Biochemical and Biophysical Research Communications 434 (2013) 363–369

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Silencing of the $P2X_7$ receptor enhances amyloid-β phagocytosis by microglia

Jiong Ni [a], Peijun Wang [a,*], Jingfa Zhang [b], Wei Chen [a], Limin Gu [b]

[a] Department of Medical Imaging, Tongji Hospital, Tongji University School of Medicine, Shanghai 200065, China
[b] Department of Regenerative Medicine, Tongji University School of Medicine, Shanghai 200092, China

ARTICLE INFO

Article history:
Received 21 March 2013
Available online 4 April 2013

Keywords:
Alzheimer's disease
Amyloid-β peptides
Microglia
$P2X_7$ receptor
RNA interference

ABSTRACT

$P2X_7$ receptor ($P2X_7R$) is an ATP-gated cation channel that promotes microglia activation and plays a critical role in the pathogenesis of Alzheimer's disease. Inhibiting $P2X_7R$ indirectly reduces the rate of amyloid-β (Aβ)-induced neurodegeneration by suppressing secretion of inflammatory factors from activated microglia. We used RNA interference to silence $P2X_7R$ in microglial cells *in vitro* and found it markedly increased microglial phagocytosis of $A\beta_{1-42}$. Increased phagocytic activity was dependent on decreasing the rate of interleukin-1β release from microglia and required inhibition of the COX-2 pathway. Modulation of microglial phagocytosis and secretion via silencing $P2X_7R$ may be a promising therapeutic option for the treatment of Alzheimer's disease.

© 2013 Elsevier Inc. All rights reserved.

1. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disease. It is pathologically characterized by senile plaques composed of amyloid-β (Aβ) peptides, which are surrounded by active microglia [1]. Microglia are thought to play a critical role in the pathogenesis of AD [2,3]. Microglia derived from mononuclear phagocytes can directly phagocytose Aβ, and microglia activated by Aβ can release an array of cytokines [4–8], including pro-inflammatory mediators, such as interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ), which can cause neuroinflammation and neuronal damage, as well as anti-inflammatory cytokines, such as interleukin-4 (IL-4), interleukin-10 (IL-10), and transforming growth factor-β1 (TGF-β1). Therapeutic strategies that inhibit the activation of potentially neurotoxic inflammatory responses by microglia or enhance microglial phagocytic activity, which may reduce the cerebral Aβ load, have been shown to limit neurodegeneration in animal models of AD and AD patients [9,10].

The $P2X_7$ receptor ($P2X_7R$) is a cell surface ATP-gated ion channel known to promote microglia activation [11,12]. $P2X_7R$ expression is significantly up-regulated in microglia surrounding Aβ plaques in the brains of patients with AD and the brains of AD model animals [13,14]. Studies have also demonstrated that brilliant blue G (BBG), a $P2X_7R$ antagonist, can improve pathological changes in the brain of AD animal models [15]. These studies indicate that $P2X_7R$ plays an important role in AD pathogenesis. However, the high doses of BBG used in animal studies are not suitable for use in humans with AD [16]. An effective therapy involving antagonism of $P2X_7R$ is urgently needed.

RNA interference (RNAi) is capable of efficiently and specifically manipulating gene expression. It can directly silence pathogenic genes by interfering with the transcribed mRNA. It may be suitable for the treatment of AD and other refractory neurodegenerative diseases [17]. Previous studies have identified several therapeutic agents, most of which target the amyloid-β protein precursor gene, that mitigate disease pathogenesis in the brains of AD animal models [18,19]. AD is a disease regulated by multiple genes. The key to treating AD using RNAi is to search for the most effective therapeutic target. It is not known whether silencing a target of abnormal cytokine expression would be effective. Furthermore, the inhibition of $P2X_7R$ is known to reduce neuroinflammation [15]. However, its effect on microglial phagocytosis has not yet been examined.

In the present study, we used RNAi to silence $P2X_7R$ *in vitro* to determine whether inhibiting $P2X_7R$ expression can influence Aβ phagocytosis and cytokine secretion by microglia.

2. Materials and methods

2.1. Cell culture

Mouse primary microglial cell cultures were prepared as described previously [20]. Briefly, hippocampi from newborn ICR mice (days 1–2 postnatal) were isolated under sterile conditions and stored at 4 °C. Cells were mechanically dissociated and plated in 75 cm^2 flasks containing RPMI 1640 media supplemented with 5% fetal calf serum, 2 mM glutamine, 100 U/ml penicillin, 0.1 μg/ml streptomycin, and 0.05 mM 2-mercaptoethanol. Primary

* Corresponding author. Address: Department of Medical Imaging, Tongji Hospital, Tongji University, School of Medicine, 389 Xincun Road, Shanghai 200065, China. Fax: +86 21 5695 2231.
E-mail address: tongjipjwang@sina.cn (P. Wang).

0006-291X/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2013.03.079

cultures were maintained for 14 d. Microglial cells were isolated by shaking the flasks at 200 rpm in a Lab-Line incubator-shaker. The purity of the microglial cells was evaluated by immunocytochemical staining using antibodies against ionized calcium binding adapter molecule 1 (Iba1, Abcam, UK). Purity was typically greater than 95%. All animals were provided by the animal center of the Chinese Academy of Sciences, Shanghai Branch (Animal Conformity Certificate No. SCXK 2008-0016). All experiments were approved by the Institutional Animal Care and Use Committee of Tongji University.

2.2. RNA interference

A small interfering RNA (siRNA) construct specific to $P2X_7R$ was designed based on the $P2X_7R$ mouse gene sequences in GenBank (NM_011027.2). Four sets of siRNA sequences (encoded as $siP2X_7R1$-4) and a non-silencing control siRNA sequence (encoded as siNC) are listed in Table 1. These siRNA sequences were searched in BLAST to verify that only the selected $P2X_7R$ mRNA gene was specifically targeted by these siRNAs. These siRNAs were synthesized by the GenePharma Company, Shanghai, China.

Microglial cells were stimulated for 2 h with 100 μM 2′,3′-(benzoyl-4-benzoyl-ATP) (BzATP, Sigma–Aldrich, USA), a $P2X_7R$ agonist, and 5 μM $A\beta_{1-42}$ (Sigma–Aldrich, $A\beta_{1-42}$ peptides were dissolved in sterile water and incubated for 5 d at 37 °C to allow fibrillization) [21]. SiRNAs were transfected into microglia using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's protocol. After transfection, cells were cultured for 48 h with Lipofectamine-$siP2X_7R1$-4 (50 μM) or Lipofectamine-siNC (50 μM).

2.3. Real-time quantitative PCR

Microglial cells were collected 48 h after siRNA transfection and total RNA was isolated using Trizol reagent (Invitrogen). Complementary DNA was synthesized using a first-strand cDNA synthesis kit (RevertAid™, Fermentas, USA). In brief, 1 μg total RNA was reverse-transcribed using MMLV reverse transcriptase (Invitrogen) and random hexamer primers according to manufacturer's instructions. The reaction mix for real-time qPCR with SYBR Green I probe (TransStart™ Gene qPCR SuperMix, Beijing TransGen Biotech, China) consisted of 12.5 μL 2× TransStar™ Green qPCR superMix, 0.5 μL of 10 μM solutions of each primer, 0.5 μL 1× Passive Reference Dye, 2 μL cDNA, and 9.5 μL nuclease-free water. Reaction conditions included 40 cycles at 95 °C for 30 s and 72 °C for 60 s. Relative $P2X_7R$ gene expression was calculated using the $2^{-(\Delta\Delta CT)}$ method with β-actin as the reference gene. Primers were synthesized by Invitrogen and the sequences were as follows:

$P2X_7R$ sense primer: 5′GTACAGCTTCCGCCGCCTGG3′
$P2X_7R$ anti-sense primer: 5′TGCACACAGTGGCCAAGCCA3′
β-actin sense primer: 5′CACCCGCGAGCACAGCTTCT3′
β-actin anti-sense primer: 5′TTTGCACATGCCGGAGCCGT3′

Table 1
$P2X_7R$-siRNA sequences and a non-silencing control siRNA.

Code	Sense sequence
$siP2X_7R1$	5′GAACGAUGUCUUGCAGUATT3′
$siP2X_7R2$	5′GGUGACGGAGAAUGUCACATT3′
$siP2X_7R3$	5′AGACGCUGUGUCCUGAGUATT3′
$siP2X_7R4$	5′GCCACAACTATACCACGAGAA3′
siNC	5′UUCUCCGAACGUGUCACGUTT3′

2.4. Detection of $P2X_7R$ protein expression by Western blot

Microglial cells, collected 48 h after siRNA transfection, were washed in cold PBS and lysed in 1 mL lysis buffer (Showbio Biotech, China) for 30 min on ice. The total protein content in lysates was determined using Nanodrop (Thermo Scientific, USA). For Western blotting, the $P2X_7R$ protein was separated on 5% SDS–PAGE, the β-actin loading control was separated on 10% SDS–PAGE, and then were electrophoretically transferred onto nitrocellulose membranes. Membranes were blocked in bovine serum albumin overnight at 4 °C, followed by incubation overnight at 4 °C with a rabbit monoclonal antibody against $P2X_7R$ (Abcam) in PBS. Blots were washed and incubated for 2 h with anti-rabbit HRP-conjugated IgG secondary antibody (Cell Signaling, USA) in PBS. Protein bands were visualized using the Image Station 2000MM System (Kodak, USA), and were scanned and analyzed with Molecular Imaging Software Standard Edition V5.0 (Kodak).

2.5. $P2X_7R$ immunocytochemical staining

Microglial cells were plated at 1×10^5 cells per well in 24-well tissue culture plates and stimulated for 2 h with 100 μM BzATP and 5 μM $A\beta_{1-42}$. In the presence of BzATP and $A\beta_{1-42}$, microglia were transfected for 48 h with 50 μM Lipofectamine-$siP2X_7R$ or 50 μM Lipofectamine-siNC. Immunocytochemical staining for $P2X_7R$ was performed using a rabbit monoclonal antibody against $P2X_7R$ (Abcam). Endogenous peroxidase activity was eliminated by treatment with 1% H_2O_2/10% methanol in Tris-buffered saline (TBS) for 60 min at room temperature. After washing with 0.1 M Tris buffer (pH 7.5) and 0.1 M TBS (pH 7.4), cells were blocked for 60 min at room temperature with 5% normal serum in 0.1 M TBS with 0.5% Triton-X-100 (TBST) to prevent non-specific protein binding. The cells were incubated for 18 h at 4 °C with the primary antibody described above (1:100) in TBST with 2% serum. Next, the cells were rinsed in 0.1 M TBS and incubated for 2 h at room temperature with the biotinylated secondary antibody in TBST with 2% serum. Finally, avidin–biotin peroxidase detection was performed using 3,3′-diaminobenzidine as a substrate (Vector Labs, CA, USA) according to the manufacturer's protocol. Microglial cells were observed using a CX31-12CO4 microscope (Olympus, Japan) equipped with a digital camera.

2.6. Cytokine analyses

Microglial cells were plated at 1×10^5 cells per well in 24-well tissue culture plates and stimulated for 2 h with 100 μM BzATP and 5 μM $A\beta_{1-42}$. In the presence of BzATP and $A\beta_{1-42}$, microglia were transfected for 48 h with 50 μM Lipofectamine-$siP2X_7R$ or 50 μM Lipofectamine-siNC, or treated for 48 h with 10 μM BBG (a $P2X_7R$ antagonist as a positive control, Sigma–Aldrich). Cell-free supernatants were collected and cytokine (IL-1β, TNF-α, IFN-γ, IL-4, IL-10, and TGF-β1) levels were examined using enzyme-linked immunosorbent assays (ELISA, R&D Systems, USA) according to the manufacturer's protocol. The results of this assay are shown as the mean of each cytokine concentration in picograms per milliliter (±SD).

2.7. Microglia Aβ phagocytosis assays

$A\beta_{1-42}$ levels were measured using ELISA as previously described [19]. Briefly, microglia were seeded at concentrations of 1×10^5 cells/well in 24-well tissue culture plates containing 0.5 mL of complete RPMI 1640 media. Cells were stimulated for 2 h with 100 μM BzATP and 20 μM $A\beta_{1-42}$ (a higher $A\beta_{1-42}$ concentration was used to study phagocytosis). In the presence of BzATP

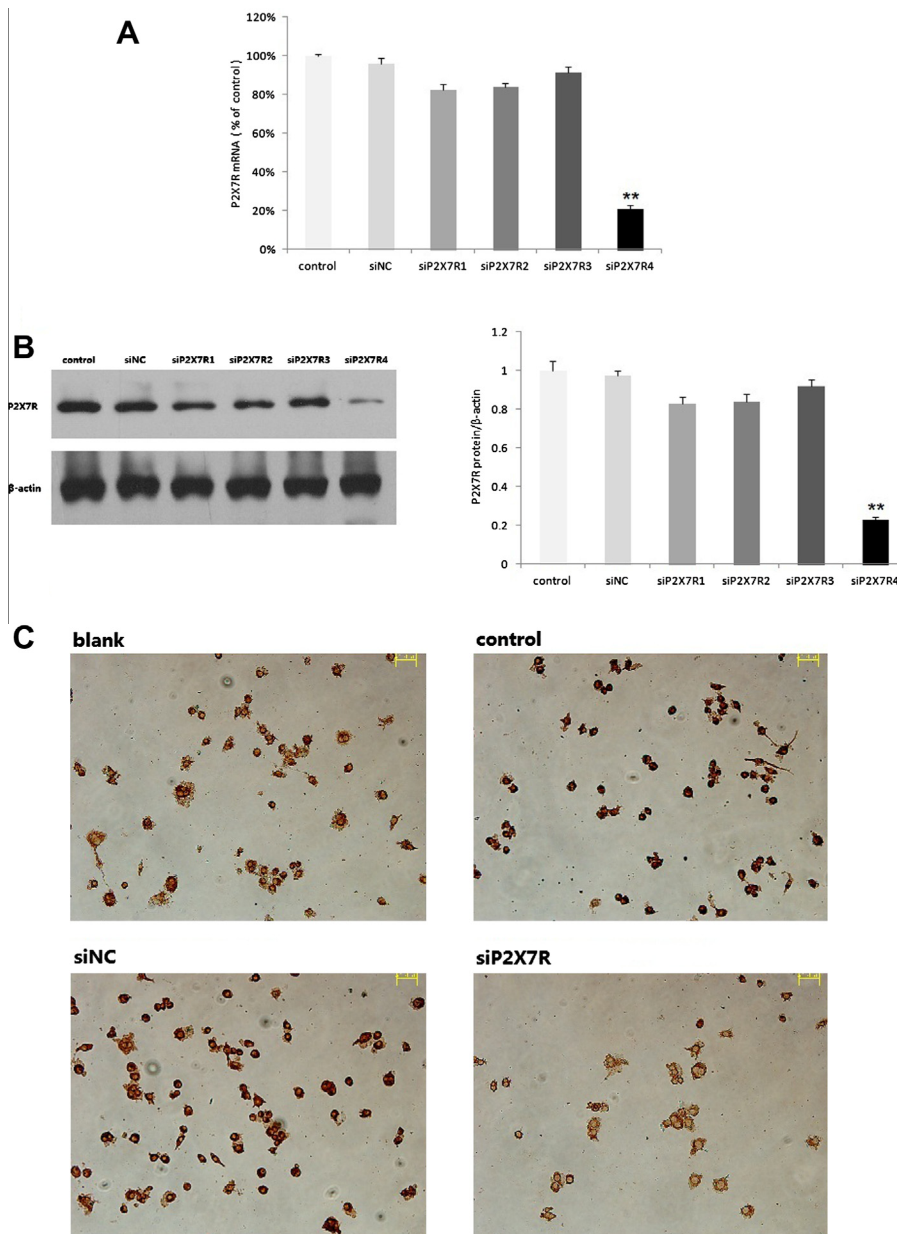

Fig. 1. $P2X_7R$ siRNA down-regulates $P2X_7R$ in microglia. (A) Quantification of $P2X_7R$ mRNA expression using real-time qPCR in control (stimulated with BzATP and $A\beta_{1-42}$, but not transfected with siRNA), siNC, and $siP2X_7R1$-4 cells (3 replicates for each). Relative $P2X_7R$ mRNA expression was assessed using the $2^{-(\Delta\Delta Ct)}$ method. Data are expressed as the mean ± SD (**$P < 0.01$ vs. control). (B) Western blots showing the levels of $P2X_7R$ protein in control, siNC, and $siP2X_7R1$-4 cells. Data are expressed as the mean ± SD (**$P < 0.01$ vs. control). (C) $P2X_7R$ immune response of microglia in blank (not stimulated with BzATP and $A\beta_{1-42}$, and not transfected with siRNA), control, siNC, and $siP2X_7R$ (transfected with $siP2X_7R4$) cells visualized by $P2X_7R$ immunocytochemical staining (Scale bar: 50 μm).

and $A\beta_{1-42}$, microglia were transfected for 48 h with 50 μM Lipofectamine-$siP2X_7R$ or 50 μM Lipofectamine-siNC, or treated for 48 h with 10 μM BBG. $A\beta_{1-42}$ levels in the supernatants and microglia were examined using specific ELISA kits (R&D Systems) according to the manufacturer's protocol. The results of the $A\beta_{1-42}$ analyses are shown in mean picograms per milliliter (±SD).

To demonstrate that $A\beta_{1-42}$ uptake involves phagocytosis, microglia were pretreated for 2 h with 5 μM cytochalasin D, an inhibitor of microglial phagocytosis (Sigma), and then exposed to 100 μM BzATP and 20 μM $A\beta_{1-42}$ as described previously [22]. To examine whether uptake of $A\beta_{1-42}$ involves the pro-inflammatory cytokines IL-1β and TNF-α, microglia were incubated overnight in the presence of 25 ng/ml IL-1β (PeproTech, USA), 25 ng/ml TNF-α (PeproTech), or 200 ng/ml IL-1ra (PeproTech) before exposure to 100 μM BzATP and 20 μM $A\beta_{1-42}$. To determine whether cyclooxygenase (COX) plays a role in the regulation of $A\beta_{1-42}$ phagocytosis, microglial cells were incubated with IL-1β overnight and then incubated for 1 h with two specific COX inhibitors: 1 μM 5-(4-chlorophenyl)-1-(4-methoxyphenyl)-3-trifluoromethyl pyrazole (SC-560, Cayman Chemical, USA) that specifically inhibits COX-1 and 1 μM 3-(4-methylsulphonylphenyl)-4-phenyl-5-trifluoromethylisoxazole (CAY10404, Cayman Chemical), which specifically inhibits COX-2 [23,24]. Cells were then stimulated with 100 μM BzATP and 20 μM $A\beta_{1-42}$.

2.8. *Aβ₁₋₄₂ immunofluorescence*

$A\beta_{1-42}$ phagocytosis by microglial cells was performed in a manner similar to that previously described [9]. Microglia were cultured at concentrations of 2×10^5/well in 24-well tissue culture plates with glass inserts for fluorescence microscopy. Cells were stimulated for 2 h with 100 μM BzATP and 20 μM $A\beta_{1-42}$. In the

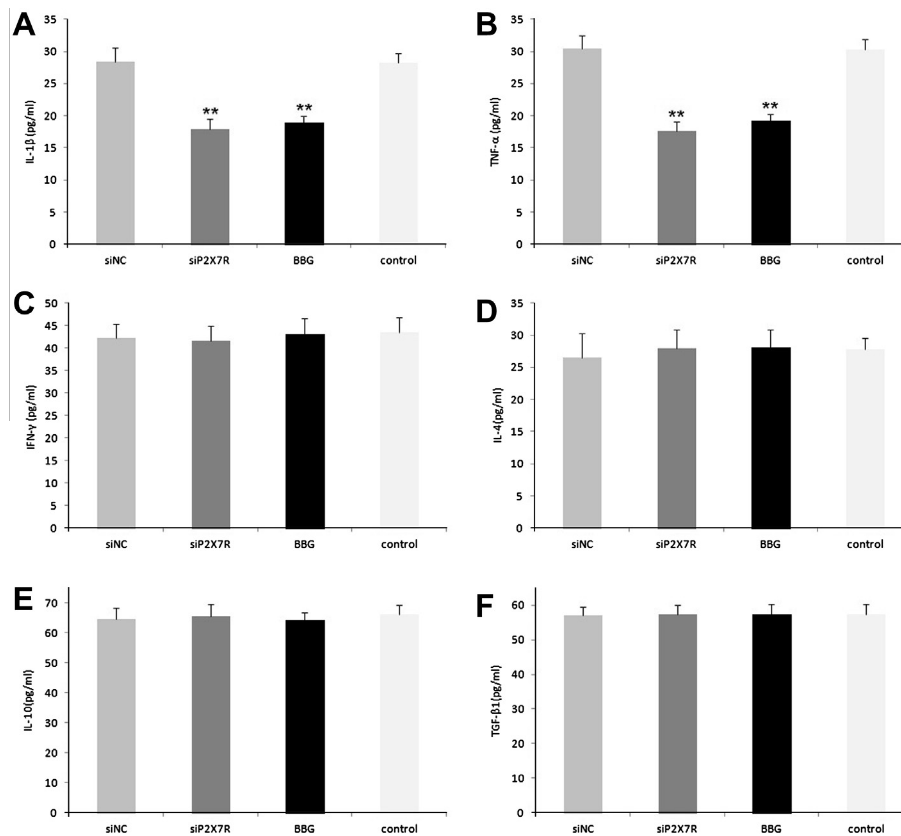

Fig. 2. Effects of $P2X_7R$ silencing on cytokine release from microglia. Detection of (A) IL-1β, (B) TNF-α, (C) IFN-γ, (D) IL-4, (E) IL-10, and (F) TGF-β1 in the supernatant of activated microglia in siNC, $siP2X_7R$, BBG, and control (stimulated by BzATP and $A\beta_{1-42}$ but not transfected with siRNA) cells by ELISA. Each panel is an average of 6 replicates. Data are presented as the mean ± SD ($^{**}P < 0.01$ vs. control).

presence of BzATP and $A\beta_{1-42}$, microglia were transfected for 48 h with 50 μM Lipofectamine-$siP2X_7R$ or 50 μM Lipofectamine-siNC, or treated for 48 h with 10 μM BBG. Immunofluorescence detection and $A\beta_{1-42}$ staining was performed using the rabbit polyclonal antibody against $A\beta_{1-42}$ (1:100, Abcam). Cells were incubated with the anti-$A\beta_{1-42}$ antibody overnight at 4 °C, rinsed in PBS, and incubated for 1 h with Alexa-fluor anti-rabbit-555 (1:400, Invitrogen). Cells were washed three times with PBS and nuclei were counterstained with DAPI (Invitrogen). Microglial cells were viewed using a CKX41-F32FL microscope (Olympus) equipped with a digital camera.

2.9. Statistical analyses

All data are expressed as the mean ± SD and were analyzed using the Statistical Program for Social Science (SPSS) (SPSS Inc., USA). Statistical analyses were performed using one-way analysis of variance (ANOVA). The least significant difference (LSD) was used for homogeneity of variance, and Tamhane's test was used for heterogeneity of variance. α Levels were set at 0.05 for all analyses.

3. Results and discussion

3.1. Effects of $P2X_7R$ siRNA on $P2X_7R$ expression in microglia

We designed siRNA for $P2X_7R$ gene silencing in microglia. To confirm the efficiency of our siRNA sequences, $siP2X_7Rs$ was transfected into microglial cells for 48 h, and the $P2X_7R$ mRNA level was quantified using real-time qPCR. As shown in Fig. 1A, $P2X_7R4$ siRNA transfection decreased the level of $P2X_7R$ mRNA to 21% of the control level. However, after transfection with $siP2X_7R1$ or $siP2X_7R2$, the $P2X_7R$ mRNA levels were 82% or 84% of control, respectively. SiP2X7R3 failed to significantly alter the $P2X_7R$ mRNA level in microglia. The negative control, siNC, had no effect on the $P2X_7R$ mRNA level in microglial cells. The effect of $P2X_7R$ siRNA silencing on the $P2X_7R$ protein level was also studied using Western blot analyses (Fig. 1B). Using β-actin as a reference, a much lower level of $P2X_7R$ protein was detected in the $siP2X_7R4$-treated group than in the groups treated with $siP2X_7R1$, $siP2X_7R2$, $siP2X_7R3$, or siNC. These results confirm that $siP2X_7R4$ was highly efficient for $P2X_7R$ gene silencing, and therefore, this $siP2X_7R$ sequence was used for further experiments.

$P2X_7R$ silencing in microglia was verified by $P2X_7R$ immunocytochemical staining (Fig. 1C). The cytoplasm of the microglial cells appeared yellow, and the cells showed negative immunostaining for $P2X_7R$. When stimulated with BzATP, a $P2X_7R$ agonist, the cytoplasm of microglia were dark brown, indicating positive $P2X_7R$ immunostaining. Transfection with siNC had no effect on $P2X_7R$ levels in microglial cells. The cytoplasm of the microglia appeared dark brown, indicating positive $P2X_7R$ immunostaining. Transfection with $siP2X_7R$ markedly reduced the $P2X_7R$ level in microglia. The cytoplasm of these cells appeared yellow and was negative for $P2X_7R$ immunostaining.

3.2. Effects of $P2X_7R$ silencing on IL-1β and TNF-α secretion by activated microglia

Activated microglia can release either pro-inflammatory or anti-inflammatory cytokines depending on the phase of activation. To examine the effects of $P2X_7R$ on cytokine secretion, the levels of pro-inflammatory cytokines (IL-1β, TNF-α, and IFN-γ) and anti-inflammatory cytokines (IL-4, IL-10, and TGF-β1) were measured in the supernatant of activated microglia in the

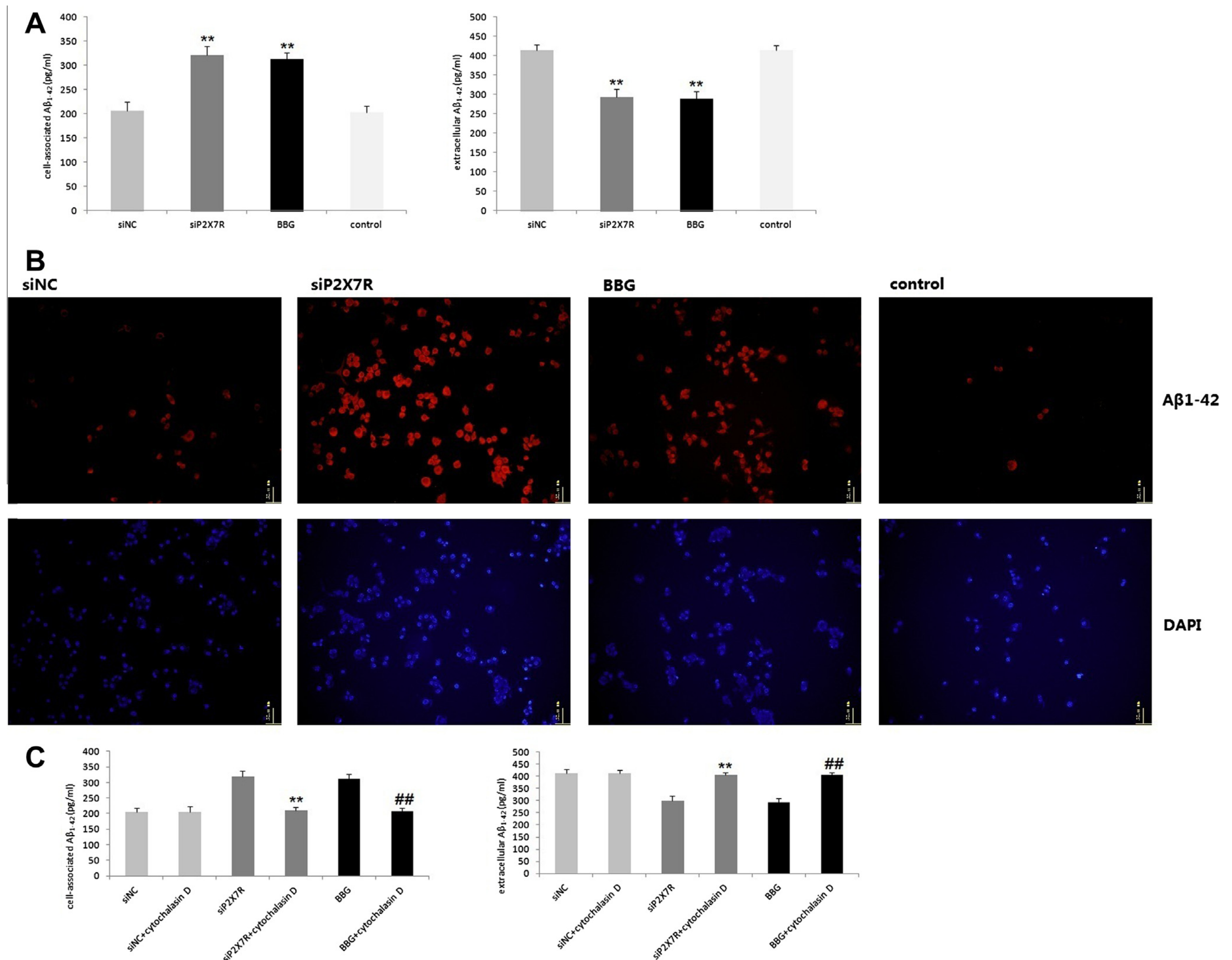

Fig. 3. Effects of $P2X_7R$ silencing on $A\beta_{1-42}$ phagocytosis by microglia. (A) Detection of $A\beta_{1-42}$ in siNC, $siP2X_7R$, BBG, and control (stimulated with BzATP and $A\beta_{1-42}$ but not transfected with siRNA) cells using ELISA ($n = 6$ for each treatment). Data are presented as the mean ± SD ($^{**}P < 0.01$ vs. control). (B) Immunofluorescence microscopy showing $A\beta_{1-42}$. Upper panels show $A\beta_{1-42}$ association with microglial cells under the various treatment conditions. Lower panels show the presence of microglial cells with DAPI-stained nuclei (Scale bar: 50 μm). (C) Detection of $A\beta_{1-42}$ in siNC, $siP2X_7R$, and BBG groups in the presence or absence of cytochalasin D using ELISA ($n = 6$ for each treatment). Data are presented as the mean ± SD ($^{**}P < 0.01$ vs. $siP2X_7R$; $^{\#\#}P < 0.01$ vs. BBG).

presence or absence of $siP2X_7R$. $SiP2X_7R$ transfection was found to inhibit IL-1β and TNF-α secretion by activated microglia (Fig. 2A and B) but did not affect IFN-γ, IL-4, IL-10, or TGF-β1 secretion (Fig. 2C–F). Similar results were observed in microglial cells treated with BBG (a $P2X_7R$ antagonist). This shows that $siP2X_7R$ inhibits the release of the pro-inflammatory cytokines IL-1β and TNF-α from activated microglia.

3.3. $P2X_7R$ silencing promotes phagocytosis of $A\beta_{1-42}$ by activated microglia

We examined the effects of $P2X_7R$ silencing on the $A\beta_{1-42}$ phagocytosis by microglia. To determine whether $P2X_7R$ silencing can modulate microglial uptake of $A\beta_{1-42}$, microglia and supernatants were examined for cell-associated and extracellular $A\beta_{1-42}$ using a specific ELISA. Transfection of $siP2X_7R$ significantly enhanced microglial phagocytosis of $A\beta_{1-42}$, as evidenced by the increase in cell-associated $A\beta_{1-42}$ levels and the corresponding reduction in extracellular $A\beta_{1-42}$ (Fig. 3A). Negative controls transfected with siNC did not show any changes in $A\beta_{1-42}$ phagocytosis. In positive controls treated with BBG, increased microglial phagocytosis of $A\beta_{1-42}$ and reduced extracellular $A\beta_{1-42}$ were observed.

Enhanced $A\beta_{1-42}$ phagocytosis was verified by immunofluorescence. Cultures were incubated with fluorescent-tagged $A\beta_{1-42}$ in the presence or absence of $siP2X_7R$, siNC, and BBG. In the presence of either $siP2X_7R$ or BBG, cultured microglia exhibited significantly higher rates of intracellular fluorescence than cultures treated with control or siNC (Fig. 3B).

To demonstrate that $A\beta_{1-42}$ uptake involves phagocytosis, microglia were pretreated with cytochalasin D, an inhibitor of microglial phagocytosis. Cytochalasin D treatment was found to reverse the increased cell-associated $A\beta_{1-42}$ levels and the decreased extracellular $A\beta_{1-42}$ caused by $siP2X_7R$ or BBG (Fig. 3C), demonstrating that the increased $A\beta_{1-42}$ uptake is due to microglial phagocytosis.

To examine the effects of the pro-inflammatory cytokines IL-1β or TNF-α on $A\beta_{1-42}$ uptake, microglial cells were incubated overnight in the presence of high levels of IL-1β or TNF-α before exposure to BzATP and $A\beta_{1-42}$. Incubation of microglia with IL-1β significantly reduced the increase in $A\beta_{1-42}$ phagocytosis caused by either $siP2X_7R$ or BBG, but incubation with TNF-α did not

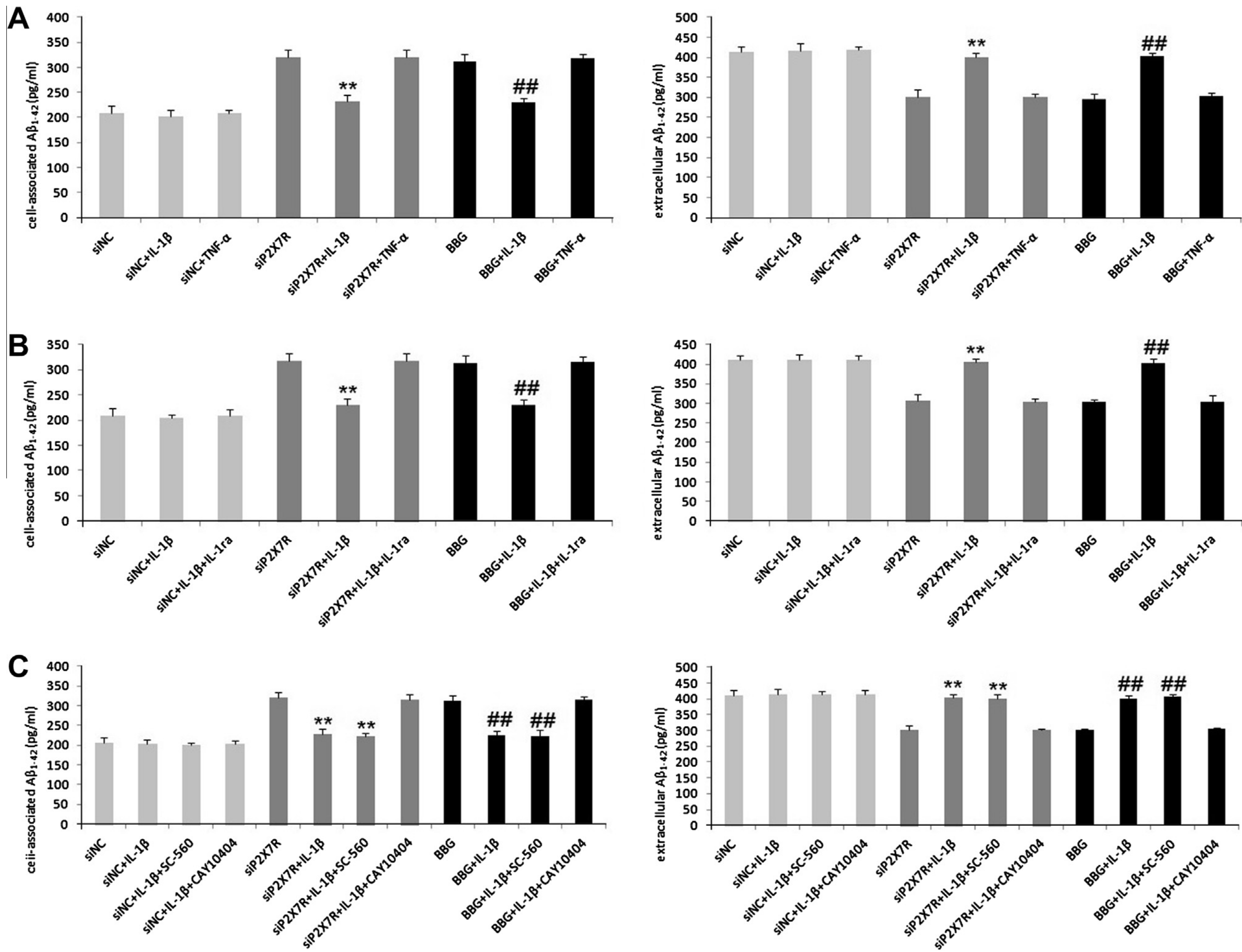

Fig. 4. Effects of IL-1β on $A\beta_{1-42}$ phagocytosis by microglia. (A) Detection of $A\beta_{1-42}$ in siNC, $siP2X_7R$, and BBG cells in the presence or absence of IL-1β and TNF-α using ELISA ($n = 6$ for each treatment). Data are presented as the mean ± SD ($^{**}P < 0.01$ vs. $siP2X_7R$; $^{\#\#}P < 0.01$ vs. BBG). (B) Detection of $A\beta_{1-42}$ in siNC, $siP2X_7R$, and BBG cells in the presence or absence of IL-1β and IL-1ra using ELISA ($n = 6$ for each treatment). Data are presented as the mean ± SD ($^{**}P < 0.01$ vs. $siP2X_7R$; $^{\#\#}P < 0.01$ vs. BBG). (C) Detection of $A\beta_{1-42}$ in siNC, $siP2X_7R$, and BBG cells in the presence or absence of IL-1β, SC-560, and CAY10404 using ELISA ($n = 6$ for each treatment). Data are presented as the mean ± SD ($^{**}P < 0.01$ vs. $siP2X_7R$; $^{\#\#}P < 0.01$ vs. BBG).

(Fig. 4A). To confirm that changes in $A\beta_{1-42}$ involve IL-1β, microglia cells were incubated in the presence or absence of IL-1β and IL-1ra (an IL-1 receptor antagonist). IL-1ra reversed the effects of IL-1β on $A\beta_{1-42}$ phagocytosis (Fig. 4B).

To determine whether COX activity is involved in the suppression of phagocytosis by IL-1β, we used two specific COX inhibitors. SC-560 is a specific inhibitor of COX-1, whereas CAY10404 is specific to COX-2. The COX-1 inhibitor was unable to rescue IL-1β-inhibition of $A\beta_{1-42}$ phagocytosis (Fig. 4C). However, COX-2 inhibition restored $A\beta_{1-42}$ phagocytosis to a similar level as that observed in $siP2X_7R$- or BBG-stimulated cells without IL-1β (Fig. 4C). We conclude that COX-2 is involved in the suppression $siP2X_7R$- and BBG-stimulated $A\beta_{1-42}$ phagocytosis by IL-1β.

IL-1β is the most important inflammatory mediator secreted by activated microglia [25]. IL-1β synthesis and secretion is a four-step process involving IL-1β gene transcription, accumulation of pro-cytokines in the cytoplasm, processing of pro-IL-1β by caspase-1, and finally the release of the mature cytokine. $P2X_7R$ can stimulate IL-1β release [26]. $P2X_7R$ triggers K^+ efflux from cells, which in turn activates caspase-1. This causes cleavage of pro-IL-1β, converting it to mature IL-1β, which is then released from the cell. Previous studies have described the ability of IL-1β to up-regulate COX-2 expression and produce prostaglandin E2 (PGE2). This limits the ability of microglia to phagocytose Aβ through the E prostanoid2 (EP2) receptor [27]. Our research is consistent with the conclusion that IL-1β inhibition of Aβ phagocytosis by microglia correlates with COX-2 activity. We have shown that COX-2 inhibition (using the specific inhibitor CAY10404) prevents the effects of IL-1β on Aβ phagocytosis.

In addition, TNF-α is a central inflammatory mediator and plays a key role in neuronal death mediated by activated microglia. Activation of the neuronal TNF-α receptor results in high levels of neuronal apoptosis [28]. Choi et al. demonstrated a reduction in the release of IL-1β and TNF-α from microglia after $P2X_7R$ inhibition [29]. These findings are similar to our results. Our study demonstrates that, unlike inhibition of Aβ phagocytosis caused by high levels of IL-1β, high levels of TNF-α had no effect on microglial phagocytosis of Aβ. This result is different from the results of Koenigsknecht-Talboo et al. [21]. We attribute these differences to the different methods used to evaluate the data. Specifically, Koenigsknecht-Talboo et al. examined the quantity of phagocytes. However, we examined the levels of intracellular and extracellular Aβ to evaluate the influence of pro-inflammatory cytokines on microglial phagocytosis of Aβ.

In conclusion, we report here for the first time that RNAi-mediated inhibition of $P2X_7R$ expression results in less IL-1β released

from microglia, thus mitigating the inhibitory effect of high concentrations of IL-1β on the phagocytic capacity of microglia, and thereby promoting Aβ phagocytosis. An important next step will be to determine whether silencing $P2X_7R$ reduces Aβ load and protects neurons in APP/PS1 transgenic mice *in vivo*.

Acknowledgments

This research project was supported by the grants from the National Natural Science Foundation of China (81071201) for Peijun Wang.

References

[1] H. Akiyama, T. Arai, H. Kondo, et al., Cell mediators of inflammation in the Alzheimer disease brain, Alzheimer Dis. Assoc. Disord. 14 (2000) S47–S53.
[2] W.J. Streit, Microglia as neuroprotective, immunocompetent cells of the CNS, Glia 40 (2002) 133–139.
[3] J.M. Sanz, P. Chiozzi, D. Ferrari, et al., Activation of microglia by amyloid-β requires P2X7 receptor expression, J. Immunol. 182 (2009) 4378–4385.
[4] K. Takata, Y. Kitamura, D. Yanagisawa, et al., Microglial transplantation increases amyloid-β clearance in Alzheimer model rats, FEBS Lett. 581 (2007) 475–478.
[5] J. Rogers, R. Strohmeyer, C.J. Kovelowski, et al., Microglia and inflammatory mechanisms in the clearance of amyloid-β peptide, Glia 40 (2002) 260–269.
[6] S. Mandrekar-Colucci, G.E. Landreth, Microglia and inflammation in Alzheimer's disease, CNS Neurol. Disord. Drug Targets 9 (2010) 156–167.
[7] L. Meda, M.A. Cassatella, G.I. Szendrei, et al., Activation of microglial cells by amyloid-β protein and interferon-β, Nature 374 (1995) 647–650.
[8] M.T. Heneka, M.K. O'Banion, Inflammatory processes in Alzheimer's disease, J. Neuroimmunol. 184 (2007) 69–91.
[9] M. Song, J.X. Xiong, Y.Y. Wang, et al., VIP enhances phagocytosis of fibrillar amyloid-β by microglia and attenuates amyloid deposition in the brain of APP/PS1 mice, PLoS One 7 (2012) e29790.
[10] A. Monsonego, H.L. Weiner, Immunotherapeutic approaches to Alzheimer' s disease, Science 302 (2003) 834–838.
[11] R.A. North, Molecular physiology of P2X receptors, Physiol. Rev. 82 (2002) 1013–1067.
[12] M. Monif, C.A. Reid, K.L. Powell, et al., The P2X7 receptor drives microglial activation and proliferation: a trophic role for the P2X7R pore, J. Neurosci. 29 (2009) 3781–3791.
[13] L.K. Parvathenani, S. Tertyshnikova, C.R. Greco, et al., P2X7 mediates superoxide production in primary microglia and is up-regulated in a transgenic mouse model of Alzheimer's disease, J. Biol. Chem. 278 (2003) 13309–13317.
[14] J.G. McLarnon, J.K. Ryu, D.G. Walker, et al., Upregulated expression of purinergic P2X7 receptor in Alzheimer disease and amyloid-β peptide-treated microglia and in peptide-injected rat hippocampus, J. Neuropathol. Exp. Neurol. 65 (2006) 1090–1097.
[15] J.K. Ryu, J.G. McLarnon, Block of purinergic P2X7 receptor is neuroprotective in an animal model of Alzheimer's disease, Neuroreport 19 (2008) 1715–1719.
[16] T. Takenouchi, K. Sekiyama, A. Sekigawa, et al., P2X7 receptor signaling pathway as a therapeutic target for neurodegenerative diseases, Arch. Immunol. Ther. Exp. 58 (2010) 91–96.
[17] P. Gonzalez-Alegre, Therapeutic RNA interference for neurodegenerative diseases: from promise to progress, Pharmacol. Ther. 114 (2007) 34–55.
[18] B. Song, K. Davis, X.S. Liu, et al., Inhibition of Polo-like kinase 1 reduces amyloid-β-induced neuronal cell death in Alzheimer's disease, Aging (Albany NY) 3 (2011) 846–851.
[19] O. Singer, R.A. Marr, E. Rockenstein, et al., Targeting BACE1 with siRNAs ameliorates Alzheimer disease neuropathology in a transgenic model, Nat. Neurosci. 8 (2005) 1343–1349.
[20] S.A. Friedle, V.M. Brautigam, M. Nikodemova, et al., The P2X7-egr pathway regulates nucleotide-dependent inflammatory gene expression in microglia, Glia 59 (2011) 1–13.
[21] J. Koenigsknecht-Talboo, G.E. Landreth, Microglial phagocytosis induced by fibrillar amyloid-β and IgGs are differentially regulated by proinflammatory cytokines, J. Neurosci. 25 (2005) 8240–8249.
[22] O.M. Mitrasinovic, G.M. Jr, Murphy, accelerated phagocytosis of amyloid-β by mouse and human microglia overexpressing the macrophage colony-stimulating factor receptor, J. Biol. Chem. 277 (2002) 29889–29896.
[23] C.J. Smith, Y. Zhang, C.M. Koboldt, et al., Pharmacological analysis of cyclooxygenase-1 in inflammation, Proc. Natl. Acad. Sci. USA 95 (1998) 13313–13318.
[24] B. Parashar, S. Latha Shankar, K. O'Guin, et al., Inhibition of human neuroblastoma cell growth by CAY10404, a highly selective COX-2 inhibitor, J. Neurooncol. 71 (2005) 141–148.
[25] C.A. Dinarello, The IL-1 family and inflammatory diseases, Clin. Exp. Rheumatol. 20 (2002) S1–S13.
[26] R. Mingam, V. De Smedt, T. Amedee, et al., In vitro and in vivo evidence for a role of the P2X7 receptor in the release of IL-1β in the murine brain, Brain Behav. Immun. 22 (2008) 234–244.
[27] F.S. Shie, R.M. Breyer, T.J. Montine, Microglia lacking E prostanoid receptor subtype 2 have enhanced Aβ phagocytosis yet lack Aβ-activated neurotoxicity, Am. J. Pathol. 166 (2005) 1163–1172.
[28] C.K. Combs, J.C. Karlo, S.C. Kao, et al., Amyloid-β stimulation of microglia and monocytes results in TNF-α-dependent expression of inducible nitric oxide synthase and neuronal apoptosis, J. Neurosci. 21 (2001) 1179–1188.
[29] H.B. Choi, J.K. Ryu, S.U. Kim, et al., Modulation of the purinergic P2X7 receptor attenuates lipopolysaccharide-mediated microglial activation and neuronal damage in inflamed brain, J. Neurosci. 27 (2007) 4957–4968.