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**Graphical abstract**

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Tau fibrils induce glial inflammation and neuropathology via TLR2 in Alzheimer’s disease–related mouse models

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Glial activation and inflammation coincide with neurofibrillary tangle (NFT) formation in neurons. However, the mechanism behind the interaction between tau fibrils and glia is poorly understood. Here, we found that tau preformed fibrils (PFFs) caused induction of inflammation in microglia by specifically activating the TLR2/MyD88, but not the TLR4/MyD88, pathway. Accordingly, the WT TLR2–interacting domain of MyD88 (wtTIDM) peptide inhibited tau PFF–induced activation of the TLR2/MyD88/NF–κB pathway, resulting in reduced inflammation. Nasal administration of wtTIDM in P301S tau–expressing PS19 mice was found to inhibit gliosis and inflammatory markers, as well as to reduce pathogenic tau in the hippocampus, resulting in improved cognitive behavior in PS19 mice. The inhibitory effect of wtTIDM on tau pathology was absent in PS19 mice lacking TLR2, reinforcing the essential involvement of TLR2 in wtTIDM-mediated effects in vivo. Studying the mechanism further, we found that the tau promoter harbored a potential NF–κB–binding site and that proinflammatory molecules increased transcription of tau in neurons via NF–κB. These results suggest that tau–induced neuroinflammation and neuropathology require TLR2 and that neuroinflammation directly upregulates tau in neurons via NF–κB, highlighting a direct connection between inflammation and tauopathy.

Introduction

Tauopathy is defined as a progressive neurodegenerative disorder caused by the abnormal accumulation of the protein tau. The characteristic tau accumulation and formation of neurofibrillary tangles (NFTs) in multiple brain regions are well manifested in diseases including Alzheimer’s disease (AD), progressive supranuclear palsy (PSP), frontotemporal dementia (FTD), Pick’s disease, and corticobasal degeneration (CBD) (1, 2). Tau proteins in humans exist in 6 different isoforms resulting from the alternative splicing of the pre–mRNA forming 3R and 4R tau species (3). In AD, both the 3R and 4R isoforms are found, but specifically in neurons. In addition to their prevalent deposition in neurons, tau aggregates are also found in astrocytes, specifically in brains affected with PSP and CBD, where isoforms of 4R tau are found in the filaments (4). The findings indicate that specific tau species found in the tangles differ among these diseases, and the mode of pathology may depend upon the brain region. In the case of AD–related tauopathy models, the existing literature indicates that tau inclusions emerge from the entorhinal cortex and travel through neuronal connections toward the hippocampus. Similarly, multiple studies using experimental animal models of AD pathology have demonstrated spreading of tau from the entorhinal cortex to the hippocampus (5, 6).

Microglia are the major brain-resident scavenger cells that actively take part in clearing pathogens, dying neurons, synapses, and aggregated proteins (7, 8). These physiological functions of microglia are indispensable for maintaining homeostasis in the developing and adult brain. However, according to Asai et al. (9), depletion of microglia halts tau propagation. Accordingly, exaggerated microglial inflammation has been well documented in preclinical animal models of tauopathy and convincingly demonstrated in human AD brains (10–14). Microglial activation is also found to coincide with the formation of phospho-tau aggregates in the hippocampus of neuron-specific tau–expressing P301S (PS19) mice (12, 15). However, the mechanism by which aggregated tau leads to microglial activation is poorly understood.

Here, we demonstrated that tau fibrils induced the activation of TLR2, but not TLR4, in microglial cells and that tau fibrils stimulated microglial inflammation via TLR2. Similarly, the WT TLR2–interacting domain of MyD88 (wtTIDM) peptide, capable of blocking the interaction of TLR2 with MyD88 (16), prevented tau–mediated TLR2 activation in microglia. Intranasal administration of wtTIDM in aged PS19 mice resulted in significant inhibition of neuroinflammation concomitant with reduced NFT formation in neurons and an improvement in cognitive behavior. Genetic deletion of TLR2 in PS19 mice also halted tau pathology in the hippocampus of PS19 mice. Most interestingly, we report that the tau promoter contained a consensus NF–κB–binding site, and therefore inflammatory molecules upregulated neuronal tau expression via NF–κB activation.

Results

Activation of microglia by fibrillar tau. Human full-length tau (2N4R) monomers were subjected to in vitro fibrillation in the presence of heparin for 7 days at 37°C, and then this solution was
centrifuged at 100,000g to precipitate the tau fibrils. This protein preparation was imaged under an electron microscope to validate successful generation of tau preformed fibrils (PFFs) (Figure 1A). By using the ToxinSensor Chromogenic LAL Endotoxin Assay Kit (GenScript), we also did not find any endotoxin contamination in PFFs (standard curve, Supplemental Figure 1A; level of LPS, 0.2 μM. (B-D) Induction of inflammatory molecules including iNOS, IL-1β, and TNF-α in WT primary microglia after treatment with different doses of tau PFFs (25, 50, and 100 nM) was measured by real-time PCR. Expression of iNOS (E), IL-1β (F), and TNF-α (G) in PFF-treated primary microglia derived from WT, TLR2−/−, and TLR4−/− pups was measured by real-time PCR. Protein expression of iNOS in PFF-induced primary microglia derived from WT (H and I), TLR2−/− (J and K), and TLR4−/− (L and M) was assessed by communostaining of iNOS and Iba1, followed by MFI analysis of iNOS (green) using ImageJ. Scale bars: 20 μm. Statistical analyses among multiple groups were conducted using 1-way ANOVA followed by Tukey’s multiple-comparison analysis, whereas unpaired 2-tailed t test was conducted for comparing 2 groups. *P < 0.05, **P < 0.01, and ***P < 0.001 compared with the untreated control group. Values are presented as mean ± SD (n = 3 different experiments).
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Figure 2. The wtTIDM peptide inhibits tau PFF-induced TLR2-MyD88 interaction and NF-κB activation in microglia. (A) BV2 cells were pretreated with wtTIDM or mTIDM (10 μM), followed by exposure to PFFs (25 nM), and after 1 hour of PFF administration, the TLR2-MyD88 interaction was monitored by IP. Input samples were probed for both anti-TLR2 and anti-MyD88 antibodies. (B) Densitometry shows the MyD88-bound TLR2 level compared with the input. (C) The interaction of TLR4 with MyD88 was also assessed in BV2 cells by IP under identical experimental conditions. NF-κB activation was measured in nuclear extracts isolated from TIDM-treated, PFF-exposed BV2 cells by EMSA (D) and by luciferase assay, wherein cells were initially transfected with luciferase reporter gene constructs (E). (F and G) The level of activated NF-κB in TIDM-treated, PFF-exposed primary mouse microglia was assessed by immunostaining of the phospho-Ser536 form of p65 in Iba1+ microglia, followed by MFI analysis of phospho-p65. (H–J) Primary microglia were pretreated with wtTIDM or mTIDM (5 and 10 μM) and then challenged with tau PFFs for 5 hours, followed by expression analysis of inflammatory genes (iNOS, IL-1β, and TNF-α) by real-time PCR. (K) The level of iNOS protein expression in TIDM-treated primary microglia was analyzed after 16 hours of PFF exposure by immunostaining. Scale bars: 20 μm. (L) MFI of iNOS expression was measured by ImageJ. Statistical analyses were performed by 1-way ANOVA, followed by Tukey’s multiple-comparison analysis. *P < 0.05, **P < 0.01 and ***P < 0.001 compared with the designated groups. Values are presented as mean ± SD (n = 3 different experiments).

The wtTIDM peptide inhibits glial inflammation in tauopathy. The inhibitory effect of wtTIDM against synthetic tau PFF– and AD-Tau-induced microglial inflammation led us to investigate its effect in an in vivo model of tauopathy. PS19 mice expressing P301S mutated human tau protein specifically in neurons are known to develop tau pathology with characteristic NFT formation by 6 months of age, whereas neuronal degeneration in the hippocampus is observed by 9 months of age (15). Interestingly, glial activation precedes formation of NFTs in the hippocampus. Given the fact these mice exhibit glial activation and inflammation prior to neuronal death, we first evaluated the status of TLR2 and MyD88 proteins in the resident microglia of hippocampus in PS19 mice 2 months ahead of the start of neurodegeneration (at 7 months of age). We found higher expression of TLR2 as well as MyD88 in Iba1+ microglia in these mice than age matched nontransgenic (nTg) mice (Supplemental Figure 6, A–D). The endogenous upregulation of TLR2 and MyD88 was also recapitulated in hippocampal astrocytes of PS19 mouse brain (Supplemental Figure 7, A–D). More importantly, enhanced expression of TLR2 and MyD88 coincided with aggravated microgliosis (Supplemental Figure 6E) as well as astrogliosis (Supplemental Figure 7E). Although this finding does not make it clear whether TLR2 and MyD88 upregulation was a result of initial tau pathology in PS19 mice, it does reveal that their upregulation paralleled glial activation in the hippocampus. Therefore, next we examined the role of TLR2/MyD88 in gliosis and inflammation. To achieve that purpose, we started wtTIDM/mTIDM nasal administration (0.1 mg/kg) in PS19 mice at the age of 7 months. After 1.5 months of TIDM treatment, multiple biochemical analyses were performed on samples from the hippocampal tissues.
Recently, we demonstrated that after intranasal administration, wtTIDM peptide is capable of entering the hippocampus (16). Here, we examined whether after intranasal administration, the wtTIDM peptide was capable of reducing the association between TLR2 and MyD88 in Iba1+ microglia in the hippocampus of untreated PS19 mice. As evident from triple-labeling of hippocampal sections with Iba1, TLR2, and MyD88, followed by imaging under super-resolution (Airyscan, Zeiss) confocal microscopy, TLR2 and MyD88 colocalized in Iba1+ microglia in the hippocampus of control untreated PS19 mice (Supplemental Figure 8, A and B). However, this association between TLR2 and MyD88 in Iba1+ microglia decreased in the hippocampus of PS19 mice treated with intranasal wtTIDM, but not mTIDM, peptide (Supplemental Figure 8, A and B).

Next we examined the effect of TIDM peptides on microglial activation in vivo in the hippocampus. Similar to that in the PFF-exposed primary microglia, the level of the activated RelA subunit of NF-κB (phospho-Ser536-p65) was higher in hippocampal microglia of PS19 brains as compared with nTg brains (Figure 3, A and B). Accordingly, expression of the downstream inflammatory protein iNOS in microglia was also higher in PS19 than in nTg mice (Figure 3, C and D). The induction of inflammation accompanied by an increased number of Iba1+ microglia signifies the occurrence of microglial activation (Figure 3E). However, treatment of PS19 mice with wtTIDM, but not mTIDM, markedly inhibited the activation of p65, upregulation of iNOS, and overall microglial activation in the hippocampus (Figure 3, A–E). Furthermore, Western blot analyses confirmed the specific inhibitory effect of wtTIDM on microglial activation and inflammation, as reduced levels of Iba1 and iNOS were found in wtTIDM-treated PS19 mice as compared with untreated PS19 mice (Figure 3, F–H). We also examined astroglial activation and found that iNOS expression and the number of GFAP+ cells were higher in astrocytes of PS19 brains as compared with nTg brains (Supplemental Figure 9, A–C). However, similar to the suppression of microglial inflammation, treatment of PS19 mice with wtTIDM, but not mTIDM, led to a reduction in iNOS in astrocytes (Supplemental Figure 9, A–C). Collectively, these data establish the fact that inhibition of TLR2 activation by wtTIDM reduced gliosis and inflammation in the hippocampus of PS19 mice.

wtTIDM peptide-mediated TLR2 inhibition mitigates NFT formation in neurons. Inhibition of glial inflammation was previously shown to attenuate tau pathology and related neurodegeneration in PS19 mice (15, 23). This prompted us to evaluate the effect of wtTIDM on NFT formation in neurons. Immunohistochemistry was performed for total tau protein using Tau-5 antibodies to monitor the level of tau deposition. The results demonstrated exaggerated NFT formation in granular cells of the DGs (Figure 4, A and B) and pyramidal neurons of CA1 (Figure 4, C and D) of PS19 mouse brains. In contrast, wtTIDM treatment drastically alleviated aggregated tau pathology in these neurons. However, mTIDM treatment did not result in any reduction in NFT formation (Figure 4, A–D), indicating the specificity of the effect. Similarly, immunohistochemistry with PHF1 antibodies indicated deposition of phospho-Ser396/Ser404 tau in CA3 and DG of PS19 mice that was reduced by intranasal treatment with wtTIDM, but not mTIDM, peptide (Supplemental Figure 10, A and B). Although we did not detect widespread phospho-Ser202-Thr205 tau in the hippocampus of 6-month-old PS19 mice by AT8 staining, we found its accumulation when the mice were 8.5 months old (Supplemental Figure 11, A and B). However, alleviation of total tau deposition in wtTIDM-treated 8.5-month-old PS19 mice was accompanied by a reduction in Ser202 and Thr205 phosphorylated tau accumulation, as revealed by immunostaining using AT8 antibodies (Supplemental Figure 12, A and B). Again, the mTIDM peptide had no such effect (Supplemental Figure 12, A and B), indicating specificity. To substantiate the data obtained from immunostaining, we conducted Western blotting on samples from hippocampal tissue fractions of experimental mice. No significant change in sarkosyl-soluble tau isomers (50–70 kDa) was found in either wtTIDM- or mTIDM-treated compared with untreated PS19 brains (Figure 4, E and F). On the other hand, a reduced level of total tau was observed in the sarkosyl-insoluble fraction isolated from the wtTIDM-treated PS19 brains in comparison to either untreated or mTIDM-treated PS19 brains (Figure 4, G and H). This finding indicates that it was not the soluble tau, but the formation of pathological tau aggregate that was affected by intranasal wtTIDM administration.

The wtTIDM peptide protects synaptic plasticity in PS19 mice. Loss of synaptic function and reduction in synaptic proteins are some important pathological features found in tauopathy brains even prior to neuronal demise (24, 25). Postsynaptic density 95 (PSD95) is one of those vital postsynaptic scaffold proteins that have been shown to be downregulated in pyramidal neurons in the hippocampus under the burden of phosphorylated tau. Here, we also found loss of PSD95 in microtubule-associated protein 2–containing (MAP2-containing) neurons of CA1 (Figure 5, A and B) and CA3 (Supplemental Figure 13, A and B) of 8.5-month-old PS19 mice. Furthermore, Western blot analysis confirmed overall loss of PSD95 in hippocampal tissues of these mice (Figure 5, C and D). However, loss of PSD95 was remarkably attenuated in CA1 (Figure 5, A and B) and CA3 (Supplemental Figure 13, A and B) of PS19 mice treated with wtTIDM, but not mTIDM, peptide.
Figure 4. wtTIDM nasal administration alleviates aggregated tau deposition in the hippocampus of PS19 animals. PS19 mice (7 months old) were given intranasal administration of wtTIDM or mTIDM (0.1 mg/kg) for 1.5 months, and content of NFTs in hippocampal neurons was assessed by immunohistochemistry using antibody specific for total tau (Tau-5). Images obtained from the DG (A) and CA1 (C) brain regions of the experimental PS19 mice are shown at 20× and 40× magnifications. Scale bars: 20 μm (left columns), 10 μm (right columns). (B and D) Relative OD of Tau-5 staining compared with the nTg mice was measured using Fiji. Two sections from each brain were included for immunostaining analysis, and the value obtained from each section is represented in the bar diagram. The total level of tau present in sarkosyl-soluble (sol) (E) and sarkosyl-insoluble (Insol) (G) tissue fractions was assessed by Western blotting. (F) The expression of total tau in the sarkosyl-soluble fraction was represented with respect to the actin present in the sarkosyl-soluble fraction. (H) On the other hand, the level of total tau in the sarkosyl-insoluble fraction was represented with respect to the actin present in the sarkosyl-insoluble fraction, as an actin band was not found in the sarkosyl-insoluble fraction. Arrows indicate the different isomers of tau, and the band near 70 kDa obtained from the sarkosyl-soluble fraction was considered for density analysis. Statistical analyses were conducted following 1-way ANOVA followed by Tukey’s multiple-comparison analysis. ***P < 0.001 compared with the respective groups. Values are presented as mean ± SEM (n = 5 different animals).
Similarly, we also observed a loss of synaptophysin in CA3 of 8.5-month-old PS19 mice that was significantly restored by intranasal administration of wtTIDM, but not mTIDM, peptide (Supplemental Figure 14, A and B). Next, the functional integrity of hippocampal neurons was measured by assessing Ca influx in hippocampal slices. Parallel to the loss of PSD95, reduced Ca influx through ionotropic glutamate receptors — including NMDA and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) — was observed in hippocampus of PS19 mice as compared with age-matched nTg animals (Figure 5, E and F). However, Ca influx through NMDA and AMPA receptors was markedly improved in wtTIDM-treated PS19 mice, and the value obtained for this group was almost the same as that in nTg mice (Figure 5, E and F). In contrast, an increase in PSD95 level or corrected Ca influx was not found in mTIDM-treated PS19 animals. The overall finding is of importance as improved synaptic functioning, which is essential for memory formation by hippocampal neurons, was achieved by wtTIDM administration, and that happened with concomitant downregulation of NFT formation in PS19 mouse brain.

The wtTIDM peptide ameliorates cognitive deficits in PS19 animals. We conducted cognitive tests to analyze the functional outcome of pathological tau reduction and improved synaptic function in the hippocampus by wtTIDM treatment. Barnes maze test exhibited impaired spatial learning and memory of untreated PS19 animals compared with the nTg group, and this was evidenced by errors and latency (Figure 6, A–C). Similarly, in the novel object recognition test (NORT), PS19 mice showed significantly lower preference for the novel object than did nTg mice (Figure 6, D–F). However, wtTIDM-treated PS19 mice made fewer errors in the Barnes maze and thereby reached the goal box much earlier than the untreated PS19 mice (Figure 6, A–C). In addition, wtTIDM-treated mice were found to have increased proclivity toward the novel object, and spent a longer time with it in the NORT (Figure 6, D–F). This specific behavioral improvement was not observed in the case of mTIDM-treated PS19 animals. We also conducted motor behavioral tests in these mice, considering the fact that PS19 mice start developing impaired motor activity as they age and eventually experience limb paralysis (15). However, in our experiments on PS19 mice, we observed no drastic or significant changes in horizontal motor activity or maintaining motor coordination. The experimental mice, including nTg, untreated PS19, and TIDM-treated PS19 mice, showed comparable distance moved (Figure 6, G and H), velocity (Figure 6I) in the arena, and time spent on the rotarod (Figure 6K). Only the time spent by PS19 animals in the center of the arena was found to be significantly shorter than that by nTg mice; however, wtTIDM treatment was not found to have any effect on these parameters (Figure 6, G–J). Together, these data indicate restoration of learning capability and memory consolidation in mice with tauopathy after intranasal treatment with wtTIDM.

The wtTIDM peptide-mediated decrease in NFT formation and improvement of cognitive functions were TLR2 dependent. To confirm that the effect of wtTIDM was mediated via TLR2 in vivo, we prepared double-transgenic mice heterozygous for mutant tau and homozygous for TLR2-null mutation by breeding PS19 mice with TLR2−/− mice. These mice were designated as PS19ΔTLR2 and validated by genotyping (Figure 7A). First, 8.5-month-old PS19 mice were compared with age-matched PS19ΔTLR2 mice in terms of glial activation, neuronal tau filament deposition, and the overall level of sarkosyl-soluble/insoluble tau. Immunohistochemical analysis of Iba1 showed a decrease in microglial activation in the hippocampus of PS19ΔTLR2 as compared with PS19 mice (Supplemental Figure 15, A and C). Similarly, GFAP staining also indicated reduced astrogial activation in the hippocampus of PS19ΔTLR2 in comparison with PS19 mice (Supplemental Figure 15, B and D). However, when 7-month-old PS19ΔTLR2 mice were treated with wtTIDM for 1.5 months, there was no further decrease in either microglial (Supplemental Figure 15, A and C) or astrogial (Supplemental Figure 15, B and D) activation.

Monitoring the status of NFTs also demonstrated that PS19ΔTLR2 mice had drastically decreased NFT formation in neuronal bodies present in both DG (Figure 7, B and C) and CA1 (Figure 7, D and E) compared with PS19 mice. Furthermore, protein analysis revealed no significant change in the sarkosyl-soluble form of total tau between these 2 groups (Figure 7, F and G), whereas markedly less tau was found in the insoluble fraction of the PS19ΔTLR2 compared with PS19 hippocampus (Figure 7, H and I). However, as with glial activation (Supplemental Figure 14), there was no further decrease in NFT level (Figure 7, B–E) or in the detergent-insoluble pathogenic tau level (Figure 7, F–I) compared with the untreated PS19ΔTLR2 mice. Similarly, Barnes maze test results also indicated attenuated impairment in spatial learning and memory in PS19ΔTLR2 mice (Figure 7, J–L). Again, although wtTIDM improved spatial learning and memory in PS19 mice (Figure 6), it was incapable of doing so in PS19ΔTLR2 mice (Figure 7, J–L). These results suggest that in the absence of functional TLR2 protein, wtTIDM remained unable to decrease pathological NFT formation and improve cognitive functions.

Proinflammatory cytokine induces tau expression in neurons via NF-κB. Our findings confirmed that TLR2 inhibition reduces both tau-mediated glial inflammation and formation of NFTs in neurons. It is still unclear how inflammatory molecules released from glial cells facilitate tau aggregation in neurons. To address this issue, we treated SH-SYSY human neuroblastoma cells with IL-1β, a proinflammatory cytokine released by activated microglia and other cells, and monitored the protein expression of tau. Interestingly, we found that tau expression was elevated in SH-SYSY cells with increasing doses of IL-1β (5–25 ng/ml) (Figure 8A). As human cells express a total of 6 alternatively spliced forms of tau, we have categorized these forms in 2 variants, where variant 1 represents the tau isoforms of higher molecular weight and variant 2 indicates the relatively lower-molecular-weight isoforms of tau; both of these variants were found to be increased following IL-1β exposure (Figure 8, B and C). IL-1β-induced tau upregulation was again validated by immunocytochemistry in SH-SYSY cells with 2 doses of IL-1β (5 and 10 ng/mL) (Figure 8, D and E). IL-1β signaling is known to activate the transcription factor NF-κB. Therefore, we examined the involvement of NF-κB activation in inflammation-induced tau expression in neurons. The DNA-binding activity of NF-κB was enhanced after IL-1β treatment, as evidenced by the formation of a distinct and specific complex in a gel shift DNA-binding assay (Figure 8F). It led to an increase in transcriptional activity of NF-κB, as shown by luciferase activity from a PBIIx-Luc construct, with maximum activation seen at a concentration of 15 ng/mL (Figure 8G). Next, we searched the promoter
region of tau using the MatInspector program and found a consensus binding site of NF-κB from 362 to 377 bp upstream of the transcription start site (Figure 8H). We cloned the tau promoter region containing the NF-κB-binding site into the PGL3 enhancer vector \([p-MAPT(WT)]\). We also mutated the core NF-κB–binding site and the mutated promoter construct \([p-MAPT(Mut)]\) was cloned into the PGL3 vector. We observed that IL-1β significantly induced luciferase activity driven by the WT \([p-MAPT(WT)]\), but not the \([p-MAPT(WT)]\)
mutated \([p\text{-MAPT(Mut)}]\), tau promoter (Figure 8I). Furthermore, ChIP coupled with real-time PCR analyses was conducted to validate NF-\(\kappa\)B-mediated transcriptional control of tau in SH-SYSY cells. Classical NF-\(\kappa\)B is a heterodimer of 2 subunits, p50 and p65, which were found to be highly recruited in the tau promoter following IL-1\(\beta\) treatment (Figure 8J and K). As transcriptional activation requires association of histone acetyltransferases, we examined recruitment of CREB-binding protein (CBP) and p300,
and found that IL-1β stimulation caused recruitment of p300, but not CBP, to the tau gene promoter; this coincided with enrichment of NF-κB in human AD brains and it is also recapitulated in relevant animal models (26, 27). Substantial aggregation of tau leads to progressive formation of NFTs, which hampers multiple cellular machineries, including ER, vescicle transport, autophagy, and mitochondrial functioning. As the excessive burden of tau progressively increases and spreads to other brain regions, the possibility arises that glial cells

Figure 7. The wtTIDM treatment fails to reduce tau pathology and improve cognitive behavior in PS19 mice lacking TLR2. (A) PS19 mice were bred with TLR2–/– mice to obtain double-transgenic PS19TLR2–/– mice. These mice were validated by genetic screening, where the 331 bp and 279 bp bands corresponded to nTg and PS19 mice, respectively. Similarly, the 499 bp and 334 bp bands indicate nTg and TLR2–/– mice, respectively. PS19TLR2–/– mice (7 months old) received wtTIDM (0.1 mg/kg/d) nasal administration for 1 month; and at 8.5 months of age, tau pathology in the hippocampus was compared with that of untreated PS19TLR2–/– and PS19 mice by conducting immunohistochemistry with Tau-5 antibodies. Tau aggregation was monitored in both DG (B) and CA1 (D) neurons. Scale bars: 20 μm (left columns), 10 μm (right columns). (C and E) OD of tau expression was calculated relative to that in nTg mice. Two sections from each brain were used for the staining and quantitative analysis of tau expression, and the values obtained from each section are shown in the bar diagram. Images are shown at 20x and 40x magnifications.

The total tau content in sarkosyl-soluble (F) and insoluble fractions (H) was assessed by Western blotting. The tau band densities obtained from the sarkosyl-soluble (G) and -insoluble fractions (I) was normalized to the loading control, actin, present in the soluble fraction. Arrows indicate the different isomers of tau, and the band near 70 kDa obtained from the sarkosyl-soluble fraction was considered for density analysis. Spatial learning and memory were tested by Barnes maze (J), heat map (K; error; L, latency). Statistical analyses were performed using 2-way ANOVA followed by Tukey’s multiple-comparison analysis. **P < 0.01 and ***P < 0.001 compared with the respective groups. Values are presented as mean ± SEM (n = 4 animals per group).

Discussion

The disease-related mutations of tau, including P301S and P301L, are known to cause reduced interaction of this protein with microtubules (28). Mutated tau has a higher tendency to self-aggregate and form paired helical filaments, which is dependent on its R2 and R3 regions at the C-terminus (29) and might also be dependent on heavy phosphorylation of certain residues (30, 31). Substantial aggregation of tau leads to progressive formation of NFTs, which hampers multiple cellular machineries, including ER, vescicle transport, autophagy, and mitochondrial functioning. As the excessive burden of tau progressively increases and spreads to other brain regions, the possibility arises that glial cells...
might also interfere in the pathogenesis. Ongoing research clearly demonstrates that microglia phagocytose extracellular tau (32) via specific receptors on microglia. This necessitated identification of the phagocytosed, or if this activation requires binding of tau to specific receptors on microglia. In that context, TLR2 is a receptor protein of tau PFFs. Our findings on tau-mediated TLR2 activation can further boost generation of inflammatory molecules. The enhanced level of TLR2 and MyD88 in glial cells of 7-month-old PS19 mice proves that the effect of wtTIDM on glial activation and tau pathology in TLR2 ablated PS19 animals proves that the effect of wtTIDM is specific to TLR2 in vivo. Last, our study provides the evidence that inflammatory molecules activate tau expression in SH-SY5Y neuronal cells and primary human neurons through NF-κB-dependent manner.

Synthetic fibrillar tau closely mimics features of tau fibrils obtained from human AD brains (37). Injection of tau PFFs in entorhinal cortex not only recapitulates the signature mode of its propagation toward hippocampus, but also forms more complex proteinase-resistant aggregates that are immunostained by TG3, a conformation-specific phospho-tau mAb, by anti-acetylated tau K280 antibody and also strongly stained for Thio-S (15, 38, 39). It explains the reason behind considering synthetic tau PFFs for studying glial activation in vitro. Efforts made by previous studies have demonstrated that tau, in monomeric, oligomeric and fibrillar forms activates microglia. However, monomeric tau-induced inflammation was not found to be as enormous as fibrillar tau as the extent of upregulation of inflammatory molecules was much less, whereas the dose required to induce inflammation was much higher compared with the PFFs used by us and also by another study (40, 41). The existing report also highlighted the involvement of p38 MAPK in mediating tau-induced inflammation. It has to be considered that kinases activated by tau exposure are present in the cytosol, whereas primary interaction of microglia with extracellular protein aggregates must involve any receptor protein in the cell membrane. In that context, TLR2 has not been hypothesized in previous studies to be a receptor protein of tau PFFs. Our findings on tau-mediated TLR2 activation fit well with the observations in previous studies that TLR2 activation can lead to activation of different kinases including p38 MAPK and can trigger microglial phagocytosis (42, 43). In addition, these reports did not show the crucial involvement of canonical NF-κB activation in inducing inflammatory genes in PFF-exposed microglia, which is revealed in the present study. There was a possibility that PFF-induced NF-κB activation might also result from activation of other TLRs, specifically TLR4, whereas primary interaction of microglia with extracellular protein aggregates must involve any receptor protein in the cell membrane. In that context, TLR2 has not been hypothesized in previous studies to be a receptor protein of tau PFFs. Our findings on tau-mediated TLR2 activation might also result from activation of other TLRs, specifically TLR4, which is known to interact with different oligomeric aggregated forms of proteins including Aβ (43, 44). However, the absence of induction of inflammatory genes in TLR2−/−, but not in TLR4−/− mice, and also wtTIDM-sensitive suppression of inflammation in PFF-exposed microglia convincingly establishes the role of TLR2/MyD88 in carrying out the inflammatory effect of tau fibrils in microglia.

The enhanced level of TLR2 and MyD88 in glial cells of 7-month-old PS19 brains provides a sharp indication that upregulation of these proteins happens concurrently with gliosis. Most importantly, these events paralleled NFT formation in the brain. It is also noteworthy that astroglial upregulation of TLR2 and MyD88 under pathological conditions can lead to astroglial inflammation, whereas microglial activation can further boost generation of inflammatory molecules by astrocytes (46). Evidence for TLR2-dependent inflammation
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in tauopathy was reinforced, as nasal administration of wtTIDM caused downregulation of microgliosis, astrogliosis, and inflammation in the brain. Moreover, reduction of neuronal tau deposition and NFT formation in the hippocampus in wtTIDM-induced mice established a strong connection between glial inflammation and tau aggregation in vivo. On the other hand, synapse loss in hippocampal neurons precedes NFT formation and neuronal death in PS19 mice (15, 47). In that context, attenuated loss of synaptic integrity by wtTIDM was proven by the increased level of PSD95 protein on CA1 neurons. PSD95 is greatly implicated in scaffolding of multiple receptor proteins activated by the excitatory neurotransmitter glutamate and in promoting glutamate receptor-mediated Ca influx required for memory formation (48, 49). Upregulation of PSD95 in wtTIDM-treated mice corrected the Ca influx in hippocampal cells and retained synaptic plasticity in PS19 animals. This finding demonstrates the indirect neuroprotective effect of TLR2 inhibition on overall functioning of the hippocampus; the effect was further replicated in the behavioral studies, in which wtTIDM-treated animals displayed better learning capability as well as retention of spatial memory. That participation of TLR2 is crucial in tau pathology was strongly supported by the finding that TLR2 ablation significantly abrogated formation of insoluble tau deposition in neurons. Furthermore, the finding that wtTIDM treatment of TLR2-ablated PS19 animals did not reduce tau pathology clearly suggested that wtTIDM peptide required TLR2 for exerting its neuroprotective effect.

Another important finding in this study is that the tau promoter contained a potential NF-κB-binding site. As a majority of the inflammatory factors employ the NF-κB pathway to activate target genes, the presence of this consensus sequence in the tau promoter clearly exhibited the bidirectional relationship between inflammation and tauopathy. More interestingly, as neurons express TLR2 on the cell membrane and TLR2 expression was also found to be increased in neurons under degenerative conditions (50), there is a possibility that extracellular tau can even activate the TLR2/MyD88/NF-κB pathway in neurons to activate tau transcription, and that facilitates irreversible tau generation and accumulation in neurons. Recently, a report elaborated the role of microglial NF-κB in tau-mediated inflammation and tau spreading (51). Our report adds knowledge to this subject by demonstrating that not only tau-mediated glial inflammation but also inflammation-mediated tau exacerbation in neurons was dependent on the NF-κB pathway. Therefore, these two events can be seen a double-edged sword: both aspects can be controlled by targeting the TLR2/MyD88/NF-κB pathway.

In the last decade, numerous studies have demonstrated antinflammatory and neuroprotective effects of different pharmacological molecules in tauopathy brains (23), but in the absence of proper knowledge about tau-induced receptor activation in glial cells, these approaches might face several challenges. This study reveals the TLR2/MyD88/NF-κB pathway as the potential link between the signature prerequisite events of progressive neurodegeneration in tauopathy brains: tau aggregation and glial inflammation. Therefore, blocking TLR2/MyD88/NF-κB-mediated inflammation with the synthetic peptide wtTIDM holds potential therapeutic value against AD, PSP, FTD, CBD, and other tauopathies.

Methods

Reagents. DMEM was purchased from Mediatech, and FBS was obtained from Atlas Biologicals. Antibiotic-antimycotic was purchased from MilliporeSigma. Recombinant human Tau-441 was purchased from AnaSpec. Primary antibodies used in the study are listed in Supplemental Table 1. Cy2- and Cy5-conjugated antibodies and fluorophore-tagged secondary antibodies were obtained from Jackson ImmunoResearch Laboratories Inc. AD brain-derived insoluble tau was provided by Virginia M.-Y. Lee, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania, USA.

Animals. Adult C57BL6, TLR2-/- (B6.129-TR2Δexik/J), TLR4-/- (B6(Cg)-Tlr4tm1.Karp/J), and PS19MAPT (B6.C3-Tg(Prnp-MAPT* P301S)PS19Vle/cj) mice were purchased from The Jackson Laboratory.

Cell culture and isolation of primary mouse microglia. Primary microglia were isolated from mixed glial cultures as described by us previously (52, 53). BV-2 murine microglial cells (a gift from V. Bocchini, University of Perugia, Perugia, Italy) was maintained in DMEM/F12 medium containing 10% FBS at 37°C in the incubator.

Culturing human neurons. Culturing of human neurons was performed as described previously (54, 55). For details, see Supplemental Methods.

TIDM peptides. TIDM peptides (>99% pure) (16, 50) were synthesized in the custom peptide synthesis facility of GenScript. TIDM peptides contained the antennapedia homeodomain (lower case) and 6-amino-acid-long MyD88 (upper case) segments: WT TIDM: dRqikiwfqnrrmkwkkPGWHQD; mTIDM: dRqikiwfqnrmkkwkkPGWHQD. Positions of mutations are underlined.

Intranasal treatment of animals with TIDM peptides. Intranasal treatment of mice with TIDM peptides was performed as described previously (16, 50). For details, see Supplemental Methods.

Preparation and validation of tau PFFs. Full-length human tau monomers (2N4R tau) were solubilized in 0.1 M acetate buffer (pH 7.4) at a concentration of 44 μM to prepare the tau stock. During fibrillation, 22 μM tau along with heparin (1:4 molar ratio) was rotated continuously in a rotary shaker at 500 rpm at 37°C for 7 days (37). Next, the solution containing tau and heparin was centrifuged at 100,000g for 30 minutes, at 4°C to precipitate the tau fibrils. The supernatant was discarded, and the pellet containing fibrils was solubilized in an equal volume of acetate buffer. The PFFs were characterized by electron microscopy (EM). For EM imaging, 1 μL stock solution was diluted in 10 μL PBS, and this solution was adsorbed to a 300-mesh copper, Formvar-coated EM grid, washed, and stained with 1% uranyl acetate, and the grid was allowed to dry for 15–20 minutes. Imaging was performed at 100,000× magnification using a JEOL JEM-1220 transmission electron microscope (operating at 80 kV). Digital micrographs were acquired using an Erlangshen ES1000W model 785 CCD camera and DigitalMicrograph software (version 1.7).


Tissue lystate preparation. Tissue lysate was prepared as described previously (16, 50). For details, see Supplemental Methods.

Western blotting. Western blotting was performed as previously described (56, 57). For details, see Supplemental Methods.

Immunostaining. Immunostaining was carried out as described previously (58, 59). For details, see Supplemental Methods.

Real-time PCR. Total RNA was isolated from primary microglia using the QIAGEN RNeasy kit following the manufacturer’s
protocol. The isolated RNA was reverse transcribed into cDNA, and real-time PCR was performed using the following the primers: mouse iNOS — sense 5’-CCCTTCGGAAGTTTCTTGCGACGAGCAGGC-3’, antisense 5’-GGCTGTCAGGCTCTGTGGCTTGGG-3’; mouse IL-1β — sense: 5’-GGATGATGCAAAAGTGCAGTGG-3’, antisense 5’-AGTACCATGGTGGGGAACCT-3’; mouse TNF-α — sense 5’-TCCTGTCATGAACTTCCGCGGAGTAC-3’, antisense 5’-GTATGAGATGCAACCTGGCTACGCTTGGAAG-3’; human MAPT — sense 5’-ACCTGGCATCCTGTGGTGGTGG-3’, antisense 5’-GAGCCTACAGCTTGGGCAAG-3’; mouse GAPDH — sense: 5’-GGTAGAGTTCGTTGGAAGGCG-3’, antisense 5’-TGACGTCACCCCTCAAGGGTG-3’. Real-time PCR was carried out in a QuantStudio 3 detection system (Thermo Fisher Scientific) using the SYBR green real-time kit obtained from Quantabio.

**ChIP assay.** Recruitment of NF-κB to the MAPT gene promoter was determined by ChIP assay as described earlier (62, 63). For details, see Supplemental Methods.

**EMSA.** EMSA was carried out as described previously (61, 63). For details, see Supplemental Methods.

**Construction of the human MAPT promoter–driven reporter construct.** Human genomic DNA isolated from primary human neurons was used as the template during PCR. The 5’-flanking sequence of human MAPT (~455/+30) gene was isolated by PCR. Primers were designed from GenBank sequences as follows: MAPT — sense 5’-acgcgtCTCCTGGCAGCCTCAGGCTCAGTACG-3’, antisense 5’-gagctcTCTCCCATACCTTGCAGGTCG-3’. While the sense primer was tagged with a MluI restriction enzyme site, the antisense primer was tagged with a XhoI restriction endonuclease site. PCR was performed using an Advantage-2 PCR kit (Clontech) according to the manufacturer’s instructions. The resulting fragments were gel-purified and ligated into the pGEM-T Easy vector (Promega). These fragments were further subcloned into the PGL3 Enhancer vector after digestion with the corresponding restriction enzymes and verification by sequencing (ACGT Inc. DNA Sequencing Services).

**Cloning of human MAPT promoter and site-directed mutagenesis.** Site-directed mutagenesis was performed as described earlier (50, 62, 64) by using a site-directed mutagenesis kit (Stratagene). Two primers in opposite orientations were used to amplify the mutated plasmid in a single PCR. The primer sequence for the mutated promoter site was as follows: sense, 5’-GTATTTTTTGAGATGTTTAGTTCTCTTGGCCAGG-3’, and antisense, 5’-CCTGGCCGACACATGTTAAATCACTCTCTAAAAATAC-3’. The PCR product was precipitated with ethanol and then phosphorylated by T4 kinase. The phosphorylated fragment was self-ligated by T4 DNA ligase and digested with the restriction enzyme DpnI to eliminate the nonmutated template. The mutated plasmid was cloned and amplified in E. coli (DH5-α strain)–competent cells.

**Luciferase assay.** The luciferase assay was performed as described previously (65, 66). For details, see Supplemental Methods.

**Ca assay.** Ca influx in hippocampal slices was measured as described earlier (67, 68). For details, see Supplemental Methods.

**Behavioral tests.** To determine cognitive function and movement abilities in mice, 4 major kinds of behavioral tests (Barnes maze, NORT, open field test, and rotarod test) were performed as described earlier (56, 58, 64, 67, 68). For details, see Supplemental Methods.

**Statistics.** Statistical analyses were performed using GraphPad Prism v9.0. Values are expressed as mean ± SD for data obtained from cellular studies and mean ± SEM for animal experiments. Statistical comparisons between 2 different samples were conducted by using unpaired 2-tailed t test. One-way ANOVA followed by Tukey’s multiple-comparison test was performed for statistical analyses among multiple groups. Two-way ANOVA was used for comparing more than 1 parameter among different groups. The criterion for statistical significance was P < 0.05.

**Study approval.** Animal housing, maintenance, and experiments were performed following the guidelines provided by the NIH and were approved by the IACUC (protocol 21-044) of the Rush University Medical Center.

**Data availability.** No new code was generated in this study; all analyses were performed using existing packages. Values for all data points in graphs are reported in the Supporting Data Values file.

**Author contributions**

KP conceived the original idea, supervised the project, acquired the funding, and edited the final version of the manuscript. DD, MJ, and KP designed the study. DD, MJ, RKP, MM, SR, and SD performed the research. DD, MJ, RKP, MM, SR, and KP analyzed the data. DD wrote the first draft of the manuscript.

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