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Transforming growth factor β1-induced astrocyte migration is mediated in part by activating 5-lipoxygenase and cysteinyl leukotriene receptor 1

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Abstract

Background: Transforming growth factor-β1 (TGF-β1) is an important regulator of cell migration and plays a role in the scarring response in injured brain. It is also reported that 5-lipoxygenase (5-LOX) and its products, cysteinyl leukotrienes (CysLTs, namely LTC_4, LTD_4 and LTE_4), as well as cysteinyl leukotriene receptor 1 ($CysLT_1R$) are closely associated with astrocyte proliferation and glial scar formation after brain injury. However, how these molecules act on astrocyte migration, an initial step of the scarring response, is unknown. To clarify this, we determined the roles of 5-LOX and $CysLT_1R$ in TGF-β1-induced astrocyte migration.

Methods: In primary cultures of rat astrocytes, the effects of TGF-β1 and CysLT receptor agonists on migration and proliferation were assayed, and the expression of 5-LOX, CysLT receptors and TGF-β1 was detected. 5-LOX activation was analyzed by measuring its products (CysLTs) and applying its inhibitor. The role of $CysLT_1R$ was investigated by applying CysLT receptor antagonists and $CysLT_1R$ knockdown by small interfering RNA (siRNA). TGF-β1 release was assayed as well.

Results: TGF-β1-induced astrocyte migration was potentiated by LTD_4, but attenuated by the 5-LOX inhibitor zileuton and the $CysLT_1R$ antagonist montelukast. The non-selective agonist LTD_4 at 0.1 to 10 nM also induced a mild migration; however, the selective agonist N-methyl-LTC_4 and the selective antagonist Bay cysLT2 for $CysLT_2R$ had no effects. Moreover, $CysLT_1R$ siRNA inhibited TGF-β1- and LTD_4-induced astrocyte migration by down-regulating the expression of this receptor. However, TGF-β1 and LTD_4 at various concentrations did not affect astrocyte proliferation 24 h after exposure. On the other hand, TGF-β1 increased 5-LOX expression and the production of CysLTs, and up-regulated $CysLT_1R$ (not $CysLT_2R$), while LTD_4 and N-methyl-LTC_4 did not affect TGF-β1 expression and release.

Conclusions: TGF-β1-induced astrocyte migration is, at least in part, mediated by enhanced endogenous CysLTs through activating $CysLT_1R$. These findings indicate that the interaction between the cytokine TGF-β1 and the pro-inflammatory mediators CysLTs in the regulation of astrocyte function is relevant to glial scar formation.

Keywords: Transforming growth factor-β1, Cysteinyl leukotriene, Cysteinyl leukotriene receptor, 5-lipoxygenase, Astrocyte, migration, Glial scar

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Background

Glial scar formation is a critical event in repair responses after injury of the central nervous system (CNS) [1,2]. The glial scar is a complex of cellular components and mainly consists of reactive astrocytes (undergoing proliferation and morphological changes). Following focal CNS injury, reactive astrocytes migrate towards the lesion and then organize into a densely packed glial scar [1,2]. As the key step of glial scar formation, astrocyte migration is regulated by various factors [3-5], among which transforming growth factor-β (TGF-β) is known as an important regulator [5,6].

TGF-β, a family of multifunctional cytokines, regulates a broad diversity of physiological and pathological processes, including wound healing, inflammation, cell proliferation, differentiation, migration and extracellular matrix synthesis [7-10]. TGF-β1 is an important mediator in the pathogenesis of several disorders in the CNS, such as in the organization of a glial scar in response to injury and in several neurodegenerative disorders [7,11,12]. After CNS injury, elevated TGF-β levels in astrocytes have been shown to induce astrocytic scar formation [13], and are also associated with ischemic brain injury [14,15].

On the other hand, cysteinyl leukotrienes (CysLTs, namely LTC_4, LTD_4, and LTE_4), the 5-lipoxygenase (5-LOX, EC 1.13.11.34) metabolites of arachidonic acid [16], are bioactive lipid mediators that modulate immune and inflammatory responses [16-19] through activating their receptors, $CysLT_1R$ and $CysLT_2R$ [17,20,21]. In the rat brain, 5-LOX is activated and the production of CysLTs is enhanced after focal cerebral ischemia, resulting in neuronal injury and astrocyte proliferation (astrocytosis). This post-ischemic astrocytosis is associated with up-regulated $CysLT_1R$ and $CysLT_2R$ [22-26]. The $CysLT_1R$ antagonist pranlukast attenuates post-ischemic astrocytosis and glial scar formation in the chronic phases of focal cerebral ischemia in mice and rats [25,27,28]. This effect suggests that $CysLT_1R$ mediates CysLT-induced astrocytosis and glial scar formation in response to *in vivo* ischemic injury. In primary astrocyte cultures, CysLTs are released after oxygen-glucose deprivation-induced ischemic injury, and the resultant activation of $CysLT_1R$ mediates astrocyte proliferation [29,30]. These findings imply that the endogenously released CysLTs might play an autocrine role in the induction of astrocytosis and resultant glial scar formation through activating $CysLT_1R$.

However, whether $CysLT_1R$ mediates astrocyte migration in the process of glial scar formation needs investigation. In the periphery, $CysLT_1R$ mediates migration in many types of cells, such as monocytes [31], dendritic cells [32], monocyte-derived dendritic cells [33], vascular smooth muscle cells [34], intestinal epithelial cells [35]

and endothelial cells [31,34-36]. Therefore, $CysLT_1R$ may also be an inducer of astrocyte migration, but many other factors have been reported to be potent inducers, such as TGF-β1 [37,38]. Thus, there may be interactions between $CysLT_1R$ and other regulators (for example, TGF-β1). TGF-β1 up-regulates $CysLT_1R$ expression and increases the production of CysLTs in several cell types such as hepatic stellate cells [39] and bronchial smooth muscle cells [37]. Based on these findings, it is possible that the regulatory role of TGF-β1 in astrocyte migration may be mediated by enhanced production of CysLTs *via* $CysLT_1R$ activation. To clarify this possibility, in the present study, we investigated the interactions between TGF-β1 and 5-LOX/$CysLT_1R$ in astrocyte migration.

Methods

Primary cultures of rat astrocytes

Primary astrocytes were isolated from the cerebral cortex of newborn Sprague–Dawley rats within 24 h as described previously [30,40]. In brief, the cortices were digested with 0.25% trypsin and plated into poly-L-lysine-coated flasks. Cells were cultured in high-glucose DMEM (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 units/mL penicillin and 100 μg/mL streptomycin at 37°C in a humidified atmosphere of 95% air/5% CO_2. After incubation for 11 to 14 days, the confluent cultures were shaken overnight at 260 rpm at 37°C, and the adherent cells were trypsinized and re-seeded in the growth medium. More than 95% of the cells were astrocytes as confirmed by immunofluorescence staining for glial fibrillary acidic protein (GFAP).

All animal experiments were carried out in accordance with the National Institutes of Heath Guide for the Care and Use of Laboratory Animals. We made every effort to minimize the number of animals used and their suffering. The experimental protocols were approved by the Ethics Committee of Laboratory Animal Care and Welfare, School of Medicine, Zhejiang University.

Cell migration (wound healing) assay

Astrocytes were grown to confluence in 24-well plates and starved in serum-free DMEM for 24 h. The monolayer cells were manually scratched with a 20-μl pipette tip to create an extended and definite scratch in the center of the dish with a bright and clear field. The detached cells were removed by washing with phosphate-buffered saline (PBS). DMEM containing 1% FBS with or without TGF-β1 (PeproTech Inc, Rocky Hill, NJ, USA) was added to each dish. In some experiments, 1 ng/ml TGF-β1 was added to each dish for 30 minutes before treatment with LTD_4 (Sigma-Aldrich Co., St Louis, MO, USA) or N-methyl LTC_4 ($NMLTC_4$, a metabolically stable LTC_4 mimetic; Cayman Chemical

Co., Ann Arbor, MI, USA). Cells were pretreated with the following inhibitor and antagonists: zileuton (0.01 to 5 μM, a 5-LOX inhibitor; Gaomeng Pharmaceutical Co., Beijing, China), montelukast (0.01 to 5 μM, a selective $CysLT_1R$ antagonist; Merck & Co., Inc., Whitehouse Station, NJ, USA), and Bay cysLT2 (0.01 to 5 μM, a selective $CysLT_2R$ antagonist; a kind gift from Dr. T. Jon Seiders of Amira Pharmaceuticals, Inc., San Diego, CA, USA) for 30 minutes, and then incubated with TGF-β1 for 24 h. Images of migratory cells from the scratch boundary were acquired at 0 and 24 h under a light microscope with a digital camera.

To continuously monitor migration time-course in live astrocytes, astrocytes were plated in 35-mm dishes and grown to confluence, and then the cells were scratched and treated with LTD_4 or/and TGF-β1 as described above. The movements of live astrocytes was traced under an inverse videomicroscope (Olympus IX81, Olympus Corp., Tokyo, Japan), and the wound was photographed at 0, 6, 12, 18 and 24 h.

The wounded areas were analyzed with ImageTool 2.0 software (University of Texas Health Science Center, San Antonio, TX, USA). The wound healing effect is determined as the initial scratch area (0 h) after wounding minus the scratch area after treatment for 24 h, or 6, 12, 18 and 24 h (live astrocytes), and reported as percentages of control values. Moreover, some astrocyte samples seeded on coverslips were visualized by GFAP immunofluorescence staining 24 h after scratching as the typical images.
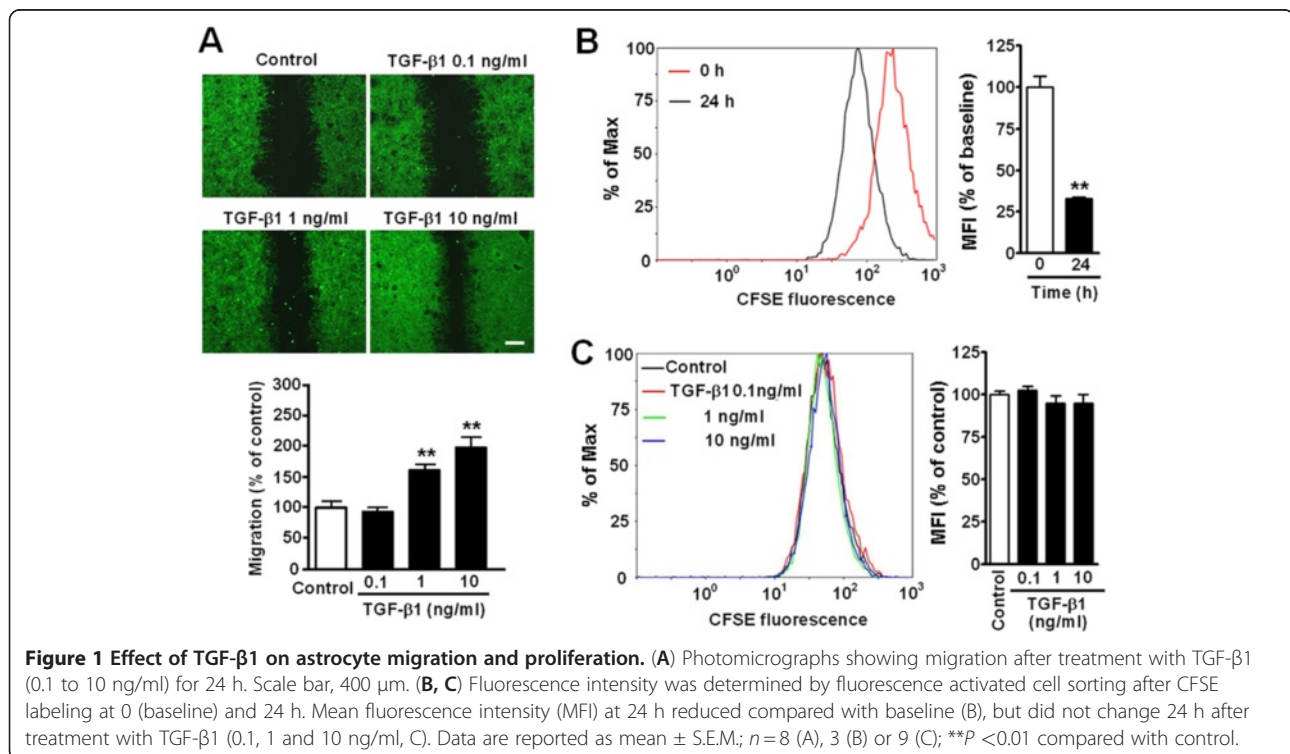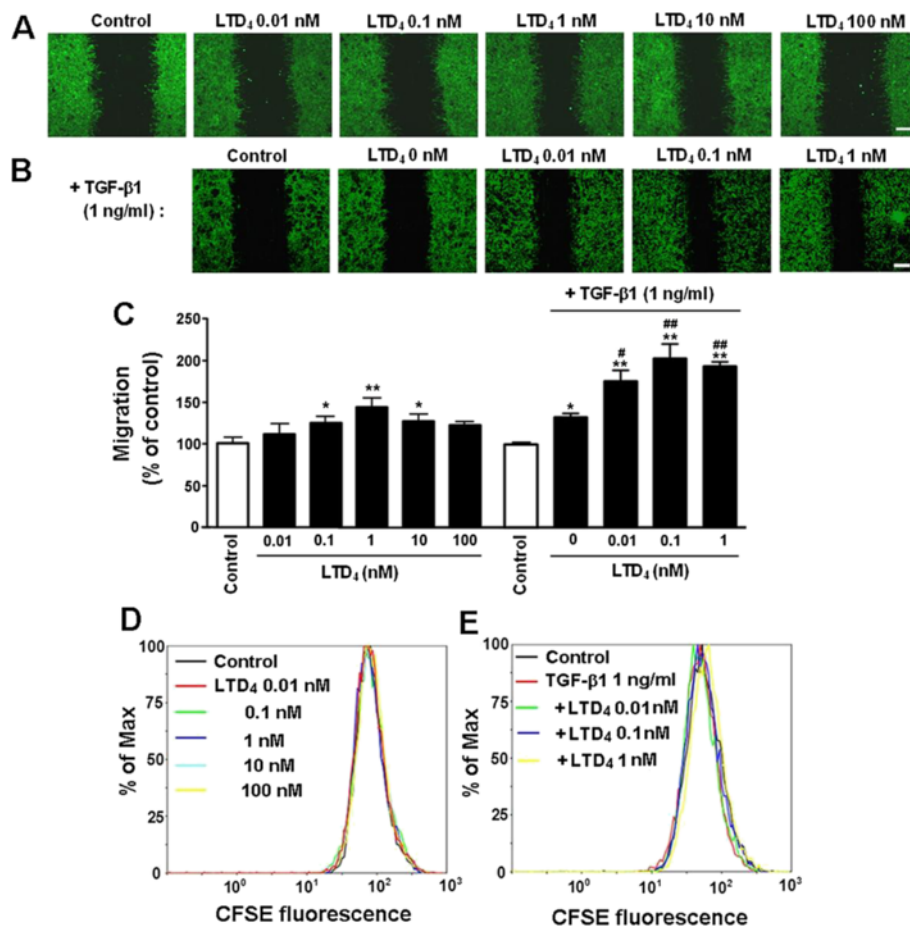
Cell proliferation assay

To measure astrocyte proliferation, carboxyfluorescein diacetate succinimidyl ester (CFSE) green fluorescent dye (Invitrogen Corp., Carlsbad, CA, USA) dilution assay was performed according to the manufacturer's instructions and the reported method [41-43]. Briefly, astrocytes were grown to confluence in six-well plates and starved in serum-free DMEM for 24 h, then the cells were washed twice with PBS and incubated in 5 μM CFSE in PBS for 15 minutes at 37°C, and subsequently washed twice with PBS. Then DMEM containing 1% FBS with or without TGF-β1 or LTD_4 was added to each plate. In some experiments, 1 ng/ml TGF-β1 was added to each plate for 30 minutes before treatment with LTD_4. The cells were harvested at 24 h, and subjected to fluorescence activated cell sorting using the FC500MCL flow cytometer (Beckman Coulter, Inc., Brea, CA, USA). Proliferation was measured by loss of CFSE dye.

CysLT₁ receptor knockdown by small interfering RNA (siRNA)

RNA duplexes of 21 nucleotides specific for rat $CysLT_1R$ sequences were chemically synthesized, together with a non-silencing negative control siRNA. The $CysLT_1R$ siRNA sense sequence was: 5´-CAG CCU UCC AAG

Figure 1 Effect of TGF-β1 on astrocyte migration and proliferation. (A) Photomicrographs showing migration after treatment with TGF-β1 (0.1 to 10 ng/ml) for 24 h. Scale bar, 400 μm. (**B, C**) Fluorescence intensity was determined by fluorescence activated cell sorting after CFSE labeling at 0 (baseline) and 24 h. Mean fluorescence intensity (MFI) at 24 h reduced compared with baseline (B), but did not change 24 h after treatment with TGF-β1 (0.1, 1 and 10 ng/ml, C). Data are reported as mean ± S.E.M.; *n* = 8 (A), 3 (B) or 9 (C); **P <0.01 compared with control.

Figure 2 Effect of LTD$_4$ on astrocyte migration and TGF-β1-induced migration and proliferation. (**A, B**), Photomicrographs showing astrocyte migration 24 h after treatment with LTD$_4$ (0.01 to 100 nmol/L) in the absence (A) or presence of TGF-β1 (1 ng/ml, B). Scale bars, 400 μm. (**C**) Data are reported as mean ± S.E.M.; $n = 8$; *$P < 0.05$ and **$P < 0.01$ compared with control, #$P < 0.05$ and ##$P < 0.01$ compared with TGF-β1 alone (LTD$_4$ 0). (**D, E**) MFI at 24 h was no significant change 24 h after treatment with LTD$_4$ (0.01 to 100 nM, D) alone or combined with TGF-β1 1 ng/ml (E) ($n = 9$ for each group, $P > 0.05$).

UAU ACA UTT-3′ and anti-sense: 5′-AUG UAU ACU UGG AAG GCU GTT-3′; the non-silencing control siRNA sense: 5′-UUC UCC GAA CGU GUC ACG UTT-3′ and anti-sense: 5′-ACG UGA CAC GUU CGG AGA ATT-3′ (GenePharma Co., Shanghai, China). Transfection of siRNA duplexes was performed according to the manufacturer's instructions. Briefly, astrocytes were seeded on the day before transfection using an appropriate medium with 10% FBS without antibiotics. They were transiently transfected with CysLT$_1$R siRNA or negative control siRNA (100 nM) for 6 h using Lipofectamine™ 2000 (Invitrogen, USA). After the transfected cells were incubated for 48 h, they were treated with LTD$_4$ or TGF-β1 for cell migration assay.

Reverse transcription-polymerase chain reaction (RT-PCR)
At the end of the experiments, total RNA was extracted from the cultured astrocytes using Trizol reagent

(Invitrogen, USA) according to the manufacturer's instructions. The cDNA synthesis and PCR reactions were performed as reported previously [29,30]. The PCR primers were: 5-LOX forward 5′-AAA GAA CTG GAA ACA GCT CAG AAA-3′ and reverse 5′-AAC TGG TGT GTA CAG GGG TCA GTT-3′; CysLT$_1$R, forward 5′- ATG TTC ACA AAG GCA AGT GG –3′ and reverse 5′-TGC ATC CTA AGG ACA GAG TCA –3′; CysLT$_2$R, forward 5′- ACC CCT TCC AGA TGC TCC A –3′ and reverse 5′- CGT GCT TTG AAA TTC TCT CCA –3′; β-actin, forward 5′-AAC CCT AAG GCC AACCGT GAA-3′ and reverse 5′-TCA TGA GGT AGT CTG TCA GGT C-3′; TGF-β1, forward 5′- GAC CGC AAC AAC GCA ATC TA –3′ and reverse 5′- AGG TGT TGA GCC CTT TCC AG –3′.

For cDNA synthesis, 2 μg total RNA was mixed with 1 mM deoxynucleotide triphosphate, 0.2 μg random primer, 20 U RNasin and 200 U M-MuLV reverse
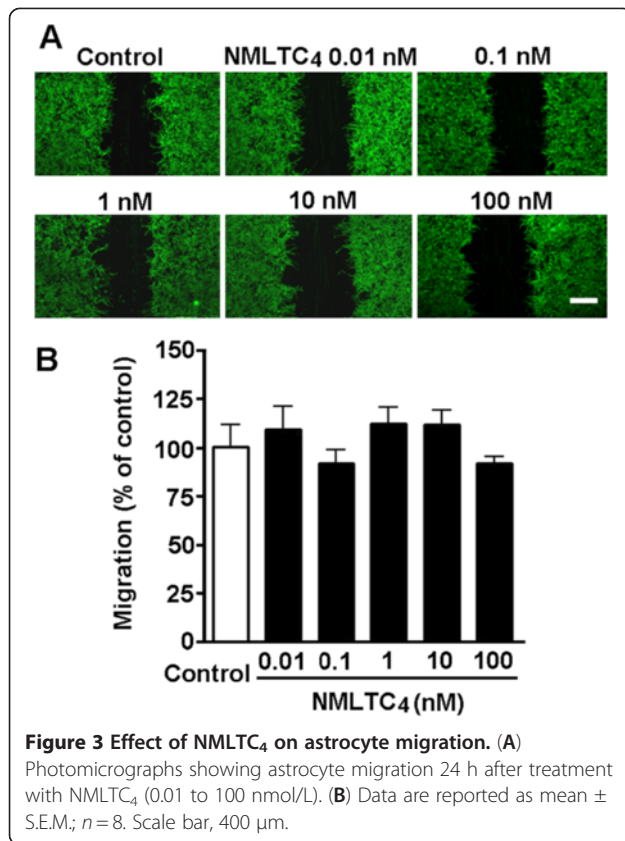
Figure 3 Effect of NMLTC$_4$ on astrocyte migration. (A) Photomicrographs showing astrocyte migration 24 h after treatment with NMLTC$_4$ (0.01 to 100 nmol/L). **(B)** Data are reported as mean ± S.E.M.; $n = 8$. Scale bar, 400 μm.

transcriptase in 20 μl reverse reaction buffer. The mixture was incubated at 42°C for 60 minutes, and then heated at 72°C for 10 minutes to inactivate the reverse transcriptase.

PCR was performed on an Eppendorf Master Cycler (Eppendorf Scientific, Inc., Westbury, NY, USA) as follows: 1 μl cDNA mixture was reacted in 20 μl reaction buffer containing 1.5 mM MgCl$_2$, 0.2 mM deoxynucleotide triphosphate, 20 pM primer and 1 U Taq DNA polymerase. The reaction mixtures were initially heated at 94°C for 2 minutes, then at 94°C for 60 sec, 56°C for 60 sec, and 72°C for 60 sec for 35 cycles and finally stopped at 72°C for 10 minutes. With the exception of TGF-β1, the reaction mixtures were initially heated at 94°C for 2 minutes, then at 94°C for 30 sec, 54°C for 30 sec, and 72°C for 60 sec for 28 cycles and finally stopped at 72°C for 10 minutes. PCR products of 20 μl were separated by 2% agarose gel electrophoresis and visualized by ethidium bromide staining. The density of each band was measured by a UVP gel analysis system (Bio-Rad Laboratories, Hercules, CA, USA). The results are expressed as the ratios to β-actin.

Western blotting analysis

Astrocytes were washed twice with ice-cold PBS and then lysed for 30 minutes on ice in Cell and Tissue Protein Extraction Solution (Kangcheng Biotechnology Inc., Shanghai, China). The homogenate was centrifuged at 12,000 g for 30 minutes at 4°C, and the supernatant was used. The protein samples (100 μg) were separated by 10% SDS-polyacrylamide gels and then transferred to nitrocellulose membranes (Invitrogen). The membranes were blocked by 10% fat-free milk, and sequentially incubated with the following antibodies: rabbit polyclonal antibody against CysLT$_1$R (1:200) [44], CysLT$_2$R (1:200) [26,45] or 5-LOX (1:300, (Chemicon International Inc. Temecula, CA, USA) and mouse monoclonal antibody against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:5,000, Kangcheng Biotechnology Inc., Shanghai, China) at 4°C overnight. After repeated wash, the membranes were incubated with anti-rabbit IRDye700DX®-conjugated antibody or anti-mouse IRDye800DX®-conjugated antibody (1:5,000, Rockland Immunochemicals, Inc., Gilbertsville, PA, USA). The immunoblot was analyzed by the Odyssey Fluorescence Scanner (LI-COR Bioscience, Inc., Lincoln, NE, USA). The protein bands were quantified using BIORAD Quantity One software (Bio-Rad, USA). The results are expressed as the ratios to GAPDH.

Immunofluorescence staining

Astrocytes seeded on coverslips were fixed in cold methanol for 5 minutes, and incubated in 10% normal goat serum for 2 h to block non-specific binding of IgG. Then the cells were reacted with a mouse monoclonal antibody against GFAP (1:500, Millipore Corp., Bedford, MA, USA) and a rabbit polyclonal antibody against CysLT$_1$R (1:200, Chemicon, USA) at 4°C overnight. After washing in PBS, astrocytes were incubated with FITC-conjugated goat anti-mouse or Cy3-conjugated goat anti-rabbit antibody (1:200, Millipore, USA) for 2 h at room temperature. Finally, the stained cells were observed under a fluorescence microscope (Olympus BX51, Olympus Corp., Tokyo, Japan). Control coverslips were treated with normal goat serum instead of the primary antibody, and did not show positive immunostaining (data not shown).

5-LOX immunocytochemistry

Astrocytes cultured on coverslips were fixed in cold methanol (−20°C) for 5 minutes and incubated for 30 minutes in PBS containing 3% H$_2$O$_2$ to eliminate endogenous peroxidase activity. Then, cells were incubated for 2 h in PBS containing 10% normal goat serum and incubated at 4°C overnight with rabbit polyclonal antibody against 5-LOX (1:200, Chemicon, USA) as the primary antibody. After three washes with PBS, cells were incubated for 2 h with biotin-conjugated goat anti-rabbit IgG antiserum (1:200) as a second antibody, followed by incubation with avidin-biotin-HRP complex. Finally, the cells were visualized with 0.01% 3, 3′-diaminobenzidine
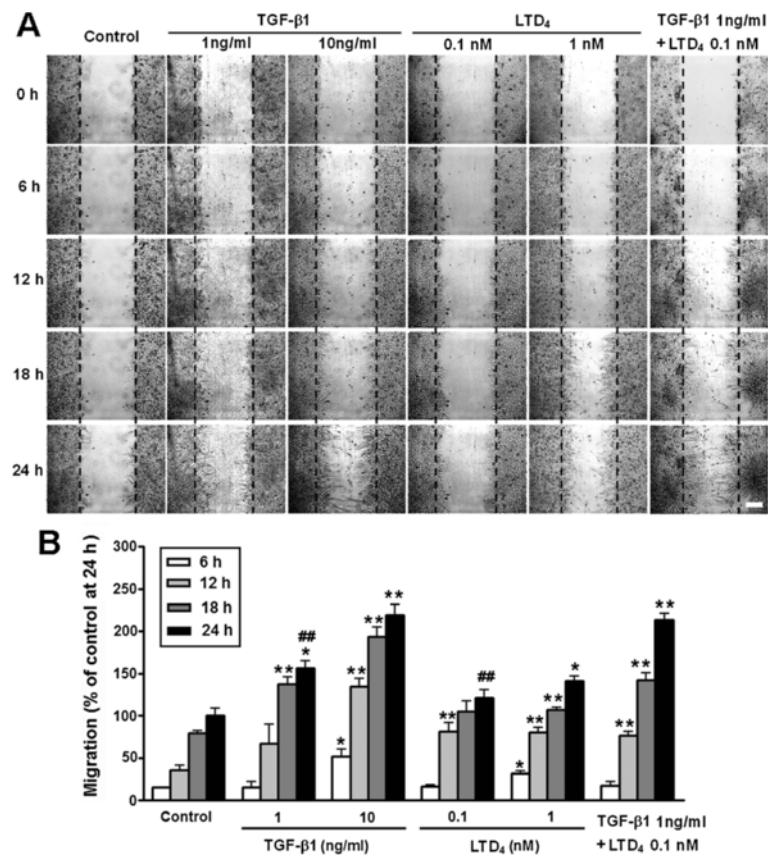
Figure 4 Time-dependent migration of live astrocytes after exposure to TGF-β1 and LTD4. Live astrocytes were continuously monitored under a videomicroscope after exposure to TGF-β1 or/and LTD4. (**A**) Representative images showing astrocyte migration traced by videomicroscopy at 6, 12, 18 and 24 h after scratching. Scale bar, 200 μm. (**B**) TGF-β1 and LTD4 concentration- and time-dependently accelerated migration. When TGF-β1 (1 ng/ml) combined with LTD4 (0.1 nM), the effect at 24 h was more potent than that of TGF-β1 or LTD4 alone. Data are reported as mean ± S.E.M.; $n = 3$; *$P < 0.05$ and **$P < 0.01$ compared with corresponding time-points of the control; ##$P < 0.01$ compared with TGF-β1 + LTD4 at 24 h.

and 0.005% H_2O_2 in 50 mM Tris–HCl, pH 7.6. Control coverslips were treated with normal goat serum instead of the primary antibody and they did not show positive immunostaining (data not shown). Then, the cells were examined under the Olympus microscope.

Measurement of extracellular cysteinyl leukotrienes and TGF-β1

According to the reported method [29,30], astrocytes were seeded into six-well culture plates at 5×10^5 cells/well in 2 ml standard culture medium for 24 h. After culture in DMEM without serum for another 24 h, astrocytes were cultured in DMEM with 1% FBS and stimulated with TGF-β1 (10 ng/mL), various concentrations of LTD4 or NMLTC4, or vehicle for the designated times. Then, cell-free supernatants were stored at −80°C. The CysLTs (LTC4, LTD4 and LTE4) in astrocyte supernatants were assayed using a commercial CysLT ELISA kit (Cayman Chemical Co., Ann Arbor, MI, USA) according to the manufacturer's instructions and

calculated as pg/mg protein. The TGF-β1 in the supernatants was assayed using a commercial TGF-β1 ELISA kit (Wuhan Boster Biological Technology Co., Ltd., Wuhan, China) according to the manufacturer's instructions, and calculated as pg/ml.

Statistical analysis

Data are reported as mean ± S.E.M. Student's *t*-test and one-way analysis of variance were used to determine the statistical significance of differences between groups. A value of $P < 0.05$ was considered statistically significant.

Results

TGF-β1- and LTD4-induced astrocyte migration

First, we confirmed the effect of TGF-β1 on astrocyte migration. TGF-β1 (1 and 10 ng/ml for 24 h) significantly accelerated the migration of astrocytes from the wound edge into the central area in a concentration-dependent manner (Figure 1A). To distinguish the effects on migration and proliferation, we determined whether TGF-β1
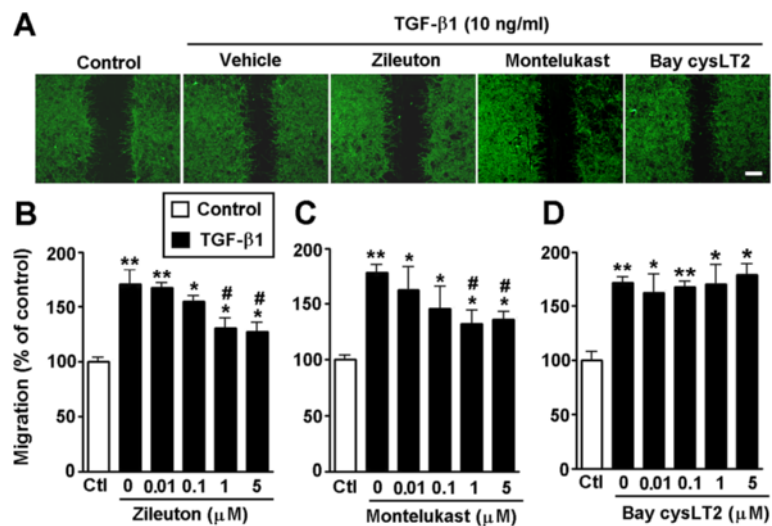
Figure 5 Effects of a 5-LOX inhibitor and CysLT receptor antagonists on TGF-β1-induced migration in astrocytes. (**A**) Photomicrographs showing TGF-β1 (10 ng/ml)-induced astrocyte migration 24 h after treatment with the 5-LOX inhibitor zileuton, the CysLT$_1$R antagonist montelukast and the CysLT$_2$R antagonist Bay cysLT2 (1 μM). Scale bar, 400 μm. (**B-D**) TGF-β1-induced migration inhibited by 0.01 to 5 μM zileuton (**B**) and montelukast (**C**), but not Bay cysLT2 (**D**). Data are reported as mean ± S.E.M.; $n = 8$; *$P < 0.05$ and **$P < 0.01$ compared with control; #$P < 0.05$ compared with TGF-β1 alone.

affects astrocyte proliferation. The results of CFSE fluorescence intensity showed that astrocyte proliferation did not differ from control level 24 h after exposure to TGF-β1 (0.1, 1 and 10 ng/ml) (Figure 1C) although the assay confirmed astrocyte proliferation at 24 h compared with 0 h (baseline) (Figure 1B).

Next, we determined whether the non-selective agonist LTD$_4$ and the CysLT$_2$R agonist NMLTC$_4$ [46] induce astrocyte migration, and LTD$_4$ potentiates the TGF-β1 effect. The results showed that LTD$_4$ significantly stimulated the migration of astrocytes at 0.1 to 10 nM but not at 0.01 and 100 nM; the maximum migration (141.7 ± 5.0%) was induced by 1 nM LTD$_4$ (Figure 2A, C). LTD$_4$ (0.01 to 1 nM) also potentiated the effect of the lower concentration of TGF-β1 (1 ng/ml); the migration rates after treatment with 1 ng/ml TGF-β1 were increased from 110.3 ± 5.4% to 175.3 ± 4.8% with 0.01 nM, from 123.5 ± 4.0% to 203.5 ± 5.3% with 0.1 nM, and from 141.7 ± 5.0% to 193.8 ± 2.9% with 1 nM LTD$_4$ (Figure 2B, C). LTD$_4$ (0.01 to 100 nM) alone (Figure 2D) or combined with TGF-β1 1 ng/ml (Figure 2E) did not affect astrocyte proliferation at 24 h. However, NMLTC$_4$ (0.01 to 100 nM for 24 h) did not have any significant effect on astrocyte migration (Figure 3). In addition, to confirm the migration and determine its temporal property, we continuously monitored migration of live astrocytes during 24 h after exposure to LTD$_4$ or/and TGF-β1. We found that TGF-β1 (1 and 10 ng/ml) and LTD$_4$ (1 nM) gradually accelerated migration during 24 h in a concentration-dependent manner. When TGF-β1 (1 ng/ml) combined with LTD$_4$ (0.1 nM), the effect at

24 h was more potent than that of TGF-β1 or LTD$_4$ alone (Figure 4).

To confirm the roles of endogenous CysLTs and CysLT$_1$R in TGF-β1-induced migration, we examined the effects of the 5-LOX inhibitor zileuton, the CysLT$_1$R antagonist montelukast, and the CysLT$_2$R antagonist Bay cysLT2 as well as CysLT$_1$R siRNA. We found that the effect of 10 ng/ml TGF-β1 was attenuated by zileuton (1 and 5 μM, Figure 5A, B) and montelukast (1 and 5 μM, Figure 5A, C), but not by Bay cysLT2 (0.01 to 5 μM, Figure 5A, D). These results indicated that endogenously released CysLTs might activate CysLT$_1$R, but not CysLT$_2$R, to induce astrocyte migration and potentiate TGF-β1-induced migration. The involvement of CysLT$_1$R was further confirmed by RNA silencing by transient transfection of CysLT$_1$R siRNA into astrocytes. The siRNA (100 nM) significantly reduced the expression of CysLT$_1$R mRNA (Figure 6A) and protein (Figure 6B, C), but the non-silencing negative control siRNA had no effect. CysLT$_1$R siRNA significantly attenuated the effects of LTD$_4$ (1 and 10 nM) and TGF-β1 (1 and 10 ng/ml) on astrocyte migration (Figure 6D, E). These results suggest that CysLT$_1$R may be associated with LTD$_4$- and TGF-β1-induced astrocyte migration.

TGF-β1-Induced Activation of 5-LOX in astrocytes
To investigate the role of endogenous CysLTs, the 5-LOX metabolites, in TGF-β1-induced astrocyte migration, we determined 5-LOX expression in astrocytes. We found that TGF-β1 10 ng/ml significantly increased 5-LOX mRNA (Figure 7A) and protein expression
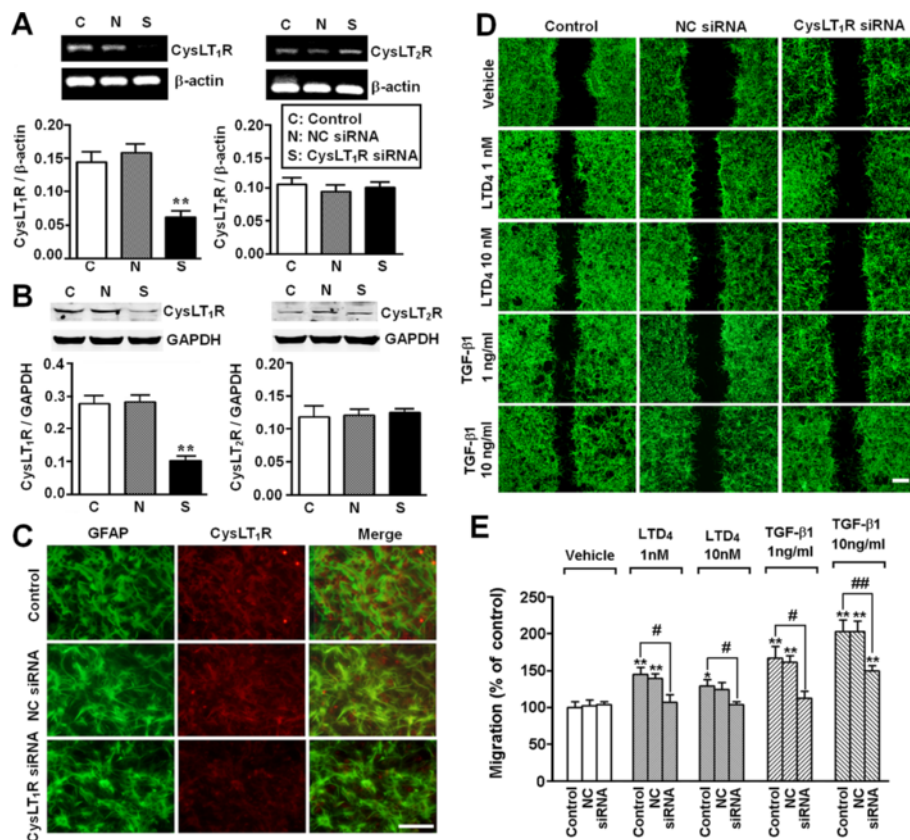
Figure 6 Effect of CysLT$_1$R siRNA on LTD$_4$- and TGF-β1-induced migration in astrocytes. (**A, B**) RT-PCR and Western blotting results showing inhibition of CysLT$_1$R, but not CysLT$_2$R, mRNA (**A**) and protein expression (**B**) by CysLT$_1$R siRNA but not by negative control (NC) siRNA. (**C**) Double immunofluorescence staining showing inhibition of CysLT$_1$R protein expression by CysLT$_1$R siRNA in GFAP-positive astrocytes. (**D**) Photomicrographs showing that the astrocyte migration induced by LTD$_4$ (1 and 10 nM) and TGF-β1 (1 and 10 ng/ml) was inhibited by CysLT$_1$R siRNA (siRNA) but not by NC siRNA. (**E**) CysLT$_1$R siRNA inhibited migration induced by LTD$_4$ and TGF-β1. Data are reported as mean ± S.E.M.; $n = 4$ (A and B) or 8 (E); *$P < 0.05$ and **$P < 0.01$ compared with control; #$P < 0.05$ and ##$P < 0.01$ compared with LTD$_4$ or TGF-β1 alone. Scale bar, 200 μm (C) or 400 μm (D).

(Figure 7B) 24 h after exposure. Immunocytochemical results showed that 5-LOX was translocated from the cytosol to the nuclear envelope 6 and 12 h after exposure to 10 ng/ml TGF-β1, and then recovered at 24 h (Figure 7C). We further determined the changes in enzymatic activity of 5-LOX by measuring its metabolites, CysLTs, in the culture medium. The levels of CysLTs increased from 1.5 h, peaked at 12 h, and were sustained over 24 h after exposure to 10 ng/ml TGF-β1 (Figure 7D). These findings revealed the involvement of 5-LOX and its metabolite CysLTs in the responses to TGF-β1.

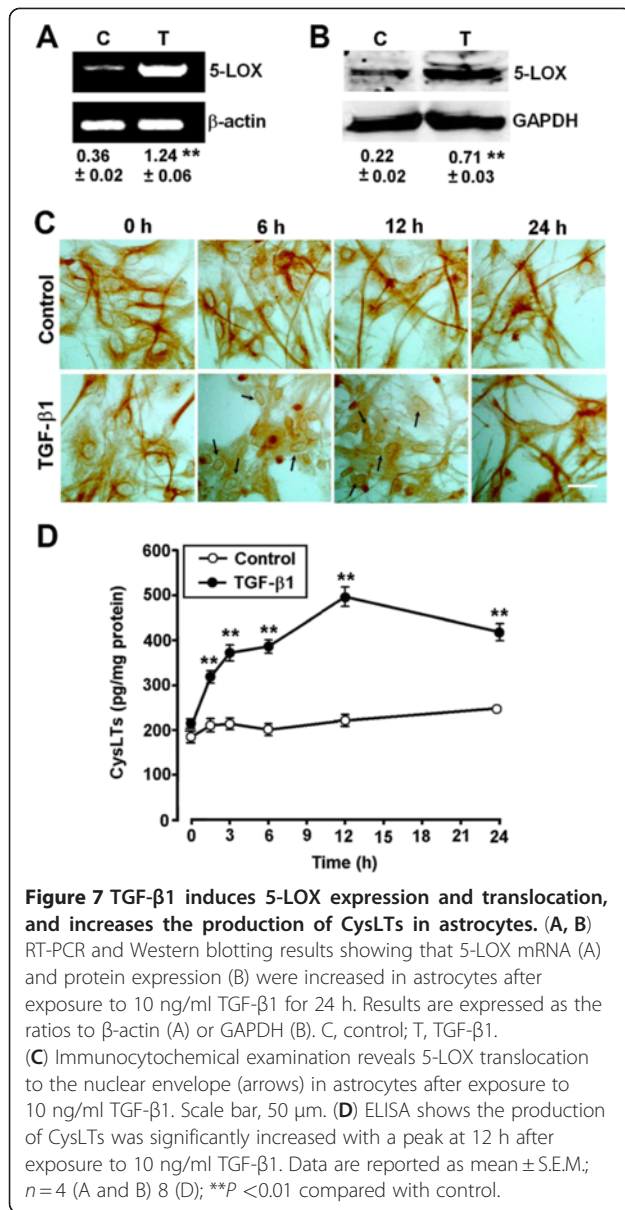## TGF-β1-regulated expression of CysLT receptor in astrocytes

Finally, we determined whether TGF-β1 regulates the expression of CysLT$_1$R and CysLT$_2$R mRNA and protein in astrocytes, and whether LTD$_4$ regulates TGF-β1 expression and release. RT-PCR and Western blot showed weak expression of CysLT$_1$R and CysLT$_2$R in control astrocytes. Exposure to 10 ng/ml TGF-β1 for 24 h

induced about three-fold increase in the mRNA (Figure 8A) and protein expression (Figure 8B) of CysLT$_1$R, but did not significantly change the expression of CysLT$_2$R. Immunofluorescence staining confirmed the enhancement of CysLT$_1$R by TGF-β1 (Figure 8C). On the other hand, treatment with various concentrations of LTD$_4$ or NMLTC$_4$ for 24 h did not affect the TGF-β1 mRNA expression in astrocytes (Figure 9A) and its content in the culture medium (Figure 9B). Thus, TGF-β1 might up-regulate CysLT$_1$R but is not regulated by LTD$_4$.

Discussion

In the present study, we revealed that TGF-β1-induced astrocyte migration is, at least in part, mediated by enhanced endogenous CysLTs through activation of CysLT$_1$R. The evidence is that TGF-β1-induced astrocyte migration was potentiated by LTD$_4$ but attenuated by a 5-LOX inhibitor and a CysLT$_1$R antagonist, and TGF-β1 activated 5-LOX and increased CysLT$_1$R

Figure 7 TGF-β1 induces 5-LOX expression and translocation, and increases the production of CysLTs in astrocytes. (A, B) RT-PCR and Western blotting results showing that 5-LOX mRNA (A) and protein expression (B) were increased in astrocytes after exposure to 10 ng/ml TGF-β1 for 24 h. Results are expressed as the ratios to β-actin (A) or GAPDH (B). C, control; T, TGF-β1. **(C)** Immunocytochemical examination reveals 5-LOX translocation to the nuclear envelope (arrows) in astrocytes after exposure to 10 ng/ml TGF-β1. Scale bar, 50 μm. **(D)** ELISA shows the production of CysLTs was significantly increased with a peak at 12 h after exposure to 10 ng/ml TGF-β1. Data are reported as mean ± S.E.M.; $n = 4$ (A and B) 8 (D); **$P < 0.01$ compared with control.

Figure 8 Effect of TGF-β1 on expression of CysLT receptors in astrocytes. (A, B) RT-PCR and Western blotting results showing that the mRNA (A) and protein expression (B) of CysLT$_1$R, but not CysLT$_2$R, in astrocytes increased after exposure to 10 ng/ml TGF-β1 for 24 h. Data are reported as mean ± S.E.M.; $n = 4$; **$P < 0.01$ compared with control. Results are expressed as the ratios to β-actin (A) or GAPDH (B). C, control; T, TGF-β1. **(C)** Double immunofluorescence staining showing that TGF-β1 increased the expression of CysLT$_1$R in GFAP-positive astrocytes. Scale bar, 100 μm.
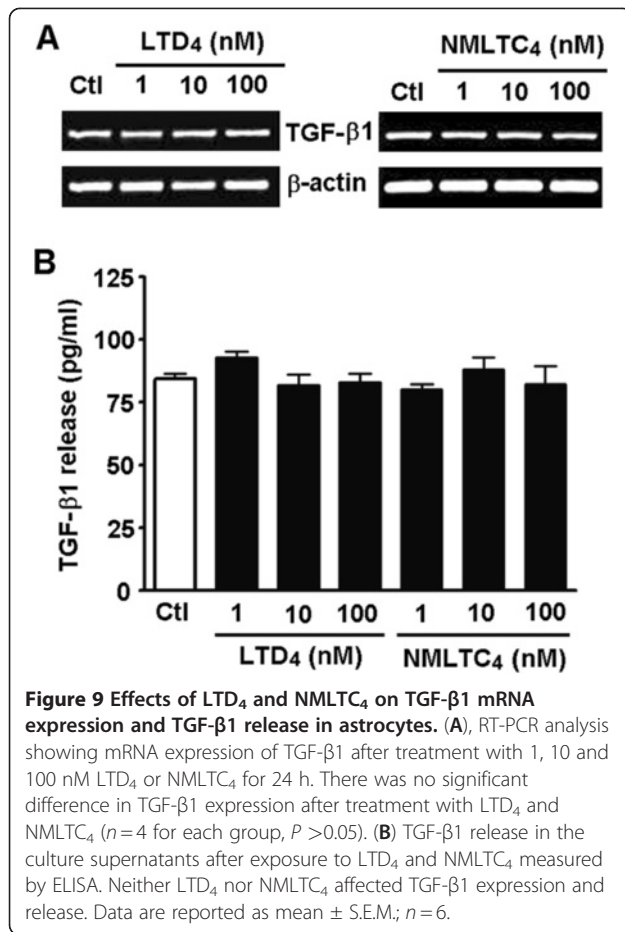
expression. Our observations have confirmed the TGF-β1-induced migration of rat astrocytes as reported [6], and indicated another mechanism underlying TGF-β1-induced astrocyte migration in addition to the pathways through activation of the Smad family [47,48] or the ROS-dependent ERK/JNK-NF-κB pathway [6]. In addition, we found that both TGF-β1 and LTD$_4$ did not alter astrocyte proliferation during 24 h. It has been reported that TGF-β1 inhibits astrocyte proliferation [47,49,50] and LTD$_4$ induces the proliferation via activating CysLT$_1$R [30]. This difference between these reported results and ours may result from different assessment timing [30] and methods [47,49,50]. However, in our experimental conditions, TGF-β1 and LTD$_4$ regulate astrocyte migration rather than proliferation.
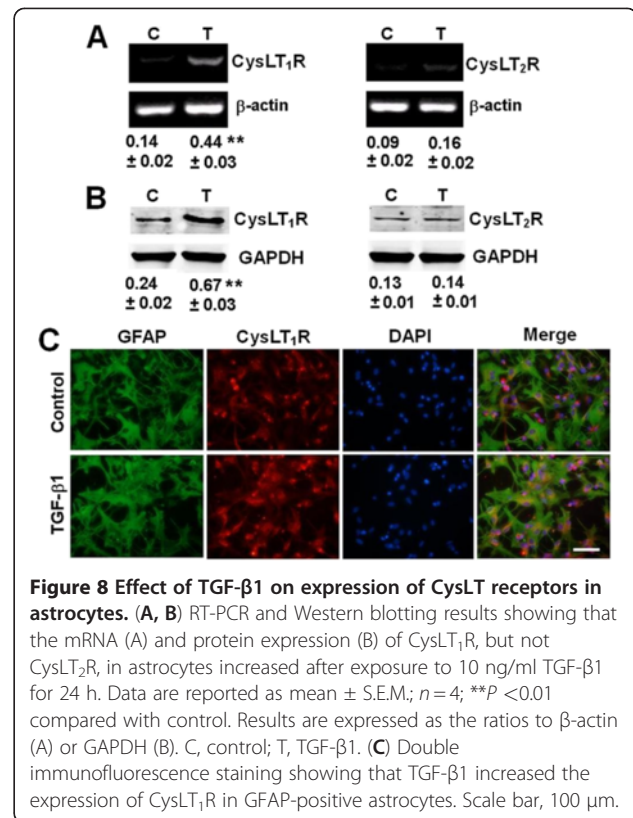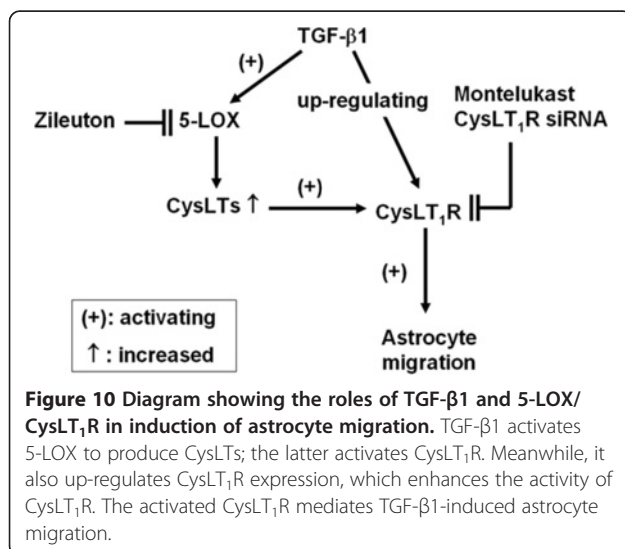
TGF-β1-induced astrocyte migration might be mediated by the CysLT signal pathway in at least two ways, that is, TGF-β1 potentiates the activity of both 5-LOX and CysLT$_1$R. On one hand, TGF-β1 increased 5-LOX expression and induced its translocation to the nuclear envelope (Figure 7C), a key step for 5-LOX activation [51-53] and, thereby, increased the production of endogenous CysLTs (Figure 7D). Consistent with this, it has been reported that TGF-β1 induces 5-LOX expression in myeloid cell lines [54-58]. The notion is also supported by the finding that the TGF-β1 effect was inhibited by the 5-LOX inhibitor zileuton (Figure 5A). On the other hand, TGF-β1 potentiates the expression of CysLT$_1$R, enhancing the activity of endogenously-produced or exogenous CysLTs as previously reported [37,39]. Therefore, one of the mechanisms underlying TGF-β1-induced astrocyte migration may be activation of endogenous 5-LOX /CysLT$_1$R signals.

Here, we demonstrated that the receptor subtype that mediated the TGF-β1 effect was CysLT$_1$R. The evidence was from the different effects of agonists and antagonists, and the effect of RNA interference. The non-selective agonist LTD$_4$ induced a moderate migration of astrocytes at lower concentrations (0.1 to 10 nM), but not at the higher concentrations 100 nM (Figure 2A, C) and 1,000 nM (data not shown). This concentration-response relationship indicated that CysLT$_1$R might

Figure 9 Effects of LTD$_4$ and NMLTC$_4$ on TGF-β1 mRNA expression and TGF-β1 release in astrocytes. (**A**), RT-PCR analysis showing mRNA expression of TGF-β1 after treatment with 1, 10 and 100 nM LTD$_4$ or NMLTC$_4$ for 24 h. There was no significant difference in TGF-β1 expression after treatment with LTD$_4$ and NMLTC$_4$ ($n = 4$ for each group, $P > 0.05$). (**B**) TGF-β1 release in the culture supernatants after exposure to LTD$_4$ and NMLTC$_4$ measured by ELISA. Neither LTD$_4$ nor NMLTC$_4$ affected TGF-β1 expression and release. Data are reported as mean ± S.E.M.; $n = 6$.

mediate the effect of LTD$_4$, because CysLT$_1$R is activated at 1 to 10 nM while CysLT$_2$R is activated at 100 to 1,000 nM in astrocytes [30]. This is also supported by the finding that the selective CysLT$_2$R agonist NMLTC$_4$ [46] had no effect on astrocyte migration (Figure 3). With regard

Figure 10 Diagram showing the roles of TGF-β1 and 5-LOX/CysLT$_1$R in induction of astrocyte migration. TGF-β1 activates 5-LOX to produce CysLTs; the latter activates CysLT$_1$R. Meanwhile, it also up-regulates CysLT$_1$R expression, which enhances the activity of CysLT$_1$R. The activated CysLT$_1$R mediates TGF-β1-induced astrocyte migration.

to receptor antagonism, the effect of TGF-β1 was attenuated by the CysLT$_1$R antagonist montelukast but not by the CysLT$_2$R antagonist Bay cysLT2. Bay cysLT2 is at least 100- to 500-fold more selective for CysLT$_2$R versus CysLT$_1$R; its pA$_2$ value indicates that at least 5 μM would act on the CysLT$_1$R [59,60]. Thus, lacking the effect of 5 μM Bay ctsLT2 in our study may be due to cell specificity and response difference. On the other hand, interference with CysLT$_1$R siRNA inhibited both TGF-β1- and LTD$_4$-induced astrocyte migration by down-regulating the expression of this receptor (Figure 6). These findings are consistent with reports that CysLT$_1$R mediates the migration of other types of cells [31-36]. Therefore, CysLT$_1$R is an important regulator of astrocyte migration in addition to its regulation of astrocyte proliferation [29,30].

The interaction between TGF-β1 and CysLTs was also investigated by determining the action of LTD$_4$ or NMLTC$_4$ on TGF-β1 expression and release. Unlike the action of TGF-β1 on the production of CysLTs and LTD$_4$ effects, LTD$_4$ or NMLTC$_4$ affected neither TGF-β1 expression nor its release in astrocytes (Figure 9). This may depend on specific cell types because LTD$_4$ induces TGF-β1 mRNA expression in human bronchial epithelial cells [61,62] and in fibroblasts from asthmatics [63], and LTC$_4$ induces TGF-β1 production in airway epithelium [62] in a CysLT$_1$R-dependent manner. Anyway, the effect of LTD$_4$ on TGF-β1 in astrocytes remains to be further investigated, especially in animal models of chronic brain injury. Since both levels of TGF-β1 and CysLTs are increased after brain injury [24,64,65] and involved in glial scar formation [25,65,66]; which of them is determinant in glial scar formation should be clarified for their therapeutic implications. Herein, our results suggest that activation of the endogenous 5-LOX/CysLT$_1$R signals might be an intermediate event in TGF-β1-regulated astrocyte migration, but not the initial event. Since TGF-β1 signaling is mainly modulated by Smad-dependent [67-75] and Smad-independent pathways [6,76-81], whether the regulation mode is mediated by the Smad or other pathways requires investigation.

Astrocyte migration is a critical step in the formation of a densely-packed glial scar [1,2], and TGF-β1 is closely associated with glial scar formation [64,66,82-84]. Thus, CysLT receptor antagonists or 5-LOX inhibitors may be beneficial in the prevention and attenuation of glial scar formation after brain injury. Actually, we have reported that the CysLT$_1$R antagonist pranlukast attenuates glial scar formation in the chronic phase of focal cerebral ischemia in mice [28] and rats [25], and the 5-LOX inhibitor caffeic acid has this effect in rats with focal cerebral ischemia [85] and in mice with brain cryoinjury [86]. Moreover, montelukast inhibits the astrocyte proliferation induced by mild ischemia-like

injury and low concentrations of LTD$_4$ [30]. The present study highlights the previous findings and clarifies the mode of action of endogenous CysLTs/CysLT$_1$R in the critical step of glial scar formation.

In conclusion, in the present study we found that TGF-β1-induced astrocyte migration is, at least in part, mediated by enhanced endogenous CysLTs through activating up-regulated CysLT$_1$R (Figure 10). These findings indicate that the interaction between the cytokine TGF-β1 and pro-inflammatory mediators (CysLTs) are involved in the regulation of astrocyte function relevant to glial scar formation. However, the detailed mechanisms underlying this interaction need investigation.

Abbreviations
5-LOX: 5-lipoxygenase; CNS: central nervous system; CysLT$_1$R: cysteinyl leukotriene receptor 1; CysLT$_2$R: cysteinyl leukotriene receptor 2; CysLTs: Cysteinyl leukotrienes; FBS: fetal bovine serum; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; GFAP: glial fibrillary acidic protein; LTD$_4$: leukotriene D$_4$; MFI: mean fluorescence intensity; NMLTC$_4$: N-methyl leukotriene C$_4$; PBS: phosphate-buffered saline; siRNA: small interfering RNA; TGF-β1: transforming growth factor- β1.

Competing interests
The authors have no competing interests.

Authors' contributions
XQH designed the study, performed the main parts of the experiments, analyzed the data, and prepared the manuscript. XYZ performed the immunofluorescence staining experiments and prepared the figures. XRW performed the cell migration experiments and analyzed the data. SYY performed the RT-PCR experiments and analyzed the data. SHF, YBL and WPZ contributed to the design of the study, to interpretation of the results and to the writing of the manuscript. EQW made essential contributions to the design of the study and interpretation of the results, and completed the manuscript. All authors read and approved the final manuscript.

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