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*J Immunol* published online 3 August 2011
http://www.jimmunol.org/content/early/2011/08/03/jimmunol.1100216

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Misfolded Truncated Protein τ Induces Innate Immune Response via MAPK Pathway

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Neuroinflammation plays a key role in the pathogenesis of Alzheimer’s disease and related tauopathies. We have previously shown that expression of nonmutated human truncated τ (151-391, 4R), derived from sporadic Alzheimer’s disease, induced neurofibrillary degeneration accompanied by microglial and astroglial activation in the brain of transgenic rats. The aim of the current study was to determine the molecular mechanism underlying innate immune response induced by misfolded truncated τ. We found that purified recombinant truncated τ induced morphological transformation of microglia from resting into the reactive phenotype. Simultaneously, truncated τ caused the release of NO, proinflammatory cytokines (IL-1β, IL-6, TNF-α), and tissue inhibitor of metalloproteinase-1 from the mixed glial cultures. Notably, when the pure microglial culture was activated with truncated τ, it displayed significantly higher levels of the proinflammatory cytokines, suggesting a key role of microglia in the τ-mediated inflammatory response. Molecular analysis showed that truncated τ increased the mRNA levels of three MAPKs (JNK, ERK1, p38β) and transcription factors AP-1 and NF-κB that ultimately resulted in enhanced mRNA expression of IL-1β, IL-6, TNF-α, and NO. Our results showed for the first time, to our knowledge, that misfolded truncated protein τ is able to induce innate immune response via a MAPK pathway. Consequently, we suggest that misfolded truncated protein τ represents a viable target for immunotherapy of Alzheimer’s disease. The Journal of Immunology, 2011, 187: 000–000.

The prominent pathological features of Alzheimer’s disease and related tauopathies are τ neuronal and/or glial lesions that correlate with clinical symptoms and disease progression (1–3). τ is an intracellular microtubule-associated protein that belongs to the family of the intrinsically disordered proteins characterized by the absence of a rigid three-dimensional structure in their natural environment (4). However, in disease condition, posttranslational modifications such as truncation and hyperphosphorylation lead to τ transformation from intrinsically disordered protein into highly ordered, soluble and insoluble misfolded structures (5–10). It has been hypothesized that endogenous intracellular τ may be released into the extracellular space upon neuron degeneration (11). Indeed, neuronal death is one of the major pathological hallmarks of Alzheimer’s disease (AD). It is noteworthy that neuronal loss has been linked to the topographic distribution of neurofibrillary tangles in several stereological studies in AD brains (12–16). Simultaneously, clinical research consistently demonstrated an increase in total and phospho-τ in the cerebrospinal fluid of AD patients (17). All these findings strongly pointed out that intracellular τ is released into the brain’s extracellular environment.

Several independent studies showed that soluble extracellular τ may promote 1) neurotoxicity by interacting with specific receptors on the surface of neurons (11, 18, 19); 2) intracellular calcium increase through M1 and M3 muscarinic receptors in neuronal cells (18); 3) synaptic impairment (20); 4) blood–brain barrier damage (21). Recent results cast a new light on the role of insoluble extracellular τ as a transmissible agent spreading τ pathology throughout the brain in “prion-like fashion” (22–24). Multiple lines of evidence also indicate that extracellular τ protein may play an important role in AD neuroinflammation. Activated microglia are present in and around neurofibrillary tangles at early (25) and at later (26–32) stages of tangle formation. Microglial activation also correlates with τ burden in other human tauopathies such as tangle-predominant dementia, Guamanian parkinsonism-dementia, progressive supranuclear palsy, and corticobasal degeneration (33–35). Moreover, activation of microglia linked to τ deposition has been documented in mice transgenic for human mutant τ protein P301S (36, 37), R406W (38), or P301L (39) and in transgenic rats expressing human nonmutated truncated τ (40, 41). Notwithstanding these findings, the mechanism underlying τ-mediated microglial activation has not been identified. To unravel this riddle, we used recombinant truncated τ protein to induce inflammatory response in the primary microglial and mixed glial cultures. In the current study, we show for the first time to our knowledge that truncated τ is able to activate microglia via the MAPK pathway leading to the release of NO, proinflammatory cytokines, and chemokines.

Materials and Methods

τ protein purification

Human truncated τ protein was purified from Escherichia coli bacterial lysates according to a published procedure (42) with modification. Briefly, the bacterial lysates were purified two times on cation-exchange HiTrap SP Sepharose HP columns. The fractions that contained truncated τ protein

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Received for publication January 21, 2011. Accepted for publication June 28, 2011.

This work was supported by Axon Neuroscience and by research grants from the Slovak Research and Development Agency of Slovak Republic (Grant APVV-0631-07 to N.Z., Grants APVV-0603-06 and APVV LPP-0363-06 to M.C.), and Grant APVV LPP 0043-09 to M.C.).

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Abbreviations used in this article: AD, Alzheimer’s disease; iNOS-2, inducible NO synthase-2; LAL, Limulus amebocyte lysate; MS, mass spectrometry; TIMP-1, tissue inhibitor of metalloproteinase-1.

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were pooled and purified by size-exclusion chromatography on a HiLoad Superdex 200 26/60 column. Then, the buffer was exchanged for DTT free buffer, and the protein was further immunoaffinity purified using the DC25 mAb column. Finally, the protein was concentrated on a cation-exchange HiTrap SP Sepharose HP column. To prevent the oxidation, prepared \( \tau \) protein was stored in PBS under argon atmosphere at \( \sim 70^\circ \text{C} \). The purity of truncated \( \tau \) protein was routinely checked by SDS gel electrophoresis followed by Coomassie blue, silver staining, or Western blot with DC25 Ab.

**MALDI-TOF**

Mass spectrometry analysis of recombinant protein was performed at the Bruker Daltonics facility (Bremen, Germany) on an HCT Ultra ion trap and an Ultraflex III MALDI-TOF/TOF (sinapinic acid as matrix). In some experiments, prepared \( \tau \) protein was subjected to nano liquid chromatography (Easy nLC; Proxeon) before the MALDI-TOF analysis.

**Detection of bacterial endotoxin in \( \tau \) protein using the Limulus amebocyte lysate (LAL) kinetic turbidimetric method.** The method is based on monitoring of the rate of the development of turbidity, which is inversely proportional to the concentration of endotoxin in the sample. Unknown concentration of endotoxin in the sample was determined by interpolating the onset time of the sample in the linear regression of the calibration curve. Reagents used for the test were purchased from Associates of Cape Cod. Control standard endotoxin (bacterial endotoxin derived from *E. coli* 0113:H10) was used as a standard. All other material used (e.g., buffer, LAL reagent water, LAL reagent, glass tubes, tips) were endotoxin-free (Associates of Cape Cod). Detection and quantification of bacterial endotoxin in \( \tau \) protein by LAL test was hindered by interference in the sample. To overcome the inhibiting activity of \( \tau \) protein, a special treatment of the sample was required. The combination dilution–heating procedure was developed for the sake of troubleshooting the sample based on the method recommended by Associates of Cape Cod for the kinetic turbidimetric method. The end-dilution used (1:180) was chosen based on the results of preliminary experiments taking into account the maximum valid dilution (1:1000). The sample was diluted only with LAL reagent water. The pH of the sample after dilution met the requirements for testing (pH 6.8). The range of the calibration curve used was 0.001–1.000 EU/ml with the sample spiked with 0.5 EU/ml as positive control product. After treatment of the sample (dilution 1:180, heating for 30 min at 60°C), all the requirements for the validity of the assay (correlation coefficient \( r^2 = 0.98 \)) and the requirements of the sample results (pH 6.8, spike recovery 50–200%) were met.

**Glial cell cultures**

Rat mixed glial cell cultures were prepared according to McCarthy and de Vellis (43). Cerebral cortices of newborn rats (0–1 d old) were dissected, stripped of their meninges, and mechanically dissociated by repeated pipetting followed by passage through a nylon mesh. Cells were plated in 96-well plates and 75-cm \(^2\) flasks precoated with poly-L-lysine (10 \( \mu \)g/ml) and 1:100 dilution of glial cell culture medium and washed with 1 ml prewarmed PBS. Total RNA was isolated using RNeasy Mini Kit according to the manufacturer’s recommendations (Qiagen, Hilden, Germany). The genomic DNA was removed by DNase I digestion during the RNA purification. RNA was eluted into 40 \( \mu \)l RNase-free water. The integrity of isolated total RNA samples was determined with an Agilent 2100 Bioanalyzer using an RNA 6000 Nano Labchip kit (Agilent Technologies, Waldbronn, Germany). For transcriptomic analysis, high-quality RNA samples were used (RNA integrity number = 8.0 to 9.5).

**Data analysis**

Values are presented as the means \( \pm \) SEM. Statistical analysis was performed using a one-way ANOVA (GraphPad Prism). Tukey’s multiple comparison test was used for post hoc comparison. Differences at \( p < 0.05 \) were accepted as statistically significant.

**Results**

**Purification and proteometric characterization of recombinant \( \tau \)**

To avoid bacterial macromolecular contamination, we included a DC25 immunoaffinity purification step in the preparation of recombinant truncated \( \tau \) with the highest purity. The results shown in Fig. 1A represent the silver, Coomassie blue, and DC25 staining
revealed the presence of two major peaks with corresponding direct infusion experiment performed on an HCT Ultra ion trap and purity mass spectrometry experiments were performed. A protein band was observed around 29 kDa and represents a small could be explained by its nonglobular character. Another minor band a DC25 immunoaffinity column.

FIGURE 1. The proteomic analyses of the purity of the human truncated τ. SDS-PAGE, WB, and MS analysis of immunoaffinity-purified truncated τ. To get truncated τ with the highest purity, we included in the purification step a DC25 immunoaffinity column. A, Silver staining, Coomassie blue staining, and immunoblotting with DC25 Ab revealed the presence of one major band with the apparent molecular mass ∼32 kDa. B, Electrospray ionization (ESI)+ mass spectrum after software deconvolution showed a major peak representing human truncated τ (25 kDa) and a small τ fragment (16 kDa; aa 1–161).

of the immunoaffinity-purified τ protein. The protein band corresponding with 32 kDa represents truncated τ protein. The discrepancy between the molecular mass of truncated τ determined on SDS-PAGE (32 kDa) and its exact theoretical mass (25 kDa) could be explained by its nonglobular character. Another minor protein band was observed around 29 kDa and represents a small τ fragment that could not be separated during the DC25 immunoaffinity purification. To confirm protein, primary structure and purity mass spectrometry experiments were performed. A direct infusion experiment performed on an HCT Ultra ion trap revealed the presence of two major peaks with corresponding mz of 16,645.8 and 25,415.3 (Fig. 1B). These were in the next experiment separated by use of nano liquid chromatography and subjected to MALDI in-source decay analysis. The obtained sequences were compared with the sequence part [1–161], and the major 25-kDa peak refers to the expected 25-kDa recombinant truncated τ sequence. No other protein peaks were observed within mass spectrometry (MS) spectra indicating very high protein purity.

Human recombinant truncated τ was not contaminated with bacterial LPS

To exclude the possibility that the glial cultures were activated by the residual contamination with LPS, we tested NO release in the presence of polymyxin B, which is widely used as an inhibitor of LPS. We treated our mixed glial cultures with truncated τ with or without the presence of polymyxin B (10 μg/ml) (Fig. 2). There was no significant effect of polymyxin B preincubation on truncated τ-stimulated NO release (7.65 ± 0.23 versus 6.38 ± 0.25, n = 16) by the cells suggesting that this effect is specific to truncated τ. As we have expected, the effect of LPS was completely abolished by the presence of polymyxin B (7.0 ± 0.48 versus 1.99 ± 0.08, n = 16). Polymyxin B alone has no effect on NO release. To identify possible endotoxin contamination in the sample, an LAL kinetic turbidimetric test was also performed. We found that the content of bacterial endotoxin in tested samples of τ protein was under detection limit of the assay (<0.001 EU/ml).

Truncated τ protein stimulates microglial transformation from resting to reactive phenotype

Activation of microglia is characterized by typical morphological changes. The activated microglia develops enlarged cell processes, which gives the cell a bushy appearance. In the late phase of activation, they became brain macrophages with typical round shape. To demonstrate whether truncated τ induces morphological changes in vitro, a mixed glial cell culture model was used. Nonstimulated microglia in mixed glial cell culture showed resting ramified morphology similar to the situation in vivo (Fig. 3A, 3C). Stimulation of mixed glial cultures with truncated τ protein (1 μM) for 24 h led to significant morphological changes. These include loss of cellular branching and transition from ramified to amoeboid (activated) state (Fig. 3B, 3D).

Misfolded truncated τ protein induced cytokine in mixed glial cultures

To characterize activation of microglia triggered by human truncated τ protein further, we measured the release of several cytokines and tissue inhibitor of metalloproteinases using ELISA assays. The results showed that stimulation of mixed glial cultures with truncated τ protein already at 100 nM concentration resulted in significant release of proinflammatory cytokines such as IL-1β (Fig. 4A, n = 2), IL-6 (Fig. 4B, n = 2), and TNF-α (Fig. 4C, n = 2) and of TIMP-1 (Fig. 4D, n = 2).

Microglial activation induced by truncated τ is mediated by MAPK pathways

Many key cellular responses to extracellular stimuli are mediated by kinase and phosphatase cascades. One of the most important kinase families involved in immune response is the MAPK family. To identify a potential MAPK signaling pathway activated by truncated τ, we incubated primary mixed glial cultures with or without preincubation with ERK1/ERK2 and p38 MAPK inhibitors. Our results clearly showed that truncated τ-induced NO production was significantly blocked by pretreatment with the ERK1/ERK2 MAPK inhibitor (PD98059) at 5 μM and 50 μM (p < 0.001, Fig. 5A). Similarly, treatment with the p38 MAPK inhibitor (SB202190) at concentrations above 0.2 μM and 2 μM markedly reduced NO production (p < 0.001, Fig. 5B). Notably, none of the tested inhibitors was able to abrogate completely the effect of truncated τ activation suggesting that both kinases, p38 and ERK1/ERK2, are activated simultaneously.
Microglial primary culture released cytokines after treatment with truncated \( \tau \) protein

Next, we analyzed production of proinflammatory cytokines by primary microglia cultures using different concentrations of human truncated \( \tau \) protein (0.1–1 \( \mu M \)).

As shown in Fig. 6, there was a significant increase in proinflammatory cytokine IL-1\( \beta \) (Fig. 6A, \( n = 2 \)), IL-6 (Fig. 6B, \( n = 2 \)), and TNF-\( \alpha \) (Fig. 6C, \( n = 2 \)) production upon addition of truncated \( \tau \) protein. The levels of the cytokines released after treatment with truncated \( \tau \) were significantly higher compared with the levels released by treated astroglia–microglia culture. On the contrary, activated microglia did not express TIMP-1. Notably, the 0.1 \( \mu M \) truncated \( \tau \) was able to induce all tested proinflammatory cytokines suggesting that truncated \( \tau \) was immunogenic already at nanomolar concentration.

Human truncated \( \tau \) stimulates activation of microglia through NF-\( \kappa \)B- and MAPK-dependent pathways

We have demonstrated that human truncated \( \tau \) protein (151–391, 4R) induces morphological changes and distinct activation of rat primary microglia. To identify the underlying molecular mechanisms involved in the truncated \( \tau \)-induced activation of microglia, we have analyzed the gene expression of several kinases, transcription factors, and target inflammatory genes. Quantitative real-time PCR analysis revealed upregulated mRNA expression of JNK1 (2.6-fold), p38\( \beta \) (2.3-fold), and ERK1 (2.2-fold) kinases upon treatment of microglia with 1 \( \mu M \) human truncated \( \tau \). No difference in the mRNA levels was observed in the case of p38\( \alpha \) (1.4-fold change) and ERK2 (1.1-fold change) kinase (Fig. 7A). Notably, peak upregulation of MAPK mRNA expression was determined after 6 h, whereas prolonged incubation (12 h) showed that the levels of mRNA either gradually decreased (ERK1) or remained stable (JNK1, p38\( \beta \)). These data indicate that JNK1, p38\( \beta \), and ERK1 are the key players in the MAPK signaling of truncated \( \tau \)-induced activation of microglia.

To identify downstream transcription factors involved in the truncated \( \tau \)-induced activation of microglia, we analyzed mRNA levels of NF-\( \kappa \)B and AP-1 factors. We found upregulated mRNA expression of c-Jun (8.7-fold) and c-Fos (3.8-fold), the integral components of AP-1 transcription factor, in microglia after 1 h of truncated \( \tau \) treatment. Levels of these factors decreased gradually in the course of time. Notably, increased levels of NF\( \kappa \)B1 (7-fold) and NF\( \kappa \)B2 (16-fold) were determined after 6 h of treatment (Fig. 7B). These data clearly indicate the sequence of molecular events upon the truncated \( \tau \) treatment, where elevation of AP-1 transcription factor precedes the NF-\( \kappa \)B signaling.
Activation of MAPK-dependent pathways and NF-κB signaling resulted in distinct elevation of target inflammatory genes already after 1 h of treatment (IL-1β, 17.8-fold; IL-6, 9.3-fold; TNF-α, 111.7-fold; iNOS-2, 2.1-fold). This effect was either sustained (TNF-α, 104-fold) or further increased (IL-1β, 322-fold; IL-6, 11,044-fold; iNOS-2, 11,520-fold) after 6 h of treatment. Prolonged (12 h) incubation of microglia in the presence of human truncated leads to additional elevation of iNOS-2 (28,323-fold) and IL-6 (15,950-fold) levels; however, the levels of TNF-α (26-fold) and IL-1β (170-fold) are gradually decreasing compared with those at 6-h incubation (Figs. 7C, 8).

Discussion

τ protein represents a key player in the pathogenesis of AD and related tauopathies (2, 3). Under physiological circumstances, τ protein is viewed as an intracellular cytoplasmic protein. However, it has been detected in extracellular biological fluids, such as human cerebrospinal fluid. Moreover, its levels in the cerebrospinal fluid are significantly elevated in AD patients, and it is used as a biomarker (17). τ is able to accumulate in the extracellular space usually as a consequence of neuronal death and may significantly contribute to neurodegeneration (11). In the current study, we report for the first time to our knowledge that misfolded truncated τ protein is a potent inflammatory mediator.

In this study, we focused on truncated τ protein, which has been shown to be a driving force behind neurofibrillary degeneration in transgenic rats expressing misfolded truncated τ (44, 45). We have previously demonstrated that in the transgenic rat brain, neurofibrillary lesions and axonal degeneration are closely associated with the distribution of reactive microglia and macrophages (40). To identify the potentially crucial role of misfolded truncated τ in neuroinflammation, we have treated microglia–astroglia culture with this pathological form of τ. To avoid the possible bacterial protein contamination, we enriched the purification procedure using pan-τ mAb DC25 for immunoaffinity purification. Mass spectrometry analysis of purified τ revealed the presence of two major peaks with corresponding m/z 16,645.8 and 25,415.3, which represent the minor 16-kDa τ form [1–161] and the major 25-kDa form of misfolded τ (151-391, 4R), respectively. No other protein peaks were observed within the MS spectra demonstrating the absence of bacterial protein contaminants. The results presented earlier demonstrate that LPS is a frequent and functionally significant contaminant in many commercial-grade preparations of proteins and peptides used commonly in research on microglial activation (46). To exclude LPS contamination, we have tested truncated τ in the absence and/or presence of the LPS inhibitor polymyxin B. We found that polymyxin B did not eliminate the microglial activation induced by truncated τ. Moreover, the LAL test confirmed the absence of bacterial endotoxins in our τ samples.

The majority of our experiments were done on the mixed glial cultures that allow us to work with highly ramified microglia, which is considered to be the predominant microglial phenotype in the normal brain (47). Microglial ramification is better developed (48–51), and the microglia is less susceptible to exterior signals when cocultured with astrocytes than a pure microglial culture (47, 52,

![Figure 5](http://www.jimmunol.org/)

**FIGURE 5.** Truncated τ activates microglial cells via p38 and ERK1/ERK2 MAPK pathways. PD98059 (A; p44/42 kinase inhibitor, 5 and 50 μM) and SB202190 (B; p38 kinase inhibitor, 0.2 and 2 μM) were added to the culture medium 30 min before treatment with truncated τ (1 μM). After 24 h, NO secretion was quantified by the Griess reaction. Values represent the mean ± SEM of two experiments conducted in quadruplicate. ***p < 0.001 (versus truncated τ-stimulated cells without pretreatment).

![Figure 6](http://www.jimmunol.org/)

**FIGURE 6.** Primary rat microglia released proinflammatory cytokines after stimulation with truncated τ. Primary microglia isolated from 20-d-old mixed glial cultures was stimulated with truncated τ protein (0.1–1 μM). Media were collected, and IL-1β (A), IL-6 (B), and TNF-α (C) concentrations were determined via commercial ELISA. Data represent the values from two independent experiments performed in duplicate.
MAPK family members are involved in the τ-induced signal transduction cascade. Using specific inhibitors of ERK1/ERK2 kinase (PD98059) and p38 kinase (SB202190), we found that MAPK pathways were involved in microglia inflammatory response induced by truncated τ. Previously, it has been shown that activation of p38 and ERK1/ERK2 MAPK contributes to the activation of transcription factors such as AP-1 or NF-κB, which in turn induces the release of proinflammatory cytokines and other inflammatory molecules (61, 62). Our transcriptomic results clearly show that human truncated τ induced upregulation of mRNA expression of several MAPKs (JNK1, p38β, ERK1) and transcription factors (c-Jun, c-Fos, NFκB1, NFκB2) that further increase transcription of proinflammatory genes ultimately leading to the release of proinflammatory cytokines IL-1β, IL-6, and TNF-α (Fig. 8).

Multiple membrane receptors have been implicated in microglial activation and intracellular signal transduction pathways (63). In particular, the class B scavenger receptor CD36 has been shown to mediate the microglial proinflammatory response to other proteins involved in neurodegeneration, amyloid β (64–66) and α-synuclein (67). Other cell surface proteins suggested to function as αβ receptors include the class A scavenger receptors SRA (68, 69) and B1 (70), the receptor for advanced glycation end products (71), heparin sulfate proteoglycans (72, 73), the serpin enzyme inhibitor complex (74), and many more. Identification of the receptors for truncated τ in microglial cells would be a further important step toward understanding the mechanism of the extracellular τ inflammatory cascade.

Previously, it has been shown that diseased-modified neuronal proteins are able to activate microglial cells at higher concentrations than those of bacterial endotoxins: amyloid β at the concentration 0.2–1 μM (69, 75, 76) and α-synuclein at the concentration 0.1–1 μM (77, 78). Similarly, the active concentration of misfolded τ was in the same range between 0.1 and 1 μM. Furthermore, τ concentration in the brain remains a matter of debate. Several authors showed that the concentration of intraneuronal τ can reach 1–2 μM (11, 79, 80, 81), whereas others argue that it could be even higher, 5–10 μM (82). Furthermore, Gómez-Ramos et al. (11) estimated that τ in the extracellular space would be ~130 nM. In this study, we showed that τ is able to activate microglia at the concentration of 100 nM,
which indicates that extracellular τ protein acts as a potent innate inflammatory stimulus. Moreover, we provide new insights into the understanding of inflammatory pathways activated by misfolded truncated τ protein in AD and related tauopathies. We suggest that misfolded truncated τ-mediated inflammatory response represents a viable target for immunotherapy of human AD.

Disclosures

The authors have no financial conflicts of interest.

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