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Specific Targeting of Tau Oligomers in Htau Mice Prevents Cognitive Impairment and Tau Toxicity Following Injection with Brain-Derived Tau Oligomeric Seeds

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Abstract. Neurodegenerative disease is one of the greatest health crises in the world and as life expectancy rises, the number of people affected will continue to increase. The most common neurodegenerative disease, Alzheimer's disease, is a tauopathy, characterized by the presence of aggregated tau, namely in the form of neurofibrillary tangles. Historically, neurofibrillary tangles have been considered the main tau species of interest in Alzheimer's disease; however, we and others have shown that tau oligomers may be the most toxic form and the species responsible for the spread of pathology. We developed a novel anti-tau oligomer-specific mouse monoclonal antibody (TOMA) and investigated the potential of anti-tau oligomer passive immunization in preventing the toxicity of tau pathology in Htau mice. We injected pure brain-derived tau oligomers intracerebrally in 3-month-old wild-type and Htau mice and investigated the protective effects of a single 60 μg TOMA injection when compared to the same dose of non-specific IgG and found that TOMA conferred protection against the accumulation of tau oligomers and cognitive deficits for up to 1 month after treatment. Additionally, we injected pure brain-derived tau oligomers intracerebrally in 3-month-old wild-type and Htau mice and treated animals with biweekly injections of 60 μg TOMA or non-specific IgG. We found that long-term administration of TOMA was effective as a preventative therapy, inhibiting oligomeric tau and preserving memory function. These results support the critical role of oligomeric tau in disease progression and validate tau oligomers as a potential drug target.

Keywords: Alzheimer's disease, immunotherapy, propagation, seeding, tau oligomers, tauopathies

INTRODUCTION

Neurodegenerative disease is one of the greatest health crises in the world today and as life expectancy rises, the number of people affected will continue to increase. The two most common neurodegenerative diseases, Alzheimer's disease (AD) and Parkinson's disease, can be classified as tauopathies, which also

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include a number of less common diseases, such as progressive supranuclear palsy and frontotemporal dementia. Tauopathies are characterized by the presence of aggregated tau, namely in the form of neurofibrillary tangles (NFTs). In its monomeric form, tau is a critical mediator of microtubule stability. However, in disease tau becomes misfolded and forms small aggregates, known as tau oligomers, possibly en route to the formation of NFTs. Historically, NFTs have been considered the main tau species of interest in pathology; however, recently it has come to light that tau oligomers may be the most toxic form of tau and the species responsible for the spread of pathology. While NFTs have been shown to correlate with severity of disease in postmortem human AD brains, neuron loss far exceeds NFT load [1, 2] and begins to occur prior to tangle formation in humans and in mouse models [3–5]. Moreover, in AD, hippocampal neurons can survive for up to 20 years with NFTs [6]. Additionally, in animal models, disease phenotypes are not associated with NFT levels [7–15], including in mice overexpressing human tau (Htau mice) [16, 17]. Therefore, it seems likely that intermediate species may underlie toxicity.

Tau oligomer levels have been shown to increase in AD brains prior to NFT formation [18–23] and disrupt the ubiquitin proteasome system at synapses [24]. When brain-derived tau oligomers are administered to wild-type (WT) mice, they induce both synaptic and mitochondrial toxicity [25]. Overexpression of a chaperone inducing tau oligomerization causes neuronal toxicity in mice [26]. Furthermore, there is evidence that tau oligomers seed the misfolding of endogenous tau [27] and are responsible for the spread of tau pathology from affected regions in the brain to unaffected areas [28]. Primary mouse neurons in culture internalize tau oligomers and small tau aggregates and spreading occurs both anterogradely and retrogradely, but when treated with tau fibrils and monomer, uptake does not occur [29]. Tau oligomers spread from injection site to connected brain regions in WT mice, while mice treated with fibrils do not show the same pattern of propagation [30].

Passive immunotherapy targeting tau oligomers may be a viable option for halting this spread of pathology, without affecting functional tau monomer [31]. Though there is a great deal of evidence for the importance of tau in AD, most of the research on therapeutics thus far has targeted amyloid-β (Aβ). Passive immunotherapy in mice overexpressing mutant amyloid-β protein precursor (PDAβPP mice) conferred cognitive benefits without decreasing amyloid plaque burden, suggesting that targeting soluble amyloid species, rather than large aggregates, is beneficial for memory [32]. While safety concerns have been implicated in passive vaccination against Aβ, it appears as though adverse effects depend upon binding to Aβ deposits [33, 34]. A few studies have been conducted using antibodies against tau, yielding cognitive benefits, however the target has mainly been phosphorylated tau, rather than tau oligomers which confer toxicity. Additionally, these immunizations saw a decrease in NFTs, which do not seem to be toxic and may even be neuroprotective [35–37]. Therefore, using passive immunotherapy to target only toxic, soluble, oligomeric tau species appears to be a promising strategy.

Here we investigate the prevention of pathological tau accumulation and cognitive deficits in Htau mice immunized with anti-tau oligomer-specific monoclonal antibody (TOMA). Htau mice overexpress human tau without the expression of endogenous mouse tau. At 3 months, tau begins to relocate from microtubules in the axons to the cell body, which is important for tau oligomerization. By 9 months, tau aggregates are seen, increasing more by 13–15 months [38, 45, 46]. In humans, tau is alternatively spliced to form six isoforms with either three or four microtubule binding repeats, 3R and 4R tau, both of which are affected in AD. However, while in humans 3R and 4R tau levels are approximately equal in adulthood, 4R tau is generally present in higher levels in adult mice [39] and only three isoforms are expressed [40]. As there is evidence for differences in the seeding and spread of 3R and 4R tau, such as research showing that 3R tau may be more likely to be secreted [41], Htau mice are a good model to study tau pathology in AD. Aggregation properties of 3R and 4R tau differ as well [42] and shifts in the normally equal ratio of 3R:4R tau have been found to be associated with neurodegeneration [43]. The ratio of 3R:4R tau is also altered in Htau mice as they age [44]. Additionally, Htau mice do not show any motor or sensory deficits, but do have memory deficits, impairment to long-term potentiation, and changes to dendritic spine morphology and volume in old age, making them an optimal animal model for study of cognitive impairment in human AD [45, 47]. Previous findings have shown that aged Htau mice treated with curcumin exhibit decreased tau dimer levels correlating with memory improvement [47]. Therefore, tau oligomers likely play a role in the phenotype of Htau mice.

In this study, 3-month-old Htau and WT mice first received intracerebroventricular (ICV) injection of brain-derived tau oligomers and a single intravenous

injection of TOMA or non-specific IgG and were tested for memory deficits at 3 days, 1 month, and 3 months post-injection using novel object recognition, as well as analyzed for tau pathology. In a second experiment, mice were given bi-weekly injections of TOMA or control IgG following administration of tau oligomers and were tested at 3 days, 3 months, 6 months, and 9 months after treatment. Additionally, two groups of 3-month-old Htau mice were given bi-weekly injections of either 60 μg of TOMA or non-specific IgG without application of brain-derived tau oligomers in order to test whether TOMA provides cognitive protection against deficits arising during aging in Htau mice.

METHODS

Preparation of brain-derived tau oligomers

Immunoprecipitation of tau oligomers from AD frontal cortex was performed as previously described [30, 49]. Tosyl-activated magnetic Dynabeads (Dynal Biotech, Lafayette Hill, PA) were coated with 20 μg of anti-tau oligomer-specific polyclonal antibody T22 (1.0 mg/ml) diluted in 0.1 M borate, pH 9.5, overnight at 37°C. Beads were washed (0.2 M Tris, 0.1% bovine serum albumin, pH 8.5) and then incubated with AD brain homogenate (PBS soluble fraction) with rotation at room temperature (RT) for 1 h. Beads were washed three times with PBS and eluted using 0.1 M glycine, pH 2.8. The pH was adjusted using 1 M Tris pH 8.0 and then fractions were centrifuged in a microcon centrifugal filter device with a molecular weight cut-off of 25 kDa (Millipore, Cat # 42415) at 14,000 g for 25 min at 4°C. Oligomers were re-suspended in sterile PBS. Protein concentration was measured using the bicinchoninic acid protein assay (Pierce). The samples were then centrifuged once more in a microcon centrifugal filter device with a cut-off of 25 kDa at 14,000 g for 25 min at 4°C. Oligomers were characterized by various methods including size-exclusion chromatography and atomic force microscopy as previously described [30, 50] and stored at −80°C. Oligomers were re-suspended in PBS in order to obtain the desired concentration (0.18–1.2 mg/ml) and kept at 4°C for 15–30 min, then at RT for 10 min before use.

Toxicity assays

Alamar blue and MTS toxicity assays were completed as described previously [9, 30, 50]. For the Alamar blue assay, SH-SY5Y human neuroblastoma cells were grown in Dulbecco's modified Eagle's medium to confluence in 96-well plates. Cells were treated with different concentrations (0.01, 0.05, 0.1, 0.2, and 0.5 μM) of either brain-derived tau oligomers, paired helical filaments (PHF), or PBS as an untreated control for 4 h. Cell survival was measured using the Alamar Blue assay kit (Serotec). Fluorescence was measured at 590 nm using a POLARstar Omega fluorescence microplate reader, with all treatments completed in triplicate. For the MTS assay, SH-SY5Y human neuroblastoma cells were grown in Dulbecco's modified Eagle's medium to confluence in 96-well plates and treated with either PBS control, brain-derived tau oligomers, brain-derived PHF, or brain-derived tau fibril mixed with TOMA at different molar ratios. After 4 h, cell viability was measured using the colorimetric Tetrazolium-based MTS assay (Promega) at 490 nm using a POLARstar Omega fluorescence microplate reader. Measurements were completed in triplicate and results were analyzed by student's *t*-test.

Animals

For Experiment 1, three-month-old homozygous Htau mice (The Jackson laboratory) were given intravenous injections of either 60 μg/animal of TOMA antibody for the treatment group or non-specific IgG (Rhodamine, Genetex cat. GTX29093) for the control group ($n = 14$ animals/group). A third group of 3-month-old C57BL/6 WT mice (The Jackson Laboratory) received saline injection. One hour after antibody treatment, mice received ICV bilateral injection of 0.6 μg/hemisphere of brain-derived tau oligomers as previously described [30]. All animals used were male in order to control for changes to cognition occurring in different hormonal states in female mice. Mice were housed at the UTMB animal care facility and experiments were performed according to IACUC-approved protocols.

Generation of tau oligomer monoclonal antibody (TOMA)

Tau oligomers were prepared in PBS as previously described [23] and purified by fast protein liquid chromatography (FPLC, Superdex 200 HR 10/30 column, Amersham Biosciences). Two-month-old BALB/c mice were immunized with tau oligomers according to standard procedures using Freund's complete adjuvant. The mice received an intraperitoneal injection of 100 μl of 1:1 (antigen: adjuvant) on the ventral side (20 μg/mouse). Two weeks later, a

second injection of antigen with Freund's incomplete adjuvant was performed, followed by 5 boosts. Anti-tau oligomer antibody response was determined by screening serial dilutions of animal sera using an enzyme-linked immunosorbant assay (ELISA). ELISA plates were coated with 50 ng or 200 ng of either tau oligomers, Aβ oligomers, or α-synuclein oligomers to rule out cross-reactivity with other amyloid oligomers. Dot blot was also used to test TOMA specificity. Selected clones were tested by Western blot using *in vitro* prepared samples, and dot blot using brain homogenates. Finally, the selected clones (TOMA) were tested using human and mouse brains. Antibody isotyping and light chain composition, κ or λ, was determined using an IsoStrip™ mouse monoclonal antibody isotyping kit (Rapid Isotyping kit Plus Kappa & Lambda-mouse, Pierce). TOMA was produced from hybridoma cells grown in X-VIVO 15 (LONZA) medium following standard conditions for cell culture. The antibody was purified from the medium by standard affinity chromatography methods followed by high-performance liquid chromatography (HPLC) purification (purity >95%). TOMA used in immunization studies was endotoxin free, as confirmed using a commercially available kit (Limulus amebocyte lysate, Chromogenic Endpoint Assay, Hycult Biotechnology). All TOMA samples were stored in appropriate endotoxin free vials at −80°C until use.

Intravenous (IV) injection

Htau mice were either immunized with 60 μg of TOMA/animal or 60 μg/animal of non-specific IgG, Rhodamine. WT mice were injected with 60 μl of saline solution. For IV injections, mice were placed in a restrainer (Braintree Scientific) and an inch of the tail was shaved and placed in warm water to dilate veins. Sixty μg were then injected into the lateral tail vein. Mice were returned to home cages and kept under observation.

Stereotaxic injection of brain-derived tau oligomers

Htau and WT mice were anesthetized with ketamine (80–100 mg/kg, ip) and xylazine (10 mg/kg, ip) and placed in a stereotactic apparatus (Motorized Stereotaxic StereoDrive, Neurostar). The scalp of each mouse was then shaved and an incision was made through the midline to expose the skull. The bregma was identified and a hole was drilled in the skull. A 5.0 μl Hamilton syringe was used to inject 2 μl of 0.3 mg/ml brain-derived tau oligomers into the hippocampi on both sides (−2.06 mm posterior, ±1.75 mm lateral, and 2.5 mm ventral to the bregma) at a rate of 0.2 μl/min as described previously [9, 30]. The incision was closed using Vet-Bond, and mice were placed on a 37°C isothermal pad and continuously observed post-surgery until recovery.

Novel object recognition

The novel object recognition task (NOR) utilizes the natural tendency of rodents to preferentially explore novel objects and environments over those that are familiar. This task has been used previously to detect memory impairment in Htau mice [45]. Three days after receiving TOMA and brain-derived tau oligomer injection, animals from Experiment 1 were habituated to the NOR task. Mice were allowed to freely explore white open-field arena (55 cm in diameter; 60 cm in height) for 15 min on the first day. The next day, mice were placed in the arena for the training phase with two identical objects, either spheres or cubes, and allowed to explore for 15 min. On the third day, mice were placed again in the arena for 15 min with one familiar object previously explored in the training phase and one novel object differing in color and shape, but sharing a common size and volume. After each trial, the apparatus was thoroughly cleaned using 70% ethanol and allowed to dry prior to placement of a new mouse. Trials were recorded and time spent exploring each object was measured using ANY-Maze software. Exploration was defined by head orientation within 2 cm of the object or physical contact with the object. The percentage of total time spent exploring the familiar object versus the novel object was measured. In order to control for any differences in exploratory behavior, the discrimination index was also calculated as the time spent exploring the familiar object subtracted from the time spent exploring the novel object, divided by the total time spent exploring both objects. In Experiment 1, the testing phase was repeated one month and three months after treatment. In Experiment 2, following testing three days post-treatment, mice were subjected to bi-weekly injections of TOMA and NOR was repeated at 3, 6, and 9 months after initial injection. Object exploration data were analyzed using Graph Pad Prism 5.04 software, either by one-way analysis of variance (ANOVA) and Tukey's *post-hoc* test for exploration time or by one-way *t*-test with a hypothesized mean of 0 for the discrimination index.

Tissue collection and immunohistochemistry

Following behavioral testing, animals were anesthetized with CO_2 and brains were collected. The right hemisphere was snap-frozen and stored at −80°C until processed for biochemical analysis. The other hemisphere was embedded in paraffin and sectioned. Immunohistochemistry was performed on paraffin-embedded sections. All sections were processed simultaneously under the same conditions. Sections (8 μm) were deparaffinized and rehydrated. After blocking in normal goat serum for 1 h, sections were incubated overnight with anti-tau oligomer-specific polyclonal antibody, T22 (1:300). The following day, sections were washed in PBS three times for 10 min each and incubated with biotinylated goat anti-rabbit IgG (1:500; Jackson ImmunoResearch) for 1 h. Sections were then washed three times for 10 min each in PBS and visualized using an ABC reagent kit (Vector Laboratories), according to the manufacturer's recommendations. Lastly, sections were counterstained with hematoxylin (Vector Laboratories) for nuclear staining and mounted. For Gallyas silver staining, we used the FD NeuroSilverKit II (FD NeuroTechnologies, Columbia MD) and brain sections were stained according to manufacturer's instructions. Sections used for fluorescent immunohistochemistry were deparaffinized and rehydrated. Sections were then blocked in normal goat serum for 1 h and incubated overnight with anti-tau oligomer-specific polyclonal antibody, T22 (1:300). The following day, sections were washed in PBS three times for 10 min each and incubated with goat anti-rabbit IgG Alexa-568 (1:500; Invitrogen) for 1 h. Sections were then washed three times for 10 min each in PBS and incubated overnight with total tau antibody, Tau-5 (1:300), or hyperphosphorylated tau antibody, AT8 (1:100). The next day the sections were washed in PBS three times for 10 min each prior to incubation with donkey anti-mouse IgG Alexa-Fluor 488 (1:500; Invitrogen). Sections were washed and mounted using Fluoromount G (Souther Biotech) mounting medium with DAPI (Invitrogen). The sections were examined using an epifluorescence microscope (Nikon Eclipse 800) equipped with a CoolSnap-FX monochrome CCD camera (Photometrics) using standard Nikon FITC and DAPI filters, and images were acquired.

Tissue processing

Frozen brains extracted from Htau and WT mice were diced and homogenized in PBS with a protease inhibitor cocktail (Roche) and 0.02% NaN_3, using a 1:3 (w/v) dilution of brain: PBS. Samples were centrifuged at 10,000 rpm for 10 min at 4°C. The supernatants were aliquoted, snap-frozen, and stored at −80°C until use. Pellets were collected for analyses of PBS-insoluble tau.

Western blot

Pre-cast NuPAGE 4–12% Bis-Tris Gels (except for 3R/4R tau analysis in which 3–8% Tris-Acetate gels were used) for SDS-PAGE (Invitrogen) were loaded with 20–25 μg of protein for each sample per well, run, and then transferred to nitrocellulose. Membranes were then blocked overnight at 4°C with 10% nonfat dried milk. The next day membranes were incubated with T22 (1:250) for tau oligomers, Tau-5 (1:1000) for total tau, AT8 for phospho tau (1:1000), and actin (1:1000) diluted in 5% nonfat dried milk for 1 h at room temperature. Tau-5 and actin immunoreactivity were detected with horseradish peroxidase-conjugated IgG anti-mouse secondary antibody (1:3000, GE Healthcare) and T22 was detected with horseradish peroxidase-conjugated IgG anti-rabbit secondary antibody (1:3000, GE Healthcare). For signal detection, ECL plus (GE Healthcare) was used. For statistics, densitometry of each band was quantified and normalized with actin using ImageJ and analyzed by one-way ANOVA.

ELISA

For ELISA analysis, 96-well plates were coated with 15 μl of samples (PBS soluble and insoluble fractions of brains) using 0.05 M sodium bicarbonate (pH 9.6) as the coating buffer and incubated overnight at 4°C. Plates were washed once with TBST (0.01% Tween 20), then blocked for 2 h at RT with 10% non-fat milk. Plates were then washed once with TBST. T22 (1:250), AT8 (1:250), or Tau-5 (1:1000) diluted in 5% nonfat milk were added and allowed to react for 1 h at RT. Plates were washed three times with TBST. T22 immunoreactivity was detected using 100 μl of HRP-conjugated anti-rabbit IgG (GE Healthcare) and AT8 and Tau-5 were detected using HRP-conjugated anti-mouse IgG (GE Healthcare) diluted 1:3000 in 5% nonfat milk and incubated for 1 h at RT. Lastly, plates were washed three times with TBST and incubated with 100ul of 3,3,5,5-tetramethylbenzidine (TMB-1 component substrate, from Dako) for 1 h in the dark. To stop the reaction, 100 μl 2 M HCl was applied and

plates were read at 450 nm in a Polar Star Omega plate reader (BMG Labtech). Each sample was measured in triplicate and results were analyzed by student's *t* test.

RESULTS

Htau mice show increased tau oligomer levels with age

Htau mice overexpress non-mutated human tau and do not express mouse tau. Researchers have previously shown that Htau mice begin to show evidence of tau mislocalization necessary to initiate oligomerization starting at 3 months of age and begin to see NFTs by 9 months of age, increasing significantly by 13–15 months [38, 45, 46]. In order to specifically test for the presence of oligomeric tau, immunohistochemistry with anti-tau oligomer antibody, T22 was completed on brain sections from Htau mice at different ages and tau knockout (KO) mice (Fig. 1A-H). Tau oligomers were detected in the cerebellum and the hippocampus of Htau mice and levels increased with age. No T22 staining was seen in the cerebellum or the hippocampus of tau KO mice, as expected. In order to quantify tau oligomer levels, brains were collected from Htau mice of various ages from 3 months to 22 months, as well as from tau KO and WT (Balb/C) mice at 3 and 6 months. Immunofluorescence staining was also completed with T22 and Tau-5 in the hippocampus of 11-month-old Htau and tau KO mice (Fig. 1I-N). Tau-5 staining was widely distributed across the hippocampus, partially colocalizing with T22-positive tau oligomers in Htau mice, while no tau was detected in tau KO mice, as expected. Immunofluorescence with AT8 and T22 in the hypothalamus of 11-month-old Htau mice (Fig. 1O-Q) revealed the presence of AT8-positive hyperphosphorylated tau and T22-positive tau oligomers present in adjacent cells. Western blot of Htau mice of different ages (6, 11-12, and 22 months) with Tau-5 and TOMA revealed an increase in higher molecular weight oligomeric tau species with age, while no tau was detected in tau KO mice (Fig. 2A-B). Direct ELISA with T22 of Htau, WT, and tau KO mice of different ages showed that 6-month-old Htau mice had a significantly higher level of tau oligomers than age-matched KO and WT mice ($p < 0.001$) (Fig. 2C). Therefore, the presence of tau oligomers increases with age in Htau mice, precedes the established time point at which NFTs appear, and may initiate toxicity prior to detection of large tau aggregates.

TOMA reacts specifically with tau oligomers

In order to verify the specificity of TOMA, we used Western blot of recombinant 3R and 4R tau and found that TOMA specifically recognizes only oligomers from both 3R and 4R tau, with no detection of tau monomer seen with Tau-5 (Fig. 2D). We used direct ELISA to test oligomeric species of various amyloid proteins and monomeric and fibrillar tau species (Fig. 2E). We found that while TOMA reacts with both 3R and 4R tau oligomers, TOMA does not react with oligomers of any other amyloid proteins (synuclein, $A\beta_{40}$, $A\beta_{42}$, or prion protein) or with tau monomer or fibril. Therefore, TOMA specifically targets only tau oligomers.

Tau oligomers, not fibrils, cause toxicity in SH-SY5Y cells and cognitive impairment in vivo

We have previously shown that tau oligomers induce toxicity *in vitro* and *in vivo* [9, 30, 50]. Using the MTS toxicity assay, we show that brain-derived tau oligomers significantly decrease cell viability compared to untreated cells and cells treated with PHFs ($p < 0.001$) (Fig. 3A). When TOMA was added along with tau treatment, cells treated with tau oligomers were protected from toxicity. Using the Alamar blue assay, we replicated findings that tau oligomers can induce toxicity (Fig. 3B). Brain-derived tau oligomer treatment significantly decreased cell viability compared to untreated cells and cells treated with brain-derived PHF ($p < 0.001$). Toxicity increased with increasing concentrations of oligomers. WT (Balb/C) were injected with PBS, tau monomers, tau fibrils, or tau oligomers ICV at 3 months of age. Using the NOR task 3 days after injection, only mice given tau oligomers were significantly impaired when compared to controls injected with PBS (data not shown).

EXPERIMENT 1

Single injection with TOMA antibody prevents object discrimination deficits in Htau mice

Previous study has shown that Htau mice do not show any motor, sensory, or general health deficits in comparison to age-matched WT mice up to 12 months of age. Additionally, young mice do not show any memory deficits as measured by the visual recognition memory task (NOR) and the spatial memory task (the Morris water maze). However, 12-month-old Htau mice are cognitively impaired [45]. In order to test

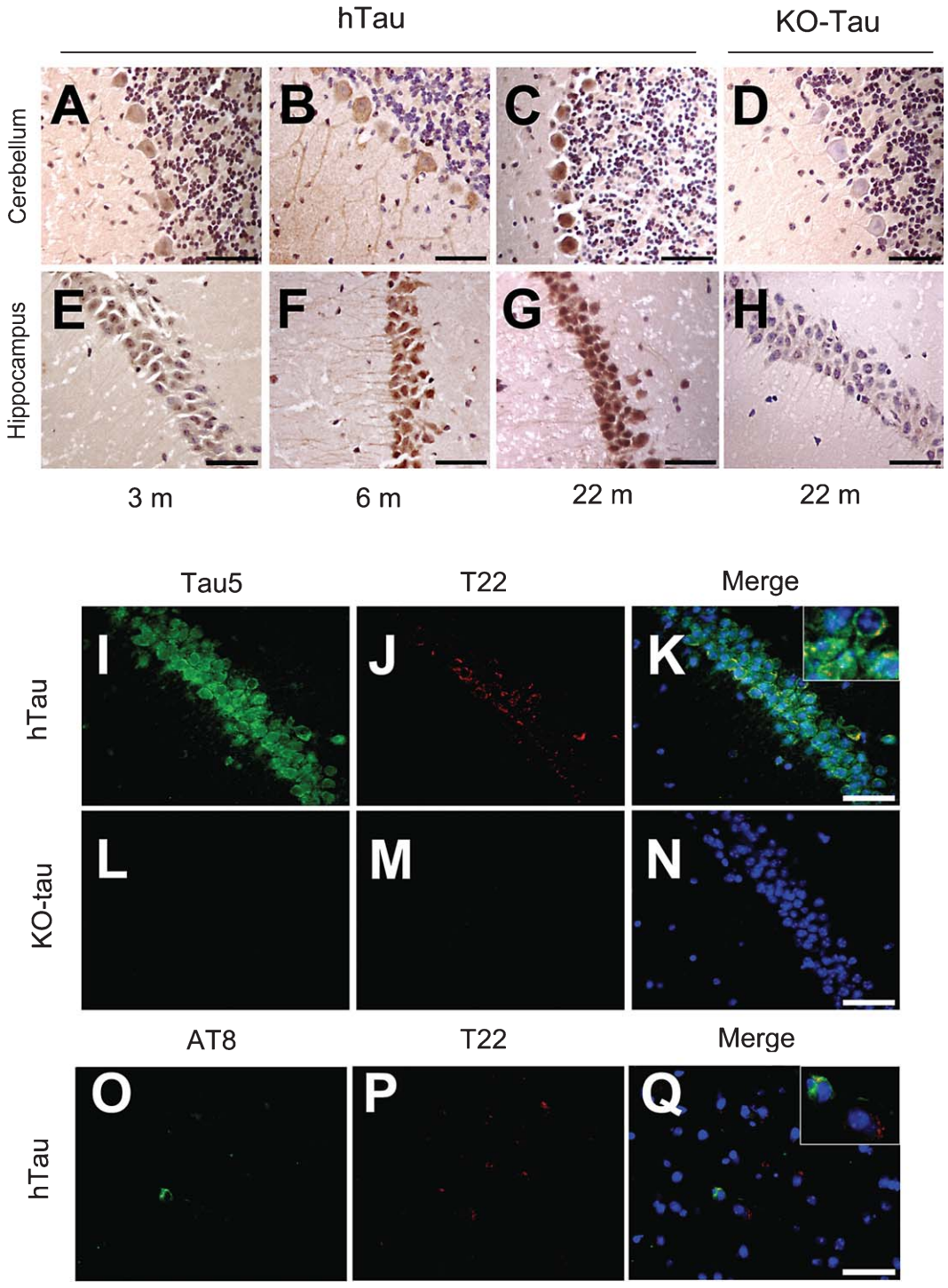

Fig. 1. Immunohistochemistry with T22 of Htau mice at different ages and tau knockout (KO) mice (A-H). T22-positive tau oligomers can be detected in the cerebellum (A-C) and the hippocampus (E-G) of Htau mice and levels increase with age. No T22 staining is observed in the cerebellum (D) or the hippocampus (H) in tau KO mice. Scale bar 50 μm. Immunofluorescence with T22 and Tau-5 in the hippocampus of 11-month-old Htau and tau KO (KO-tau) mice (I-N). Tau-5 staining was widely distributed across the hippocampus (I), partially colocalizing with T22-positive tau oligomers in Htau mice (J-K), while no tau was detected in tau KO mice (L-N). Immunofluorescence with AT8 and T22 in the hypothalamus of 11-month-old Htau mice (O-Q) revealed the presence of AT8-positive hyperphosphorylated tau (O) and T22-positive tau oligomers (P) present in adjacent cells. Scale bar 25 μm.

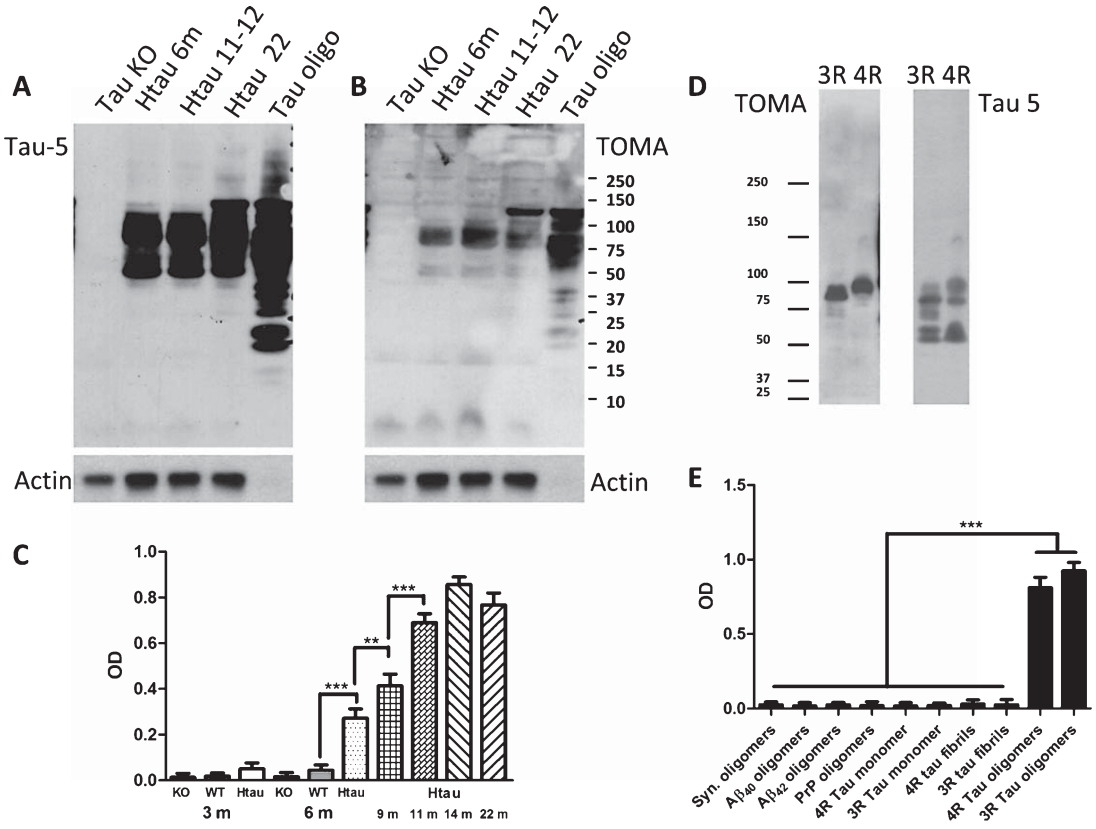

Fig. 2. Western blot of Htau mice of different ages (6, 11-12, and 22 months) with Tau-5 (A) and TOMA (B) revealed an increase in higher molecular weight oligomeric tau species with age. No tau was detected in tau KO mice. Direct ELISA with T22 of Htau, WT, and tau KO mice of different ages (C). 6-month-old Htau mice had a significantly higher level of tau oligomers than age-matched KO and WT mice (***$p < 0.001$). Oligomer levels continued to rise until 14 months of age (**$p < 0.01$; ***$p < 0.001$) and then did not significantly change up to 22 months. Western blot of recombinant 3R and 4R tau showed that TOMA specifically recognizes only oligomers from both 3R and 4R tau, with no detection of tau monomer seen with Tau-5 (D). Direct ELISA with TOMA of amyloid oligomers and tau (E). TOMA reacts with both 3R and 4R tau oligomers, but not with oligomers of synuclein, $A\beta_{40}$, $A\beta_{42}$, or prion protein or with tau monomers or fibrils (***$p < 0.001$).

whether young Htau mice which have been injected with tau oligomeric seeds show deficits and whether targeting oligomers can attenuate detrimental effects, we used the NOR task (Fig. 3C). Once mice had been habituated to the test environment, mice from Experiment 1 were tested on the apparatus at 3 days, 1 month, and 3 months after receiving TOMA injection IV and brain-derived tau oligomer injection ICV. At 3 days and 1 month following treatment, Htau mice treated with TOMA spent a significantly higher percentage of time exploring the novel object than the familiar object ($p < 0.001$). However, Htau mice treated with control IgG showed no preference for the novel object over the familiar object. After 3 months, both groups were impaired, showing no differences between time spent investigating the two objects (Fig. 4A), while WT mice were still protected by TOMA after 3 months (data not shown). This difference in cognitive benefit between WT and Htau mice highlights the importance of using mice expressing human tau and is likely due to the heightened ability of human brain-derived tau oligomers to seed the misfolding of tau in Htau mice compared to WT mice. Differences were not due to exploratory behavior as there were no differences in total exploration time (data not shown). Similar results were found with the discrimination index, calculated as the time spent exploring the familiar object subtracted from the time spent exploring the novel object, divided by the total time spent exploring both objects. Htau mice receiving IgG had a discrimination index ~0, performing at chance levels and showing no preference for the novel object over the familiar object, while Htau mice receiving TOMA exhibited preference for the novel object approaching significance and performed significantly better than IgG-treated animals at 3 days ($p < 0.001$) and 1 month ($p < 0.05$) post-

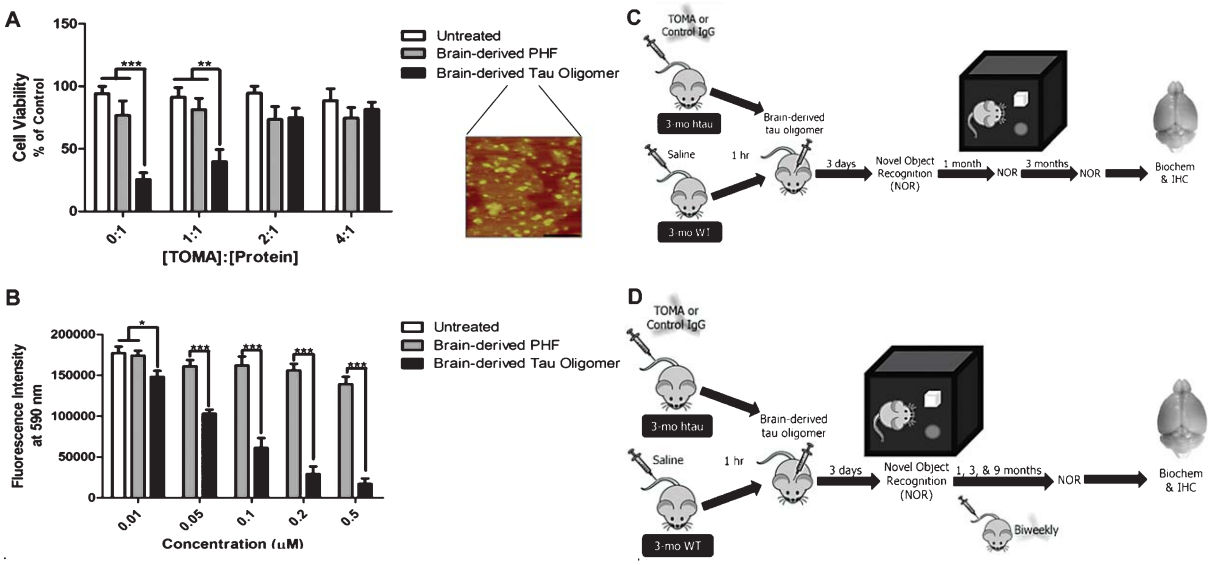

Fig. 3. Cell viability measured by the MTS toxicity assay (A). Brain-derived tau oligomers significantly decrease cell viability compared to untreated cells and cells treated with PHF (***$p < 0.001$). When TOMA is added along with tau treatment, cells treated with tau oligomers are protected from toxicity. At a 1:1 ratio of TOMA to tau oligomers, cell viability is still significantly lowered (**$p < 0.01$), while at a 2:1 ratio, tau oligomer toxicity is prevented and there is no significant difference between treatment with tau oligomers or PHF or with untreated cells. Inset image shows characterization of brain-derived tau oligomers by atomic force microscopy. Scale bar 100 nm. Tau oligomer toxicity detected with the Alamar Blue assay (B). Brain-derived tau oligomer treatment at the lowest concentration (0.01 μM) significantly decreased cell viability compared to untreated cells and cells treated with brain-derived PHF (*$p < 0.05$). Toxicity increased with increasing concentrations of oligomers (***$p < 0.001$). Schematic depicting the timeline for Experiment 1 (C) and Experiment 2 (D).

treatment (Fig. 4B). Similarly, WT mice injected with tau oligomers and non-specific IgG were impaired after 3 days and TOMA injection was protective (data not shown). Therefore, the application of oligomeric seeds induced memory deficits in young Htau mice, which were protected when treated with a single injection of TOMA for up to 1 month.

Single TOMA injection reduces tau oligomers, but not total tau or hyperphosphorylated tau

Following behavioral testing, brains were collected and divided for biochemical and immunohistochemical analysis. Immunohistochemistry with T22 revealed a decrease in tau oligomer-positive cells in the CA2 and dentate gyrus regions of the hippocampus in mice treated with TOMA compared to control IgG and tau KO mice (Fig. 5A-F), while no difference was seen in AT8-positive NFTs (data not shown). Brains were homogenized and the PBS soluble and insoluble fractions were collected. ELISA of the PBS soluble fraction with T22 showed that mice treated with TOMA had significantly lower levels of tau oligomers than those treated with control IgG ($p < 0.001$) (Fig. 5G). However, no differences were detected by ELISA with Tau-5 which recognizes all forms of tau (Fig. 5H). The PBS insoluble fraction was analyzed for hyperphosphorylated tau NFT levels as measured with AT8 (Ser202/Thr205 phosphorylated tau) by ELISA and no differences were seen between TOMA and non-specific IgG mice (Fig. 5I). Therefore, a single injection with TOMA lowers levels of toxic tau oligomers, but does not affect total tau levels or NFT levels.

EXPERIMENT 2

Biweekly injections with TOMA antibody prevent object discrimination deficits in Htau mice

In order to test whether bi-weekly injections of TOMA can confer protection against cognitive deficits seen in young Htau mice injected with tau oligomeric seeds, the NOR task was again used (Fig. 3D). In Experiment 2, mice were tested at 3 days, 3 months, 6 months, and 9 months following the initial treatment and received bi-weekly TOMA injections throughout testing. Htau mice receiving TOMA injections spent significantly more time exploring the novel object than the familiar object at 3 days, 3 months, 6 months, and 9 months after initial treatment ($p < 0.001$), while mice receiving control IgG were unable to discriminate

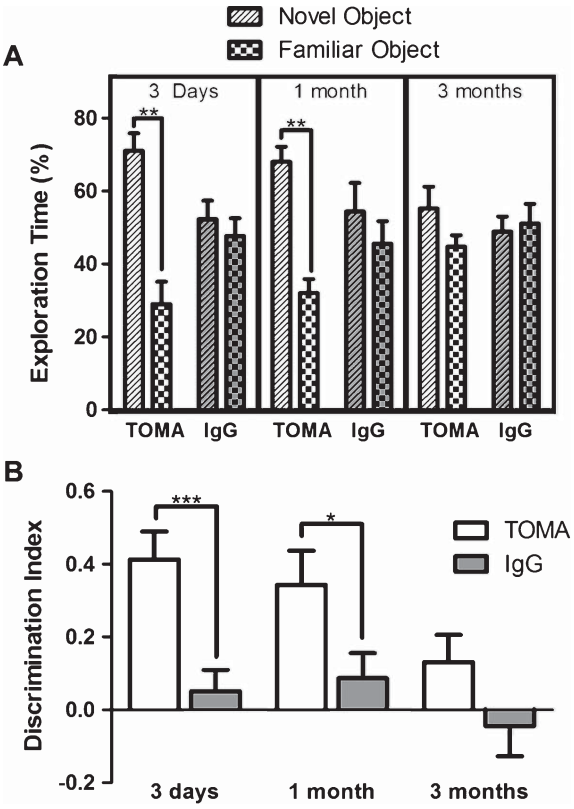

Fig. 4. Sensory recognition memory was impaired in Htau mice injected with brain-derived tau oligomers and was protected in mice receiving TOMA treatment (white bars) rather than non-specific IgG (grey bars). Comparison of the percent exploration time spent on the novel object (striped bars) versus the familiar object (checkered bars) (A). Htau mice receiving control IgG showed no preference for the novel object over the familiar object, while mice receiving TOMA spent significantly more time exploring the novel object than the familiar object at 3 days and 1 month after injection (**$p < 0.01$). Comparison of the discrimination index, calculated as the time spent exploring the familiar object subtracted from the time spent exploring the novel object divided by the total time spent exploring both objects (B). Htau mice receiving IgG had a discrimination index ~0, performing at chance levels and showing no preference for the novel object over the familiar object, while Htau mice receiving TOMA exhibited preference for the novel object approaching significance ($p = 0.05$) and performed significantly better than IgG-treated animals at 3 days (***$p < 0.001$) and 1 month (*$p < 0.05$) post-treatment.

between the two objects (Fig. 6A). In order to control for overall exploratory behavior, the discrimination index was also measured in tau-oligomer treated mice receiving control IgG or TOMA. Htau mice receiving bi-weekly IgG injections had a discrimination index ~0, performing at chance levels and showing no preference for the novel object over the familiar object, while Htau mice receiving bi-weekly TOMA exhibited preference for the novel object ($p < 0.001$) and performed significantly better than IgG-treated animals from 3 days to 9 months post-treatment ($p < 0.05$) (Fig. 6B). Similar results were observed in WT mice receiving either TOMA or control IgG (data not shown). Additionally, 3-month-old Htau mice were given bi-weekly injections of either 60 μg of TOMA or non-specific IgG without application of brain-derived tau oligomers in order to test whether TOMA provides cognitive protection against deficits due to normal aging in these mice and to control for any effects TOMA treatment may have on cognition or inflammation. Htau mice receiving bi-weekly control IgG and TOMA displayed preference for the novel object over the familiar object up to 6 months of age ($p < 0.001$) (Fig. 6C). At 9 months of age, memory began to decline in Htau mice receiving bi-weekly non-specific IgG, while mice receiving bi-weekly TOMA still appeared cognitively normal, spending significantly more time exploring the novel object than the familiar object up to 12 months of age ($p < 0.001$). No evidence of inflammation was seen in TOMA-treated mice. Based on these results, it appears that TOMA treatment is capable of counteracting cognitive detriment due to the toxicity of tau oligomers without resulting in any negative effects on general health.

Biweekly TOMA injections reduce tau oligomers, but not total tau or hyperphosphorylated tau

Brains were collected and divided for biochemical and immunohistochemical analysis. ELISA of the PBS soluble fraction with T22 showed that mice treated with TOMA had significantly lower levels of tau oligomers than those treated with control IgG ($p < 0.001$) (Fig. 7A). Lowered levels of tau oligomers in TOMA-treated mice were also seen by Western blot with T22 of the PBS soluble fraction of Htau brains (Fig. 7B-C). However, no differences were detected by ELISA or Western blot in total tau levels as measured with Tau-5 (Fig. 7D-F). No differences in hyperphosphorylated tau levels as measured with AT8 were seen by ELISA or Western blot between TOMA and non-specific IgG-treated mice (Fig. 7G-I). Similarly, immunohistochemistry with AT8 (Ser202/Thr205 phosphorylated tau) and Gallyas silver staining did not reveal any changes to levels of NFTs in Htau mice treated with TOMA versus non-specific IgG (Fig. 7J-M). Therefore, as expected, TOMA treatment selectively targeted oligomeric tau, without lowering levels of hyperphosphorylated NFTs or tau monomer and decreasing tau oligomers alone was enough to confer benefits to memory.

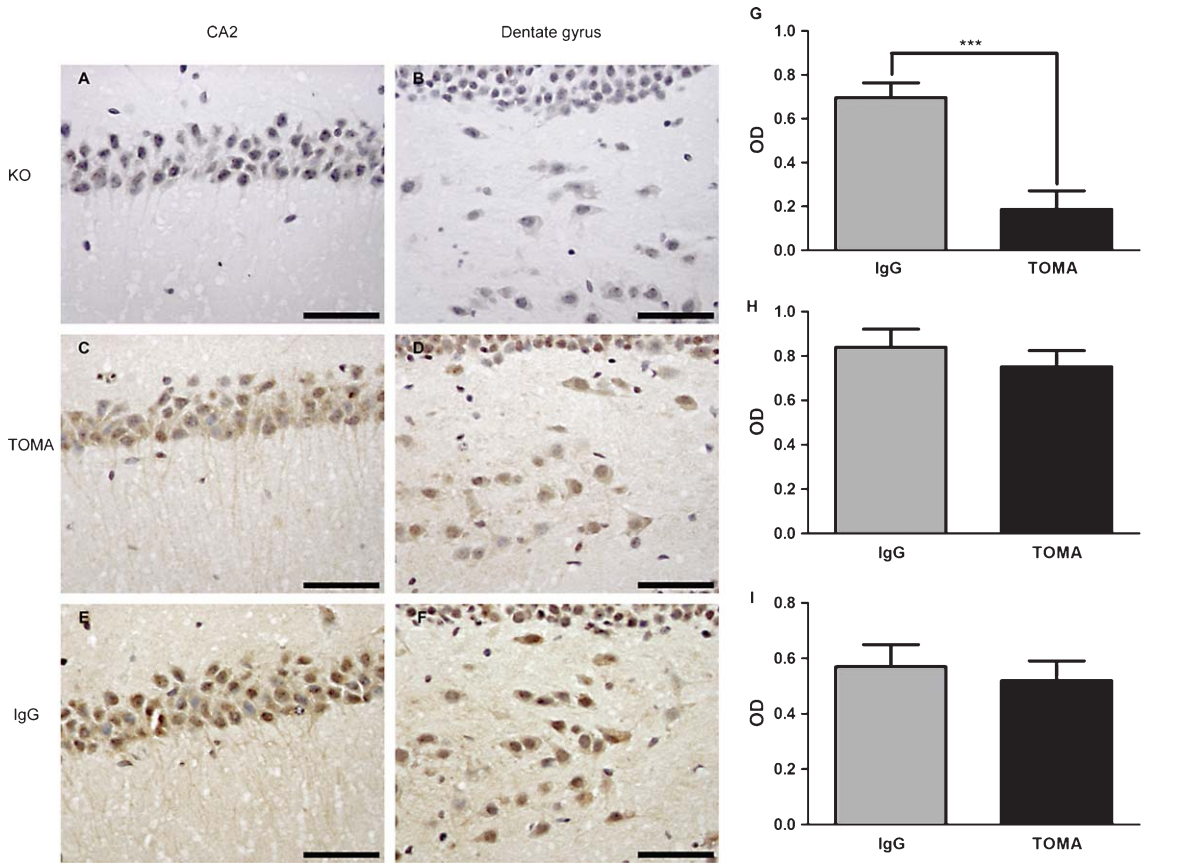

Fig. 5. Immunohistochemistry with T22 (A-F) showed a decrease in tau oligomers in the CA2 and the dentate gyrus regions of the hippocampus in mice treated with TOMA (C-D) compared to control IgG (E-F) and tau KO mice (A-B). Scale bar 50 μm. ELISA of the PBS soluble fraction with T22 (G) showed that mice treated with TOMA had significantly lower levels of tau oligomers than those treated with control IgG (***$p < 0.001$). No differences were detected by ELISA in total tau levels as measured with Tau-5 (H) or in hyperphosphorylated tau levels as measured with AT8 (I).

DISCUSSION

Passive immunotherapy is a promising mode of treatment for neurodegenerative disease [31]. While studies have traditionally focused on targeting Aβ to prevent and treat AD, more research is now being dedicated to tau. However, most of the studies conducted using tau passive immunotherapy have targeted phosphorylated tau species known to be present in the late stages of aggregation and have resulted in a decrease of NFTs [35–37]. While NFTs have historically been the main tau species of interest, more results are emerging to suggest that toxicity and neuronal loss exceeds and occurs prior to NFT accumulation [1–6], thereby implicating the importance of intermediate tau species in toxicity. Evidence is growing suggesting that tau oligomers are the most toxic tau species in neurodegenerative tauopathies and that they may also seed the spread of tau pathology from cell to cell [9, 18, 19, 23, 28–30]. Therefore, in order to stop the initial toxicity and the spread of disease, tau oligomers may be the best target. Moreover, it was recently shown that antibodies confer beneficial effects by targeting tau found extracellularly, implying that antibodies do not need to enter cells in order to reduce the toxic effects of tau, but may instead target tau which may be involved in the propagation of pathology [37, 51]. Additionally, studies of tau therapeutics for neurodegenerative disease have primarily used transgenic mouse models expressing mutant tau. While these models are comparable to diseases such as frontal temporal lobe dementia, AD and Parkinson's disease do not arise due to mutations to tau, making the Htau mouse a good model for investigating immunotherapy.

TOMA is a monoclonal antibody specific to tau oligomers, but not recognizing tau monomers or

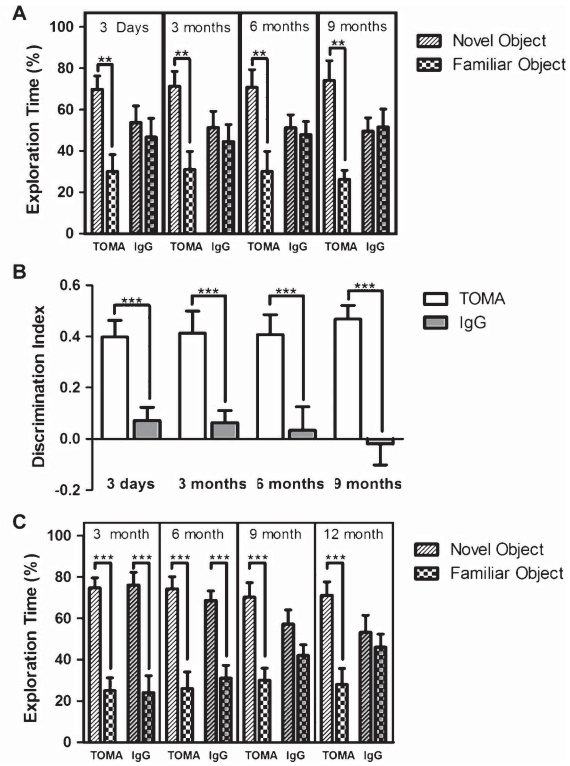

Fig. 6. Sensory recognition memory was impaired in Htau mice injected with brain-derived tau oligomers and was protected in mice receiving TOMA treatment (white bars) rather than non-specific IgG (grey bars). Comparison of the percent exploration time spent on the novel object (striped bars) versus the familiar object (checkered bars) (A). Htau mice receiving bi-weekly control IgG showed no preference for the novel object over the familiar object after 3 days, while mice receiving bi-weekly TOMA spent significantly more time exploring the novel object than the familiar object up to 9 months after initial injection (**$p < 0.01$). Comparison of the discrimination index (B). Htau mice receiving bi-weekly non-specific IgG had a discrimination index ~0, performing at chance levels and showing no preference for the novel object over the familiar object, while Htau mice receiving TOMA exhibited preference for the novel object (***$p < 0.001$) and performed significantly better than control IgG-treated animals from 3 days to 9 months (***$p < 0.001$) post-treatment. Comparison of the percent exploration time spent on the novel object (striped bars) versus the familiar object (checkered bars) in control Htau mice not receiving injection of brain-derived tau oligomers (C). Htau mice receiving bi-weekly control IgG and TOMA displayed preference for the novel object over the familiar object up to 6 months of age (***$p < 0.001$). At 9 months of age, memory began to decline in Htau mice receiving bi-weekly non-specific IgG, while mice receiving bi-weekly TOMA were protected and spent significantly more time exploring the novel object than the familiar object up to 12 months of age (***$p < 0.001$).

fibrils. Here we show that tau oligomer levels increase with age in Htau mice and are reduced by passive immunotherapy with TOMA in Htau mice injected with brain-derived tau oligomers from AD cortex, as measured by ELISA, Western blot, and immunohistochemistry. A single injection of TOMA antibody protected 3-month-old Htau mice injected intracerebrally with brain-derived tau oligomers from cognitive deficit for up to 1 month, while bi-weekly TOMA treatments prevented memory decline for 9 months when compared to animals injected with non-specific IgG. However, NFTs and total tau levels were not affected by treatment, implying that reducing oligomeric species of tau alone are enough to confer benefits to cognition. TOMA successfully halted the induction of cognitive impairment occurring naturally with age in Htau mice which were not injected with tau oligomers. There was no evidence of negative effects to general health observed due to TOMA treatment. These results are in support of a study using curcumin treatment in Htau mice which found that a decrease in tau dimers ameliorated memory impairment as defined by improved performance in NOR, as well as a spatial memory task, without affecting insoluble tau [47]. The ability to target only pathogenic tau prevents any negative side effects which may occur from inhibiting the endogenous function of functional tau monomer.

We have shown previously that tau oligomers induce toxicity and seed the aggregation of endogenous tau in WT mice when injected intracerebrally [30] and show here that tau oligomers, but not monomers or fibrils induce toxicity in neuroblastoma cells and impair memory in WT mice. The prevention of cognitive deficit due to tau oligomer injection by TOMA is consistent with these results. As Htau mice express all six isoforms of human tau, rather than mouse tau and do not express any mutations, these results are particularly applicable to sporadic AD. Furthermore, Htau mice do not have any motor, sensory, or general health impairments, but do acquire memory deficits with old age [45]. As the only tau species affected in these mice was tau oligomers, which induced premature memory deficits, it appears as though oligomeric tau alone may underlie cognitive changes in Htau mice. In addition, studies have implicated tau pathology in traumatic brain injury (TBI), another growing health concern worldwide affecting millions, the repetitive occurrence of which has also been shown to increase the likelihood of acquiring neurodegenerative disease later in life [51–53]. The sudden onslaught of tau oligomers induced by intracerebral injection is comparative to the increase in pathological tau seen in humans following TBI [54]. Aged Htau mice subjected to repeated closed head mild TBI exhibit increased tau pathology compared to sham Htau mice [55]. Moreover, we have shown that in rats subjected to fluid percussion injury, levels of tau oligomers are significantly

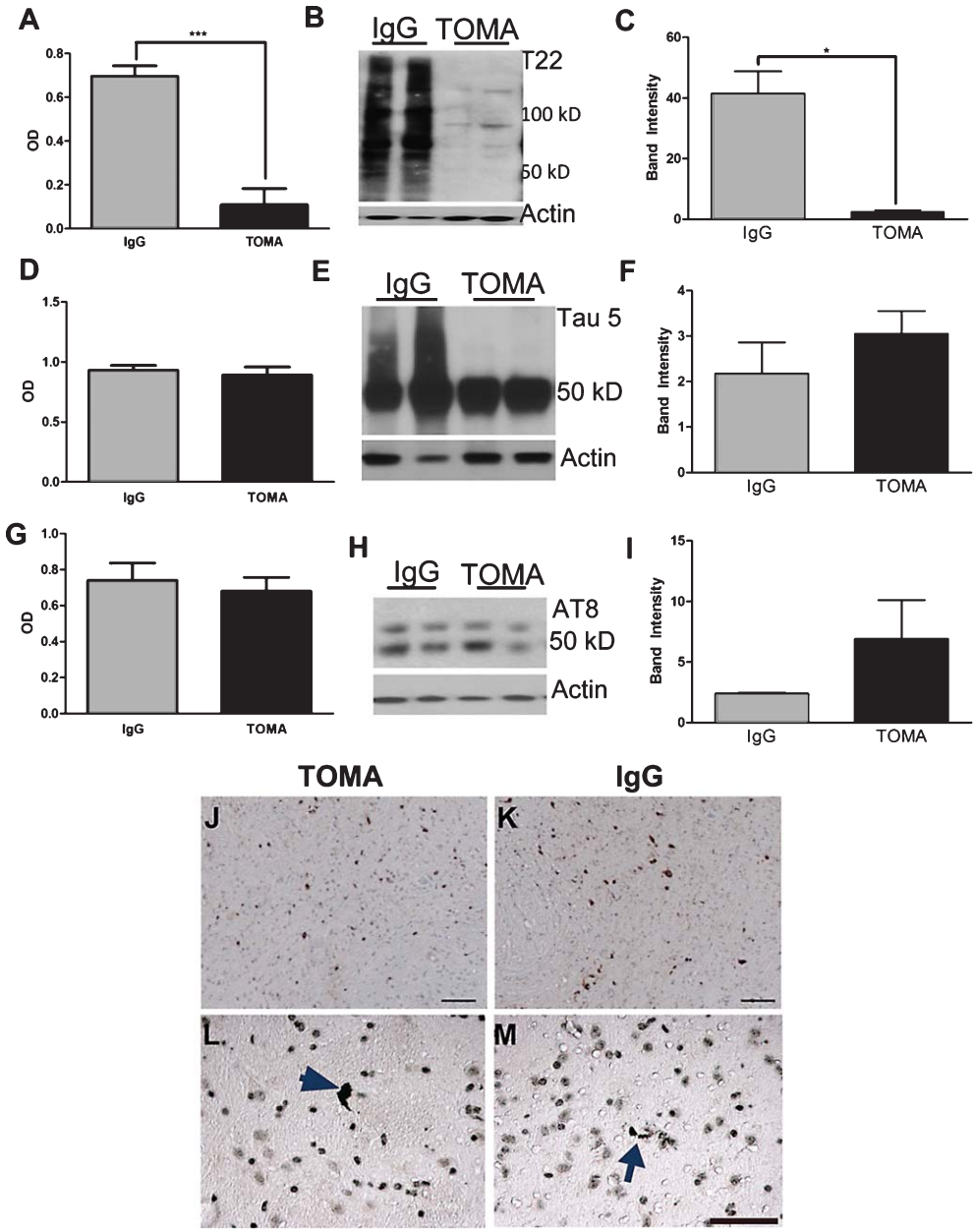

Fig. 7. ELISA of the PBS soluble fraction with T22 showed that mice receiving bi-weekly TOMA injections had significantly lower levels of tau oligomers than those treated with control IgG (***$p < 0.001$) (A). Significantly lowered levels of tau oligomers in TOMA-treated mice were also seen by Western blot with T22 of the PBS soluble fraction of Htau brains (B) and densitometric quantification (*$p<0.05$) (C). No differences were detected in total tau levels measured with Tau-5 by ELISA (D) or Western blot (E-F). No differences in hyperphosphorylated tau levels measured with AT8 were seen by ELISA (G) or Western blot (H-I). Immunohistochemistry with AT8 (Ser202/Thr205 phosphorylated tau) showed no difference in levels of hyperphosphorylated tau in Htau mice treated with TOMA (J) versus non-specific IgG (K). Scale bar 50 μm. Gallyas silver staining revealed equal NFT load (blue arrows) in Htau mice treated with TOMA (L) and non-specific IgG (M).

increased in comparison to sham animals, while levels of total tau remain the same, indicating an induction of tau oligomerization after injury [56]. Therefore these results are relevant for neurodegenerative tauopathies, such as AD which are not dependent upon mutations to tau, as well as TBI. Targeting oligomeric tau seeds with anti-tau oligomer-specific antibodies, such as TOMA, may be an effective means of prevention and reversal of cognitive deficits associated with tau pathology.

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