Two Novel Tau Antibodies Targeting the 396/404 Region Are Primarily Taken Up by Neurons and Reduce Tau Protein Pathology*

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Background: Tau immunotherapy is an emerging approach to treat Alzheimer disease. Results: Two novel Tau antibodies targeting the 396/404 region are primarily taken up by neurons and reduce Tau pathology. Conclusion: Receptor-mediated endocytosis and the intracellular endosome/autophagosome/lysosome system are likely involved in antibody-mediated clearance of pathological Tau. Significance: This study provides valuable insight into development of Tau immunotherapy.

Aggregated Tau proteins are hallmarks of Alzheimer disease and other tauopathies. Recent studies from our group and others have demonstrated that both active and passive immunizations reduce Tau pathology and prevent cognitive decline in transgenic mice. To determine the efficacy and safety of targeting the prominent 396/404 region, we developed two novel monoclonal antibodies (mAbs) with distinct binding profiles for phospho and non-phospho epitopes. The two mAbs significantly reduced hyperphosphorylated soluble Tau in long term brain slice cultures without apparent toxicity, suggesting the therapeutic importance of targeting the 396/404 region. In mechanistic studies, we found that neurons were the primary cell type that internalized the mAbs, whereas a small amount of mAbs was taken up by microglia cells. Within neurons, the two mAbs were highly colocalized with distinct pathological Tau markers, indicating their affinity toward different stages or forms of pathological Tau. Moreover, the mAbs were largely co-localized with endosomal/lysosomal markers, and partially co-localized with autophagy pathway markers. Additionally, the Fab fragments of the mAbs were able to enter neurons, but unlike the whole antibodies, the fragments were not specifically localized in pathological neurons. In summary, our Tau mAbs were safe and efficient to clear pathological Tau in a brain slice model. Fc-receptor-mediated endocytosis and the endosome/autophagosome/lysosome system are likely to have a critical role in antibody-mediated clearance of Tau pathology.

The pathological hallmarks of Alzheimer disease (AD) include aggregates of Aβ peptides and hyperphosphorylated Tau proteins in brain regions that are important for cognitive functions. The Aβ-containing plaques and Tau-containing neurofibrillary tangles, as well as their precursors, are generally believed to play a critical role in disruption of neuronal functions and subsequent loss of synapses and neurons. Therefore, to prevent or remove the Aβ and Tau aggregates is the key to develop treatments for AD, among which immunotherapy is an emerging and promising approach.

Disappointingly, several clinical trials of Aβ immunotherapy in AD patients reduced Aβ deposits but failed to generate substantial improvements in cognitive functions (1–5). These therapies were likely initiated too late to slow the progression of the cognitive decline in those patients (6). Tau lesions may precede Aβ deposition (7), and accumulating evidence indicates that the synergistic effects of Aβ and Tau pathologies lead to neurodegeneration (8–17). Furthermore, studies in human patients have shown that Tau pathology exhibits better correlation with severity of dementia than Aβ deposits (18–21). It has also been reported that Tau proteins are required for Aβ-induced neurodegeneration (10, 22). Last, several tauopathies exist without Aβ pathology. Therefore it is critical to develop Tau targeting therapies.

Studies from our group and others have demonstrated the efficaciousness of Tau immunotherapy in animal studies (23–30) and in a cell culture model (31). We have shown that both active and passive immunization targeting the Ser(P)-396/404 sites of the Tau protein reduced Tau pathology in two different transgenic mouse models (23, 25, 26). Importantly, immunized mice exhibited significant improvements in Tau-associated functional deficits including cognition, which are well correlated with antibody titers in animals.

Understanding the cellular mechanisms of antibody-mediated clearance of Tau aggregates is necessary to improve the efficacy and safety for further development of Tau immunotherapy. Our previous studies demonstrated that Tau antibodies injected into the carotid artery can reach and bind to Tau aggregates within the brain (23). The compromised blood-brain barrier in AD and related mouse models may facilitate entry of antibodies into the brain, which has also been reported in Aβ immunotherapy studies (32). Inside the neurons, we have...
detected the co-existence of Tau antibodies and pathological Tau proteins in lysosomes, suggesting the involvement of the endosome/lysosome pathway in antibody-mediated clearance of Tau aggregates (33). Similar intracellular co-localization has been observed by others studying antibodies targeting αB and α-synuclein aggregates (34, 35).

In our previous studies, we used purified antibodies from actively immunized mice to examine their subcellular location (33). However, although the mice were immunized with a phosphorylated Tau peptide (Tau 379–408, Ser(P)-396/404), the purified antibodies are polyclonal and do not specifically target one epitope. It is important to determine whether/how epitope recognition may influence the efficacy and safety profile of targeting the 396/404 region. Hence, we have developed two new monoclonal antibodies, 4E6G7 and 6B2G12, against the Tau 396/404 region. The two mAbs had distinct binding profiles: 4E6G7 is phospho-selective, and 6B2G12 is partially phospho-selective and partially conformational. First, we utilized a brain slice model of Tau pathology and found both mAbs were safe and effective to reduce pathological Tau in the slices. We then investigated the potential cellular mechanisms of antibody-mediated clearance of Tau pathology. Antibody uptake primarily occurred in neurons, whereas some was detected in microglia as well. Besides the endosome/lysosome pathway, the intracellular autophagy pathway also appeared to participate in the clearance. Finally, we found that the Fc portion of the antibodies was involved in the specific uptake of antibodies by neurons containing Tau aggregates.

EXPERIMENTAL PROCEDURES

Materials—Minimum essential media, Hanks’ balanced salt solution, horse serum, Alexa 568 antibody labeling kit, and Alexa 488-conjugated dextran were purchased from Invitrogen. Protease inhibitor mixture tablets were from Roche Diagnostics. The following primary antibodies were used for Western blot and immunocytochemistry: PHF1, MC1, CP13, and CP27 (gifts from Dr. Peter Davies, Albert Einstein College of Medicine, Bronx, NY); Tau5 (Fisher Scientific, Pittsburgh, PA); NeuN (Millipore, Billerica, MA); Iba1 (Wako Chemicals, Richmond, VA); glial fibrillary acidic protein(Dako, Carpinteria, CA); myelin basic protein (Covance, Princeton, NJ); EEA1, Rab7, and p62 (Cell Signaling, Danvers, MA); LC3 (Novus Biologicals, Littleton, CO); and Lamp2 (Abcam, Cambridge, MA). Pooled mouse IgG was purchased from Equitech-Bio (Kerrville, TX). Fab fragments were made from whole monoclonal antibodies by the Fab preparation kit from Fisher. Monoclonal antibodies were generated by GenScript Inc. (Piscataway, NJ).

Animals—Aged mice (12–15 months) from the JNPL3 line (36), which express human 0N4R Tau with the P301L mutation, and age-matched wild-type (WT) mice with the same background that do not express human Tau were used for antibody characterization and short term slice cultures. Pups (11–13 days) of JNPL3 mice were used in long term slice cultures. All animals were housed in accordance with NYU School of Medicine IACUC regulations in AAALAC approved facilities.

ELISA—Purified antibodies were tested for affinity to various phosphorylated and non-phosphorylated peptides containing the sequence of the Tau immunogen. Plates were coated overnight at 4 °C with the following Tau peptides (1 µg/well) in 50 mm carbonate buffer: 1) Ser(P)-396/404 (TDHGAEIVYK-[Ser(P)]PVVSGDT[Ser(P)]PRHL); 2) Ser(P)-404 (TDHGAEIVYKSPV[S]GDT[Ser(P)]PRHL); 3) Ser(P)-396 (TDHGAEIVYK-[Ser(P)]PVVSGDTSPRHL); or 4) non-Ser(P)-396/404 peptide (RENAKAKTDHGAEIVYKSPV[S]GDTSPRHL). Several washes were performed between all steps (0.1% TBS-T). Following coating, the plate was blocked with SuperBlock (Fisher Scientific) for 1 h at room temperature. Serial dilutions of 1 mg/ml of antibodies (1/1,000 to 1/243,000) were added in triplicate and incubated for 3 h at room temperature. Subsequently, HRP-conjugated anti-mouse IgG (1/3,000) was added and incubated for 1 h at room temperature. The plate was developed with TMB peroxidase EIA reagent (Fisher Scientific) and stopped by 2 N sulfuric acid. Absorbance at 450 nm was read on a BioTek Synergy 2 plate reader.

For competition ELISA, the same amount of peptides were coated in each well. mAbs (37 ng/ml) were preincubated with various concentrations of peptides for 30 min at room temperature. The preincubated mixtures were then added to the plate and incubated for 3 h at room temperature. The remaining procedure was the same as the regular ELISA described above.

Brain Slice Culture—Long term organotypic brain slice culture was used for antibody screening. Whole brain slices (400 μm) were obtained from 11–13-day-old pups of JNPL3 mice and plated on Millicell cell culture inserts (Millipore, Billerica, MA). The culture medium contains 50% minimum essential media (MEM) with Glutamax, 25% heat-inactivated horse serum, and 25% Hanks’ balanced salt solution. The slice culture was maintained at 37 °C with 5% CO₂ and the media was replaced every 3–4 days. After a 2-week stabilization period, Tau mAbs and control IgGs were added into culture medium at 10 μg/ml. An equal amount of slices were collected every week from 2 to 6 weeks.

Acute brain slice culture was used for the mechanistic studies. Coronal brain slices (400 μm) were prepared from aged JNPL3 mice (12–15 months old), and allowed to recover for 1 h at room temperature in brain slice buffer (all in mM: 124 NaCl, 3 KCl, 1.25 KH₂PO₄, 4 MgSO₄, 2 CaCl₂, 3.5 NaHCO₃, 10 glucose, 2 ascorbic acid, 0.075 adenosine, pH 7.4, bubbled with oxygen). For Western analysis, the brain slices were then incubated with 10 μg/ml of monoclonal antibodies in slice buffer at 37 °C for 2 h, and collected for Tau extraction. For immunocytochemistry analysis, the slices were incubated with 10 μg/ml of Alexa 568-labeled monoclonal antibodies in slice buffer at 37 °C for 2 h, and then fixed in 4% paraformaldehyde with 0.2% picric acid.

Tau Extraction and Western Blot—Brain slices were homogenized in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, pH 7.4) with protease and phosphatase inhibitors (1 μg/ml of protease inhibitor mixture, 1 mM NaF, 1 mM Na₂VO₄, 1 mM PMSF, 0.25% sodium deoxycholate). Homogenized tissue was centrifuged at 20,000 × g for 20 min and the supernatant was collected as the soluble Tau fraction. Equal amounts of protein from the soluble fraction was mixed with 1% Sarkosyl solution for 30 min and centrifuged at 100,000 × g for 1 h. The pellet was dissolved in O buffer (62.5 mM Tris-HCl, 10% glycerol, 5% β-mercaptoethanol, 2.3% SDS,
1 mM EDTA, 1 mM EGTA, 1 mM NaF, 1 mM Na<sub>2</sub>VO<sub>4</sub>, 1 mM PMSF, 1 µg/ml of protease inhibitor mixture) as the Sarkosyl-insoluble Tau fraction.

An equal amount of protein (10 µg) from both fractions was prepared in O+ buffer, boiled, and electrophoresed on 10% SDS-PAGE gels and transferred to nitrocellulose membranes. The blots were blocked in 5% milk in 0.1% TBS-T, incubated with various primary antibodies and HRP-conjugated secondary antibodies, and detected with ECL substrates (Fisher Scientific). Images of immunoreactive bands were acquired by Fuji LAS-4000 imaging system.

Immunocytochemistry—Fixed brain slices were cut into 40-µm sections on a cryostat. The sections were permeabilized in 0.3% Triton X-100 for 30 min, blocked in MOM blocking reagent (for primary antibodies generated in mouse) or 5% normal goat serum (for primary antibodies generated in other species), then incubated with various primary and corresponding secondary antibodies. Fluorescent imaging was performed on a Nikon C1 confocal system or a Zeiss LSM 700 confocal system.

Image Analysis—All image analysis was performed with Imagej software. To count the neurons and microglial cells containing mAbs or Fab fragments, at least 10 random images of cortical region from each mouse were taken. There were 3 mice in each treatment group. NeuN/Iba1-positive and mAb/Fab-positive cells were manually counted on each image.

RESULTS

Tau Monoclonal Antibodies Reduce Tau Hyperphosphorylation in Slice Culture—Our group first reported an active immunization study targeting a prominent pathological Tau epitope, Ser(P)-396/404. The active immunization by a peptide containing these two phosphorylated sites (Tau 379–408, Ser(P)-396/404) successfully reduced Tau pathology and improved related behavioral impairments in JNPL3 mice and hTau/PS1 mice (23, 25). In a passive immunization study targeting the same epitope with PHF1 antibody, we found similar beneficial effects (26). Now we have generated our own monoclonal antibodies, 4E6G7 and 6B2G12, against the same peptide Tau 379–408 (Ser(P)-396/404). The affinity of the two mAbs toward different Tau epitopes was characterized by ELISA. 4E6G7 exhibited strong binding to the Ser(P)-396/404 and the Ser(P)-404 peptides, with little binding to the Ser(P)-396 peptide. 4E6G7 also showed moderate binding to the non-Ser(P)-396/404 peptide, but significantly less compared with the Ser(P)-396/404 and Ser(P)-404 peptides at lower concentrations (p < 0.01, from 1/3,000 to 1/243,000). 6B2G12 recognized both phospho and non-phospho epitopes, with no significant differences among different peptides, suggesting that it is binding to the non-phospho region of these peptides (Fig. 1A). Additionally, we performed competitive ELISA by preincubation of each antibody with various Tau peptides to determine their selectivity. Preincubation of 4E6G7 with the Ser(P)-396/404 peptide blocked its binding to the same peptide as well as the Ser(P)-404 and the non-Ser(P)-396/404 peptides. Meanwhile, preincubation of 4E6G7 with the non-Ser(P)-396/404 peptide did not affect its binding to all 3 peptides, suggesting that 4E6G7 is phospho-selective (Fig. 1B). Interestingly, preincubation of 6B2G12 with the Ser(P)-396/404 peptide also inhibited its binding to all Tau peptides. Preincubation of 6B2G12 with the non-Ser(P)-396/404 peptide partially blocked its binding to the same peptide as well as the Ser(P)-396 and the Ser(P)-404 peptides, but not to the Ser(P)-396/404 peptide (Fig. 1B). These data suggested that 6B2G12 may be partially phospho-selective and partially conformational antibody that has high affinity toward the Ser(P)-396/404 epitope and moderate affinity toward the non-phospho Tau 379–408 region.

Both mAbs were able to detect pathological Tau in JNPL3 (Tau P301L) mouse brain by immunostaining (Fig. 1C). On Western blot, 4E6G7 preferentially detected pathological Tau proteins from JNPL3 mouse brains; whereas 6B2G12 was bound to Tau in both JNPL3 and wild-type mice (Fig. 1D). JNPL3 mice had a significantly higher 4E6G7 signal level (p < 0.05) and 4E6G7/Tau5 ratio (p < 0.05) compared with WT mice, although the differences were not as strong as for PHF1 (p < 0.01) and PHF1/Tau5 (p < 0.01). The significant difference in the 4E6G7/Tau5 ratio indicated that the preferential binding of 4E6G7 to pathological Tau did not result from Tau overexpression in JNPL3 mice.

To quickly screen the efficacy of the monoclonal Tau antibodies, we have established a brain slice system by culturing organotypic whole brain slices from pups of JNPL3 (Tau P301L) mice. After a 2-week stabilization period, monoclonal antibodies and control IgGs were added into culture media twice per week and an equal amount of slices was harvested every week from 2 to 6 weeks. A previous study that used a similar system showed that Tau pathology can be observed in the slices as early as 4 weeks in culture (37). Consistently, we detected a steady increase of PHF1 signal (Ser(P)-396/404) and the ratio of PHF1 over Tau5 (total Tau) in the control slice cultures treated with pooled mouse IgG, reaching 160 ± 33% at 6 weeks (Fig. 2, A and C). Meanwhile, we did not detect any significant increase in the percentage of total human Tau, as shown by the ratio of CP27 signal over Tau5, suggesting the increase of hyperphosphorylated Tau was not merely resulting from overexpression of human Tau (Fig. 2, A and C). However, Sarkosyl-insoluble Tau fractions did not exhibit any significant changes in PHF1 or CP27 signals from 2 to 6 weeks (Fig. 2, B and D). It should be noted that minimal and variable amounts of insoluble Tau proteins were detected in these slice cultures.

Compared with 6-week-old control slice culture, 4E6G7 and 6B2G12 significantly reduced the PHF1 level by 50 and 49%, and the PHF1/total Tau ratio by 31 and 39%, respectively (p < 0.05) (Fig. 2, A and C). The antibodies did not decrease the level of total Tau proteins (shown by Tau5) over the entire course of experiment, indicating no adverse effects by the treatment (Fig. 2, A and C). The amount of Sarkosyl-insoluble Tau in treated slices did not differ significantly from control slices (Fig. 2, B and D). Again, the very low and variable amount of insoluble Tau protein may have limited our ability to accurately detect any changes.

In summary, these results indicated the usefulness of the long term brain slice model to screen monoclonal antibodies for their efficacy in clearing soluble forms of pathological Tau protein. Both mAbs reduced hyper-phosphorylated Tau despite their different binding profiles, suggesting the Tau 396/404 region is an effective target to reduce Tau pathology.
Tau Antibodies Are Primarily Taken Up by Neurons and Co-localize with Distinct Pathological Tau Markers—To examine the cellular mechanisms of the antibody-mediated clearance of Tau pathology in the brain slice model, we labeled the two mAbs, 4E6G7 and 6B2G12, as well as pooled mouse IgG (control IgG), with a fluorescent dye, Alexa 568, to examine their cellular and subcellular distributions by confocal microscopy. The brain slices in long term culture become softer, thinner, and adhesive to the culture membrane after a few weeks, which makes them very difficult to collect for immunocytochemical studies. Therefore, we adapted a different short term slice culture system for the mechanistic studies (38). Whole brain slices were obtained from 12–15-month-old JNPL3 mice, which have extensive Tau pathology, and age-matched WT mice. The slices were allowed to recover for 1 h and then treated with mAbs for 2 h. In this short term culture system, the slices maintained their structural integrity and thickness after 3 h, which were better suited for immunocytochemistry analyses.

We first tested whether a 2-h treatment of non-labeled mAbs in acute slices can lower Tau pathology as well. However, antibody-treated slices did not show any decreases of hyperphosphorylated Tau in both soluble and insoluble fractions (Fig. 2E). The 2-h treatment was likely too short for the antibodies to clear Tau aggregates.

On the other hand, the 2-h treatment was enough for the Alexa 568-labeled mAbs to bind to intracellular Tau aggregates. Red fluorescence was widely seen throughout JNPL3 brain slices, indicating substantial antibody uptake. A majority of the labeled antibodies were located in neurons, as revealed by NeuN co-staining (Fig. 3, A and B). In control experiments, labeled mouse IgG was not detected in JNPL3 brain slices (Fig. 3C). Additionally, minimal uptake of mAbs was found in WT brain slices (Fig. 3D and E). These data suggested that the Tau mAbs were able to enter neurons and that intracellular pathological Tau protein facilitated the uptake and/or retention of antibody. Furthermore, a small amount of antibodies was found in microglia, as revealed by Iba-1 co-staining (Fig. 4, A and B). Staining for glial fibrillary acidic protein and myelin basic protein found virtually no antibodies in astrocytes or oligodendrocytes, respectively (Fig. 4, C–F). Overall, we estimated that ~85% of antibody signals were in neurons, 10% in microglia, and the remaining 5% associated with unidentified structures. The small amount of mAbs in microglia may be derived from antibodies binding...
to extracellular Tau proteins, which would then be endocyto-
tosed by microglia.

Subsequently, we co-stained the brain sections with patho-
logical Tau markers to determine whether the Tau antibodies
in the neurons bind to Tau aggregates. Indeed, most antibody-
containing neurons were positive for pathological Tau markers
PHF1 (Ser(P)-396/404), MC1 (conformational), and CP13
(Ser(P)-202) (Fig. 5). However, not all pathological Tau-positive
neurons had labeled antibodies, indicating that the antibodies
are not uniformly internalized. Interestingly, within the neu-
rons, the two mAbs exhibited different distribution patterns.
Most 4E6G7 signals were concentrated in puncta around the

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FIGURE 2. The monoclonal antibodies reduced Tau hyperphosphorylation in long term brain slice culture. A and B, representative images of Western blot analysis of soluble and Sarkosyl-insoluble Tau fractions from brain slices treated with the respective antibodies. The blots were detected by PHF1 recognizing Ser(P)-396/404 epitope, CP27 for total human Tau, and Tau5 for total Tau. C, quantified results showing the change of hyperphosphorylated Tau level and human Tau level. In control slices, the soluble Tau fraction exhibited a ~60% increase of PHF1/total Tau ratio. Our two monoclonal antibodies, 4E6G7 and 6B2G12, reduced PHF1 immunoreactivity by 50 and 49%, PHF1/Tau5 ratio by 31 and 39%, and PHF1/CP27 ratio by 15 and 43%, respectively, after 4 weeks of treatment (*, p < 0.05, two-way analysis of variance, Bonferroni’s post hoc test, n = 4 in each group). There were no differences in CP27, Tau5, or CP27/Tau5 ratios among different groups. D, quantified results of Sarkosyl-insoluble Tau detected by PHF1 antibody. The amount of Sarkosyl-insoluble Tau in antibody treated slices did not differ significantly from control slices. E, quantified results of the short term slice cultures treated with monoclonal antibodies for 2 h. Shown here is that neither mAbs had significant effects on the PHF1/Tau5 ratio in soluble and insoluble fractions. There was also no difference in PHF1 or Tau5 immunoreactivity in both fractions (data not shown).
Efficacy and Mechanistic Studies of Two Novel Tau Antibodies

The autophagy pathway has also been linked to intracellular Tau pathology. We probed the treated brain slices with autophagy pathway markers. However, no co-localization between autophagosome marker LC3 and labeled antibodies was detected in the cells (Fig. 7, A and B). This is probably because of the transient nature of LC3 on the autophagosomes, especially in neurons, where it is quickly degraded (39). We then stained the brain sections with another autophagy marker p62, which binds to protein aggregates and sorts them to autophagosomes (40). There was a partial co-localization of p62 with labeled antibodies, suggesting some involvement of the autophagy pathway (Fig. 7, C and D). The endocytic pathway and the autophagy pathway do not exclude each other, and actually the two pathways converge on lysosomes for protein degradation. Therefore, the whole endosome/autophagosome/lysosome system in the cell appears to be involved in the antibody-mediated reduction of intracellular Tau pathology.

**Tau Antibody Fab Fragments Do Not Specifically Bind to Tau Aggregates**—As shown above, and supported by our previous findings (33), endocytosis could be the major mechanism for the antibodies to gain access to intracellular Tau aggregates. Because it is known that a number of Fc receptors exist on the surface of neurons and microglia (41), we explored the role of Fc receptor-mediated endocytosis. To investigate this possibility, we generated Fab fragments from the two labeled mAbs and applied them to brain slices. Surprisingly, extensive Fab uptake was observed after a 2-h incubation in brain slices from JNPL3 mice. Most Fab fragments were taken up by neurons, similar to the intact antibody treated slices (Fig. 8, A and B). Moreover, control slices from WT mice also showed extensive uptake of Fab fragments (Fig. 8, C and D). The latter finding is in contrast with uptake of intact Tau mAbs, which was minimal in WT slices (Fig. 3, D and E). We counted that about 25% of total neurons in the cortical region of JNPL3 brain slices contained whole antibody signals, whereas WT slices had less than 10% of neurons taking up the mAbs (Fig. 8E). However, intracellular Fab fragments were detected in more than 60% of neurons in both WT and JNPL3 slices, which was significantly more than intact mAbs (Fig. 8E). It indicated that the Fab fragments were not specifically taken up by Tau pathology positive neurons.

Co-staining of PHF1 and MC1 confirmed that, in contrast to the intact mAbs, Fab fragments were present in many neurons that were negative for pathological Tau markers (Fig. 9, A–D). Inside the neurons, Fab fragments exhibited a generally diffuse pattern. In those neurons positive for Tau pathology, there was a partial co-localization between the fragments and PHF1 or MC1 signals (Fig. 9, E–H). These data suggest that the Fab fragments are likely internalized by different mechanisms than receptor-mediated endocytosis. Eventually, the Fab fragments end up in the lysosomes as evident by the co-staining with Lamp2.

One possible alternate pathway of the internalization is via bulk endocytosis. To examine this possibility, we treated brain slices together with fluorescence (Alexa 488)-labeled dextran and mAbs or Fab fragments. We found a partial co-localization between the mAbs and dextran (Fig. 10, A and B). The Fab fragments showed a much higher co-localization with dextran,

nucleus, and they were highly colocalized with PHF1 signals, indicating the high selectivity of 4E6G7 toward hyperphosphorylated Tau (Fig. 5A). On the other hand, 6B2G12 showed a more diffused pattern with a few small aggregates in the cells, which was similar to MC1 staining. Taken together with the ELISA data (Fig. 1, A and B), 6B2G12 may recognize earlier altered conformation in the 396/404 region that may or may not be phosphorylated but has not formed large aggregates (Fig. 5D). Both mAbs partially co-localized with CP13 signals, and 6B2G12 had a better match. It is likely that its epitope is more prominent in Tau lesions than the phospho epitope recognized by 4E6G7.

**Antibody-mediated Clearance of Hyperphosphorylated Tau Primarily Occurs in the Endosome/Autophagosome/Lysosome System**—Next, we investigated the relationship between the internalized antibodies and subcellular organelles to explore the cellular pathways involved in antibody uptake and clearance. 4E6G7 had a high degree of co-localization with endosome/lysosome markers, including EEA1 for early endosomes, Rab7 for late endosomes and lysosomes, and Lamp2 for lysosomes (Fig. 6, A, C, and E). The more diffuse distribution of the 6B2G12 antibody showed a partial co-localization with endosome/lysosome markers (Fig. 6, B, D, and F). These data suggested that antibody-mediated clearance of hyperphosphorylated Tau is likely to predominantly occur within the endosome/lysosome system. The autophagy pathway has also been linked to intracellular clearance of protein aggregates. We probed the treated

FIGURE 3. Co-staining of antibody or control IgG-treated brain slices with neuronal markers. Representative images of brains sections show the distribution of monoclonal antibodies or control IgG in neurons. A and B, labeled mAbs were widely detected in neurons in JNPL3 brain slices; C, labeled control IgG was not detected in JNPL3 slices; D and E, minimal uptake of mAbs was found in WT slices. Nuclei are stained blue with DAPI. Scale bar = 20 μm.
although their subcellular distributions were slightly different (Fig. 10, C and D). This indicated that the Fab fragments entered the neurons primarily via bulk endocytosis. Importantly, uptake and/or retention of Fab fragments are largely independent of intracellular Tau pathology, but anti-Tau Fab fragments appear to interact with Tau aggregates in the lysosomes.

**DISCUSSION**

In this study we developed a brain slice culture model to assess the efficacy and safety of monoclonal Tau antibodies for potential Tau passive immunotherapy. Two mAbs, 4E6G7 and 6B2G12 raised against Tau 379–408 (Ser(P)-396/404), significantly reduced the amount of hyperphosphorylated Tau in the brain slices after a 4-week treatment. Importantly, neither mAbs showed apparent toxicity, as evident by the stable total Tau level. These results were consistent with our previous active and passive immunization studies targeting the same epitope (23, 25, 26). The two mAbs had distinct properties based on ELISA data, with 4E6G7 being phospho-selective and 6B2G12 partially phospho-selective and partially a conformational antibody. Subsequent experiments in short term slices indicated that both mAbs specifically targeted neurons with Tau pathology. However, immunocytochemistry data sug-
gested their distinct binding properties toward intracellular Tau. 4E6G7 was highly specific for PHF1-positive Tau aggregates in the perinuclear region, which were likely already in the endosome/autophagosome/lysosome system. Despite binding to normal Tau protein on Western blot, 6B2G12 was specifically taken up/retained by pathological neurons, whereas only a limited uptake/retention was observed in WT tissue. The co-localization between 6B2G12 and MC1 staining and the partial block by the non-Ser(P)-396/404 peptide on ELISA suggested that it may recognize some abnormal conformation of Tau. Neutralizing this abnormal conformation may lead to reduced Tau pathology as well. Unfortunately, the long term brain slice model may not be appropriate to detect changes in insoluble Tau. Culture of brain slices from pups for several weeks shows a clear increase in soluble phosphorylated Tau over time but limited amounts of insoluble Tau aggregates. Alternatively, the aggregates may be lost during frequent medium change over the course of the study. Nonetheless, as suggested by our data, this brain slice model appears well suited to evaluate the efficacy and safety of antibodies before lengthy in vivo studies.

One key question regarding the mechanism of Tau immunotherapy is how the Tau antibodies cross the cell membrane and gain access to Tau aggregates in the neurons. Early reports have shown neuronal uptake of exogenous antibodies, probably via

- **FIGURE 5. Co-staining of antibody-treated brain slices with different pathological Tau markers.**

  A and B, 4E6G7 was highly co-localized with PHF1 signal. 6B2G12 exhibited a more diffused pattern in cytosol and was only partially co-localized with PHF1. C and D, 6B2G12 had better co-localization with MC1, which recognizes early abnormal conformation of Tau pathology, whereas 4E6G7 had a partial co-localization. E and F, both 4E6G7 and 6B2G12 showed a partial co-localization with CP13, which recognizes Tau Ser(P)-202. Arrows indicate colocalization. Nuclei are stained blue with DAPI. Scale bar = 10 μm.
Our previous studies found the ability of polyclonal mouse antibodies to bind to intracellular Tau aggregates after intracarotid injection in transgenic mice or incubation with brain slices (23, 33). In this study we performed more detailed and systematic studies on the uptake of two newly generated mAbs in JNPL3 brain slices that recognized different forms of Tau. The mAbs can indeed enter the neurons and bind to the intracellular Tau aggregates, as shown by their co-localization with pathological Tau markers inside the neurons. Importantly, most antibodies were taken up by pathology positive neurons, whereas not all pathological neurons contain antibodies. The presence of antibodies was minimal in control brain slices from WT animals. The mAbs may enter the neurons via receptor-mediated endocytosis (44). There is an increase in endocytic activities in AD brain (45), which could partially explain the uptake of mAbs by pathological neurons. Alternatively, it has been reported that AD neurons have a significantly higher amount of membrane-associated Tau protein (46). Membrane-associated pre-fibrillary Tau oligomers have been found to disrupt cell membrane integrity (47), which may allow the entry of Tau antibodies. We found that the two mAbs had different distribution inside neurons: 4E6G7 was more aggregated around the nucleus, whereas 6B2G12 was more diffused in the cell body. 6B2G12 likely rec-
ognized more pre-fibrillary Tau proteins with abnormal conformation, which were membrane-associated or in the cytosol. The binding of 6B2G12 to this misfolded Tau that has yet to form large aggregates may prevent its further aggregation, which will lead to reduced Tau pathology. Hyperphosphorylated Tau is prone to dissociate from the cell membrane and concentrate in the perinucleus region (48), which matched the aggregated appearance of phospho-selective 4E6G7. Regardless of the mechanisms of cell entrance, the intraneuronal Tau aggregates appear to promote the uptake/retention of antibodies, leading to the higher amount of mAbs observed in pathological neurons. This is consistent with our other study showing that the uptake of 4E6G7 strongly correlates with Tau pathology.3

Alternatively, it has been reported that extracellular Tau proteins exist and may be responsible for the propagation of Tau pathology in the brain (49–53). It has recently been shown that neurons continuously secret a small amount of Tau protein via the exosome pathway and other unconventional exocytosis pathways (52, 54). Furthermore, extracellular Tau aggregates can be internalized by adjacent cells and lead to Tau aggregation in healthy cells (55). In this situation, Tau antibodies can neutralize extracellular Tau aggregates and stop the spread of Tau pathology, as recently reported (31). Additionally, the depletion of extracellular Tau could lead to increased secretion and indirectly clear the intracellular Tau aggregates (44). The extracellular Tau antibody-aggregate complex should be cleared by microglial phagocytosis, which would explain our finding of antibodies in microglia. Although only a small portion of our mAbs was found in microglia, we cannot determine the relative extent of intracellular and extracellular clearance of Tau pathology in the brain slice model. The extracellular Tau aggregates and antibody-aggregate complexes may be released into media and not be detected.

Previous studies from our group have indicated the involvement of the endosome/lysosome pathway in antibody-mediated clearance of Tau aggregates (33). An increase in the lysosome protease cathepsin D was found in α-synuclein immunization studies, suggesting the clearance of intracellular α-synuclein aggregates via lysosomal activation (34). However, another Tau active immunization study reported a decrease in lysosome proteases (24). In the current study, we found co-localizations among endosomal/lysosomal markers, pathological Tau markers, and monoclonal antibodies. Lysosome dysfunction, including abnormal morphology and protease activity, is found in AD and other tauopathies (56). Once bound to early misfolded Tau and/or Tau aggregates, our mAbs could facilitate their disassembly and access of lysosomal enzymes, thus promote their degradation and improve lysosome function. This scenario could also increase the turnover

3 Congdon, E. E., Gu, J., Sait, H. B. R., and Sigurdsson, E. M. (October 25, 2013) J. Biol. Chem. 10.1074/jbc.M113.491001.
rate of intracellular endosomes and lysosomes, which may lead to enhanced endocytosis of the mAbs into the neurons. However, because our data from long term slice studies did not show any decrease in insoluble Tau aggregates, further studies are necessary to determine the role of the endosome/lysosome/autophagosome system in the clearance of Tau aggregates.

p62 is known as a selective substrate of the autophagy pathway, which is responsible for the degradation of intracellular bulk proteins (40). Interestingly, it has been found to be associated with neurofibrillary tangles in AD brains (57). Thus at least some of the Tau aggregates could be brought to the lysosome system via the autophagy pathway. We found a partial co-localization between p62 and the antibodies. It is likely that the endocytosed mAbs interact with p62 and Tau aggregates in the endosome/lysosome pathway. It is also possible that portions of the mAbs enter the neurons via a compromised cell membrane or are released from endosomes and bind to the Tau aggregates in the cytosol; then the complexes are sorted to autophagosomes by p62. Regardless of where the mAbs bind to Tau aggregates, the endocytic and autophagic pathways will converge on the lysosomes, where the degradation of Tau aggregates occurs.

FIGURE 8. Co-staining of Fab fragment treated wild-type (WT) and transgenic (P301L) brain slices with neuronal markers. A–D, representative images of brains sections show the distribution of Fab fragments in neurons: A and B, transgenic; C and D, wild-type. The majority of Fab fragments were detected in neurons. E, quantified data shows the percentage of neurons that are positive for antibodies (4E and 6B) or Fab fragments (4E Fab and 6B Fab) in the cortical region of treated brain slices from P301L and WT mice. Fab fragments were taken up by a significantly larger portion of neurons in both P301L and WT slices, which were not different between the two groups (**, p < 0.01, t test. n = 3 mice in each group). Nuclei are stained blue with DAPI. 4E, 4E6G7; 6B, 6B2G12. Scale bar = 20 μm.
FIGURE 9. Co-staining of Fab fragment-treated brain slices with pathological tau markers and endosome and lysosome markers. Fab fragments had a partial co-localization with pathological Tau markers PHF1 (A and B) and MC1 (C and D). Note that many neurons containing Fab fragments are negative for Tau pathology. Fab fragments co-localized infrequently with early endosomes (EEA1), but showed a partial co-localization with lysosomes (Lamp2) (E–H). Nuclei are stained blue with DAPI. Scale bar = 10 μm.
Furthermore, we explored the role of Fc receptor-mediated endocytosis in the uptake of antibodies. Different subtypes of Fc receptors are found in all major cell types in the central nervous system, including neurons, microglia, astrocytes, and oligodendrocytes (41). Findings from our laboratory indicate that the primary pathway of antibody uptake is via low affinity Fc receptors. Analogous to that report, using different methodologies, we found that the majority of antibodies are located in neurons, with a smaller portion in microglia cells. Some microglia cells could take up nearby extracellular Tau antibody-aggregate complex via Fc receptor-mediated endocytosis for clearance. Intriguingly, we detected extensive neuronal uptake of Fab fragments of Tau antibodies without specificity toward Tau pathology, as evidenced by the similar percentage of neurons containing Fab fragments in both JNPL3 and wild-type brain slices. The percentage (~60% in both JNPL3 and WT) was much greater than that of uptake of whole Tau antibodies (~25% in JNPL3, ~10% in WT). The co-localization between Fab fragments and dextran suggested the involvement of bulk endocytosis. The Fab fragments, which are much smaller (~50 kDa) than intact antibodies (~150 kDa), could be more easily internalized by bulk endocytosis. Importantly, the Fc receptor-mediated endocytosis seems to be crucial for directing the Tau antibodies specifically toward the Tau aggregates.

In conclusion, our new Tau mAbs were safe and efficient to reduce Tau pathology in the brain slice model. We found that the mAbs were able to access intraneuronal Tau aggregates and a large portion of antibodies and Tau aggregates co-localized with the endosome/autophagosome/lysosome system. Furthermore, the Fab fragments of the mAbs could also enter the neurons, but did not show specificity for toward Tau pathology. Hence, whole Tau antibodies, instead of their smaller Fab fragments, seem to be more appropriate as therapy for AD and other tauopathies.

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