Gnetin C suppresses double-stranded RNA-induced C-C motif chemokine ligand 2 (CCL2) and CCL5 production by inhibiting Toll-like receptor 3 signaling pathway

Hidemi YOSHIDA¹, Tadaatsu IMAIZUMI¹, Tomoh MATSUMIYA¹, Kazuhiko SEYA¹, Shogo KAWAGUCHI², and Hiroshi TANAKA^{3,4}

¹Department of Vascular Biology, Institute of Brain Science; ²Department of Gastroenterology and Hematology; ³Department of Pediatrics, Hirosaki University Graduate School of Medicine, 5 Zaifu-cho, Hirosaki 036-8562; and ⁴Department of School Health Science, Faculty of Education, Hirosaki University, Hirosaki 036-8560, Japan

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ABSTRACT

The innate immune system is a prerequisite for biophylactic ability, but its dysregulation can cause inflammatory and autoimmune diseases. To determine a safe method of controlling inflammatory reactions in the brain, we examined the effects of gnetin C, a natural resveratrol dimer, on C-C motif chemokine ligand 2 (CCL2) and CCL5 (pro-inflammatory chemokines) production observed after treatment with polyinosinic-polycytidylic acid [poly IC; a synthetic analog of dsRNA as a Toll-like receptor 3 (TLR3) ligand, 30 µg/mL] in cultured human astrocytoma U373MG and neuroblastoma SH-SY5Y cells. The addition of gnetin C (10 µM) to the media moderately reduced the CCL2 production and markedly suppressed CCL5 production in both cells. In the TLR3–interferon (IFN)-β–phosphorylated-STAT1 (signal transducer and activator of transcription protein 1)–RIG-I (retinoic acid-inducible gene-I) pathway that mediates CCL2 and CCL5 production, gnetin C first inhibits *IFN-β* expression in SH-SY5Y cells and primarily inhibits STAT1 phosphorylation in U373MG cells. In any case, gnetin C attenuated the dsRNA-activated TLR3 signaling resulting in CCL2 and CCL5 production, thus, may be useful for controlling TLR3-mediated inflammation in the brain.

Innate immune responses play a key role in controlling infection of the brain by pathogens including viral nucleic acid patterns (14, 23). Viral nucleic acids are recognized by pattern recognition receptors, which include the following three major families: Toll-like receptors (TLRs), Nod-like receptors, and retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs). In the central nervous system (CNS), parenchymal microglia as well as astrocytes, oligodendrocytes, and endothelial cells, and even neurons

express functional levels of some of these receptors (2, 18, 28). The engagement of such receptors results in the induction of specific pathways and the release of specific cytokines that play a role in resolving the injury (14). Upon infection, viral double-stranded (ds)RNA activates TLR3 and RIG-I, leading to production and secretion of chemokines, such as C-C motif chemokine ligand 2 [CCL2, also known as monocyte chemoattractant protein-1 (MCP-1)] and CCL5 [also known as regulated and normal T-cell expressed and secreted (RANTES)] (24). These chemokines enhance migration of circulating leukocytes, such as monocytes and lymphocytes, through the blood–brain barrier (BBB). These CNS-infiltrating immune cells play cytoprotective roles together with CNS-resident cells, such as microglia and astrocytes. Meanwhile, they can also contribute to

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Address correspondence to: Hidemi Yoshida, Ph.D., D.M.Sc., Department of Vascular Biology, Institute of Brain Science, Hirosaki University Graduate School of Medicine, 5 Zaifu-cho, Hirosaki 036-8562, Japan
Tel: +81-172-39-5145, Fax: +81-172-39-5145
E-mail: hidemi_yoshida@gakushikai.jp

harmful CNS inflammation which can cause neurodegeneration and/or autoimmunity (33).

Polyinosinic–polycytidylic acid (poly IC), an authentic dsRNA ligand for TLR3, is used as a mimic of viral RNAs or endogenous RNA ligands. We have previously found that human astrocytoma U373MG cells, used as a model of astrocytes, release CCL5 following the induction of RIG-I when treated with poly IC (31). We also reported that poly IC activates TLR3 signaling in U373MG cells (8) and that the TLR3–interferon (IFN)- β –phosphorylated STAT1 (signal transducer and activator of transcription protein 1)–RIG-I signaling pathway mediates the poly IC-induced CCL5 expression (6, 22).

Gnetin C is one of the resveratrol dimers (16) and is known to have cytoprotective functions (4), including radical scavenging activity, similar to that of ascorbic acid and *dl*- α -tocopherol (11). We recently found that gnetin C decreases neurotoxic amyloid- β (A β) production and ameliorates A β -induced neurotoxicity in cultured human neuroblastoma cells (22). However, the effect of gnetin C on inflammatory signaling pathways leading to chemokine production has not been reported. In this study, we examined the effect of gnetin C on the dsRNA-induced production of pro-inflammatory chemokines, CCL2 and CCL5, using cultured human astrocytoma and neuroblastoma cells as models of human astrocytes and neurons, respectively.

MATERIALS AND METHODS

Reagents. Dulbecco's Modified Eagle's Medium (DMEM) containing GlutaMAX™-I, Advanced DMEM, DMEM/Nutrient F-12 Ham (F12) (1 : 1) containing GlutaMAX™-I, Advanced DMEM/F12, and fetal bovine serum (FBS) were purchased from Invitrogen™/Thermo Fisher Scientific Inc. (Frederick, MD, USA). Bovine serum albumin (BSA), poly IC (#P9582), a protease inhibitor cocktail (#P8340), and an anti-actin antibody (rabbit polyclonal, #5060) were purchased from Sigma-Aldrich Co. LLC (St. Louis, MO, USA). Gnetin C [standard $\geq$ 97.0% (HPLC), #070-06141], dimethyl sulfoxide (DMSO), and sodium dodecyl sulfate (SDS) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Enzyme-linked immunosorbent assay (ELISA) kits for CCL2 (#DC00) and CCL5 (#DRN00B) were obtained from R&D Systems (Minneapolis, MN, USA). The NucleoSpin® RNA II total RNA isolation kit was obtained from Macherey-Nagel GmbH & Co. KG (Düren, Germany). An iScript™ Advanced cDNA synthesis kit and SsoAdvanced™

Universal SYBR® Green Supermix solution for reverse transcription-quantitative polymerase chain reaction (RT-qPCR) were obtained from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). Oligonucleotide primers for qPCR were custom synthesized by Fasmac/Greiner Japan (Atsugi, Japan). A bicinchoninic acid (BCA™) protein assay kit from Pierce®/Thermo Scientific Inc. (Waltham, MA, USA) was also used. Polyvinylidene fluoride (PVDF) membranes and the Luminata™ Crescendo Western horseradish peroxidase (HRP) substrate were from Millipore Corporation (Billerica, MA, USA). Mouse monoclonal IgG against phosphorylated signal transducer and activator of transcription protein 1 (STAT1) (pSTAT1) (pY701.4A, #sc-136229) and rabbit polyclonal IgG against STAT1 (p84/p91, E-23, #sc-346) were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). An antibody against β -actin [ACTN (C4), mouse monoclonal, #ab3280] was obtained from Abcam plc (Cambridge, UK). Anti-mouse (#7076) and anti-rabbit (#7074) IgG and HRP-linked antibodies were obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA).

Cell culture. Human astrocytoma U373MG cells (ECACC No. 89081403) and human neuroblastoma SH-SY5Y cells (ECACC No. 94030304) were purchased from the European Collection of Authenticated Cell Cultures (Porton Down, UK) and cultured in DMEM (for U373MG) and DMEM/F12 (for SH-SY5Y) containing GlutaMAX™-I supplemented with 10% FBS (22, 32). U373MG and SH-SY5Y cells have properties of astrocytes (7) and catecholaminergic neurons (30), respectively. Once the cells reached 80% confluence, they were used for experimental purposes; immediately, the medium was replaced with fresh Advanced DMEM (for U373MG) or Advanced DMEM/F12 (for SH-SY5Y) containing GlutaMAX™-I supplemented with 3% FBS. In experiments with gnetin C, cells were pretreated with this biophenol for 1 h prior to the addition of poly IC. Gnetin C was dissolved in DMSO; the maximum final concentration of DMSO in the culture medium was 0.1%. Meanwhile, control cells were treated with the vehicle alone.

ELISA. Aliquots of cell culture supernatants were stored at -80°C until further use. Levels of CCL2 and CCL5 in the cell-conditioned medium were determined using ELISA kits and an iMark/Bio-Rad microplate reader.

RT-qPCR. Total RNA was extracted from cells, and

Table 1 Oligonucleotide primers used for real-time quantitative PCR

cDNA	Forward sequence (5' to 3')	Reverse sequence (5' to 3')
IFN- β	CCTGTGGCAATTGAATGGGAGGC	CCAGGCACAGTGACTGTACTCCTT
RIG-I	GTGCAAAGCCTTGGCATGT	TGGCTTGGGATGTGGTCTACTC
CCL2	AAACTGAAGCTCGCACTCTCGC	ATTCTTGGGTTGTGGAGTGAGT
CCL5	CTACTCGGGAGGCTAAGGCAGGAA	GAGGGGTTGAGACGGCGGAAGC
18S rRNA	ACTCAACACGGGAAACCTCA	AACCAGACAAATCGCTCCAC

Abbreviations used: IFN- β , interferon- β ; CCL2, C-C motif chemokine ligand 2, also known as monocyte chemoattractant protein-1 (MCP-1); CCL5, C-C motif chemokine ligand 5, also known as regulated on activation normal T-cell expressed and secreted (RANTES); RIG-I, retinoic acid-inducible gene-I.

single-stranded cDNA was synthesized using the iScript™ Advanced cDNA synthesis kit for RT-qPCR (5). A CFX96™ Real-Time PCR System (Bio-Rad) was used for quantitative analyses of 18S rRNA and mRNAs of the genes listed in Table 1. Primers were used at 500 nM. Amplification was performed in SsoAdvanced™ Universal SYBR® Green Supermix solution, and the following reaction conditions were used: 95°C for 30 s; 40 cycles of 95°C for 5 s, 55°C for 10 s, and 60°C for 15 s; and an increase from 65°C to 95°C (at 0.5°C/5 s) for the melting step. Data were analyzed with CFX Manager™ Version 2.1 software.

Western blot analysis. Cells cultured in a 35-mm-diameter dish were washed twice with ice-cold 20 mM phosphate-buffered saline (PBS, pH 7.4). Cells were then lysed in 50 μ L hypotonic cell lysis buffer [10 mM Tris (pH 7.4), 100 mM NaCl, 1.5 mM MgCl₂, and 0.5% NP-40] containing 0.2% proteinase inhibitors, and immediately placed at -80°C until use. Thawed and vortex-mixed lysates were centrifuged at 10,000 $\times$ g for 10 min at 4°C, and the supernatants were transferred to fresh tubes. After the BCA protein assay, aliquots of the solutions containing 10 μ g of the protein were mixed with 6 $\times$ loading buffer and boiled for 6 min. The samples were subjected to electrophoresis on 7.5% SDS-polyacrylamide gels, and proteins were transferred to PVDF membranes. The membranes were blocked by incubation in Tris-buffered saline with Tween 20 (TBS-T, pH 7.4; 50 mM Tris-HCl, 250 mM NaCl, and 0.1% Tween-20) containing 3% nonfat dry milk (blocking buffer) for 1 h at 25°C. The membranes were then incubated with a mouse anti-pSTAT1, or rabbit anti-STAT1 antibody (1 : 1000 in TBS-T containing 3% BSA) overnight at 4°C, followed by a third incubation with the appropriate secondary antibodies (HRP-conjugated anti-mouse, or anti-rabbit IgG, 1 : 10,000 in blocking buffer) for 1 h at 25°C. The immunoreactive bands were detected using an en-

hanced chemiluminescence (ECL) detection system. The same blot was then reprobed with an anti- β -actin antibody (1 : 5000).

Densitometric quantification. Integrated optical density of the immunoreactive band was quantified using ImageJ 1.49i software (National Institute of Health, Bethesda, MD, USA) and was normalized to the β -actin band density.

Statistics. Values are expressed as mean $\pm$ SD, and statistical significance was analyzed using unpaired two-tailed *t*-test or one-way analysis of variance. Significance was considered at a probability (*P*) value of <0.05.

RESULTS

Gnetin C reduces poly IC-induced CCL2 and CCL5 production

First, we examined whether gnetin C has suppressive activity against CCL2 and CCL5 production that was enhanced by dsRNA in cultured human astrocytoma U373MG cells and neuroblastoma SH-SY5Y cells (Fig. 1). The cells were pretreated with gnetin C at the indicated concentrations for 1 h and then treated with 30 μ g/mL poly IC for 24 h. Control cells were treated with DMSO during the experiment. In both U373MG and SH-SY5Y cells, gnetin C suppressed poly IC-induced CCL2 and CCL5 production in a concentration-dependent manner. Gnetin C pretreatment (10 μ M) of U373MG cells effectively suppressed poly IC-induced CCL2 and CCL5 secretion by 79% and 96%, respectively. In SH-SY5Y cells, gnetin C at 10 μ M moderately lowered the CCL2 and CCL5 secretion by 35% and 53%, respectively. Because the 10- μ M dose of gnetin C did not affect cell viability (19), we used this dose for the following experiments.

Fig. 1 Effect of gnetin C (GNC) on CCL2 or CCL5 secretion from cultured U373MG or SH-SY5Y cells. After pretreatment with 1 to 10 μ M GNC for 1 h, the cells were incubated with 30 μ g/mL of polyinosinic-polycytidylic acid (poly IC) for 24 h. Dimethyl sulfoxide (DMSO) was used as a solvent for GNC and was also used as a vehicle control [0.1% (v/v) DMSO]. The levels of CCL2 and CCL5 secreted into the cell-conditioned medium were analyzed by enzyme-linked immunosorbent assay (ELISA) and normalized to total cell protein determined by the BCA assay. The mean $\pm$ SD of three experiments is shown. * denotes $P < 0.05$.

Time- and concentration-dependent poly IC-induced mRNA expression

To set our next experimental conditions, we confirmed the time courses of representative mRNA expression in poly IC-induced TLR3 signaling (Fig. 2). In U373MG cells, *IFN- β* , *RIG-I*, *CCL2*, and *CCL5* expression reached its peak at 4, 8, 16, or 24 h, respectively; in SH-SY5Y cells, the expression reached its peak at 4 (and another 16), 16, 16, and 24 h or more, respectively. These phase-shift patterns apparently confirmed the existence of the poly IC–TLR3–*IFN- β* –*RIG-I*–*CCL2/5* axis (6) not only in U373MG cells but also in SH-SY5Y cells. The concentration dependence of poly IC (up to 100 μ g/mL)-induced *IFN- β* or *RIG-I* expression was also confirmed at the peak time (Fig. 3), and we used the 30- μ g/mL dose of poly IC in the following experiments.

In SH-SY5Y cells, gnetin C first inhibited *IFN- β* expression, whereas in U373MG cells, it first inhibited *STAT1* phosphorylation

Based on the aforementioned results, we performed step-by-step investigation of the suppressive effect of gnetin C (10 μ M) on the poly IC (30 μ g/mL)-activated TLR3 signaling pathway in U373MG and SH-SY5Y cells (Figs. 4 and 5). In SH-SY5Y cells, gnetin C first inhibited poly IC-enhanced *IFN- β* mRNA expression, but in U373MG cells, gnetin C did not affect this expression step (Fig. 4). In both cells, gnetin C markedly inhibited the poly IC-induced *RIG-I* mRNA; however, in SH-SY5Y cells, gnetin C moderately inhibited CCL2 and CCL5 production. Because *IFN- β* is known to activate *STAT1* phosphorylation in U373MG cells (8), next, we examined whether gnetin C affects *STAT1* phosphorylation (Fig. 5). Gnetin C mostly inhibited the poly

Fig. 2 Time course of *IFN-β*, *RIG-I*, *CCL2*, or *CCL5* mRNA expression in U373MG or SH-SY5Y cells stimulated with 30 μg/mL poly IC. mRNA expression was analyzed by real-time quantitative PCR and was normalized to 18S rRNA levels (relative value). The mean ± SD of three experiments is shown. * denotes $P < 0.05$ vs. control (0 h).

Fig. 3 Concentration-dependent stimulation of *IFN-β* or *RIG-I* mRNA expression by poly IC in U373MG or SH-SY5Y cells. The cells were treated with 1–100 µg/mL poly IC for 4, 8, or 16 h. mRNA expression was analyzed by real-time quantitative PCR and normalized to 18S rRNA expression. The mean ± SD of three experiments is shown. * denotes $P < 0.05$ vs. control (0 µg/mL).

IC-induced pSTAT1 in U373MG cells, and moderately inhibited the enhanced pSTAT1 in SH-SY5Y cells. In SH-SY5Y cells, the rate of decrease of the inhibited pSTAT1 (45%) was comparable with that of the inhibited *IFN-β* mRNA (60%, Fig. 4B) or *CCL5* mRNA (53%, Fig. 4H), whereas the rate was relatively low for the inhibited *CCL2* mRNA (30%, Fig. 4F).

DISCUSSION

In the present study, we examined a *trans*-resveratrol dimer, gnetin C, as a possible means of controlling pro-inflammatory chemokine production induced by a TLR3 ligand. This is the first study to state that gnetin C suppressed CCL2 and CCL5 production in both human astrocytoma U373MG and human neuroblastoma SH-SY5Y cells treated with poly IC which mimics a viral dsRNA.

We have previously reported that poly IC activates TLR3 signaling (8, 10) and enhances production of chemokines including CCL5 via the TLR3–*IFN-β*–pSTAT1–RIG-I signaling pathway in human

astrocytoma cells (22) and in human mesangial cells (6), although findings have suggested that the poly IC-induced TLR3–NF-κB/IRF3–CCL5 pathway is active in a case of human biliary epithelial cells (21). In this study, we confirmed that the poly IC-induced TLR3–*IFN-β*–pSTAT1–RIG-I pathway involved in upstream of CCL2/5 production is active not only in astrocytoma cells but also in neuroblastoma cells. We found that gnetin C first inhibits *IFN-β* expression in neuroblastoma cells and primarily inhibits STAT1 phosphorylation in astrocytoma cells.

A high level of RIG-I expression has been observed in the poly IC-stimulated astrocytoma cells in culture and resident astrocytes in the mouse brain injected with poly IC (31). RIG-I is one of the *IFN*-stimulated genes (ISGs). STAT1 is a transcription factor that mediates *ISG* mRNA expression involved in downstream of TLR3–*IFN-β* signaling. We have previously shown that, in astrocytoma cells, inhibition of STAT1 phosphorylation can decrease RIG-I expression (9). In this case, gnetin C markedly inhibited poly IC-induced pSTAT1 at initial stage and also completely suppressed *RIG-I* and *CCL2/5*

Fig. 4 Effect of gnetin C (GNC) on *IFN- β* , *RIG-I*, *CCL2*, or *CCL5* mRNA expression in U373MG or SH-SY5Y cells. After pretreatment with 10 μ M GNC for 1 h, the cells were incubated with 30 μ g/mL of poly IC for 4 or 8 h. Dimethyl sulfoxide (DMSO) was used as a solvent for GNC and was also used as a vehicle control [0.1% (v/v) DMSO]. mRNA expression was analyzed by real-time quantitative PCR and was normalized to 18S rRNA levels (relative value). The mean $\pm$ SD of three experiments is shown. * denotes $P < 0.05$.

Fig. 5 Effect of gnetin C (GNC) on STAT1 phosphorylation in U373MG or SH-SY5Y cells. After pretreatment with 10 μ M GNC for 1 h, the cells were incubated with 30 μ M/L of poly IC for 16 h. Dimethyl sulfoxide (DMSO) was used as a solvent for GNC, and also used as a vehicle control [0.1% (v/v) DMSO]. (**A**, **B**) Phosphorylated STAT1 (pSTAT1) or STAT1 in hypotonic cell lysates was detected by Western blotting (samples were separated in a 7.5% SDS-polyacrylamide gel and probed with a mouse anti-pSTAT1 monoclonal antibody or a rabbit anti-STAT1 polyclonal antibody), and β -actin was used as a loading control. Data shown are representative of three experiments. (**C**, **D**) The integrated optical density of the blot for pSTAT1 or STAT1 in (**A**, **B**) was quantified using ImageJ 1.49i and normalized to that of β -actin. The mean $\pm$ SD of three experiments is shown. * denotes $P < 0.05$.

in astrocytoma cells. On the other hand, in neuroblastoma cells, gnetin C also markedly inhibited poly IC-enhanced *RIG-I* expression, but its upstream *IFN- β* -pSTAT1 and downstream *CCL2* or *CCL5* induced by poly IC were not completely but moderately inhibited by gnetin C. These findings suggest that gnetin C directly inhibits *RIG-I* and that *RIG-I*-independent pathways, such as the TLR3-NF- κ B/IRF3-CCL5 pathway (21) or other unknown pathways, are to some extent involved in poly IC-induced *CCL2/5* expression in neuroblastoma cells. Differences in the inhibitory effects of gnetin C between astrocytoma and neuroblastoma cells may be due to the different cell types, but the detailed underlying mechanisms involved in this should be investigated further in future studies.

As our results indicate, the suppressive effect of gnetin C on *CCL2/5* production is more efficient in astrocytoma cells than in neuroblastoma cells. Gnetin C ($C_{28}H_{22}O_6$, 454.47 g/mol) has a lipophilic property, which makes it possible to cross BBB. These suggest that gnetin C may be effective in the whole brain because astrocytes are widely distributed over there and, in particular, constitute BBB. BBB is one of the most important components of the first line of innate immunity (12, 14, 25).

Immune and inflammatory reactions mediated by TLR3 signaling are suggested to be important in host defenses against viral or "pseudoviral" infections (6, 23). Cross-talk between functional molecules induced by TLR3 activation, such as type I IFNs and pro-inflammatory chemokines or cytokines stimu-

Fig. 6 Suppressive potentials of gnetin C on pro-inflammatory chemokine production. We focused on the double-stranded RNA-induced CC chemokine, CCL2 and CCL5, production in cultured human astrocytoma U373MG and neuroblastoma SH-SY5Y cells. Gnetin C may act against neuroinflammation by suppressing the IFN- β -pSTAT1-RIG-I pathway, which regulates the subsequent CCL2 and CCL5 production in both astroglial and neuronal cells, although its primary target signal differs (IFN- β in SH-SY5Y cells; pSTAT1 in U373MG). Abbreviations used: poly IC, polyinosinic-polycytidylic acid; TLR3, Toll-like receptor 3; IFN- β , interferon- β ; STAT1, signal transducer and transcription 1; pSTAT1, phosphorylated STAT1; RIG-I, retinoic acid-inducible gene-I; CCL2, C-C motif chemokine ligand 2 (also known as monocyte chemoattractant protein-1, MCP-1); CCL5, also known as regulated on activation normal T-cell expressed and secreted (RANTES).

lated by type I IFNs, plays protective or deleterious roles during the pathogenesis of brain inflammation (12, 13). Chemokines are small, 8- to 12-kDa cytokines that have the ability to induce the chemotaxis of leukocytes and are divided into four groups based on their initial cysteine residue: XC, CC, CXC, and CX3C (24). Among them, CCL2 and CCL5 are produced by various cell types in the brain, such as neurons, astrocytes, and microglia (15, 20), and are suggested to be involved in viral and autoimmune inflammatory neurological disorders (1, 13). Previous infection with Epstein–Bar virus may contribute to the development of multiple sclerosis (17), and elevated levels of CCL2 and CCL5 are associated with the activity of this disease (3, 29).

Gnetin C as well as other melinjo-derived biophenols are free from harmful side effects (26, 27), leading to high expectations that it could be used for many neuroinflammatory and neurodegenerative dis-

eases. Although the clinical and prophylactic efficacy of gnetin C in pathologies remains to be evaluated, its appropriate use could play a role in preventing excess or prolonged inflammation after TLR3 activation in the CNS.

In conclusion, gnetin C was found to negatively regulate IFN- β expression and STAT1 phosphorylation in poly IC-treated human astroglial and neuronal cells, respectively, and finally suppressed their downstream CCL2 or CCL5 production in both cell types (Fig. 6). Gnetin C could potentially be used for controlling the progression of virus-mediated neuroinflammation. Further studies are needed to confirm these preliminary findings.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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REFERENCES

1. Bose S and Cho J (2013) Role of chemokine CCL2 and its receptor CCR2 in neurodegenerative diseases. *Arch Pharm Res* **36**, 1039–1050.
2. Carty M, Reinert L, Paludan SR and Bowie AG (2014) Innate antiviral signaling in the central nervous system. *Trends Immunol* **35**, 79–87.
3. Conductier G, Blondeau N, Guyon A, Nahon JL and Rovère C (2010) The role of monocyte chemoattractant protein MCP1/CCL2 in neuroinflammatory diseases. *J Neuroimmunol* **224**, 93–100.
4. Espinoza JL and Inaoka PT (2017) Gnetin-C and other resveratrol oligomers with cancer chemopreventive potential. *Ann N Y Acad Sci* **1403**, 5–14.
5. Fukui K, Yachi K, Yoshida H, Tanji K, Matsumiya T, Hayakari R, Tsuruga K, Tanaka H and Imaizumi T (2017) Rebamipide reduces amyloid- β 1–42 (A β 42) production and ameliorates A β 43-lowered cell viability in cultured SH-SY5Y human neuroblastoma cells. *Neurosci Res* **124**, 40–50.
6. Imaizumi T, Hayakari R, Watanabe S, Aizawa T, Matsumiya T, Yoshida H, Tsuruga K, Kawaguchi S and Tanaka H (2017) Cylindromatosis (CYLD), a deubiquitinase, attenuates inflammatory signaling pathways by activating Toll-like receptor 3 in human mesangial cells. *Kidney Blood Press Res* **42**, 942–950.
7. Imaizumi T, Murakami K, Ohta K, Seki H, Matsumiya T, Meng P, Hayakari R, Xing F, Aizawa-Yashiro T, Tatsuta T, Yoshida H and Kijima H (2013) MDA5 and ISG56 mediate CXCL10 expression induced by Toll-like receptor 4 activa-

- tion in U373MG human astrocytoma cells. *Neurosci Res* **76**, 195–206.
8. Imaizumi T, Numata A, Yano C, Yoshida H, Meng P, Hayakari R, Xing F, Wang L, Matsumiya T, Tanji K, Tatsuta T, Murakami M and Tanaka H (2014) ISG54 and ISG56 are induced by TLR3 signaling in U373MG human astrocytoma cells: possible involvement in CXCL10 expression. *Neurosci Res* **84**, 34–42.
  9. Imaizumi T, Sakashita N, Mushiga Y, Yoshida H, Hayakari R, Xing F, Wang L, Matsumiya T, Tanji K, Chiba Y, Furudate K, Kawaguchi S, Murakami M and Tanaka H (2015) Desferrioxamine, an iron chelator, inhibits CXCL10 expression induced by polyinosinic-polycytidylic acid in U373MG human astrocytoma cells. *Neurosci Res* **94**, 10–16.
  10. Imaizumi T, Yoshida H, Hayakari R, Xing F, Wang L, Matsumiya T, Tanji K, Kawaguchi S, Murakami M and Tanaka H (2016) Interferon-stimulated gene (ISG) 60, as well as ISG56 and ISG54, positively regulates TLR3/IFN- β /STAT1 axis in U373MG human astrocytoma cells. *Neurosci Res* **105**, 35–41.
  11. Jia Y, Wang N and Liu X (2017) Resveratrol and amyloid- β : mechanistic insights. *Nutrients* **9**, E1122.
  12. Klein RS and Hunter CA (2017) Protective and pathological immunity during central nervous system infections. *Immunity* **46**, 891–909.
  13. Kothur K, Wienholt L, Brilot F and Dale RC (2016) CSF cytokines/chemokines as biomarkers in neuroinflammatory CNS disorders: a systematic review. *Cytokine* **77**, 227–237.
  14. Lampron A, Elali A and Rivest S (2013) Innate immunity in the CNS: redefining the relationship between the CNS and its environment. *Neuron* **78**, 214–232.
  15. Lanfranco MF, Mocchetti I, Burns MP and Villapol S (2018) Glial- and neuronal-specific expression of CCL5 mRNA in the rat brain. *Front Neuroanat* **11**, 137.
  16. Lins AP, Ribeiro MN de S, Gottlieb OR and Gottlieb HE (1982) Gnetins: resveratrol oligomers from *Gnetum* species. *J Nat Prod* **45**, 754–761.
  17. Márquez AC and Horwitz MS (2015) The role of latently infected B cells in CNS autoimmunity. *Front Immunol* **6**, 544.
  18. Nair S and Diamond MS (2015) Innate immune interactions within the central nervous system modulate pathogenesis of viral infections. *Curr Opin Immunol* **36**, 47–53.
  19. Seino S, Kimoto T, Yoshida H, Tanji K, Matsumiya T, Hayakari R, Seya K, Kawaguchi S, Tsuruga K, Tanaka H and Imaizumi T (2018) Gnetin C, a resveratrol dimer, reduces amyloid- β 1–42 (A β 42) production and ameliorates A β 42-lowered cell viability in cultured SH-SY5Y human neuroblastoma cells. *Biomed Res (Tokyo)* **39**, 105–115.
  20. Semple BD, Kossmann T and Morganti-Kossmann MC (2010) Role of chemokines in CNS health and pathology: a focus on the CCL2/CCR2 and CXCL8/CXCR2 networks. *J Cereb Blood Flow Metab* **30**, 459–473.
  21. Shimada T, Imaizumi T, Shirai K, Tatsuta T, Kimura T, Hayakari R, Yoshida H, Matsumiya T, Kijima H, Mizukami H and Hakamada K (2017) CCL5 is induced by TLR 3 signaling in HuCCT1 human biliary epithelial cells: possible involvement in the pathogenesis of biliary atresia. *Biomed Res (Tokyo)* **38**, 269–276.
  22. Shirai K, Shimada T, Yoshida H, Hayakari R, Matsumiya T, Tanji K, Murakami M, Tanaka H and Imaizumi T (2017) Interferon (IFN)-induced protein 35 (IFI35) negatively regulates IFN- β -phosphorylated STAT1-RIG-I-CXCL10/CCL5 axis in U373MG astrocytoma cells treated with polyinosinic-polycytidylic acid. *Brain Res* **1658**, 60–67.
  23. Shives KD, Tyler KL and Beckham JD (2017) Molecular mechanisms of neuroinflammation and injury during acute viral encephalitis. *J Neuroimmunol* **308**, 102–111.
  24. Sokol CL and Luster AD (2015) The chemokine system in innate immunity. *Cold Spring Harb Perspect Biol* **7**, a016303.
  25. Swanson PA 2nd and McGavern DB (2015) Viral diseases of the central nervous system. *Curr Opin Virol* **11**, 44–54.
  26. Tani H, Hikami S, Iizuna S, Yoshimatsu M, Asama T, Ota H, Kimura Y, Tatefuji T, Hashimoto K and Higaki K (2014) Pharmacokinetics and safety of resveratrol derivatives in humans after oral administration of melinjo (*Gnetum gnemon* L.) seed extract powder. *J Agric Food Chem* **62**, 1999–2007.
  27. Tatefuji T, Yanagihara M, Fukushima S and Hashimoto K (2014) Safety assessment of melinjo (*Gnetum gnemon* L.) seed extract: acute and subchronic toxicity studies. *Food Chem Toxicol* **67**, 230–235. Erratum in: *Food Chem Toxicol* **69**, 360.
  28. Tohidpour A, Morgun AV, Boitsova EB, Malinovskaya NA, Martynova GP, Khilazheva ED, Kopylevich NV, Gertsog GE and Salmina AB (2017) Neuroinflammation and infection: molecular mechanisms associated with dysfunction of neurovascular unit. *Front Cell Infect Microbiol* **7**, 276.
  29. Tomioka R and Matsui M (2014) Biomarkers for multiple sclerosis. *Intern Med* **53**, 361–365.
  30. Xicoy H, Wieringa B and Martens GJ (2017) The SH-SY5Y cell line in Parkinson's disease research: a systematic review. *Mol Neurodegener* **12**, 10.
  31. Yoshida H, Imaizumi T, Lee SJ, Tanji K, Sakaki H, Matsumiya T, Ishikawa A, Taima K, Yuzawa E, Mori F, Wakabayashi K, Kimura H and Satoh K (2007) Retinoic acid-inducible gene-I mediates RANTES/CCL5 expression in U373MG human astrocytoma cells stimulated with double-stranded RNA. *Neurosci Res* **58**, 199–206.
  32. Yoshida H, Meng P, Matsumiya T, Tanji K, Hayakari R, Xing F, Wang L, Tsuruga K, Tanaka H, Mimura J, Kosaka K, Itoh K, Takahashi I and Imaizumi T (2014) Carnosic acid suppresses the production of amyloid- β 1–42 and 1–43 by inducing an α -secretase TACE/ADAM17 in U373MG human astrocytoma cells. *Neurosci Res* **79**, 83–93.
  33. Waisman A, Liblau RS and Becher B (2015) Innate and adaptive immune responses in the CNS. *Lancet Neurol* **14**, 945–955.