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RESEARCH ARTICLE

Magnesium ion influx reduces neuroinflammation in Aβ precursor protein/Presenilin 1 transgenic mice by suppressing the expression of interleukin-1β

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Alzheimer's disease (AD) has been associated with magnesium ion (Mg^{2+}) deficits and interleukin-1β (IL-1β) elevations in the serum or brains of AD patients. However, the mechanisms regulating IL-1β expression during Mg^{2+} dyshomeostasis in AD remain unknown. We herein studied the mechanism of IL-1β reduction using a recently developed compound, magnesium-L-threonate (MgT). Using human glioblastoma A172 and mouse brain D1A glial cells as an *in vitro* model system, we delineated the signaling pathways by which MgT suppressed the expression of IL-1β in glial cells. In detail, we found that MgT incubation stimulated the activity of extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) and peroxisome proliferator-activated receptor gamma (PPARγ) signaling pathways by phosphorylation, which resulted in IL-1β suppression. Simultaneous inhibition of the phosphorylation of ERK1/2 and PPARγ induced IL-1β upregulation in MgT-stimulated glial cells. In accordance with our *in vitro* data, the intracerebroventricular (i.c.v) injection of MgT into the ventricles of APP/PS1 transgenic mice and treatment of Aβ precursor protein (APP)/PS1 brain slices suppressed the mRNA and protein expression of IL-1β. These *in vivo* observations were further supported by the oral administration of MgT for 5 months. Importantly, Mg^{2+} influx into the ventricles of the mice blocked the effects of IL-1β or amyloid β-protein oligomers in the cerebrospinal fluid. This reduced the stimulation of IL-1β expression in the cerebral cortex of APP/PS1 transgenic mice, which potentially contributed to the inhibition of neuroinflammation.
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INTRODUCTION

Alzheimer's disease (AD) is the most common cause of dementia in people aged 65 years and above, and is characterized clinically by cognitive decline and pathologically by the accumulation of amyloid β-protein (Aβ) plaques and the presence of neurofibrillary tangles (NFTs) in the brain.[1] It has been suggested that various factors may be involved in the etiology, pathogenesis, and progression of AD.[2] Previous studies have shown that brain magnesium ion (Mg^{2+}) levels[3] and serum Mg^{2+} concentrations[4] appear to be significantly lower in AD patients compared with age-matched normal subjects. Consequently, the dysregulation of Mg^{2+} levels may play a key role in the pathogenesis and progression of AD. The present study was initiated to determine Mg^{2+} homeostasis in AD.

Although the mechanisms regulating Mg^{2+} concentrations in AD have yet to be defined, there are a few studies concerning the potential roles of Mg^{2+} in the brain. It has been shown that Mg^{2+} is associated with the decreased production of cytokines in brain disease. Sugimoto *et al.*[5] reported that $MgSO_4$ exposure reduced the number of monocytes producing tumor necrosis factor alpha (TNF-α) and interleukin-1β (IL-1β) in intrapartum women. Moreover, $MgSO_4$ also inhibited cytokine production in toll-like receptor ligand-treated human peripheral and cord blood mononuclear cells, thereby indicating the broad anti-inflammatory activity of Mg^{2+}. More recently, accumulating evidence suggests that neuroinflammatory mechanisms are critical for the progression of AD. For example, superinduction of one of the Mg^{2+} targets, IL-1β,

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in the AD brain is directly related to the excessive expression of neuronal Aβ precursor protein (APP) and the induction of astrocytes/microglial cell activation,[6] which contribute to the pathogenesis of AD. In addition, blocking IL-1 signaling rescues cognition by attenuating tau phosphorylation in APP/PS1/Tau transgenic mice.[7] These changes may, in part, explain the mechanistic link between Mg^{2+} and AD. However, the mechanisms underlying the role of Mg^{2+} elevation in suppressing the expression IL-1β during the course of AD development remain unknown.

To understand the functional significance of Mg^{2+} in the neuroinflammation of AD, we treated glial cells with magnesium-L-threonate (MgT) for 48 h. We found that MgT treatment markedly inhibited the expression of IL-1β via activating extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) and peroxisome proliferator-activated receptor gamma (PPARγ) pathways in microglia and astroglia cells. Consistent with these *in vitro* data, our *in vivo* results reinforced the notion that the oral administration or injection (intracerebroventricular (i.c.v)) of MgT to APP/PS1 mice clearly inhibited the expression of IL-1β in the cerebral cortex by reducing the production and deposition of Aβ oligomers. These *in vitro* and *in vivo* observations may be instrumental to understanding the roles of Mg^{2+} elevation in suppressing neuroinflammation, which potentially contributes to regulating the pathogenesis of AD.

MATERIALS AND METHODS

Reagents

$A\beta_{1\text{-}42}$, GW9662, and the inhibitor for ERK1/2, PD98059, were obtained from Sigma-Aldrich Corp (St. Louis, MO, USA). MgT was purchased from Soyoung Biotechnology Company (Shanghai, China). Antibodies against β-actin, ERK1/2, p-ERK1/2 (Thr 202/Tyr 204), PPARγ, Aβ, IL-1β, NeuN, glial fibrillary acidic protein (GFAP), Alexa Fluor 488-labeled, Fluor 555-labeled, and horseradish peroxidase -labeled secondary antibody were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Iba-1, p-PPARγ (Ser 82) antibody was from Merck Millipore (Bedford, MA, USA). ERK1/2, PPARγ, or scramble siRNA was obtained from Cell Signaling Technology, Inc. 4,6-diamidino-2-phenylindole was obtained from Beyotime Institute of Biotechnology (Haimen, JS, China). The IL-1β and IL-1β enzyme immunoassay kits were from Raybiotech, Inc. (Norcross, GA, USA). The $A\beta_{1\text{-}42}$ enzyme immunoassay kits were from Invitrogen (Carlsbad, CA, USA). All reagents for the quantitative real time polymerase chain reaction (qRT-PCR) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) experiments were purchased from Bio-Rad Laboratories (California, USA). All other reagents were from Invitrogen unless otherwise specified.

Transgenic mice and treatments

The male wild-type (WT) or APP/PS1 transgenic mice (B6C3-Tg (APPswe, PSEN1dE9) 85Dbo/J (Stock Number: 004462)) (Tg) were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). Genotyping was performed 3–4 weeks after birth. Five mice per cage were housed in a controlled environment under standard room temperature, relative humidity, and 12 h light/dark cycle with free access to food and water. In selected experiments, 4 months old mice were treated with Mg^{2+} (100 mg/kg/d) in drinking water for 2 or 5 months before determining the expression of IL-1β or the deposition of Aβ plaques. The general health and body weights of animals were monitored everyday. The brains of animals in different groups ($n = 6$) were collected under anesthesia and perfusion fixed as previously described.[8]

Cerebrospinal fluid collection

Cerebrospinal fluid (CSF) was collected as previously described.[9] In brief, the mice were anesthetized and placed prone on the stereotaxic instrument. A sagittal incision of the skin was made inferior to the occiput. Under the dissection microscope, the subcutaneous tissue and neck muscles through the midline were bluntly separated. A microretractor was used to hold the muscles apart. Next, the mouse was laid down so that the body made a 135° angle with the fixed head. In this angle, the dura and spinal medulla were visible, had a characteristic glistening and clear appearance, and the circulatory pulsation of the medulla (i.e., a blood vessel) and adjacent CSF space could be seen. The dura was then penetrated with a 6-cm-long glass capillary that had a tapered tip with an outer diameter of 0.5 mm. Following a noticeable change in resistance to the capillary insertion, the CSF flows into the capillary. The average volume of CSF obtained was approximately 7 μl. All samples were stored in polypropylene tubes at –80 °C until injection.

Intracerebroventricular injection

MgT, IL-1β, Aβ oligomers, or vehicle (phosphate-buffered saline (PBS) were injected (i.c.v.) into WT or APP/PS1 transgenic mice as previously described.[10] In selected experiments, the WT mice were injected (i.c.v.) with the CSF of APP/PS1 in the absence or presence of Aβ antibody. Briefly, stereotaxic injections were placed at the following coordinates from bregma: mediolateral: –1.0 mm; anteroposterior: –0.22 mm; dorsoventral: –2.8 mm. Following injection, each mouse recovered spontaneously on a heated pad. The reliability of injection sites was validated by injecting trypan blue dye (Invitrogen) in separate cohorts of mice and observing staining in the cerebral ventricles; 24 h after injection, mice were killed under anesthesia and perfused as previously described.[8]

Organotypic slice culture of brain tissue

Parts of brain tissues were freshly collected from 3-month-old WT C57BL/6 mice. Serial sections (400 μm thick) were cut in a chopper without fixation. The slices were immediately cultured in Dulbecco's Modified Eagle's medium/high glucose medium with 10% fetal bovine serum. In a separate set of experiments, the slices were grown in serum-free medium for an additional 24 h before incubation with MgT in the absence or presence of IL-1β or Aβ oligomers, as previously described.[11,12] After 24 h,

the tissue sections were fixed, and immunostained with IL-1β antibody by immunohistochemistry staining kit (Invitrogen).

Luciferase assays and live animal imaging

1×10^3 D1A cells pre-transfected with IL-1β promoter plasmids were injected in the right cerebral ventricle. MgT or vehicle (PBS) was then injected (i.c.v.) into the left cerebral ventricle. At different time intervals, mice were anesthetized and injected (i.c.v.) with luciferin in the right cerebral ventricle. The scan was performed exactly 5 min following luciferin introduction. All images were analyzed using Bruker *in vivo* imaging systems (MS FX PRO, Carestream, Billerica, MA, USA).

Two-photon imaging

The mice were anesthetized and placed on a heating pad in order to maintain a body temperature of 37 °C before surgery. Residual fur was removed by shaving and a midline scalp incision was made that extended approximately from the neck region (between the ears) to the frontal portion of the head (between the eyes). The left part of the skull was carefully taken off using a drill and the right part of the skull was left untouched. The skull chamber was adhered on the top of skull. The membrane of the cerebral cortex was permeabilized with 0.3% triton X-100 solution. In selected experiments, MgT was injected (i.c.v.) into the right ventricles of mice brains. Using the skull chamber, the surface of the cerebral cortex was immunostained with IL-1β antibody before two-photon scanning, which was performed using a custom-built two-photon microscope based on a chameleon excitation laser operating at 690–1064 nm. The laser-scanning unit was mounted on an upright microscope equipped with a water-immersion objective (Zeiss; 20× W), and the fluorescence was detected using specific antibody staining. The brain surface was stained and scanned before and after the injection (i.c.v.) of MgT or vehicle (PBS).

Cell culture

Human glioblastoma A172 or mouse astrocytes/microglia D1A cells were grown (37 °C and 5% CO_2) on 6 cm tissue culture dishes (10^6 cells per dish) in appropriate media. In a select set of experiments, the cells were grown in serum-free medium for an additional 24 h before incubation with inhibitors in the absence or presence of MgT, as previously described.[11,12] In separate experiments, the astrocytes were separated from the neopallia of the cerebral hemispheres as previously described.[13] In distinct experiments, astrocytes and neuron cells were co-cultured using transwell, which seeded astrocytes in upper chamber and neuron cells in lower chamber of transwell. After 24 h, the neuron cells were immunostained with IL-1β.

Quantitative real-time PCR

qRT-PCR assays were performed with the MiniOpticon Real-Time PCR detection system (Bio-Rad) using total RNA and the GoTaq one-step Real-Time PCR kit with SYBR green (Promega, Madison, Wisconsin, USA) and the appropriate primers as previously described.[14] The GenBank accession number and forward and reverse primers for human IL-1β[12] and GAPDH[14] are provided in our previous publications; for mouse IL-1β (NM_008361.3) F-TTCAAATCTCGCAGCAGC AC, R-GTGCAGTTGTCTAATGGGAACG; GAPDH (NM_00 1289726.1) F-AACTTTGGCATTGTGGAAGG, R-ACACATT GGGGGTAGGAACA. The gene expression values were normalized to those of GAPDH.

Western blot analysis

Tissues or cells were lysed in radio-immune precipitation assay buffer (25 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, and 0.1% SDS) containing protease inhibitor cocktail (Pierce Chemical Company, Rockford, Illinois, USA). The protein content of the cell lysates was determined using a bicinchoninic acid protein assay reagent (Pierce Chemical Company). The total cell lysates (4 μg) were subjected to SDS-PAGE, transferred to a membrane, and probed with a panel of specific antibodies. Each membrane was only probed with one antibody. β-actin was used as a loading control. All of the western experiments were performed at least in triplicate using a different cell preparation each time.

Immunohistochemistry

Mouse brains were collected from 3-month-old WT or APP/PS1 Tg mice and immobilized with 4% paraformaldehyde. Serial sections (10 μM thick) were cut using a cryostat (Leica, CM1850, Germany). Slides were first rehydrated in a graded series of ethanol and submerged in 3% hydrogen peroxide to eliminate endogenous peroxidase activity. IL-1β levels were determined using immunohistochemical staining kit, following the manufacturer's instructions (Invitrogen). In selected experiments, the slices from the brains of human and mouse were double-stained with IL-1β (Alexa Fluor 555-labeled secondary IgG) or one of NeuN, Iba-1, or GFAP (Alexa Fluor 488-labeled secondary IgG) antibody as previously described.[15]

Measurement of the IL-1β and Aβ concentration

The IL-1β and $A\beta_{1-42}$ levels were determined using IL-1β and Aβ enzyme immunoassay kits following the manufacturer's instructions. The total protein in the medium was used as a loading control, and the results are expressed as pg of IL-1β or Aβ per μg or mg of total protein.

Transfection

Cells were transfected with 100 nM of an ERK1/2 or PPARγ-specific siRNA oligonucleotide. In control experiments, the cells were transfected with 100 nM of scrambled siRNA. The transfected cells were allowed to recover for at least 12 h in growth medium and then incubated overnight in serum-free medium before extraction. For live animal imaging experiments, cells were transfected with IL-1β promoter plasmids for 48 h before screening with puromycin (1 μg/ml). The stable cell lines were then seeded in the ventricles of C57BL/6 mice.

Animal committee

All animals were handled according to the care and use of medical laboratory animals (Ministry of Health, Peoples Republic of China, 1998) and the guidelines of the laboratory animal ethical standards of China Medical University.

Human brain samples

Human brain samples were obtained from New York Brain Bank, serial numbers P535-00 (normal), TT4263 (early stage of AD), T4308 (middle stage of AD), and T4339 and T4304 (late stage of AD).

Statistical analysis

All data are represented as the mean ± SE of at least three independent experiments. The statistical significance of the differences between the means was determined using Student's *t*-test or one-way analysis of variance where appropriate. If the means were found to be significantly different, multiple pairwise comparisons were performed using Tukey's post hoc test.[16]

RESULTS

IL-1β is markedly upregulated in APP/PS1 transgenic mice and AD patients

In light of previous studies suggesting critical roles for IL-1β in the pathogenesis of AD,[6,17] we evaluated the expression levels of IL-1β in the brains of AD patients and 3-month-old APP/PS1 Tg mice. As shown in Figure 1a, IL-1β immunostaining was evident in the human cerebral cortex, and the positive staining gradually increased with the progression of AD. In accordance with these data, IL-1β immunostaining was also highly enhanced in the cerebral cortex and the dentate gyrus region of the hippocampus in 3-month-old APP/PS1 Tg mice compared with WT C57BL/6 mice (Figure 1b). These data reveal that IL-1β is upregulated with the development/progression of AD. To further confirm this finding, we examined the mRNA and protein levels of IL-1β in the APP/PS1 Tg mice. In agreement with the immunostaining data, our results showed that mRNA levels of IL-1β were upregulated only in the cerebral cortex (Figure 1c and d upper panel), whereas IL-1β protein

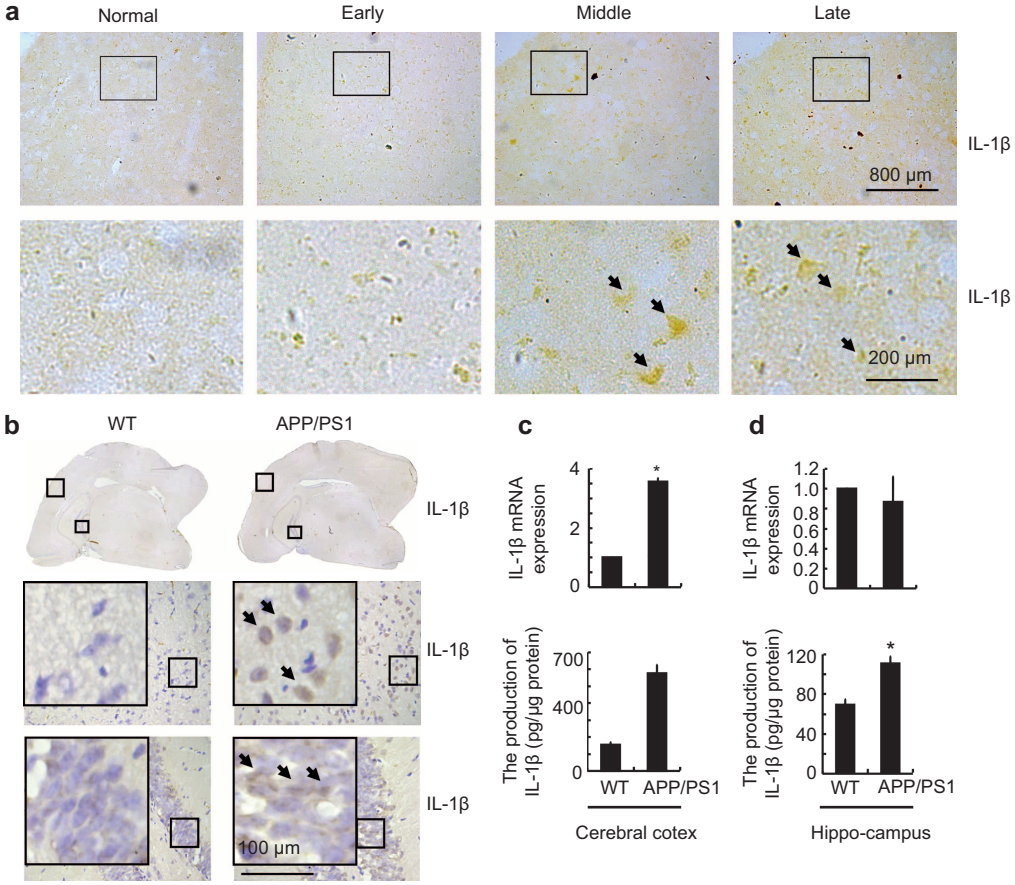

Figure 1 IL-1β was upregulated in AD patients and APP/PS1 transgenic mice. The tissue blocks of human brains at different stages of AD were collected from the New York Brain Bank at Columbia University. From 40 μm free-floating slices were prepared using a cryostat. (**a**) In select experiments, the brains of 3-month-old APP/PS1 transgenic mice were collected after anesthesia and perfusion. (**b**–**d**) The immunoreactivity of IL-1β was determined by immunohistochemistry using an anti-IL-1β antibody (**a** and **b**). These images are representative of six independent experiments, all with similar results. IL-1β mRNA levels were determined by qRT-PCR, and total amounts of GAPDH served as an internal control (**c** and **d** upper panels). The production of IL-1β in culture medium was determined using IL-1β enzyme immunoassay kits (**c** and **d** lower panels). The data represent the means ± SE of three independent experiments. $*P < 0.05$ compared with the WT controls.

was upregulated in both the cerebral cortex and the hippocampus of the APP/PS1 mice (Figure 1c and d lower panel). These observations further suggested the potential involvement of IL-1β in AD.

In light of our observations, which showed that only the protein levels of IL-1β were upregulated in the hippocampus of APP/PS1$^{+/-}$ Tg mice, it is easy to speculate that IL-1β may not be transcriptionally synthesized by neuronal cells. As a type of secreted cytokine, it is possible that IL-1β is secreted from other types of cells and exerts its effects on neuronal cells by binding to their receptors. We thus carried out experiments to determine the localization of IL-1β using localization of fluorescence staining. As a first step, we labeled the neurons, microglia, or astrocytes with NeuN, Iba-1, or GFAP antibodies (green), respectively. The brains of humans and C57BL/6 mice were then double-stained with IL-1β antibody (red). The results revealed that IL-1β co-localized with the neuronal and microglia cells, but not with the astrocytes (Figure 2a and b). Nevertheless, we still could not exclude the possibility of IL-1β expression in neuronal cells based on these findings. We thereby further determined the mRNA expression of IL-1β in neuronal and microglial cells. The results demonstrated that IL-1β was highly expressed in both primary cultured astrocytes (Figure 2c) or D1A cells, but not in neuronal cells (Table 1).

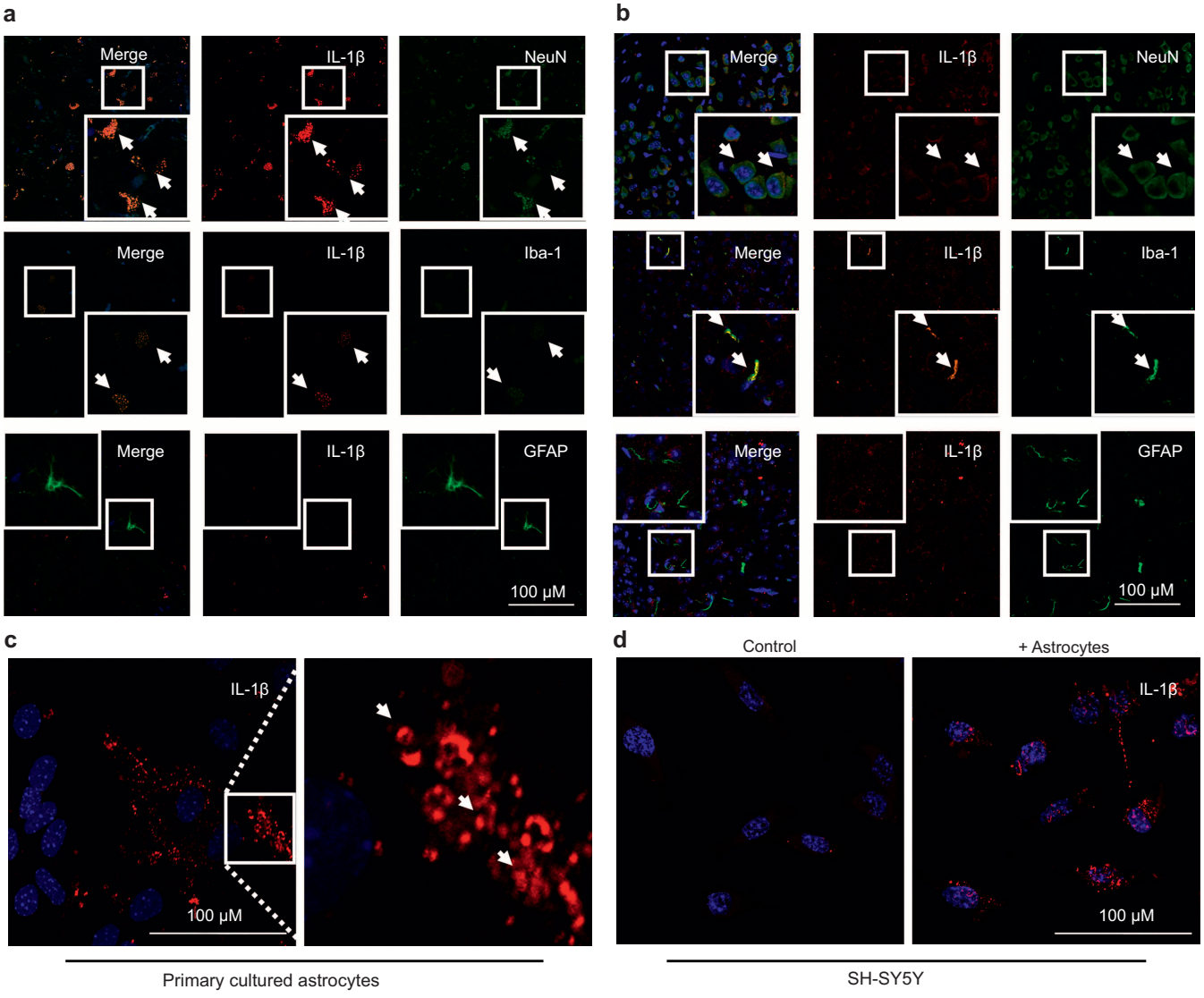

Figure 2 Localization of IL-1β in the brains of AD patients. The tissue blocks of human brains at a late stage of AD were collected from the New York Brain Bank at Columbia University. From 40 μm free-floating slices were prepared using a cryostat. (**a**) In select experiments, the brains of 3-month-old APP/PS1 transgenic mice were collected after anesthesia and perfusion. (**b**) The slices of human (a) or mouse (b) brains were double-stained with NeuN, Iba-1, GFAP (green), or IL-1β (red) antibody before observation using confocal microscopy. In separate experiments, primary cultured astrocytes were immunostained with IL-1β antibody before observation under confocal microscopy. (**c**) In distinct experiments, the astrocytes were co-cultured with neurons using transwell experiments for 24 h before staining the neurons with IL-1β antibody. The cells were then observed under confocal microscopy. (**d**) These images are representative of six independent experiments, all with similar results.

In addition, both neuronal cells and astrocytes expressed IL-1 receptors, including IL-1 receptor types I and II (Table 1). In view of these observations, we questioned where IL-1β comes from in neuronal cells. To resolve this problem, transwell experiments were carried out to co-culture astrocytes and neurons as described in the *Materials and Methods* section. Interestingly, by immunostaining neuron cells with IL-1β antibody, the results demonstrated that IL-1β translocated from the astrocytes to the neurons (Figure 2d).

Additionally, our data and observations not only revealed that IL-1β is not produced by neuronal cells but also explained the reason why we could observe IL-1β-positive staining in neuronal cells.

Mg^{2+} attenuates the synthesis of IL-1β in MgT-treated glial cells

Previous studies have revealed that brain Mg^{2+} levels[3] and serum Mg^{2+} concentrations[4] appear to be significantly lower in AD patients compared with aged-matched normal subjects. These observations suggest a potential protective effect for Mg^{2+} against AD. Given the essential roles of IL-1β in the pathogenesis and progression of AD,[6,17] we initially determined the effects of MgT treatment on the expression of IL-1β in glial cells. Using human A172 cells as a model system for glial cells, we detected the effects of MgT treatment on the expression of IL-1β. As shown in Figure 3a, MgT (50 μM) treatment markedly suppressed the mRNA and protein expression of IL-1β in A172 cells. To further exclude the possibility of cell-specific inhibition of IL-1β by MgT (50 μM), we also carried out similar experiments in mouse astrocytes/microglia D1A cells, and similar results were obtained (Figure 3b). Taken together, our results reveal a key role for Mg^{2+} in inhibiting the synthesis of IL-1β in glial cells (Figure 3a and b).

Pivotal roles of ERK1/2 and PPARγ signaling pathways in Mg^{2+}-mediated suppression of IL-1β expression in glial cells

We next aimed to elucidate the signaling pathways of IL-1β regulation in MgT-treated glial cells. In light of a previous work suggesting that Mg^{2+} deprivation inhibits the MEK-ERK signaling cascade,[18] we evaluated the effects of exogenous MgT (50 μM) on ERK1/2 activation. In line with these *in vivo* data, our *in vitro* results show that MgT treatment induced the phosphorylation of ERK1/2 without altering the total protein levels of ERK1/2 in A172 cells (Figure 3c and d). To further elucidate the potential role of ERK1/2 in the regulation of the expression of IL-1β we treated A172 cells with the pharmacological ERK1/2 inhibitor PD98059 (10 μM) in the absence or presence of MgT (50 μM). The incubation of A172 cells with PD98059 (10 μM) not only suppressed the MgT-induced phosphorylation of ERK1/2 but also reversed the MgT-dependent decrease of IL-1β synthesis (Figure 3c). To eliminate any potential non-specific effects of the pharmacological ERK1/2 inhibitor PD98059, experiments were carried out using A172 cells transfected with an siRNA oligonucleotide sequence specific for ERK1/2. ERK1/2-knockdown and scramble control cells were treated with MgT (50 μM) or the vehicle control for 48 h. ERK1/2 knockdown markedly reversed the inhibitory effects of MgT on the mRNA and protein expression of IL-1β in A172 cells (Figure 3d).

Treatment of A172 cells with MgT (50 μM) induced PPARγ phosphorylation (Figure 3e). PD98059 (10 μM) abrogated the MgT-induced phosphorylation of PPARγ in A172 cells (Figure 3e). To decipher the role of PPARγ in regulating IL-1β expression, we next treated A172 cells with the PPARγ antagonist GW9662 (1 μM). The results indicate that GW9662 treatment reversed the effects of the MgT-induced suppression of IL-1β expression in A172 cells (Figure 3e'). To eliminate any non-specific effects of the PPARγ antagonist, the experiments were performed using cells transfected with an siRNA oligonucleotide specific for PPARγ. The efficiency of the PPARγ knockdown was demonstrated by assessing the PPARγ mRNA levels in the A172 cells (Figure 3f upper panel). The PPARγ knockdown reversed the inhibitory effects of MgT on the mRNA and protein expression of IL-1β in A172 cells (Figure 3f lower panel). To further confirm the above observations, similar experiments were carried out in primary cultured astrocytes. The results demonstrated that the ERK1/2 and PPARγ signaling pathways are critical for mediating the MgT-dependent downregulation of the expression of IL-1β in primary cultured astrocytes (Figure 3g and h). Taken together, these observations indicate that the activation of the ERK1/2 and PPARγ signaling pathways plays a critical role in regulating MgT-induced suppression of the expression of IL-1β in human glial cells.

Table 1 The enrichment of IL-Iβ and IL-I receptors in glia and neuron cells.

Ct value	IL-Iβ	IL-1R1	IL-1R2
SH-SY5Y	N/A	N/A	25.47
A172	26.94	31.85	N/A
n2a	N/A	30.46	26.98
D1A	28.94	25.11	30.82

Mg^{2+} elevation inhibits the mRNA and protein expression of IL-1β in APP/PS1 transgenic mice

Although Mg^{2+} elevation suppresses the expression of IL-1β *in vitro*, little is known about the effects of Mg^{2+} elevation in the *in vivo* setting. This is particularly important because Mg^{2+} homeostasis may be disturbed in AD Tg mice. To delineate the relationship between Mg^{2+} elevation and the expression of IL-1β *in vivo*, we first employed immunohistochemistry to evaluate the expression of IL-1β in 3-month-old Tg mice. We found that Mg^{2+} administration (100 mg/kg/d) for 2 months decreases IL-1β expression in the cerebral cortex of APP/PS1 Tg mice (Figure 4a left panel). In addition, Mg^{2+} administration (100 mg/kg/d) for 2 months suppressed the mRNA and protein expression of IL-1β in the cerebral cortex (Figure 4a right panel). To validate the inhibitory effects of MgT on the expression of IL-1β, an organotypic slice culture was carried out in the absence or presence of MgT (50 μM) for 24 h.

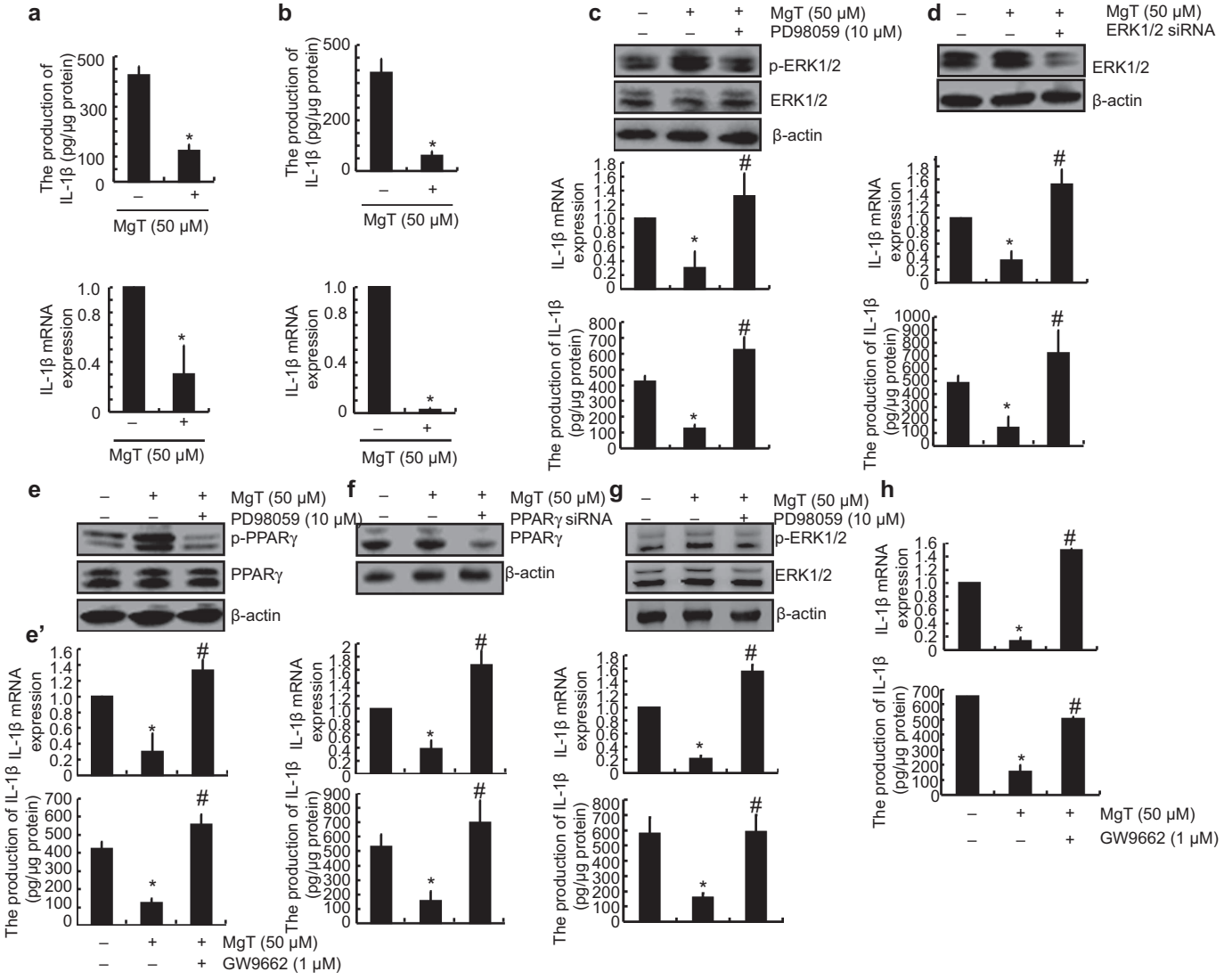

Figure 3 Involvement of ERK1/2 and PPARγ pathways in regulating the expression of IL-1β in MgT-treated A172 or D1A cells. Human glioblastoma A172 (**a**) or mouse astrocytes/microglia D1A cells (**b**) were treated with MgT (50 μM) for 48 h. In select experiments, A172 cells were treated with PD98059 (10 μM) in the absence or presence of MgT (50 μM) for 48 h (**c** and **e**). In separate experiments, the cells were transfected with ERK1/2 (**d**) or PPARγ siRNA (**f**) before incubation with MgT (50 μM) for 48 h. In distinct experiments, A172 cells were treated with GW9662 (1 μM) in the absence or presence of MgT (50 μM) for 48 h (**e'**). In other experiments, primary cultured astrocytes were treated with MgT (50 μM) in the absence or presence of PD98059 (10 μM) (g) or GW9662 (1 μM) for 48 h (h). Total ERK1/2 (**c**, **d** and g upper panel), phosphorylated ERK1/2 levels (**c** and g upper panel), total PPARγ (**e** and **f** upper panel), and phosphorylated PPARγ (**e**) were detected by immunoblotting using specific Abs. Equal lane loading is demonstrated by the similar intensities of total β-actin. IL-1β protein and mRNA levels were determined by IL-1β enzyme immunoassay kits and qRT-PCR, respectively. The total amounts of protein and GAPDH served as internal controls. The data represent the means ± SE of three independent experiments. $^{*}P < 0.05$ compared with the vehicle-treated control. $^{\#}P < 0.05$ compared with MgT treatment alone.

Imunohistochemistry experiments were then carried out to determine the expression of IL-1β after 24 h of culture. The results reveal that MgT (50 μM) treatment abolishes the upregulation of IL-1β in APP/PS1 Tg mice (Figure 4b).

To further verify the key role of MgT in suppressing the expression of IL-1β *in vivo*, we combined i.c.v. injection with live animal/two-photon imaging. As described in Figure 4c upper panel, D1A cells transfected with IL-1β promoter constructs were pre-seeded into the right cerebral ventricle, whereas Mg^{2+} (2 μg/5 μl) was injected into the left cerebral ventricle of 3-month-old APP/PS1 Tg mice. After 24 h, luciferin was injected (i.c.v.) into the right cerebral ventricle of APP/PS1 Tg mice before live animal imaging. The results show that MgT decreased the luciferase activity of the IL-1β promoter (Figure 4c lower panel). In addition, the cerebral cortex of APP/PS1 Tg mice was immunostained with IL-1β before or after injection (i.c.v.) of MgT for 24 h (Figure 4d upper panel). The two-photon scanning results revealed that Mg^{2+} (2 μg/5 μl) injection (i.c.v.) decreases the immunofluorescence of IL-1β on the surface of the cerebral cortex of APP/PS1 Tg mice (Figure 4d lower panel). Collectively, our results demonstrate that Mg^{2+} influx *in vivo* transcriptionally suppresses the expression of IL-1β in APP/PS1 Tg mice.

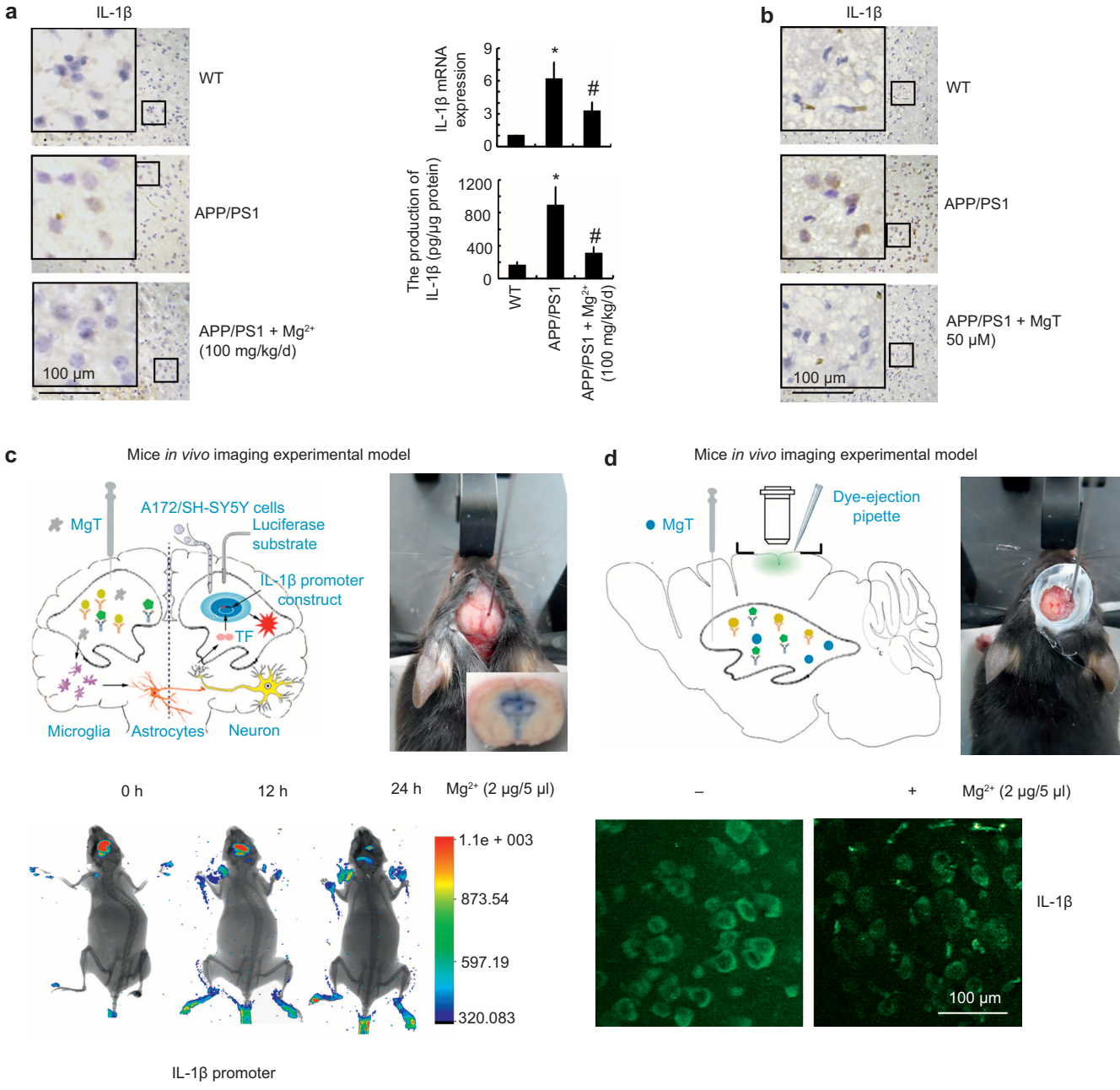

Figure 4 Elevated levels of Mg^{2+} in APP/PS1 transgenic mice decrease the expression of IL-1β. The APP/PS1 transgenic mice at the age of 4 months were administered Mg^{2+} (100 mg/kg/d) for 2 months before collecting the brain (**a**). In select experiments, the brains of APP/PS1 transgenic mice at the age of 3 months were harvested and sectioned (400 μm) using a cryostat (**b**). In separate experiments, the left cerebral ventricle was injected with Mg^{2+} (2 μg/5 μl) or vehicle (PBS) and the right cerebral ventricle was injected (i.c.v.) with D1A cells, which was pre-transfected with IL-1β promoter in the right cerebral ventricle (**c**). In distinct experiments, the left cerebral ventricle was injected with Mg^{2+} (2 μg/5 μl) or vehicle (PBS) before staining with IL-1β antibody and scanning under two-photon microscopy (**d**). The immunoreactivity of IL-1β was determined by immunohistochemistry using an anti-IL-1β antibody (a left panel, b). These images are representative of six independent experiments, all with similar results. IL-1β protein and mRNA levels were determined by qRT-PCR and IL-1β enzyme immunoassay kits, respectively (a right panel). The total amounts of GAPDH and protein served as an internal control. The experimental cartoon and real surgery images are shown (c, d upper panel). Luciferase activities from the different groups of mice were measured using a live animal imaging system (c lower panel). The immunofluorescence of IL-1β was scanned using a two-photon microscope (d lower panel). The data represent the means ± S.E. of three independent experiments. *$p < 0.05$ compared with wild type mice. #$p < 0.05$ compared with APP/PS1 transgenic mice.

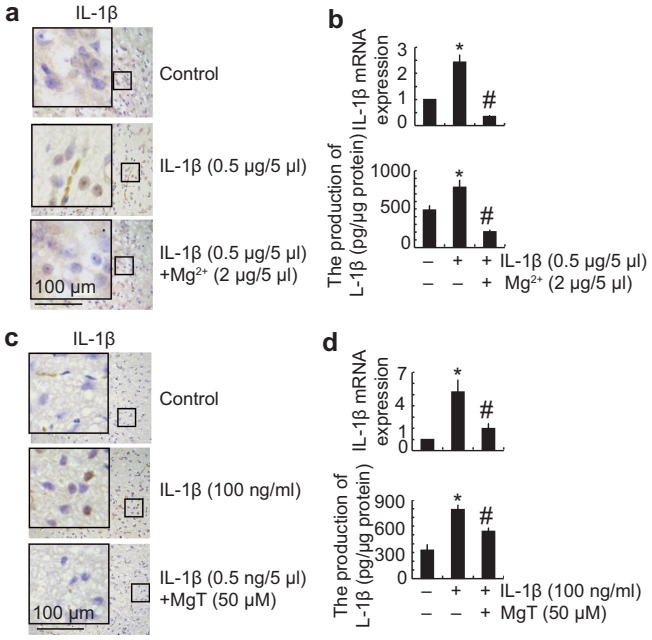

Figure 5 MgT treatment diminished the effects of IL-1β in the CSF with respect to inducing the expression of IL-1β in the cerebral cortex. WT C57BL/6 mice at the age of 3 months were intracerebroventricularly injected with IL-1β (0.5 μg/5 μl) in the absence or presence of Mg^{2+} (2 μg/5 μl). The brains were then collected and sectioned after 24 h (**a** and **b**). In select experiments, the brains of WT C57BL/6 mice at the age of 3 months were harvested and freshly sectioned (400 μm) before treatment with IL-1β (100 ng ml^{-1}) in the absence or presence of MgT (50 μM) for 24 h (**c**). In separate experiments, A172 cells were treated with IL-1β (100 ng ml^{-1}) in the absence or presence of MgT (50 μM) for 24 h (**d**). The immunoreactivity of IL-1β was determined by immunohistochemistry using an anti-IL-1β or -APH-1 antibody (**a** and **c**). These images are representative of six independent experiments, all with similar results. IL-1β mRNA and protein levels were determined by qRT-PCR and IL-1β enzyme immunoassay kits, respectively (**b** and **d**). The total amounts of GAPDH and protein served as an internal control. The data represent the means ± SE of three independent experiments. $^{*}P < 0.05$ compared with respect to vehicle-treated controls. $^{\#}P < 0.05$ compared with IL-1β-treated alone.

MgT suppresses the effects of the secreted form of IL-1β and Aβ oligomers in the ventricles by inducing the expression of IL-1β in the cerebral cortex

In view of our findings, which showed that MgT incubation decreases the expression of IL-1β *in vitro* and *in vivo*, we next examined the potential contribution of the secreted form of IL-1β and Aβ in the pathogenesis and progression of AD. Interestingly, the injection of IL-1β (0.5 μg/5 μl) into the cerebral ventricles of WT C57BL/6 mice for 24 h increased the expression of IL-1β in the cerebral cortex, whereas the addition of Mg^{2+} (2 μg/5 μl) effects the IL-1β in the ventricles by inducing the expression of IL-1β in the cerebral cortex (Figure 5a and b). Similar results were observed in the organotypic slice cultures of brain tissue (Figure 5c). These findings are in line with *in vitro* results showing that incubation with MgT (50 μM) suppressed the expression of IL-1β in IL-1β (100 ng ml^{-1})-stimulated A172 cells (Figure 5d).

In view of the critical roles of Aβ deposition during neuroinflammation,[19,20] we questioned whether Aβ production in APP/PS1 transgenic mice has the ability to regulate the expression of IL-1β. As a first step, we determined the production and deposition of Aβ in APP/PS1 mice. The results demonstrated that the levels of $Aβ_{1-42}$ in the CSF were decreased in APP/PS1 transgenic mice and restored by MgT treatment (Figure 6a). However, the deposition or aggregation of $Aβ_{1-42}$ was highly induced and deposited in the APs of 9-month-old APP/PS1 transgenic mice and was suppressed by MgT administration (Figure 6b and c). These observations demonstrated that the majority of $Aβ_{1-42}$ was progressively aggregated and deposited in the cerebral cortex during the course of AD development. To further evaluate the involvement of Aβ in the regulation of IL-1β expression, *in vivo* experiments were performed. CSF (5 μl) was collected from APP/PS1 Tg mice and then injected into WT C57BL/6 mice for 2 weeks before killing. Compared with the control subjects, the expression of IL-1β in the cerebral cortex was markedly induced by the injection of APP/PS1 CSF (Figure 6d–f). Moreover, Aβ antibody injection blocked the effects of APP/PS1 CSF with respect to inducing the expression of IL-1β in the cerebral cortex of mice (Figure 6d–f). These observations were clearly in contrast to the total levels of $Aβ_{1-42}$ in the CSF. In light of the ability of $Aβ_{1-42}$ aggregation, we suspect that the proportion of aggregated $Aβ_{1-42}$ was elevated, although the total amount of $Aβ_{1-42}$ was downregulated in the CSF of APP/PS1 mice. To this end, oligomeric $Aβ_{1-42}$ (2 μg/5 μl) was injected (i.c.v.) into the ventricles of 3-month-old WT C57BL/6 mice, and the results demonstrated that oligomeric Aβ injection increased the expression of IL-1β in the cerebral cortex (Figure 7a and b). In addition, the upregulation of IL-1β was suppressed by Mg^{2+} (2 μg/5 μl) injection (i.c.v) in the Aβ oligomers-stimulated C57BL/6 mice (Figure 7a and b). Similar results were obtained by organotypic slice cultures of brain tissue (Figure 7c). These *in vivo* observations were in concert with *in vitro* data showing that Aβ (1 μM) treatment stimulated the expression of IL-1β, which was blocked by MgT incubation in glial cells (Figure 7d). Collectively, these data revealed that $Aβ_{1-42}$ is a downstream molecule of MgT and regulates the expression of IL-1β in APP/PS1 transgenic mice.

DISCUSSION

AD is pathologically characterized by a marked induction of neuroinflammation.[21] Although Mg^{2+} has been implicated in reducing neuroinflammation during the course of AD development,[22,23] the underlying mechanisms between Mg^{2+} and IL-1β expression are still elusive. Thus, this study aimed to decipher the suppressive effects of Mg^{2+} on IL-1β expression. The major findings of this work were as follows: (i) the elevation of Mg^{2+} in microglia cells suppressed the synthesis of IL-1β; (ii) the ERK1/2 and PPARγ pathways are involved in mediating the suppressive effects of Mg^{2+} on IL-1β expression in microglia cells; (iii) the secreted form of IL-1β in the CSF is critical for self-induction of IL-1β in the cerebral cortex of APP/PS1 transgenic mice; and (iv) the Aβ oligomers in the CSF mediated the effects of MgT on suppressing the expression

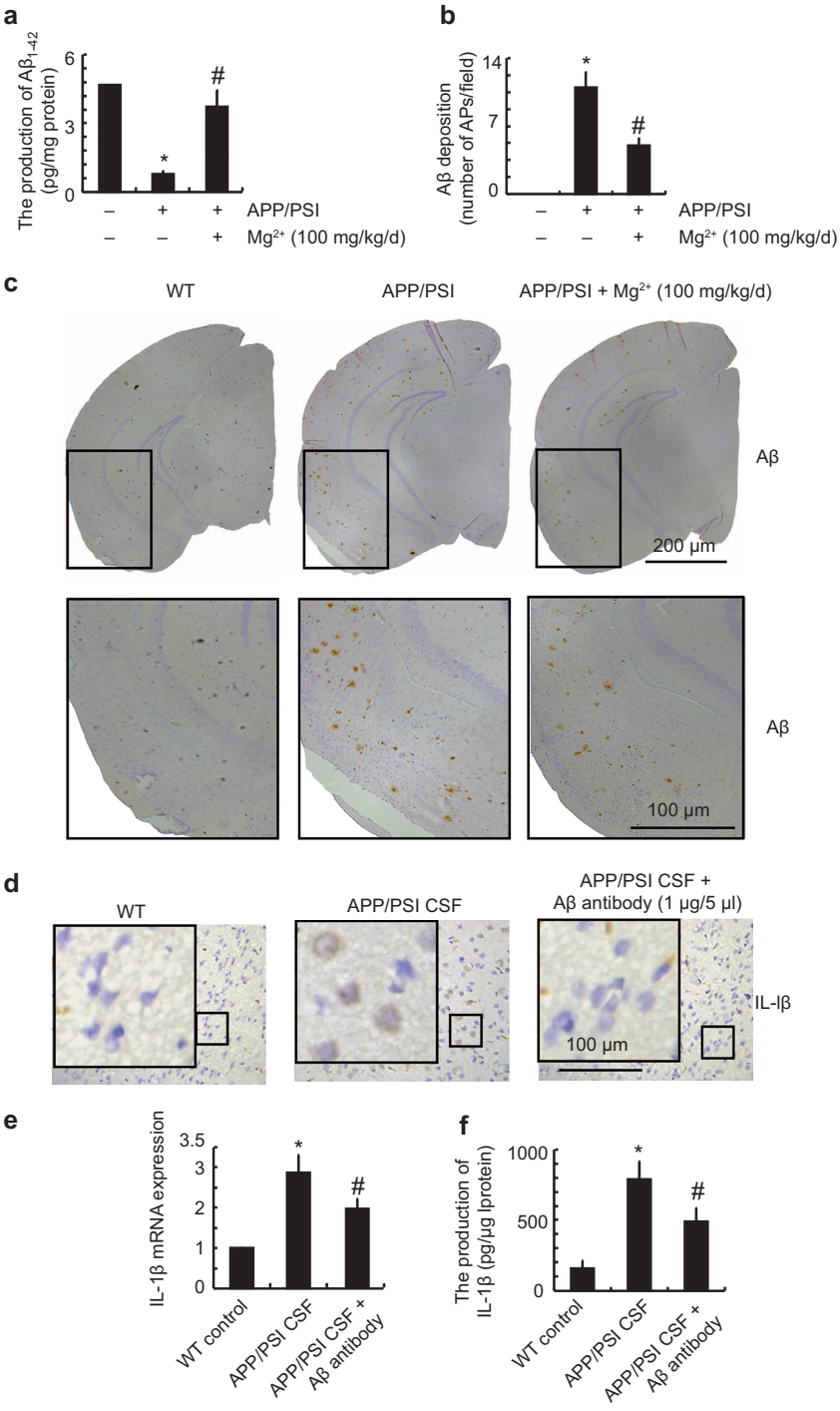

Figure 6 Aβ oligomers in the CSF of APP/PS1 mice have the ability to stimulate the expression of IL-1β in the cerebral cortex of WT mice. The APP/PS1 Tg mice at the age of 4 months were treated with Mg^{2+} (100 mg/kg/d) for 5 months before collecting the CSF and brains (**a-c**). Cerebrospinal fluid (CSF) was then injected into the wild type C57BL/6 mice in the absence or presence of Aβ antibody (1 μg/5 μl) for two weeks before scarifice (**d-f**). The production of $Aβ_{1-42}$ was determined by ELISA kits, and the total amount of protein served as an internal control (**a**). The immunoreactivity of Aβ and IL-1β was determined by immunohistochemistry using an anti-Aβ or IL-1β antibody (**c, d**). These images are representative of six independent experiments, all with similar results. The number of AP/fields was calculated according to the images of IHC (**b**). IL-1β mRNA and protein levels were determined by qRT-PCR and IL-1β enzyme immunoassay kits, respectively (**e, f**). The total amounts of GAPDH and protein served as an internal control. *$p < 0.05$ compared with respect to wild type mice. #$p < 0.05$ compared with APP/PS1 alone.

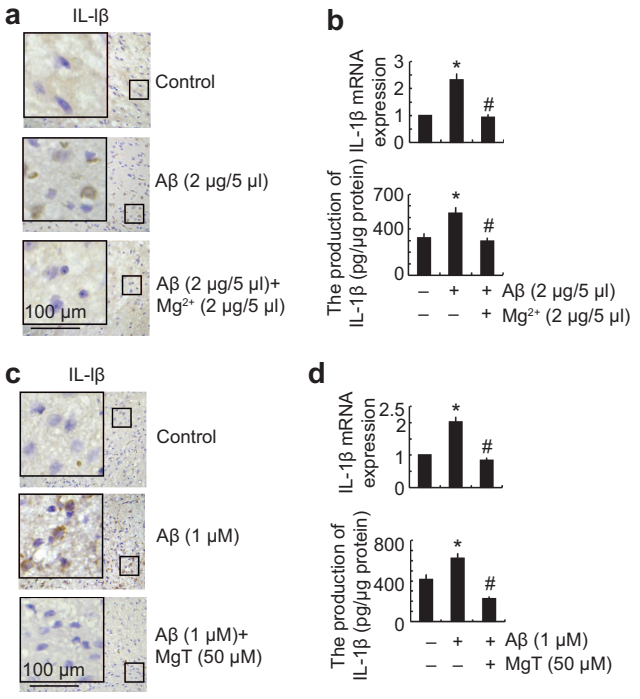

Figure 7 MgT treatment attenuated the effects of Aβ oligomers in the CSF for inducing the expression of IL-1β in the cerebral cortex. The WT C57BL/6 mice at the age of 3 months were injected (i.c.v) with Aβ oligomers (2 μg/5 μl) in the absence or presence of Mg^{2+} (2 μg/5 μl). The brains were then collected and sectioned after 24 h (**a** and **b**). In select experiments, the brains of WT C57BL/6 mice at the age of 3 months were harvested and freshly sectioned (400 μm) before treatment with Aβ oligomers (1 μM) in the absence or presence of MgT (50 μM) for 24 h (**c**). In distinct experiments, A172 cells were treated with Aβ oligomers (1 μM) in the absence or presence of MgT (50 μM) for 24 h (**d**). The immunoreactivity of IL-1β was determined by immunohistochemistry using an anti-IL-1β antibody (**a** and **c**). These images are representative of six independent experiments, all with similar results. IL-1β mRNA and protein levels were determined by qRT-PCR and IL-1β enzyme immunoassay kits, respectively (b and d). The total amounts of GAPDH and protein served as an internal control. The data represent the means ± SE of three independent experiments. $^{*}P < 0.05$ compared with respect to vehicle-treated controls. $^{\#}P < 0.05$ compared with Aβ-treated alone.

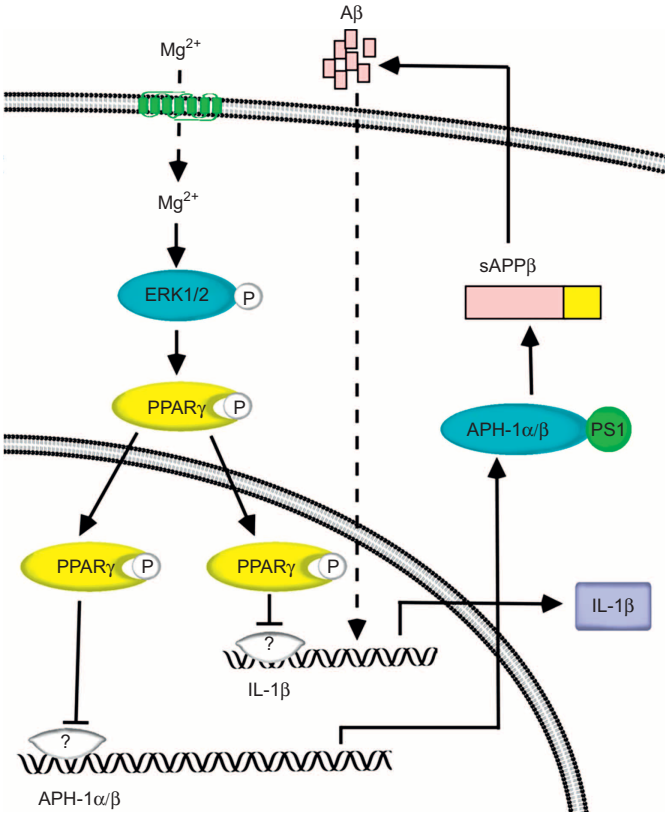

Figure 8 Proposed cascade of the signaling events regulating the expression of IL-1β by MgT. In detail, attenuated levels of Mg^{2+} in APP/PS1 transgenic mice will elevate the production of IL-1β and Aβ via ERK1/2- and PPARγ-dependent pathways in glial cells of the APP/PS1 transgenic mouse brain, which in turn will potentially regulate the pathogenesis of AD. Interestingly, the highly secreted IL-1β and Aβ in the CSF are able to regulate the synthesis of IL-1β in the cerebral cortex of APP/PS1 mice. These observations might be instrumental for understanding the roles of Mg^{2+} in suppressing the neuroinflammation of AD.

of IL-1β in the cerebral cortex of APP/PS1 transgenic mice, which potentially improved the neuroinflammation of AD (Figure 8).

Mg^{2+} is tightly regulated and kept at basal levels under physiological conditions. Accumulating evidence suggests that Mg^{2+} is markedly downregulated during the course of AD progression.[3,4] Indeed, MgT administration prevents synapse loss and reverses memory deficits in aged rats.[24] These observations suggest that Mg^{2+} elevation in the brain may exert neuroprotective effects against the onset of AD. In addition, Lipinski *et al.*[25] also reported that Mg^{2+} has the ability to disrupt the aggregation between red blood cells and fibrillar $A\beta_{1\text{-}42}$, which protects the brain from the risk of AD. Along these lines, $MgSO_4$ treatment decreases maternal and fetal inflammation following lipopolysaccharide (LPS) injection.[26]

On the other hand, magnesium deficiency results in cardiac dysfunction and inflammation, as evidenced by the marked induction of cytokine production in rats.[27–29] Moreover, $MgSO_4$ also decreases inflammation-associated brain injury in fetal mice,[30] supporting a link between magnesium, inflammation, and neurological injury in rodents. This evidence prompted us to hypothesize that MgT exerts its neuroprotective effect by downregulating inflammatory cytokine production. Indeed, our findings reveal that MgT treatment obviously suppressed the synthesis of IL-1β both *in vitro* and *in vivo*. In line with our data, Sugimoto *et al.*[5] reported that Mg^{2+} reduces the expression of IL-1β in human monocytic cells. Others[31] have found that the administration of $MgSO_4$ to pre-eclamptic placentas resulted in an attenuation of the increased secretion of IL-1β into the maternal circulation and in a reduction of IL-1Ra.

In light of the suppressive effects of MgT on IL-1β expression, we were prompted to reveal the mechanisms. In agreement with our data, Li *et al.*[32] reported that pioglitazone, a PPARγ

agonist, suppresses the expression of IL-1β in LPS-stimulated microglia cells. In addition, Valles *et al.*[33] revealed that increased PPARγ expression prevents Aβ-induced IL-1β expression in cultured astrocytes. More specifically, the PPARγ agonist, 1,1-bis(3'-indolyl)-1-(p-trifluoromethylphenyl) methane suppresses Mg^{2+}-induced production of nitric oxide in astrocytes.[34] We extended the prior works to ERK1/2, which is responsible for PPARγ phosphorylation. Our data were supported by the study showing that active PPARγ was recruited to phosphorylated ERK1/2, which in turn improves the cognitive decline in AD patients or Tg2576 mice.[35] Moreover, Denner *et al.*[36] found a significant overlap between PPARγ target genes and ERK1/2-regulated, cAMP response elements containing target genes in Tg2576 mice. Although they did not extend their study to find the regulatory relationship between ERK1/2 and PPARγ, these prior works have implied the important roles of ERK1/2 and PPARγ signaling pathways in AD development. Along these lines, our data provide a mechanistic understanding of the previous phenomenological studies and establish the key roles of ERK1/2 and PPARγ pathways in mediating IL-1β suppression by MgT exposure.

Because AD is characterized by the production and aggregation of Aβ, we further found that Aβ oligomers in the CSF have the ability to stimulate the expression of IL-1β in the cerebral cortex of APP/PS1 transgenic mice. In line with our data, it has been recently reported that misfolded and aggregated Aβ and tau bind to pattern recognition receptors on microglia and astroglia, which potentially contribute to neuroinflammation by releasing inflammatory cytokines (pro-IL-1β, IL-6, and TNFα).[19,20] Others[37] have suggested that Aβ oligomeric clusters exacerbate the processing of pro-IL-1β into mature IL-1β in microglial cells, which in turn enhances microglia neurotoxicity and the progression of AD. In addition, Lindberg *et al.*[38] revealed that both oligomer $Aβ_{1-42}$ peptides and $Aβ_{1-40}$ are able to upregulate the secretion of IL-1β in primary cultures of microglia from the rat neonatal cerebral cortex. All of these observations revealed a relationship between IL-1β and Aβ during the course of AD progression.

In addition, neuroinflammation is not a passive system activated by the formation of AP and NFTs but instead exacerbates the pathogenesis of AD by inducing Aβ deposition and tau phosphorylation.[39] For example, IL-1β has been implicated in promoting the synthesis and amyloidogenic accumulation of APP in thyroid cells and skeletal muscle.[40,41] Grilli *et al.*[42] further identified nuclear factor-κB as a critical transcriptional factor of the APP promoter that drives the synthesis of APP in response to IL-1β stimulation in primary mouse neurons. In line with these observations, IL-1β stimulates γ-secretase-mediated cleavage of APP, which in turn controls Aβ production in HEK293 cells.[43] All of these observations pointed to the possible roles of IL-1β in accelerating Aβ formation. However, some studies have revealed that IL-1β treatment also increases α-secretase-dependent APP processing,[44–46] which in turn decreases Aβ deposition in $APP/PS1^{+/-}$ transgenic mice.[47] Thus, the ratio of α-, β-, or γ-secretase alteration in response to IL-1β treatment in various microenvironments would be a critical factor in determining the effects of IL-1β on Aβ deposition or AD development.

Although we still could not completely determine the roles of IL-1β in Aβ deposition, especially during the course of AD development, IL-1β has been regarded as a biomarker of AD. Cacabelos *et al.*[48] first reported that IL-1β levels were significantly higher in the CSF of AD patients than in multi-infarct dementia, normal pressure hydrocephalus, and multiple sclerosis patients. Because IL-1β was found to be elevated in the brain of AD patients,[48] the pathological role of IL-1β in AD development has been extensively investigated over the past two decades. Three theories about the mechanisms by which IL-1β affects AD progression have emerged as follows: (i) neuroinflammation,[49] (ii) tau; (Ghosh *et al.* 2013;[50] Kitazawa *et al.* 2011[7]), and (iii) cell death.[51] Unfortunately, most of these studies were phenomenological in nature. Novel components or drugs need to be developed elevate the levels of Mg^{2+} in AD brains, which may result in improved treatment options for AD patients.

Using MgT as a model drug, we revealed that Mg^{2+} elevation suppresses the expression and production of IL-1β in glial cells and the cerebral cortex of APP/PS1. In addition, the phosphorylation of ERK1/2 and PPARγ by MgT-stimulation suppresses the expression of IL-1β in glial cells. Importantly, the production of IL-1β and Aβ oligomers in the CSF is critical for stimulating the expression of IL-1β in the cerebral cortex of APP/PS1 transgenic mice. Based on these novel mechanistic findings, it may be easier to monitor the effects of MgT on combating AD via suppressing the expression of IL-1β in AD patients.

CONFLICT OF INTEREST

The authors declare no competing financial interests.

ACKNOWLEDGEMENTS

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