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# Lactosylceramide Mediates the Expression of Adhesion Molecules in TNF-$\alpha$ and IFN$\gamma$-stimulated Primary Cultured Astrocytes

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**Here we have investigated how lactosylceramide (LacCer) modulates gene expression of adhesion molecules in TNF-$\alpha$ and IFN$\gamma$ (CM)-stimulated astrocytes. We have observed that stimulation of astrocytes with CM increased the gene expression of ICAM-1 and VCAM-1. D-Threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) and N-butyldeoxynojirimycin (NBDNJ), inhibitors of glucosylceramide synthase (GLS) and LacCer synthase (galactosyltransferase, GalT-2), inhibited the gene expression of ICAM-1 and VCAM-1 and activation of their gene promoter induced by CM, which were reversed by exogenously supplied LacCer. Silencing of GalT-2 gene using its antisense oligonucleotides also attenuated CM-induced ICAM-1 and VCAM-1 expression, which were reversed by LacCer. PDMP treatment and silencing of GalT-2 gene significantly reduced CM-induced luciferase activities in NF-$\kappa$B, AP-1, GAS, and STAT-3 luciferase vectors-transfected cells. In addition, LacCer reversed the inhibition of NF-$\kappa$B and STAT-1 luciferase activities by PDMP. Taken together, our results suggest that LacCer may play a crucial role in the expression of ICAM-1 and VCAM-1 via modulating transcription factors, such as NF-$\kappa$B, AP-1, STAT-1, and STAT-3 in CM-stimulated astrocytes.**

**Key Words: ICAM-1, VCAM-1, D-Threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol, Lactosylceramide, Astrocytes**

## INTRODUCTION

The cell adhesion molecules, intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1 and E-selectin, are important mediators of immune interactions within the central nervous system (CNS). A wide variety of pro-inflammatory insults to the brain results in upregulation of these molecules in brain endothelial cells, astrocytes, and microglia [1]. Astrocytes are critical players in the innate immune response of the CNS. Cytokines, such as TNF-$\alpha$, IL-1$\beta$ and IFN$\gamma$, may be released in the CNS by either infiltrating leukocytes or resident cells, such as astrocytes and microglial cells. These cytokines cause the expression of ICAM-1 and VCAM-1, all of which are mechanisms involved in leukocytes recruitment. Thus, factors affecting the expression of endothelial adhesion molecules are important in regulating vascular inflammatory processes in the CNS [1,2].

Glycosphingolipid biosynthesis is initiated by transfer of UDP-glucose onto ceramide by the action of glucosylceramide synthase (GLS) to form glucosylceramide (GluCer). Lactosylceramide (LacCer) is generated from GluCer by the action of LacCer synthase (galactosyltransferase, GalT-2). LacCer is a precursor for complex GSLs, including gangliosides [3]. Previous studies have shown the involvement of sphingolipids such as ceramide and psychosine in the cytokine-mediated inflammatory disease of CNS, such as Krabbe disease and spinal cord injury [4-7].

Earlier studies reported the involvement of ceramide in the regulation of cytokine-mediated ICAM-1 and VCAM-1 expression in endothelical cells [8-10]. The induction of ICAM-1 depends on the transcription factors (TFs), such as AP-1 and NF-$\kappa$B, which are activated by proinflamatory cytokines [11-13]. However, the involvement of LacCer and TFs, such as AP-1, NF-$\kappa$B and STATs on ICAM-1 and VCAM-1 expression in astrocytes is still unknown.

In the present study, the involvement of LacCer in the regulation of TNF-$\alpha$ and IFN$\gamma$-mediated ICAM-1 and VCAM-1 gene expression was investigated in astrocytes. Our results imply that LacCer may play an important role an inflammatory mediator through adhesion molecules expression by activating TFs, such as NF-$\kappa$B, AP-1, STAT-1 and STAT-3 in the CNS.

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**ABBREVIATIONS:** ICAM-1, intercellular cell adhesion molecule-1; VCAM-1, Vascular cell adhesion molecule-1; PDMP, D-Threo-1-henyl-2-decanoylamino-3-morpholino-1-propanol; NBDNJ, N-butyldeoxynojirimycin.

## METHODS

### *Cell culture and reagents*

These experiments were approved by the University of Hallym Animal Care and Use Committee (Hallym 2009-05-01). Primary astrocyte-enriched cultures were prepared from the whole cortex of 1-d-old Sprague Dawley rats as described earlier [6]. Briefly, the cortex was dissected rapidly in ice-cold calcium/magnesium-free HBSS (Invitrogen, Seoul, Korea), pH 7.4, as described previously. Then the tissue was minced, incubated in HBSS containing trypsin (2 mg/ml) for 20 min, and washed twice in plating medium containing 10% FBS and 10 $\mu$g/ml gentamicin; next it was disrupted by triturating through a Pasteur pipette, after which the cells were seeded in 75 $cm^2$ culture flasks (Falcon, Franklin, NJ, USA). Following incubation at 37°C in 5% $CO_2$ for 1 d, the medium was changed completely to the culture medium (DMEM containing 5% FBS and 10 $\mu$g/ml gentamicin). The cultures received half-exchanges with fresh medium twice a week. After 14~15 d the cells were shaken for at least 24 hr on an orbital shaker to remove the microglia and then seeded on multi-well tissue culture dishes. The cells were incubated with serum-free DMEM for 24 hr before the incubation with drugs. Recombinant IFN $\gamma$ and TNF-$\alpha$ (CM: cytokine mixture) were obtained from R&D systems (Minneapolis, MN, USA). D-Threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) and Lactosylceramide (LacCer) obtained from Matreya (Pleasant Gap, PA, USA). N-butyldeoxynojirimycin (NBDNJ) was obtained from Calbiochem (La Jolla, CA, USA).

### *Transient transfection and luciferase assay*

ICAM-1 and VCAM-1 promoter luciferase vectors were a kind gift from S. Giri (MUSC, SC) and NF-$\kappa$B, AP-1, GAS, and STAT-3 luciferase reporter vectors were purchased from panomics (Fremont, CA, USA). Cells ($5\times10^5$) were cultured in 12-well plates for 1 d before the transfection. Transfection was performed with plasmid concentration (2 $\mu$g) and 8 $\mu$l of FuGENE®6 transfection reagent (Roche, Seoul, Korea). One day after transfection, the cells were placed in serum-free media overnight. Transfection efficiency was about 30%. After PDMP and CM treatment, the cells were washed with PBS, scraped, and resuspended with 100 $\mu$l of lysis buffer contain in luciferase assay kit (Promega, Madison, WI, USA). After incubation at room temperature for 15 min with occasional vortexing, the samples were centrifuged and isolated supernatants. The luciferase activity was measured by using a luciferase assay kit. The emitted light and optical absorbance was measured with GloMax® 20/20 luminometer (Promega).

### *GalT-2 antisense oligonucleotides*

A 20-mer antisense oligonucleotide (AS-GalT-2) of the following sequence, 5'-CGC TTG AGC GCA GAC ATC TT-3', targeted against rat GalT-2 was synthesized by Bioneer (Daejeon, Korea). A scrambled oligonucleotide (5'-CTG ATA TCG TCG ATA TCG AT-3') also was synthesized and used as control. Cells ($1\times10^6$) were cultured in six-well plates for 1 d before the transfection. Transfection was performed with FuGENE®6-oligonucleotide complexes (final: 200 nM) under serum-free conditions. At 48 hr after transfection the protein levels of GalT-2 were analyzed by using polyclonal antibodies raised against rat GalT-2 (Data not shown). The mRNA level of ICAM-1 and VCAM-1 was examined at 6 hr after stimulation of CM and LacCer in the transfected cells.

### *Total RNA isolation and reverse transcription*

Cells were cultured in six-well culture plates. After drugs treatment, the cells were homogenized in TRIzol® reagent (Invitrogen). Total RNA was extracted from the cells according to the manufacturer's suggested protocol. Total RNA concentration was determined from spectrophotometric optical density measurement (260 and 280 nm). Total RNA (2 $\mu$g) was treated with 1 U DNase I (Promega) for 15 min at room temperature in 18 $\mu$l of volume containing 1× PCR buffer and 2 mM $MgCl_2$. Then it was inactivated by incubation with 2 $\mu$l of 25 mM EDTA at 65°C for 15 min. Reverse transcriptase reactions were carried out using MuLV Reverse Transcriptase (Promega) according to the manufacturer's protocol. Each reaction tube contained 2 $\mu$g of total RNA in a volume of 25 $\mu$l containing 5 $\mu$l of MuLV 5× RT buffer, 1 $\mu$g of Oligod(T)15 (promega), 2.5 $\mu$l of dNTP Mixture (Promega), 40 U of RNasin (Promega), 20 U of MuLV Reverse Transcriptase and nuclease-free water to volume. Reverse transcriptase reactions were carried out in a DNA Thermal Cycler 480 (Perkin Elmer, Branchburg, NJ, USA) at 25°C for 20 min, 42°C for 60 min and 95°C for 10 min. The cDNA was then stored at −20°C.

### *Quantitative real-time PCR*

Real-time PCR for the analysis of ICAM-1 and VCAM-1 mRNA levels were performed in a Rotor-Gene Q (Qiagen, Hilden, Germany). The primer sets for real-time PCR were designed using PrimerQuest (Integrated DNA Technologies, Coralville, IA, USA) and synthesized from Bioneer (Daejeon, Korea). The primer sequences for ICAM-1, VCAM-1 and GAPDH were as follows: rat ICAM-1 (forward, 5'-aaacgggagatgaatggtacctac-3', and reverse, 5'-tgcacgtccctggtgatactc- 3'); rat VCAM-1 (forward, 5'-ggctcgtacaccatccgc-3', and reverse, 5'-cggttttcgattcacactcgt-3'); and GAPDH (forward, 5'-cctacccccaatgtatccgttgtg-3', and reverse, 5'-ggaggaatgggagttgctgttgaa-3'). QuantiTect SYBR Green PCR kit was purchased from Qiagen. The reaction mixture consisted of 2 $\mu$l of cDNA template, 10 $\mu$l of SYBR Green PCR master mix and 10 pmol of primers in total volume of 20 $\mu$l. The cDNA was denatured at 95°C for 10 min followed by 45 cycles of PCR at 95°C for 10 sec, 58°C for 15 sec and 72°C for 20 sec. Data acquisition and analysis of real-time PCR were performed using the Rotor-Gene Q series software (version 1.7). Delta-delta Ct method was used to calculate the relative quantitation of each target gene normalized with GAPDH level in each individual sample.

### *Western blot analysis*

The cells were washed with ice-cold Tris-buffered saline (TBS; 20 mM Trizma base and 137 mM NaCl, pH 7.5) and lysed in 1× SDS sample loading buffer (62.5 mM Trizma base, 2% w/v SDS, 10% glycerol); after sonication and centrifugation at 15,000×g for 5 min, the supernatant was used for Western Blot assay. The protein concentration of samples was determined with the detergent-compatible protein assay reagent (Bio-Rad, Hercules, CA, USA), using bovine serum albumin (BSA) as standard. Samples were boiled for

3 min with 0.1 volume of 10% β-mercaptoethanol and 0.5% bromophenol blue mix. Fifty micrograms of total cellular protein was resolved by electrophoresis on 8% polyacrylamide gels, electro-transferred to a PVDF membrane (Amersham Pharmacia, Buckinghamshire, UK) and blocked with TBST (10 mM Trizma base (pH 7.4), 150 mM NaCl, and 1% Tween 20) with 5% skim milk. After incubation with antiserum against ICAM-1 and VCAM-1 (Santacruz biotechnology, Santa Cruz, CA, USA) for 2 hr at room temperature, the membranes were washed with TBST three times and then incubated with horseradish peroxidase conjugated anti-rabbit IgG for 1 hr. The membranes were visualized by using ECL-plus (Amersham Pharmacia) after washing with TBST buffer.

### *Statistical analysis*

All values shown in the figures are expressed as the mean±SD obtained from at least three independent experiments. Statistical analysis was carried out by t test (Fig. 1) and one-way analysis of variance (ANOVA) with Tukey's post- hoc test (Fig. 2∼5) using GraphPad Prism version 4.03 (GraphPad Software, San Diego, CA, USA). p values less than 0.05 were considered to indicate statistical significance.

## RESULTS

### *Expression of adhesion molecules in CM-stimulated astrocytes*

In the present study, we first examined the expression of adhesion molecules, such as ICAM-1, VCAM-1, and E-selectin in CM-stimulated primary astrocytes. As shown in Fig. 1, ICAM-1 mRNA level increased at 3 hr and returned to the basal level at 24 hr after CM treatment (Fig. 1A). Its protein level also significantly increased at 9∼24 hr after CM stimulation (Fig. 1C). VCAM-1 mRNA level began to increase at 1 hr and further significantly increased at 3∼24 hr after CM treatment (Fig. 1B). Protein level of VCAM-1 greatly increased much earlier than ICAM-1 level at 6∼24 hr in CM-treated astrocytes (Fig. 1D). CM also induced mRNA level of E-selectin, the other adhesion molecule, in astrocytes (data not shown).

### *Effect of PDMP and NBDNJ on expression of ICAM-1 and VCAM-1 in CM-stimulated astrocytes*

We next examined whether LacCer was invloved in the expression of ICAM-1 and VCAM-1 in CM-treated astrocytes. The cells pretreated with inhibitors of GalT-2, PDMP (20 μM) and NBDNJ (20 μM) showed a decrease in CM-induced ICAM-1 (Fig. 2A, B) and VCAM-1 (Fig. 3A, B) mRNA levels as well as their protein levels. However, PDMP- and NBDNJ-mediated inhibitions of ICAM-1 and VCAM-1 expression were reversed by exogenously supplied LacCer (10 μM). In addition, CM-induced promoter luciferase activities were also inhibited by PDMP and the inhibition was reversed by LacCer in the cells transiently transfected with ICAM-1 (Fig. 2D) and VCAM-1 promoter (Fig. 3D)-luciferase gene constructs, respectively.

### *Effect of GalT-2 silencing on expression of ICAM-1 and VCAM-1 in CM-stimulated astrocytes*

Inhibition of GalT-2 gene using AS-GalT-2 also attenuated mRNA level of ICAM-1 (Fig. 2C) and VCAM-1 (Fig.

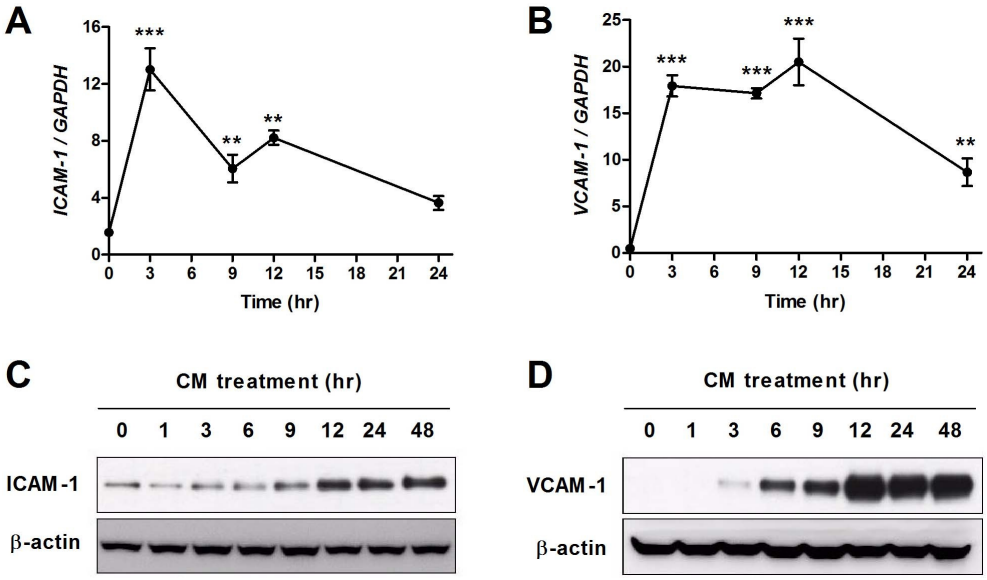


**Fig. 1.** The expression of ICAM-1 and VCAM-1 in CM-stimulated primary cultured astrocytes. The cells were treated cytokine mixture (CM), TNF-α (10 ng/ml) and IFNγ (10 ng/ml). Total RNA and protein were isolated from 1, 3, 6, 9, 12, 24 and 48 hr in CM-treated astrocytes. Gene expression of ICAM-1 (A) and VCAM-1 (B) were measured by quantitative real-time PCR using Rotor-Gene Q. The expression of each gene was normalized with GAPDH gene expression. Protein levels of ICAM-1 (C) and VCAM-1 (D) were examined by Western blot analysis. Data were obtained from triplicated PCR reactions of three different cultures, and values are mean±SD (**$p<0.01$, ***$p<0.001$, vehicle vs. CM).

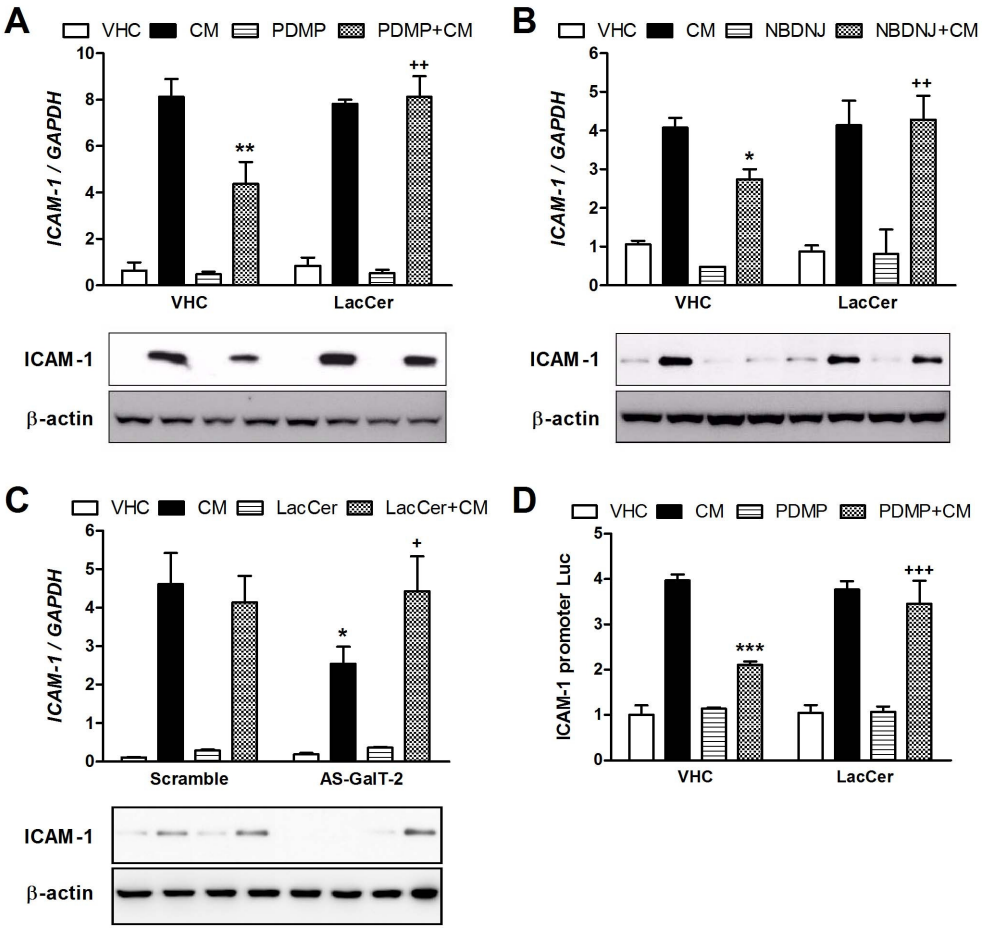


**Fig. 2.** Lactosylceramide mediates the expression of ICAM-1 in CM-stimulated astrocytes. The cells were pretreated with PDMP (A) and NBDNJ (B) and LacCer for 0.5 hr before CM. Gene expression of ICAM-1 (A～C; top) was measured by quantitative real-time PCR at 6 hr after CM stimulation. The expression of each gene was normalized with GAPDH gene expression. For the silencing of GalT-2 gene (C), the cells were transfected with AS-GalT-2 and scrambled DNA oligomer (scramble). At 48 hr after transfection with AS and scrambled oligonucleotides, the cells were stimulated with CM and LacCer. Protein levels of ICAM-1 (A～C; bottom) was examined by Western blot analysis at 24 hr after CM stimulation. (D) At 24 hr after transient transfection of cells with ICAM-1 promoter-luciferase gene construct, the cells were pretreated with PDMP for 0.5 hr before stimulation with CM. The cellular luciferase activity was measured. All graph indicate mean±SD (A: $^{**}p<0.01$, CM vs. CM+PDMP, $^{++}p<0.01$, CM+PDMP vs. CM+PDMP+LacCer; B: $^{*}p<0.05$, CM vs. CM+NBDNJ, $^{++}p<0.01$, CM+NBDNJ vs. CM+NBDNJ+LacCer; C: $^{*}p<0.05$, scramble-CM vs. AS-GalT-2-CM, $^{+}p<0.05$, AS-GalT-2-CM vs. AS-GalT-2-CM+LacCer; D: $^{***}p<0.001$, CM vs. CM+PDMP, $^{+++}p<0.001$, CM+PDMP vs. CM+PDMP+LacCer, n=3 independent experiments).

3C) in CM-stimulated astrocytes. In addition, AS-GalT-2-mediated inhibition of ICAM-1 and VCAM-1 gene expression was reversed by exogeneous LacCer. These results indicate LacCer may play a role in the regulation of CM-mediated ICAM-1 and VCAM-1 gene expression.

### *Inhibition of GalT-2 attenuated the activation of transcription factors in CM-stimulated astrocytes*

To examine which TFs were involved in CM-induced ICAM-1 and VCAM-1 expression, luciferase assay was performed using several cis-acting element luciferase vectors. CM treatment showed an increase of luciferase activities and then elevated luciferase activities were inhibited by PDMP in the cells transient transfected with NF-κB, AP-1, GAS and STAT-3 luciferase vectors (Fig. 4A, C, E, G). In addition, AS-GalT-2 significantly attenuated the increase of luciferase activities compared to the scrambled co-transfected group (Fig. 4B, D, F, H). Furthermore, CM-induced NF-κB and GAS luciferase activities were also inhibited by PDMP and the inhibition was reversed by LacCer in the cells transiently transfected with NF-κB (Fig. 5A) and GAS (Fig. 5B)-luciferase gene constructs, respectively. These data indicate that activation of LacCer may play a role on the activation of NF-κB, AP-1, STAT-1 and STAT-3 in CM-treated astrocytes.

## DISCUSSION

In the present study, we have investigated the effect of PDMP, NBDNJ on the expression of ICAM-1 and VCAM-1 genes in CM-stimulated astrocytes. Our results indicate that mRNA and protein expression of ICAM-1 and VCAM-1 were significantly increased by CM treatment in primary cultured astrocytes (Fig. 1). Interestingly, gene expression

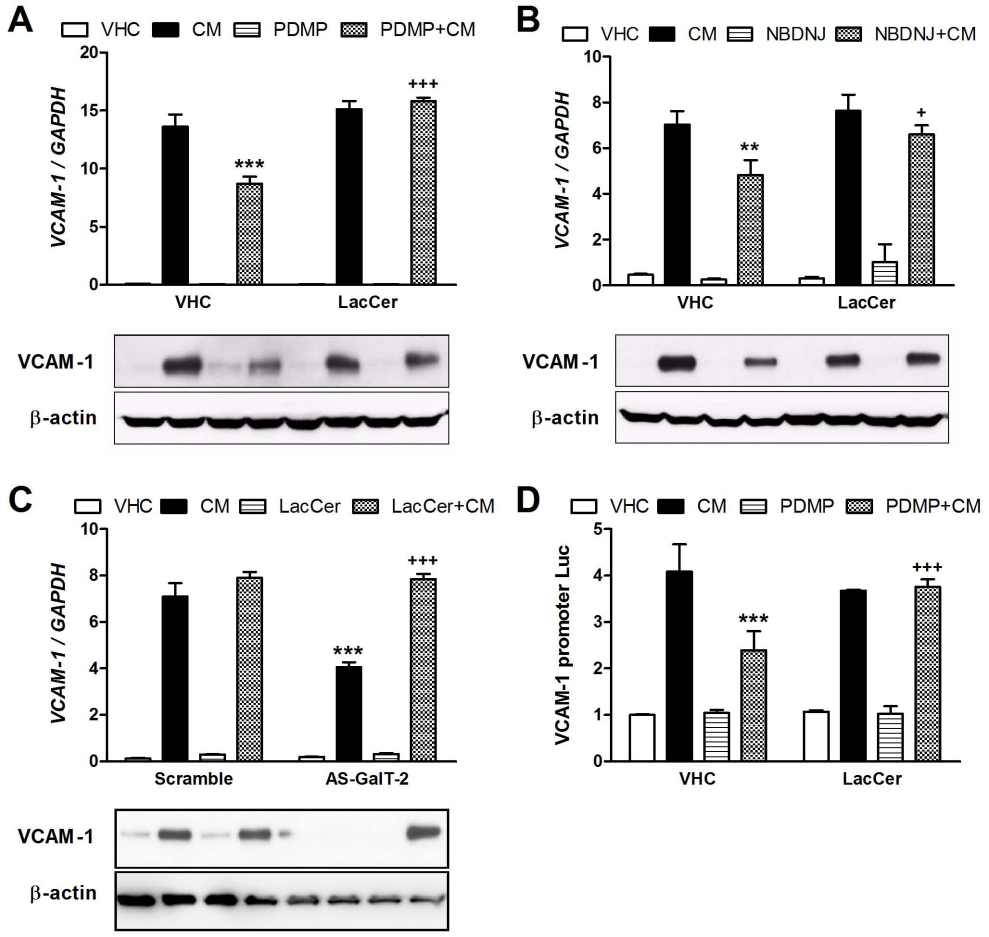


**Fig. 3.** Lactosylceramide mediates the expression of VCAM-1 in CM-stimulated astrocytes. The cells were pretreated with PDMP (A) and NBDNJ (B) and LacCer for 0.5 hr before CM. Gene expression of VCAM-1 (A∼C; top) was measured by quantitative real-time PCR at 6 hr after CM stimulation. The expression of each gene was normalized with GAPDH gene expression. For the silencing of GalT-2 gene (C), the cells were transfected with AS-GalT-2 and scrambled DNA oligomer (scramble). At 48 hr after transfection with AS and scrambled oligonucleotides, the cells were stimulated with CM and LacCer. Protein levels of VCAM-1 (A∼C; bottom) was examined by Western blot analysis at 24 hr after CM stimulation. (D) At 24 hr after transient transfection of cells with VCAM-1 promoter-luciferase gene construct, the cells were pretreated with PDMP for 0.5 hr before stimulation with CM. The cellular luciferase activity was measured. All graph indicate mean±SD (A: ***$p<0.001$, CM vs. CM+PDMP, $^{+++}p<0.001$, CM+PDMP vs. CM+PDMP+LacCer; B: **$p<0.01$, CM vs. CM+NBDNJ, $^{+}p<0.05$, CM+NBDNJ vs. CM+NBDNJ+LacCer; C: ***$p<0.001$, scramble-CM vs. AS-GalT-2-CM, $^{+++}p<0.001$, AS-GalT-2-CM vs. AS-GalT-2-CM+LacCer; D: ***$p<0.001$, CM vs. CM+PDMP, $^{+++}p<0.001$, CM+PDMP vs. CM+PDMP+LacCer, n=3 independent experiments).

of VCAM-1 detected much higher and earlier than ICAM-1 gene expression. Since initial identification as an endothelial cell surface glycoprotein, previous studies reported ICAM-1 and VCAM-1 expression in nonvascular CNS cells, such as astrocytes, neuroblastoma and glioblastoma [1,2, 12,14-18]. The interaction of astrocytes with endothelial cells in the formation of BBB implies a critical role in the neuroinflammation response [1]. Thus, ICAM-1 and VCAM-1 expression beyond the vasculature may therefore provide significant environmental cues to recruiting immune cells in CNS.

Several studies demonstrate that glycosphingolipids, such as ceramide and sphingosine are involved in inflammatory responses, such as astrogliosis, ROS generation, cytokine production, and iNOS gene expression in the CNS [4,6,7,19]. Pharmacological or genetic inhibition of GalT-2 gene causes an inhibitory effect of ROS production and iNOS gene expression induced by TNF-$\alpha$ and reversed by LacCer [6]. Our results showed that treatment of PDMP and NBDNJ and transfection of AS-GalT-2 inhibited ICAM-1 and VCAM-1 genes expression and their promoter activation. In addition, exogeneous LacCer reversed the inhibition of ICAM-1 and VCAM-1 gene expression induced by inhibition of GalT-2 gene (Fig. 2C, 3C). In line with our results, Bhunia et al [9] reported that LacCer mediates TNF-$\alpha$-induced ICAM-1 expression in human umbilical vein endothelial cells. Taken together, LacCer biosynthesis is important to ICAM-1 and VCAM-1 expression in neuroinflammatory conditions, such as monocyte infiltration and astrogliosis.

The involvement of TFs, such as AP-1, NF-$\kappa$B and STATs on ICAM-1 and VCAM-1 expression in astrocytes is still unknown. AP-1 and NF-$\kappa$B are well known as important TFs in the expression of several inflammatory-related genes in glial cells. The STAT-1 binding site (GAS) located in the promoter region of ICAM-1 gene is required

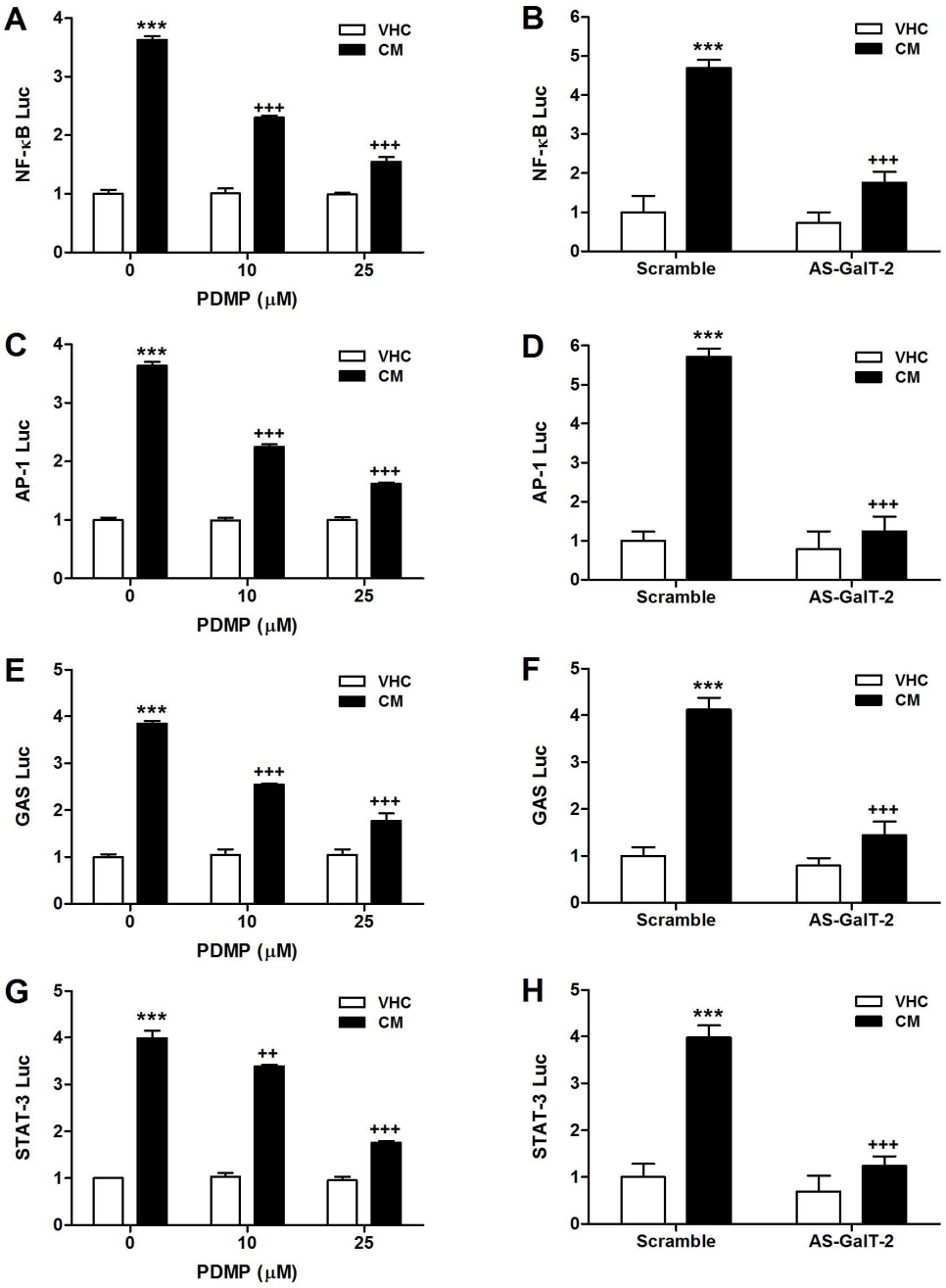


**Fig. 4.** Inhibition of GalT-2 attenuates the activation of transcription factors in CM-stimulated astrocytes. At 24 hr after transient transfection of cells with NF-κB (A), AP-1 (C), GAS (E), and STAT-3 (G) luciferase reporter vectors, the cells were pretreated with PDMP (10 and 25 μM) for 0.5 hr before stimulation with CM. For the silencing of GalT-2 gene, the cells were co-transfected with AS-GalT-2 (or scramble) and NF-κB (B), AP-1 (D), GAS (F), and STAT-3 (H) luciferase reporter vectors. At 48 hr after transfection with AS-GalT-2 (or scramble) and luciferase reporter vectors the cells were stimulated with CM. The cellular luciferase activity was measured. All graph indicate mean± S.D (A, C, E, G: ***$p<0.001$, vehicle vs. CM, $^{++}p<0.01$, $^{+++}p<0.001$, CM vs. CM+ PDMP, B, D, F, H: ***$p<0.001$, vehicle vs. CM in scramble-transfected group, $^{+++}p<0.001$, scamble+CM vs. AS- GalT-2+CM, n=3 independent experiments).

for induction by cytokines [11-13]. In this study, we examined which TFs are involved in CM-stimulated astrocytes using several cis-acting element luciferase vectors. Our results clearly show that PDMP and AS-GalT-2 significantly attenuated the elevated luciferase activities, indicating involvement of AP-1, NF-κB, STAT-1, and STAT-3 (Fig. 4). Exogenously supplied LacCer reversed the inhibition of NF-κB and STAT-1 (GAS) luciferase activities induced by PDMP (Fig. 5). TNF-α and IFNγ can induce an activation of STAT-1 and STAT-3 in astrocytes [20-22]. Several lines of evidence suggest that AP-1 and NF-κB mediate the expression of ICAM-1 and VCAM-1 in several cell types [12, 23-27]. Furthermore, NF-κB DNA binding activity and luciferase repoter activity were inhibited by PDMP in LPS and IFNγ-treated astrocytes [6]. Taken together, these results indicate that LacCer mediates an activation of AP-1, NF-κB, STAT-1 and STAT-3 in CM-stimulated astrocytes.

Previous studies reported that LacCer mediates a series of processes, such as ROS generation, Ras activation, ERK and JNK activation, AP-1 and NF-κB activation, and ICAM-1 expression in inflammatory or shear stress conditions [3,5,6,9,10,28]. Interestingly, LacCer itself increased expression of ICAM-1 in HUVECs [9]. However, in this study, LacCer itself had no effect on expression of ICAM-1 and VCAM-1 genes, their promoter activities, or activation of transcription factors in primary cultured astrocytes. In

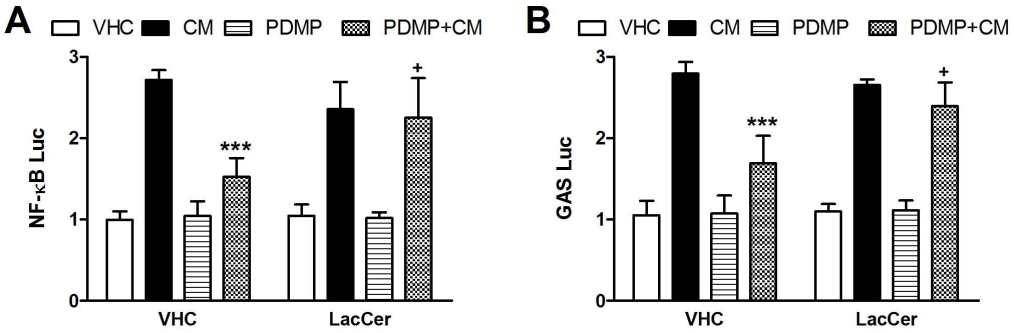


**Fig. 5.** Lactosylceramide mediates the activation of NF-kB and STAT-1 in CM-stimulated astrocytes. At 24 hr after transient transfection of cells with NF-kB (A) and GAS (B) luciferase reporter vectors, cells were pretreated with PDMP (20 $\mu$M) and LacCer (5 $\mu$M) for 0.5 hr before CM. The cellular luciferase activity was measured as described in Materials and Methods. All graph indicate mean±SD (***$p<0.001$, CM vs. CM+PDMP, $^{+}p<0.05$, CM+PDMP vs. CM+PDMP+LacCer, n=3 independent experiments).

line with our results, LacCer itself had no effect on iNOS expression and reversed the PDMP-mediated inhibition of iNOS expression in astrocytes [6]. Although further studies will be necessary to understand this phenomenon, it can be explained by the following: (1) LacCer may induce a synergistic expression of adhesion molecules in the presence of cytokines, such as TNF-$\alpha$ and IFN$\gamma$ in astrocytes; (2) Resulting from cell-type specificity, LacCer-mediated expression of adhesion molecules may be differently regulated in vascular and non-vascular cells.

Inhibitor of GLS, miglustat (OGT 918, NBDNJ, Zavesca®) is used primarily to treat Type 1 Gaucher disease (GD1) [29]. Earlier studies reported the involvement of sphingolipids such as ceramide and psychosine in the cytokine-mediated inflammatory disease of CNS, such as Krabbe disease and spinal cord injury [4-7]. Recent studies showed that PDMP increased imatinib-induced cell death in drug-sensitive and drug-resistant chronic myeloid leukemia cells via enhancing ceramide accumulation [30,31]. These data also suggest the potential utility of inhibiting GSL biosynthesis pathways in CNS disease as well as other diseases.

In summary, the present results indicate that LacCer mediate ICAM-1 and VCAM-1 expression through NF-$\kappa$B, AP-1, STAT-1 and STAT-3 activation in CM-stimulated astrocytes. Thus, we propose that elevated expression of ICAM-1 and VCAM-1 on astrocytes during certain CNS diseases can contribute to ongoing inflammatory responses by inducing the expression of proinflammatory cytokines and chemokines.

## ACKNOWLEDGEMENTS

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