Tetrahydroxystilbene glycoside antagonizes β-amyloid-induced inflammatory injury in microglia cells by regulating PU.1 expression

Chenli Jiao*, Feng Gao*, Li Ou, Jinhua Yu, Min Li, Peifeng Wei and Feng Miu

Inhibiting β-amyloid (Aβ)-induced microglial activation is proposed as an effective strategy for the treatment of Alzheimer's disease. Tetrahydroxystilbene glycoside (TSG) is the main active ingredient of *Polygonum multiflorum* and has a wide range of biological properties, including antiinflammation. Here, we focused on the function and regulatory mechanism of TSG in Aβ-induced N9 and BV2 cells. The results showed that Aβ treatment induced the activation of microglia cells and the production of inflammatory molecules, including inducible nitric oxide synthase, nitric oxide, cyclooxygenase 2, and prostaglandin E2, which were significantly inhibited by TSG pretreatment. Furthermore, we found Aβ exposure increased the levels of microglial M1 markers, interleukin (IL)-1β, IL-6, and tumor necrosis factor α, and the pretreatment of TSG suppressed the increase of M1 markers and enhanced the levels of M2 markers, including IL-10, brain-derived neurotrophic factor, glial cell-derived neurotrophic factor, and arginase-1. PU.1 overexpression was found to eradicate the anti-inflammatory effects of TSG in Aβ-induced microglial cells. Taken together, these findings indicate that TSG attenuates Aβ-induced microglial activation and polarizes microglia towards M2 phenotype, which may be closely associated with the regulation of PU.1. *NeuroReport* 29:787–793

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Introduction

β-Amyloid (Aβ) deposition has been proposed as a crucial pathogenic event in the progression of Alzheimer's disease (AD) [1]. Increasing evidence indicates that innate immunity may play an important role in the disorder, a factor mainly represented by microglial cells [2]. Under ordinary conditions, microglia are in an inactive state and they repeatedly monitor the neighboring environment for any changes to maintain the homeostasis of the brain. When microglia are activated by proinflammatory stimuli, they can repair brain lesions by phagocytosis or secretion of various inflammatory mediators; however, these inflammatory mediators may be harmful when microglia are continuously activated [3]. As Aβ level is increased in AD, microglia are overstimulated and thus produce inflammatory cytokines. Inhibiting aspects of Aβ-induced microglial activation is considered to be an effective strategy for AD treatment.

Tetrahydroxystilbene glucoside (2,3,5,4-tetrahydroxystilbene-2-glucoside, TSG) is the main active ingredient from the root of *Polygonum multiflorum*, which has a wide range of biological functions based on its antioxidant [4], anti-inflammatory [5], antidepressant [6], antiatherosclerotic [7], and antiaging [8] properties. Recently, many investigations have revealed that TSG is effective in the treatment of AD [9,10]. However, the functional roles and the molecular mechanisms of TSG in AD are as yet unclear. We aimed to investigate the effects of TSG on the microglial activation induced by Aβ in this study.

Here, we pretreated human N9 or BV2 microglial cell lines with TSG, and then exposed them to $A\beta_{1-42}$. We found that TSG pretreatment successfully attenuated $A\beta_{1-42}$-induced microglial activation and polarized towards M2 phenotype in these cells. The protective effects of TSG may be attributable to the regulation of PU.1 signaling.

Materials and methods

Cell culture

The mouse N9 and BV2 microglial cells were cultured in Dulbecco's Modified Eagle's medium containing 5% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were cultured in a humidified atmosphere containing 5% CO_2 at 37°C.

Construction of vector and cell transfection

Full-length *Spi1* (protein name: PU.1; gene name: *Spi1*) cDNA sequence was amplified by PCR from cDNA template, which were obtained from N9 cells. The 5′-GCTGGATGTTACAGGCGTGCAAAATG-3′ and

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5′-CCGGGCGAGGGCTTAATGCTATGGCC-3′ are used as primer sequences as previously described [11]. The *Spi1* cDNA fragments were cloned into pcDNA3.1 vector, named pcDNA3.1-PU.1. The empty pcDNA3.1 vector was used as a negative control. Microglial cells were seeded into six-well plates or 96-well plates 24 h before transfection, and then transiently transfected with pcDNA3.1-PU.1 or empty vectors using lipofectamine 2000 (Invitrogen, Carlsbad, California, USA) according to the manufacturer's instructions.

MTT assay

The cell viability was assessed by MTT assay (Millipore, Boston, Massachusetts, USA) according to the manufacturer's protocol. In summary, 10 μl of MTT solution was added to the different treated microglial cells and then incubated for 4 h. The absorbance was determined at 570 nm with a microplate reader (Wallace; PerkinElmer, Waltham, Massachusetts, USA).

Western blot

The total protein samples were lysed with the RIPA buffer (Sigma-Aldrich; St. Louis, Missouri, USA). The protein concentration was determined using a BCA Protein Assay Kit (Pierce; Rockford, Illinois, USA). Protein samples were separated by SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membrane (Millipore). After blocking in 5% skim milk at room temperature for 1 h, the membranes were probed with the anti-ionized calcium-binding adapter molecule (IBA) (1 : 200), anti-inducible nitric oxide synthase (iNOS) (1 : 500), anti-cyclooxygenase 2 (COX-2) (1 : 500), anti-interleukin (IL)-1β (1 : 1000), anti-IL-6 (1 : 1000), anti-tumor necrosis factor α (TNF-α) (1 : 1000), anti-IL-10 (1 : 1000), anti-arginase-1 (Arg-1) (1 : 500), and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1 : 1000) overnight at 4°C. The membranes were then incubated with horseradish peroxidase conjugated secondary antibodies for 1 h at room temperature. Then the blots were visualized using an Enhanced Chemiluminescence Detection Kit (Pierce; Rockford, Illinois, USA) and normalized to GAPDH signals.

Determination of nitric oxide production

The level of nitric oxide (NO) in the cell culture media was measured using the Griess method. The culture medium of the different treated microglial cells was removed and mixed with an equal volume of Griess reagent. The NO concentration was determined by the absorbance at 540 nm and compared with a standard curve of sodium nitrite absorbance.

Measurement of prostaglandin E2

The level of prostaglandin E2 (PGE2) in the cell culture media was quantified using an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, Minnesota, USA) according to the manufacturer's protocol. The PGE2 concentration was determined by the absorbance at 490 nm.

Enzyme-linked immunosorbent assay

The supernatants of the different treated microglial cells were collected, and the concentrations of IL-1β, IL-6, IL-10, TNF-α, brain-derived neurotrophic factor (BDNF), and glial cell-derived neurotrophic factor (GDNF) were determined using corresponding ELISA kits according to the manufacturer's instructions.

Statistical analysis

Statistical analysis was carried out using the GraphPad Prism 5 software (GraphPad Software Inc., La Jolla, California, USA). One-way analysis of variance was performed followed by the Bonferroni test for multiple group comparisons. Data are presented as the mean ± SEM. *P* values less than 0.05 were considered statistically significant.

Results

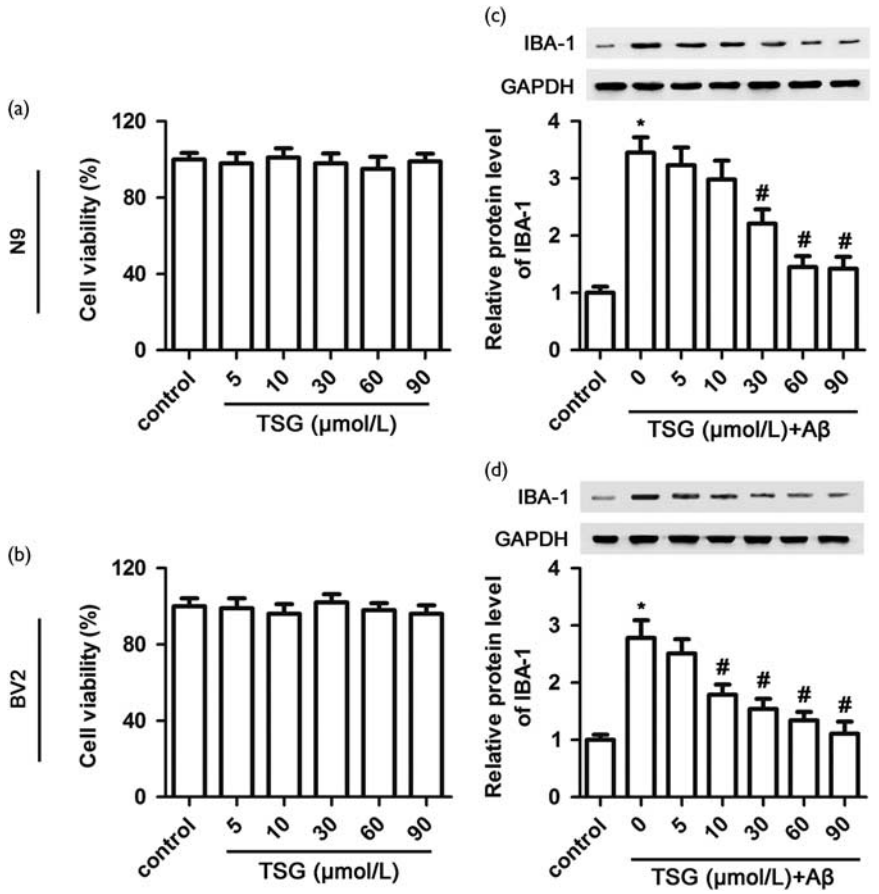
Tetrahydroxystilbene glycoside suppresses β-amyloid-induced microglia cell activation in a concentration-dependent manner

To assess the cytotoxicity of TSG to the microglia cells, we treated mouse N9 and BV2 cells with increasing concentrations of TSG for 48 h, and performed MTT assay to measure the cell viability. As shown in Fig. 1a and b, the cell viability in TSG groups (5, 10, 30, 60, and 90 μM) exhibits no significant difference compared with that in control group, suggesting that 5–90 μM TSG treatment has no cytotoxicity in N9 and BV2 cells. To investigate the effects of TSG on the microglia cell activation induced by Aβ, N9, and BV2 cells were treated with different concentrations of TSG for 24 h, followed by Aβ treatment for another 24 h. Then western blot was performed to evaluate the protein expression of IBA-1, a microglial activation marker. As shown in Fig. 1c and d, compared with control group, Aβ treatment induced significantly elevated expression of IBA-1. Compared with the Aβ group, IBA-1 expression showed a clear reduction in the TSG + Aβ group as TSG concentrations increased. These results suggest that TSG suppresses Aβ-induced microglia cell activation in a concentration-dependent manner, and 90 μM TSG was used in the following experiments.

Tetrahydroxystilbene glycoside suppresses β-amyloid-induced production of nitric oxide and prostaglandin E2 in microglia cells

To investigate the effects of TSG on the production of Aβ-induced inflammatory molecules, N9 and BV2 cells were treated with Aβ or the combination of TSG and Aβ. Then western blot assay was performed to measure the protein expression of iNOS and COX-2. Compared with the control group, Aβ treatment significantly upregulated the expression of iNOS and COX-2, which were

Fig. 1



TSG suppresses Aβ-induced microglia cell activation in a concentration-dependent manner. (a, b) N9 and BV2 cells were treated with 0, 5, 10, 30, 60, and 90 μM of TSG for 48 h, correspondingly. Cell viability was measured by MTT assay. (c, d) N9 and BV2 cells were treated with 0, 5, 10, 30, 60, and 90 μM TSG for 24 h, and then treated with 10-μM Aβ for another 24 h, correspondingly. Expression of IBA-1 was measured by western blot assay and normalized to GAPDH level. The relative protein level was shown in a bar graph. $^{*}P < 0.05$ versus control group; $^{\#}P < 0.05$ versus Aβ group. Aβ, β-amyloid; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IBA-1, ionized calcium-binding adapter molecule; TSG, tetrahydroxystilbene glycoside.

markedly inhibited by the pretreatment of TSG (Fig. 2a and b). Furthermore, we assessed the levels of NO and PGE2. As shown in Fig. 2c and d, the pretreatment with TSG significantly reduced the Aβ-induced increased production of NO and PGE2. These results suggest that TSG suppresses Aβ-induced expression of iNOS and COX-2 and inhibits production of NO and PGE2 in microglia cells.

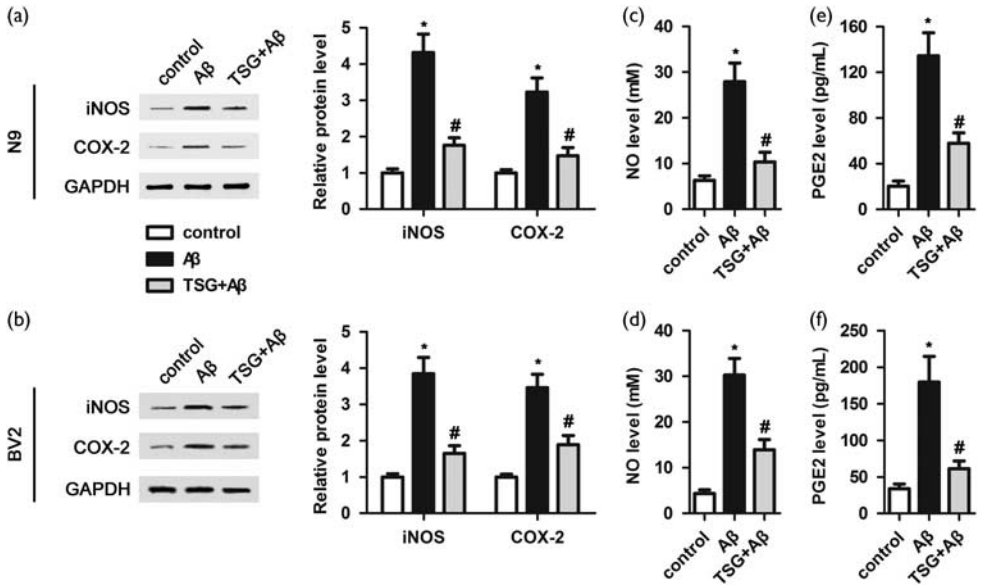
Tetrahydroxystilbene glycoside suppressed β-amyloid-induced production of microglial M1 markers

To investigate the effects of TSG on the microglial M1 state induced by Aβ, ELISA assay and western blot were performed to detect the expression levels of M1 markers, including IL-1β, IL-6, and TNF-α. First, the ELISA data showed that compared with control cells, the concentrations of IL-1β, IL-6, and TNF-α were significantly greater in the cells treated with Aβ alone, which showed an obvious reduction in the cells treated with the combination of TSG and Aβ (Fig. 3a and b). Similarly, the western blot assay also revealed that the Aβ-induced production of IL-1β, IL-6, and TNF-α was significantly inhibited by the pretreatment with TSG (Fig. 3c and d). These findings suggest that TSG suppresses Aβ-induced production of IL-1β, IL-6, and TNF-α and inhibits the proinflammatory M1 state in microglia cells.

Tetrahydroxystilbene glycoside promoted the production of M2 markers in β-amyloid-induced microglia cells

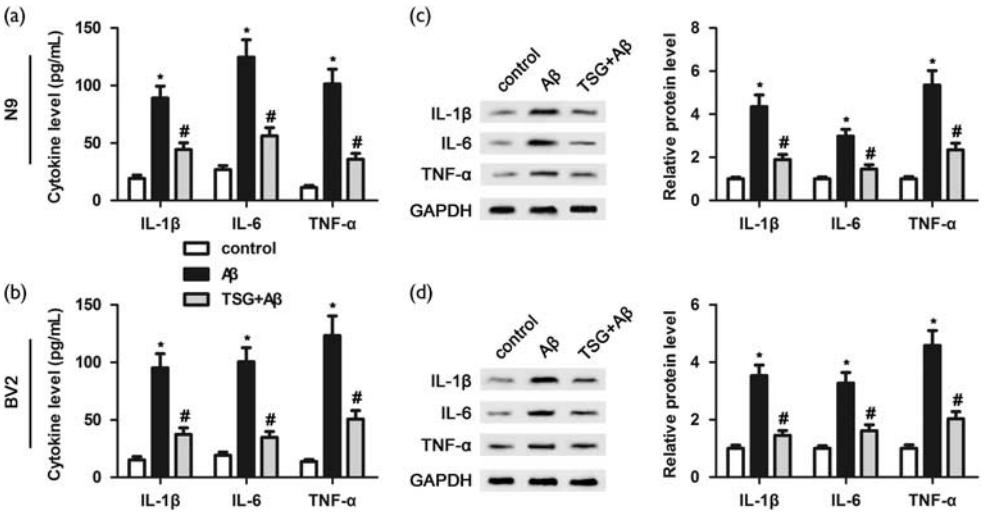
We further determine the effects of TSG on the anti-inflammatory M2 state in Aβ-induced microglia cells. We measured the expression levels of microglial M2 markers, including IL-10, BDNF, GDNF, and Arg-1 by ELISA and western blot assay. As shown in Fig. 4a and b, the concentrations of IL-10, BDNF, and GDNF showed no obvious changes between the Aβ group and the control group. However, the TSG pretreatment significantly elevated the levels of IL-10, BDNF, and GDNF in

Fig. 2



TSG suppresses Aβ-induced production of NO and PGE2 in microglia cells. N9 and BV2 cells were treated with or without 90-μM TSG for 24 h, and then treated with 10-μM Aβ for another 24 h, respectively. (a, b) Expression of iNOS and COX-2 was measured by western blot assay and normalized to GAPDH level. The relative protein levels are shown in bar graphs. (c, d) The production of NO was measured by Griess method. (e, f) The production of PGE2 was measured by enzyme-linked immunosorbent assay. $^{*}P < 0.05$ versus control group; $^{\#}P < 0.05$ versus Aβ group. Aβ, β-amyloid; COX-2, cyclooxygenase 2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; iNOS, inducible nitric oxide synthase; NO, nitric oxide; PGE2, prostaglandin E2; TSG, tetrahydroxystilbene glycoside.

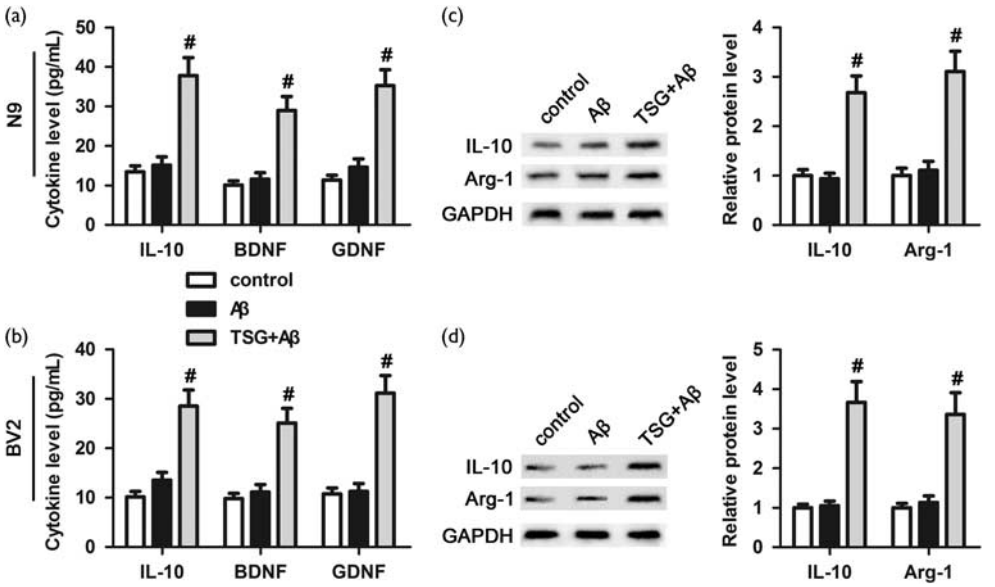
Fig. 3



TSG suppressed Aβ-induced production of IL-1β, IL-6, and TNF-α. (a, b) The concentration levels of IL-1β, IL-6, and TNF-α were detected by enzyme-linked immunosorbent assay. (c, d) The protein expression of IL-1β, IL-6, and TNF-α was measured by western blot assay and normalized to GAPDH level. The relative protein levels are shown in bar graphs. $^{*}P < 0.05$ versus control group; $^{\#}P < 0.05$ versus Aβ group. Aβ, β-amyloid; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IL, interleukin; TNF-α, tumor necrosis factor α; TSG, tetrahydroxystilbene glycoside.

Aβ-induced microglia cells. Furthermore, the protein expression of IL-10 and Arg-1 showed a significant increase when N9 and BV2 cells were treated with the combination of Aβ and TSG compared with the cells treated with Aβ alone (Fig. 4c and d). These data indicate that TSG promotes the production of IL-10, BDNF, GDNF, and Arg-1 and facilitates the anti-inflammatory M2 state in Aβ-induced microglia cells.

Fig. 4



TSG promoted the production of IL-10, BDNF, GDNF, and Arg-1 in Aβ-induced microglia cells. (a, b) The concentration levels of IL-10, BDNF, and GDNF were detected by enzyme-linked immunosorbent assay. (c, d) The protein expression of IL-10 and Arg-1 was measured by western blot assay and normalized to GAPDH level. The relative protein levels are shown in bar graphs. $^{\#}P < 0.05$ versus Aβ group. Aβ, β-amyloid; Arg-1, arginase-1; BDNF, brain-derived neurotrophic factor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GDNF, glial cell-derived neurotrophic factor; IL, interleukin; TSG, tetrahydroxystilbene glycoside.

The anti-inflammatory effects of tetrahydroxystilbene glycoside in β-amyloid-induced microglia cells were partly mediated by PU.1

To study the molecular mechanism mediating the anti-inflammatory effects of TSG in Aβ-induced microglia cells, we focused on PU.1, a transcription factor that is essential for the regulation of the immune response. First, we detected the protein expression of PU.1 by western blot assay and found that compared with the control group, Aβ treatment significantly upregulated the protein expression of PU.1, which showed an obvious reduction when the cells were pretreated with TSG (Fig. 5a and b). These results suggest that PU.1 may be involved in the regulation of TSG in Aβ-induced microglia cells. To confirm this suggestion, we transfected N9 and BV2 cells with overexpression vector pcDNA3.1-PU.1 or the empty vector, followed by the treatment of TSG and Aβ. Western blot assay revealed that the downregulation of IBA-1, iNOS, and COX-2, and the upregulation of Arg-1 induced by the pretreatment of TSG were partly reversed by PU.1 overexpression (Fig. 5c and d). Furthermore, we found that PU.1 overexpression in part reversed the reduction of IL-1β, IL-6, and TNF-α, and the increase in IL-10, BDNF, and GDNF induced by the pretreatment with TSG (Fig. 6). These data indicate that the biological effects of TSG on microglia cell activation and inflammation induced by Aβ are partly mediated by PU.1.

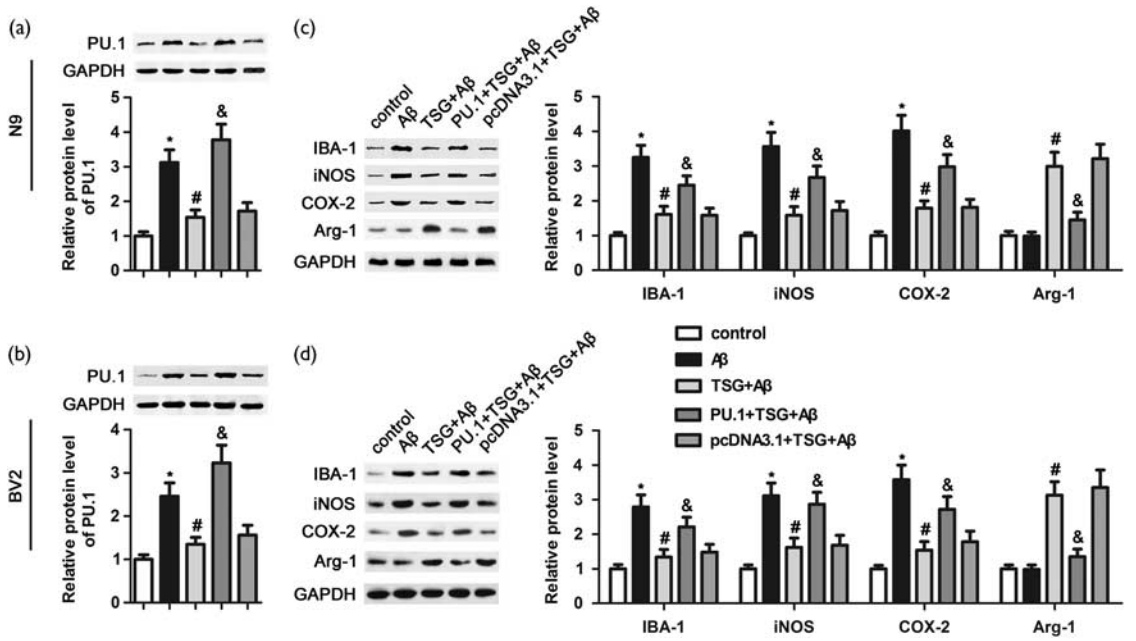
Discussion

Aβ deposition-induced microglia activation has been found to be a crucial event in the pathology of AD [12]. Inhibiting aspects of microglial activation is believed to be an effective therapy for the treatment of AD. To investigate the effects of TSG on the microglia cell activation induced by Aβ, western blot was performed to evaluate the protein expression of IBA-1, and the results showed that Aβ treatment significantly elevated the expression of IBA-1, which showed an obvious reduction in a concentration-dependent manner when the cells were pretreated with TSG. These results suggest that TSG suppresses Aβ-induced microglia activation.

Aβ deposition activates microglia cells to secrete inflammatory molecules, such as COX-2 and iNOS, and long-term inflammation is neurotoxic, inducing neuron injury and even death [13]. PGE2 is one of the inflammatory products of COX-2. Elevated levels of NO produced by iNOS have been found in response to a wide variety of proinflammatory stimuli in AD [14]. In this study, we found that Aβ treatment significantly upregulated the expression of iNOS and COX-2, and elevated the production of NO and PGE2, which were markedly inhibited by the pretreatment with TSG in N9 and BV2 cells. These results suggest that TSG suppresses the production of inflammatory molecules induced by Aβ in microglia cells.

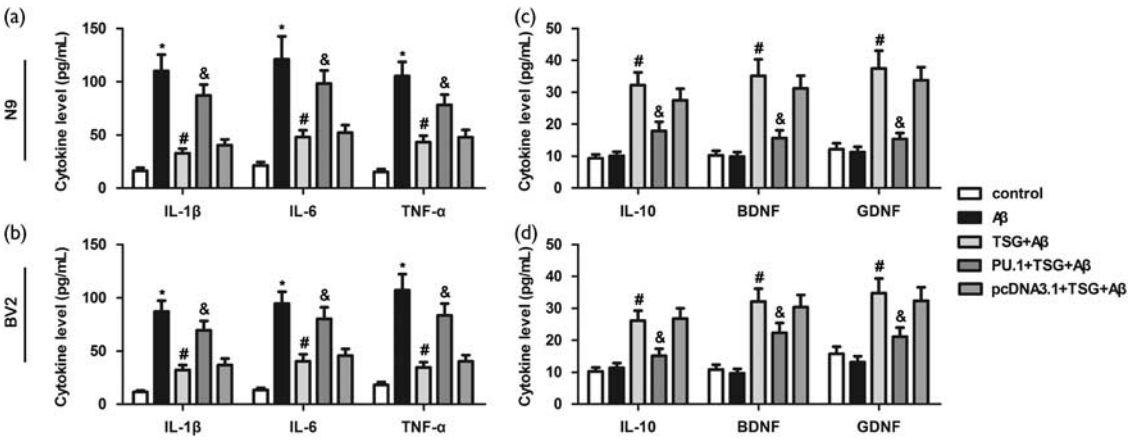
Microglia can be activated in a classic activated state (M1 state) or alternative activated state (M2 state) depending

Fig. 5



PU.1 overexpression reversed the effects of TSG on microglia cell activation and neurotoxic molecule production induced by Aβ. (a, b) The protein expression of PU.1 was measured by western blot assay and normalized to GAPDH level. The relative protein levels are shown in bar graphs. (c, d) The protein expression of IBA-1, iNOS, COX-2, and Arg-1 was measured by western blot assay and normalized to GAPDH level. The relative protein levels are shown in bar graphs. $^{*}P < 0.05$ versus control group; $^{\#}P < 0.05$ versus Aβ group; $^{\&}P < 0.05$ versus TSG + Aβ group. Aβ, β-amyloid; Arg-1, arginase-1; COX-2, cyclooxygenase 2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IBA-1, ionized calcium-binding adapter molecule; iNOS, inducible nitric oxide synthase; TSG, tetrahydroxystilbene glycoside.

Fig. 6



PU.1 overexpression reversed the effects of TSG on microglial M1 and M2 markers. (a, b) The concentration levels of IL-1β, IL-6, and TNF-α were detected by enzyme-linked immunosorbent assay. (c, d) The concentration levels of IL-10, BDNF, and GDNF were detected by enzyme-linked immunosorbent assay. $^{*}P < 0.05$ versus control group; $^{\#}P < 0.05$ versus Aβ group; $^{\&}P < 0.05$ versus TSG + Aβ group. Aβ, β-amyloid; BDNF, brain-derived neurotrophic factor; GDNF, glial cell-derived neurotrophic factor; IL, interleukin; TNF-α, tumor necrosis factor α; TSG, tetrahydroxystilbene glycoside.

on the microenvironment [15]. The M1 state is believed to be proinflammatory and the M2 state may produce anti-inflammatory cytokines and neurotrophins involved in tissue repair and remodeling [16]. Therefore, polarizing the microglia from M1 phenotype to the M2 phenotype may be effective in treating microglial activation-induced degenerative disorders. In the study, Aβ-induced increased levels of IL-1β, IL-6, and TNF-α showed an obvious

reduction in the cells treated with the combination of TSG and Aβ. Meanwhile, TSG pretreatment significantly elevated the levels of IL-10, BDNF, and GDNF in Aβ-induced microglia cells. These findings suggest that TSG inhibited the proinflammatory M1 phenotype and facilitated the anti-inflammatory M2 phenotype in Aβ-induced microglia cells.

PU.1 has been reported as a critical regulator within the hematopoietic system, and also has a great effect on the immune system [17,18]. One recent study revealed that PU.1 plays a crucial role in the regulation of the genes relevant to specialized functions of microglia [19]. Actually, PU.1 is identified as a central hub of AD-modified gene expression and is associated with AD pathology, especially in terms of microglia activation [20,21]. As PU.1 has received increasing attention for its role in inflammation [22,23], we evaluated whether the anti-inflammatory role of TSG in Aβ-induced microglia cells was mediated by PU.1. Aβ treatment significantly upregulated PU.1 protein expression, which showed an obvious reduction when the cells were pretreated with TSG, suggesting that PU.1 may be involved in the anti-inflammatory effects of TSG in Aβ-induced microglia cells. We further revealed that the PU.1 overexpression partly reversed the downregulation of IBA-1, iNOS, and COX-2, and the upregulation of Arg-1 induced by the pretreatment of TSG. Moreover, we found that PU.1 overexpression in part reversed the reduction of IL-1β, IL-6, and TNF-α, and the increase in IL-10, BDNF, and GDNF induced by the pretreatment with TSG. These data suggest that PU.1 overexpression partly reverses the effects of TSG on microglia activation and M1/M2 polarization induced by Aβ.

Taken together, we found that TSG attenuated Aβ-induced microglial activation and inflammation, reduced the levels of microglial M1 state markers, and increased the levels of M2 markers in N9 and BV2 cells. PU.1 overexpression partly eliminated the anti-inflammatory effects of TSG, suggesting that the roles of TSG in Aβ-induced microglial cells were mediated by PU.1 expression. Our findings indicate that TSG may be an effective drug, and PU.1 may be a new target for reducing Aβ-induced microglia inflammation in AD. In addition, except for M1/M2 phenotypes, activated microglia can also be defined by morphology, phagocytosis (phagocytic receptors including CD36, SRA, and RAGE), and marker expression (including CD11b and CD45) [24,25]. Therefore, to better understand the role of TSG on Aβ-induced microglial activation and inflammatory injury, more careful analysis of the aforementioned different activation states is necessary in the future.

Acknowledgements

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Conflicts of interest

There are no conflicts of interest.

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