e4 allele. In particular exaggerated post treatment CD68/Th-S ratio in APP/e4 mice implies less effective Aβ degradation, which can be tied to a recent discovery showing that excessive apoE accumulation within Aβ plaques impairs ability of microglia to contain plaque load [35]. In fact, we have previously demonstrated that APP/e4 and PDAPP/e4 mice have greatly increased content of apoE within Aβ deposits compared to the same Tg mouse lines expressing other human APOE alleles [19, 36]. Though microglial cells play pivotal role in maintaining immunoproteostasis through phagocytosis and degradation of misfolded proteins, their protective function is inseparably associated with immune activation exerting a chronic, potentially harmful effect on the regional milieu of neuronal networks [35, 37–39]. Although, APP/e4 mice show the greatest absolute reduction in the load of parenchymal Aβ plaques in response to anti-Aβ immunization as compared to mice of other APOE genotypes, this occurs at the expense of unparalleled microglia response making the post treatment Iba1 and CD68 loads the highest among all experimental groups. Thus both higher level of microglia activation driven by higher Aβ plaque load and exaggerated microglia effector response exerted by anti-Aβ immunotherapy may constitute independent deleterious effects associated with the e4 allele, in AD pathogenesis. Increased microglia activation and associated reduction in Aβ plaque load was found only in 10D5 mAb immunized groups, while administration of the isotype control antibody TY11-15 changed neither expression of microglia markers nor parenchymal plaque load as compared to untreated age-matched animals of matching APOE genotypes.

Both CAA and CCA variants of VAB pathology have been described in AD autopsies and their relative preponderance has been linked to various APOE genotypes [40–43]. CAA comprises of fibrillar Aβ sandwiched between the adventitia and the tunica media of cerebral arteries, while CCA comprises of non-fibrillar Aβ deposits closely associated with the capillary outer basement membrane. Both development of CAA and CCA is primarily driven by neuron derived Aβ, while Aβ produced by myocytes locally in the tunica media further contributes to CAA development (reviewed in [42]). Presence of apoE within CAA lesions is critical for formation of Aβ fibrillar assemblies [25]. In this study we found that APOE genotype modulates development of both CAA and CCA pathology in APP Tg mice. In accordance to previously published data concerning e4 allele effect, we found increased CAA load in APP/e4 mice [25, 44–46]. Our novel observation here is that the e2 allele was associated with greatly reduced CAA load, but also with enhanced incidence of CCA. Reduced CAA load in APP/e2 mice likely reflects limited effect of apoE2 isoform on promoting Aβ fibrillar assemblies within arterial walls as compared to other human apoE isoforms, which are associated with higher load of CAA and fibrillar Aβ plaques [47]. In turn, greater incidence of CCA may be related to increased amount of soluble Aβ cleared across the BBB in APP/e2 animals, which deposit less Aβ in the brain while maintaining the same level of Aβ production [32]. In
fact increased rate of soluble Aβ clearance across the BBB has been experimentally confirmed in the setting of the APOE ε2 allele [32, 48]. Vascular disease risk factors including hypercholesterolemia and volume overload occurring in aged APP/ε2 mice effect deterioration of capillary wall structure and function and likely in tandem with greater load of soluble Aβ passing across the BBB contribute to CCA development.

The largest percentage of AD patients with both CAA and CCA was found among ε4 allele carriers, while CAA without CCA was more frequent among ε2 and ε3 carriers [40, 42]. In these studies ε3 allele carriers and in particular ε3 allele homozygotes constituted the largest percentage among CAA/CCA free cases. Accordingly, we found that APP/ε3 mice have the lowest incidence of CCA among all APOE genotypes and their CAA load is significantly lower than that in APP/ε4 mice demonstrating protective effect of the ε3 allele against development of Aβ angiopathy. However, mechanisms underlying Vαβ deposition including preferential development of CAA and CCA remain elusive and require further elucidation. They likely are a resultant of multiple genetic features extending beyond APOE genotype as no APOE allelic variant confers absolute protection against Vαβ deposition [42, 43].

Vαβ is detrimental to the function of cerebrovascular circulation through compromising blood–brain-barrier integrity [49], effecting hemodynamic dysfunction [29, 50], and facilitating occurrence of spontaneous hemorrhages [25, 26, 36]. We found a significant number of perivascular hemosiderin deposits, reflecting ensued microhemorrhages, in brains of control mice representing all APOE genotypes. They were associated both with CAA and CCA affected vessels. Strikingly the lowest incidence of perivascular hemosiderin deposits was observed among untreated age-matched and TY11-15 control APP/ε3 mice. In APP/ε4 and APP/ε2 controls the incidence of perivascular hemosiderin deposits was comparable and statistically significant higher than that in APP/ε3 controls. In human subjects prevalence of spontaneous microhemorrhages is high and positively correlates with advancing age. MRI studies detected microbleeds in 17.8% of subjects aged 60–69 years and in 38.3% in subjects who were more than 80 years old [51, 52]. For comparison an autopsy study reported histological evidence of ensued microhemorrhages in 92.9% of elderly individuals with mean age of 81.1 ± 10.8 years, which included both AD and non-demented subjects [53]. Evidence independently supports link of both the APOE ε4 and ε2 allele with increased risk for microhemorrhages in humans [51, 52, 54–57]. Both Vαβ and hypertensive or atherosclerotic microangiopathy are recognized clinical risk factors for cerebral microbleeds, with the former correlating stronger with bleeding in cortical/subcortical distribution [58, 59] while the later with bleeding to deep brain structures including basal ganglia and thalamic nuclei [60, 61]. APOE ε2 targeted replacement mice develop type III hyperlipoproteinemia and spontaneous atherosclerosis [16] as well as volume overload as a function of their obesity, all of which are recognized vascular risk factors and may explain increased preponderance to thalamic localization of hemosiderin deposits in APP/ε2 mice found in our study. However, these striking vasculotropic effects observed here in APP/ε2 mice may not be robustly seen in human subjects, where ε2 homozygotes constitute only 1% of the population, with less than 10% of them developing type III hyperlipoproteinemia, what is linked to distinctly different contribution of apoE to VLDL formation between humans and mice [62].

Aβ immunotherapy can ameliorate Vαβ burden and improve compromised vascular reactivity [12, 21, 63], but it is well known to escalate incidence of brain microhemorrhages [21, 26]. Both CAA load and CCA incidence were reduced in 10D5 mAb treated mice with the strongest effect on CAA load reduction seen in APP/ε4 animals and the strongest effect on CCA incidence seen in APP/ε2 mice. We also found a profound effect of APOE genotype on Aβ immunization related microhemorrhages. Surprisingly not the ε4 allele but the ε2 allele was associated with the greatest increase in the number of perivascular hemosiderin deposits. 10D5 mAb treated APP/ε2 mice had the highest increase in the number of all perivascular hemosiderin deposits and also in the number of deposits in small and large (≥15 μm in diameter) subcategories. The increase in the total number of hemosiderin deposits in APP/ε2 mice was approximately two-fold higher than these in APP/ε3 and APP/ε4 mice. APP/ε2 mice represented the only APOE genotype where 10D5 mAb immunization increased incidence of large hemosiderin deposits, bringing their number to three-fold value of the control group. Several human autopsy studies have shown that the ε2 allele is an exacerbating factor of microhemorrhages among subjects with Vαβ pathology and that this effect is linked to fibrinoid necrosis of vascular wall specifically associated with the ε2 allele [55–57]. As mentioned above APP/ε2 mice also develop hypercholesterolemia and atherosclerosis, hence increased susceptibility to cerebral microhemorrhages and in particular high incidence of large hemosiderin deposits in APP/ε2 mice associated with immunotherapy can be a resultant of prevalent Vαβ pathology, anti-Aβ mAb effect, and cerebrovascular risk factors all cooperatively compromising vascular wall integrity. In contrast, 10D5 mAb treated APP/ε3 and APP/ε4 mice showed only an increase in the number of small hemosiderin deposits. Again the post-10D5 treatment number of hemosiderin deposits in APP/ε3 mice was the lowest among all APOE genotypes, indicating a relative vasculoprotective effect of the ε3 allele, which has not been previously
described in AD patients or APP Tg mice expressing human APOE alleles.

Exacerbation of VAB associated microhemorrhages appears to be associated with propensity of anti-Aβ mAbs to bind deposited Aβ. Thus, application of N-terminus specific mAbs, which like10D5 binds epitopes exposed in Aβ parenchymal plaques and VAB, but not mAbs, which like m266 bind the Aβ central domain and do not bind to deposited Aβ effect increased hemorrhage incidence [64, 65]. However mAbs binding deposited Aβ are generally more effective in clearing VAB [12]. Time course analysis of VAB-related events during immunotherapy in PDAPP mice, revealed that a time window for increased incidence of microhemorrhages can last up to six months, but then their incidence diminishes drastically, which correlates with achieving significant clearance of both CAA and CCA [21]. Thus, the risk of brain microhemorrhages related to Aβ immunotherapy may be a transient complication and possibly reduced in individuals with modest VAB burden, who also have lower prevalence of spontaneous microbleeds [58, 59].

Conclusions

Utilizing APOE humanized APPSW/PS1mice and APP humanized APPmice we demonstrated that APOE alleles differentially modulate various aspects of response to anti-Aβ immunotherapy including Aβ load reduction, microglia response, and incidence of cerebral microhemorrhages. This information can be utilized in design of clinical trials of anti-Aβ mAbs and other Aβ targeting therapeutics in order to maximize potency of studied approaches and minimize adverse reaction to the treatment.

Additional files

Additional file 1: Figure S1. Comparative analysis of Aβ plaque load immunostained against N-terminal and central Aβ epitopes revealed no significant differences. a Representative microphotographs of coronal brain sections through the somatosensory cortex from the same TY11-15 control, and 10DS mAAb treated mice of APPx4background, which were immunostained using H3J4 mAAb directed against the N-terminus of Aβ and 4G8 mAAb directed against the mid-portion of Aβ. b Unbiased analysis of the parenchymal Aβ plaque load in the brain cortex revealed by H3J4 and 4G8 immunostaining. Values represent mean ± SEM from 10 to 12 animals per group. *p < 0.0001 (one-way analysis of variance); ***p < 0.0001, TY11-15 control vs. 10DS mAAb treatment for matching anti-Aβ immunostains (Sidak’s post hoc test). Differences between H3J4 and 4G8 immunostaining in TY11-15 control and 10DS mAAb treatment groups were non-significant; not shown on the graph. Scale bar 50 μm (a). (PDF 423 kb)

Additional file 2: Figure S2. Regional analysis of perivascular hemosiderin deposits identifies the brain cortex in mice of all APOE genotypes and the thalamus in APPx4 mice as brain structures susceptible to microbleeds associated with anti-Aβ immunization. Shown are means (± SEM) for counts of all brain perivascular hemosiderin deposits in the brain cortex, the hippocampus, the basal ganglia, the corpus callosum, the thalamus, the septum and the midbrain in APPx4 (a), APPx3 (b), and APPx4 mice (c) (n = 5-11/group). Perivascular hemosiderin deposits were counted on every tenth brain coronal cross-section along the entire rostro-caudal axis of the brain. a through (c) p < 0.0001 (one-way analysis of variance); *p < 0.005, ***p < 0.0001, and ****p < 0.0001, TY11-15 control vs. 10DS mAAb treatment for matching brain structures and APOE genotypes (Sidak’s post hoc test). For brain structures where the differences between TY11-15 control and 10DS mAAb treatment groups were not statistically significant p values are not shown. Differences between Age control and TY11-15 groups were non-significant for all structures (Sidak’s post hoc test); p values not shown on the graph. (PDF 419 kb)

Abbreviations

AD: Alzheimer’s disease; APOE: human apolipoprotein E gene; Apoe: murine apolipoprotein E gene; apoe: apolipoprotein E; APP: APPsw/PS1mice transgene; ARIA: amyloid related imaging abnormalities; Aβ: β-amyloid; BBB: blood-brain barrier; CAA: cerebral amyloid angiopathy; CCA: cerebral capillary Aβ; DAB: 3,3′-diaminobenzidine; FA: formic acid; h: hippocampus; mAAb: monoclonal antibody; PBS: phosphate buffered saline; PET: positron emission tomography; SEM: standard error of the mean; t: thalamus; Tg: transgenic; Th-S: Thioflavine-S; VAB: vascular Aβ

Acknowledgements

We thank Dr. Frederique Bard of Janssen Alzheimer Immunotherapy for her advice in study design.

Funding

This work was supported by the following awards: NIA R01 AG047644 (DWH), NIA R01 AG031221 (MJS), NIA R01 AG053990 (MJS) and by Janssen Alzheimer Immunotherapy Sponsored Research Agreement No. 379851 (MJS).

Availability of data and materials

All data generated or analyzed during this study is included in this manuscript and its Additional files 1, and 2. The datasets used and/or analyzed during the current study available from the corresponding author on reasonable request.

Authors’ contributions

JEP carried out morphometric analyses of Aβ deposits, microglia, and hemosiderin deposits, and contributed to data analysis and manuscript preparation. JBB and SS performed husbandry and genotyping of APOE humanized APPsw/PS1mice and their treatment with 10D5 and TY11-15 antibodies. JLC carried out histological and immunohistochemical procedures. JK, PMS, and DWH developed APOE humanized APPsw/PS1mice and contributed to manuscript preparation. MJS analyzed the data and wrote the manuscript. All authors have read and approved the final version of the manuscript.

Competing interests

This study was supported in part by Janssen Alzheimer Immunotherapy, which owns 10D5 mAAb. Financial and in kind support (donation of 10D5 and TY11-15 mAAbs) was provided under Sponsored Research Agreement No. 379851 to NYU (PI: MJDadowski).

Consent for publication

All authors agree to publication of this study.

Ethical approval and consent to participate

All mouse care and experimental procedures were approved by Institutional Animal Care and Use Committees of the New York University School of Medicine and the Washington University School of Medicine.

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