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TGFβ Contributes to the Anti-inflammatory Effects of Tauroursodeoxycholic Acid on an Animal Model of Acute Neuroinflammation

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Abstract The bile acid conjugate tauroursodeoxycholic acid (TUDCA) is a neuroprotective agent in various animal models of neuropathologies. We have previously shown the anti-inflammatory properties of TUDCA in an animal model of acute neuroinflammation. Here, we present a new anti-inflammatory mechanism of TUDCA through the regulation of transforming growth factor β (TGFβ) pathway. The bacterial lipopolysaccharide (LPS) was injected intravenously (iv) on TGFβ reporter mice (Smad-binding element (SBE)/Tk-Luc) to study in their brains the real-time activation profile of the TGFβ pathway in a non-invasive way. The activation of the TGFβ pathway in the brain of SBE/Tk-Luc mice increased 24 h after LPS injection, compared to control animals. This activation peak increased further in mice treated with both LPS and TUDCA than in mice treated with LPS only. The enhanced TGFβ activation in mice treated with LPS and TUDCA correlated with both an increase in TGFβ3 transcript in mouse brain and an increase in TGFβ3 immunoreactivity in microglia/macrophages, endothelial cells, and neurons. Inhibition of the TGFβ receptor with SB431542 drug reverted the effect of TUDCA on microglia/macrophages activation and on TGFβ3 immunoreactivity. Under inflammatory conditions, treatment with TUDCA enhanced further the activation of TGFβ pathway in mouse brain and increased the expression of TGFβ3. Therefore, the induction of TGFβ3 by TUDCA might act as a positive feedback, increasing the initial activation of the TGFβ pathway by the inflammatory stimulus. Our findings provide proof-of-concept that TGFβ contributes to the anti-inflammatory effect of TUDCA under neuroinflammatory conditions.

Keywords Lipopolysaccharide · Neuroinflammation · SBE/Tk-Luc mice · TGFβ · TGFβ3 · TUDCA

Introduction

The blood-brain barrier (BBB) is a physical and functional barrier, composed of endothelial cells, pericytes, and the end-feet of astrocytes that restricts the crossing of substances and cells between the blood and the central nervous system (CNS) parenchyma and *vice versa*. CNS homeostasis is maintained by the BBB, as well as by the active role of CNS resident cells (mainly glial cells). CNS insults that disrupt homeostasis, such as infections, toxins, trauma, or stroke, induce an innate immune response to protect the CNS. This acute neuroinflammatory response causes the activation of astrocytes and microglia to counterbalance the changes in tissue homeostasis [1, 2]. Reactive glial cells show enhanced migration to the insult site, as well as increased phagocytic activity and release of inflammatory mediators, including pro- and anti-inflammatory cytokines and chemokines. The pro-inflammatory mediators increase BBB permeability and induce the activation and recruitment of leukocytes to the

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inflammation site in the CNS parenchyma [2, 3]. If this initial response cannot restore homeostasis, the inflammatory response is maintained long after the initial insult. This chronic neuroinflammation is detrimental and causes the loss of white and gray matter that characterizes many CNS pathologies [4, 5]. Due to the crucial role of microglial cells in the development of the neuroinflammatory response, the modulation of microglia activation has been postulated to be a potential therapeutic approach to different types of neurodegenerative diseases [6].

TGFβ is a pleiotropic cytokine involved in a wide variety of physiological and pathological conditions [7]. TGFβs bind to the TGFβ serine/threonine kinase receptor type II, which recruits and phosphorylates type I receptor (activin receptor-like kinase 5, ALK5). The type I receptor then phosphorylates Sma and Mad family member 2 (Smad2) and Sma and Mad family member 3 (Smad3), which further bind to Sma and Mad family member 4 (Smad4) to form a heteromeric complex that translocates into the nucleus to regulate the expression of target genes [8, 9]. Three TGFβ isoforms (TGFβ1, TGFβ2, and TGFβ3), encoded by unique genes located on different chromosomes, have been described in the mammalian CNS [10]. Under physiological conditions, TGFβ1 is expressed by meningeal cells, choroid plexus epithelial cells, and glial cells, whereas TGFβ2 and TGFβ3 are expressed by both glia and neurons [11]. The basal TGFβ activity is higher in the brain than in other organs [12] and has been related to neuronal differentiation [13] and synaptic transmission [14].

All TGFβ isoforms have key roles in injury response and wound repair of the CNS [10]. Under neuropathological conditions, the TGFβ pathway has been related to astrogliosis and glial scar formation [15, 16], neuroprotection [17], and brain tumor formation [18]. Moreover, TGFβ is a key modulator of inflammation [19], by a direct inhibitory effect on immune cells and CNS resident cells under both basal conditions [20, 21] and neuropathological conditions [22–24]. TGFβ also induces acquired deactivation of macrophages through inhibition of pro-inflammatory cytokine production and regulation of inflammatory signaling pathways [25]. It also plays an important role in the alternative activation of microglia [26].

Previous work from our laboratory has shown that the bile acid conjugate tauroursodeoxycholic acid (TUDCA) exerts a potent anti-inflammatory effect on the neuroinflammatory process by inhibiting NFκB activation [27]. Therefore, TUDCA inhibited different key proteins involved in NFκB-regulated processes induced by pro-inflammatory stimuli, required for blood leukocyte transmigration to the CNS parenchyma, such as glia activation (i.e., interferon gamma, IFNγ), microglial migration (i.e., monocyte chemoattractant protein-1, MCP-1), and endothelium activation (i.e., vascular cell adhesion molecule-1, VCAM-1) [27].

Bile acids are an interesting therapeutic tool, because they can be administered either orally, intravenously (iv), or intraperitoneally (ip), as they cross the BBB. The bile acid ursodeoxycholate (UDCA) is a common prescription drug for the treatment of primary biliary cirrhosis and shows not relevant side effects during chronic treatment [28]. Besides, recent studies have described that TUDCA has a beneficial effect on amyotrophic lateral sclerosis (ALS) patients [29].

Here, we have studied the time course and cell specificity of TGFβ signaling in an acute neuroinflammation model, and the relevance of early activation of the TGFβ pathway in the anti-inflammatory response induced by TUDCA. The results showed that the TGFβ signaling was increased in the brain of mice suffering neuroinflammation, and that TUDCA treatment further increased that activation. This effect correlated with an increase in TGFβ3 transcript in mouse brain and an increase of TGFβ3 immunoreactivity in microglia/macrophages, endothelial cells, and neurons. Selective blockade of the TGFβ receptor early after induction of neuroinflammation reverted the inhibitory effect of TUDCA on microglia/macrophages activation and reduced TGFβ3 transcripts and immunoreactivity in mouse brain.

These results suggest that after acute neuroinflammation, TGFβ signaling plays a crucial role in the anti-inflammatory effect of TUDCA.

Materials and Methods

Reagents

TUDCA sodium salt and SB431542 were purchased from Calbiochem (La Jolla, CA, USA), *Escherichia coli* LPS isotype 055:B5 was purchased from Sigma-Aldrich (St. Louis, MO, USA), normal goat serum and horse serum were purchased from Gibco BRL (Gaithersburg, MD, USA), and D-Luciferin was from Caliper (Madrid, Spain).

Acute Inflammation in a Transgenic Mouse Model

Transgenic Smad-binding element (SBE)/Tk-Luc mice (B6.Cg-Tg(SBE/TK-luc)7Twc/J) were purchased from Jackson (Jackson Laboratories, Maine, USA) and expanded at the Instituto Cajal Animal Care House. Animal handling and care was performed in compliance with European Union guidelines (2010/63/EU) and Spanish regulations (BOE67/8509–12; BOE 1201/2005) for the use and care of laboratory animals. All the protocols were approved by the Ethics and Scientific Commitees of the Instituto Cajal, CSIC, and the Hospital Nacional de Parapléjicos, SESCAM.

All the mice were screened and genotyped by PCR, with specific primers for the SBE sequence according to manufacturer's protocol (Primer sequences 5′-3′: Transgene Forward cgcatgccagagatcctatt, Transgene Reverse primer tacctggcagatggaacctc, Internal Positive control Forward

caaatgttgcttgtctggtg, Internal Positive control Reverse gtcagtcgagtgcacagttt). Only the positive mice were used for the luciferase assay.

Acute inflammation in SBE/Tk-Luc mice was induced by iv injection of LPS from E. coli isotype 055:B5 (Sigma-Aldrich, St Louis, MO, USA, 2 mg/kg, 1:1 LPS solution/heparin, final volume 75 μL) in the tail vein of 23 mice, 11 of which were further injected ip with TUDCA (500 mg/kg) every 24 h after bioluminescence detection.

The concentration of TUDCA used in the present study was selected on the basis of previous reports [30–32] and according to our previous work [27].

Bioluminiscence Imaging

The bioluminiscence of SBE/Tk-Luc mice was detected with the in vivo Imaging System (IVIS; Xenogen, Alameda, CA). SBE/Tk-Luc mice are TGFβ reporter mice that express firefly luciferase [3] under the control of 12 repeats of Smad-binding element (SBE). Mice were ip injected with 150 mg/kg D-Luciferin (Caliper, Madrid, Spain) 10 min before imaging and anesthetized with isoflurane during the imaging procedure. Photons emitted from living mice were acquired as photons per second per square centimeter per steradian (sr) and integrated for 5 min. The obtained pseudo-colored representation was then superimposed using LIVINGIMAGE Software overlay (Xenogen), the region of interest was manually selected, and the area was kept constant within the experiment for the different mice. Baseline bioluminiscence imaging was determined for each mouse, and bioluminiscence was expressed as fold induction related to the baseline levels for each mouse.

Acute Brain Inflammation in a Mouse Model

Adult C57BL/6 male mice (8–10 week-old) were purchased from Harlan® Interfauna Ibérica (Sant-Feliu-de-Codines, Spain). They were housed in the Instituto Cajal animal house at a constant room temperature of 22 °C with 50 % ± 10 % relative humidity and with a 12 h light/12 h dark cycle and given food and water ad libitum. Animal handling and care was performed following European Union guidelines (2010/63/EU) and Spanish regulations (BOE67/8509–12; BOE 1201/2005) for use and care of laboratory animals. All the protocols were approved by the ethics and scientific Commitees of the Instituto Cajal, CSIC and the Hospital Nacional de Parapléjicos, SESCAM.

Acute brain neuroinflammation was induced, after anesthesia with 3 mL/Kg of equitesin, by icv injection of 2 mg/kg LPS from *E. coli* isotype 055:B5 (Sigma-Aldrich, St Louis, MO, USA) dissolved in 5 μL of PBS, in the left ventricle, at stereotaxic coordinates: AP −0.46, ML −1.0, and DV −1.8 from Bregma [33] with a Hamilton syringe.

Two experimental procedures were used to determine the effect of TUDCA on TGFβ pathway activation, in the acute brain inflammation model. For the first procedure, a group of mice (n = 11) received an icv injection of LPS (2 mg/kg) and an ip injection of TUDCA at 500 mg/kg, every 24 h, starting after the icv LPS injection. Another group of mice (n = 8) received only an icv injection of LPS (2 mg/kg). The control group (n = 5 mice) received an icv injection of 5 μL of PBS at the same brain coordinates. Three days after the icv injection, the animals were sacrificed with an overdose of sodium pentobarbital (50 mg/kg, ip), and perfused with 60 mL of saline buffer and 60 mL of 4 % paraformaldehyde (PFA, Merck, Darmstatd, Germany). Brains were extracted, postfixed for 24 h in 4 % PFA, at 4 °C, left 48 h in 30 % sucrose at 4 °C, embedded in O.C.T.™ compound (Tissue-Tek®, Sakura Finetek Europe, Alphen aan den Rijn, The Netherlands) and stored at −20 °C until use.

To study the effect of TUDCA on the transcription of TGFβ isotypes in mouse hippocampus, a group of animals (n = 5) received an icv injection of LPS as described above. Other group of mice (n = 7) were treated with an icv injection of LPS and an ip injection of TUDCA (500 mg/kg) every 8 h. A control group of animals (n = 6) received an icv injection of PBS at the same coordinates. Some mice (n = 7) received an icv injection of PBS and were also treated with an ip injection of TUDCA (500 mg/kg) every 8 h. Mice were sacrificed 24 h after LPS injection, and the brains were extracted and put into a small culture plate. Then, the left hippocampus of each mouse was processed for messenger RNA (mRNA) extraction.

For the second procedure, mice were distributed in four experimental groups: control (icv PBS, n = 6), control with TUDCA (n = 6), icv LPS (n = 10), and icv LPS with ip TUDCA (n = 10). All mice were anesthetized with equitesin (3 mL/kg). LPS-treated mice were injected in the left ventricle, at stereotaxic coordinates: AP −0.46, ML −1.0, and DV −1.8 from Bregma, with a Hamilton syringe, 2 mg/kg of LPS from *E. coli* isotype 055:B5 (Sigma-Aldrich, St Louis, MO, USA), dissolved in 5 μL of PBS [33]. Control mice were injected 5 μL PBS in the same stereotaxic coordinates. The groups of mice receiving TUDCA (500 mg/kg) were injected ip every 24 h. Half the mice in each experimental group received 5 μL of the selective inhibitor of the TGFβ receptor, SB431542 (0.625 mM stock solution dissolved in 0.1 M PBS containing 4 % DMSO [34]), injected in the right ventricle (stereotaxic coordinates: AP −0.46, ML +1.0, and DV −1.8 from Bregma [33]). We estimated the appropriate SB431542 dose for mice, by converting previously tested doses in rats based on the assumptions and constants in the paper of Freireich et al. [35]. Control mice were injected with 5 μL of vehicle in the same stereotaxic coordinates.

The concentration of TUDCA used in the present study was selected on the basis of previous reports [30–32] and according to our previous work [27].

Three days after the icv injection, the animals were sacrificed with an overdose of sodium pentobarbital (50 mg/kg, ip), and perfused with 60 mL of saline buffer and 60 mL of 4 % PFA (Merck, Darmstatd, Germany). Brains were extracted, postfixed for 24 h in 4 % PFA, at 4 °C, left 48 h in 30 % sucrose at 4 °C, embedded in O.C.T.™ compound (Tissue-Tek®, Sakura Finetek Europe, Alphen aan den Rijn, The Netherlands) and stored at −20 °C until use.

RNA Purification and Quantitative Real-Time PCR

Quantitative real-time PCR (qPCR) in vivo was performed by dissecting the mouse hippocampus 1 day after injecting icv LPS (n = 5), and further treating with ip TUDCA (500 mg/kg), every 8 h (n = 7). Control mice were injected icv PBS instead (icv PBS n = 6; icv PBS and ip TUDCA n = 7). After tissue extraction, each hippocampus was homogenized in TRIzol reagent (Invitrogen, Carlsbad, CA, USA; 500 μL) with a blender. Total RNA was extracted from these samples and reverse transcribed with RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas, Vilnius, Lithuania). Specific primers for different mRNA were obtained with Primer Express 3.0 software (Applied Biosystems, Warrington, UK), and the pair of primers with less secondary structures for all the mRNA were selected (see Table 1), once analyzed by Gene Runner 3.05 software (Hastings Software Inc.). qPCR was developed in a 7500 Real-Time PCR System (Applied Biosystems, Warrington, UK) with Power SYBR® Green reagent (Applied Biosystems). Gene expression was determined with 7500 Software v2.0.4, using a relative standard curve for each pair of primers, with ROX as passive reference gene and RPS29 as a housekeeping gene to normalize the data.

Immunohistochemistry

Serial coronal sections (of 15 μm width) were mounted on gelatin-coated slides (n = 6 sections per slide), were cut on a LEICA CM1900 cryostat (Nussloch, Germany), and were stored at −20 °C until use. Sections were permeabilized with 0.5 % Tween-20 in PBS for 30 min at room temperature. Endogenous peroxidase was quenched with a solution of peroxide (1 % methanol, 1 % hydrogen peroxide in PBS with 0.1 % Tween-20). After washing the sections with 0.1 % Tween-20 in PBS and blocking with 5 % normal serum in PBS with 0.1 % Tween-20 (blocking solution) at room temperature for 1 h, sections were incubated with the primary antibody diluted in blocking solution (for more information see Table 2), overnight, at 4 °C.

A specific antibody against Iba-1 or TGFβ3 was used to detect the expression of these proteins in different regions of the mouse brain. After washing with 0.1 % Tween-20 in PBS, slides were incubated for 90 min, at room temperature, with the corresponding biotinylated secondary antibody diluted in blocking solution (for more information see Table 2). The signal was amplified with Vectastain ABC reagent (Vectastain ABC kit, Vector Laboratories, Burlingame, CA, USA) following the manufacturer's protocol, and the immunohistochemical stain was developed with 3,3′-diaminobenzidine (DAB). Slides were mounted with DePeX mounting medium (BDH, Poole, England) and photographed using an Olympus Provis AX70 microscope, coupled to an Olympus PD50 photography system.

The staining for each slice was quantified under double-blind conditions using the Image J software (NIH; Bethesda, MD, USA). Microscopy images were transformed into 8-bit images, and the background was subtracted with the subtract background plug-in. One section from each mouse included in the study was stained with the secondary antibody alone. The average immunoreactivity from all the sections stained with the secondary antibody alone was considered as background and was substracted from the images. After splitting the channels, we set the selected brain area (the hippocampus, corpus callosum, frontal cortex, or lateral cortex), and set the threshold for the stained area. The threshold settings remained unchanged for every slice analyzed. The area fraction (the ratio of stained area versus the total selected area) was quantified with the corresponding Analyze plugin. The results (arbitrary units) are shown as percentage of the ratio of stained area versus the total area selected.

To determine the cells expressing TGFβ3, brain slices were co-stained overnight, at 4 °C, with Iba-1 antibody to detect microglia, anti-type IV collagen to stain endothelial cells, anti-β-III-tubulin for neurons, or glial fibrillary acidic protein (GFAP) antibody to detect astrocytes and anti-TGFβ3. Slides were incubated for 90 min, at room

Table 1 Primers for quantitative PCR in mouse hippocampus

Gene	Accession #	Forward primer 5′-3′	Reverse primer 5′-3′	Product length
TGFβ1	NM_011577.1	ccagccgcgggactct	ttccgtttcaccagctccat	56
TGFβ2	NM_009367	ccacctcccctccgaaa	gagagcatcaaaagcggacgatt	60
TGFβ3	NM_009368.3	ctgtccacttgcaccacgtt	cctaatggcttccaccctctt	63
RPS29	NM_009093.2	gccgcgtctgctccaa	acatgttcagcccgtatttgc	54

Table 2 Antibodies

Antibody	Host	Distributor	Working dilution
Iba-1	Rabbit	WAKO	1:2000
GFAP (096)	Rabbit	DAKO	1:2000
Collagen IV	Rabbit	Abcam	1:200
β-III-tubulin	Rabbit	Abcam	1:2000
TGFβ3	Mouse	Iowa Hybridoma Bank	1:500
Hoechst			1:5000
α-Mouse biotinylated	Goat	Jackson Immunoresearch	1:200
α-Mouse Alexa 488	Goat	Invitrogen Molecular Probes	1:1000
α-Rabbit biotinylated	Goat	Jackson Immunoresearch	1:200
α-Rabbit IgG Alexa 594	Goat	INVITROGEN Molecular Probes	1:1000

temperature, with the corresponding Alexa-conjugated secondary antibody. After immunofluorescence labeling, the nuclei were stained with Hoechst, washed and mounted with Fluoromount-G T, Electron Microscopy Sciences (EMS, Hatfield, PA), and visualized on a Leica TCS-SP5 confocal system.

Statistical Analysis

Results were analyzed with GraphPad Prism 5.0 statistical package. The differences between variances of the treatments were compared with a one-way ANOVA and a Tukey *post-hoc* test for multiple comparisons. Statistical significance was considered when p values were lower than 0.05. Data in the graphs are presented as the mean ± SEM of each experimental group.

Results

TUDCA Enhances the TGFβ Pathway Activation in the Brain of LPS-Treated Mice

TGFβ has been described as a constitutive signal that keeps the cells in a resting state, both by exerting a direct effect on the target cells and by counteracting the effect of pro-inflammatory cytokines [19, 36]. TGFβ receptor activation induces Smad2/3 phosphorylation, complex formation, and translocation into the nucleus, where they regulate the transcription of target genes through the binding to Smad-binding elements (SBE). SBE/Tk-Luc mice are TGFβ reporter mice that express firefly luciferase under the control of 12 SBE repeats [12]. After injection of a luciferase substrate (D-Luciferin) in these transgenic mice, bioluminescence imaging was used to study the time course and tissue specificity of the Smad2/3 cascade and the TGFβ pathway activation, after iv injection of LPS [12, 37]. We used this model for initial experiments because it is easy, reliable, does not require brain surgery (avoid postoperatory inflammation due to the surgery), and it has been described before inducing the TGFβ pathway in SBE/Tk-Luc mice [12]. Both neuroinflammatory models, icv injection of LPS and iv injection of LPS, have similar neuroinflammatory response: induce early microglia activation [12, 27, 38, 39] and progressive neuronal cell death [38, 40].

The bioluminescence emission analysis of the brain of LPS-treated SBE/Tk-Luc mice (Fig. 1a) showed an increase in the TGFβ pathway activation in the brain, 24 h after the iv injection of LPS, and a decay of the activation for the next 2 days ($p < 0.05$) compared to control mice. Mice treated with LPS and an ip injection of TUDCA (500 mg/kg) showed a further increase in the activation of this pathway 24 h ($p < 0.05$) and 48 h after the iv injection of LPS, compared to mice treated with LPS alone. The bioluminescence signal decayed to basal levels after 72 h in both experimental groups.

TUDCA specifically Induces TGFβ3 Expression in the Brain of LPS-Treated Mice

The greatest increase in the activation of the TGFβ pathway was seen 24 h after LPS injection. We determined the levels of the transcripts for the three isoforms of TGFβ in the hippocampus of mice treated with TUDCA 24 h after LPS injection (Fig. 1b). The results showed an upward trend in the TGFβ1 transcript in the hippocampus of mice receiving icv LPS compared to control animals. Mice treated with TUDCA alone did not show any increase in the levels of the transcripts for any TGFβ isoforms, compared to control mice. However, when mice were treated with both LPS and TUDCA, there was an upward trend in the TGFβ2 transcript and a significant increase ($p < 0.05$) in the TGFβ3 mRNA expression, compared to mice treated with LPS alone.

On the basis of these results, we studied TGFβ3 immunoreactivity in mouse brain 3 days after icv LPS

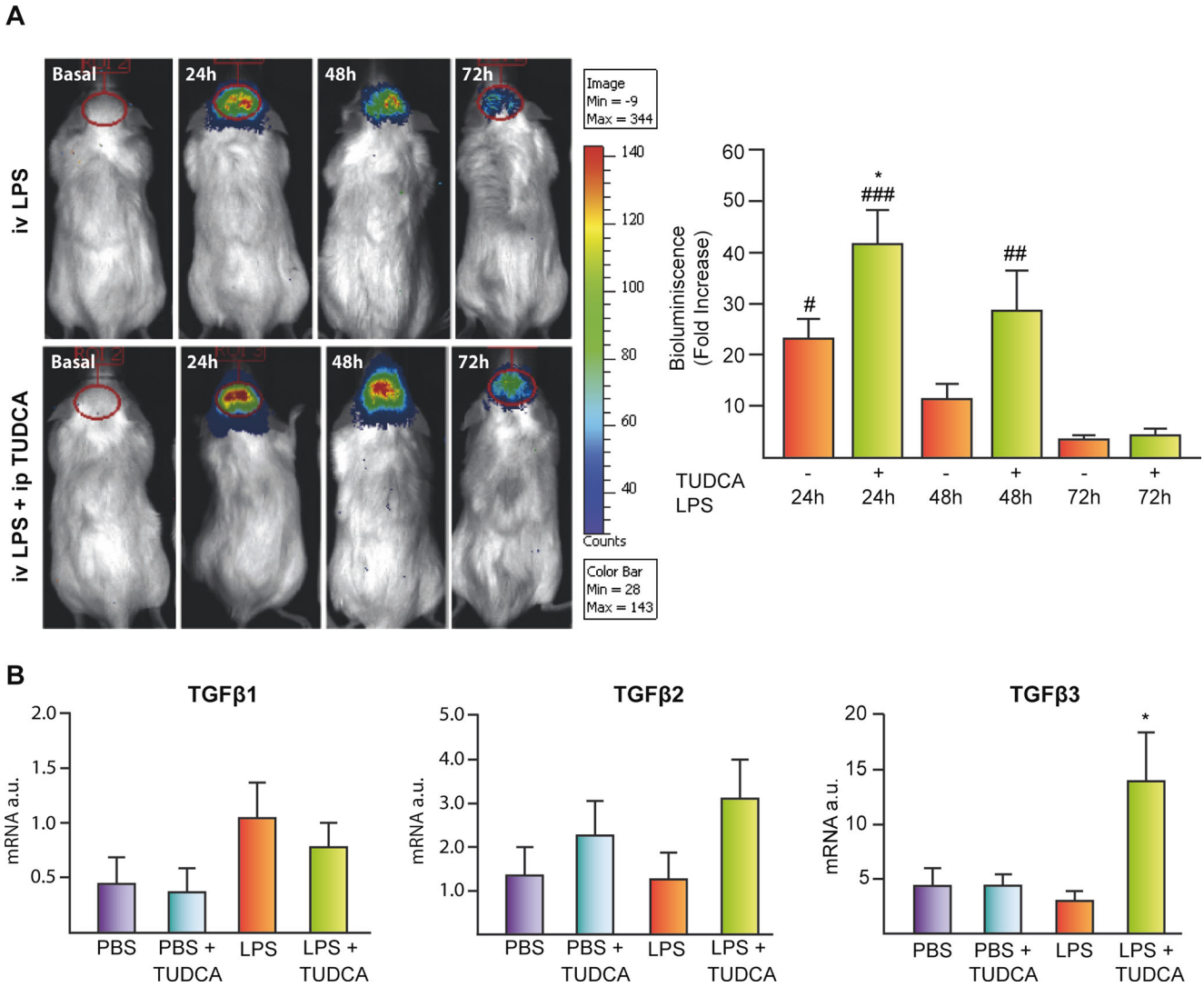
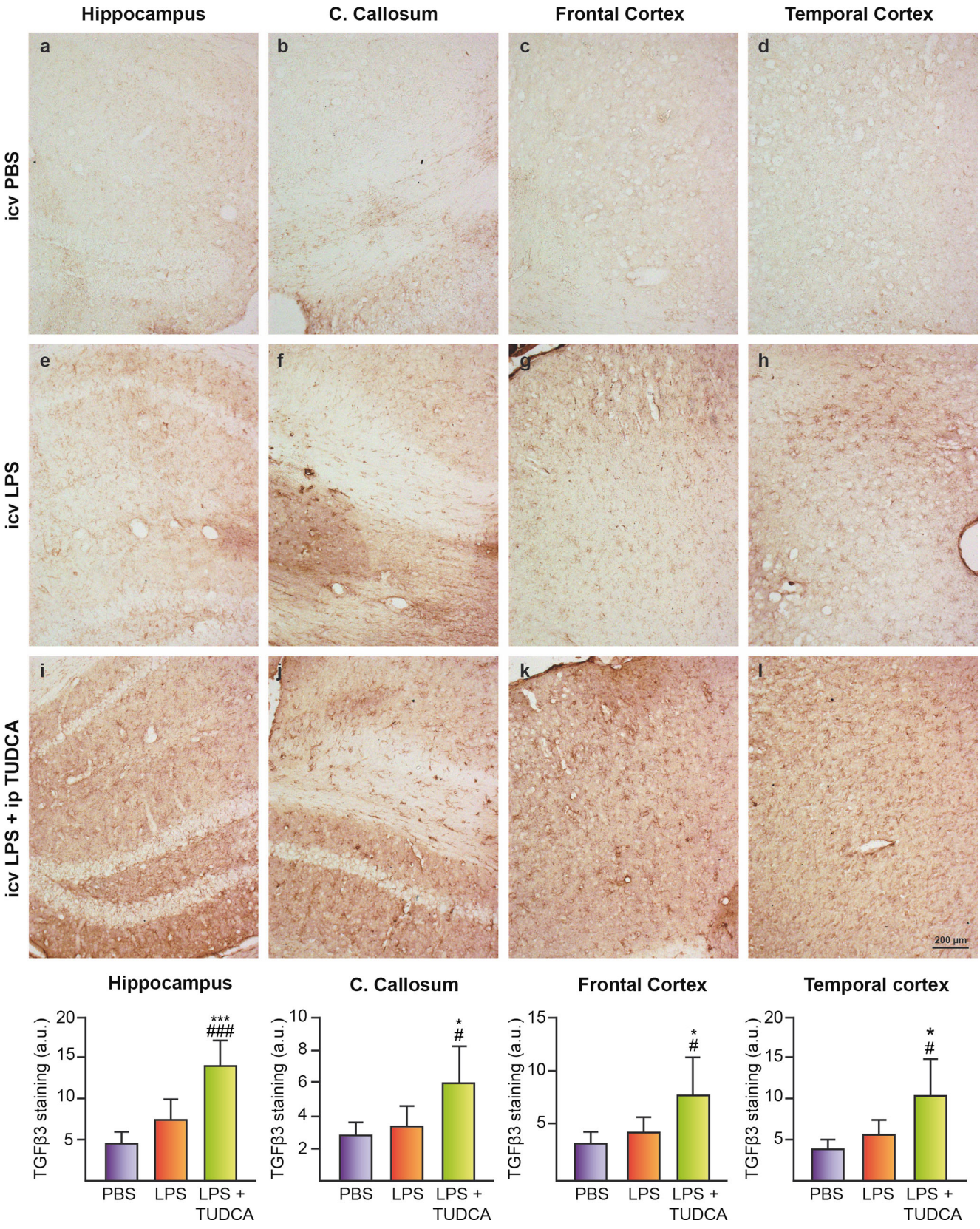


Fig. 1 TUDCA activates the TGFβ pathway in an animal model of acute neuroinflammation. **a** The activation of Smad2/3-TGFβ pathway was determined in the brain of SBE/TK-luc transgenic mice after iv LPS by measuring the emission of bioluminiscence. *Bar graphs* represent the mean ± SEM of the bioluminiscence, measured as the fold increase in bioluminiscence compared to the basal emission for each mouse, in 12 mice injected iv with LPS (2 mg/kg), and 11 mice that were also treated with TUDCA (500 mg/kg) ip. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$ compared to control; $^{*}p < 0.05$ compared to LPS-treated mice. **b** Under pro-inflammatory conditions, TUDCA treatment induces TGFβ2 and TGFβ3 transcription in wild-type mice. We determined the transcription of TGFβ isotypes in the hippocampi of mice 24 h after treatment. The results represent the ratio between the mRNA expression of the target gene and the mRNA of RPS29 (used as a housekeeping gene). *Bar graphs* represent the mean ± SEM of icv PBS ($n = 6$), icv PBS + TUDCA ($n = 7$), icv LPS ($n = 9$), and icv LPS + ip TUDCA ($n = 10$). $^{*}p < 0.05$ compared to icv LPS

injection (Fig. 2). We found a significant increase in TGFβ3 staining in the frontal cortex ($p < 0.05$), the hippocampus ($p < 0.001$), the corpus callosum ($p < 0.05$), and the temporal cortex ($p < 0.05$) of mice treated with both LPS and TUDCA, compared to control mice. Moreover, we found a significant increase in TGFβ3 staining in mice treated with both LPS and TUDCA compared to mice treated with LPS alone in the temporal cortex ($p < 0.05$), hippocampus ($p < 0.001$), corpus callosum ($p < 0.05$), and frontal cortex ($p < 0.05$).

We determined the cell types involved in this response by co-staining for TGFβ3 and specific cell markers: Iba-1 for microglia, type IV collagen for endothelial cells, GFAP for

Fig. 2 TUDCA induces TGFβ3 expression in the brain of LPS-treated mice. The effect of TUDCA on TGFβ3 expression was determined by the immunoreactive area in several brain regions of the mice 3 days after icv injection with PBS ($n = 5$), LPS ($n = 8$), or LPS and ip TUDCA ($n = 11$). *Bar graphs* represent the mean ± SEM of TGFβ3 staining in the hippocampus, corpus callosum, and frontal or temporal cortex of five sections per animal. $^{\#}p < 0.05$ and $^{\#\#\#}p < 0.001$ compared to control; $^{*}p < 0.05$ and $^{***}p < 0.001$ compared to LPS treatment. Scale bar 200 μm



astrocytes, and β-III-tubulin for neurons, in the brain of mice treated both with LPS and TUDCA (Fig. 3). TGFβ3 staining co-localized with microglial cells, endothelial cells, and neurons, but not with astrocytes.

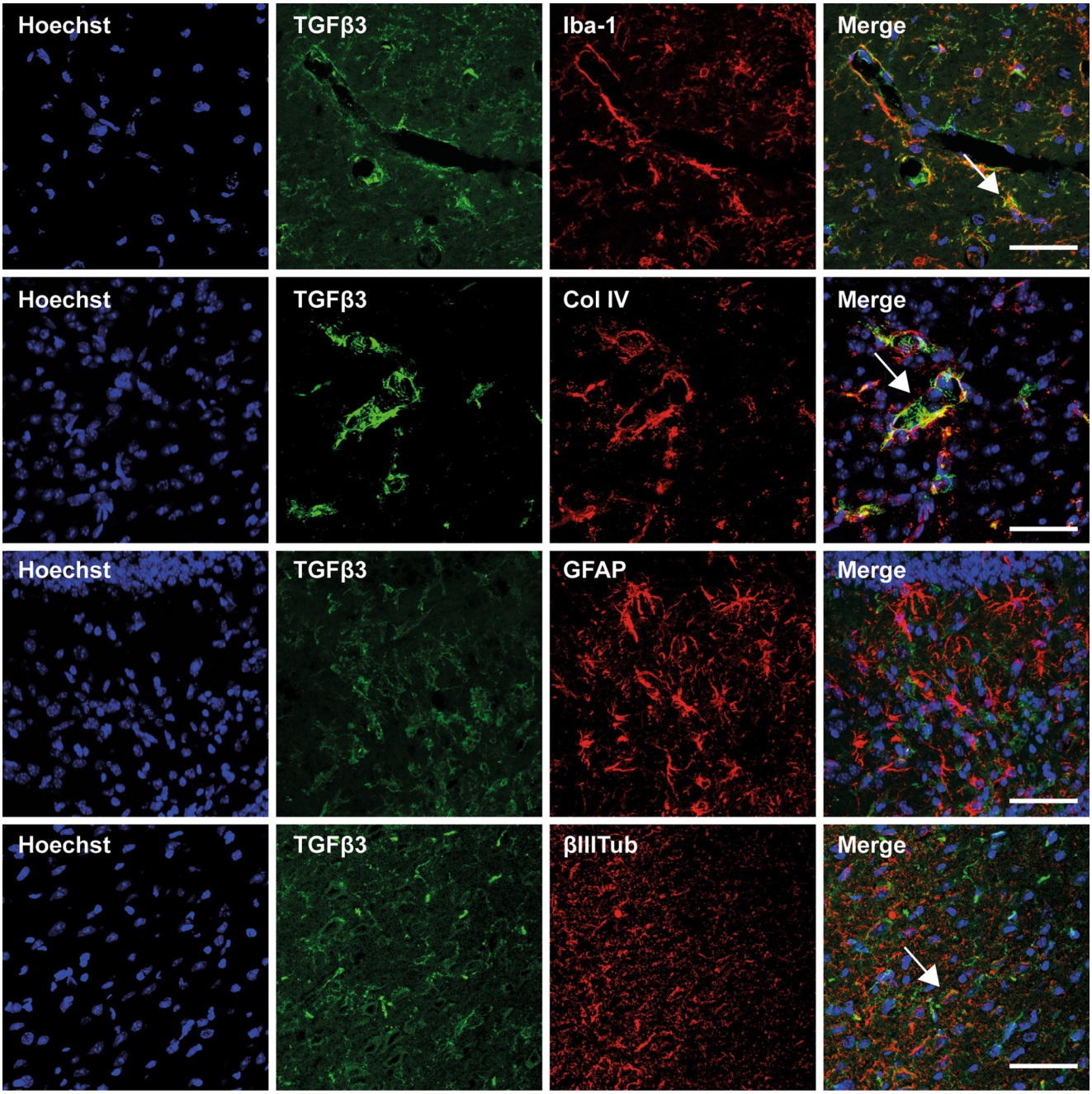


Fig. 3 TUDCA induces TGFβ3 expression in the microglia, endothelial cells, and neurons, but not in astrocytes, from the hippocampus of LPS-treated mice. Confocal microscopy images of cell types expressing TGFβ3. Double staining of the hippocampal sections with TGFβ3 antibody together with Iba-1 antibody (for microglia), collagen type IV (Col IV) antibody (for endothelial cells), GFAP antibody (for astrocytes), or β-III-Tubulin (βIIITub) antibody (for neurons). *Scale bar* 100 μm

TUDCA Reduces Microglial Reactivity in the Brain of LPS-Treated Mice Through TGFβ Activation

To study whether the TGFβ pathway activation was involved in the anti-inflammatory response to TUDCA, we injected icv the selective inhibitor of the TGFβ receptor, SB431542, in our acute neuroinflammation model. SB431542 was injected in the contralateral ventricle of the mice, right after the PBS or LPS injections in the course of the same surgical procedures. The analysis of TGFβ3 staining in coronal sections from the brain did not show any differences in the TGFβ3 expression in the hippocampus, corpus callosum, frontal cortex, and temporal cortex of control or LPS-treated mice, when they were also treated with SB431542 (Fig. 4). The staining for TGFβ3 increased in those areas in mice

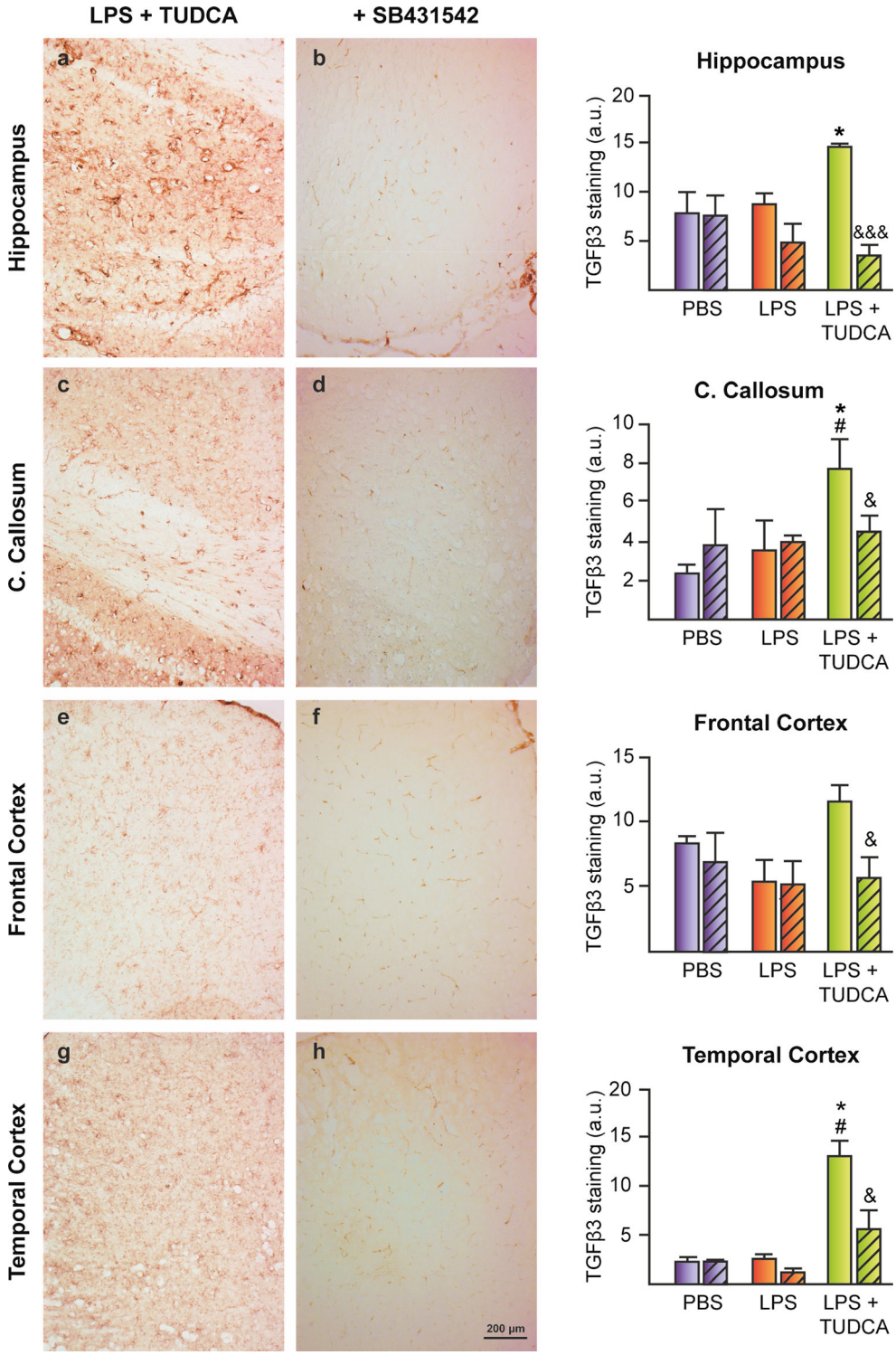


Fig. 4 TGFβ receptor inhibitor SB431542 reduces TUDCA induction of TGFβ3 expression in the brain of LPS-treated mice. The effect of TGFβ receptor blockade by SB431542 on TGFβ3 expression was determined in three experimental groups: icv PBS as a control, icv LPS, and icv LPS treated with TUDCA ip (500 mg/kg). The expression of TGFβ3 was determined in different brain regions by measuring the immunoreactive area 3 days after the induction of neuroinflammation. Brain sections show representative TGFβ3 staining pictures from the hippocampus (**a**), corpus callosum (**c**), frontal cortex (**e**), and temporal cortex (**g**) of LPS plus TUDCA-treated mice, and in the same areas in LPS and TUDCA-treated mice which were also injected with icv SB431542 (**b**, **d**, **f**, and **h**, respectively). *Bar graphs* represent the mean ± SEM of TGFβ3 staining in the hippocampus, corpus callosum, and frontal or temporal cortex of five sections per animal of at least three mice per experimental group. $^{\#}p < 0.05$ compared to control; $^{*}p < 0.05$ compared to LPS treatment; $^{\&}p < 0.05$ $^{\&\&\&}p < 0.001$ compared to the same treatment without SB431542 (). *Scale bar* 200 μm

treated with both LPS and TUDCA, compared to mice treated with LPS alone. However, this staining was significantly decreased in the hippocampus ($p < 0.001$), corpus callosum ($p < 0.05$), frontal cortex ($p < 0.05$), and temporal cortex ($p < 0.05$), when these mice were also injected icv with SB431542. These data show that selective blockade of the TGFβ receptor (by icv injection of SB431542) effectively blocked the increase in the TGFβ3 immunoreactivity of mice treated with both TUDCA and LPS.

Previous work from our lab demonstrated that TUDCA reduced microglial activation in the hippocampus of mice

treated with LPS [27]. To study whether the inhibition of the TGFβ pathway had any effect on microglial activation, we studied Iba-1 immunoreactivity in coronal sections from mouse hippocampus, 3 days after icv injection, in eight experimental groups (Fig. 5): control (icv PBS, a), control treated with TUDCA (icv PBS and ip TUDCA, b), LPS injection (icv LPS, c), LPS treated with TUDCA (icv LPS and ip TUDCA, d), and the same treatments injected SB431542 in the contralateral ventricle (e–h, respectively). The results showed that icv SB431542 injection did not increase the immunoreactivity for Iba-1 in control mice (whether treated with TUDCA or not) or in the hippocampus of mice treated with LPS alone (Fig. 5a–c and e–f). However, icv injection of SB431542 significantly reverted TUDCA inhibition of microglial reactivity in LPS-treated mice ($p < 0.01$, Fig. 5d, h). These results suggest that the anti-inflammatory effect of TUDCA is mediated, at least partially, through the activation of the TGFβ pathway.

Discussion

Several studies have demonstrated that TUDCA is neuroprotective for neurodegenerative diseases (e.g., amyotrophic lateral sclerosis [29], Alzheimer's disease [41] , Parkinson's disease [42], Huntington's disease [30], and stroke [31, 32]). In addition to this cytoprotective effect, we have previously shown that the potent anti-inflammatory effect of TUDCA on neuroinflammation proceeds through inhibition of NFκB activation [27]. The inhibitory effect on the NFκB pathways reduced endothelium activation, leukocyte transmigration to the CNS parenchyma, glia activation, and microglia migration in an animal model of acute neuroinflammation [27]. Here, we show an additional anti-inflammatory effect of TUDCA through the regulation of the TGFβ pathway.

TGFβ is a pleiotropic cytokine involved in various physiological and pathological processes in the CNS [10]. Signaling through the TGFβ pathway is enhanced in LPS-driven neuroinflammation and brain damage [24, 30, 31, 37]. Here, we

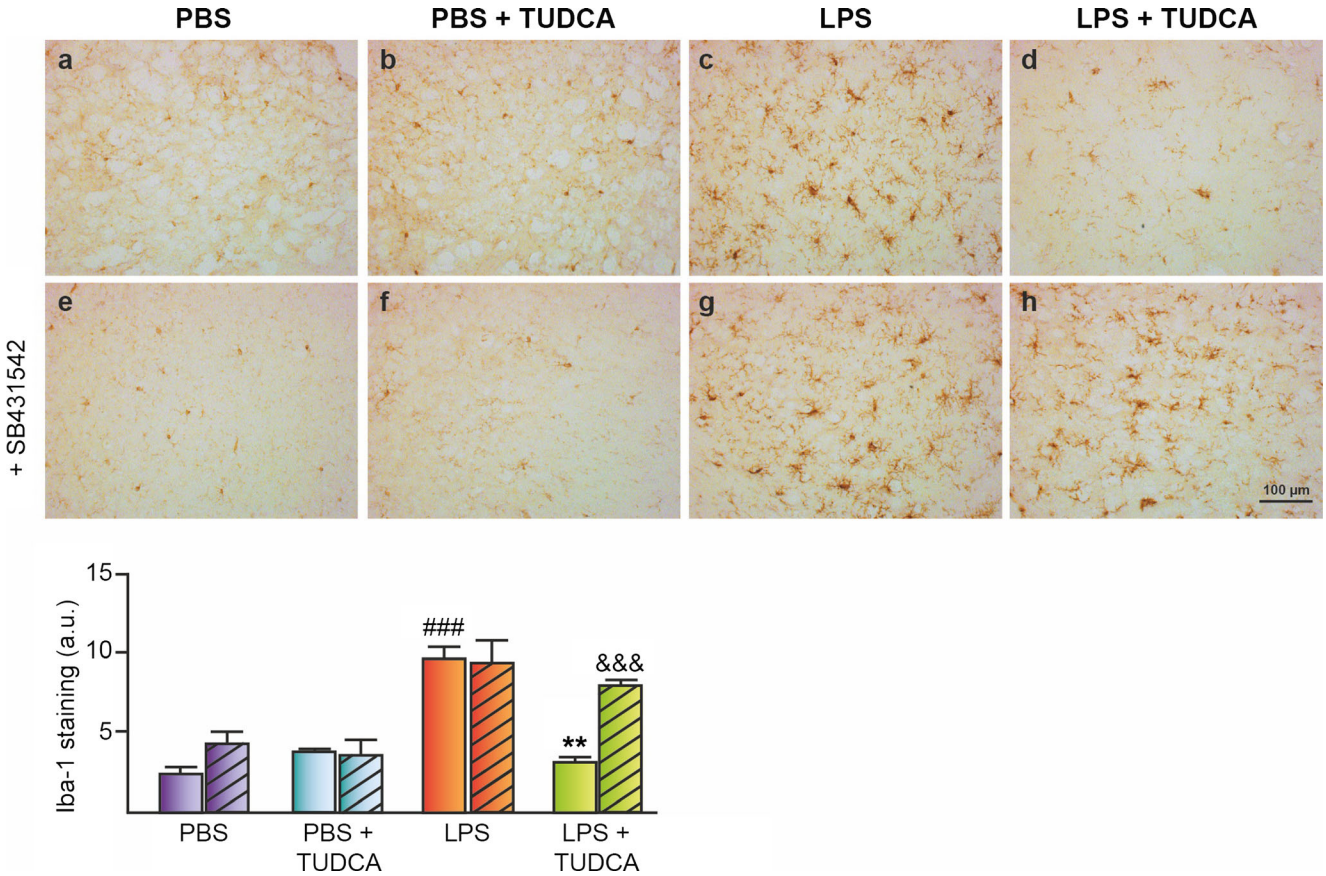


Fig. 5 Selective blockade of the TGFβ receptor reverts the inhibitory effect of TUDCA on microglial activation. The effect of SB431542 (a selective inhibitor of the TGFβ receptor) on microglial activation was determined by the immunoreactive area for Iba-1 in the hippocampus of control mice (PBS), control mice treated with TUDCA (PBS + TUDCA), icv LPS-injected mice (LPS), and icv LPS-injected mice treated with TUDCA (icv LPS and ip TUDCA). To this aim, half of the mice in each experimental group received an icv injection of SB431542 in the contralateral ventricle. *Bar graphs* represent the mean ± SEM of Iba-1 staining in the hippocampus of five sections per animal of at least three mice per experimental group. $^{###}p < 0.001$ compared to control; $^{**}p < 0.05$ compared to LPS treatment; $^{\&\&\&}p < 0.001$ compared to the same treatment without SB431542. *Scale bar* 100 μm

studied TUDCA effect on the activation of the TGFβ pathway in acute neuroinflammation, using a SBE/Tk-Luc transgenic mice model. The Smad-responsive luciferase reporter construct in these mice allows to study, using non-invasive bioluminescent imaging, the temporal and spatial patterns of Smad2/3-dependent signaling, and therefore the TGFβ pathway activation [12]. As reported earlier [12], we noticed a significant increase in the activation of the TGFβ pathway 1 day after iv LPS administration compared to basal levels. This activation was lower 2 days after LPS injection and decayed completely after 3 days. Activation of the TGFβ pathway with LPS might be due to an early release of TGFβ1 from the extracellular matrix [43, 44]. When mice were treated with both LPS and TUDCA, the TGFβ pathway activation increased further compared to mice treated with LPS alone. Because mice treated with TUDCA alone did not show any effect on the TGFβ pathway, our results suggest that treatment with both LPS and TUDCA activated an additional mechanism of activation of the TGFβ pathway.

Three different TGFβ isotypes have been described in mammals, TGFβ1, TGFβ2, and TGFβ3. Although the three isoforms show high sequence homology and bind to the same receptors, they have different functions in the regulation of the inflammatory process in vivo [45–47]. Besides, depending on the cell type, TGFβ3 may transmit signals similar to those transmitted by TGFβ1 and TGFβ2 but more potent [48].

Our results show an increase in the transcription of TGFβ2 and TGFβ3 in the hippocampus of mice treated with both LPS and TUDCA, compared to mice treated with LPS alone. However, only the increase in the TGFβ3 transcript in mice treated with both LPS and TUDCA was statistically significant, and the induction of TGFβ3 expression required the concomitant activation of both LPS and TUDCA pathways.

The inhibition of the TGFβ pathway with its receptor inhibitor SB431542 reduced the expression of TGFβ3 in mice treated with both LPS and TUDCA. SBE/Tk-Luc mice treated with LPS alone activated the TGFβ pathway, but this treatment did not activate TGFβ3 transcription and expression in wild-type mice. In the same way, mice treated with TUDCA alone did not show an increase of TGFβ3 transcription and expression. The activation of the TGFβ pathway was required for TGFβ3 expression, but it was not enough. The study of the TGFβ3 promoter agrees with these results, pointing to the critical role of transcription factors Smad3 and CREB-1 (cAMP response element binding protein 1) for the TGFβ induction of TGFβ3 expression [49]. The TGFβ induction of TGFβ3 expression is common in other TGFβ isotypes by an auto-feedback loop [50]. The auto-induction of TGFβ1transcript requires the activator protein-1(AP-1), Smad3, and Smad4 transcription factors, and the activation of the c-Jun terminal kinases (JNKs), and the extracellular signal-regulated kinases (ERKs) cascades in epithelial cells [51]. Conversely, the auto-induction of TGFβ3 transcript requires Smad3 and CREB-1 transcription factors, and the activation of JNKs and p38 reactivating kinases (p38RKs) cascades in epithelial cells [49]. These results suggest that the activation of a TGFβ isotype (e.g., TGFβ1) and the consequent activation of the TGFβ receptor might induce the activation other TGFβ isotypes through the activation of Smad3. As the transcriptional induction of Smad3 target genes requires the cooperation of other transcription factors [52], this additional signaling might be responsible for the induction of specific TGFβ isotype. Together with the TGFβ pathway induction of Smad3, TUDCA may activate additional pathways to induce TGFβ3 transcription and expression.

TGFβ is a key modulator of inflammation [19], inhibiting immune and CNS resident cells under both basal [20, 21] and neuropathological conditions [22–24]. TGFβ inhibited the expression of chemokines in microglia, chemokine receptors, and other genes mediating cell migration (e.g., metalloproteases) induced by pro-inflammatory cytokines [53]. This study concluded that one of the main effects of TGFβ was to impair cell entry into the CNS and hinder microglia migration to the CNS parenchyma [53]. Previous work from our lab showed that TUDCA reduced Iba-1 expression in the hippocampus of mice treated with LPS, compared to mice treated with LPS alone. We concluded that TUDCA exerted a direct effect on microglia, inhibiting their activation and hindering their migratory capacity under pro-inflammatory conditions [27]. Here, we show that, under pro-inflammatory conditions, treatment of mice with both TUDCA and the TGFβ receptor inhibitor SB431542 increased Iba-1 expression, compared to mice treated with TUDCA alone. These results suggest that TUDCA, in addition to its direct anti-inflammatory effect through the NFκB pathway [27], may have an additional anti-inflammatory effect through the induction of TGFβ3 and the concomitant enhancement of the TGFβ pathway. Besides, as both microglia and macrophages express Iba-1, we cannot exclude that TUDCA may affect the infiltration of blood monocytes into the brain parenchyma through the induction of TGFβ3. Under pro-inflammatory conditions, TGFβ3 expression in mice treated with TUDCA was limited to certain cell types, such as microglia, endothelial cells, and some neurons, but not astrocytes. In addition to the important regulatory role of TGFβ3 on the immune response [54, 55], TUDCA-induced expression of TGFβ3 in endothelial cells may participate, under pro-inflammatory conditions, in the reduction of blood monocyte infiltration into the brain.

Under pro-inflammatory conditions, microglial activation [40, 56] and the infiltration of leukocytes into the brain [57] might produce a detrimental environment for neurons inducing neuronal cell death. Our work suggests that TUDCA might have an indirect effect on neuroprotection inducing the expression of TGFβ3. This anti-inflammatory cytokine might be responsible for the reduction of microglial activation and leukocytes infiltration into the brain. Moreover, as TGFβ3 has

neuroprotective effects [58, 59], we cannot exclude that the neuroprotective effect of TUDCA in different neuropathology models [30–32, 41, 42] might be partially due to the induction of the TGFβ pathway. In the acute neuroinflammation model used in this study, most of the neuronal cell death is a consequence of the activation of microglial cells [38, 40, 56] and occurs much later than the time points studied (>7 days after icv injection of LPS, [38] [40]). To determine whether TUDCA has a direct effect on neuroprotection inducing TGFβ3 expression further studies must be conducted using other animal models.

In summary, we have presented evidence of an anti-inflammatory effect of TUDCA, additional to its previously described inhibitory effect on NFκB [27], now increasing the activation of the TGFβ pathway. This may have therapeutic implications for the treatment of those neuropathologies that course with neuroinflammation.

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Compliance with ethical standards Animal handling and care was performed following European Union guidelines (2010/63/EU) and Spanish regulations (BOE67/8509–12; BOE 1201/2005) for use and care of laboratory animals. All the protocols were approved by the Ethics and Scientific Commitees of the Instituto Cajal, CSIC, and the Hospital Nacional de Parapléjicos, SESCAM.

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