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Galectin-3 Exerts Cytokine-Like Regulatory Actions through the JAK–STAT Pathway

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Galectin-3, a β-galactoside–binding lectin, has been proposed to have multifaceted functions in various pathophysiological conditions. However, the characteristics of galectin-3 and its molecular mechanisms of action are still largely unknown. In this study, we show that galectin-3 exerts cytokine-like regulatory actions in rat and mouse brain-resident immune cells. Both the expression of galectin-3 and its secretion into the extracellular compartment were significantly enhanced in glia under IFN-γ–stimulated, inflamed conditions. After exposure to galectin-3, glial cells produced high levels of proinflammatory mediators and exhibited activated properties. Notably, within minutes after exposure to galectin-3, JAK2 and STAT1, STAT3, and STAT5 showed considerable enhancement of tyrosine phosphorylation; thereafter, downstream events of STAT signaling were also significantly enhanced. Treatment of the cells with pharmacological inhibitors of JAK2 reduced the galectin-3–stimulated increases of inflammatory mediators. Using IFN-γ receptor 1–deficient mice, we further found that IFN-γR 1 might be required for galectin-3–dependent activation of the JAK–STAT cascade. However, galectin-3 significantly induced phosphorylation of STATs in glial cells from IFN-γ–deficient mice, suggesting that IFN-γ does not mediate activation of STATs. Collectively, our findings suggest that galectin-3 acts as an endogenous danger signaling molecule under pathological conditions in the brain, providing a potential explanation for the molecular basis of galectin-3–associated pathological events. ***The Journal of Immunology*****, 2010, 185: 7037–7046.**

Galectins are a growing family of β-galactoside–binding proteins with carbohydrate-recognition domains (CRDs). To date, 15 galectins have been identified in mammals, each having one or two CRDs of ~130 aa. Galectins have been classified into three subgroups based on their structures and how many CRDs they include; these subgroups are the prototype, chimera type, and tandem repeat type (1). Different galectins have specific characteristics in their distributions, expression patterns, and binding abilities and have distinctive functions as modulators in a variety of phenomena. Galectin-3, a unique chimera-type member of the β-galactoside–binding soluble lectin family, has been proposed to have multifaceted functions (2). It has been found in the nucleus and cytoplasm, at the cell surface, and even in the extracellular fluid surroundings of several cell types although lacking classical signal sequence required for secretion. Both extracellular and intracellular galectin-3 have been shown to exert diverse functions in cell adhesion, migration, chemotaxis, phagocytosis, and apoptosis (3, 4). Indeed, the association of galectin-3 with pathological conditions has been demonstrated both in patients and in several experimental models of immune diseases and cancer (5–7).

In recent years, galectin-3 has attracted attention as a potent immune regulator (1, 8). Increasing evidence suggest that galectin-3 can activate various types of immune-associated cells, including neutrophils, monocytes, dendritic cells, macrophages, and mast cells (9–12). In addition, increased expression and secretion of galectin-3 have been observed in inflammatory milieu, including renal fibrosis, hepatic fibrosis, myocarditis, diabetes, allergic airway inflammation, and experimental pneumococcal meningitis (13–17). Moreover, studies in galectin-3–deficient mice strongly indicated that galectin-3 plays important roles as a proinflammatory mediator in the immune and inflammatory responses associated with pathological conditions. For example, galectin-3–deficient mice displayed defective neutrophil recruitment and decreased macrophage survival (18). It has also been reported that galectin-3–deficient mice exhibited reduced NF-κB responses and decreased cytokine production in several cell types (19, 20). However, although considerable progress has been made in understanding the functions of galectin-3 in immune systems, the molecular mechanisms by which galectin-3 is regulated under pathophysiological conditions and by which galectin-3 mediates immune and inflammatory responses remain to be elucidated.

The JAK family and STAT family are activated by numerous cytokines and stimuli. They are responsible for regulating the initiation, propagation, and resolution of inflammation and are closely linked to diverse cellular events, including cell growth, survival, and differentiation. Indeed, accumulating evidence indicates that dysregulation of the JAK–STAT signaling pathway can cause diverse disease states, including cancer and viral infections (21). Upon ligands binding to their cognate cell-surface receptors, the receptors undergo conformational changes, leading to auto- or cross-phosphorylation of tyrosine residues on receptor-associated JAKs. The activated JAKs phosphorylate tyrosine residues on the cytoplasmic tail of the receptor, thereby providing a docking site for the STAT family. After being recruited to the receptor complex,

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Abbreviations used in this paper: CRD, carbohydrate-recognition domain; GAS, γ-IFN–activated sequence; IFNGR1, IFN-γ receptor 1; iNOS, inducible NO synthase; KO, knockout; MFI, mean fluorescence intensity; PA, primary astrocytes; pS-STAT, phospho-serine STAT; pY-STAT, phospho-tyrosine STAT; WT, wild-type.

STATs become phosphorylated on conserved tyrosine residues by JAKs and then are released from the receptor complex and form dimers. These dimers, with or without additional serine phosphorylation, translocate to the nucleus where they directly bind to specific transcription elements and regulate expression of inflammation-associated genes (22).

We questioned whether galectin-3 could act as a regulatory molecule under pathological conditions in the brain, especially during inflammation-associated pathological events. In an effort to address this, we investigated the characteristics of galectin-3 and its roles in rat and mouse brain-resident immune cells, microglia, and astrocytes. In this study, we show that galectin-3 may act as an endogenous regulator that could activate brain-resident immune cells, especially under IFN-γ–stimulated, inflamed conditions. Furthermore, we found that extracellular galectin-3 is able to activate immune and inflammatory signaling events through phosphorylation of STAT1, STAT3, and STAT5 as well as JAK2. Our results reveal, to our knowledge, a novel function of galectin-3 in the brain and provide insight into the molecular basis of its regulatory properties in the inflammatory context. These findings may contribute to our understanding of how galectin-3 acts as a modulator under pathological conditions, particularly in the case of inflammation-associated brain diseases including cancer.

Materials and Methods

Animals

Sprague Dawley rats were purchased from SamTako Bio Korea (Osan, Korea). C57BL/6, B6.129S7-*Ifngr*^{tm1Agt}/J, and B6.129S7-*Ifng*^{tm1Ts}/J mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All animal procedures were performed according to the National Cancer Center guidelines for the care and use of laboratory animals.

Reagents

Recombinant galectin-3 was purchased from Cell Science (Canton, MA). IFN-γ, AG490, and JSI-124 were purchased from Calbiochem (San Diego, CA). Abs against phospho-tyrosine STAT1, phospho-tyrosine STAT3, phospho-serine STAT3, phospho-tyrosine STAT5, phospho-tyrosine STAT6, total STAT1, and total STAT5 were purchased from Cell Signaling Technology (Beverly, MA). Galectin-3 and tubulin Abs were obtained from Affinity BioReagents (Golden, CO) and Sigma (St. Louis, MO), respectively. Inducible NO synthase (iNOS), phospho-JAK2, and total JAK2 Abs were purchased from Millipore (Temecular, CA).

Cell culture

Primary microglia and astrocytes were cultured from the cerebral cortices of 1- to 3-d-old mice or Sprague Dawley rats (SamTako Bio Korea). Briefly, cortices were triturated into single cells in MEM (Sigma) containing 10% FBS (Hyclone, Logan, UT), plated in 75-cm^2 T-flasks (4 hemispheres/flask for mice, 0.5 hemisphere/flask for rats) and incubated for 2 wk. The microglia were detached from the flasks by mild shaking and applied to a nylon mesh to remove astrocytes and cell clumps. Cells were plated in 6-well plates (5×10^5 cells/well), 60 mm^2 dishes (8×10^5 cells/dish), or 100 mm^2 dishes (2×10^6 cells/dish). One hour later, unattached cells were removed by washing, and the remaining cells were used in experiments. After removal of the microglia, primary astrocytes were prepared by trypsinization. Adult brain glial cells were cultured from the cerebral cortices of 8-wk-old mice (SamTako Bio Korea). Briefly, cortices were incubated with 0.1% trypsin for 10 min and then triturated into single cells in MEM containing 10% FBS. The cells were plated in 75-cm^2 T-flasks (1 hemisphere/flask) and incubated for 2 wk. Rat B35 glioma and BV2 murine microglial cell lines were cultured in DMEM (Life Technologies, Grand Island, NY) supplemented with 5% FBS.

RT-PCR analysis

Total RNA was extracted using RNAzol B (TEL-TEST, Friendswood, TX), and cDNA was prepared using reverse transcriptase that originated from avian myeloblastosis virus (TaKaRa), according to the manufacturer's instructions. Oligonucleotide primers were purchased from Bioneer (Seoul, Korea). The sequences of PCR primers were previously reported (23) or as follows: (F) 5′-ATC TGG AGG AAC TGG CAA AAG GAC G-3′ and (R) 5′-CCT TAG GCT AGA TTC TGG TGA CAG C-3′ for rat IFN-γ; (F) 5′-TGG CTT CTA GTG CTG ACG C-3′ and (R) 5′-TAG TTT GGA CAG GAT CTG GC-3′ for rat TGF-β; (F) 5′-TGC CTT CAG TCA AGT GAA GAC-3′ and (R) 5′-AAA CTC ATT CAT GGC CTT GTA-3′ for rat IL-10; (F) 5′-GCA GAA TGT GAC CAT CAT GG-3′ and (R) 5′-ACA ACC TTG GTG TTG AAG GC-3′ for rat iNOS; (F) 5′-CTC ACC TGC TGC TAC TCA TTC-3′ and (R) 5′-GCT TGA GGT GGT TGT GGA AAA-3′ for mouse MCP-1; (F) 5′-TTT AAT GAA AGC GTT TAG CC-3′ and (R) 5′-TTC GGC AGT TAC TTT TGT CT-3′ for mouse IP-10; (F) 5′-TCA CTG GGA CAG CAC AGA AT-3′ and 5′-TGT GTC TGC AGA TGT GCT GA-3′ for mouse iNOS; (F) 5′-CAT GTT TGA GAC CTT CAA CAC CCC-3′ and (R) 5′-GCC ATC TCC TGC TCG AAG TCT AG-3′ for mouse actin.

Western blot analysis

Cells were washed twice with cold PBS and then lysed in ice-cold modified RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM Na_3VO_4, and 1 mM NaF) containing protease inhibitors (2 mM PMSF, 100 μg/ml leupeptin, 10 μg/ml pepstatin, 1 μg/ml aprotinin, and 2 mM EDTA). The lysates were centrifuged at 12,000 × *g* for 10 min at 4°C, and the supernatant was collected. Proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane, which was incubated with primary Abs and peroxidase-conjugated secondary Abs (Vector Laboratories, Burlingame, CA). Blots were first probed with Abs against the target protein; the blot was then stripped and sequentially reprobed with Abs against loading-control proteins. The results were visualized using an ECL system (Sigma).

Flow cytometry

Cells were washed twice with PBS containing 1% FBS, collected, and stained with FITC-conjugated anti-mouse ICAM-1 or isotype control Ab (eBioscience, San Diego, CA) for 30 min at 4°C. After washing, the cells were analyzed with a FACSCalibur (BD Biosciences, San Jose, CA). The data analyses were done with CellQuest software (BD Biosciences), FlowJo (Tree Star, Ashland, OR), and Win-MDI software (The Scripps Research Institute, La Jolla, CA).

Phagocytosis assay

Cells were plated in 60 mm^2 dishes (2.5×10^4 cells/dish) and then either treated or left untreated with galectin-3. Phagocytic capacity was measured by incubating cells with FITC-conjugated phagocytic beads (8×10^6 beads/ml; FluoSpheres polystyrene microspheres; Molecular Probes, Eugene, OR) at 37°C. Subsequently, cells were washed three times with PBS and then gently removed from the wells using cell scrapers. Then, cells containing FITC-conjugated phagocytic beads were analyzed by FACSCalibur or fluorescence microscopy (Zeiss, Jena, Germany).

Immunofluorescence and confocal microscopy

Cells were plated onto 12-mm round coverslips (Fisher Scientific, Pittsburgh, PA), untreated or treated with IFN-γ, and fixed with ice-cold 100% methanol. The cells were permeabilized with PBS containing 0.1% Triton X-100 and blocked with 1% BSA (Sigma). The samples were then washed with PBS containing 0.1% Triton X-100 and incubated overnight at 4°C with galectin-3 Ab and then secondary Ab. DAPI (DAPI hydrochloride) was used to stain nuclei. Images were obtained with a Carl Zeiss LSM510 confocal microscope and analyzed by LSM software release 3.2 (Carl Zeiss, Jena, Germany).

Electrophoretic mobility shift assay

Cells were stimulated with 1 μg/ml galectin-3 for 30 min, and nuclear extracts were prepared as previously described (23). The NF-κB gel shift oligonucleotide used had the following sequence: 5′-AGT TGA GGG GAC TTT CCC AGG C-3′ (sc-2505; Santa Cruz Biotechnology, Santa Cruz, CA). The probes were 5′-end labeled with 40 μCi [γ-^{32}P]ATP using T4 polynucleotide kinase (Promega, Madison, WI) and purified on Sephadex G-25 Quick Spin Columns (Roche Molecular Biochemicals, Indianapolis, IN).

Statistical analysis

All data were expressed as the mean ± SD and analyzed by one-way ANOVA followed by post hoc comparisons (Student–Newman–Keuls test) using the Statistical Package for Social Sciences 8.0 (SPSS, Chicago, IL).

Results

Galectin-3 expression and its secretion into the extracellular space are distinctively upregulated by IFN-γ treatment in microglia and astrocytes

Galectin-3 has recently attracted attention as a multifunctional regulatory molecule in diverse biological events. In an effort to

explore the function of galectin-3 under pathological conditions in the brain, we investigated the characteristics of galectin-3 after exposure to representative inflammatory stimuli in rat and mouse brain-resident immune cells, microglia, and astrocytes. Notably, we found that galectin-3 expression was distinctively upregulated by IFN-γ exposure in both microglia and astrocytes. Confocal microscopy showed that treatment of primary microglia from rats and mice with 10 U/ml IFN-γ considerably increased the expression level of galectin-3 (Fig. 1*A*, Supplemental Fig. 1 and data not shown). Similar patterns of galectin-3 expression were observed in primary astrocytes (Fig. 1*A* and data not shown). Western blot analysis also showed that IFN-γ treatment induced expression of galectin-3 at the protein level in both mouse and rat glial cells (Fig. 1*B* and data not shown). To examine further the effects of IFN-γ on galectin-3, we investigated whether galectin-3 could be secreted from IFN-γ–exposed glial cells, as galectin-3 is reportedly secreted from inflammatory cells (3, 15). As shown in Fig. 1*B* and 1*C*, IFN-γ treatment significantly augmented the intracellular expression of galectin-3 within 6 h, followed by its secretion into extracellular space in BV2 microglial cells and rat primary astrocytes. These findings indicate that both the expression and the secretion of galectin-3 are affected by IFN-γ in brain-resident immune cells, supporting the notion that galectin-3 plays a role in the inflamed brain.

To validate these results, we examined the expression and secretion levels of galectin-3 in the B35 rat glioma cell line, as tumor can be considered a representative IFN-γ–overproducing pathological condition in the brain. Consistent with the previously described results, confocal microscopy and Western blot analysis revealed that the basal expression and secretion levels of galectin-3 were obviously higher in B35 rat glioma cells compared with those in normal rat primary astrocytes (Fig. 1*D*). In addition, Western blot analysis revealed that adenovirus-infected BV2-microglial cells, which have significantly upregulated IFN-γ levels due to the viral infection, also show increased secretion of galectin-3 into the culture media (Supplemental Fig. 2). Collectively, these findings indicate that IFN-γ may regulate galectin-3 in the brain and suggest a possible mechanism through which galectin-3 could be modulated under pathological conditions.

Galectin-3 stimulates the expression of proinflammatory mediators and triggers glial activation

A great deal of attention has recently focused on the cytokine-like actions of galectin-3 and the contribution of secreted galectin-3 to inflammation-associated diseases (15, 24). Having determined that exposure to IFN-γ greatly enhanced the secretion of galectin-3 from microglia and astrocytes, we postulated that galectin-3 might act as a cytokine-like modulator in the brain. To test this possibility, we examined whether galectin-3 could affect the expression level of inflammatory mediators in primary cultured brain-resident immune cells. Rat primary microglia were treated with the indicated concentrations of exogenous galectin-3 for 3 h, and total RNA was extracted for RT-PCR analysis. Our results revealed that galectin-3 treatment markedly induced the transcription of various proinflammatory mediators including TNF-α, IL-6, IL-1β, IL-12p40, IP-10, and even IFN-γ. Using ELISA, we confirmed the increased production of TNF-α, IL-1β, and IL-12 proteins in galectin-3–treated microglia (Fig. 2*A* and data not shown). In contrast, we did not detect any change in the mRNA or protein levels of the anti-inflammatory mediators IL-10, IL-13, or TGF-β (Fig. 2*B* and data not shown). These results suggest that galectin-3 may promote the production of proinflammatory mediators in brain-resident immune cells, thereby potentially contributing to inflammatory conditions in the brain.

To provide further evidence for the proinflammatory action of galectin-3 in the brain, we examined whether galectin-3 could affect the binding activity of NFs to the binding elements of NF-κB, which is a key transcription factor responsible for coordinating innate immunity and inflammation. After rat primary microglia were treated with 1 μg/ml galectin-3 for 30 min, nuclear extracts were prepared and analyzed by EMSA. Our results revealed that galectin-3 significantly stimulated the binding of NFs

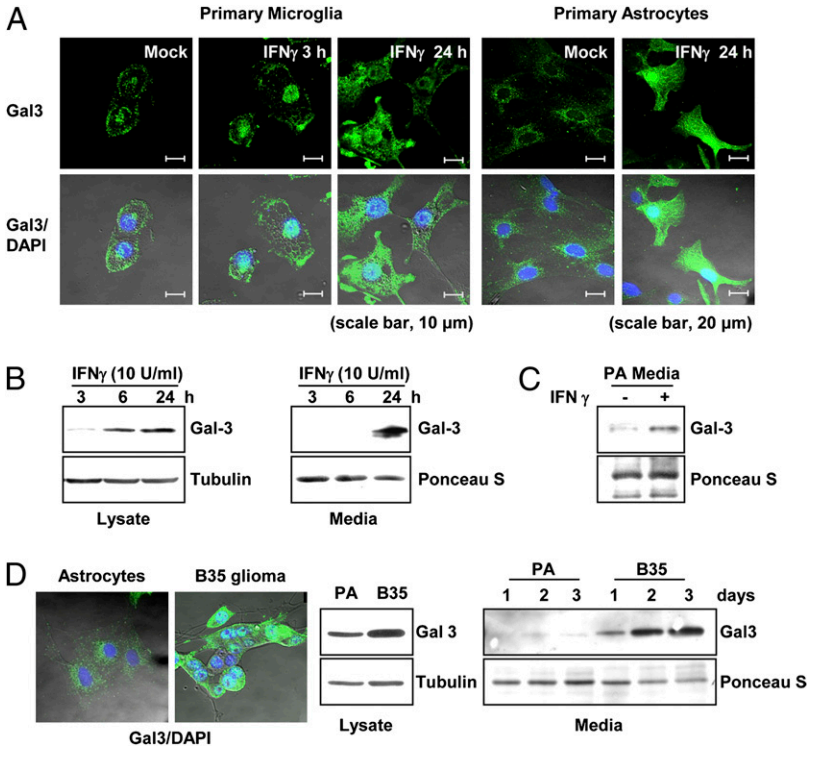

FIGURE 1. IFN-γ stimulates galectin-3 expression and its secretion from glial cells. *A*, Rat primary microglia and astrocytes were treated with 10 U/ml IFN-γ for the indicated times. Cells were stained with an Ab against galectin-3 (green) and Hoechst dye (blue) and photographed under confocal microscope (original magnification ×1260 for microglia and ×630 for astrocytes). All images are representative of at least four independent experiments. *B*, BV2 murine microglial cells were incubated with 10 U/ml IFN-γ for 3, 6, or 24 h. Cell lysates and media were collected and subjected to Western blot analysis using Abs against galectin-3. The blots were stripped and reprobed with tubulin Ab. The membranes were stained with ponceau S as a loading control for media fraction. *C*, Rat primary astrocytes were mock-treated or treated with 10 U/ml IFN-γ for 24 h. Conditioned media were collected and subjected to Western blot analysis using an anti–galectin-3 Ab. The membranes were stained with ponceau S as a loading control. *D*, Rat primary astrocytes (PA) and B35 rat glioma cells were analyzed by immunofluorescence with an anti–galectin-3 Ab (*left*). DAPI is a nuclear staining marker (original magnification ×400). Western blot analysis was performed on cell lysates and conditioned media using an anti–galectin-3 Ab (*right*). Data are representative of at least three independent experiments.

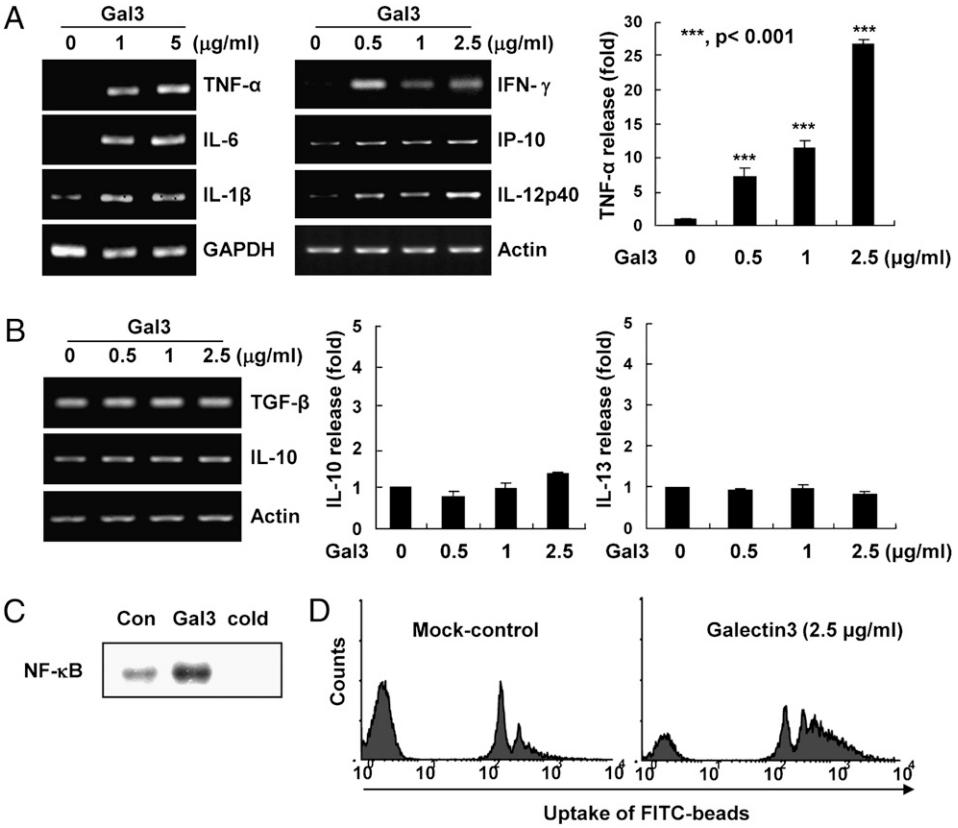

FIGURE 2. Galectin-3 triggers inflammatory events in rat primary microglia. *A* and *B*, Rat primary microglia were mock-treated or treated with the indicated concentrations of galectin-3 for 3 h, and the mRNA levels of TNF-α, IL-6, IL-1β, IFN-γ, IP-10, IL12-p40, TGF-β, and IL-10 were determined by an RT-PCR–based assay (*left*). Cells were incubated with the indicated concentrations of galectin-3 for 18 h, and the levels of TNF-α, IL-10, and IL-13 secreted to the media were measured by ELISA (*right*). The results shown represent the mean ± SD of triplicate wells and are representative of at least four individual experiments. *C*, Rat primary astrocytes were mock-treated or treated with 1 μg/ml galectin-3 for 30 min, after which nuclear extracts were prepared, and EMSA was used to assay the amount of binding activity to the NF-κB binding element. The specificity of the binding complex was confirmed by a competition assay using an excess amount of unlabeled oligonucleotides (20×). Data are representative of three independent experiments. *D*, BV2 microglial cells were mock-treated or treated with 2.5 μg/ml galectin-3 for 15 h. The cells were incubated with FITC-conjugated fluorescent beads for 1 h, and the cellular uptake of fluorescent beads was determined by flow cytometric analysis. $***p < 0.001$ when compared with mock-treated cells.

to the NF-κB elements within 30 min (Fig. 2*C*). This supports our hypothesis that galectin-3 may play proinflammatory roles in the brain.

An attribute of activated glia is phagocytosis, which is accompanied by secretion of inflammatory mediators. To assess the functional activity of galectin-3–exposed glia, we used FITC-conjugated fluorescent beads to examine phagocytic activity. FACS analysis and fluorescent microscopy showed that treatment of BV2 microglial cells with galectin-3 significantly increased the cellular uptake of fluorescent beads, indicating that galectin-3 treatment triggered microglial activation (Fig. 2*D* and data not shown). Overall, these results show that galectin-3 is capable of activating inflammatory mediators and promoting phagocytic activity, resulting in inflammatory conditions in the brain.

Galectin-3 triggers the phosphorylation of JAK2 and STAT1, STAT3, and STAT5 in primary microglia and astrocytes

The above findings led us to question how galectin-3 could trigger the activation of glia. To investigate the molecular mechanism(s) underlying the proinflammatory action of galectin-3, we tested for links between galectin-3 and various signaling molecules. Notably, Western blot analysis showed that galectin-3 treatment rapidly induced tyrosine phosphorylation of STAT1 within 5 min in rat primary microglia (Fig. 3*A*). In addition, STAT3 and STAT5 also showed marked phosphorylation on tyrosine-residues in galectin-3–treated rat primary microglia (Fig. 3*A*, 3*B*). Similar results were observed in rat primary astrocytes and mouse primary glia (data not shown). In contrast, we did not observe any significant change in the serine-phosphorylation of STAT3 (Fig. 3*A*). To confirm that galectin-3 stimulates phosphorylation of STATs in glial cells, we examined the effects of galectin-3 in primary glial cells from 8-wk-old mice. Galectin-3 significantly enhanced the phosphorylation of STAT1 and STAT3 in glial cells from adult mice and in those from newborn mice (Fig. 3*C*).

Phosphorylation of STATs depends on the phosphorylation of JAKs. In particular, activation of JAK2 has been linked with phosphorylation of STAT1, STAT3, and STAT5 (25). Thus, we next examined whether JAK2 could be involved in the galectin-3–induced phosphorylation of the noted STATs. As shown in Fig. 3*D*, phosphorylation of JAK2 was also enhanced by treatment with galectin-3 in rat primary microglia. To our knowledge, this is the first report to show that galectin-3 activates the tyrosine-phosphorylation of JAK2 and STAT1, STAT3, and STAT5. This finding suggests that galectin-3 may trigger the activation of microglia and astrocytes by activating the JAK–STAT signaling pathway.

Galectin-3 activates signaling events downstream of JAK–STAT phosphorylation

STATs are essential transcription factors that regulate the expression of a number of inflammatory mediators, including cytokines and chemokines (21, 22). To evaluate the functional importance of the galectin-3–stimulated JAK–STAT cascade, we in-

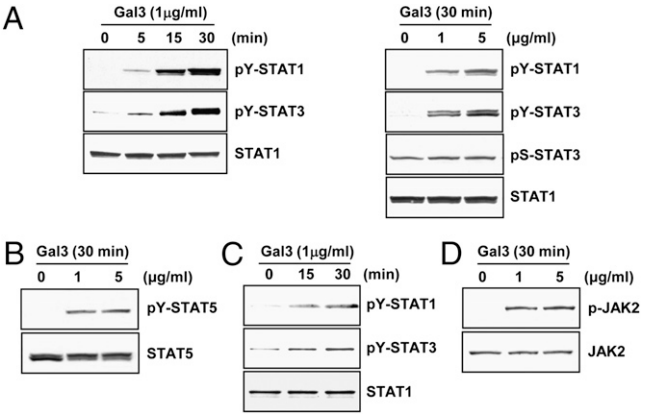

FIGURE 3. Galectin-3 rapidly triggers phosphorylation of JAK2 and STAT1, STAT3, and STAT5 in primary glia. *A*, Primary microglia from 1-d-old newborn rat were mock-treated or treated with the indicated concentrations of galectin-3 for the indicated times. Cell lysates were subjected to Western blot analysis using Abs against phospho-tyrosine STAT1 (pY-STAT1), phospho-tyrosine STAT3 (pY-STAT3), phospho-serine STAT3 (pS-STAT3), and total STAT1. *B*, Rat primary microglia were mock-treated or treated with the indicated concentrations of galectin-3 for 30 min, followed by Western blot analysis using phospho-tyrosine STAT5 (pY-STAT5) Ab. The blot was stripped and reprobed with an Ab against total STAT5. *C*, Primary glial cells were prepared from 8-wk-old mice, and Western blot assays were performed to determine the level of pY-STAT1. The blot was stripped and sequentially reprobed with pY-STAT3 and total STAT1. *D*, Rat primary microglia were treated with the indicated concentrations of galectin-3 for 30 min, followed by Western blot analysis using Abs against pY-JAK2. The blot was stripped and reprobed with total JAK2. Data shown are representative of at least three independent experiments.

vestigated the events downstream of the STATs that were activated by galectin-3 treatment. First, we examined the expression levels of several inflammatory mediators whose promoters contain STAT binding sites. Rat primary microglia were treated with galectin-3 for 18 h, and the cell surface expression levels of ICAM-1, an inflammation-associated chemokine, were determined by FACS analysis. We found that galectin-3 treatment notably increased the cell surface expression of ICAM-1 (Fig. 4*A*). We then used RT-PCR analysis to examine the mRNA levels of several inflammatory mediators in cells treated with galectin-3. Compared with mock-treated control cells, the mRNA levels of MCP-1 and IL-8 were significantly elevated within 3 h after treatment with galectin-3 (Fig. 4*B*). We also observed elevated expression of iNOS at both the mRNA and protein levels after exposure to galectin-3 (Fig. 4*C*). Similar results were obtained in rat primary astrocytes (data not shown). To demonstrate more clearly the effects of galectin-3 on STAT-dependent transcription, we examined the promoter activity of the γ-IFN–activated sequence (GAS), which is the representative binding site for STATs. In agreement with the above results, GAS promoter activity was markedly increased by treatment of primary astrocytes with galectin-3 (Fig. 4*D*). These findings support the notion that galectin-3 is able to activate STATs, thereby promoting STAT-dependent gene transcription.

Next, we assessed the effects of AG490 and JSI-124, pharmacological inhibitors of JAKs, on the expression of galectin-3–induced inflammatory mediators at the mRNA and protein levels. Compared with cells treated with galectin-3 alone, the mRNA levels of IL-6 were reduced in cells treated with AG490 or JSI-124. In addition, both AG490 and JSI-124 significantly reduced galectin-3–enhanced iNOS expression at the protein and transcript levels (Fig. 4*E* and data not shown). These results indicate that the JAK–STAT cascade is closely involved in galectin-3–triggered expression of IL-6 and iNOS. Collectively, these findings convincingly demonstrate that galectin-3 activates phosphorylation of JAK2 and various STATs, thus regulating immune and inflammatory events in the brain.

The CRD of galectin-3 is not essential for activation of STATs

We next examined how galectin-3 might activate the JAK–STAT pathway in primary microglia and astrocytes. As previous reports have shown that galectin-3 can interact with several proteins through its CRD domain (26–28), we investigated whether the galectin-3 CRD was required for activation of JAK–STAT signaling. Primary microglia were treated with 1 μg/ml galectin-3 in the absence or presence of lactose, a competitive natural inhibitor for the CRD (29, 30). Unexpectedly, the presence of the competitor did not change the galectin-3–induced phosphorylation of STAT1 or STAT3, regardless of the tested concentration of lactose (Fig. 5). Similarly, we did not observe reduced phosphorylation of STAT1 in the presence of mannose, which has a very low affinity to the CRD of galectin-3 (31, 32). These data suggest that the CRD of galectin-3 is not essential for galectin-3–induced phosphorylation of JAK2 and the STATs.

Galectin-3–triggered phosphorylation of STATs is not observed in Ifngr1$^{-/-}$ *mice*

In the extracellular context, galectin-3 has been reported to bind to cell surface receptor (33–35). Because IFN-γ receptor 1 (IFNGR1) has been shown to be responsible for ligand binding and activation of the JAK–STAT pathway (36), we questioned whether IFNGR1 could be associated with galectin-3–triggered phosphorylation of the JAK2-STAT cascade. To address this, we examined galectin-3–dependent phosphorylation of JAK2 and STATs in IFNGR1 knockout (KO) mice (*Ifngr1*$^{-/-}$ mice). Primary astrocytes were cultured from *Ifngr1*$^{-/-}$ and wild-type (WT) mice and treated with 1 μg/ml galectin-3 for the indicated times. Intriguingly, galectin-3 treatment markedly enhanced the phosphorylation of STAT1 in primary astrocytes from normal mice but not in those from *Ifngr1*$^{-/-}$ mice (Fig. 6*A*). To demonstrate more clearly the involvement of IFNGR1 in galectin-3–induced activation of the JAK2-STAT pathway, we investigated the level of phosphorylated STAT1 after treatment of primary astrocytes from *Ifngr1*$^{-/-}$ and WT mice with galectin-3, IFN-γ, and LPS (Fig. 6*B*). In astrocytes from WT mice, phosphorylation of STAT1 was upregulated by all three tested stimuli. However, in *Ifngr1*$^{-/-}$ mice, STAT1 phosphorylation was triggered by LPS, but not by galectin-3 or IFN-γ. In addition, we did not detect phosphorylation of STAT1, STAT3, and STAT5 in primary microglia from *Ifngr1*$^{-/-}$ mice (Fig. 6*C*). Similarly, iNOS expression was induced in primary glia from *Ifngr1*$^{-/-}$ mice after their exposure to LPS but not galectin-3 or IFN-γ (Fig. 6*D*). These results show that IFNGR1 is necessary for activation of JAK–STAT signaling by galectin-3 and IFN-γ but not LPS.

We then examined the transcription of representative inflammatory mediators that contain STAT-binding sites in their gene promoters in the context of WT mice and *Ifngr1*$^{-/-}$ mice. The mRNA levels of iNOS were elevated within 3 h after galectin-3 treatment of primary microglia from normal mice but not in those from *Ifngr1*$^{-/-}$ mice (Fig. 7*A*). In addition, the galectin-3–triggered enhancement of MCP-1 mRNA levels was considerably reduced in cells from *Ifngr1*$^{-/-}$ mice compared with those in WT mice, and we did not observe significant galectin-3–induced enhancement of IP-10 mRNA expression in cells from *Ifngr1*$^{-/-}$ mice (Fig. 7*B*). Taken together, these results show that both

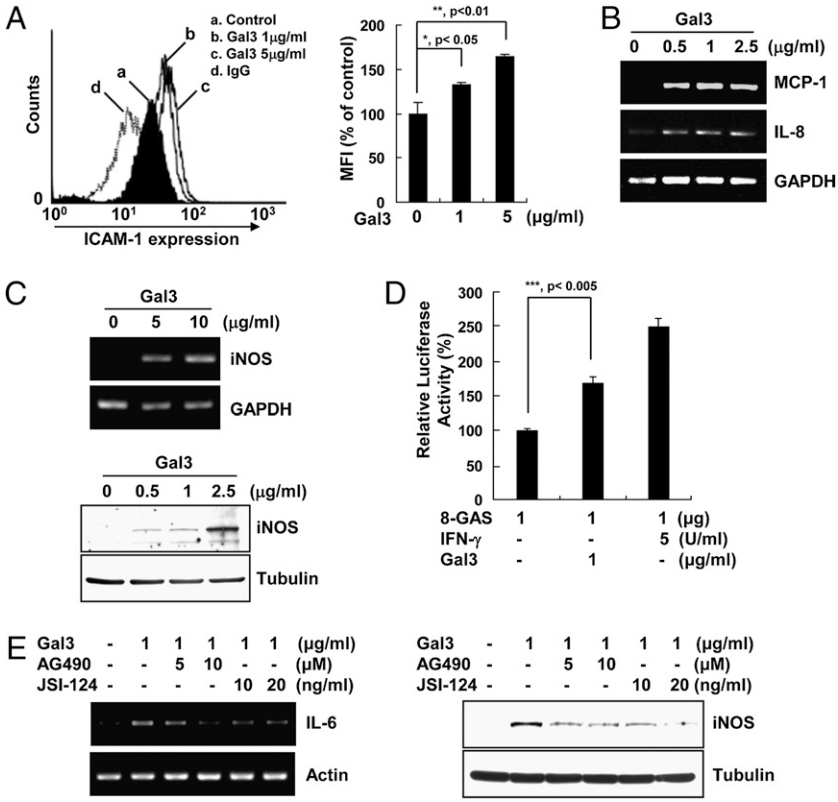

FIGURE 4. Downstream events of the STATs are also activated by exogenously added galectin-3. *A*, Rat primary microglia were treated with 1 or 5 μg/ml galectin-3 for 18 h, and the cell surface expression of ICAM-1 was analyzed using flow cytometry. The mean fluorescence intensity (MFI) values represent the mean ± SD of three independent experiments. *B*, Rat primary microglia were treated with the indicated concentrations of galectin-3 for 3 h, and the mRNA levels of MCP-1, IL-8, and GAPDH were determined by RT-PCR. *C*, Rat primary microglia were treated with the indicated concentrations of galectin-3, followed by RT-PCR analysis and Western blot analysis. *D*, Rat primary astrocytes were transfected with an 8-GAS luciferase construct, and the cells were untreated or treated with 1 μg/ml galectin-3 or 5 U/ml IFN-γ (as a positive control) for 24 h. Cell extracts were then subjected to a luciferase activity assay. The results shown represent the mean ± SD of triplicate experiments and are representative of four individual experiments. *E*, Rat primary microglia were pretreated with AG490 or JSI-124, and then left untreated or treated with 1 μg/ml galectin-3 for 3 h. The mRNA level of IL-6 was determined by RT-PCR (*left*). Rat primary microglia were incubated with galectin-3 for 18 h in the absence or presence of AG490 or JSI-124, and Western blot analysis was performed using Abs against iNOS. The blots were stripped and reprobed with tubulin Ab (*right*). Data shown are representative of at least three independent experiments. $*p < 0.05$; $**p < 0.01$; $***p < 0.005$ when compared with mock-treated cells.

galectin-3–triggered phosphorylation of STATs and downstream events of the JAK–STAT cascade are not observed in *Ifngr1*$^{-/-}$ mice. These findings suggest that galectin-3 might activate phosphorylation of the JAK–STAT pathway through IFNGR1, thereby activating brain-resident immune cells.

IFN-γ does not mediate the galectin-3–triggered phosphorylation of STATs

Because the effects of galectin-3 on STATs phosphorylation were not observed in *Ifngr1*$^{-/-}$ mice, we considered the possibility that galectin-3 might enhance endogenous IFN-γ–inducing signaling in glial cells. To address it, we examined the effects of galectin-3 on phosphorylation of STATs in IFN-γ KO (*Ifng*$^{-/-}$) mice. Primary cultured microglia or astrocytes from WT or *Ifng*$^{-/-}$ mice were mock-treated or treated with 1 μg/ml galectin-3 for 30 min. As shown in Fig. 8*A*, phosphorylation of STAT1 and STAT3 was significantly enhanced by galectin-3 in microglia from both WT mice and *Ifng*$^{-/-}$ mice. Similar results were obtained in primary astrocytes from *Ifng*$^{-/-}$ mice (data not shown). In addition, iNOS expression was markedly induced by exposure to galectin-3 in primary microglia and astrocytes from *Ifng*$^{-/-}$ mice (Fig. 8*B* and data not shown). These results indicate that galectin-3 does not activate the JAK–STAT cascade through IFN-γ in glial cells.

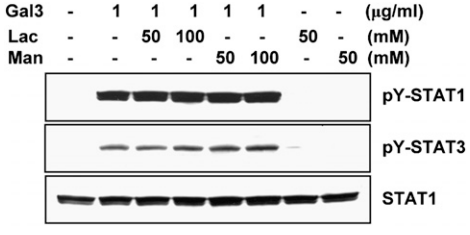

FIGURE 5. CRD does not mediate galectin-3–stimulated phosphorylation of STAT. Primary microglial cells were incubated with 50–100 mM lactose or mannose and then treated with 1 μg/ml galectin-3 for 30 min. Western blot analysis was performed using Abs for pY-STAT1, pY-STAT3, and total STAT1, respectively.

Discussion

Accumulating experimental and clinical evidence has revealed that galectin-3 has distinctive expression patterns and plays critical roles in various pathological conditions, including cancer (6, 37–40). Galectin-3 appears to function through both intracellular and extracellular actions, thereby modulating health and disease (1). However, the cellular and molecular mechanisms through which galectin-3 contributes to pathophysiological events remains poorly understood. In this study, we have focused our attention on the function of extracellular galectin-3 and its signaling cascade in two major brain-resident immune cell types: microglia and astro-

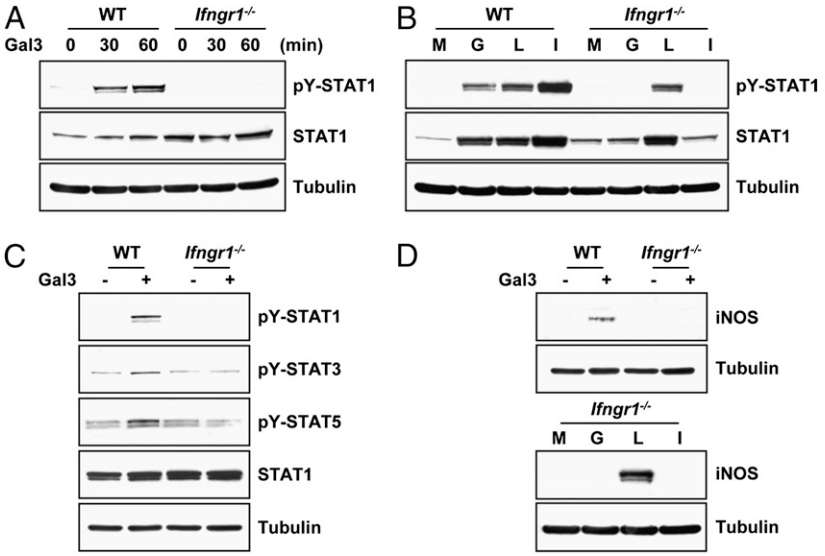

FIGURE 6. Galectin-3–dependent phosphorylation of STATs is not observed in *Ifngr1*$^{-/-}$ mice. *A*, Primary astrocytes from WT or *Ifngr1*$^{-/-}$ mice were treated with 1 μg/ml galectin-3 for the indicated times, after which the phosphorylated levels of STAT1 were analyzed by Western blot analysis. Data shown are representative of at least four independent experiments. *B*, Primary astrocytes from WT or *Ifngr1*$^{-/-}$ mice were mock-treated (M) or treated with 1 μg/ml galectin-3 (G), 50 ng/ml LPS (L), or 10 U/ml IFN-γ (I) for 24 h, followed by Western blot analysis using Abs against pY-STAT1. The blot was stripped and reprobed with Abs against total STAT1 and tubulin, respectively. *C*, Primary microglia from WT or *Ifngr1*$^{-/-}$ mice were mock-treated or treated with 1 μg/ml galectin-3 for 30 min, and Western blot analysis was performed using pY-STAT1, pY-STAT3, pY-STAT5, total STAT1, and tubulin antibodies. *D*, Primary microglia from WT or *Ifngr1*$^{-/-}$ mice were mock-treated (M) or treated with 1 μg/ml galectin-3 (G), 50 ng/ml LPS (L), or 10 U/ml IFN-γ (I) for 24 h, followed by Western blot analysis using Abs against iNOS and tubulin.

cytes. These cells, which are responsible for orchestrating immune and inflammatory responses in the brain, can recognize subtle changes in the brain milieu and mount immune responses against pathological stimuli (41). Our current study shows that galectin-3 is secreted into the extracellular compartment under IFN-γ–overproducing pathological conditions, where it modulates the inflammatory responses in brain-resident immune cells, thereby affecting disease states in the brain.

The expression levels of galectins have been shown to depend on cell status, with expression changes observed during processes such as differentiation and activation (42). For example, clinical evidence has shown a strong correlation between galectin-3 expression levels and the malignant properties of several types of cancer (40). In the brain, studies have shown that galectin-3 is highly expressed in brain tumor and that this expression may be associated with various tumor attributes, such as migration and invasion (43). We also found that galectin-3 expression is markedly upregulated in a brain tumor xenograft rat model compared with that in normal regions (S.B. Jeon, H.J. Yoon, and E.J. Park, unpublished data). In addition, other studies have shown that galectin-3 is upregulated in prion-infected brain tissue (44), experimental autoimmune encephalomyelitis (7), and experimental pneumococcal meningitis (14). Based on these findings, we hypothesized that galectin-3 might play crucial roles in the inflammatory environment of the brain and investigated which condition could be associated with expression and function of galectin-3 in brain-resident immune cells. In a series of screenings, we found that IFN-γ treatment increased both expression and secretion of galectin-3 in primary cultured microglia and astrocytes (Fig. 1). These results suggest that galectin-3 may be upregulated in response to IFN-γ, which is produced under pathological conditions, thereby affecting disease states. Consistent with this notion, we observed enhanced expression and secretion of galectin-3 in rat B35 glioma cells and adenovirus-infected BV2 microglial cells overexpressing IFN-γ (Fig. 1, Supplemental Fig. 2).

In the extracellular space, galectin-3 can bind to multiple binding partners, potentially allowing it to mediate communication between cells or transmit extracellular signals into intracellular regions (1, 24). Recent findings suggest that galectins have cytokine-like properties that could modulate inflammatory processes

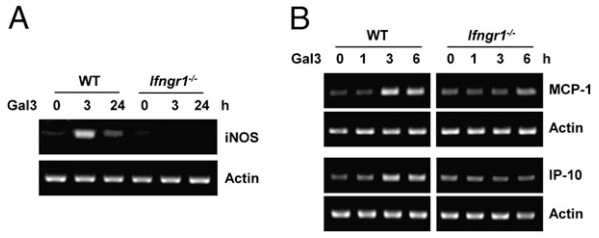

FIGURE 7. Galectin-3–induced transcription of proinflammatory mediators is not observed in primary microglia from *Ifngr1*$^{-/-}$ mice. *A* and *B*, Primary microglia (*A*) and astrocytes (*B*) from WT or *Ifngr1* $^{-/-}$ mice were mock-treated or treated with 1 μg/ml galectin-3 for the indicated times. The mRNA levels of iNOS, MCP-1, IP-10, and actin were determined by RT-PCR analysis.

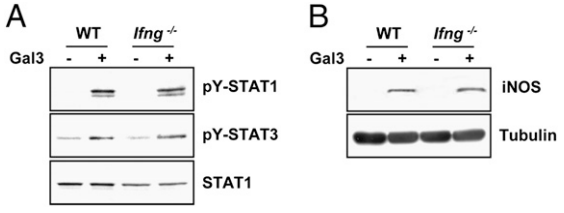

FIGURE 8. Galectin-3 induces phosphorylation of STAT1 and STAT3 in primary glial cells from IFN-γ KO mice. *A*, Primary microglial cells from WT or *Ifng*$^{-/-}$ mice were treated with 1 μg/ml galectin-3 for 30 min, followed by Western blot analysis using Abs against pY-STAT1. The blot was stripped and reprobed with Abs against pY-STAT3 and total STAT1. *B*, Primary astrocytes from WT or *Ifng*$^{-/-}$ mice were incubated with 1 μg/ml galectin-3 for 24 h. Western blot analysis was performed using an Ab against iNOS. The blot was reprobed for tubulin as a loading control.

(19, 45, 46). Consistent with this, our current data indicate that galectin-3 displays cytokine-like properties in brain-resident immune cells, regulating immune and inflammatory responses. Treatment of cultured microglia and astrocytes with galectin-3 considerably increased expression of proinflammatory cytokines and chemokines but not anti-inflammatory mediators. In addition, galectin-3 significantly enhanced the binding of NFs to the NF-κB binding site and increased the phagocytic activities of the tested cells, two characteristics that are representative of activated glia (Fig. 2). These results suggest that secreted galectin-3 from activated glia might act as an endogenous stimulus, which in turn could affect the inflammatory environment of the brain.

The above results led us to have a question regarding the possible mechanism underlying the effects of galectin-3 on the activation of microglia and astrocytes. Notably, we found that galectin-3 treatment activated phosphorylation of JAK2, STAT1, STAT3, and STAT5 within minutes (Fig. 3). In addition, we observed that STAT-dependent transcription of target genes (including cytokines) was significantly enhanced after galectin-3 treatment of microglia and astrocytes. Pharmacological inhibitors of JAKs considerably reduced the downstream events of STAT signaling in galectin-3–treated primary glia. To our knowledge, this is the first demonstration that galectin-3 is able to induce directly phosphorylation of JAK2 and several STATs. The JAK–STAT cascade is a critically important signaling response that regulates diverse physiological and pathological processes, including cancer and immunes diseases. Indeed, recent attention has focused on the development of JAK–STAT cascade-based therapeutic strategies for various inflammation-associated diseases. Our results suggest that there may be a link between extracellular galectin-3 and the JAK–STAT pathway, which is, to our knowledge, a novel insight into the potential regulation of inflamed pathological conditions. These findings not only provide a possible molecular basis for the regulatory roles of galectin-3 but also further support the function of galectin-3 as an amplifier of inflammatory cascades.

Regarding the mechanistic basis for this effect, we questioned whether the IFN-γR could be involved in the galectin-3–dependent phosphorylation of JAK2 and STATs, as this receptor is a receptor that mediates phosphorylation of JAK2, STAT1, STAT3, and STAT5 (47, 48). Intriguingly, galectin-3–dependent phosphorylation of JAK2 and STATs was not detected in *Ifngr1*-deficient mice (Fig. 6). These results strongly suggest that IFNGR1 may be intimately involved in galectin-3–dependent activation of the JAK–STAT cascade. Using IFN-γ KO mice, we further investigated the possibility that galectin-3 affected endogenous IFN-γ and thereby stimulated phosphorylation of STATs. However, we detected no difference in the level of phosphorylated STATs between WT mice and *Ifng*$^{-/-}$ mice. These results indicate that IFNGR1 might be required for galectin-3–dependent activation of the JAK–STAT cascade, but IFN-γ does not mediate activation of STAT signaling in brain glia.

A number of studies have shown that galectin-3 exhibits various extracellular activities through its carbohydrate binding activity (26–28). To gain an insight into the possible mechanism underlying galectin-3–stimulated activation of the JAK–STAT pathway, we tested whether galectin-3 could affect STAT signaling via the CRD domain. Notably, galectin-3–induced phosphorylation of STAT1 and STAT3 was unchanged in the presence of a lactose competitor, whereas galectin-3–induced phosphorylation of Akt was significantly reduced under the same condition (Fig. 5 and data not shown). Considering these competitive experimental results, it seems unlikely that the CRD of galectin-3 is essential for galectin-3–induced phosphorylation of STATs. To date, the majority of previously reported extracellular galectin-3 activities are dependent on its CRD domain (35, 49–52), although some intracellular galectin-3 activities are reported to be independent of carbohydrate-mediated mechanism (53, 54). For example, Markowska et al. (52) have recently shown that galectin-3 modulates vascular endothelial growth factor and basic fibroblast growth factor-mediated angiogenesis by carbohydrate-mediated interaction between galectin-3 and complex *N*-glycans on αvβ3 integrin. In addition, it has been reported that galectin-3 functions by interacting with cell surface receptors including CD98, CD11b/18, and TCR complex at the cell surface (33, 55). Although further research will be required for definite elucidation, our findings suggest that extracellular galectin-3 activates the JAK–STAT pathway in a fashion that might be distinct from the previous findings.

There are a few reports that extracellular galectin-3 can interact with cellular molecules in a CRD-independent manner (50, 56, 57). Mey et al. (56) have reported that galectin-3 interacts with *Salmonella minnesota* R7 LPS independently of the carbohydrate binding activity, whereas it interacts with LPS from *Klebsiella pneumoniae* through the CRD domain. In addition, galectin-3 has been shown to interact with hensin, an extracellular matrix protein, independently of the CRD activity, thus affecting terminal differentiation in epithelial cells (57). However, a careful search of the literature failed to find any experiment or evidence showing the CRD-independent interaction between extracellular galectin-3 and cell surface receptor. To our knowledge, this is the first report suggesting the possibility that extracellular galectin-3 interacts with cell surface receptor in a carbohydrate-independent manner, thus modulating intracellular signaling. Studies have shown that galectin-3 oligomerizes and forms lattice, thus exhibiting various extracellular activities including modulation of the threshold of ligand concentration for signaling (2, 33, 58). Because both the carbohydrate domain and the N-terminal domain are required for oligomerization, it is unlikely that galectin-3 induces phosphorylation of STATs through lattice formation or cross-linking receptors. Although additional detailed studies will be needed to define precisely the interactive mechanism between galectin-3 and IFNGR1, the current study suggests the possibility that galectin-3 may directly interact with IFNGR1 or bind to IFNGR1-binding molecule as monomer, thereby modulating the JAK–STAT pathway.

Gaectin-3 serum levels were reported to be significantly elevated in cancer patients compared with those in healthy individuals, indicating that galectin-3 might function as an endogenous regulator (59). Galectin-3 secreted from activated cells may be able to activate neighboring cells in a paracrine fashion by interacting with molecules in extracellular space or at the cell surface, resulting in the modulation of cell signaling. Our data convincingly support the idea that galectin-3 could act as a cytokine-like regulator, amplifying the inflammatory cascade in the brain. In addition, the current data provide a possible molecular mechanism through which galectin-3 could modulate immune and inflammatory events under pathological conditions. These findings suggest a new direction for the development of interventions targeting galectin-3 for the treatment of pathological inflammatory diseases. Gaining further understanding of the interaction between JAK–STAT signaling and galectin-3 might allow us to identify new molecular targets for the regulation of inflammatory conditions in the brain, leading to novel clinical applications.

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Disclosures

The authors have no financial conflicts of interest.

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