Targeting Amyloid-β Peptide (Aβ) Oligomers by Passive Immunization with a Conformation-selective Monoclonal Antibody Improves Learning and Memory in Aβ Precursor Protein (APP) Transgenic Mice

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Passive immunization of murine models of Alzheimer disease amyloidosis reduces amyloid-β peptide (Aβ) levels and improves cognitive function. To specifically address the role of Aβ oligomers in learning and memory, we generated a novel monoclonal antibody, NAB61, that preferentially recognizes a conformational epitope present in dimeric, small oligomeric, and higher order Aβ structures but not full-length amyloid-β precursor protein or C-terminal amyloid-β precursor protein fragments. NAB61 also recognized a subset of brain Aβ deposits, preferentially mature senile plaques, and amyloid angiopathy. Using NAB61 as immunotherapy, we showed that aged Tg2576 transgenic mice treated with NAB61 displayed significant improvements in spatial learning and memory relative to control mice. These data implicated Aβ oligomers as a pathologic substrate for cognitive decline in Alzheimer disease.

The Aβ peptide has been hypothesized to cause the pathologic and behavioral manifestations of Alzheimer disease (AD), including synaptic dysfunction and loss, neurofibrillary tangle formation, neuronal degeneration, and impaired memory. A variety of methods designed to inhibit the production or enhance the clearance of Aβ are being developed as potential AD therapies. Indeed, immunization of murine models of Aβ amyloidosis inhibits senile plaque formation and ameliorates associated cognitive impairments (1–6). Despite the development of meningoencephalitis in 6% of individuals immunized with the Aβ1–42 peptide during a phase II clinical human trial (7, 8), immunotherapy, especially passive immunization, remains a compelling potential treatment for AD. Interestingly, passive immunization of mouse models of AD-like Aβ plaques has been shown to rapidly reverse learning and memory deficits without affecting Aβ plaque pathology, indicating that neutralization of toxic Aβ species can quickly restore neuronal function in vivo (9, 10).

The lack of learning and memory deficits in young APP transgenic mice indicates that monomeric Aβ is not responsible for behavioral impairments in vivo (11, 12). Furthermore, levels of soluble monomeric Aβ do not increase with age or with the onset of cognitive defects in transgenic mice (13). Therefore, if Aβ is responsible for learning and memory deficits in vivo, then Aβ must gain one or more of its toxic properties as a function of time. One potential mechanism for this toxic gain of function is a change in the conformation of Aβ such that it exerts its pathologic effects as an oligomeric or fibrillar macromolecule.

To specifically target toxic forms of Aβ, we developed a monoclonal antibody named NAB61 that recognizes a pathologic conformation present in Aβ dimers, soluble oligomers, and higher order species of Aβ. Using this antibody, we found that neutralization of pathologic Aβ by passive immunization of transgenic mice resulted in rapid improvement in spatial learning and memory. These results suggest that pathologic Aβ conformers produced in vivo are capable of disrupting neuronal function, and our data have substantiated the therapeutic potential of targeting Aβ oligomers for the treatment of AD.

MATERIALS AND METHODS

Generation of NAB61—Synthetic Aβ1–40 (from D. Teplow, Boston, MA or from W. M. Keck Foundation Biotechnology Resource Laboratory, Yale University, New Haven, CT) was treated with peroxynitrite, UV light, or 4-hydroxynonenal (HNE) as described (14–16). BALB/c mice were immunized with 100 μg of these Aβ species emulsified with complete Freund’s adjuvant followed by three additional injections of 25 μg of Aβ emulsified with incomplete Freund’s adjuvant. Isolated lymphocytes were used to generate hybridomas by fusion with Sp2/O-Ag14 myeloma cells with polyethylene glycol 1500.

Immunoprecipitation and Immunoblotting—Synthetic Aβ preparations (0.5 μg) were electrophoresed on 16% Tris-Tricine gels and immunoblotted with NAB228 or NAB61 or immunoprecipitated using NAB61 or NAB228 with protein A/G agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) prior to immunoblotting with NAB228. For radiolabeled APP and C-terminal APP fragments, CHO Pro5 cells transfected with pcDNA3.1 containing the cDNA for either green fluorescent protein or APP harboring the Swedish Mutation (APPsw) were radiolabeled with [35S]methionine for 2 h in the presence of 200 μM MG132 (Peptides International, Louisville, KY) to enhance the accumulation of C-terminal APP fragments. RIPA buffer cell lysates


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(0.5% sodium deoxycholate, 0.1% SDS, 1% Nonidet P-40, 5 mM EDTA in Tris-buffered saline, pH 8.0) containing protease inhibitors (1 µg/ml peptatin A, leupeptin, 1-1-tosylamido-2-phenylethyl chloromethyl ketone, 1-chloro-3-tosylamido-7-amino-2-heptanone, and soybean trypsin inhibitor and 0.5 mM phenylmethylsulfonyl fluoride) were centrifuged at 100,000 × g for 20 min at 4 °C and immunoprecipitated with a panel of monoclonal antibodies that recognize Aβ_{1–11} (NAB14, NAB61, NAB89, NAB228, Ban50), a rabbit polyclonal antibody raised against the C terminus of APP (antibody number 2493), or a goat polyclonal antibody raised against the N-terminal ectodomain of APP (Karen). NAB antibodies were generated using the oligomeric Aβ preparations as antigens but do not exhibit oligomer specificity. Immunoprecipitates were electrophoresed on 10/16.5% step gradient Tris-Tricine gels, fixed with methanol, dried, and exposed to a phosphorimaging screen for visualization. APP and APP fragments from mouse cortical extracts were detected as described (17), using the following antibodies: rabbit polyclonal antibody raised against the C terminus of APP (antibody number 5685); goat anti-N-terminal APP antibody (Karen); rabbit anti-sAPPβsw (antibody number 54); and NAB228. An anti-tubulin antibody (TUB21, Sigma) was used to detect tubulin. To detect peripheral Aβ, 200 µl of plasma was diluted with RIPA buffer and immunoprecipitated with 4G8 (anti-Aβ_{1–11}), which was covalently conjugated to protein A/G beads with dimethyl pimelimidate to prevent competition with endogenous IgG. Immunoprecipitates were electrophoresed on a 10/16.5% Tris-Tricine gel and immunoblotted with 4G8.

**Immunocytocchemistry and Immunoelectron Microscopy**—Neuro2A, CHO Pro5, and NT2N neurons were transduced with a Simliki Forest Virus encoding APPsw (SFV-APPsw). After 16 h, cells were fixed with cold 95% ethanol, 5% acetic acid for 10 min followed by further permeabilization with 0.2% Triton X-100 in PBS for 10 min. Cells were stained with a goat polyclonal N-terminal APP antibody (Karen) and NAB61 followed by fluorescein isothiocyanate-conjugated anti-goat IgG and Texas Red-conjugated anti-mouse IgG. For immunoelectron microscopy, fibrillar synthetic Aβ_{42} was absorbed to 300 mesh carbon-coated copper grids, washed with PBS, and blocked with 1% bovine serum albumin in PBS. Aβ fibrils were stained with NAB61 followed by antimouse IgG conjugated to 5-nm colloidal gold particles. Grids were then stained with 1% uranyl acetate, dried, and visualized with a Joel (Peabody, MA) 1010 transmission electron microscope.

**Immunohistochemistry**—Tissue blocks from human subjects or mice were immersion-fixed in 70% ethanol with 150 mM NaCl or 10% buffered formalin. Samples were dehydrated through graded ethanol solutions to xylene and infiltrated with paraffin as described (18). Sections (6 µm) were stained using standard avidin-biotin-peroxidase methods using 3,3′-diaminobenzidine. Ban50 (mouse anti-Aβ_{1–10}, NAB228 (mouse anti-Aβ_{1–11}), and NAB61 (mouse anti-oligomer Aβ_{1–11}) were used as primary antibodies followed by HRP-conjugated anti-mouse secondary antibody (Vector, Burlingame, MD). Quantification of Aβ plaque burden in the Tg2576 mice overexpressing human APP harboring the Swedish mutation (11) was conducted as described (17). For immunofluorescence, sections were stained with a rabbit polyclonal anti-Aβ_{42} antibody (BIOSOURCE International, Camarillo, CA) and with NAB61 followed by fluorescein isothiocyanate-conjugated anti-rabbit IgG and Texas Red-conjugated anti-mouse IgG.

**ELISA Analysis**—For solid-phase experiments, ELISA plates (Nunc, Rochester, NY) were coated with Aβ at 1 µg/ml in PBS and blocked with 5% fetal bovine serum in PBS. Antibodies diluted in 5% fetal bovine serum/PBS were incubated at 4 °C overnight, and bound antibodies were detected with HRP-conjugated anti-mouse IgG (Jackson Immunoresearch, West Grove, PA). For blocking experiments, ELISA plates were coated with Aβ at 0.1 µg/ml, and antibodies were preincubated with the indicated blocking peptides at 10 µg/ml. For capturing experiments, ELISA plates were coated either NAB61 or Ban50 at 10 µg/ml in PBS and blocked with 1% casein in PBS. Aβ peptides diluted in blocking buffer at 10 µg/ml were incubated at 4 °C overnight, and bound Aβ was detected with HRP-conjugated BA27 (mouse anti-Aβ_{40}). For Aβ quantification, detergent-soluble fractions of cortical and hippocampal regions were obtained by sonicating samples in 1 ml of RIPA buffer containing protease inhibitors for every 150 mg of tissue. After centrifugation at 100,000 × g for 20 min at 4 °C, the resulting pellet was solubilized by sonication in 70% formic acid followed by another round of centrifugation to obtain detergent-insoluble Aβ. Supernatants were assayed by sandwich ELISA as described previously (19). Briefly, ELISA plates were coated with either JRF/c40 or JRF/c42 to capture Aβ_{40} and Aβ_{42}, respectively. After application of diluted samples and a standard curve consisting of serially diluted synthetic Aβ (Bachem Biosciences, King of Prussia, PA), the concentration of Aβ was determined by using horseradish peroxidase-conjugated m266 (anti-Aβ_{13–28}) as a reporting antibody. These antibodies do not recognize the N terminus of Aβ and therefore do not compete with NAB61 for Aβ.

**Immunization and Behavioral Analysis**—Tg2576 mice were maintained on a C57B6/SJL F2 background by successive backcrossing to wild-type C57B6/SJL F1 females. All mice were generated and handled according to University of Pennsylvania Institutional Animal Care and Use Committee guidelines. To determine whether immunization improves learning and memory, 17–19-month-old Tg littermates were administered an initial dose of 400 µg of NAB61 or nonspecific mouse IgG (Sigma) intraperitoneally followed by maintenance doses of 200 µg as described in Fig. 5A. Wild-type littermates were administered either IgG or NAB61, and these two wild-type groups were analyzed as one group after treatment was determined to have no effect in non-Tg mice as described under “Results.” The visible and hidden versions of the Morris water maze (MWM) were performed as described by Westerman et al. (12) and are described in detail in the Supplemental Methods. Briefly, mice were subjected to the visible platform MWM in which latency to reach a visibly marked platform was measured over six consecutive training blocks (four trials/block, two blocks/day). Mice were then subject to the hidden platform MWM in which the latency to reach a submerged platform was measured over 9 days (four trials/block, one block/day). Three probe trials were interspersed throughout the hidden platform MWM to test for acquisition of visual reference memory by removing the hidden platform and recording swimming behavior for 60 s. Data were recorded using a video tracking system (HVS Image, San Diego, CA). Mice were tested in a blinded manner, with groups balanced for genotype and treatment. Mice were sacrificed 3 days after the termination of the MWM for biochemical and histological assessment. Experiments were analyzed with one-way ANOVA and two-way ANOVA, with Fischer’s test for post-hoc analysis. To determine whether passive immunization removed Aβ plaques, long term passive immunization was performed on a cohort of 8-month-old Tg2576 mice, which were administered weekly doses of intraperitoneal NAB61 or nonspecific IgG (500 µg) for 6 months and sacrificed at 14 months of age. Changes in Aβ deposits by immunohistochemistry and Aβ levels by ELISA were conducted as described above.

**RESULTS**

**Generation of an Oligomer-selective Monoclonal Antibody**—The role of Aβ oligomers is difficult to assess in vivo due to the dearth of conformation-specific molecular tools. Toward this end, we generated a monoclonal antibody that selectively recognizes oligomeric Aβ by using
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FIGURE 1. Conformational epitope of NAB61, an Aβ oligomer-selective monoclonal antibody. A, NAB61 preferentially recognizes Aβ oligomers by immunoblotting. Aβ1–40 was treated with a peroxynitrite, UV light, or 4-HNE, electrophoresed on a 16% Tris-Tricine gel, and blotted with either NAB61 (left) or NAB228 (right). B, NAB61 preferentially recognizes Aβ oligomers by immunoprecipitation. Aβ1–40 was treated with a peroxynitrite, UV light, or 4-HNE and subject to immunoprecipitation with NAB61 (left) or NAB228 (right). Immunoprecipitates were electrophoresed on a 16% Tris-Tricine gel and blotted with NAB228. C, solid-phase immunoreactivity of NAB61. NAB61, NAB228, and non-specific mouse IgG were serially diluted and tested for immunoreactivity on ELISA plates coated with Aβ1–40 (1 μg/ml). D, NAB61 recognizes an N-terminal epitope. Solid-phase ELISA immunoreactivity was blocked by preincubation of NAB61 and NAB228 with peptides corresponding to full-length Aβ (residues 1–40), the N terminus of Aβ (residues 1–11), or the C terminus of Aβ (11–40). Only peptides containing the N terminus of Aβ were able to block NAB61 and NAB228 immunoreactivity. E, NAB61 preferentially recognizes oligomeric Aβ when used in a sandwich ELISA as a capturing antibody. ELISA plates coated with either Ban50 or NAB61 were tested for their ability to capture Aβ or UV-cross-linked Aβ. Captured peptides were detected with HRP-conjugated BA27 (anti-Aβ1–40). A two-tailed test was performed on OD readings performed in duplicate from two independent experiments (***, p < 0.001).

a stable oligomeric Aβ preparation as antigen. Treating synthetic Aβ1–40 with peroxynitrite, a reactive species generated by the reaction between superoxide and nitric oxide, resulted in the formation of SDS-stable Aβ oligomers, as shown by SDS-PAGE followed by immunoblotting with NAB228, a monoclonal antibody that recognizes a linear N-terminal Aβ epitope (Fig. 1A). Additional stable Aβ oligomers were also generated by treating Aβ1–40 with UV light and with the lipid-derived reactive aldehyde, 4-HNE (gifts from D. Teplow and T. Montine). Over 5,500 hybridoma supernatants were tested for the presence of Aβ antibodies, and one hybridoma was generated from a mouse immunized with nitrated Aβ that produced an IgG1, named NAB61, with selectivity toward oligomeric Aβ species. As shown by both immunoblotting and immunoprecipitation, NAB61 showed selectivity toward SDS-stable Aβ oligomers relative to monomeric Aβ (Fig. 1, A and B, left panels), in contrast with other Aβ antibodies such as NAB228 (Fig. 1, A and B, right panels).

To further characterize this novel monoclonal antibody, we tested NAB61 in a variety of additional immunologic assays. When tested in a solid-phase ELISA format in which Aβ1–40 was coated onto plastic, NAB61 titers were very low relative to NAB228 (Fig. 1C). Despite this low immunoreactivity, peptides corresponding to Aβ1–11 and Aβ1–40 were able to block the signal generated by NAB61, whereas Aβ1–40 did not, indicating that NAB61 recognizes an N-terminal Aβ epitope (Fig. 1D). When used as a capturing antibody in a sandwich ELISA format, NAB61 had greater affinity for oligomeric Aβ relative to non-oligomeric Aβ, in contrast with other anti-Aβ monoclonal antibodies such as Ban50 (Fig. 1E). These in vitro studies indicated that NAB61 recognizes a complex conformational epitope found in the N terminus of oligomeric forms of Aβ.

Although the primary sequence of Aβ is present in full-length APP and C-terminal APP fragments, an antibody that recognizes a pathologic Aβ conformation should be specific for the Aβ peptide. Therefore, we hypothesized that NAB61 does not recognize full-length APP or C99, akin to human anti-Aβ antibodies generated upon active immunization (20). Immunoprecipitations from radiolabeled CHO cells overexpressing either green fluorescent protein or APP were performed with a panel of monoclonal antibodies that recognize the N terminus of Aβ (designated NAB antibodies), a polyclonal N-terminal APP antibody, and a polyclonal C-terminal APP antibody. All of the NAB antibodies recognized both full-length APP and C99 with the notable exception of NAB61 (Fig. 2A). The lack of cross-reactivity with APP was confirmed by double immunofluorescence staining of Neuro2A, NT2N, and CHO cells overexpressing APPswe, which showed that NAB61 staining did not co-localize with staining of total APP by a polyclonal C-terminal APP antibody. All of the NAB antibodies recognized both full-length APP and C99 with the notable exception of NAB61 (Fig. 2A). The lack of cross-reactivity with APP was confirmed by double immunofluorescence staining of Neuro2A, NT2N, and CHO cells overexpressing APPswe, which showed that NAB61 staining did not co-localize with staining of total APP by a polyclonal C-terminal APP antibody. All of the NAB antibodies recognized both full-length APP and C99 with the notable exception of NAB61 (Fig. 2A). The lack of cross-reactivity with APP was confirmed by double immunofluorescence staining of Neuro2A, NT2N, and CHO cells overexpressing APPswe, which showed that NAB61 staining did not co-localize with staining of total APP by a polyclonal C-terminal APP antibody.
NAB61 Immunoreactivity against Fibrillar Aβ Amyloid—To ensure that NAB61 recognized bona fide Aβ amyloid, immunohistochemistry was performed on a variety of tissues containing Aβ amyloid plaques. Individuals with pathologic aging (no history of cognitive impairment despite the presence of Aβ amyloid deposits), mild cognitive impairment (MCI) as demonstrated by psychometric testing, Down syndrome, and AD all contained Aβ plaques and other Aβ deposits that were NAB61-immunoreactive (Fig. 3). Interestingly, diffuse amyloid plaques were poorly stained by NAB61, despite robust staining of amyloid angiopathy (see Fig. 3, insets, for pathologic aging). Compact amyloid plaques from Tg2576 transgenic mice overexpressing APPswe were also recognized by NAB61 (Fig. 3).

The initial stages of AD pathology are generally characterized by Aβ amyloid pathology in association cortices such as the mid-frontal cortex (21). NAB61 immunoreactivity generally exhibited regional selectivity, which reflected the regional progression and severity of Aβ amyloid pathology. For example, many diffuse amyloid deposits in the hippocampus and entorhinal cortex of AD brains were poorly stained by NAB61 (Fig. 4A, left and middle panels) despite strong staining of mature senile plaques and amyloid angiopathy (Fig. 4A, arrowheads, and 4B), whereas many Aβ amyloid plaques in the mid-frontal cortex showed more robust NAB61 immunoreactivity (Fig. 4A, right panels).

The regional selectivity of NAB61 immunoreactivity suggested that NAB61 recognizes a conformation that is found in advanced, pathologic Aβ deposits, namely mature senile plaques and amyloid angiopathy. These inclusions are distinct from diffuse Aβ deposits, which are not associated with neuritic alterations, tau pathologies, or neuronal loss. Double immunofluorescence staining showed that many amorphous Aβ deposits, which were recognized by a conventional anti-Aβ42 antibody, were not recognized by NAB61 (Fig. 4B). In contrast, mature senile plaques were labeled by both antibodies (Fig. 4B). Given that Aβ fibrils are the ultrastructural building blocks of senile plaques, NAB61 staining of synthetic Aβ fibrils by immunoelectron microscopy further corroborated the ability of NAB61 to recognize pathologic forms of Aβ (Fig. 4B). Therefore, NAB61 appears to recognize a pathologic confor-
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FIGURE 4. NAB61 recognizes a subset of fibrillar Aβ pathology. A, regional distribution of NAB61 immunoreactivity. Sections from the hippocampus, entorhinal cortex, and mid-frontal cortex from individuals with AD display different amounts of NAB61 immunoreactivity. In cases of AD in which limbic regions contain predominantly diffuse-type Aβ deposits, NAB61 immunoreactivity was not abundant, although amyloid angiopathy and dense senile plaques were strongly stained (arrowheads). In contrast, abundant staining was observed in regions such as the mid-frontal cortex, where dense, mature senile plaques predominate. B, a higher magnification view of serial sections from the entorhinal cortex of an individual with AD clearly demonstrates that NAB61 does not recognize all Aβ deposits (left panels). Double immunofluorescence with a polyclonal anti-Aβ42 antibody (green) and NAB61 (red) of the entorhinal cortex demonstrates that NAB61 recognizes a mature, dense, corel senile plaque. In contrast, a diffuse Aβ deposit was not stained by NAB61 (upper right panels). NAB61 recognizes Aβ fibrils by immunoelectron microscopy. NAB61 staining of synthetic Aβ fibrils was detected with 5 nm colloidal gold-conjugated anti-mouse IgG (lower right panel).

mation present in dimeric and oligomeric Aβ, which is maintained during fibrillization and coalescence into senile plaques. NAB61 did not recognize other inclusions consisting of amyloidogenic proteins such as neurofibrillary tangles or Lewy bodies (data not shown), indicating that NAB61 is specific for Aβ and does not recognize a pathologic conformation common to other amyloidogenic proteins.

NAB61 Improves Spatial Learning and Memory—Learning and memory impairments have been shown to normalise rapidly upon neutralization of Aβ following passive immunization of murine models of Aβ amyloidosis (9, 10). To probe the role of Aβ oligomers on cognitive dysfunction, 17–19-month-old Tg2576 mice were immunized with NAB61 (n = 14) or nontarget IgG (n = 16) and tested in the MWM for spatial learning and memory, using the schedule shown in Fig. 5A. Non-transgenic mice were also treated with either IgG (n = 7) or NAB61 (n = 7). However, for statistical analysis, data from IgG- and NAB61-treated non-transgenic control mice were pooled after performing a two-way ANOVA, which revealed no effect of treatment on performance in the hidden water maze for non-Tg mice (treatment, p = 0.8974; block, p = 0.0251; interaction, p = 0.8942).

To test for potentially confounding sensorimotor or motivational defects, latencies to reach a visible platform were measured over successive training blocks (four trials/block). Non-transgenic, NAB61-treated Tg2576, and IgG-treated Tg2576 mice showed no deficits in the visible water maze (Fig. 5B). Although latencies for both NAB61-treated and IgG-treated Tg2576 mice on the second and third training blocks of the visible water maze tended to be higher than latencies for non-transgenic mice, differences in overall performance were insignificant (repeated measures ANOVA, p = 0.0814). Furthermore, the latency to reach the visible platform and swim speeds on both the first trial and the first block were not statistically different between the three groups (data not shown), arguing against the presence of confounding sensorimotor or motivational deficits.

Spatial learning and memory were then tested using the hidden water maze in which the primary measure of learning and memory was latency to reach the hidden platform (Fig. 5C). IgG-treated Tg2576 mice showed no significant improvement in latencies over the testing period (one-way ANOVA, p = 0.4402), whereas NAB61-treated Tg2576 mice and non-transgenic mice both showed a significant decrease in latency with training (one-way ANOVA: NAB61, p = 0.0004; non-transgenic, p = 0.0184). Furthermore, comparisons between the three groups of mice demonstrated that both non-transgenic and NAB61-treated Tg2576 mice performed significantly better than IgG-treated Tg2576 mice (repeated measures ANOVA, p = 0.0002; non-transgenic versus IgG, p = 0.0014; NAB61 versus IgG p = 0.0006). Therefore, passive immunization with NAB61 ameliorates behavioral deficits in the hidden water maze.

To confirm that the improved behavior on the hidden water maze was due to the acquisition of spatial reference memory, three probe trials were interpolated throughout the training period (Fig. 5A) in which the platform was removed, and the percentage of time spent searching in the target quadrant (where the platform is usually located) was determined. During the first probe trial, the three groups of mice exhibited spatially oriented swimming behavior, indicating that all three groups have acquired some degree of a spatial reference for the general location of the hidden platform (Fig. 5D). However, the time spent in the target quadrant relative to adjacent quadrants was only significantly different for NAB61-treated Tg2576 and non-transgenic mice. After further training, this behavioral measure became saturated and thus was unable to discern any differences between the three groups of mice in the final two probe trials (Fig. 5, E and F).

Since the time spent in the target quadrant appeared to plateau by the second of the three probe trials, we used a third measure of spatial learning to confirm the improved acquisition of spatial reference memory upon NAB61 immunization. A platform crossing index was calculated that measures the number of crossings over the exact location of the platform subtracted by the average number of crossings over the platform locations in non-target quadrants (Fig. 5G). Using this measure, both NAB61-treated Tg2576 mice and non-transgenic mice performed significantly better than IgG-treated Tg2576 mice (repeated measures ANOVA, p = 0.0301; NAB61 versus IgG, p = 0.0332; non-transgenic versus IgG, p = 0.0426). Again, this effect was not due to the presence of motor deficits as all three groups of mice exhibited similar swim speeds regardless of the probe trial (Fig. 5H, two-way ANOVA: group, p = 0.4033; probe trial, p = 0.8911; interaction, p = 0.9804). Therefore, three statistical measures (latency, percentage of time in target quadrant, and platform crossing index) all indicated that short term immunization with NAB61 improved spatial learning and memory in aged Tg2576 mice.

NAB61 Immunization Does Not Affect APP Processing or Aβ Accumulation—To show that the NAB61-mediated neutralization of Aβ oligomers was independent of effects on APP processing or the extent of Aβ amyloid pathology, we examined levels of APP and APP fragments in mice after NAB61 treatment. Steady-state levels of full-length APP, sAPPβ, and C99 were not different among IgG-treated and NAB61-treated Tg2576 mice, arguing that NAB61 did not affect proteolytic processing of APP (Fig. 6A). Furthermore, no differences in amyloid plaque morphology, distribution, or density were noted upon NAB228 or NAB61 immunohistochemistry (Fig. 6B). Additionally, quantification of detergent-soluble and insoluble levels of cortical and hippocampal Aβ by sandwich ELISA indicated that Aβ levels were not statistically different upon short term NAB61 immunization (Fig. 6C). Surprisingly, we also found that 6 months of peripheral passive immu-
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**FIGURE 5. NAB61 improves spatial learning and memory.** A, immunization and behavioral testing. 17–19-month-old Tg2576 mice were administered an initial dose of 400 µg of NAB61 or nonspecific mouse IgG on day –3 with maintenance doses of 200 µg on days 0, 6, and 12. Performance on the visible water maze was tested on days 1–3 (four trials/block, two blocks/day) followed by the hidden water maze on days 4–13 (four trials/block, one block/day). Probe trials were interpolated throughout the hidden water maze on days 7, 10, and 13. B, performance in the visible water maze. NAB61-treated Tg2576, IgG-treated Tg2576, and non-transgenic mice all learned the visible water maze task. No significant differences were found between the three groups. C, performance in the hidden water maze. Within-group analysis indicated that NAB61-treated Tg2576 and non-transgenic mice both showed significant improvements in escape latencies with time, whereas data from IgG-treated Tg2576 mice did not reach statistical significance. Between-group analysis indicated that both NAB61-treated Tg2576 and non-transgenic mice both performed significantly better than IgG-treated Tg2576 mice. (**, p < 0.01; *** p < 0.001). D–F, spatial reference memory, percentage of time in quadrant. The percentage of time spent in each quadrant for a 60-s probe trial conducted early (D), middle (E), and late (F) during training indicated that all three groups of mice acquired some level of spatial reference memory. However, only NAB61-treated Tg2576 and non-transgenic mice spent significantly more time in the target quadrant relative to adjacent quadrants during the first probe trial (D). ns, not significant; * p < 0.05; ** p < 0.01; *** p < 0.001. G, spatial reference memory, platform crossing index. The number of crosses over the exact location of the hidden platform subtracted by the average number of crosses over non-target platforms was measured throughout the three probe trials. NAB61-treated Tg2576 and non-transgenic mice performed significantly better than IgG-treated Tg2576 mice. (* p < 0.05). H, motor function in the Morris water maze. All three groups of mice displayed similar swim speeds in all three probe trials.

**DISCUSSION**

Despite the use of a stable Aβ oligomer preparation, the generation of an anti-Aβ oligomer-selective monoclonal antibody was a low probability event, with less than 0.02% of tested hybridomas generating an antibody with selectivity toward Aβ oligomers. Indeed, although several monoclonal antibodies that recognize Aβ or nitrated epitopes were also isolated, none of these antibodies showed any Aβ oligomer selectivity. Given its unique epitope, we have thoroughly characterized NAB61 using multiple methods including immunoblotting, immunoprecipitation, solid-phase ELISA, sandwich ELISA, immunocytochemistry, immunohistochemistry, immunoelectron microscopy, and immunotherapy. In contrast with other reported oligomer- or amyloid fibril-specific antibodies (22, 23), NAB61 does not recognize other amyloidogenic proteins and is specific for Aβ amyloid pathology.

Finally, passive immunization against Aβ has been postulated to enhance the efflux of Aβ from the central nervous system into the periphery. However, the presence of Aβ oligomers has not been demonstrated in the periphery, and therefore, NAB61 would not be expected to affect peripheral pools of Aβ. Immunoprecipitation of Aβ from sera obtained from passively immunized Tg2576 mice failed to demonstrate an increase in peripheral Aβ (Fig. 6D). Furthermore, naturally occurring Aβ oligomers were not immunoprecipitated from sera samples, although very low concentrations of synthetic Aβ oligomers could be immunoprecipitated when added to sera samples (Fig. 6D). Having ruled out effects on APP processing, Aβ amyloid accumulation, and peripheral pools of Aβ, the selectivity toward oligomeric Aβ exhibited by NAB61 suggested that direct neutralization of Aβ oligomers by immunization with NAB61 can reverse learning and memory deficits in Tg2576 mice.

**Table 1**

| Condition          | Day 3 | Day 6 | Day 12 |
|--------------------|-------|-------|--------|
| Tg2576 IgG         | 40    | 40    | 40     |
| Tg2576 NAB61       | 40    | 40    | 40     |
| Non-transgenic     | 40    | 40    | 40     |

**FIGURE 6. Aβ oligomer selectivity of NAB61.** A, detection of Aβ oligomers by nitrocellulose dot blotting. B, immunoprecipitation of Aβ from sera obtained from NAB61- or IgG-treated Tg2576 mice and non-transgenic mice. C, immunoprecipitation of Aβ from passively immunized Tg2576 mice failed to demonstrate an increase in peripheral Aβ (D). ns, not significant; * p < 0.05; ** p < 0.01; *** p < 0.001.
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FIGURE 6. Sub-chronic NAB61 treatment does not affect APP or Aβ levels. A, APP processing in NAB61 immunized mice. Full-length APP was immunoprecipitated with a C-terminal APP antibody (5865) and immunoblotted with an N-terminal APP antibody (Karen). sAPP(III) levels were assessed by immunoblotting with an end-specific polyclonal antibody (546). C99 was immunoprecipitated with NAB228, run on a 10/16.5% Tris-Tricine gel, and immunoblotted with NAB61. β-tubulin was immunoblotted with TUB2.1. B, Aβ amyloid pathology in NAB61 immunized mice. Serial sections from Tg2576 mice treated with either nonspecific IgG or NAB61 were subject to immunohistochemistry using either NAB228 (left) or NAB61 (right) as primary antibodies, showing that short term NAB61 immunization did not clear amyloid deposits. C, quantification of Aβ accumulation in NAB61 immunized mice. Cortical and hippocampal regions were subject to sequential extraction with RIPA and then 70% formic acid (FA). Aβ concentrations were measured by sandwich ELISA specific for Aβ42 and Aβ40. The short NAB61 immunization protocol did not alter levels of Aβ accumulation. D, peripheral pools of Aβ in NAB61 immunized mice. Plasma samples from Tg2576 mice treated with either nonspecific IgG or NAB61 were subject to immunoprecipitation and immunoblotting with 4G8, demonstrating that NAB61 immunization does not result in the peripheral accumulation of Aβ. Although Aβ oligomers (1 ng of untreated Aβ and 1 ng of UV-cross-linked Aβ) could be detected when spiked into plasma from non-transgenic mice, no Aβ oligomers could be detected in plasma from Tg2576 mice. wt, wild type.

TABLE 1

|            | Aβ40 | Aβ42 |
|------------|------|------|
|            | IgG  | NAB61 | p value |
|            | pmol/g | pmol/g |       |
| RIPA       |       |       |       |
| Cortex     | 25.3 ± 1.5 | 23.9 ± 0.9 | 0.43  |
| Hipp       | 24.4 ± 1.0 | 22.8 ± 0.8 | 0.22  |
| FA         |       |       |       |
| Cortex     | 2830.7 ± 529.7 | 3170.8 ± 531.8 | 0.66  |
| Hipp       | 1449.5 ± 221.8 | 1039.5 ± 172.2 | 0.16  |

* Aβ levels were quantified using IFA-m266 sandwich ELISA, and are presented as average values ± standard error.

Statistical analysis using two-tailed t-tests showed no significant differences between treatment groups.

(7, 8). Treatment using anti-Aβ antibodies has been proposed as a safer alternative to active immunization since immunotherapy-induced meningoencephalitis appears to be due to the activation of autoreactive T cells and does not correlate with the presence of anti-Aβ antibodies (8). Furthermore, Aβ oligomer-selective antibodies are decreased in AD patients, suggesting that replacement therapy with such antibodies may be appropriate (27). A priori, the selectivity of NAB61 for pathologic forms of Aβ, in addition to the lack of cross-reactivity with APP or C99, indicated that NAB61 may be a safer alternative for therapy when compared with other monoclonal anti-Aβ antibodies. However, we have reported one case of meningoencephalitis after peripheral immunization of a 19-month-old Tg2576 mouse with NAB61 (28). This isolated case was not part of the behavioral cohort presented here, and the mechanism triggering Aβ vaccine-related meningoencephalitis is still unknown. Nonetheless, current passive immunization trials should continue with careful regard toward unwanted complications. Additionally, the low titer of NAB61 by solid-phase ELISA indicated that the measurement of antibody response in actively immunized individuals by ELISA methods may lead to false negative results.

Although we have shown that NAB61 treatment improves spatial learning and memory in 17–19-month-old Tg2576 mice through the measurement of three different behavioral indices, NAB61 has been ineffective thus far in clearing Aβ pathology even in Tg2576 mice treated for 6 months. The successful clearance of amyloid pathology by passive immunization has been reported in PDAPP mice and very old (>16 months) Tg2576 mice (2, 29–32), both of which are characterized by the presence of considerable amounts of diffuse Aβ. However, our long term passive immunization trial was performed in younger Tg2576 mice (<14 months) in which compact amyloid deposits predominate, perhaps accounting for the lack of effect on the burden of Aβ pathology following the schedule used here. Alternatively, the Aβ oligomers recognized by NAB61 may not be easily cleared by immune-mediated mechanisms. NAB61 is also an IgG1, that has been shown to be less effective in reducing Aβ amyloid burden in transgenic mice (33).
Indeed, distinct morphological subtypes of Aβ amyloid plaques are differentially cleared depending on the anti-Aβ antibody isotype (34). Thus, NAB61 may exert its behavioral effect by blocking the biological activity of pathologic Aβ oligomers, and at the same time, being ineffective in promoting the immune-mediated clearance of Aβ.

Finally, soluble oligomeric forms of Aβ have been postulated to contribute to the onset of AD, and they may affect neuronal function initially by impairing synaptic function (35–39). Furthermore, although insoluble amyloid plaques are found very early in the disease process in patients with early AD or mild cognitive impairment, soluble Aβ levels are also increased in these individuals, and soluble Aβ levels correlate better with neurofibrillary degeneration and the loss of synaptic markers than do amyloid plaques (40–42). Similarly, decreases in synaptophysin immunoreactivity and impairments in synaptic transmission in APP transgenic mice precede the onset of microscopic Aβ amyloid pathology (43, 44). With mounting evidence for the synaptotoxic effects of soluble Aβ oligomers, our study corroborates previous reports indicating that immunization can ameliorate cognitive defects independent of APP processing and levels of insoluble Aβ (9, 10). Furthermore, since NAB61 recognizes a conformation found on oligomeric forms of Aβ, we have provided strong evidence that Aβ oligomers generated in situ disrupt neuronal function. Thus, we propose that targeting the pathologic conformation recognized by NAB61 may be useful in the treatment of AD and that further elucidation of the conformation recognized by NAB61 may yield insights into the mechanisms underlying the synaptotoxic effects of Aβ as well as assist in determining the roles of different conformational pools of Aβ to the development of dementia.

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