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Elevated β1,4-galactosyltransferase-I induced by the intraspinal injection of lipopolysaccharide

Aiguo Shen · Jianping Chen · Ji Qian · Jianchun Zhu · Ling Hu · Meijuan Yan · Dan Zhou · Ying Gao · Junling Yang · Fei Ding · Chun Cheng

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Abstract β1,4-Galactosyltransferase-I (β1,4-GalT-I) is one of the best studied glycosyltransferases. Previous studies demonstrated that β1,4-GalT-I was a major galactosyltransferase responsible for selectin-ligand biosynthesis and that inflammatory responses of β1,4-GalT-I deficient mice were impaired. In this study, we investigate the expression of β1,4-GalT-I in lipopolysaccharide (LPS)-induced neuroinflammatory processes. The results of this study demonstrated that β1,4-GalT-I was strongly induced by intraspinal administration of LPS. More than 90% galactose-containing glycans and β1,4-GalT-I were expressed in immune cells. The ELISA assay shows focal injection LPS also induces TNF-α alteration. Double staining indicated β1,4-GalT-I overlapped with TNF-α. Moreover, RT-PCR for β1,4-GalT-I mRNA showed that β1,4-GalT-I mRNA in microglia *in vitro* was affected in a dose- and time dependent manner in response to LPS or TNF-α stimulation. All these results indicated that the increase of β1,4-GalT-I might attribute to the effect of TNF-α excreting during inflammation. E-selectin, which ligand was modified by β1,4-GalT-I, was correlated with galactose-containing glycans following injecting LPS into spinal cord. We therefore suggest that β1,4-GalT-I may play an important role in regulating immune cell migration into the inflammatory site.

Keywords β1,4-Galactosyltransferase-I · Lipopolysaccharide · Neuroinflammation · Tumor necrosis factor-alpha · HAPI · Spinal cord · Rat

Introduction

Lipopolysaccharide (LPS) is the component of a gram-negative bacterial cellular wall. It breaks out after bacterial invasion into host circulation, and cause a serious illness with inflammation in the whole body. It stimulates the production of a potent inflammatory mediator, and causes circulatory injury, shock, multiple organ failure, and death [34]. Experimentally, inflammation can be induced in most tissues by the local injection of LPS, and typically large numbers of neutrophils and monocytes invade the tissue from the bloodstream within minutes [4, 19]. Recruitment of leukocytes proceeds along a cascade of events, beginning with the capture of free flowing leukocytes to the vessel wall. This is followed by leukocyte rolling along the endothelium. Capture and rolling are mediated by a group of glycoproteins, called selectins, which bind to carbohydrate determinants on selectin ligands [14]. Injection LPS into central nervous system (CNS) evokes inflammatory reaction typically [3, 33]. In recent years, increasing evidence has strongly suggested inflammation in the CNS may be a basic mechanism driving the progressive nature of multiple neurodegenerative disease, including Parkinson's

Aiguo Shen and Jianping Chen contributed equally to this work.

A. Shen · M. Yan · Y. Gao · F. Ding (✉) · C. Cheng (✉)
The Jiangsu Province Key Lab of Neuroregeneration,
Nantong University,
Nantong 226001, People's Republic of China
e-mail: dingfei@ntu.edu.cn
e-mail: Cheng_Chun@yahoo.com.cn

J. Chen · J. Qian · J. Zhu · L. Hu · D. Zhou · J. Yang
Department of Microbiology and Immunology,
Nantong University,
Nantong 226001, People's Republic of China

and Alzheimer's disease, multiple sclerosis and the AIDS dementia complex [5, 21, 27, 31].

β1,4-galactosyltransferase I (β1,4-GalT-I) is one of at least seven polypeptides that synthesize β1,4-galactosyl linkages [16]. It is a type II membrane glycoprotein, similar to other glycosyltransferase, in the presence of the sugar donor UDP-galactose, it is responsible for the galactosylation of complex glycoconjugates terminating in *N*-acetylglucosamine (GlcNAc) [10]. It resides in two distinct subcellular compartments, the trans-Golgi network and the cell surface. β1,4-GalT-I is expressed ubiquitously and strongly in almost all tissues, suggesting that β1,4-GalT-I is involved in β4 galactosylation of many glycoproteins. β4 galactosylation of glycoproteins is widely distributed in mammalian tissues and is involved in various physiological functions through interactions with selectins, galectins, asialoglycoprotein receptor and so on. It shows reduced inflammatory responses [2] and delayed skin wound healing [26] attributable to impaired leukocyte infiltration caused by the reduced biosynthesis of selectin ligands in β1,4-GalT-I deficient mice. Toshikazu Nishie and colleagues demonstrated that β1,4-GalT-I$^{+/-}$ mice developed an immunoglobulin A nephropathy (IgAN)-like disease consistent with the pathological diagnosis of human IgAN [29]. In addition, β1,4-GalT-I serves as a recognition molecule and participates in a number of cellular interactions, including neurite extension, cell growth, sperm–egg interaction, cell spreading, and migration [6, 8, 15, 18, 24, 28].

Selectin ligands belong to a growing number of glycoproteins, where protein function is closely linked to its proper post-translational glycosylation. Post-translational glycosylation is mainly performed in the Golgi apparatus, involving a group of Golgi resident enzymes termed glycosyltransferases [23]. β1,4-GalT-I has been identified to participate directly in the synthesis of functional selectin ligands *in vivo* [2]. Our previous studies have shown that β1,4-GalT-I may play an important role in the peripheral organs during the inflammation reaction induced by intraperitoneal injection of LPS [30]. However, the expression of β1,4-GalT-I and its biological function in CNS during inflammation and infection remain to be elucidated.

Multiple sclerosis (MS) is the most prevalent inflammation-mediated demyelinating disease. The neurodegeneration associated with MS occurs as lesions in white matter of the CNS. Intraspinal injection of LPS results in inflammation and subsequently in prominent demyelination, which is a feature of the pattern III lesion. LPS-induced demyelination serves as an experimental model available for the study of MS, particularly in pattern III lesion [9]. In the present study, we examined β1,4-GalT-I expression in inflammatory stage following the injection of LPS into rat spinal cord. These results might contribute to further understanding of the function of β1,4-GalT-I in the inflammation in the CNS.

Materials and methods

Experimental animals and treatments

Male Sprague-Dawley rats (180–220 g), provided by the Animal House, Nantong Medical College, were caged in groups of three with food and water given ad libitum. The animals were kept in a temperature-controlled environment (21°C) on a 12 h light–dark cycle. The surgery was performed as described [9]. Briefly, rats were deeply anesthetized with a cocktail of xylazine (10 mg/kg), ketamine (95 mg/kg) and acepromazine (0.7 mg/kg) administered intraperitoneally. Then using sterile technique, a quarter laminectomy was performed at the T12 vertebral level in rats. Two small holes, 1 mm apart, were made in the dura over the left dorsal column and a drawn glass micropipette (tip diameter 10 μm) was inserted into the dorsal column and 0.5 μl of LPS (E. coli 055:B5, Sigma) (100 ng/μl in saline) was injected at each of the two sites at depths of 0.7 and 0.4 mm (2 μl in total). The injection sites were marked by placing a small amount of sterile charcoal on the adjacent dura. Control animals were injected by saline alone. All animal experiments were carried out in accordance with the United States National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Cell culture and treatment

HAPI microglial cells were provided by professor Qin Shen (Nantong University, Jiangsu province, China). Cells were maintained in RPMI-1640 medium (Sigma-Aldrich, Rehovot) supplement with 10%FCS, L-glutamine (1 mM), sodium pyruvate (1 mM), β-mercaptoethanol (50 μM), penicillin (100 U/ml), and streptomycin (100 μg/ml). All cells were maintained at 37°C in a humidified incubator under 5% CO_2 and 95% air. After cells were allowed to reach 80% confluence, culture medium was switched to serum-free RPMI-1640 medium and experiments were initiated 24 h later. LPS or TNF-α was administered directly into growth media for the indicated incubation times. Non-treated cells were included as controls in all experiment.

Real-time PCR

Total RNA was isolated using the TRIzol method (Invitrogen, Burlington, ON) according to the manufacturer's protocol. Single-stranded cDNA was synthesized from total RNA using the RevertAid First Strand cDNA Synthesis Kit (Fermentas). Transcript levels of β1,4-GalT-I in rat spinal cord were measured in 36-well microtiter plates using Rotogene 2000 Sequence Detector Systems (Perkin-Elmer/Applied Biosystems, Foster City, CA). The thermal cycling program consisted of 3 min at 94°C, followed by 40 cycles

of 20 s at 94°C and 1 min at 60°C. All runs were accompanied by the TaqMan β_2-microglobulin (β_2-M) control Reagents. Initial experiments demonstrated identical realtime amplification efficiencies of target and reference gene. The expression level of each target gene was calculated by standardizing the target gene copy number with the β_2-M copy number in a sample. Specific mRNA transcript levels were expressed as fold difference. Analysis of results is based on three independent experiments. PCR primers for β1,4-GalT-I, and β_2-M were designed corresponding to the coding region of the genes as follows: β1,4-GalT-I primers, sense 5′-TATTTGCATCCAGTCTTT CAGC-3′ and antisense 5′-CAGCTTAGCTCGATTAAAC ATGG-3′; β_2-M primers, sense 5′-GTCTTTCTACATCCT GGCTCACA-3′ and antisense 5′-GACGGTTTTGGG CTCCTTCA-3′. PCR probes for β1,4-GalT-I and β_2-M as follows: β1,4-GalT-I probe, 5′(FAM)-TCCAGCCTGA TTGATGACGTAGATGC-(TAMRA)3′ and β_2-M probe, 5′(FAM)-CACCCACCGAGACCGATGTATATGCTTGC-(TAMRA)3′.

RT-PCR

Integrity of RNA of microglia was checked by agarose gel electrophoresis and ethidium bromide staining. PCR (30 cycles) was performed as follows: denaturation for 30 s, annealing at 57°C for 40 s, and elongation at 72°C for 40 s. PCR products were analyzed by agarose (2%) gel electrophoresis and ethidium bromide staining. Primer sequences were described as above. The relative differences in expression between groups were expressed using optical density normalized with GAPDH, and the relative differences between control and treatment groups were calculated. Values are responsible for at least four independent reactions.

Immunofluorescent staining

Animals whose tissues were to be examined by immunohistochemistry were perfused with ice-cold saline followed by 4% paraformaldehyde in phosphate buffer (pH 7.4) at postinjection times of 8, 12 and 24 h. The spinal cord was then removed and serial coronal frozen sections of 8 μm thickness were cut on a freezing sliding microtome and stored at −20°C until use. For single label immunohistochemistry, all of sections were blocked with 10% donkey serum with 0.1% Triton X-100 and 1% bovine serum albumin (BSA) for 2 h at room temperature (RT) and incubated overnight at 4°C with anti-β1,4-GalT-I antibody (Goat Anti-β1,4-GalT-I, 1:100, Santa Cruz). After washing in phosphate-buffered saline (PBS) three times, the second antibodies were added (FITC–Donkey-anti-Goat, 1:100, Jackson Laboratory) and the mixture was placed in a dark room and incubated for 2 h at 4°C. For double labeling, sections were first blocked with 10% normal serum blocking solution—species the same as secondary antibody overnight at 4°C to avoid unspecific staining. Then, the sections were incubated with both antibody specific for β1,4-GalT-I and antibody for different markers as follows: OX-42 (Mouse Anti-OX-42, 1:50, Serotec), ED-1 (Mouse Anti-ED1, 1:100, Serotec), MPO (Rabbit Anti-MPO, 1:100, NeoMarkers), GFAP (Mouse Anti-GFAP,1:100, Sigma), NeuN (Mouse Anti-NeuN,1:600, Sigma), CNPase (Mouse Anti-CNPase, 1:50, Sigma), CD62E (Rabbit Anti-CD62E, 1:200, Abcam) and TNF-α (Mouse Anti-TNF-α, 1:100, Santa Cruz) overnight at 4°C. After washing in PBS three times for 10 min each, the secondary antibodies (FITC–Donkey-anti-Goat; TRITC–Donkey-anti-Mouse, 1:100, Jackson Laboratory; TRITC–Donkey-anti-Rabbit, 1:100, Jackson Laboratory; FITC–Donkey-anti-Rabbit, 1:100, Jackson Laboratory) were added in the dark room and incubated for 2–3 h at 4°C. The images were captured by Leica fluorescence microscope (Germany).

Lectin-fluorescent staining with RCA-I

To eliminate terminal sialic acid moieties, sections were digested with sialidase for 5 h at 37°C. Frozen sections were first blocked with 1% BSA overnight at 4°C in order to avoid unspecific staining. Then the sections were incubated with isothiocyanate (FITC)-conjugated RCA-I (1:400, Vector) in the dark room for 2 h at 4°C. For double staining analysis, the sections were incubated with monoclonal primary antibody for different cell markers (OX-42, ED-1, MPO, GFAP, NeuN, CNPase, CD62E) overnight at 4°C. After wash in PBS for three times, the second antibodies above-mentioned and FITC-RCA-I were added in dark room and incubated for 2 h at 4°C. The fluorescence was detected by Leica fluorescence microscope.

Lectin blotting

Spinal cord tissues at different time points postoperatively (n=6 for each point) were homogenized in a lysate buffer containing 1 M Tris–HCl pH 7.5, 1% Triton X-100, 1% NP-40 (nonidet p-40), 10% SDS, 0.5% Sodium deoxycholate, 0.5 M EDTA, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM PMSF, were then centrifuged at 10,000 ×g for 30 min to collect the supernatants. Each supernatant was diluted in 2×SDS loading buffer and boiled. Proteins were separated with SDS-PAGE (10% gradient gels) and then transferred onto a PVDF membrane. After being blocked with 5% BSA, the membrane was incubated with 1:300 dilution of HRP-RCA-I or 1:1,000 diluted antibody to E-selectin followed by a 1:500 HRP-labeled secondary antibody for 2 h at room temperature. The blots were washed and developed with the ECL detection system using x-ray film (Fuji Photo Film).

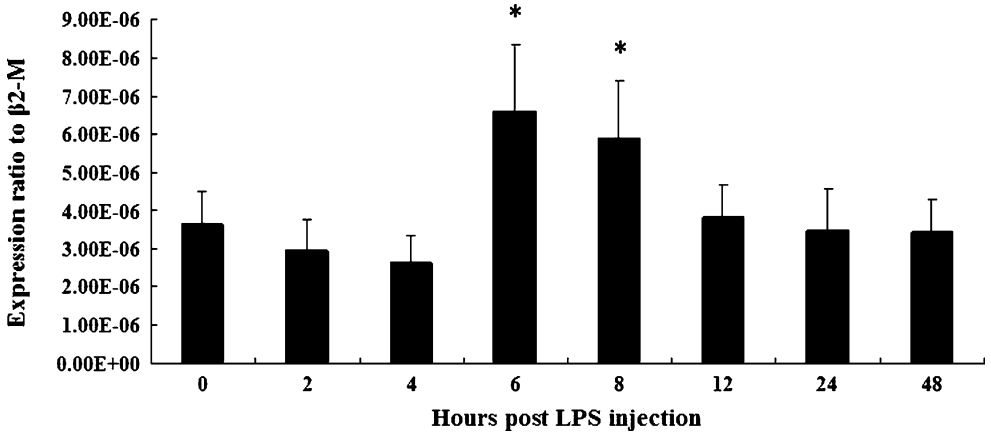


Fig. 1 Quantification of β1,4-GalT-I mRNA in the rat spinal cord at various times after focal injection of LPS. There was a significant increase in β1,4-GalT-I mRNA at 6 and 8 h after intraspinal injection of LPS ($p<0.05$). Total RNA of the spinal cord was isolated at 0, 2, 4, 6, 8, 12, 24 and 48 h post-injection. For quantitative analysis, the β1,4-GalT-I mRNA level was normalized by β_2-microglobulin mRNA. Data were from six animals at each time point, and the experiments were tripled under the same condition. Values are mean± SEM ($n=5$). *$p<0.05$

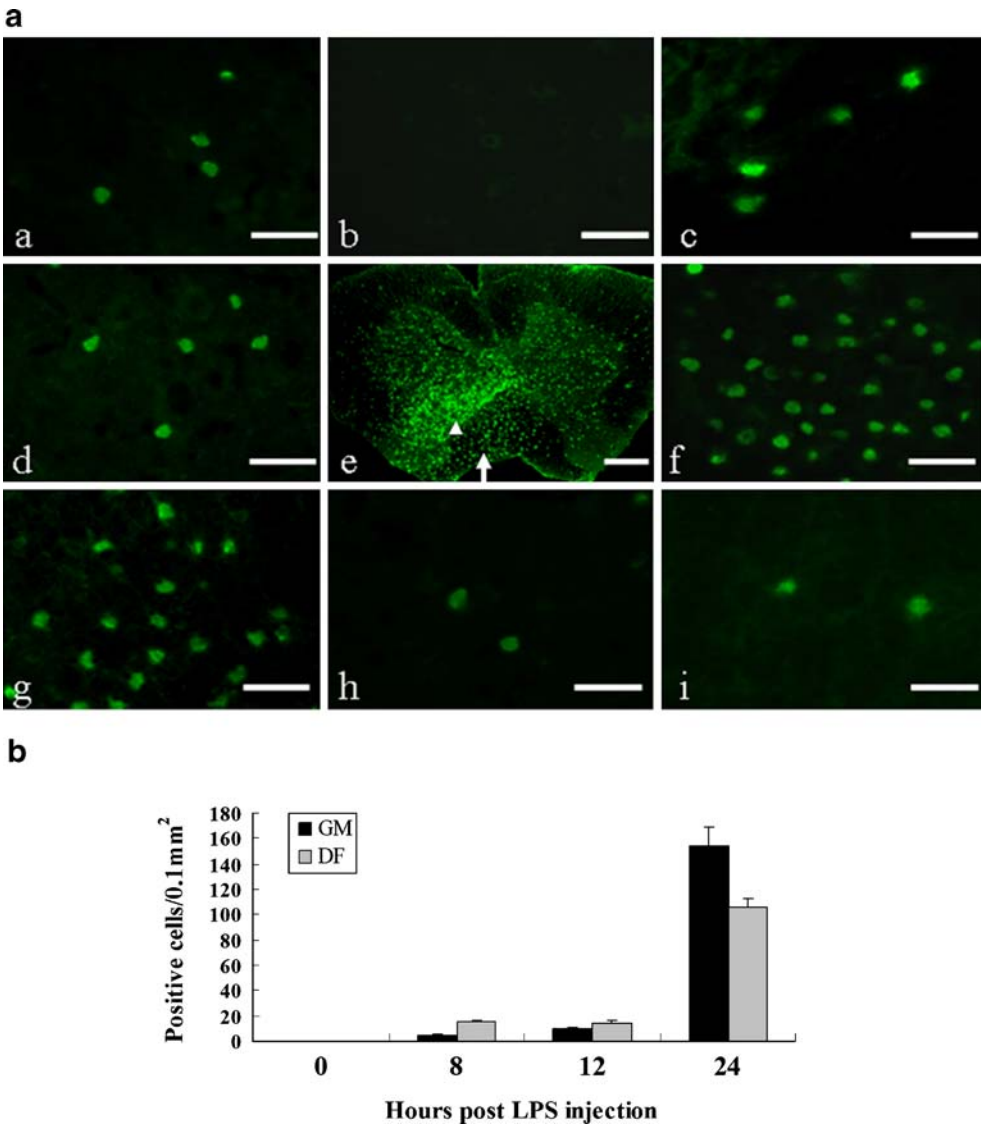


Fig. 2 β1,4-GalT-I expression in the spinal cord of rats as control and focal injection of LPS. **A** label with β1,4-GalT-I. *a*: grey matter adjacent to the deep portions of the left dorsal column after intraspinal injection of saline; *b*: grey matter (GM) of the normal spinal cord; *c*, *d*: grey matter adjacent to the injection site after administration of LPS for 8 and 12 h respectively; *e–i*: spinal cord 1 d after LPS injection; *e*: low-magnification of cross-section of the spinal cord at the level of injection; β1,4-GalT-I staining was significant, particularly in the left dorsal funiculus (*arrow-head*, indicate higher magnification in *f*) and grey matter adjacent to the left dorsal funiculus (*arrow*, indicate higher magnification in *g*); *h*: grey matter adjacent to the right dorsal column; *i*: a portion of the right dorsal funiculus. **B** the number of β1,4-GalT-I positive cells in the left grey matter and dorsal column (*$p<0.01$). *Scale bars*: (*a–d*, *f–i*)=20 μm, (*e*)=50 μm

Immunoprecipitation

Tissues were homogenized in lysis buffer (1% NP-40, 50 mmol/L Tris, pH 7.4, 0.15 mol/L NaCl, 1% sodium deoxycholate, 1% Triton-X100, 0.1% SDS, 1 mmol/L PMSF, 10 mg/ml aprotinin, and 1 μg/ml leupeptin) and clarified by centrifuging for 30 min in a microcentrifuge at 4°C. 0.2 ml of aliquots of the supernatant were incubated with 25 ml of protein G-agarose (Santa Cruz Biotechnology, Inc.), at 4°C for 3 h followed by centrifugation at 10,000 ×*g* for 10 min, 0.1 ml aliquots of supernatant were incubated with the polyclonal antibody to E-selectin (CD62E) antiserum (3 μl, Abcam, Rabbit Anti-CD62E) overnight at 4°C. Thereafter, 25 μl protein G Sepharose beads were added and the incubation was continued with gentle rotation for an additional 2 h. The beads were washed five times with PBS; finally, the protein of E-selectin samples was adjusted to the same concentration (30 μg/ml). Then, the immunoprecipitated E-selectin subunits were subjected to lectin blotting.

ELISA

The spinal cord were homogenized as described [12]. TNF-α levels were determined using ELISA kit (Biosource Europe, SA), according to the manufacturer's instructions. The detection limits of the assays were determined to be 15 pg/ml.

Statistical analysis

At least three repetitive assessments were performed, and for each assessment, all groups were tested in quadruplicate. All data were given in terms of relative values and expressed as mean±standard error. One-way ANOVA was used to compare differences between the operated groups and the control group. All statistical analyses were conducted with a STATA 7.0 software package (Stata Corp., College Station, TX, USA), and all significance levels were set at $p<0.05$.

Results

Upregulation of β1,4-GalT-I expression after intraspinal injection of LPS

The mRNA expression of β1,4-GalT-I in spinal cord was investigated by Real-time PCR analysis. Prior to this study, β_2-microtubulin (β_2M) was determined as the optimal endogenous reference gene for normalization in the experimental conditions used in the present work. Real-time PCR results showed that expression of β1,4-GalT-I mRNA was in a time-dependent manner after intraspinal injection of LPS (Fig. 1). The relative abundance of β1,4-GalT-I transcripts increased 1.5–2 fold at 6–8 h after intraspinal injection of LPS, and then decreased to normal levels at 12–48 h.

Now that β1,4-GalT-I was altered in a time-dependent manner by the local injection of LPS (Fig. 1), then we identify the distribution of β1,4-GalT-I in the spinal cord, single-labeled immunohistochemical analysis was performed. Spinal cord injection with saline (Fig. 2A.a) showed a very small number of β1,4-GalT-I expressed in the grey matter and dorsal column. Weak signals of staining were detected in the normal spinal cord (Fig. 2A.b). The weak expression of β1,4-GalT-I was detected in the left grey matter (Fig. 2A.c,d) and the left dorsal column (Data not shown) at 8 h and 12 h after intraspinal injection of LPS. By 1 d after the LPS injection into the left dorsal column there were signs of an intense response, where β1,4-GalT-I expressed widespreadly and strongly both in the left grey matter (Fig. 2A.e,f) and the dorsal column (Fig. 2A.g). However, very little β1,4-GalT-I appeared in the right grey matter (Fig. 2A.h) and dorsal column (Fig. 2A.i). Quantitative analysis showed that injection of LPS increased β1,4-GalT-I expression at 1 d significantly compared with uninjected spinal cord (Fig. 2B).

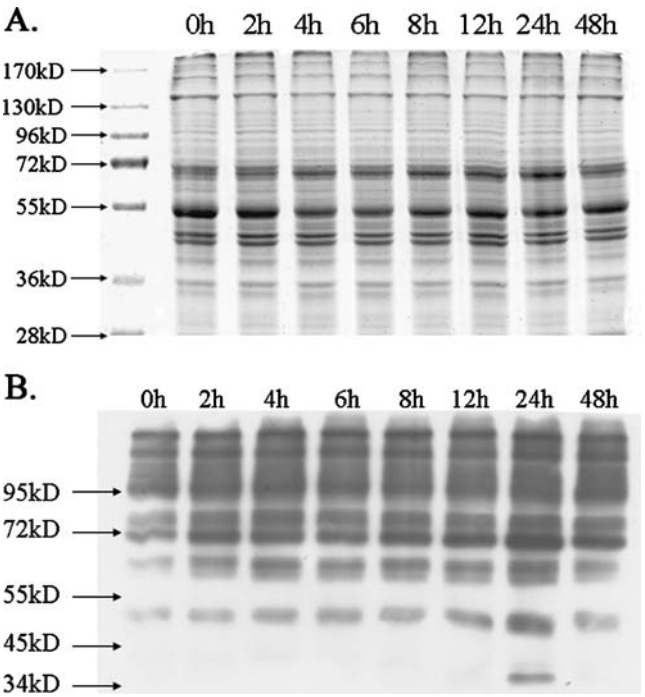


Fig. 3 RCA-I lectin assay in the spinal cord after intraspinal injection of LPS. At 2 h, 4 h, 6 h, 8 h, 12 h, 24 h, 48 h after injection, protein extraction of each brain regions was separated by SDS-PAGE and analyzed by Coomassie Blue staining (**A**) and RCA lectin (**B**). A significant increase of the binding of total glycoprotein with RCA-I was observed for 34–45 kD, 45–55 kD and 72–95 kD protein at 1 d after intraspinal injection of LPS

Changes of glycoproteins in rat lesion spinal cord

Since the gene expression and protein levels of β1, 4-GalT I were changed after intraspinal injection of LPS, as revealed in Figs. 1 and 2, we examined the total galactosylated glycoproteins using RCA lectin blotting. Spinal cord tissues from different time points postoperative were separated by electrophoresis and labeled with biotinylated RCA lectin, which interacts specifically with oligosaccharides terminating with the Galβ1→4GlcNAc group. Consistent with accrescence in the expression of GalT I, a significant increase of the binding of total glycoprotein with RCA-I was observed for 34–45 kD, 45–55 kD and 72–95 kD protein at 1 d after intraspinal injection of LPS (Fig. 3B).

Alternatively, FITC–RCA-I staining indicated that LPS injection caused cell surface galactose-containing glycans to increase sharply. Its distribution was similar with β1, 4-GalT I. Weak staining was observed in the spinal cord at 8 h and 12 h after LPS administration (Fig. 4A.c,d). Enhanced immunoreactivity was observed in the grey matter adjacent to the deep portions of the left dorsal column (Fig. 4A.e,f) and the dorsal column (Fig. 4A.g) at 1 d after LPS administration. This finding is in agreement with lectin blotting results demonstrating significant levels of galactose-containing glycans in the lesion site at 1 d. The effects of intraspinal injection of LPS at different time on galactose-containing glycans are summarized in Fig. 4B.

Cellular localization of β1,4-GalT-I and RCA

To further determine in which kind of cell type β1,4-GalT-I and galactose-containing glycans are expressed after administration of LPS, we used double immunofluorescent staining to definite more accurate location of the molecular. Since previous study showed that in response to LPS and

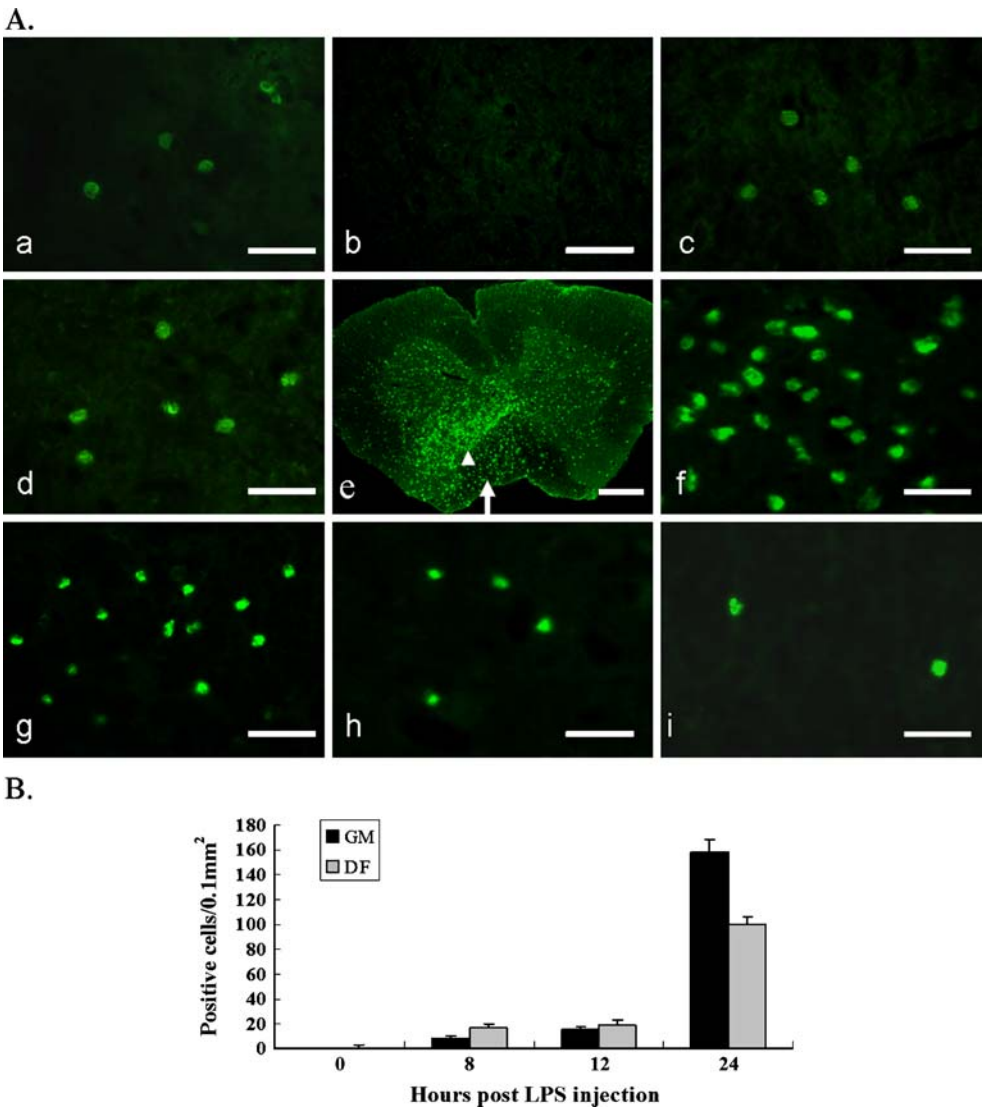


Fig. 4 Lectin-fluorescent staining with RCA-I analysis of glycoproteins from frozen sections of rat spinal cord. **A** single label with FITC-RCA-I. *a*: grey matter adjacent to the deep portions of the left dorsal column after intraspinal injection of saline; *b*: grey matter (GM) of the normal spinal cord; *c*, *d*: grey matter adjacent to the injection site after administration LPS 8 and 12 h, respectively; *e–i*: spinal cord after injection LPS 1 d; *e*: low-magnification of cross-section of the spinal cord at the level of injection; glycoproteins was present, particularly in the left dorsal funiculus (*arrowhead*, shown at higher magnification in *f*) and grey matter adjacent to the left dorsal funiculus (*arrow*, shown at higher magnification in *g*). *h*: grey matter adjacent to the right dorsal column; *i*: a portion of the right dorsal funiculus. **B**: the number of galactose-containing glycans positive cells in the left grey matter and dorsal column (*$p<0.01$). *Scale bars*: (*a–d*, *f–i*)=20 μm, (*e*)=50 μm

inflammatory cytokines, the endothelium altered vascular tone and permeability, absorbed infectious insults, and directed leukocytes such as neutrophils and macrophages into the areas of inflammation [19], so we examined the immunohistochemical colocalization of β1,4-GalT-I, galactose-containing glycans with ED1 (a marker for macrophage), MPO (a marker for neutrophil), and CNS resident cells, including OX42 (a marker for microglia), CNPase (a marker for oligodendrocytes), NeuN (a marker for neuron) and GFAP (a marker for astrocytes). Laser scanning

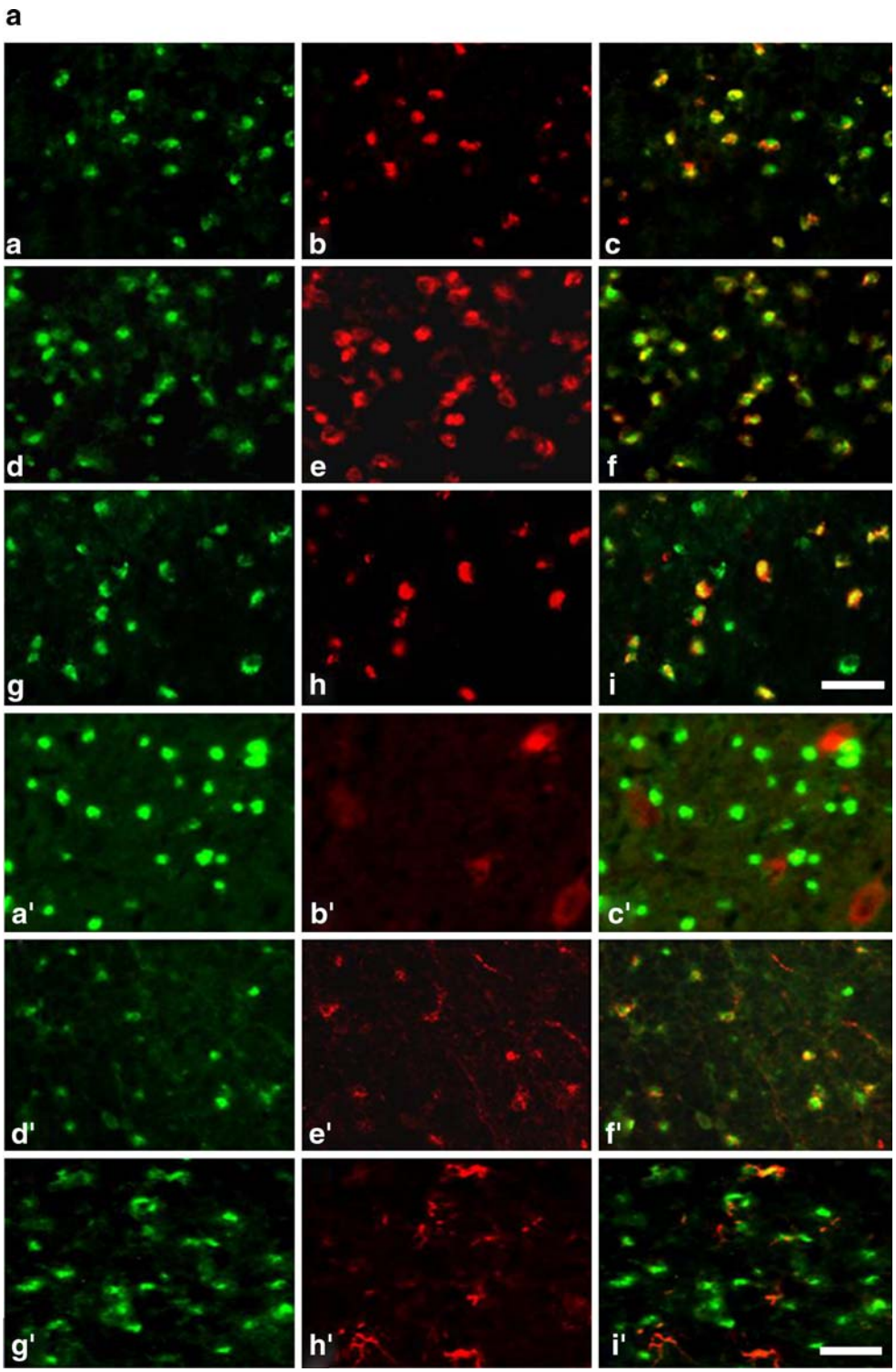
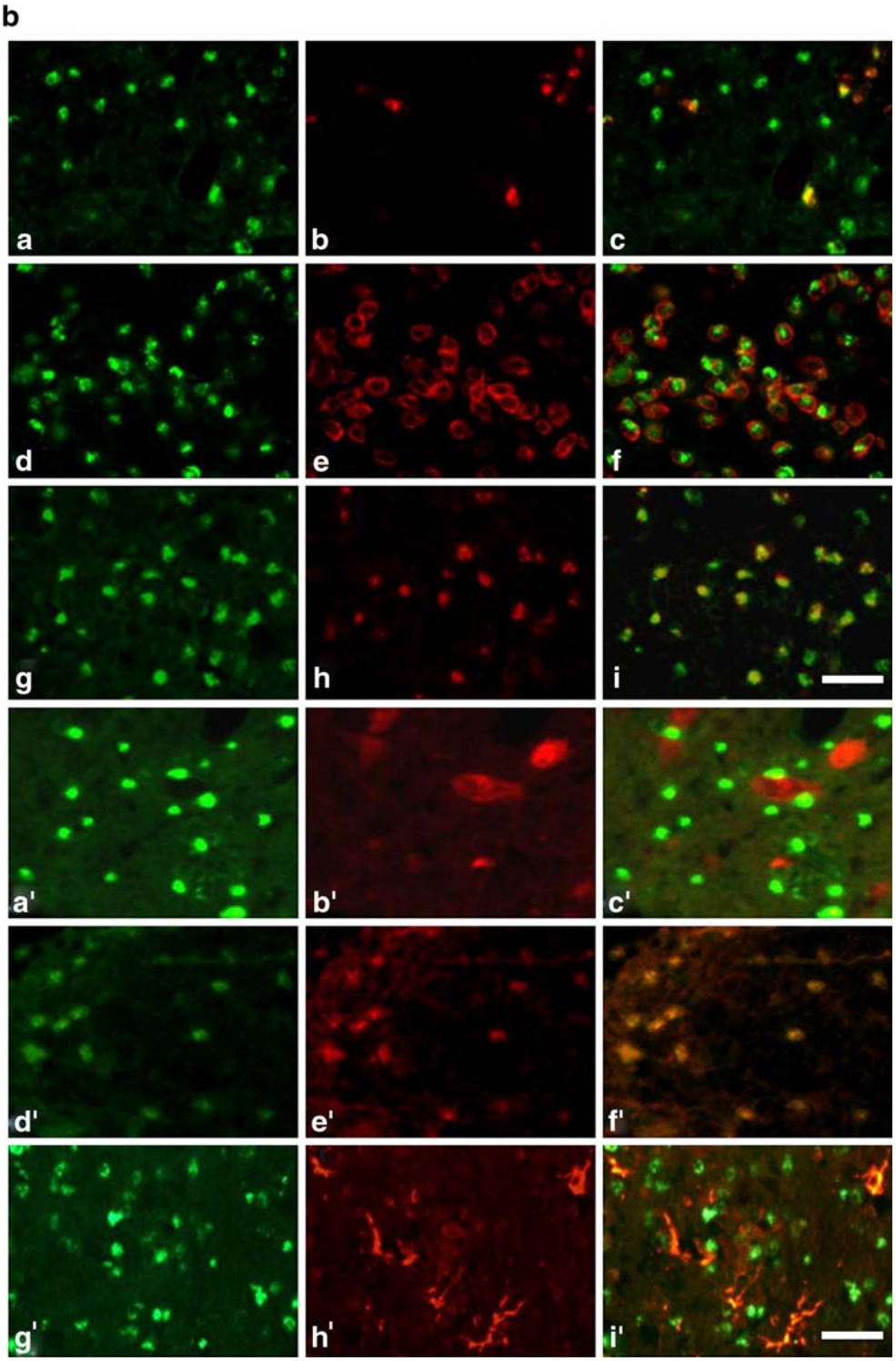


Fig. 5 The distribution of β1,4-GalT-I or FITC-RCA-I in the rats spinal cord at 1 d after focal injection of LPS. Immunofluorescent staining was performed on sections of the spinal cord after LPS treatment. FITC (*green*) signals indicate β1,4-GalT-I (**A**. *a*, *d*, *g*, *a′*, *d′*, *g′*) or RCA-I (**B**. *a*, *d*, *g*, *a′*, *d′*, *g′*). TRITC (*red*) signals indicate ED1 (**A**. *b*, **B**. *b*), OX-42 (**A**. *e*, **B**. *e*), MPO (**A**. *h*, **B**, *h*),CNPase (**A**. *e′*, **B**. *e″*), NeuN (**A**. *b′*, **B**. *b′*), GFAP (**A**. *h′*, **B**. *h′*). Merge picture (**A**. *c*, *f*, *I*, *c′*, *f′*, *i′*; **B**. *c*, *f*, *I*, *c′*, *f′*, *i′*) showed that β1,4-GalT-I and RCA-I mostly localized in macrophage, microglia cell, neutrophils, and oligodendrocytes, while scanty with neuron, astrocytes after intraspinal injection of LPS. *Scale bars*: 20 μm

Fig. 5 (continued)



microscopic observation revealed that β1,4-GalT-I and galactose-containing glycans of rat spinal cord mostly colocalized with macrophage (Fig. 5A.c,B.c), actived microglia (Fig. 5A.f,B.f), neutrophils (Fig. 5A.i,B.i) and oligodendrocytes (Fig. 5A.f′,B.f″) after intraspinal injection of LPS, accounting to 93%, and scanty with neuron (Fig. 5A.c′,B.c″) and astrocytes (Fig. 5A.i′,B.i′).

The association of E-selectin and galactose-containing glycans in spinal cord

Because inflammation could induce significant increase of E-selectin expression in CNS, we sought to determine whether the expression of this molecule correlates with the adhesion of microglia to the lesion spinal cord.

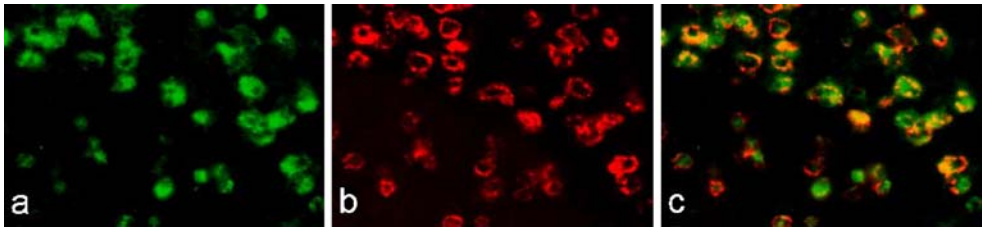


Fig. 6 The expression of E-selectin in the spinal cord 1 d after intraspinal injection of LPS. Immunohistochemistry was performed on sections of 1 d lesioned spinal cord using antibody E-selectin (**a**, *green*) and antibody OX-42 (**b**, *red*) specific for microglia. **c** picture indicated that E-selectin localized in microglia. *Scale bars*: 20 μm

Representative photograph of double labelling of E-selectin and microglia were shown in Fig. 6, indicating that E-selectin was expressed in microglia. This section was taken from an animal that received intraspinal injection of LPS 1 d.

Previous results have demonstrated that β1,4-GalT-I is a major galactosyltransferase responsible for selectin-ligand biosynthesis and that inflammatory responses of β1,4-GalT-I deficient mice are impaired because of the defect in selectin-ligand biosynthesis [2]. In this study, we found that E-selectin localized in inflammatory cells. To study whether β1,4-GalT-I is associated with E-selectin during the pathological process of intraspinal injection of LPS, we performed double labeling immunochemistry and immunoprecipitation to assess the specificity of interactions between E-selectin and galactose-containing glycans in the spinal cord at 1 d after intraspinal injection of LPS. The colocalization of the two molecules was observed in the grey matter adjacent to the deep portions of the left dorsal column (Fig. 7A) and the dorsal column (data not show). To further address the interaction E-selectin and galactose-containing glycans, we did immunoprecipitation in spinal cord on post-injection 1 d (Fig. 7B). The result showed that E-selectin precipitated with galactose-containing glycans, whereas no galactose-containing glycans were detected in the control rabbit IgG immunoprecipitation. These data indicated that these two proteins interacted with each other in the spinal cord.

LPS Induces β1,4-GalT-I mRNA expression in HAPI in a time- and dose-dependent manner

Now that β1,4-GalT-I expressed predominantly in inflammatory cells, especially in microglia, during local inflam-

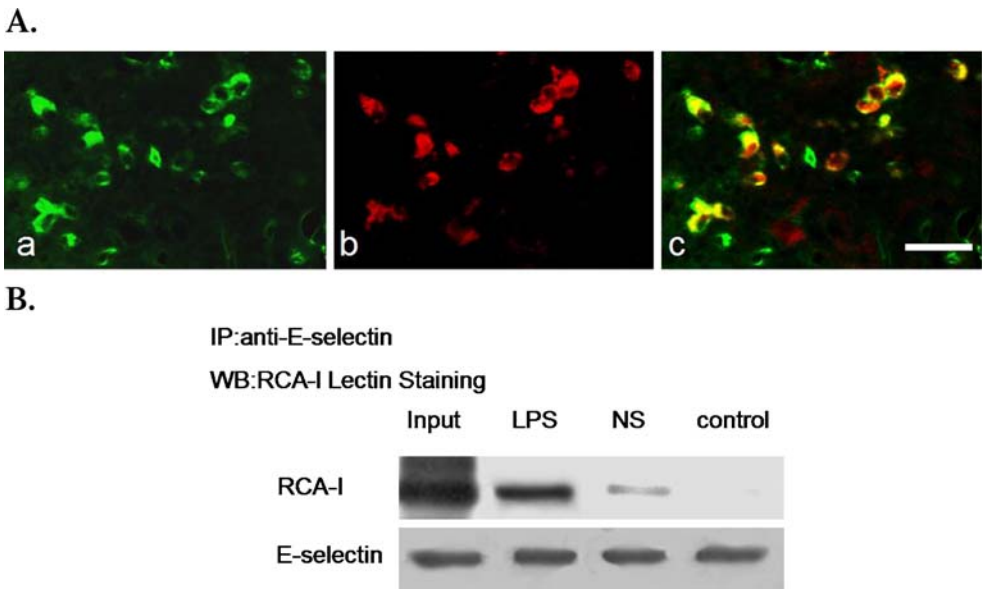


Fig. 7 Association of galactose-containing glycans with E-selectin in spinal cord. **A** Immunofluorescence analysis of galactose-containing glycans and E-selectin in impaired spinal cord. FITC (**a**); TRITC (*red*) signals indicate E-selectin (**b**); Merge (**c**). Colocalization of RCA-I and E-selectin could be found in rat spinal cord. *Scale bars*: 20 μm. **B** Immunoprecipitations (IP) were done in the damaged spinal cord. Lysates were immunoprecipitated using a rabbit anti-E-selectin polyclonal antibody. The immunoprecipitates were subjected to lectin blotting. "*Input*" indicates samples before IP procedure; "*NS*" indicates spinal cord focal injection with saline; "*control*" indicates IP procedure with control rabbit IgG. *Scale bars*: 20 μm

matory responses in the CNS, we investigated its expression in microglia *in vitro*. The demonstration of an increase in β1,4-GalT-I expression in microglia after LPS stimulation was confirmed *in vitro* using cultured microglia cell line HAPI. When stimulated with 0.1 ng/ml–10 μg/ml LPS, microglia transcribed mRNA for β1,4-GalT-I was determined by RT-PCR. The expression level of β1,4-GalT-I mRNA in cultured microglia was increased obviously compared with the control group. It increased from 0.1 ng/ml and kept on the high level (Fig. 8A). In addition, the expression of β1,4-GalT-I mRNA in microglia was time-dependent. It increased from 0.5 h, peaked at 5 h to 6 h, then decreased gradually after exposure to 100 ng/ml LPS (Fig. 8B).

Increase in protein expression for TNF-α after LPS injection

To assess whether β1,4-GalT-I is related to LPS-induced cytokine production, we examine the expression of TNF-α in after LPS injection, ELISA assay was used. TNF-α protein content of the spinal cord increased with time after intraspinal injection of LPS. In control rats, TNF-α level was low (132±9 pg/ml). Post LPS administration, the expression of TNF-α elevated. The most robust increase was found at 8 h, reached 1,780 pg/ml ($p<0.05$), and then gradually recovered to the baseline level at 48 h.

TNF-α level was increased after intraspinal injection of LPS, suggesting that TNF-α may play a part in this context of events. To study whether the elevation of β1,4-GalT-I is associated with TNF-α, we performed immunofluorescence experiments in animals killed at 1 d after surgery. Fluorescence microscope observation showed that coexpression of β1,4-GalT-I and TNF-α was observed in the left grey matter (Fig. 9) and the dorsal column (data not show).

TNF-α affects β1,4-GalT-I production in HAPI microglia cell line

To establish the kinetics of the TNF-α mediated expression level of β1,4-GalT-I mRNA *in vitro*, confluent monolayers of microglia were incubated in the presence of increasing concentrations of TNF-α and for different times. The expression level of β1,4-GalT-I mRNA in cultured microglia was dose-dependant compared with the control group. It increased from 0.1 ng/ml, reached at a peak at 5 ng/ml and then restored gradually to normal level at 7.5–10 ng/ml (Fig. 10A). In addition, the expression of β1,4-GalT-I mRNA in cultured microglia was time-dependent. It increased from 0.5 h, peaked at 4 h, then decreased to normal level after exposure to 5 ng/ml LPS (Fig. 10B).

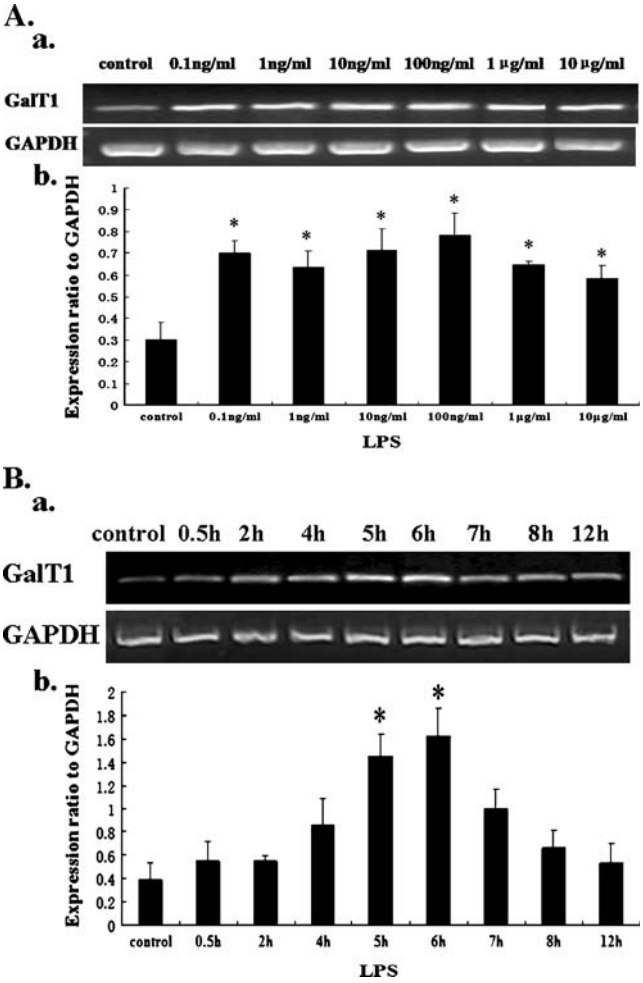


Fig. 8 Effects of LPS treatment on the levels of β1,4-GalT-I and GAPDH mRNAs in microglia. Serum-starved microglia were exposed to LPS (0.1 ng/ml–10 μg/ml) for the time period indicated. β1,4-GalT-I were detected by RT-PCR. **A** Concentration dependence of LPS-induced β1,4-GalT-I expression in rat microglia. **B** Time course of LPS-induced β1,4-GalT-I expression in rat microglia. **A**. *a*, **B**. *a*: *Upper blot* shows β1,4-GalT-I induced by LPS; *lower blot* demonstrates equal loading by detecting GAPDH. **B** bands were quantified by densitometer. The amount of β1,4-GalT-I was normalized by referring to the amount of GAPDH (**A**. *b*, **B**. *b*). *$p<0.05$, **$p<0.01$

Discussion

Inflammation, a common denominator among the diverse list of neurodegenerative disease, has recently been implicated as a critical mechanism responsible for the progressive nature of neurodegeneration. During inflammation, leukocytes sense the presence of infecting microbes, attach to endothelial cells lining the capillaries in the affected regions, penetrate between the endothelial cells out into the surrounding tissues and destroy the invading microbes. In this study, we found that β1,4-GalT-I played an important role in neuroinflammatory processes.

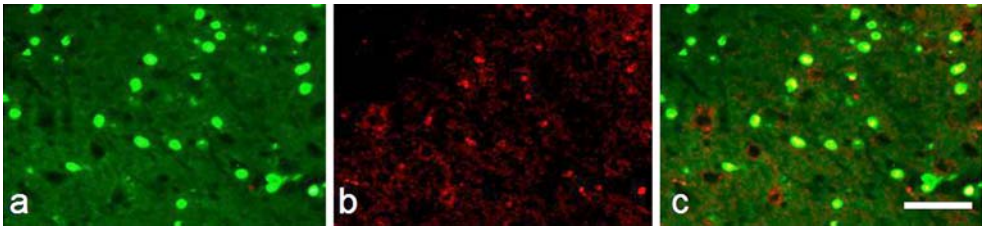


Fig. 9 Double immunofluorescent staining with β1,4-GalT-I and TNF-α. FITC (*green*) signal indicate β1,4-GalT-I (**a**); TRITC (*red*) signal indicate TNF-α (**b**); **c** picture indicated that β1,4-GalT-I co-localized with TNF-α. *Scale bars*: 20 μm

In the present study, we demonstrate that β1,4-GalT-I is induced by intraspinal administration of LPS. It is interesting that expression of β1,4-GalT-I mRNA reached peak at 6-8 h after focal injection of LPS, yet β1,4-GalT-I protein was not upregulated in the spinal cord at this time point, suggesting that this increase of β1,4-GalT-I mRNA expression does not translate to their protein expression. Furthermore, the Gal β1,4GlcNAc group also increased significantly at 1 d after lesion, which is consistent with the expression of β1,4-GalT-I protein. It is plausible that up-regulation of β1,4-GalT-I described in the present study can be expected to contribute to the change in glycan synthesis.

Based on the results of double immunofluorescent staining, β1,4-GalT-I and galactose-containing glycans are overlapped with leukocytes such as actived microglia, macrophages, neutrophils, and scanty with astrocytes and neuron. Leukocyte emigration is known to be mediated by E- and P-selectin on endothelial cells and their oligosaccharide ligands such as sialyl Lewis^x (sLe^x) on leukocytes [22, 25]. Selectin ligands such as sLe^x and sulfated sLe^x are mainly expressed at the terminus of *N*-acetyllactosamine repeats on serine/threonine (O)-Linked oligosaccharides (O-glycans) [17, 37]. β1,4-GalT-I catalyzes the addition of UDP-galactose to terminal *N*-acetylgalactosamine and acts in concert with β1,3-*N*-acetylglucosaminyltransferase to synthesize polylactosamine extensions of core 2 decorated O-glycans. In addition, β1,4-GalT-I also participates in the generation of sLe^x. Using β1,4-GalT-I deficient mice, Asano and colleagues investigated the contribution of β1,4-GalT-I on selectin ligand activity. They found that binding of soluble selectin to neutrophils and monocytes of β1,4-GalT-I deficient mice was significantly impaired [2], suggesting a role of β1,4-GalT-I in selectin mediate rolling *in vivo*. Ryoichi Mori and his colleagues also found that skin wound healing was significantly delayed in the β1,4-GalT-I$^{-/-}$ mice, neutrophil and macrophage recruitment at the wound site in the inflammatory phase was reduced, probably because of the reduced expression of selectin ligands in the β1,4-GalT-I$^{-/-}$ mice compared with β1,4-GalT-I$^{+/-}$ mice [26]. These results indicated that β1,4-GalT-I may participate in immune cells recruiting into the areas of inflammation. Interestingly, we also observed β1,4-GalT-I and galactose-containing glycans are co-localized with oligodendrocytes. Felts and colleagues has reported oligodendrocyte numbers appear to be reduced in the later demyelination stage after intraspinal injection of LPS [9]. Zejuan Li and her colleagues reported that β1,4-GalT-I involved in CDK11p58-mediated apoptotic signaling in hepatocarcinoma cells [20]. Therefore, we will further

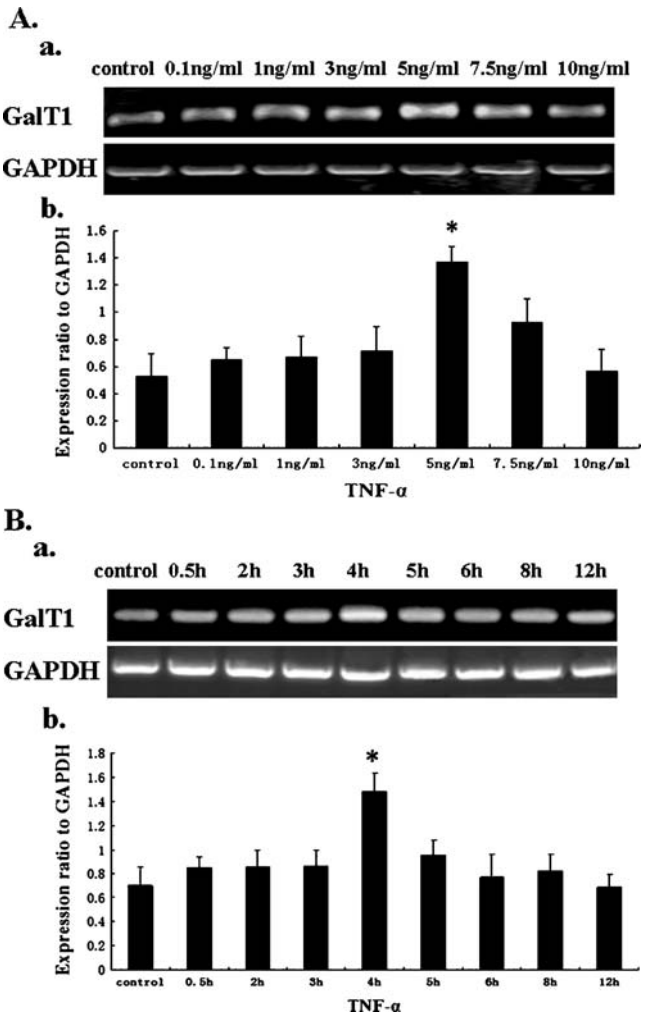


Fig. 10 Effects of TNF-α treatment on the levels of β1,4-GalT-I and GAPDH mRNAs in microglia. Serum-starved microglia were exposed to TNF-α (0.1 ng/ml–10 ng/ml) for the time period indicated. β1,4-GalT-I were detected by RT-PCR. **A** Concentration dependence of TNF-α-induced β1,4-GalT-I expression in rat microglia. **B** Time course of TNF-α-induced β1,4-GalT-I expression in rat microglia. **A**. *a*, **B**. *a*: *Upper blot* shows β1,4-GalT-I induced by TNF-α; *lower blot* demonstrates equal loading by detecting GAPDH. **B** bands were quantified by densitometer. The amount of β1,4-GalT-I was normalized by referring to the amount of GAPDH (**A**. *b*, **B**. *b*). *$p<0.05$, **$p<0.01$

study whether the decrease of oligodendrocytes related with β1,4-GalT-I by the injection.

We also observed in this study that E-selectin was colocalized with microglia and macrophages. Typically, leukocytes are arrested to the wall of the vasculature prior to their transmigration into the tissue proper. In general, this process is known to be mediated sequentially by selectins, chemokines, and integrins [1]. Of the three members of the selectin family, P- and E-selectins are known to be critical for the binding of leukocytes to the vascular wall [7]. Paul A. Felts has shown that intraspinal injection of LPS could induce leukocytes infiltration [9]. Not surprisingly, the results of this study show that E-selectin expression is also associated with intraspinal injection of LPS induced leukocyte infiltration. β1,4-GalT-I has been shown to exert various functions other than a catalytic enzyme [6, 8, 15, 18, 24, 28]. We then asked whether β1,4-GalT-I acts catalytically or in a lectin-like fashion. Double labeling immunohistochemistry and immunoprecipitation of E-selectin and galactose-containing glycans provided the interaction between them in the lesion spinal cord. Asano and colleagues has reported β1,4-GalT-I has a considerable role in P-selectin mediated rolling *in vivo* [2]. Here, the specific binding between E-selectin and galactose-containing glycans suggests galactosyltransferase plays a pivotal role in E-selectin mediated leukocyte influx into the damaged spinal cord.

Microglia are bone marrow-derived monocytes that are found in CNS early in development. Under normal physiological conditions, microglia are involved in immune surveillance and host defense against infectious agents. In response to inflammatory stimuli, microglia cells which like all myeloid cells assume an actived phenotype associated with adhesion, proliferation, migration to the site of injure, phagocytosis of cellar debris, and elaboration of both neurotoxic and neurotrophic factors [13]. In this report, we provide that LPS administration affects β1,4-GalT-I expression in microglia in a time- and dose-dependent manner *in vitro*. All these results suggest that β1,4-GalT-I play an important role in inflammation wherever they are located in microglia *in vitro* or *in vivo*.

TNF-α, a major pro-inflammatory cytokine, is produced during the inflammatory response by a variety of immune cells and nonimmune cells (fibroblast, smooth muscle cells, epithelial cells, glial cells, neuron) [36]. In early stages of inflammation TNF-α promotes blood brain barrier permeability, resulting in damaging edema [32]. The induction of adhesion molecules on endothelia promotes the migration of leukocytes into a lesioned area [35]. In present study we found TNF-α protein level is induced after LPS injection, and earlier than β1,4-GalT-I expression, as assessed by ELISA. We also found TNF-α located with β1,4-GalT-I. *in vitro*, RT-PCR analysis showed that the expression level of β1,4-GalT-I mRNA in microglia was dose- and time-dependent after exposure to TNF-α compared with the normal untreated microglia. Of note, treatment with 7.5–10 ng/ml TNF-α appeared to induce less β1,4-GalT-I biosynthesis than treatment with 5 ng/ml TNF-α, this may be a loss of cell viability or numbers at the highest LPS concentration. Juan Garcia-Vallejo *et al.* reported that β1,4-GalT-I in primary human endothelial cell (HUVECs) was up-regulated in a time- and concentration-dependent manner in response to TNF-α stimulation, due to an increase in the stability of the mRNA transcript [11]. It indicates that expression of β1,4-GalT-I mRNA is affected by the concentration and time of proinflammatory factors. Findings from this study and other reports have revealed that the up-regulation of β1,4-GalT-I is attribute to a direct effect of TNF-α signaling by the local injection of LPS.

This study has established the role of β1,4-GalT-I in LPS-induced neuroinflammatory processes. It is known neuroinflammatory processes participate in the propagation of the neurodegenerative process in many disease including multiple sclerosis, Alzheimer's disease, Parkinson's disease. Drugs targeting specific expression of β1,4-GalT-I may prove effective in slowing or even halting the progressive neurodegeneration.

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References

1. Alon, R., Feigelson, S.: From rolling to arrest on blood vessels: leukocyte tap dancing on endothelial integrin ligands and chemokines at sub-second contacts. Semin. Immunol. **14**, 93–104 (2002), doi:10.1006/smim.2001.0346
2. Asano, M1., Nakae, S., Kotani, N., Shirafuji, N., Nambu, A., Hashimoto, N., *et al.*: Impaired selectin-ligand biosynthesis and reduced inflammatory responses in beta-1,4-galactosyltransferase-I-deficient mice. Blood **102**, 1678–1685 (2003), doi:10.1182/blood-2003-03-0836
3. Bell, M.D., Perry, V.H.: Adhesion molecule expression on murine cerebral endothelium following the injection of a proinflammagen or during acute neuronal degeneration. J. Neurocytol. **24**, 695–710 (1995), doi:10.1007/BF01179819
4. Cybulsky, M.I., McComb, D.J., Movat, H.Z.: Neutrophil leukocyte emigration induced by endotoxin. Mediator roles of interleukin 1 and tumor necrosis factor alpha 1. J. Immunol. **140**, 3144–3149 (1988)
5. Dickson, D.W., Lee, S.C., Mattiace, L.A., Yen, S.H., Brosnan, C.: Microglia and cytokines in neurological disease, with special reference to AIDS and Alzheimer's disease. Glia. **7**, 75–83 (1993), doi:10.1002/glia.440070113

6. Eckstein, D.J., Shur, B.D.: Cell surface beta-1,4-galactosyltransferase is associated with the detergent-insoluble cytoskeleton on migrating mesenchymal cells. J. Exp. Cell Res. **201**, 83–90 (1992), doi:10.1016/0014-4827(92)90350-H
7. Engelhardt, B., Vestweber, D., Hallmann, R., Schulz, M.: E- and P-selectin are not involved in the recruitment of inflammatory cells across the blood–brain barrier in experimental autoimmune encephalomyelitis. Blood **90**, 4459–4472 (1997)
8. Evans, S.C., Lopez, L.C., Shur, B.D.: Dominant negative mutation in cell surface beta-1,4-Galactosyltransferase inhibits cell–cell and cell–matrix interactions. J. Biol. Chem. **120**, 1045–1057 (1993)
9. Felts, P.A., Woolston, A.M., Fernando, H.B., Asquith, S., Gregson, N.A., Mizzi, O.J., *et al.*: Inflammation and primary demyelination induced by the intraspinal injection of lipopolysaccharide. Brain **128**, 1649–1666 (2005), doi:10.1093/brain/awh516
10. Furukawa, K., Sato, T.: Beta-1,4-Galactosylation of N-glycans is a complex process. J. Biochim. Biophys. Acta **1473**, 54–66 (1999)
11. Garcia-Vallejo, J.J., van Dijk, W., van Die, I., Gringhuis, S.I.: Tumor necrosis factor-alpha up-regulates the expression of beta 1,4-galactosyltransferase I in primary human endothelial cells by mRNA stabilization. J. Biol. Chem. **280**(13), 12676–12682 (2005), doi:10.1074/jbc.M410093200
12. George, A., Schmidt, C., Weishaupt, A., Toyka, K.V., Sommer, C.: Serial determination of tumor necrosis factor-alpha content in rat sciatic nerve after chronic constriction injury. Exp. Neurol. **160**, 124–132 (1999), doi:10.1006/exnr.1999.7193
13. Garden, G.A., Möller, T.: Microglia biology in health and disease. J. Neuroimmune Pharmacol. **1**, 127–137 (2006)
14. Hamann, A., Engelhardt, B.: Leukocyte trafficking. Wiley, Weinheim (2005)
15. Hathaway, H.J., Shur, B.D.: Cell surface beta-l,4-galactosyltransferase functions during neural crest cell migration and neurulation *in vivo*. J. Cell Biol. **117**, 369–382 (1992), doi:10.1083/jcb.117.2.369
16. Hennet, T.: The galactosyltransferase family. Cell. Mol. Life Sci. **59**, 1081–1095 (2002), doi:10.1007/s00018-002-8489-4
17. Hiraoka, N., Petryniak, B., Nakayama, J., Tsuboi, S., Suzuki, M., Yeh, J.C., *et al.*: A novel, high endothelial venule-specific sulfotransferase expresses 6-sulfo sialyl Lewis(x), an L-selectin ligand displayed by CD34. Immunity **11**, 79–89 (1999), doi:10.1016/S1074-7613(00)80083-7
18. Huang, Q.L., Shur, B.D., Begovac, P.C.: Overexpression cell surface β1,4-galactosyltransferase-I in PC12 cells increases neurite outgrowth on laminin. J. Cell Sci. **108**, 839–847 (1995)
19. Issekutz, T.B., Chin, G.W., Hay, J.B.: Lymphocyte traffic through chronic inflammation lesions: differential migration versus differential retention. Clin. Exp. Immunol. **45**, 604–614 (1981)
20. Li, Z., Wang, H., Zong, H., Sun, Q., Kong, X., Jiang, J., *et al.*: Downregulation of β1,4-galactosyltransferase 1 inhibits CDK11p58-mediated apoptosis induced by cycloheximide. Biochem. Biophys. Res. Commun. **327**, 628–636 (2005), doi:10.1016/j.bbrc.2004.12.047
21. Liu, B., Hong, J.S.: Role of microglia in inflammation-mediated neurodegenerative disease: mechanisms and strategies for therapeutic intervention. J. Pharmacol. Exp. Ther. **304**, 1–7 (2003), doi:10.1124/jpet.102.035048
22. Lowe, J.B.: Selectin ligands, leukocyte trafficking, and fucosyltransferase genes. Kidney Int. **51**, 1418–1426 (1997), doi:10.1038/ki.1997.194
23. Lowe, J.B., Varki, A.: Glycosyltransferases. In: Varki, A., Cummings, R.D., Esko, J., Freeze, H.H., Hart, G., Marth, J.D. (eds.) Essentials of glycobiology, pp. 253–266. Cold Spring Harbor Laboratory Press, Cold Spring Harbor (1999)
24. Maillet, C.M., Shur, B.D.: Perturbing cell surface β1,4-galactosyltransferase on F9 embryonal carcinoma cells arrests cell growth and induces laminin synthesis. J. Cell Sci. **107**, 1713–1724 (1994)
25. McEver, R.P., Moore, K.L., Cummings, R.D.: Leukocyte trafficking mediated by selectin–carbohydrate interactions. J. Biol. Chem. **270**, 11025–11028 (1995), doi:10.1074/jbc.270.19.11025
26. Mori, R., Kondo, T., Nishima, T., Asano, M.: Impairment of skin wound healing in β1,4-galactosyltransferase-deficient mice with reduced leukocyte recruitment. Am. J. Pathol. **164**, 1303–1314 (2004)
27. McGeer, P.L., Itagaki, S., Boyes, B.E., McGeer, E.G.: Reactive microglia are positive for HLA-DR in the substantia nigra of Parkinson's disease brains. Neurology **38**, 1285–1291 (1988)
28. Miller, D.J., Macek, M.B., Shur, B.D.: Complementarity between sperm surface beta-l,4-galactosyltransferase and egg-coat ZP3 mediates sperm–egg binding. J. Nat. **357**, 589–593 (1992), doi:10.1038/357589a0
29. Nishie, T., Miyaishi, O., Azuma, H., Kameyama, A., Naruse, C., Hashimoto, N.: Development of immunoglobulin a nephropathy-like disease in β1,4-galactosyltransferase-I-deficient mice. Am. J. Pathol. **170**, 447–456 (2007), doi:10.2353/ajpath.2007.060559
30. Qian, J., Cheng, C., Liu, H., Chen, J., Yan, M., Niu, S.: Expression of β1,4-Galactosyltransferase-I in rat during inflammation. Inflammation **30**, 59–68 (2007), doi:10.1007/s10753-007-9022-6
31. Sherman, M.P., Griscavage, J.M., Ignarro, L.J.: Nitric oxide-mediated neuronal injury in multiple sclerosis. Med. Hypotheses **39**, 143–146 (1992), doi:10.1016/0306-9877(92)90175-C
32. Shohami, E., Bass, R., Wallach, D., Yamin, A., Gallily, R.: Inhibition of tumor necrosis factor-a (TNF-a) activity in rat brain is associated with cerebroprotection after closed head injury. J. Cereb. Blood Flow Metab. **16**, 378–384 (1996), doi:10.1097/00004647-199605000-00004
33. Stern, E.L., Quan, N., Proescholdt, M.G., Herkenham, M.: Spatiotemporal induction patterns of cytokine and related immune signal molecular mRNAs in response to intrastriatal injection of lipopolysaccharide. J. Neuroimmunol. **106**, 114–129 (2000), doi:10.1016/S0165-5728(00)00194-6
34. Ulevitch, R.J., Tobias, P.S.: Receptor-dependent mechanisms of cell stimulation by bacterial endotoxin. Annu. Rev. Immunol. **13**, 437–457 (1995), doi:10.1146/annurev.iy.13.040195.002253
35. Vastag, M., Skopal, J., Voko, Z., Csonka, E., Nagy, Z.: Expression of membrane-bound and soluble cell adhesion molecules by human brain microvessel endothelial cells. Microvasc. Res. **57**, 52–60 (1999), doi:10.1006/mvre.1998.2115
36. Wang, H., Czura, C.J., Tracey, K.J.: Tumor necrosis factor. In: Thomson, A.W., Lotze, M.T. (eds.) From the cytokine handbook, vol 2, 4th edn. pp. 837–860. Academic, Amsterdam (2003)
37. Wilkins, P.P., McEver, R.P., Cummings, R.D.: Structures of the O-glycans on P-selectin glycoprotein ligand-1 from HL-60 cells. J. Biol. Chem. **271**, 18732–18742 (1996), doi:10.1074/jbc.271.6.3255