NEURAL REGENERATION RESEARCH
September 2016, Volume 11, Issue 9
www.nrronline.org

RESEARCH ARTICLE

Effects of triptolide on hippocampal microglial cells and astrocytes in the APP/PS1 double transgenic mouse model of Alzheimer's disease

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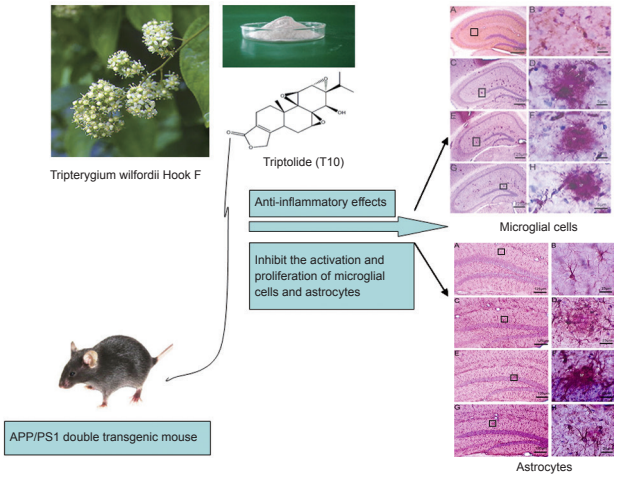
How to cite this article: *Li JM, Zhang Y, Tang L, Chen YH, Gao Q, Bao MH, Xiang J, Lei DL (2016) Effects of triptolide on hippocampal microglial cells and astrocytes in the APP/PS1 double transgenic mouse model of Alzheimer's disease. Neural Regen Res 11(9):1492-1498.*

Funding: *This study was supported by China Postdoctoral Science Foundation, No. 2016M590757; the Postdoctoral Science Foundation of Xiangya Hospital of Central South University of China, No. 20; the Hunan Provincial Natural Science Foundation of China, No. 2015JJ6010; a grant from the Basic Research Program of Science and Technology Commission Foundation of Hunan Province of China, No. 2015JC3059; the Project Fund of the Department of Education in Hunan Province of China, No. 15A023, 13C1107; the Scientific Research Project Fund of Health Department of Hunan Province of China, No. B2011-071, B2016096; a grant from the Construction Program of the Key Discipline in Hunan Province of China.*

Graphical Abstract

Triptolide inhibits activation and proliferation of microglial cells and astrocytes in the hippocampus following Alzheimer's disease



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doi: *10.4103/1673-5374.191224*

Accepted: *2016-08-16*

Abstract

The principal pathology of Alzheimer's disease includes neuronal extracellular deposition of amyloid-beta peptides and formation of senile plaques, which in turn induce neuroinflammation in the brain. Triptolide, a natural extract from the vine-like herb *Tripterygium wilfordii* Hook F, has potent anti-inflammatory and immunosuppressive efficacy. Therefore, we determined if triptolide can inhibit activation and proliferation of microglial cells and astrocytes in the APP/PS1 double transgenic mouse model of Alzheimer's disease. We used 1 or 5 μg/kg/d triptolide to treat APP/PS1 double transgenic mice (aged 4–4.5 months) for 45 days. Unbiased stereology analysis found that triptolide dose-dependently reduced the total number of microglial cells, and transformed microglial cells into the resting state. Further, triptolide (5 μg/kg/d) also reduced the total number of hippocampal astrocytes. Our *in vivo* test results indicate that triptolide suppresses activation and proliferation of microglial cells and astrocytes in the hippocampus of APP/PS1 double transgenic mice with Alzheimer's disease.

Key Words: *nerve regeneration; neurodegenerative disease; traditional Chinese medicine; Tripterygium wilfordii Hook F; triptolide; Alzheimer's disease; amyloid plaques; amyloid-β; amyloid precursor protein; inflammation; microglia; astrocytes; neural regeneration*

Introduction

The pathological changes seen in Alzheimer's disease (AD) brain reflect a series of chronic inflammatory processes. The chronic inflammatory response is characterized by the presence of abundant activated microglia. Moreover, reactive astrocyte proliferation is associated with deposition of amyloid-beta (Aβ), formation of amyloid plaques (senile plaques) and neurofibrillary tangles (Gil-Bea et al., 2012), and increased expression of proinflammatory cytokines and complement components (Salminen et al., 2009; Villegas-Llerena et al., 2016). Non-steroidal anti-inflammatory drugs not only reduce the risk of AD, but also delay AD progression (Pasinetti, 2002; Mcgeer and Mcgeer, 2007). Animal studies have shown that non-steroidal anti-inflammatory drugs suppress Aβ deposition and senile plaque formation, inhibit microglial activation and reactive astrocyte proliferation, and decrease expression and secretion of proinflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and interleukin-1β (IL-1β) in AD brain (Weggen et al., 2001; Smith et al., 2002). Therefore, the anti-inflammatory effects of these drugs appear to play an important role in AD. Nonetheless, there are serious and poisonous side effects associated with their use (Vane 2000), which limits their clinical application.

Triptolide (T10) compound is an active component derived from a traditional Chinese medicine extract, *Tripterygium wilfordii* Hook F. It possesses anti-inflammatory and immunosuppressive functionality, and has been used to treat rheumatism, rheumatism arthritis, systematic lupus erythematosus, and other autoimmune and inflammatory responsive diseases (Han et al., 2012; Ho et al., 2013; Huang et al., 2013; Peng et al., 2014). Previous *in vitro* research has suggested that the anti-inflammatory and immunosuppressive effects of T10 are due to suppression of expression and release of pro-inflammatory cytokines (such as IL-1β and TNF-α), as well as suppression of inducible nitric oxide synthase and cyclooxygenase-2 induction (Dai et al, 2005; Tong et al., 2007; Zhou et al., 2007; Tang et al., 2012; Wu et al., 2013; Cui et al., 2016). T10 has also been shown to have a neurotrophic function, and protects mesencephalic dopaminergic neurons from lipopolysaccharide injury (Li et al., 2004; Zhou et al., 2005). The predicted neuronal protective mechanism of T10 involves inhibition of microglial activation and suppression of the inflammatory reaction (Li et al., 2004; Zhou et al., 2005). To date, there are no reports on the effect of T10 on the inflammatory and immune cascade of AD brain. Accordingly, in the present study, we are the first to use T10 to treat the APP/PS1 double transgenic mouse model of AD (APP/PS1 dtg). Our study is preliminary, to assess the effect of T10 on APP/PS1 dtg mice. We also discuss the possibility and putative mechanism for T10 to treat or prevent AD.

Materials and Methods

Animals

Eighteen male dtg APP/PS1 mice and five C57BL/6JNNIA (B6) male wild-type control mice aged 4–4.5 months and weighing 25–30 g were obtained from the Animal Center of Xiangya School of Medicine, China (animal license No: SYXK (Xiang) 2014-0023). Two to five mice were housed in plastic cages with corncob bedding, and free access to food and filtered water in a vivarium maintained on a 12-hour light/dark cycle at 22.0 ± 0.5°C.

Animal use was in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. All experimental procedures were approved by the Ethics Committee of Central South University Xiangya School of Medicine, China, for animal care and use [Ethic Committee (EC) number: EC/11/018, 6/15/2011].

Treatments

APP/PS1 dtg mice were randomly divided into three groups: high-dose T10 treatment (purity: 99%, Sigma, St. Louis, MO, USA) (T10-H group, $n = 6$, 5 μg/kg/d, intraperitoneal injection), low-dose T10 treatment (T10-L group, $n = 6$, 1 μg/kg/d, intraperitoneally), or placebo (PLC group) (intraperitoneal injection of an equivalent volume of normal saline). Triptolide was dissolved in dimethyl sulfoxide as 100 × stock solution and diluted with normal saline for the concentration used. Treatments were administered at approximately 10:00 a.m. every day for 45 days. Five wild-type male mice received no treatment and were used as controls.

Tissue preparation

After 45 days of treatment, mice were deeply anesthetized by CO_2 inhalation, and transcardially perfused with 0.1 M phosphate-buffered saline (PBS), followed by standard fixation using 4% paraformaldehyde in 0.1 M PBS (pH 7.4). After removal, the brain was post-fixed in 4% paraformaldehyde overnight. Next, brains were transferred to 30% sucrose in 0.1 M PBS until they sank, then frozen in dry ice/isopentane, and stored at −80°C until sectioning. Each brain was serially sectioned along the coronal plane using a sliding freezing microtome (Walldorf, Germany). Sections were cut using an instrument setting of 50 μm and sampled in a systematic-random manner, *i.e.*, the first five sections were randomly selected, and then every fifth section systematically cut.

Immunohistochemistry

For microglia visualization, systematic-random sections through the hippocampus (10–12 sections per brain), were collected in 12-well plates and washed in 0.1 M PBS. Sections were then incubated in 1% hydrogen peroxide for 30 minutes at room temperature, washed again in 0.1 M PBS, and incubated in 0.3% Triton X-100 for 10 minutes at room temperature. Sections were washed in 0.1 M PBS and transferred to 5% normal goat serum in 0.1 M PBS for 30 minutes at room temperature to block non-specific binding. Sections were not subjected to antigen retrieval, but incubated overnight in rat anti-mouse monoclonal antibody to mouse macrophage antigen-1 (Mac-1) (1:3,000 in 2% normal goat serum and 0.3% Triton-X-100 in PBS; CR3 receptor-CD11b;

Serotec, Washington, DC, USA) at 4°C. Afterwards, sections were washed in 0.1 M PBS and incubated in biotinylated secondary goat anti-rat antibody (1:200 in normal goat serum and 0.1 M PBS; Vector Laboratories, Burlingame, CA, USA) for 90 minutes at room temperature. Sections were washed in 0.1 M PBS and re-incubated for another 90 minutes at room temperature in ABC solution from the Vectorstain Kit (Vector Laboratories, Burlingame, CA, USA). Sections were rinsed in 0.1 M PBS and reacted using 3,3′-diaminobenzidine (10 mg 3,3′-diaminobenzidine, 0.1 M PBS, 40 mL) for 6–10 minutes. On an adjacent systematic-random set of sections, astrocytes were immunostained using rabbit anti-bovine glial fibrillary acidic protein (GFAP) (1:15,000; Dako, Carpenteria, CA, USA), with goat anti-rabbit biotinylated IgG against cow GFAP as the secondary antibody. Sections were mounted on slides and dried overnight. For counterstaining, sections were dehydrated through a graded alcohol series, followed by a dH_2O rinse for 1 minute. Cresyl violet (Sigma) counterstaining of basophilic structures was applied for 2 minutes, followed by color adjustment in 5% acetic acid, with a final rinse in dH_2O. Sections were rehydrated using a reverse ethanol series, rinsed twice in xylene for 10 minutes each, and then coverslipped with Cytoseal 60 (Richard-Allan Scientific, Kalamazoo, MI, USA) (O'Neil et al. 2007).

Stereological analysis

Modern stereological methods were used to determine stereological parameters on an adjacent series of randomly sampled sections through the entire hippocampus using the Stereologer computerized stereology system (Systems Planning and Analysis, Inc., Alexandria, VA, USA). The system hardware consisted of a X-Y-Z motorized stage, color video camera interfaced to a Zeiss Axioskop(Oberkochen, Germany), high-resolution video card, and a personal computer and monitor. The stereology software allowed an outline of the region of interest (hippocampus) to be mapped while viewing the section under low magnification (4×). In addition, an estimate of the total number of immunopositive microglial cells and astrocytes was obtained using optical fractionator methods under a high magnification objective lens (60× oil with 1.4 numerical aperture) (Long et al., 1998; Mouton, 2002). Every fifth section was selected from the total number of hippocampal sections in a systematic-random manner, generating 10–12 sections per reference space. The sampling fractions included: (i) section sampling fraction (ssf): number of sections sampled divided by total number of sections for each hippocampus; (ii) area sampling fraction (asf): area of the sampling frame divided by the area of the x-y sampling step; and (iii) thickness sampling fraction (tsf): height of the dissector divided by the section thickness. A guard volume of 2.0 mm was used during cell counting to avoid sectioning artifacts (including lost caps and uneven section surfaces). Here, the sampling parameters for microglia and astrocyte counting were: (1) ssf = 1/5; (2) asf = 0.01040 {a(frame) = 0.0049 mm^2]/ {[a(step) = 0.0047089 mm^2]}; and (3) tsh = 0.6936 (height of the dissector (12 μm)/average post-processing section thickness (17.3 μm). N = ΣQ-•1/ssf•1/asf•1/tsf, where N is total number estimated and ΣQ- is cell number counted.

Statistical analysis

Data are expressed as the mean ± SD, and analyzed with SPSS 13.0 software (SPSS, Chicago, IL, USA). Statistical analysis was performed by one-way analysis of variance with *post hoc* evaluation using Scheffe's *F* test. Statistical significance was set at $P < 0.05$.

Results

Effect of T10 on microglial cells

Immunoreactive Mac-1 microglia were recognized as small, deeply basophilic, oval-to-round shaped cell bodies, approximately 5–7 μm in diameter, with a clear halo around the cell body and immunoreactive processes that extended into the surrounding parenchyma. Immunohistochemical staining showed that Mac-1-positive microglial cells in the hippocampus of wild-type mice were characterized by small cell bodies with slender processes and multi-branched shapes. This pattern is indicative of microglial cells in the resting state. Distribution of microglial cells in the PLC, T10-L, and T10-H groups was different from wild-type mice. These three groups of mice showed clusters of microglial cells around Aβ deposits and senile plaques. The microglia had large cell bodies, short and thick processes, and some had no processes, a phenotype known as the amoeboid shape (**Figure 1**). These features indicate the microglial cells are in the activated state. Comparing all three groups, there were more amoeboid-shaped microglial cells in the PLC group, and more microglia with processes in the T10-H group, which tended to change into the resting state.

Quantitative analysis found that the total number of hippocampal microglial cells decreased by 30% ($P < 0.01$) and 18% ($P < 0.05$) in the T10-H and T10-L groups, respectively, compared with the PLC group. Further, the T10-H group showed a 15% reduction in total microglia number compared with the T10-L group ($P < 0.05$; **Table 1**). These data indicate that T10 reduces the total number of microglial cells and inhibits microglial proliferation.

Effect of T10 on astrocytes

GFAP is a specific marker for labeling the intermediate filament protein of astrocytes. GFAP-immunoreactive cells show typical morphological features of fibrous astrocytes. The cell bodies have variable shapes with processes that project into the parenchyma. GFAP-positive astrocytes in the hippocampus of wild-type mice were evenly scattered with small cell bodies and slender processes, indicating these astrocytes are in the static state. In the PLC, T10-L, and T10-H groups, astrocyte distribution was similar to microglial cells, and showed that activated astrocytes were distributed in senile plaque clusters with large cell bodies and thick processes (**Figure 2**). Compared with the PLC group, astrocytes in the T10-H group had more thin processes, which tended to change into the resting state (**Figure 2**).

Table 1 Effect of T10 on total microglial cells and astrocyte number in the hippocampus

Group	*n*	Microglial cells	Astrocytes
PLC	6	91,605±15,857	315,237±27,376
T10-L	6	75,126±3,105*	271,328±21,460
T10-H	6	62,295±2,898$^{**\#}$	251,243±11,978*
Wild-type	5	55,686±3,850**	234,356±38,076*

*$P < 0.05$, **$P < 0.01$, *vs.* PLC group; #$P < 0.05$, *vs.* T10-L group. Data are expressed as the mean ± SD (one-way analysis of variance and Scheffe *F* test). PLC: Placebo treatment group (PLC, normal saline, intraperitoneally); T10-L: low-dose T10 treatment group (T10-L, 1 μg/kg/d, intraperitoneally); T10-H: high-dose T10 treatment group (T10-H, 5 μg/kg/d, intraperitoneally); wild-type: normal mice (no treatment). T10: Triptolide.

Quantitative analysis found that the total number of hippocampal astrocytes decreased by 20% ($P < 0.05$) and 13% ($P > 0.05$) in the T10-H and T10-L groups, respectively, compared with the PLC group. No significant difference was detected in total astrocyte number in the hippocampus between the T10-H group and wild-type mice (**Table 1**).

Discussion

Here, we show that T10 treatment reduces the total number of microglial cells, and inhibits microglial activation and proliferation in a dose-dependent manner. Moreover, high-dose T10 treatment also reduces the total number of astrocytes, and inhibits astrocyte activation.

T10 inhibited activation and proliferation of microglial cells in the hippocampus of APP/PS1 dtg mice

Microglial cells are the macrophages of the central nervous system, and play an important role in the inflammatory and immune response in AD brain. In this study, 6-month-old APP/PS1 dtg mice showed activated microglial cells that clustered around Aβ plaque deposits and senile plaques in the hippocampus. These cells showed marked activation, including morphological characteristics of an activated state (*e.g.*, large cell bodies, and short or no processes). Our results are consistent with other studies on transgenic mouse models of AD (Wright et al., 2013; Brendel et al., 2016). Further, we show that after 45 days of T10 treatment in 4.5-month-old APP/PS1 dtg mice, total microglial cells number in the hippocampus was reduced. Additionally, we observed that microglia tended to change to a resting state, suggesting that T10 inhibits microglial activation and proliferation in the hippocampus of the APP/PS1dtg mouse model of AD.

The effect of T10 and other components of the Chinese herb *Tripterygium wilfordii* on microglial cells have previously been reported. In an earlier study, lipopolysaccharide (LPS) was injected into the CA3 area of the hippocampus in rats that had been pretreated with T10 for 3 days. Observations and analysis were performed 24 hours later, and showed that T10 dose-dependently suppressed activation and proliferation of microglial cells (Dai et al., 2006). Moreover, long-term T10 administration was also shown to suppress microglial activation and improve retinal ganglion cell survival in DBA/2J mice (Yang et al., 2013). The mechanism of this protection is associated with suppression of activation and proliferation of microglial cells, together with reduction of IL-1β and TNF-α production (Zhou et al., 2005).

During the pathological process of AD, Aβ deposition and senile plaque formation from fibrotic Aβ attracts and activates microglial cells, thereby resulting in chronic inflammation (Alarcón et al., 2005; Jiang et al., 2014). In chronic inflammation in AD brain, activated microglial cells produce several proinflammatory cytokines, such as IL-1β, IL-6, and TNF-α, and complements such as C1q (Alam et al., 2016). These components may act as a trigger for an inflammatory reaction in the brain, and accelerate microglial and astrocyte proliferation, together with Aβ deposition and senile plaque formation, leading to a vicious cycle. Activated microglial cells can also produce glutamate (Huang et al., 2011) and N-methyl-D-aspartate receptor 1 (Zheng et al., 2010), both of which are excitatory neurotoxins. These excitatory neurotoxins do not cause complete cell death, but may damage neural dendrites, resulting in synaptic loss, which may be associated with AD-related cognitive dysfunction. Altogether, based on these previous studies and our findings, we believe that T10 exerts its protective effect against AD by suppressing activation and proliferation of microglial cells, and reducing proinflammatory cytokine production related to activation of microglial cells.

Activated microglial cells function as phagocytic cells, which has been confirmed by *in vivo* and *vitro* experiments (Pan et al., 2011; Zhang et al., 2014). Microglial cells can engulf Aβ and digest it using scavenger receptors and advanced glycation end product receptors on the cell surface, to combine Aβ with the cell. Taken together, T10 inhibition of activated microglial cells may also suppress phagocytosis and clearance of Aβ. However, microglial phagocytosis of Aβ may launch a neurocytopathic state. Indeed, after engulfment of some foreign bodies, peripheral macrophages release certain cytotoxins that sharply increase respiratory rate in cells. Further, after the foreign body is phagocytosed, microglial cells release reactive oxygen species (Wang et al., 2013) such as TNF-α. All of these components damage nerve cells and have a close relationship with deprivation of AD.

T10 inhibited activation and proliferation of astrocytes in the hippocampus of APP/PS1 dtg mice

Our findings in the hippocampus of 6-month-old APP/PS1 dtg mice show that astrocytes are arranged around Aβ deposits and senile plaques. A similar phenomenon has been observed in other studies (Furman et al., 2012; Vromman et al. 2013; Miklossy et al., 2016). After intraperitoneal T10 injection for 45 days, activation and proliferation of astrocytes are suppressed, and total astrocyte number reduced. In our previous study in rats, we used T10 pretreatment for 3 days, and subsequently injected LPS into the hippocampus. We found that T10 inhibits astrocyte activation (Dai et al., 2005, 2006). These results suggest that T10 inhibits different

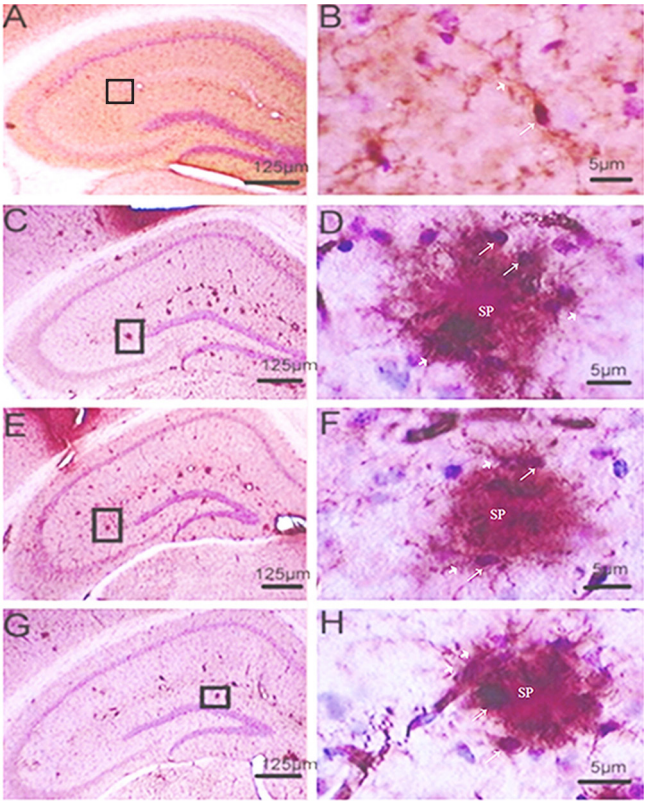

Figure 1 Effect of T10 on microglial cells in the hippocampus of APP/PS1 double transgenic mice (immunohistochemical staining).
Mac-1-positive microglial cells in the hippocampus of wild-type mice were characterized by small cell bodies (fine arrows) and slender processes (bold arrows) with a multi-branched shape. This pattern is indicative of microglial cells in the resting state (A, B). Distributions of microglial cells in the following groups (C–H) were different from this resting state. These three groups of mice had Aβ deposits and senile plaques with clusters of microglial cells around them. The microglia had large cell bodies (fine arrows) and short, thick (bold arrows) processes. Some had no processes and are known as the amoeboid shape. (A, B) Wild-type group (no treatment); (C, D) placebo treatment group (PLC, normal saline, intraperitoneally); (E, F) low-dose T10 treatment group (T10-L, 1 μg/kg/d, intraperitoneally); (G, H) high-dose T10 treatment group (T10-H, 5 μg/kg/d, intraperitoneally); T10: triptolide. B, D, F, and H are magnified images of the squares in A, C, E, and G, respectively. Scale bars in A, C, E, G: 125 μm; B, D, F, H: 5 μm. Aβ: Amyloid-beta.

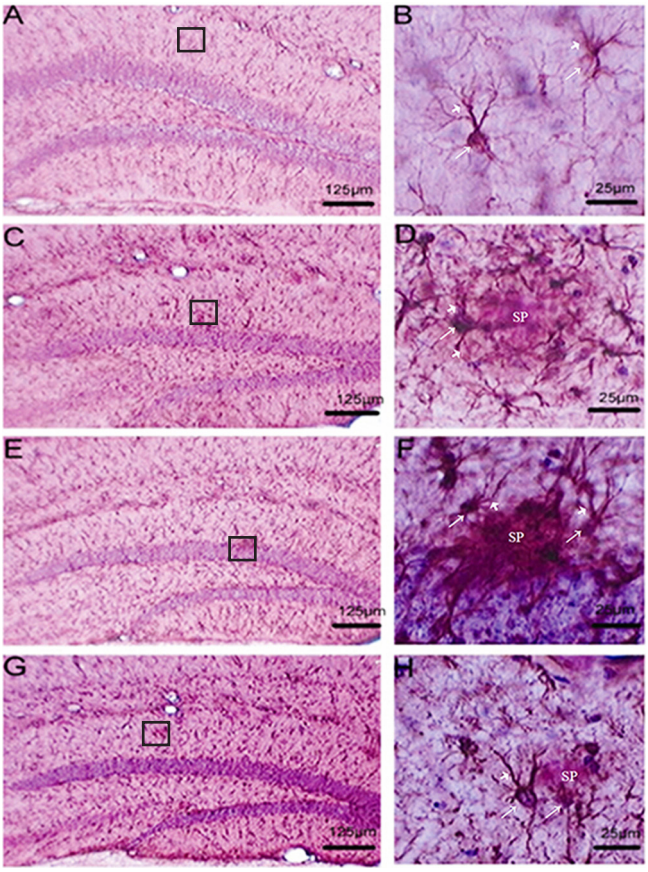

Figure 2 Effect of T10 on astrocytes in the hippocampus of APP/PS1 double transgenic mice (immunohistochemical staining).
(A, B) Wild-type group (no treatment); (C, D) placebo treatment group (PLC, normal saline, intraperitoneally); (E, F) low-dose T10 treatment group (T10-L, 1 μg/kg/d, intraperitoneally); (G, H) high-dose T10 treatment group (T10-H, 5 μg/kg/d, intraperitoneally). GFAP immunohistochemical staining shows that GFAP-immunoreactive astrocytes in the hippocampus of wild-type mouse are evenly scattered with small cell bodies (fine arrows) and slender processes (bold arrows), indicating these astrocytes are in the static state (A, B). In the three groups of transgenic mice, astrocyte distribution is similar to microglial cells: activated astrocytes are distributed around senile plaques with large cell bodies (fine arrows) and short, thick processes. B, D, F, and H are magnified images of the squares in A, C, E, and G, respectively. Scale bars in A, C, E, G: 125 μm; B, D, F, H: 5 μm. T10: Triptolide.

pathological conditions caused by activation of astrocytes.

Astrocytes play crucial roles in the maintenance of neuronal structure and function. As such, they respond actively to brain injury caused by trauma, stroke, or neuronal degeneration (Wilhelmsson et al., 2006; Chvatal et al., 2007; Dong et al., 2013; Johann et al., 2013; Wagner et al., 2013; Wajima et al., 2013; Torrente et al., 2014). Astrocytes that respond to injury *in vivo*, increase in size, and increase expression of the astrocyte specific intermediate filament protein, GFAP, *i.e.*, become hypertrophic. These astrocytes are described as reactive and/or activated (Myer et al., 2006).

The hallmark of AD pathology is the presence of an increased number of reactive astrocytes near senile plaques composed of Aβ aggregates. During AD progression, astrocytes undergo both morphological and functional changes, giving rise to the term "reactive gliosis". The function of astrocytes in the pathological process of AD is still uncertain. Further research is needed on the advantages and disadvantages of T10 suppression on astrocytes in the brain of APP/PS1 dtg mice. Activated astrocytes are thought to provide support for damaged neural tissue through several mechanisms, including release of neurotrophic factors and degradation of amyloid deposits (Schubert et al., 2001; Burbach et al., 2004; Pihlaja et al., 2011; Thal et al. 2012; Lee et al., 2013; Wakabayashi et al., 2013; Scardigli et al., 2014; Yamamoto et al., 2014).

Astrocyte activation forms a glial scar that envelopes Aβ deposits and senile plaques, forming a barrier between senile plaques and nerve cells, and preventing a toxic effect by protecting nerve cells from Aβ. As a suppressor of astrocyte activation, T10 may weaken this protective effect. However, Schubert et al. (2001) verified that reactive astrocytes contribute to the

pathomechanisms underlying AD by favoring oxidative neuronal damage. Reactive astrocytes may promote transformation of Aβ into toxic forms (Abramov et al., 2004). Activated astrocytes produce several proinflammatory cytokines (*e.g.*, IL-1) (Cekanaviciute et al., 2014; Deng et al., 2014), complement, other components, and S100β. These components accelerate not only activation of microglial cells and astrocytes, but production of proinflammatory cytokines. Such acceleration will aggravate inflammation and may even accelerate Aβ deposition and senile plaque formation. Overall, astrocyte activation causes several insalubrious effects. By suppressing astrocyte activation, T10 may reduce the damaging effect caused by activation.

In conclusion, the suppressive effect of T10 on activation and proliferation of glial cells is a double-edged sword. It can help relieve brain inflammation in the transgenic mouse model of AD and AD patients. Although the inflammatory reaction accelerates Aβ deposition and senile plaque formation, as T10 reduces Aβ deposition and senile plaque formation, this may be attributable to its suppressive effect on glial cells.

Author contributions: *JML designed the project, performed experiments and wrote the paper. DLL directed the research and revised the paper writing. YZ, LT, YHC, QG, MHB, and JX were responsible for implementation of the experiment. All authors approved the final version of the paper.*
Conflicts of interest: *None declared.*
Plagiarism check: *This paper was screened twice using Cross-Check to verify originality before publication.*
Peer review: *This paper was double-blinded and stringently reviewed by international expert reviewers.*

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Copyedited by James R, Haase R, Wang J, Qiu Y, Li CH, Song LP, Zhao M