Targeting of nonlipidated, aggregated apoE with antibodies inhibits amyloid accumulation

Fan Liao
Washington University School of Medicine in St. Louis

Aimin Li
Washington University School of Medicine in St. Louis

Monica Xiong
Washington University School of Medicine in St. Louis

Hong Jiang
Washington University School of Medicine in St. Louis

Mary Beth Finn
Washington University School of Medicine in St. Louis

See next page for additional authors

Follow this and additional works at: https://digitalcommons.wustl.edu/open_access_pubs

Recommended Citation
Liao, Fan; Li, Aimin; Xiong, Monica; Jiang, Hong; Finn, Mary Beth; Hoyle, Rosa; Keyser, Jennifer; Lefton, Katheryn B; Robinson, Grace O; Serrano, Javier Remolina; Leyns, Cheryl Eg; Gallardo, Gilbert; Ulrich, Jason D; Holtzman, David M; and et al, "Targeting of nonlipidated, aggregated apoE with antibodies inhibits amyloid accumulation." The Journal of Clinical Investigation. 128, 5. 2144 - 2155. (2018).
https://digitalcommons.wustl.edu/open_access_pubs/8479

Please let us know how this document benefits you.
Targeting of nonlipidated, aggregated apoE with antibodies inhibits amyloid accumulation

Fan Liao,1 Aimin Li,1 Monica Xiong,1 Nga Bien-Ly,2 Hong Jiang,1 Yin Zhang,2 Mary Beth Finn,1 Rosa Hoyle,1 Jennifer Keyser,1 Kathryn B. Lefton,1 Grace O. Robinson,1 Javier Remolina Serrano,1 Adam P. Silverman,2 Jing L. Guo,1 Jennifer Getz,1 Kirk Henne,2 Cheryl E.G. Leyns,1 Gilbert Gallardo,1 Jason D. Ulrich,1 Patrick M. Sullivan,3 Eli Paul Lerner,4 Eloise Hudry,4 Zachary K. Sweeney,2 Mark S. Dennis,3 Bradley T. Hyman,3 Ryan J. Watts,2 and David M. Holtzman1

1Department of Neurology, Hope Center for Neurological Disorders, Charles F. and Joanne Knight Alzheimer’s Disease Research Center, Washington University School of Medicine, St. Louis, Missouri, USA. 2Department of Medicine, Duke University, Durham, North Carolina, USA. 3MassGeneral Institute for Neurodegenerative Disease, Massachusetts General Hospital and Harvard Medical School, Charlestown, Massachusetts, USA. 4Department of Medicine, Hope Center for Neurological Disorders, Washington University School of Medicine, St. Louis, Missouri, USA. 5Denali Therapeutics Inc., South San Francisco, California, USA. 6Department of Medicine, Duke University, Durham, North Carolina, USA. 7MassGeneral Institute for Neurodegenerative Disease, Massachusetts General Hospital and Harvard Medical School, Charlestown, Massachusetts, USA.

The apolipoprotein E E4 allele of the APOE gene is the strongest genetic factor for late-onset Alzheimer disease (LOAD). There is compelling evidence that apoE influences Alzheimer disease (AD) in large part by affecting amyloid β (Aβ) aggregation and clearance; however, the molecular mechanism underlying these findings remains largely unknown. Herein, we tested whether anti-human apoE antibodies can decrease Aβ pathology in mice producing both human Aβ and apoE4, and investigated the mechanism underlying these effects. We utilized APPPS1-21 mice crossed to apoE4-knockin mice expressing human apoE4 (APPPS1-21/APOE4). We discovered an anti-human apoE antibody, anti-human apoE 4 (HAE-4), that specifically recognizes human apoE4 and apoE3 and preferentially binds nonlipidated, aggregated apoE over the lipidated apoE found in circulation. HAE-4 also binds to Aβ in amyloid plaques in unfixed brain sections and in living APPPS1-21/APOE4 mice. When delivered centrally or by peripheral injection, HAE-4 reduced Aβ deposition in APPPS1-21/APOE4 mice. Using adeno-associated virus to express 2 different full-length anti-apoE antibodies in the brain, we found that HAE antibodies decreased amyloid accumulation, which was dependent on Fcγ receptor function. These data support the hypothesis that a primary mechanism for apoE-mediated plaque formation may be a result of apoE aggregation, as preferentially targeting apoE aggregates with therapeutic antibodies reduces Aβ pathology and may represent a selective approach to treat AD.

Introduction

Alzheimer disease (AD) is the most common form of dementia and it affects more than 5 million people in the United States (www.alz.org). Compelling evidence has shown that amyloid β (Aβ) plays a key role in the pathogenesis of AD (1, 2). In autosomal dominant AD, missense mutations in amyloid precursor protein (APP) or components of the γ-secretase complex presenilin 1 (PS1) or presenilin 2 (PS2) result in early onset amyloid deposition in the brain due to a relative increase in production of longer Aβ species such as Aβ42, an increase in all Aβ species, or an alteration of Aβ aggregation propensity and clearance (3). Also, a protective mutation in AD was recently described near the N-terminus of the Aβ secretase (Aβ42) gene is the strongest genetic risk factor for LOAD. In humans, the 3 common isoforms of apoE — E2, E3, and E4 — are encoded by a 299 amino acid protein. Compared with the most common form of apoE, apoE3 (Cys112, Arg158), each allele of apoE4 (Arg112, Arg158) strongly increases the risk for AD (~3.7-fold for one E4 allele and ~12-fold for 2 E4 alleles relative to the E3/E3 genotype); in contrast, apoE2 (Cys112, Cys158) is protective for AD (5, 6). How apoE impacts AD pathogenesis is not entirely clear; however, evidence shows that apoE influences Aβ aggregation and clearance (7) and is also found in amyloid plaques (8, 9).

Previously, our group has shown that passive immunotherapy using HJ6.3, an antibody targeting endogenous murine apoE, strongly suppresses Aβ pathology in the APPswe/PS1ΔE9 mouse brain when treatment is started prior to plaque onset (10). When administered after plaque onset, HJ6.3 reduced brain Aβ plaque load, restored resting-state functional connectivity, and mildly improved spatial performance in the water maze (11). However, in order to further explore whether such an approach could potentially translate into an immunotherapy for humans, studies with apoE antibodies targeting human apoE in animals expressing human Aβ and apoE are critical. It is also important to understand the mechanism(s) underlying the therapeutic effects.

The majority of AD cases are known as late-onset AD (LOAD), which clinically begins after the age of 65. The apolipoprotein E (APOE) gene is the strongest genetic risk factor for LOAD. In humans, the 3 common isoforms of apoE — E2, E3, and E4 — are encoded by a 299 amino acid protein. Compared with the most common form of apoE, apoE3 (Cys112, Arg158), each allele of apoE4 (Arg112, Arg158) strongly increase the risk for AD (~3.7-fold for one E4 allele and ~12-fold for 2 E4 alleles relative to the E3/E3 genotype); in contrast, apoE2 (Cys112, Cys158) is protective for AD (5, 6). How apoE impacts AD pathogenesis is not entirely clear; however, evidence shows that apoE influences Aβ aggregation and clearance (7) and is also found in amyloid plaques (8, 9).

Previously, our group has shown that passive immunotherapy using HJ6.3, an antibody targeting endogenous murine apoE, strongly suppresses Aβ pathology in the APPswe/PS1ΔE9 mouse brain when treatment is started prior to plaque onset (10). When administered after plaque onset, HJ6.3 reduced brain Aβ plaque load, restored resting-state functional connectivity, and mildly improved spatial performance in the water maze (11). However, in order to further explore whether such an approach could potentially translate into an immunotherapy for humans, studies with apoE antibodies targeting human apoE in animals expressing human Aβ and apoE are critical. It is also important to understand the mechanism(s) underlying the therapeutic effects.

Authorship note: FL and AL contributed equally to this work.

Conflict of interest: NBL, YZ, APS, JLG, XK, ZS, MSD, and RW are employees of Denali. FL, HJ, and DMH are inventors on a patent filed by Washington University on the topic of anti-apoE antibodies that was licensed by Denali. DMH cofounded and is on the scientific advisory board of C2N Diagnostics. DMH consults for Genentech, AbbVie, Eli Lilly, Proclara, and Denali. Washington University receives research grants to the lab of DMH from C2N Diagnostics, Eli Lilly, AbbVie, and Denali. Submitted: July 20; 2017; Accepted: February 1, 2018.


https://doi.org/10.1172/JCI96429.

Related Commentary: p. 1734
In the present study, we tested the effects of anti–human apoE (HAE) antibodies, a new series of apoE antibodies targeting human apoE, on Aβ pathology in APPPS1-21/APOE4-knockin (KI) mice. These mice were generated by crossing human apoE4-KI mice expressing human apoE4 under the control of endogenous murine apoE regulatory elements (APOE4) (12) to line APPPS1-21 (13). We found that HAE-4, an antibody specific for apoE3 and apoE4, was able to reduce Aβ deposition when infused directly into the brain, delivered by i.p. injection starting at the time of plaque onset, or expressed in the brain via adeno-associated virus–mediated (AAV–mediated) delivery. Here we present in detail the features of antibody HAE-4 that facilitate its ability to decrease Aβ deposition and propose a likely mechanism of action.

Results
Characterization of the HAE series of apoE antibodies using recombinant human apoE. We used a direct ELISA to test whether the HAE series of apoE antibodies binds to different recombinant apoE isoforms (Figure 1, A–D). HAE-2 and HAE-3 recognized all 3 isoforms of human apoE (Figure 1, B and C), whereas HAE-4 had much higher affinity for apoE3 and apoE4 with no appreciable binding to apoE2 (Figure 1D). HAE-1 was apoE4-specific (Figure 1A). Using surface plasmon resonance (SPR) (Figure 1, E–G), the apparent $K_D$ values were determined for HAE-1 (6.02 × 10^{-8} M), HAE-2 (3.19 × 10^{-10} M), and HAE-4 (1.98 × 10^{-7} M) for their interaction with recombinant apoE4 (Table 1). The $K_D$ value for HAE-3 was not determined because it showed binding characteristics similar to those of HAE-2 in ELISA (Figure 1, B and C). Oddly, the maximum response units for HAE-1 and HAE-4 as measured by SPR were only 10% of the maximum value seen for HAE-2, despite greater apoE4 capture levels for experiments with HAE-1 and HAE-4. This suggested the possibility that these 2 antibodies were recognizing a minor fraction of the immobilized apoE, a hypothesis that was later validated (see below).

Effects of intracerebroventricularly administered anti–apoE antibodies on Aβ pathology in APPPS1-21/APOE4 mice. apoE is present in Aβ-containing amyloid plaques in both human AD as well as in mouse models of amyloidosis (8, 9, 14). To test whether HAE antibodies are able to decrease Aβ pathology in APPPS1-21/APOE4 mice, we directly infused the antibodies into the lateral ventricle of the mice before plaque onset, starting at the age of 2 months. Antibodies were infused continuously (0.3 μg/h) via an osmotic pump for 6 weeks and mice were assessed at the age of 3.5 months (females, n = 10–11 per group). Infusions of PBS or a control mouse IgG2ab into the mouse brain were included as negative controls, and anti–Aβ antibody HJ5.1 (15) was used as a positive control. Aβ plaques were stained using anti–Aβ antibody HJ3.4 (Supplemental Figure 1A; supplemental material available online with this article; Table 1. Apparent $K_D$ values of HAE-1, HAE-2, and HAE-4 calculated based on the SPR experiment

<table>
<thead>
<tr>
<th>Antibody</th>
<th>$K_a$ (1/Ms)</th>
<th>$K_d$ (1/s)</th>
<th>Apparent $K_D$ (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HAE-1</td>
<td>2.0E+04</td>
<td>1.2E-03</td>
<td>6.0E-08</td>
</tr>
<tr>
<td>HAE-2</td>
<td>6.6E+05</td>
<td>2.1E-04</td>
<td>3.2E-10</td>
</tr>
<tr>
<td>HAE-4</td>
<td>2.6E+04</td>
<td>5.2E-03</td>
<td>2.0E-07</td>
</tr>
</tbody>
</table>
load in APPPS1-21/APOE4 mice, we tested whether peripheral administration of HAE-1 and HAE-4 could impact Aβ pathology in these mice. Beginning at the age of 2 months, APPPS1-21/APOE4 mice (females, n = 10–13 per group) were treated weekly with i.p. injections (50 mg/kg body weight) of antibodies until 3.5 months of age. A group of untreated mice were harvested at 2 months to determine baseline Aβ pathology. As determined by Aβ immunostaining, HAE-4 significantly reduced Aβ plaque load compared with the mouse IgG2ab control (Figure 2, A and B). X-34 staining suggested that HAE-4 also reduced fibrillar plaque load, but the effect was not significant (Figure 2, A and C). However, HAE-4 significantly reduced insoluble Aβ40 (Figure 2D) and Aβ42 (Figure 2E) in the guanidine fraction of the cerebral cortex, as compared with both the PBS- and IgG2ab-treated control groups at the same age. There were no signifi-
animals, we performed a dose-response study with HAE-4. At 2 months of age, the mice (mixed sexual phenotype, \(n = 17–18/\) group) received weekly i.p. injections of control IgG2ab (50 mg/kg), anti–A\(\beta\) antibody HJ3.4, which recognizes amyloid plaques (50 mg/kg), or HAE-4 (2, 10, and 50 mg/kg) for 2.5 months prior to sacrifice. There was a significant dose-dependent effect of HAE-4 on reducing insoluble A\(\beta\) levels in the brain (Supplemental Figure 4).

Binding profile of HAE antibodies to lipidated apoE. Given that the antibodies were chronically injected into the peripheral compartment at high levels, we were somewhat surprised that HAE-1 and HAE-4 did not affect levels of plasma apoE or brain apoE, as apoE is abundant in both locations. This prompted us to ask whether some of the anti–apoE antibodies bound differentially to lipidated apoE versus other forms of the protein. In order to assess binding to lipidated apoE, we characterized the binding of HAE antibodies to plasma-derived apoE, the majority of which is lipidated. Plasma that contained intact plasma lipoproteins from apoE-KO (EKO), APOE2\(^-\), APOE3\(^-\), and APOE4-KI mice was

![Image of graph](A.png)

**Figure 3. Binding profile of HAE-1, HAE-2, and HAE-4 with lipidated apoE.** (A and B) Antibody binding to mouse plasma from EKO, APOE2, APOE3, and APOE4 mice was coated onto the plates. Titrations of chi–HAE-2 and chi–HAE-4 were incubated and antibody that was captured was detected with HRP-goat anti–human IgG antibody. (C and D) HAE-2 or HAE-4 was immobilized on the plate followed by the addition of plasma from mice with different genotypes. apoE, captured from the plasma, was detected with HRP-goat polyclonal anti-apoE. (E) Plasma inhibition of anti–apoE binding to immobilized recombinant apoE4. HAE-1 (50 nM), HAE-2 (4 nM), and HAE-4 (50 nM) were preincubated with serially diluted plasma from APOE4-KI mice and then added to plates coated with recombinant apoE4. The HAE antibodies bound to the plates were detected with HRP-goat anti–mouse IgG antibodies. (F) Plasma antibody concentrations of HAE-4 or control IgG following i.p. injection into APOE4-KI or EKO mice. HAE-4 was dosed at 2 mg/kg, 10 mg/kg, and 50 mg/kg and plasma samples were collected by submandibular puncture. Control murine IgG2a (msIgG2a) was anti-Her2 and dosed at 10 mg/kg. Quantification of dosed antibodies in plasma was by antigen-capture ELISA using coated recombinant apoE4 to detect HAE-4, with recombinant Her2 used to detect the control IgG.
In contrast, plasma did not inhibit the binding of HAE-1 or HAE-4, suggesting that these antibodies do not bind lipidated forms of apoE (Figure 3E).

The difference in ability to recognize plasma-derived apoE was further supported by the pharmacokinetic profiles of HAE-1, HAE-2, HAE-3, and HAE-4. Following i.p. injection (10 mg/kg) into APOE4 mice, HAE-2 and HAE-3 were rapidly cleared from plasma within 4 hours (Supplemental Figure 5), whereas HAE-4 was present in the plasma 14 days after injection at levels similar to a control mouse IgG2a antibody (Figure 3F). The likely target-mediated clearance observed with HAE-2 and HAE-3 suggests that these antibodies bind to plasma-derived lipidated apoE, which is abundant in the plasma (~50 μg/ml), whereas HAE-1 and HAE-4 do not. HAE-1 and HAE-4 appear to be selective for nonlipidated apoE, which is low or absent in plasma.

Binding of HAE-4 to plaque and aggregated apoE. Using unfixed frozen brain sections from APPPS1-21/APOE4 or APPPS1-21/EKO mice, we evaluated whether HAE-1 and HAE-4 could bind to aggregated apoE. The presence of Aβ plaques was confirmed with anti-Aβ antibody HJ3.4 on sections from the same brain. Given the lower total signal seen by SPR for HAE-1 and HAE-4 compared with HAE-2 (Figure 4A), we then tested the ability of plasma to inhibit the binding of HAE-1, HAE-2, and HAE-4 to immobilized recombinant nonlipidated apoE4. Antibodies were preincubated with serially diluted plasma from APOE4 mice and subsequently added to the recombinant apoE4-coated plate. The results showed that plasma inhibited the binding of HAE-2 to immobilized apoE4, suggesting that HAE-2 bound both lipidated and nonlipidated forms of apoE. In contrast, plasma did not inhibit the binding of HAE-1 or HAE-4, suggesting that these antibodies do not bind lipidated forms of apoE (Figure 3E).

The likely target-mediated clearance observed with HAE-2 and HAE-3 suggests that these antibodies bind to plasma-derived lipidated apoE, which is abundant in the plasma (~50 μg/ml), whereas HAE-1 and HAE-4 do not. HAE-1 and HAE-4 appear to be selective for nonlipidated apoE, which is low or absent in plasma.
ure 1, E–G), we hypothesized that HAE-1 and HAE-4 bound to a less-abundant subspecies of the recombinant apoE4 that had been immobilized on the sensor chip. To assess whether HAE-4 binds to aggregated forms of apoE, we compared the binding of HAE-1, HAE-2, or HAE-4 to untreated apoE4 or apoE4 preincubated overnight at 40°C. HAE-2 bound to both forms of apoE similarly, whereas the binding of HAE-1 and HAE-4 to heat-treated apoE was dramatically enhanced (Figure 4B). HAE-1 preferentially binds to nonlipidated apoE4 and HAE-4 preferentially binds to nonlipidated apoE3 and apoE4. The binding preferences of HAE-1 to apoE4 and HAE-4 to apoE3 and apoE4 are retained with heat treatment (Supplemental Figure 6). In other words, HAE-1 and HAE-4 show strong preferential binding to aggregated apoE, which may be the predominant form of apoE found in the plaques of APPPS1-21 mouse and human brain sections.

Figure 5. Binding of HAE-1, HAE-4, and control antibody to human apoE4 in the brains of living mice. (A) Control IgG2Ab (n = 7), HAE-1 (n = 5), and HAE-4 (n = 6) conjugated with Alexa 594 were applied directly onto the surface of the brain in living APPPS1-21/APOE4 mice that were 6 months of age, and antibody localization was observed using 2-photon microscopy. Amyloid was labeled using methoxy-X04. The signal from Alexa 594 and methoxy-X04 was merged (MERGE) to show the colocalization of antibodies and plaques. (B) Control human IgG (n = 2) or chi–HAE-4 at 50 mg/kg body weight was injected i.p. in 1 dose (0 hour, n = 3) or 2 doses (0 and 48 hour, n = 3). APPPS1-21/APOE4 mice were sacrificed 48 hours after final injection. The antibodies in the brain were detected by biotinylated rabbit anti–human IgG followed by DAB. Left panel, bar = 1 mm. Right panel, high-power image of the indicated areas shown in the left panel; bar = 300 μm.

Binding of HAE-4 to brain apoE in vivo. Next, we tested whether HAE-1 and HAE-4 could bind to apoE in the brains of living mice. A mouse IgG2ab (control), HAE-1, and HAE-4 were conjugated with Alexa 594 and applied to the brain surface (40 μl of 1 mg/ml) of 6-month-old APPPS1-21/APOE4 mice. Antibody localization was monitored through a cranial window using 2-photon microscopy (Figure 5A). HAE-1 and HAE-4 were found to localize to amyloid plaques. To test whether peripherally administered apoE antibodies could enter the brain and bind to apoE in plaques, chi–HAE-4 or control human IgG were i.p. injected (50 mg/kg) for 1 or 2 doses into 5- to 6-month-old APPPS1-21/APOE4 mice. Two days after the final injection, chi–HAE-4 was detected bound to plaques in the dosed mice (Figure 5B). This indicated that chi–HAE-4 was able to enter the brain and bind to apoE in the Aβ plaques in living animals.

The effects of HAE-1 and HAE-4 on microglial activation and Aβ deposition: requirement of a microglial response. To determine whether HAE-1 and HAE-4 antibodies can increase activated microglia or infiltrating monocytes, we quantified CD45+ cells relative to the amount of fibrillar plaques after short-term treatment of HAE-1 and HAE-4 antibodies (4 doses by i.p. injection every 3 days) in 4-month-old APPPS1-21/APOE4 mice that already had existing plaques (Figure 6A and Supplemental Figure 7). After acute passive immunization, HAE-1 had no effect on the amount of CD45+ cells, whereas HAE-4 significantly increased the CD45+ cells compared with the controls (Figure 6A). Interestingly, there was no increase in the number of Iba1+ cells around plaques following HAE-4 treatment (1.02 × 10^4 ± 8.63 × 10^6 cells/μm^2 for...
In the present study, we report that both i.c.v. and i.p. administration of the anti-human apoE antibody HAE-4 (specific for apoE3 and apoE4) into APP transgenic mice expressing human apoE4 significantly reduced Aβ plaque load and Aβ accumulation in the brain. HAE-4 preferentially binds to nonlipidated forms of apoE as compared with lipidated apoE, which is the major form of apoE in vivo. When monomeric and aggregated forms of apoE were compared, HAE-4 preferentially bound to apoE aggregates. HAE-4 bound to apoE in amyloid plaques after direct application to the brain surface or after peripheral administration into living animals. Importantly, HAE-4 did not significantly alter the levels of total apoE in the brain or plasma, and both HAE-1 and HAE-4 were able to reduce plaque accumulation by direct central administration or expression via AAV. This effect appears to require microglial activation, as the corresponding antibodies with mutations in the Fc effector domain of the antibodies that ablate Fc receptor effector function. (A) At the age of 4 months, the APPPS1-21/APOE4 mice received 4 i.p. injections of 50 mg/kg of antibodies every 3 days. The mice were sacrificed 24 hours after the final injection and the fibrillar plaques were stained with X-34 and the activated microglia was stained with CD45. The ratio of percentage of area covered by CD45 staining/percentage of area covered by X-34 staining was quantified (equal numbers of male and female mice, n = 8-9/group). (B-E) APPPS1-21/APOE4 mice were injected at day P0 with AAV 2/8 into the lateral ventricle (equal numbers of male and female mice, n = 17-25/group). AAV 2/8 is able to express and secrete full-length HAE-1 and HAE-4 antibodies as well as the same constructs with a D265A mutation in the Fc domain (HAE-1Δ and HAE-4Δ). At the age of 3.5 months, the Aβ plaques (B) were stained with antibody HJ3.4, the fibrillar plaques were stained with X-34 (C), and the insoluble Aβ42 or Aβ40 (D) and Aβ42 or Aβ40 (E) were measured by ELISA. One-way ANOVA followed by Tukey’s t test was performed to compare different groups shown in A–E. Data are mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

Discussion
In the present study, we report that both i.c.v. and i.p. administration of the anti-human apoE antibody HAE-4 (specific for apoE3 and apoE4) into APP transgenic mice expressing human apoE4 significantly reduced Aβ plaque load and Aβ accumulation in the brain. HAE-4 preferentially binds to nonlipidated forms of apoE as compared with lipidated apoE, which is the major form of apoE in vivo. When monomeric and aggregated forms of apoE were compared, HAE-4 preferentially bound to apoE aggregates. HAE-4 bound to apoE in amyloid plaques after direct application to the brain surface or after peripheral administration into living animals. Importantly, HAE-4 did not significantly alter the levels of total apoE in the brain or plasma, and both HAE-1 and HAE-4 were able to reduce plaque accumulation by direct central administration or expression via AAV. This effect appears to require microglial activation, as the corresponding antibodies with mutations in the Fc effector domain of the antibodies that ablate Fcγ receptor binding are not effective in plaque clearance. Overall, these findings demonstrate that certain anti-apoE antibodies selective for nonlipidated, aggregated apoE, such as HAE-1 and HAE-4, can preferentially bind to apoE in plaques and have very similar effects to anti-Aβ antibodies that also bind to and decrease Aβ accumula-
tion via a microglial-mediated clearance mechanism (18, 19). Further studies will be required to determine potential advantages and disadvantages to this approach versus the use of anti-Aβ antibodies as a treatment modality.

Although apoE has been established as the strongest genetic factor for LOAD for more than 20 years, the mechanism(s) by which apoE modifies AD pathogenesis is still not entirely clear. However, a large body of literature shows that apoE affects AD at least in part through exacerbating Aβ aggregation and reducing its clearance (7, 20, 21). The effects of apoE isoform, level, or lipidation status on Aβ pathology have been extensively studied in vitro and in vivo (7, 21). Whether increasing or decreasing of apoE function or expression is beneficial for reducing Aβ pathology has long been debated. Overexpression of apoE2 using viral vectors reduces Aβ pathology, whereas overexpression of apoE4 increases Aβ pathology in APP transgenic mice (22–25). Increasing apoE4 expression prior to but not after Aβ deposition enhances plaque burden (26). Genetic removal of endogenous murine apoE or reduction of apoE with antisense oligonucleotides prior to the onset of Aβ deposition decreases Aβ plaque load and fibrillar plaques in APP transgenic mice (27–29). Lowering apoE levels after Aβ deposition had started had no effect on Aβ levels (29). APP mice that express 1 copy of human apoE have markedly less Aβ pathology as compared with those expressing 2 copies of the same apoE isoform (30, 31). Blocking the interaction between apoE and Aβ using Aβ12-28p suppresses Aβ plaque deposition in APP transgenic mice (32). Taken together, only the overexpression of the apoE2 isoform appears beneficial, at least with respect to reducing Aβ accumulation. Based on the effects we describe, the ability to target specific pools of apoE might be an ideal therapeutic strategy to address the more than 60% of the patients with LOAD who have apoE4 carriers. Importantly, this approach of specifically targeting apoE in plaques allows for a direct antibody-mediated decrease in Aβ accumulation, in contrast to the alternate approach of simply lowering apoE levels. Whether anti–apoE antibodies such as HAE-4 will clear existing apoE-containing plaques will need to be tested in future studies.

In previous studies, we found that anti–mouse apoE antibody HJ6.3 reduced Aβ plaque load in APPPS1 mice expressing murine apoE (10, 11). In those studies, we were not able to determine the mechanism whereby anti-apoE modulated plaque pathology. To better explore this issue, as well as to develop antibodies that bind to human apoE, we generated a series of anti-human apoE antibodies and assessed their efficacy and mechanism of action in mice that develop Aβ deposition and express human apoE4. We focused most of these studies on antibody HAE-4, as it was most effective at reducing plaques when given peripherally. We first analyzed the apoE levels in the treated animals. Similar to the findings in our previous study using anti–mouse apoE antibody HJ6.3 (10, 11), peripheral administration of HAE-4 did not change the level of plasma apoE or the level of total brain apoE in the tissue lysates. Therefore, the effects of HAE-4 on Aβ plaques were not mediated simply by lowering total apoE levels. It was previously reported that a plaque-specific Aβ antibody (mE8) that targets only pyro-glutamated Aβ, a small proportion of Aβ in amyloid plaques, was able to localize to Aβ plaques and remove them by triggering microglia-mediated phagocytosis (19). In the present study, we found that HAE-4 is able to bind to apoE in amyloid plaques, both on unfixed brain sections and in the brain of living APPPS1-21/APOE4 mice following central or peripheral injection. Further, we found that the ability of HAE-4 to decrease plaque accumulation was dependent on microglial activation. This suggests that the key features of this antibody’s ability to decrease Aβ plaques are its selectivity toward binding a conformation of apoE in amyloid plaques, specifically apoE aggregates, and its ability to directly microglial-driven apoE-amyloid phagocytosis.

To better understand whether the antibody most effective at decreasing plaques was binding to a particular form of apoE that was selectively present in plaques and not normally found in brain or blood, we assessed the binding affinity of HAE-4 to lipoprotein apoE with antisense oligonucleotides prior to and after plaque accumulation. Whether increasing or decreasing of apoE function or expression is beneficial for reducing Aβ pathology has long been debated. Overexpression of apoE2 using viral vectors reduces Aβ pathology, whereas overexpression of apoE4 increases Aβ pathology in APP transgenic mice (22–25). Increasing apoE4 expression prior to but not after Aβ deposition enhances plaque burden (26). Genetic removal of endogenous murine apoE or reduction of apoE with antisense oligonucleotides prior to the onset of Aβ deposition decreases Aβ plaque load and fibrillar plaques in APP transgenic mice (27–29). Lowering apoE levels after Aβ deposition had started had no effect on Aβ levels (29). APP mice that express 1 copy of human apoE have markedly less Aβ pathology as compared with those expressing 2 copies of the same apoE isoform (30, 31). Blocking the interaction betweenapoE and Aβ using Aβ12-28p suppresses Aβ plaque deposition in APP transgenic mice (32). Taken together, only the overexpression of the apoE2 isoform appears beneficial, at least with respect to reducing Aβ accumulation. Based on the effects we describe, the ability to target specific pools of apoE might be an ideal therapeutic strategy to address the more than 60% of the patients with LOAD who have apoE4 carriers. Importantly, this approach of specifically targeting apoE in plaques allows for a direct antibody-mediated decrease in Aβ accumulation, in contrast to the alternate approach of simply loweringapoE levels. Whether anti–apoE antibodies such as HAE-4 will clear existing apoE-containing plaques will need to be tested in future studies.

In previous studies, we found that anti–mouse apoE antibody HJ6.3 reduced Aβ plaque load in APPPS1 mice expressing murine apoE (10, 11). In those studies, we were not able to determine the mechanism whereby anti-apoE modulated plaque pathology. To better explore this issue, as well as to develop antibodies that bind to human apoE, we generated a series of anti-human apoE antibodies and assessed their efficacy and mechanism of action in mice that develop Aβ deposition and express human apoE4. We focused most of these studies on antibody HAE-4, as it was most effective at reducing plaques when given peripherally. We first analyzed the apoE levels in the treated animals. Similar to the findings in our previous study using anti–mouse apoE antibody HJ6.3 (10, 11), peripheral administration of HAE-4 did not change the level of plasma apoE or the level of total brain apoE in the tissue lysates. Therefore, the effects of HAE-4 on Aβ plaques were not mediated simply by lowering total apoE levels. It was previously reported that a plaque-specific Aβ antibody (mE8) that targets only pyro-glutamated Aβ, a small proportion of Aβ in amyloid plaques, was able to localize to Aβ plaques and remove them by triggering microglia-mediated phagocytosis (19). In the present study, we found that HAE-4 is able to bind to apoE in amyloid plaques, both on unfixed brain sections and in the brain of living APPPS1-21/APOE4 mice following central or peripheral injection. Further, we found that the ability of HAE-4 to decrease plaque accumulation was dependent on microglial activation. This suggests that the key features of this antibody’s ability to decrease Aβ plaques are its selectivity toward binding a conformation of apoE in amyloid plaques, specifically apoE aggregates, and its ability to directly microglial-driven apoE-amyloid phagocytosis.

To better understand whether the antibody most effective at decreasing plaques was binding to a particular form of apoE that was selectively present in plaques and not normally found in brain or blood, we assessed the binding affinity of HAE-4 to lipoprotein apoE with antisense oligonucleotides prior to and after plaque accumulation. Whether increasing or decreasing of apoE function or expression is beneficial for reducing Aβ pathology has long been debated. Overexpression of apoE2 using viral vectors reduces Aβ pathology, whereas overexpression of apoE4 increases Aβ pathology in APP transgenic mice (22–25). Increasing apoE4 expression prior to but not after Aβ deposition enhances plaque burden (26). Genetic removal of endogenous murine apoE or reduction of apoE with antisense oligonucleotides prior to the onset of Aβ deposition decreases Aβ plaque load and fibrillar plaques in APP transgenic mice (27–29). Lowering apoE levels after Aβ deposition had started had no effect on Aβ levels (29). APP mice that express 1 copy of human apoE have markedly less Aβ pathology as compared with those expressing 2 copies of the same apoE isoform (30, 31). Blocking the interaction betweenapoE and Aβ using Aβ12-28p suppresses Aβ plaque deposition in APP transgenic mice (32). Taken together, only the overexpression of the apoE2 isoform appears beneficial, at least with respect to reducing Aβ accumulation. Based on the effects we describe, the ability to target specific pools of apoE might be an ideal therapeutic strategy to address the more than 60% of the patients with LOAD who have apoE4 carriers. Importantly, this approach of specifically targeting apoE in plaques allows for a direct antibody-mediated decrease in Aβ accumulation, in contrast to the alternate approach of simply loweringapoE levels. Whether anti–apoE antibodies such as HAE-4 will clear existing apoE-containing plaques will need to be tested in future studies.
to the process of decreasing Aβ accumulation needs to be sorted out in future studies. In addition to efficacy, it will be important to determine whether anti–apoE antibodies with features such as HAE-4 have any potential side effects. We were not able to determine whether there was an increase in microhemorrhages in the APPPS1-21/APOE4 mice following treatment with HAE-4, as there was very little amyloid angiopathy in this mouse model. Future studies addressing this issue may support the development of anti–apoE antibodies for the treatment of AD.

Methods

Animals. APPPS1-21 mice on a C57BL/6J background (gift from Mathias Jucker, Hertie Institute for Clinical Brain Research, Tübingen, Germany) coexpress human APP with a Swedish mutation (KM670/671NL) and mutant PS1 with the L1669 mutation under control of a Thy1 promoter (I3). APOE2-, APOE3-, and APOE4-knock-in mice express APOE2, E3, and E4 under control of the endogenous mouse regulatory elements on a C57BL/6J background (S8). EKO mice were purchased from Taconic. APPPS1-21/APOE4 mice were generated by breeding APPPS1-21 with APOE4 mice. The Aβ plaque pathology in APPPS1-21/APOE4 mice begins at the age of 2 months.

Generation of antibodies. The HAE antibodies were generated by injecting recombinant apoE4 with complete Freund’s adjuvant into mice. For an initial screening of antibodies, supernatants from hybridoma clones were added to 96-well plates coated with recombinant apoE4 and the HAE that bound to apoE4 was detected using anti–mouse IgG antibodies. All 4 anti–apoE antibodies studied are mouse IgGs. HAE-1 contained similar amounts of endotoxin as compared with control purified on a protein G column. All HAE antibodies utilized in vivo experiments were generated by breeding APPPS1-21 with APOE4 mice. The Aβ plaque pathology in APPPS1-21/APOE4 mice begins at the age of 2 months.

 apoE binding ELISAs. Recombinant apoE (Leinco Technologies) was coated to half-area 96-well plates at 0.5 μg/ml in PBS overnight at 4°C (25 μl/well). After 3 washes with PBS, the wells were blocked with 1% BSA-PBS for 1 hour at room temperature with shaking at 500 rpm. The blocked wells were washed once with PBS and subsequently loaded with HAE antibodies at serial concentrations (starting at 300 nM with 3-fold dilutions thereafter). Bound HAE antibodies were detected with HRP-labeled goat anti–mouse IgG HRP. The antibodies that performed well in the initial screening were further characterized and selected for in vivo studies. For the in vivo study, antibodies were generated from cultured hybridoma cells and purified on a protein G column. All HAE antibodies utilized in vivo contained similar amounts of endotoxin as compared with control antibodies. All 4 anti–apoE antibodies studied are mouse IgGs. HAE-1 is an IgG1. HAE-2, HAE-3, and HAE-4 are IgG2ab subtypes.

 apoE antibody administration and sample collection. At the age of 2 months, APPPS1-21/APOE4 mice underwent surgical implantation of a subcutaneous osmotic minipump (Alzet, model 2006) connected to a catheter into the left lateral cerebral ventricle (Bregma −0.4 mm, 1.0 mm lateral to midline, 2.5 mm below the skull). The apoE antibodies or control antibodies (2 mg/ml) were filled into the osmotic minipump and continuously infused (i.e., at the speed of 0.15 μl/h for 6 weeks). At the age of 3.5 months, the mice were perfused with ice-cold PBS containing 0.3% heparin. The right hemi-brain was dissected and flash-frozen on dry ice for biochemical assays. The left hemi-brain was fixed in 4% paraformaldehyde for histological analysis. Serial coronal sections at 50-μm thickness were collected from the rostral to the caudal end of each brain hemisphere using a freezing sliding microtome (Leica Biosystems).

 ELISA for tissue lysates. To extract Aβ in different fractions, brain cortices were sequentially homogenized with cold PBS, 1% Triton-X 100, and 5 M guanidine buffer in the presence of 1× protease inhibitor mixture (Roche). The levels of Aβ40, Aβ35-40, and apoE were measured by sandwich ELISA. For Aβ40 or Aβ35-40, anti-Aβ35-40, HJ2 (produced in-house) or anti-Aβ35-40, HJ7.4 (produced in-house) were used as capture antibodies, and anti-Aβ35-40, HJ5.1-biotin (produced in-house) was used as a detecting antibody (39). For apoE ELISA, HJ6.2 (produced in-house) (10) was used as the capture antibody and HJ6.1-biotin (produced in-house) (10) was used as the detecting antibody. Recombinant apoE4 was used as the standard for the apoE ELISA. For assessment of the concentration of rHAE-1, rHAE-1a, rHAE-4, and rHAE-4A following expression via AAV2/8, recombinant apoE4 was coated on 96-well ELISA plates overnight at 4°C. After washing, individual puri-
fied hybridoma-derived and purified HAE-1 or HAE-4 were added to wells at different concentrations for a standard curve or the PBS-soluble fraction of mouse cortex was added to the wells. Following washing, the antibodies were detected with HRP-coupled anti-mouse IgG.

**Immunohistochemistry for Aβ pathology.** Aβ plaques were immunostained using biotinylated anti-Aβ
t

produced in-house) (11). Fibrillar plaques were stained with 0.025% Thioflavin S (Sigma-Aldrich) or 10 nM X-34. Quantitative analysis of immunopositive staining was performed as previously described (39). All quantitation of Aβ and Thioflavin S staining was done by an investigator who was blind to both mouse genotype and treatment condition. Briefly, images of immunostained sections were exported with an NDP viewer (Hamamatsu Photonics), converted to 8-bit grayscale using ACDSee Pro 2 software (ACD Systems), thresholded to highlight positive staining, and analyzed using ImageJ software (NIH). Three sections per mouse (Bregma, -1.4 mm caudal to Bregma, and -2.0 mm caudal to Bregma) were quantified (cortex dorsal to hippocampus) and the average was used to represent each mouse.

**Binding of HAE antibodies to lipided apoE.** Blood was collected from the right atrium of 6-week-old, apoE-targeted replacement mice or EKO mice and centrifuged in plasma collection tubes (containing EDTA) at 14,000 g for 5 minutes. For the plasma-coat ELISA, plasma was diluted 50-fold in PBS and coated to the bottom of half-well ELISA plates overnight at 4°C. Plates were washed with PBS and blocked in 5% BSA-PBS for 2 hours. Antibodies were serially diluted 5-fold (starting at 500 nM) in 5% BSA-PBS and incubated for 1 hour at room temperature with shaking (500 rpm). Plates were next washed extensively in PBS and bound antibody was detected with an HRP-goat anti-human secondary antibody (catalog 109-035-003, Jackson ImmunoResearch Laboratories). TMB was used as the chromogenic reagent and reactions were stopped with BioFX stop solution. Plates were read at OD

Replicate assays were performed and data from 1 experiment in duplicate are shown. For the plasma-coat ELISA, plasma was coated at 500 ng/ml overnight at 4°C. Plates were washed extensively with PBS and bound antibody was detected with an HRP-goat anti-human secondary antibody (catalog 109-035-003, Jackson ImmunoResearch Laboratories). TMB was used as the chromogenic reagent and reactions were stopped with BioFX stop solution. Plates were read at OD

Replicate assays were performed and data from 1 experiment in duplicate are shown.

**Pharmacokinetics of HAE antibodies in mice.** Murine HAE-4 and control mouse Ig2a anti-HER2 were injected i.p. at 2 mg/kg, 10 mg/kg, and 50 mg/kg, and plasma samples were collected by submandibular puncture at various time points. Assessment of plasma antibody concentrations was performed using coated recombinant apoE4 (5 μg/ml) to capture dosed HAE-4, and recombinant Her2 protein (R&D) (1 μg/ml) to capture the control antibody. Plates were blocked with 3% BSA in TBS/0.1%Tween for 1 hour and washed 3 times before incubation with plasma samples at a 1:2500 dilution. Bound antibodies were detected with HRP-anti-mouse IgG (catalog 115-035-003, Jackson ImmunoResearch Laboratories). The standard curve range was 0.49–1000 ng/ml and fit with a 4-parameter logistic function.

**Binding of HAE-1 and HAE-4 to apoE in the unfixed brain tissue.** Brain tissue from APPPS1/APOE4 and APPPS1/EKO mice was sectioned at 20-μm thickness using a cryotome (ULTRApro 5000, Vibratome) and mounted on slides. The sections were stained with biotinylated HJ3.4, HAE-1, or HAE-4. The antibodies bound to the sections were detected using an ABC kit (Vector Laboratories) followed by DAB (Sigma-Aldrich).

**Cranial window implantation, topical application of antibodies and multiphoton imaging.** APPPS1-21/APOE4 mice (5-7 months old) were anesthetized under 1.5% isoflurane, and a 4-mm cranial window was drilled in order to expose the cortex, as previously described (24). After removing the dura matter, 40 μl of each antibody stock (1.0 mg/ml), previously conjugated with Alexa 594 (Alexa Fluor 568 Antibody Labeling Kit, ThermoFisher Scientific), was topically applied for 30 minutes. After 2 washes with sterile PBS, a glass coverslip was cemented on the skull in order to seal the window before imaging. Fluorescein dextran (70,000 Da; 12.5 mg/ml in sterile PBS; Invitrogen) was also injected retroorbitally to provide a fluorescent angiogram. After a first set of images was taken in order to detect the signal from each antibody alone, methoxy-XO4 (5 mg/kg) was injected intravenously in order to label amyloid plaques as well as cerebral amyloid angiopathy, as previously described (40). In vivo multiphoton imaging was performed using an Olympus FluoView FV1000MPe multiphoton laser-scanning system mounted on an Olympus BX61WI microscope and an Olympus ×25 objective (numerical aperture = 1.05). A DeepSee Mai Tai Ti: sapphire mode-locked laser (Mai Tai; Spectra-Physics) generated 2-photon excitation at 800 nm, and detectors containing 3 photomultiplier tubes (Hamamatsu) collected emitted light in the range of 420–460 nm, 495–540 nm, and 575–630 nm. Mice were placed on the microscope stage, heated using a heating pad, and feedback regulation was obtained from a rectal temperature probe (Harvard Apparatus). Z-series images (2 μm steps, depth of ~200 μm, 512 × 512 pixels) were taken to cover a large surface of the window. The laser power was measured and adjusted before each imaging and the settings of the photomultiplier tubes were unchanged throughout the different imaging sessions and between all antibodies applied.

**Binding of peripherally administered chi-HAE-4 to apoE in the brain.** Chi-HAE-4 and control human IgG were injected i.p. at 50 mg/kg into 4- to 6-month-old APPPS1-21/APOE4 mice for 1 (0 hour) or 2 (0 and 48 hours) doses and the mice were sacrificed 48 hours after the final injection. The antibodies were detected using biotinylated rabbit anti-human IgG (catalog ab97158, Abcam) followed by DAB.
Acute injection of HAE antibodies and microglial activation staining. APPPS1-21/APOE4 mice received 4 doses of antibodies every 3 days at the age of 4 months. Each dose was 50 mg/kg (administered i.p.). The mice were sacrificed 24 hours after final injection. The fibrillar plaques were stained using X-34. Activated microglia were immunostained using rat-anti CD45 (catalog MCA1388, BioRad) followed by biotinylated goat anti-rat IgG secondary antibody (catalog A10517, Life Technologies). Iba-1+ cells were detected using rabbit anti-Iba-1 (catalog 019-19741, Wako) followed by donkey anti-rabbit IgG Alexa Fluor 647 (catalog A-31573, ThermoFisher Scientific). For quantification, 3 Z-stack images were captured per animal on the Nikon A1R+ confocal microscope using ×20 objective at 1024 × 1024 pixel resolution with a Z-step size of 1.1 μm at 32-μm thickness. These images were then processed using ImageJ (Bitplane). The coordinates of X-34+ plaques and Iba-1+ microglia were determined using the Spots function and imported into Matlab (Mathworks). An automated script was used to determine the microglial density around plaque surfaces, and the number of microglia within a 30 μm radius was calculated.

Preparation and injection of AAV2/8 vectors expressing control antibody and HAE antibodies. The variable regions of heavy and light chain cDNA sequences of control IgG, HAE-1, and HAE-4 were cloned from hybridoma cells. The single open reading frame (ORF) of the heavy chain, Furin cleavage site, P2A, and the light chain was assembled as a cDNA construct. On P0, APPPS1-21/APOE4 mice (mixed sexual phenotype, n = 17–25/group) received a bilateral i.c.v. injection of 2 μl AAV vectors. On P50 AG05681, P01 AG03991, and P01 AG026276, and the BrightFocus Foundation (A2013037F to FL). The authors acknowledge Maya Leabman (independent scientific consultant) for discussions on pharmacokinetics, Katherine Jenkins (Denali) for animal support, and Wilbur Song and Marco Colonna (Washington University) for the methods of Iba-1 immunostaining quantification.

Address correspondence to: David M. Holtzman, Department of Neurology, Hope Center for Neurological Disorders, Charles F. and Joanne Knight Alzheimer’s Disease Research Center, Washington University School of Medicine, 660 S. Euclid Avenue, Box 8111, St. Louis, Missouri 63110, USA. Phone: 314.747.0644; Email: holtzman@neuro.wustl.edu.

Acknowledgments

This work was supported by a research grant from Denali (to DMH), the NIH (AG R01 AG047644 to DMH and BTH, and grants P50 AG05681, P01 AG03991, and P01 AG026276), and the BrightFocus Foundation (A2013037F to FL). The authors acknowledge Maya Leabman (independent scientific consultant) for discussions on pharmacokinetics, Katherine Jenkins (Denali) for animal support, and Wilbur Song and Marco Colonna (Washington University) for the methods of Iba-1 immunostaining quantification.

Author contributions

FL, AL, YZ, APS, JLG, CEGL, GG, NBL, RJW, JDU, PMS, EH, KH, ZKS, MSD, BTH, and DMH designed the research studies. HJ, FL, AL, YZ, APS, NBL, JG, MSD, RJW, and DMH generated and characterized the anti–apoE antibodies. FL, AL, MX, HJ, MFB, RH, KBL, JK, GOR, YZ, JRS, APS, JLG, EPL, and EH conducted experiments and acquired data. FL, AL, MX, YZ, APS, JLG, NBL, RJW, EPL, EH, JG, KH, MSD, ZKS, BTH, and DMH analyzed the data. FL, AL, YZ, APS, JLG, NBL, MSD, EH, BTH, RJW, and DMH wrote the manuscript.

REFERENCES

2155