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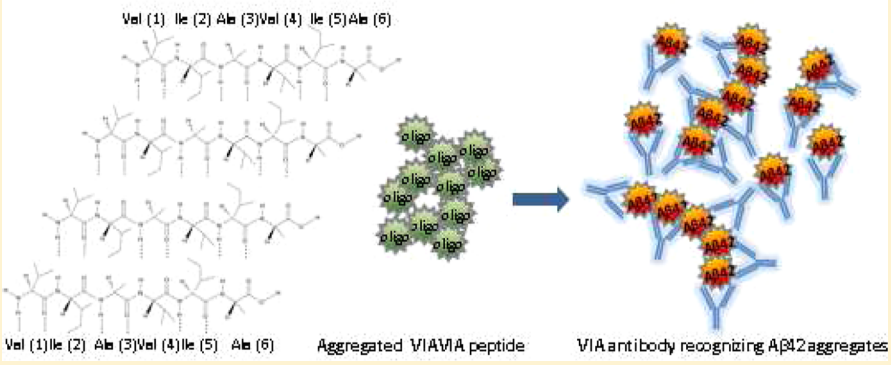
Antibody against Small Aggregated Peptide Specifically Recognizes Toxic Aβ-42 Oligomers in Alzheimer's Disease

Riddhi U. Bodani,[†] Urmi Sengupta,[†,‡] Diana L. Castillo-Carranza,[†,‡] Marcos J. Guerrero-Muñoz,[†,‡] Julia E. Gerson,[†,‡,∥] Jai Rudra,[§] and Rakez Kayed*[,†,‡,∥]

[†]Mitchell Center for Neurodegenerative Diseases, [‡]Departments of Neurology and Neuroscience and Cell Biology, [§]Department of Pharmacology and Toxicology, and [∥]Sealy Center for Vaccine Development, University of Texas Medical Branch, Galveston, Texas 77555, United States



ABSTRACT: Amyloid-beta (Aβ) oligomers have emerged as the most toxic species in Alzheimer's disease (AD) and other amyloid pathologies. Also, Aβ-42 peptide is more aggregation-prone compared to other Aβ isoforms. Thus, we synthesized a small peptide of repeated sequence containing the last three amino acids, Val-40, Ile-41, and Ala-42 of Aβ-42 that was subsequently aggregated and used to generate a novel antibody, VIA. In this study, we examined human AD and Tg2576 mouse brain samples using VIA in combination with other amyloid-specific antibodies and confirmed the specificity of VIA to oligomeric Aβ-42. Moreover, we found that VIA does not recognize classic amyloid plaques composed of fibrillar Aβ or Aβ-40 ex vivo. Since VIA recognizes a distinct epitope specific to Aβ-42 oligomers, it may have broad use for examining the accumulation of these oligomers in AD and other neurodegenerative diseases. VIA may also be used in immunotherapy studies to prevent neurodegenerative effects associated with Aβ-42 oligomers.

KEYWORDS: *Amyloid oligomers, repeated sequence, small peptide*

The presence of extracellular plaques composed of amyloid-beta (Aβ) protein and intracellular neurofibrillary tangles composed of tau protein pathologically characterizes Alzheimer's disease (AD), a progressive, irreversible neurodegenerative disorder. Aβ is a 39−42 amino acid peptide formed by the proteolytic cleavage of amyloid precursor protein (APP) by β and γ secretases. The two predominant isoforms of Aβ formed through this processing, Aβ-40 and Aβ-42, have very distinct properties even though they have high sequence similarity.[1] Aβ-42 has a much faster aggregation rate compared to Aβ-40 due to the two unique carboxy-terminal residues of Aβ-42, isoleucine and alanine, which promote Aβ-42 polymerization.[2,3] Furthermore, Aβ-40 primarily exists as a monomer, while Aβ-42 is in trimer/tetramer and monomer equilibrium.[4] It has also been shown that diffuse plaques are comprised primarily of Aβ-42, while mature plaques are comprised of Aβ-40, indicating that Aβ-42 is processed to Aβ-40 as plaques mature and that amyloid deposition begins with Aβ-42.[5] In addition, unlike Aβ-40, Aβ-42 has been shown to promote the phosphorylation and aggregation of tau in vivo.[6] These results support the idea that Aβ-42 is more aggregation-prone and the more pathogenic species compared to Aβ-40.

Furthermore, insoluble Aβ fibrils constituting classic plaques were thought to be responsible for the neurodegenerative changes associated with AD for many years.[7] However, growing evidence implicates soluble oligomers as the more toxic species, and the extent of oligomer formation and assembly correlates better with disease progression and cognitive dysfunction.[8,9] Aβ oligomers have been shown to bind membrane receptors, form pores in cell membranes, and form intracellular aggregates that lead to pathological events including mitochondrial dysfunction and proteasome impairment.[10] These Aβ oligomers are able to induce other aggregation-prone proteins, including α-synuclein (α-syn), prion protein (PrP), and TDP-43, to assume oligomeric conformations. These proteins can then seed tau aggregation, resulting in neurodegeneration.[11]

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Considering the propensity of Aβ-42 aggregation and the increasing evidence pointing to the toxicity of Aβ oligomers, we aimed to develop a conformation-dependent antibody, called VIA, against oligomeric Aβ-42. Here we determined that VIA is reactive to Aβ-42 aggregates and does not detect Aβ-40 oligomers, Aβ monomer or APP. The transgenic $APP_{K670L,M671N}$ Tg2576 mouse is one of the most extensively studied AD models and is characterized by the overproduction and deposition of Aβ protein.[12] Similar to other amyloid precursor protein (APP) models, the Tg2576 exhibits hyperphosphorylated tau, but it does not develop neurofibrillary tangles (NFTs).[13,14] In the present study, we established the specificity of the novel VIA antibody to Aβ-42 oligomers in vitro and ex vivo in human AD and Tg2576 mice, and laid the groundwork for an anti-Aβ-42 oligomer-specific immunotherapy approach for the treatment of AD progression.

RESULTS

In Vitro Specificity of VIA to Aβ-42 Oligomers. Specificity of VIA to Aβ-42 oligomers was determined by dot blot and Western blot analysis using prepared recombinant Aβ samples. Dot blot analysis using various amounts of Aβ oligomers indicated that VIA is specific for Aβ-42 oligomers (Figure 1A). Signal was obtained using 0.1 and 0.05 μg of oligomeric Aβ-42 protein sample, although no signal was detected using 0.001 μg of sample or same concentartions of Aβ-40 oligomers. As expected, 6E10 recognized both Aβ-40 and 42. Western blot analysis verified VIA specificity to Aβ-42 and indicated that VIA does not recognize monomeric Aβ-42, which has a weight of approximately 4.5 kDa. VIA recognized SDS-stable conformational variants of Aβ-42 of high molecular weight between 100 and 150 kDa (Figure 1B).

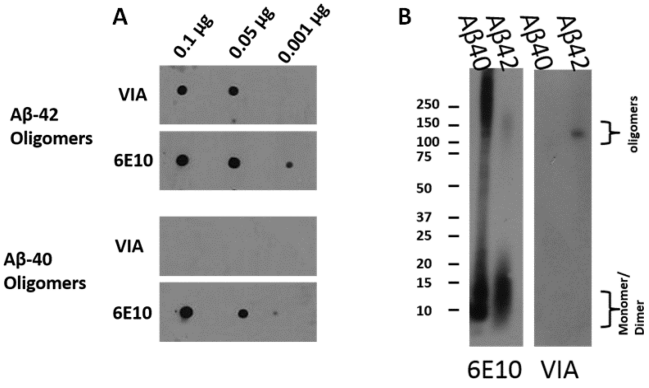

Figure 1. Specificity of anti-Aβ-42 oligomeric VIA antibody. Dot blot using VIA and 6E10 against varying concentrations of prepared recombinant Aβ-42 and 40 oligomers indicates that VIA is specific to Aβ-42 oligomers and does not recognize Aβ-40 (A). Western blot using VIA and 6E10 against Aβ-40 and 42 confirms VIA specificity and indicates that VIA does not recognize monomeric Aβ-42 (B).

In addition, SH-SY5Y cells were treated with Aβ-42 oligomers, Aβ-42 fibrils, and Aβ-42 oligomers incubated with VIA antibody to examine the toxicity of these preparations. Aβ-42 oligomers were much more toxic to cells with 66.9% of cells affected after treatment compared to Aβ-42 fibrils, with only 44.4% of cells affected (Figure 2A, B, and D). Moreover, Aβ-42 oligomers and VIA antibody complex induced less cell toxicity with 2.9% of cells affected, indicating that VIA binds to Aβ-42 oligomers and neutralizes their toxic effects (Figure 2C and 2D).

Ex Vivo Specificity of VIA to Aβ-42 Oligomers. Immunohistochemistry and double immunofluorescence labeling using VIA in combination with other amyloid antibodies were used to determine VIA specificity in human AD brain samples. Immunohistochemical analysis with VIA staining revealed significant perinuclear aggregates of Aβ-42 in the frontal cortex and hippocampus of these brain samples (Figure 3B, C). Staining with VIA and amyloid-specific 6E10 antibodies indicated that VIA does not recognize classic amyloid plaques (Figure 3I). Furthermore, VIA-positive oligomers showed some colocalization with 6E10 in certain regions of the frontal cortex, suggesting that the 6E10 epitope is exposed in these oligomers (Figure 4I). Also, significant colocalization was observed between VIA and anti-Aβ-42 antibody, confirming VIA specificity to aggregates of Aβ-42 rather than Aβ-40 (Figure 3O).

Previous studies have suggested that at least two different types of amyloid oligomers with distinct conformations exist: prefibrillar or Type 1 oligomers that are reactive to A11 antibody, and fibrillar or Type 2 oligomers that are reactive to OC antibody which specifically identifies amyloid fibrils.[15−17] Fibrillar oligomers have a parallel β-sheet structure similar to that present in amyloid plaques while prefibrillar oligomers have a unique structure distinct from that of fibrillar oligomers.[15,16] To determine VIA immunoreactivity to these different classes of Aβ oligomers, control and human AD brains were stained with VIA and A11 or OC. Significant colocalization was observed between VIA and A11 in the frontal cortex of human AD brains, while minimal VIA and A11-positive signal was observed in age-matched controls, suggesting that VIA recognizes Aβ-42 prefibrillar aggregates in these diseased brains (Figure 4C and F). Double staining with VIA and OC antibodies indicated that OC-positive amyloid plaques are not recognized by VIA (Figure 4I and L). However, colocalization was observed between VIA and OC in microdeposits comprised of fibrillar oligomers that are OC-positive, indicating that VIA recognizes Aβ-42 fibrillar oligomers (white arrows). Thus, VIA recognizes both prefibrillar and fibrillar Aβ-42 oligomers.

In addition, AD brains and age-matched control brains were stained with VIA and two neuroinflammmatory markers, GFAP, a marker for activated astrocytes and Iba1, a marker for activated microglia. Colocalization was found between VIA and GFAP (Figure 5F) and Iba1 (Figure 5L) in AD brains. This observation is in accordance with previous studies suggesting the role of Aβ oligomers in microglia and astrocyte activated inflammation. Therefore, our results indicate that VIA recognizes pathologically toxic Aβ oligomers that are associated with inflammation in AD brains.

To determine VIA specificity to Aβ-42 oligomers in Tg2576 mice, coronal sections of 6, 10, and 14 month-old Tg2576 mice were stained with VIA and amyloid-specific 4G8 antibodies. Microdeposits of Aβ-42 oligomers were found in the coronal sections of 6, 10, and 14 month-old Tg2576 mice (Figure 6B and 6D) but were not observed in C57BL/6 mice (Figure 6A). Significant colocalization was observed between VIA and 4G8 in Tg2576 mice, suggesting that the 4G8 epitope is exposed in these oligomers. Furthermore, classic amyloid plaques observed in 14-month-old Tg2576 mice were negative for VIA, confirming that VIA does not recognize Aβ fibrils, although VIA-positive oligomeric Aβ-42 deposits were found in the vicinity of the plaques (Figure 6D). Furthermore, to confirm VIA specificity to Aβ-42 in Tg2576 mice, brain sections of 14-month-old Tg2576 mice were stained with VIA and an anti-Aβ-

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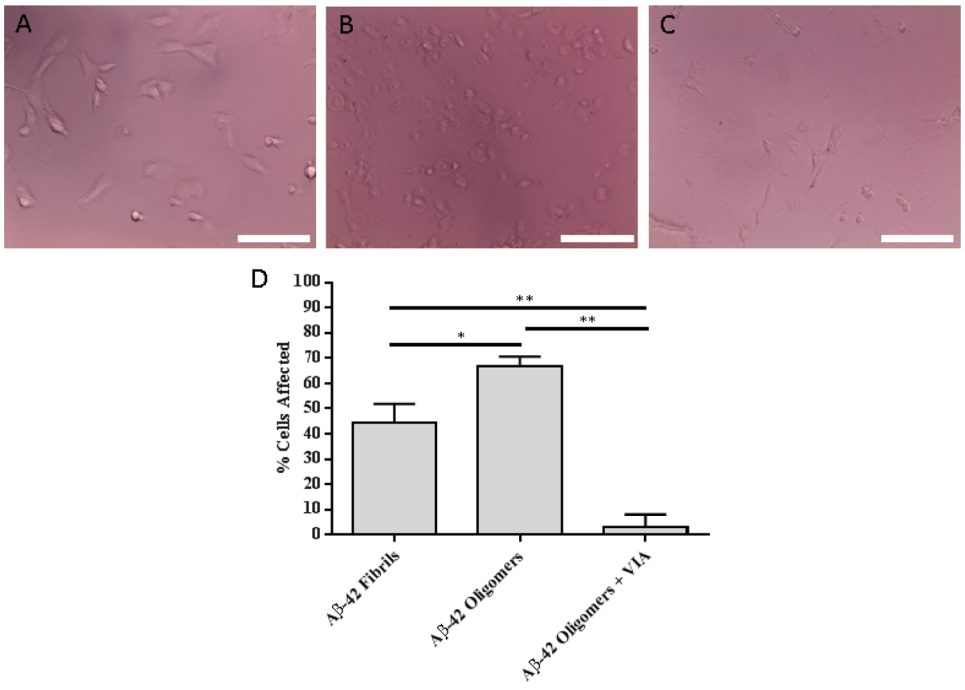

Figure 2. Toxicity assay of SH-SY5Y cells treated with preparations of Aβ-42. Images of cells treated with 2 μM Aβ-42 fibrils, Aβ-42 oligomers, and Aβ-42 oligomers with VIA (A, B, and C respectively). Percentage of cells affected with each sample is shown in (D). Aβ-42 oligomers were more toxic to cells compared to Aβ-42 fibrils and Aβ-42 oligomers alone were more toxic than oligomers blocked with VIA. Each group of treatment was performed in triplicate (n = 3). Bars and error bars represent means and standard deviations, respectively (*p < 0.05, **p < 0.01). Scale bar = 50 μm.

40 antibody. VIA showed no colocalization with Aβ-40 and did not recognize Aβ-40 plaques (Figure 6G).

To examine the localization of Aβ-42 oligomers specifically in the hippocampus of Tg2576 mice, the hippocampi of 10 and 14 month old Tg2576 and control 12 month old wild-type C57BL/6 mice were stained with VIA. Significant VIA immunoreactivity was observed in the CA1 region of the hippocampus of Tg2576 mice (Figure 7B and C), while no VIA immunoreactivity was detected in the CA1 region of C57BL/6 mice (Figure 7A). Furthermore, the hippocampi of 10 and 14 month old Tg2576 mice were stained with VIA and 4G8 or OC. Microdeposits of Aβ-42 oligomers positive for VIA and 4G8 were found in the CA1 region of the hippocampus in Tg2576 mice (Figure 7E and F) while 4G8-positive amyloid plaques seen in the hippocampus were VIA-negative (Figure 7G). Also, microdeposits positive for both VIA and OC were observed in the hippocampus of Tg2576 mice (Figure 7I and J). These VIA and OC-positive deposits were found in the vicinity of OC-positive amyloid plaques in 14 month old mice, although the plaques were not recognized by VIA (Figure 7J). The VIA and OC-positive deposits are most likely composed of fibrillar oligomers since these oligomers are OC-positive.[15] Thus, our results suggest that VIA is able to recognize Aβ-42 fibrillar oligomers in Tg2576 mice.

DISCUSSION

Amyloid peptides and proteins adopt various distinct assembly states that differ in their degree of pathogenicity. Conformation-dependent antibodies that are specific for different assembly states can provide insight into their unique characteristics and significance in disease. In this study, we developed a novel conformation-dependent polyclonal antibody called VIA that successfully recognizes recombinant preparations of Aβ-42 oligomers and is specific to Aβ-42 oligomers in human AD brains and Tg2576 mice. Furthermore, VIA does not recognize inert amyloid plaques and is not reactive to Aβ-40 in human AD brains and Tg2576 mice. Since VIA recognizes a distinct epitope specific to toxic Aβ-42 oligomers, it may have broad applications for detecting and studying the accumulation of Aβ-42 oligomers in AD and other neurodegenerative diseases.

Amyloid oligomers have been previously classified into two distinct categories based on their unique conformations. Prefibrillar or Type 1 amyloid oligomers are A11-reactive and have been suggested to spread throughout different regions of the brain causing neural dysfunction and leading to cognitive deficits.[15,17] Fibrillar or Type 2 oligomers are OC-reactive and have a parallel β-sheet structure similar to amyloid plaques.[15,17] It has been suggested that fibrillar oligomers seed the formation of amyloid plaques and that these plaques subsequently are involved in the formation of microdeposits of fibrillar oligomers.[17] These oligomers are confined in the vicinity of amyloid plaques and are thus considered less toxic compared to prefibrillar oligomers, which have been shown to spread.[17] Our results are consistent with these previous findings. VIA and OC staining in mice showed significant deposits of Aβ-42 fibrillar oligomers in the vicinity of amyloid plaques in human AD and Tg2576 mice. Furthermore, we showed that VIA is specific to Aβ-42 species of both oligomer classes, and thus may be used in conjunction with various antibodies to further study these oligomeric species.

Also, neuroinflammation is often associated with AD pathology contributed by microglial cells and astrocytes in the brain. Activated microglia and accumulation of astrocytes are found surrounding Aβ deposits.[18] VIA-positive Aβ-42 oligomers were also found associated with astrocytes and microglia, indicating that these oligomers may play a role in inflammation in AD. Astrocytes most likely take up Aβ oligomers, which are later formed into Aβ annular protofibrils (APFs). APFs cause harmful metabolic changes within astrocytes and thus

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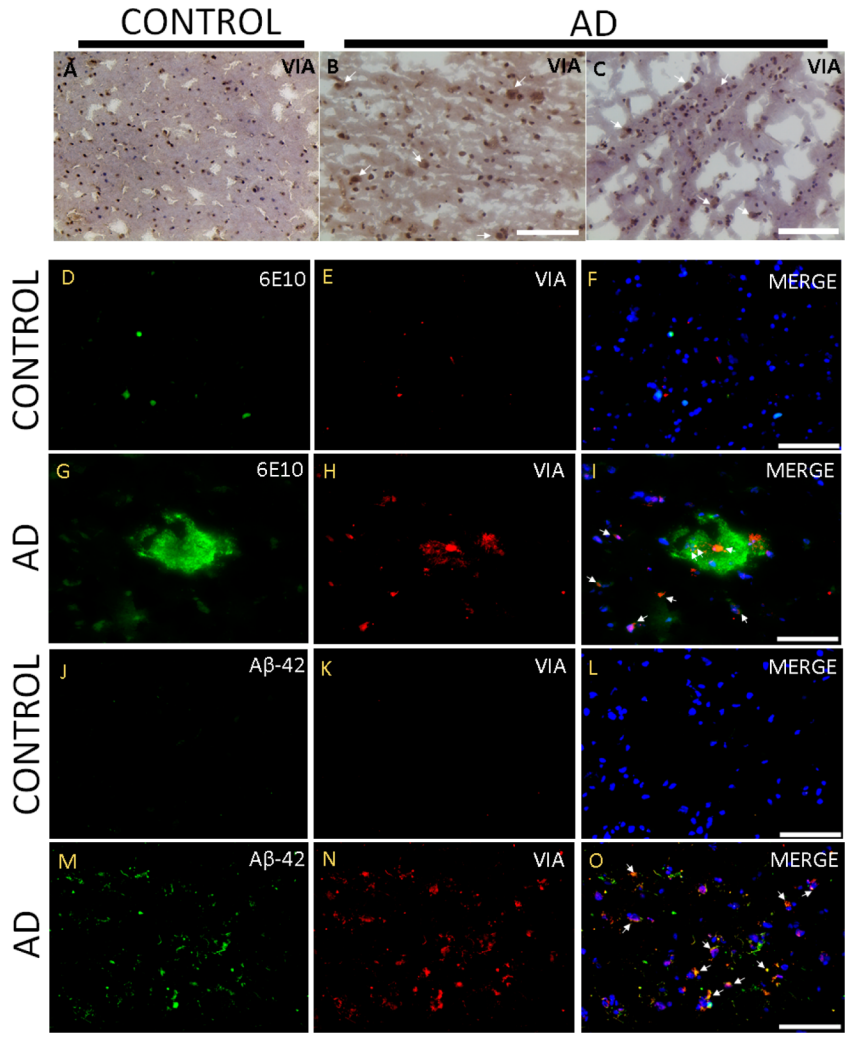

Figure 3. VIA recognizes intracellular perinuclear aggregates of Aβ-42 and does not recognize amyloid plaques in human AD brains. Single-labeling, bright-field images with antioligomeric Aβ-42 antibody VIA in control (A), frontal cortex, and hippocampus of AD brains (B and C respectively). Epifluorescence double immunofluorescence images (D−O) of AD frontal cortex and control brain sections labeled with VIA (E, H, K, and N), 6E10 (D and G), and anti-Aβ-42 antibody (J and M). Some colocalization was observed between 6E10 and VIA in AD brains, and VIA did not recognize classic amyloid plaques (I). Significant colocalization was observed between VIA and anti-Aβ-42 antibody in AD brains (O). Minimal immunoreactivity was detected with VIA antibody (E and K) in control brain samples. Scale bar = 50 μm.

compromise neuronal health.[19] Moreover, previous studies have shown that Aβ oligomers stimulate primary microglia and that activated human microglia increase the expression of Aβ-42 in neuroblastoma cells.[20,21] Our observations and these previous results suggest that Aβ-42 oligomers activate astrocytes and microglia, which release inflammatory markers that increase the formation of Aβ-42 oligomers, promoting neurodegeneration. Injured neurons further activate astrocytes and microglia resulting in a feed-forward cycle of inflammation and cell death contributing to AD.[22] However, though accumulation of intraneuronal Aβ-42 has been found in human AD brain prior to plaque deposition,[23,24] further experiments will be required to localize the cell-specific distribution of VIA-immunoreactive Aβ-42 aggregates in these brain tissues. Also microglial cells and astrocytes have been shown to possess the capability to internalize Aβ deposits,[25,26] therefore, it will be of great siginificance to study whether these aggregates exist inside or outside the cell.

Our results suggest that Aβ-42 oligomers are a viable therapeutic target for AD. Aβ is considered to be critical since it lies upstream of tau pathology.[27] Furthermore, it has been well-established that aggregated Aβ induces tau phosphorylation, mislocalization, and aggregation in animal models and cell cultures.[28−32] In addition, Aβ oligomers seed tau oligomerization, an important event in AD pathogenesis since tau oligomers cause mitochondrial and synaptic dysfunction.[33,34]

The results of this study indicate that the novel VIA antibody may be used successfully in future passive immunotherapy studies to target Aβ-42 oligomers in Tg2576 mice. The same aggregated antigen can be used to generate Aβ-42 oligomer-specific mouse monoclonal antibodies as described previously.[35] VIA was shown to be specific to aggregated Aβ-42 in human AD brains and Tg2576 mice in this study. Since VIA does not recognize classic amyloid plaques, a passive immunotherapy approach using VIA can prevent plaque disruption and potential side effects, including inflammation.[36] Furthermore, since VIA is specific to Aβ-42 and does not recognize Aβ-40, which is associated with vascular amyloid, a VIA-based immunotherapy approach would lower the risk hemorrhagic stroke.[37] Although previous Aβ active and passive immunotherapy approaches for

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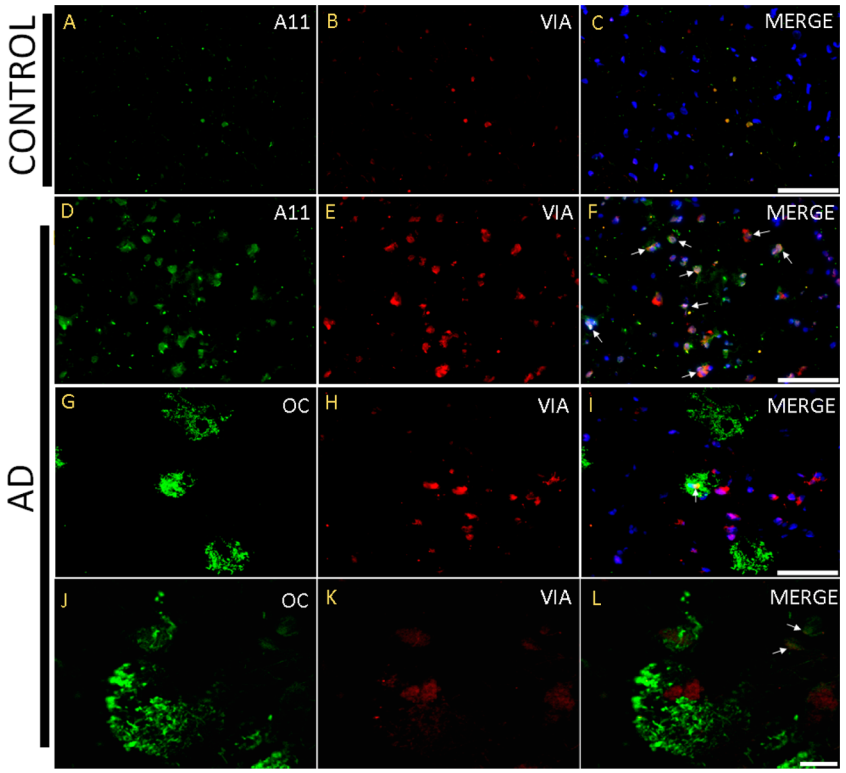

Figure 4. VIA recognizes both prefibrillar and fibrillar Aβ-42 oligomers in human AD brains. Epifluorescence double immunofluorescence images (A–I) and confocal images (J-L) of AD frontal cortex and control brain sections labeled with VIA (B, E, H, and K), A11 (A and D), and OC (G and J). Significant colocalization was observed between A11 and VIA in AD brains. Colocalization was observed between OC and VIA in microdeposits in AD brains (I); however, VIA did not recognizes OC-positive amyloid plaques (I and L). Significant minimal immunoreactivity was detected with VIA antibody (B) in control brain samples. Scale bar = 50 μm (C, F, and I). Scale bar = 10 μm (L).

the treatment of AD have shown mixed results in clinical trials, we believe that targeting a highly specific aggregation or assembly state of Aβ, in this case, Aβ-42 oligomers, rather than all forms of Aβ, may be a more beneficial approach.[38,39]

METHODS

Oligomer Preparation. Lyophilized peptides of Aβ-40 and Aβ-42 were resuspended in 50% acetonitrile/water mixture and relyophilized. Oligomers were prepared by dissolving 0.3 mg of peptide in 250 μL of hexafluoroisopropanol (HFIP) and incubating for 10–20 min at room temperature as described previously.[40] Then 200 μL of the resulting solution was added to 700 μL of double-distilled H_2O (dd H_2O) in a siliconized Eppendorf tube. The samples were then stirred at 500 rpm using a Teflon-coated microstir bar for 24–48 h at room temperature (RT). Tube caps contained holes to allow slow evaporation of HFIP.

Generation of Antigen and Oligomer-Specific VIA Antibody. The amino acid sequence VIA (valine, isoleucine, and alanine) represents the last three amino acids of Aβ-42. Aggregated Aβ-40 and Aβ-42 share similar β-sheet structural architecture. The last two amino acids, Ile-41 and Ala-42 in Aβ-42 peptide are exposed to the solvent,[41] thus representing a potential binding site for antibodies targeted to its aggregated form. Although the β-sheet exhibits parallel in-register organization, some structural studies have shown that it has a tendency to shift forming different polymorphisms.[42,43] To account for this potential shift, we have extended our antigen from Ala-42 and Ile-41 to Val-40. The aggregation of single and triplicate sequences (VIA and VIAVIAVIA, respectively) were not reproducible. Moreover, these aggregates produced low immunogenicity. Therefore, to obtain maximum immunogenicity and appropriate peptide length for aggregation, we used repeated sequence VIAVIA (Figure 8). Likewise, we tested various combinations of different amino acid sequences among which VIAVIA sequence produced consistent aggregates that were immunogenic. This small peptide was synthesized, subsequently aggregated and used as the antigen to generate antibody by immunizing New Zealand white rabbits (Pacific Immunology Corp. Ramona, CA). Antibody was purified from the sera obtained from these immunized animals using affinity chromatography.

Immunoblotting. Protein samples (recombinant amyloid oligomers) were electrophoresed on NuPAGE 4–12% Bis-Tris gels (Invitrogen). Samples were transferred onto nitrocellulose membranes, which were subsequently blocked overnight at 4 °C using 10% nonfat dry milk in 1× TBST buffer and then washed once for 5 min with 1× TBST buffer. Membranes were incubated with VIA serum or purified polyclonal antibody (1:50) and 6E10 antibody (1:1000, Covance) in 5% milk-TBST for 1 h at RT. Following washing, the membranes were then incubated with corresponding antirabbit or antimouse secondary antibodies conjugated with horseradish peroxidase (1:3000, GE Healthcare) at RT for 1 h. Membranes were washed several times and finally developed with Super Signal West Pico chemiluminescence reagents (Thermo Scientific). For dot blots, 0.3–1.2 μL of sample was spotted, blocked, and immunolabeled as described above for Western blotting.

Toxicity Assay. SH-SY5Y neuroblastoma cells were maintained in DMEM with 10 mM HEPES, 10% fetal bovine serum, 4 mM glutamine, penicillin (200 U/mL), and streptomycin (200 μg/mL) in 5% CO_2 at 37 °C. The medium was replaced every 2 days. Cells were differentiated in serum-free DMEM with N2 supplement and 1× 10^{-5} M all-*trans* retinoic acid before use. Cells were plated at 10 000 cells/well in 96-well plates and grown overnight. The medium was removed, and 2 μM of Aβ-42 oligomers, fibrils, and oligomers incubated with VIA antibody (2:1 ratio of antibody to oligomers) were added in 80 μL of fresh medium. After incubation for 90 min at 37 °C, cells were subjected to MTT colotimetric assay using Cell Proliferation I (MTT) assay kits (Roche) according to the manufacturer's directions. Each data point was determined in triplicate.

Human and Mouse Brain Selection. Frozen brain tissue from AD patients and age-matched controls was obtained from the

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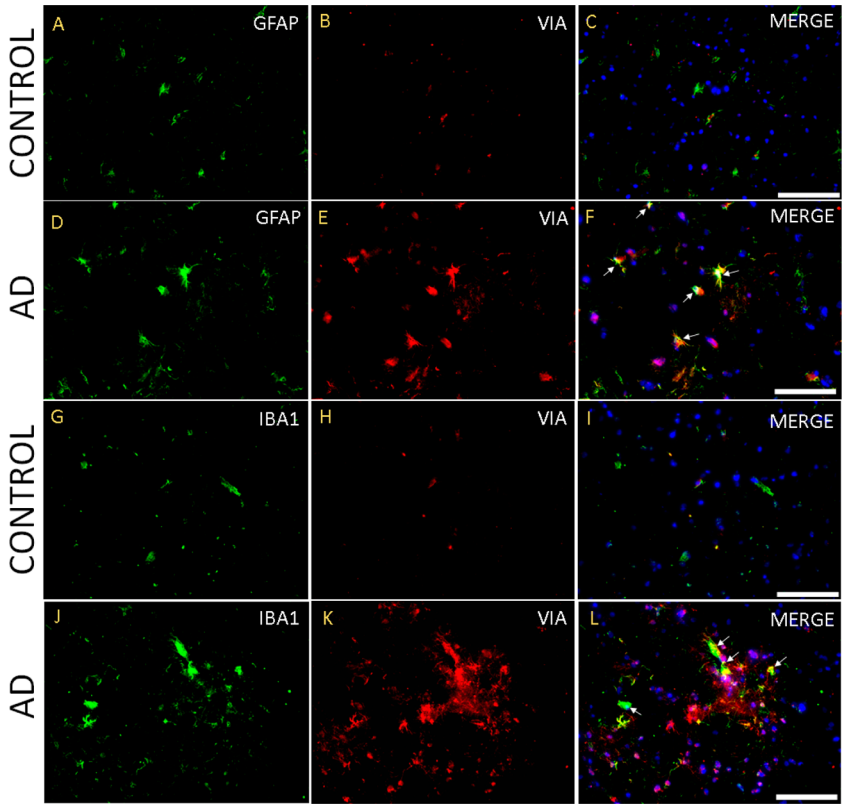

Figure 5. VIA recognizes intracellular aggregates of Aβ-42 in activated astrocytes and microglia in the frontal cortex of human AD brains. Epifluorescence microscopy images of AD and control brain sections labeled with antioligomeric Aβ-42 antibody VIA (B, E, H, and K), GFAP (A and D), and Iba1 (G and J). Significant colocalization was observed between VIA and GFAP (F) and Iba1 (L) in AD brains. Minimal immunoreactivity was detected with VIA antibody in age-matched control brain samples (B and H). Scale bar = 50 μm.

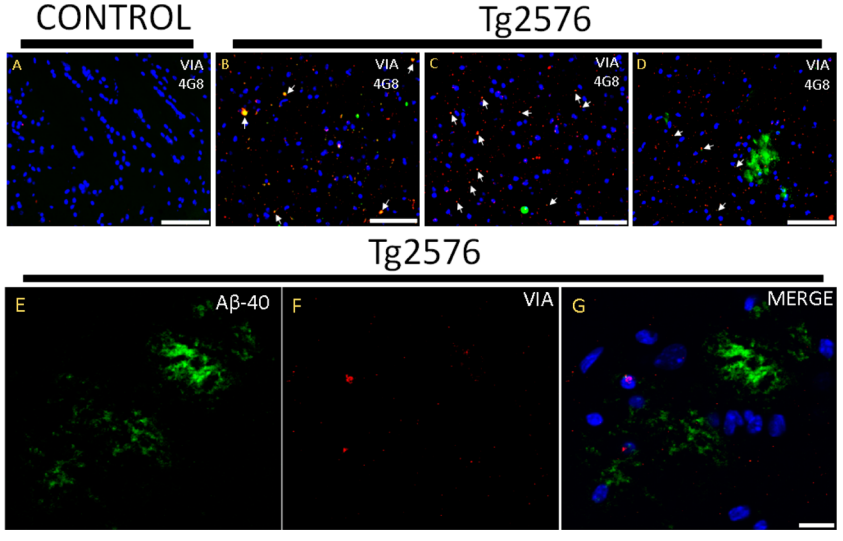

Figure 6. VIA recognizes Aβ-42 oligomers but not amyloid plaques in coronal sections of Tg2576 mice. Aβ-42 aggregates were seen in sections of 6, 10, and 14 month-old Tg2576 mice (B, C, and D, respectively) stained with VIA (red) and 4G8 (green). VIA-negative amyloid plaques were observed at 14 months (D). No VIA immunoreactivity was detected in control C57BL/6 mice (A). Confocal microscopy images of 14 month Tg2576 brain sections labeled with Aβ-40 (E) and VIA (F). No colocalization was observed between Aβ-40 and VIA (G). Scale bar = 50 μm (A–D). Scale = 10 μm (G).

University of California at Irvine Alzheimer's Disease Research Center (UCI-ADRC). Sections of frontal cortex and hippocampus samples from moderate-to-severe Braak stage AD cases and nondemented elderly controls were used in this study.

Sections that included the hippocampus from Tg2576 mice at 6, 10, and 14 months of age that were bred at University of Texas Medical Branch (UTMB) were compared with 12 month old C57BL/6 wild-type mice (stock # 1638M; Taconic).

Immunohistochemistry. For single-labeling, bright-field immunohistochemistry, all frozen sections were fixed in chilled methanol and treated with 3% hydrogen peroxide in 1× PBS to quench endogenous peroxidase. Sections were subsequently washed twice for 5 min each

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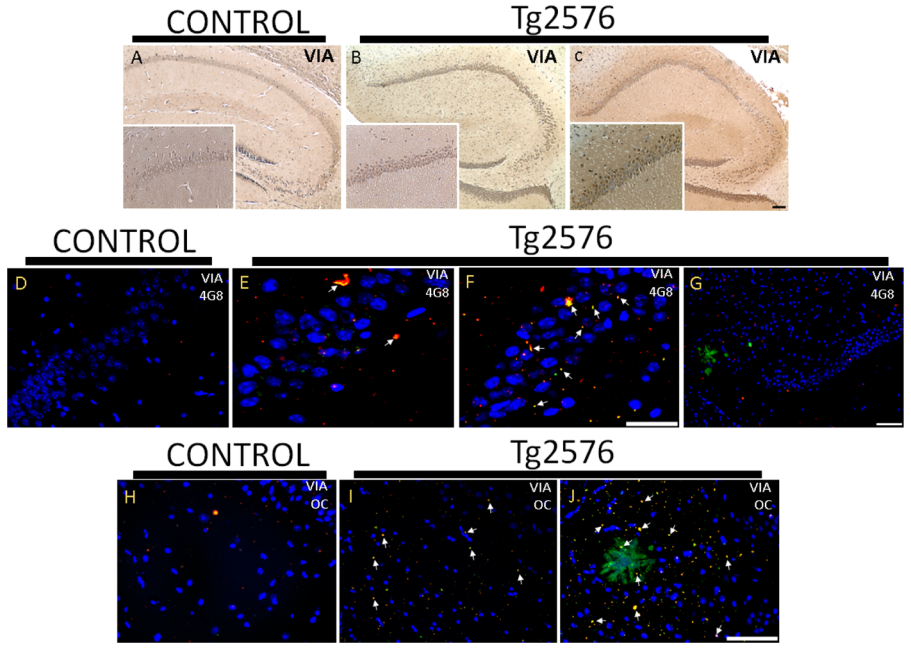

Figure 7. VIA recognizes Aβ-42 oligomers in the hippocampus of 10 and 14 month old Tg2576 mice. Bright-field images emphasizing the CA1 region of the hippocampus labeled with VIA antibody in control wild-type C57BL/6 (A), 10 month old Tg2576 (B), and 14 month old Tg2576 mice (C). Epifluorescence microscopy double fluorescence images of control wild-type C57BL/6 (D and H), 10 month Tg2576 (E and I), and 14 month Tg2576 mice (F–G, and J) stained with VIA (red, D–J) and 4G8 (green, D–G) or OC (green, H–J). Note image (G) is a less magnified view (20× image) of the same brain section shown in (F) and shows a VIA-negative plaque at the end of the CA1 region. VIA does not recognize inert plaques observed in 14 month old mice (G and J). Scale bar = 50 μm.

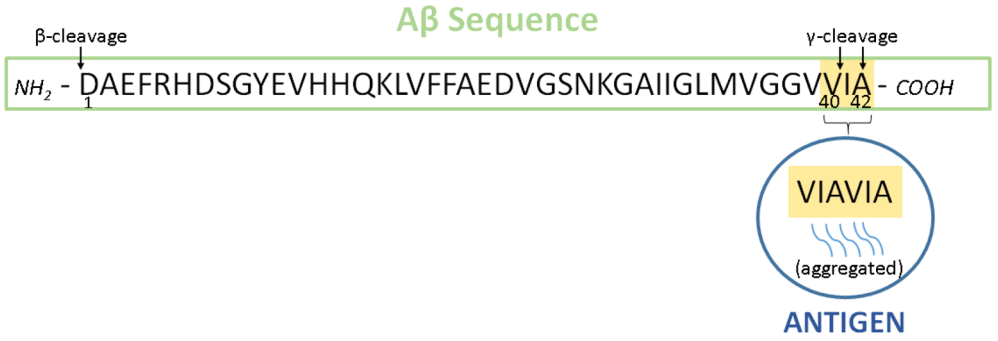

Figure 8. Schematic representation of antigen design used to generate an Aβ-42 oligomer-specific antibody. The amino acid sequence VIA represent the last three amino acids of Aβ-42. Thus, a repeated sequence of six amino acids, VIAVIA was synthesized and subsequently aggregated to produce a highly specific immunogenic antigen. This aggregated VIAVIA antigen was used to produce antibody specific for aggregated Aβ-42.

with 1× PBS and then blocked with 5% goat serum in PBS for 60 min at RT. Sections were incubated with VIA serum (1:150) overnight at 4 °C. The next day, sections were washed three times with 1× PBS and were incubated with goat anti-rabbit biotinylated anti-IgG and then with avidin–biotin complex (ABC) (Vectastain ABC kit, Vector Laboratories). This was followed by hematoxylin staining for nuclei.

Paraffin sections were first deparaffinized and rehydrated using xylene, 100% ethanol, 95% ethanol, 80% ethanol, and distilled water. The sections were then treated for retrieving antigenic sites. Subsequent hydrogen peroxide treatment, blocking, and antibody labeling were conducted as described above.

Double Immunofluorescence. A series of immunofluorescence experiments were conducted using VIA antibody in combination with various antibodies. Frozen brain tissue was fixed in chilled methanol followed by blocking with 5% goat serum in 1× PBS for 1 h at RT. Sections were incubated with VIA serum (1:150) overnight at 4 °C. After washing three times with 1× PBS, sections were incubated with a secondary goat anti-rabbit IgG antibody (Alexa-Fluor 568 [1:350]) for 1 h at RT for visualization. Sections were washed three times for 10 min each and subsequently incubated with a second primary antibody overnight at 4 °C. The amyloid-specific antibodies used were 6E10 (1:300, Covance) which recognizes amino acids 1–16 of Aβ, 4G8 (1:200, Covance) which is specific to 17–24 of Aβ, OC (1:300) which is specific for fibrillar Aβ, A11 (1:500) which recognizes prefibrillar Aβ oligomers, anti Aβ-42 (1:200, Abcam), and anti Aβ-40 (1:500, Covance). Anti-GFAP (1:200) and anti-Iba1 (1:100) were also used to detect activated astrocytes or microglia, respectively. After washing three times, sections were incubated with the appropriate species-specific secondary antibody for 1 h at RT. Sections were washed three times to remove unbound antibodies. Nuclei were stained with DAPI in 1× PBS (4′,6′-diamidino-2-phenylindole; [1:3000], Molecular Probes) and subsequently washed with 1× PBS and mounted (Fluoromount G, Southern Biotech).

Paraffin sections were first deparaffinized and rehydrated using xylene, 100% ethanol, 95% ethanol, 80% ethanol, and distilled water. The sections were treated for antigen retrieval followed by washing. Subsequent blocking and antibody labeling were conducted as described.

Image Collection. Images were collected using an epifluorescence microscope (Nikon Eclipse 800) equipped with a CoolSnap-FX

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monochrome CCD camera (Photometrics) using standard Nikon fluorescein isothiocyanate (FITC), Texas Red, and DAPI filters. Image acquisition and analysis were performed using cellSens software (Olympus Life Science).

Images were also collected using a Zeiss LSM-510 Meta confocal microscope with a 63 × 1.20 numerical aperture water immersion objective (UTMB Optical Microscopy Core). Images were sequentially obtained using three different excitation lines (364, 488, and 543). After excitation with 364, 488, and 543 lasers, line emissions were collected with 385−470, 505−530, and 560−615 nm filters, respectively. An 8-frame Kallman averaging with a pixel time of 2.51 μs and a pixel size of 1.60 nm was used to collect images.

Statistical Analysis. Statistical analysis on the data was performed by Excel (Microsoft). Significant p value was set to <0.05. Bars and error bars represent means and standard deviations, respectively.

AUTHOR INFORMATION

Corresponding Author

*Mailing address: Mitchell Center for Neurodegenerative Disorders, Department of Neurology, 301 University Blvd, Route 1045, Galveston, TX 77555-1045. Phone: (409) 772-0138. Fax: (409) 747-0015. E-mail: rakayed@utmb.edu.

Author Contributions

R.K. conceived and planned the project. R.K., J.R., and M.J.M. performed peptide synthesis and purification. R.K and U.S performed oligomerization and preparation of the antigen. R.U.B. and U.S. performed the in vitro and IHC experiments. R.U.B. performed the fluorescence staining experiments. D.L.C. performed the confocal imaging. R.U.B., U.S., D.L.C., J.E.G., and R.K. analyzed the results. R.U.B. prepared the figures. R.U.B., R.K., U.S., and J.E.G. wrote the manuscript.

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Notes

The authors declare no competing financial interest.

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