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Galectin-3 Deficiency Reduces the Severity of Experimental Autoimmune Encephalomyelitis[1]

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Galectin-3 (Gal-3) is a member of the β-galactoside-binding lectin family and plays an important role in inflammation. However, the precise role of Gal-3 in autoimmune diseases remains obscure. We have investigated the functional role of Gal-3 in experimental autoimmune encephalomyelitis (EAE) following immunization with myelin oligodendrocyte glycoprotein $(MOG)_{35–55}$ peptide. Gal-3 deficient (Gal-$3^{-/-}$) mice developed significantly milder EAE and markedly reduced leukocyte infiltration in the CNS compared with similarly treated wild-type (WT) mice. Gal-$3^{-/-}$ mice also contained fewer monocytes and macrophages but more apoptotic cells in the CNS than did WT mice. Following Ag stimulation in vitro, lymph node cells from the immunized Gal-$3^{-/-}$ mice produced less IL-17 and IFN-γ than did those of the WT mice. In contrast, Gal-$3^{-/-}$ mice produced more serum IL-10, IL-5, and IL-13 and contained higher frequency of $Foxp3^+$ regulatory T cells in the CNS than did the WT mice. Furthermore, bone marrow-derived dendritic cells from Gal-$3^{-/-}$ mice produced more IL-10 in response to LPS or bacterial lipoprotein than did WT marrow-derived dendritic cells. Moreover, Gal-$3^{-/-}$ dendritic cells induced Ag-specific T cells to produce more IL-10, IL-5, and IL-12, but less IL-17, than did WT dendritic cells. Taken together, our data demonstrate that Gal-3 plays an important disease-exacerbating role in EAE through its multifunctional roles in preventing cell apoptosis and increasing IL-17 and IFN-γ synthesis, but decreasing IL-10 production. ***The Journal of Immunology,*** **2009, 182: 1167–1173.**

Galectins are a family of lectins composed of 15 members that are conserved in the animal kingdom. They recognize galactose-containing oligosaccharides and share sequence similarities in their carbohydrate-binding domain. Many immune cells express galectins that have diverse functions, including triggering cell growth, cell death, and cell migration (1, 2), while galectin-1 is a negative regulator of inflammatory and autoimmune responses in vivo (3). The function of galectin-3 (Gal-3)[3] in inflammatory responses is less clear. Gal-3 expression is increased in inflammatory tissues in patients with inflammatory ocular diseases (4), rheumatoid arthritis (5), or atherosclerosis (6). So far, most in vitro and in vivo studies suggest that Gal-3 may increase inflammatory responses through its functions on cell activation, cell migration, or inhibition of apoptosis (7). Studies of Gal-3-deficient mice have provided support for a role of Gal-3 in promoting inflammatory responses (8–10). In the CNS, Gal-3 expression is highly up-regulated in prion-infected brain tissue (11, 12), and Gal-3 seems to play a detrimental role in brain prion infections (13). However, the role of Gal-3 in autoimmune neurological disease remains unclear.

Experimental autoimmune encephalomyelitis (EAE) is an animal model of CNS inflammatory demyelination, and it is commonly used as a model of multiple sclerosis in humans (14). EAE is thought to be a T cell-mediated autoimmune disease, and both Th1 and Th17 cells were thought to be responsible for the inflammatory demyelination in multiple sclerosis and EAE (15–19). Th2 and $Foxp3^+$ regulatory T (Treg) cells and related cytokines such as IL-5 and IL-10 have been shown to be important in the resolution stages of the disease (20–22).

We report herein that Gal-$3^{-/-}$ mice immunized with myelin oligodendrocyte glycoprotein $(MOG)_{35–55}$ peptide developed markedly attenuated EAE compared with similarly immunized wild-type (WT) mice. The disease attenuation is accompanied by reduced cellular infiltration in the CNS and decreased production of IL-6, IL-17, and IFN-γ, but increased apoptosis in the CNS infiltrates and increased synthesis of IL-10 and IL-5. Furthermore, the CNS of the Gal-$3^{-/-}$ mice contained higher frequency of $Foxp3^+$ Treg cells, and DC from Gal-$3^{-/-}$ mice produced more IL-10 and induced the production of more IL-10 and IL-5 by Ag-specific T cells. Our data therefore demonstrate that Gal-3 plays an important pathogenic role in EAE by preventing cell apoptosis in the CNS and enhancing IL-17 and IFN-γ synthesis but decreasing IL-10 production, and it may therefore be a potential target for therapeutic intervention in autoimmune neurological diseases.

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[3] Abbreviations used in this paper: Gal-3, galectin-3; DLN, draining lymph node; BMDC, bone marrow-derived dendritic cell; EAE, experimental autoimmune encephalomyelitis; iNOS, inducible NO synthase; MOG, myelin oligodendrocyte glycoprotein; Treg, regulatory T; WT, wild-type.

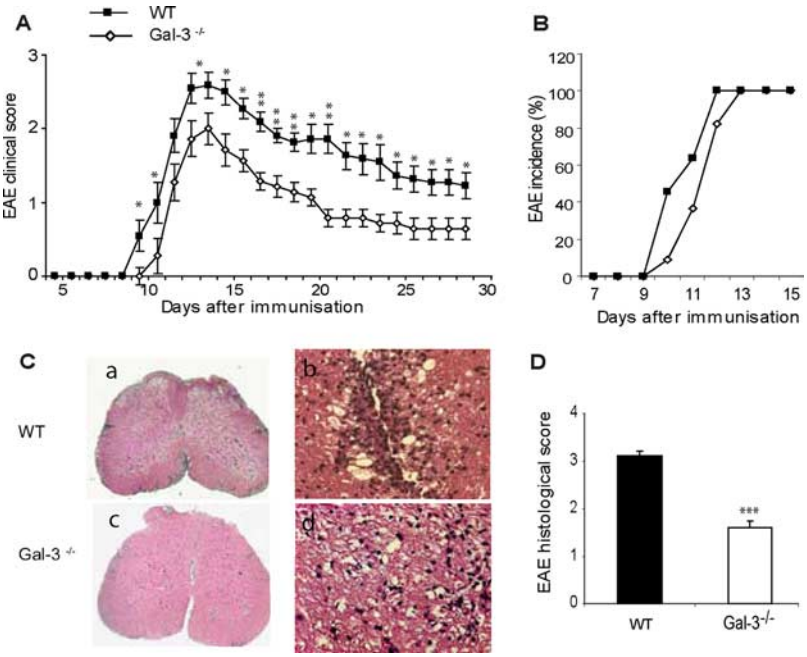

FIGURE 1. Gal-3$^{-/-}$ mice developed less severe EAE than did WT mice. Mice were immunized with $MOG_{35–55}$ peptide and CFA. *A*, Clinical scores show that Gal-3$^{-/-}$ mice had a reduced EAE severity. However, all mice in both groups developed EAE (*B*). Results are mean clinical scores ± SEM ($n = 11$/group). *, $p < 0.05$ and **, $p < 0.01$, representative of four experiments. *C*, Pathology of spinal cord sections of WT (*a* and *b*) and Gal-3$^{-/-}$ mice (*c* and *d*) were examined 18 days after immunization using H&E staining at low (*a* and *c*) and high magnification (*b* and *d*). WT mice exhibited typical perivascular and subpial infiltrates of different sizes, while Gal-3$^{-/-}$ developed only single cell and rare small subpial mononuclear cell infiltrates. *D*, Semiquantitative assessment of the histological sections from the spinal cord of WT and Gal-3$^{-/-}$ mice. Ten noncontiguous sections per spinal cord were scored. ***, $p < 0.001$ ($n = 3$).

Materials and Methods

Mice

C57BL/6 mice were purchased from Harlan Laboratories. Gal-3$^{-/-}$ mice of the C57BL/6 background were generated as described previously (23). DO.11.10 mice were on C57BL/6 background. All mice were maintained in the Biological Services Facilities, University of Glasgow under U.K. Home Office guidelines. Gal-3$^{-/-}$ mice were also maintained in the Faculty of Medicine & Health Sciences (FMHS) animal facilities at the United Arab Emirates University. All experiments conducted were approved by the FMHS, United Arab Emirates University Animal Research Ethics Committee. Male mice 7–12 wk old were used in all experiments.

EAE induction and clinical evaluation

Mice were immunized s.c. on the back with 100 mg of $MOG_{35–55}$ peptide (Sigma-Aldrich) in 50 ml of PBS emulsified with an equal volume of CFA (2.5 mg/ml of *Mycobacterium tuberculosis*, strain H37RA, Difco). Each mouse also received i.p. 100 ng/200 μl of pertussis toxin (Sigma-Aldrich) in PBS on days 0 and 2 postimmunization. EAE was scored according to a 0–5 scale as follows: 0, no clinical sign; 1, complete loss of tail tone; 2, hind limb weakness; 3, hind limb paralysis; 4, forelimb involvement; 5, moribund.

Histological evaluation of disease

Mice were killed by cervical dislocation and their spinal cords excised. The tissues were placed in 10% buffered formalin fixative overnight and routinely embedded in paraffin wax. Spinal cord sections (5–7 mm) were stained with H&E. The level of mononuclear cell infiltration was graded using the following semiquantitative score as described previously (24): 0, no infiltration; 1, mild infiltration around pial vessels; 2, single-cell infiltration within the CNS; 3, infiltration with mild perivascular cuffing; and 4, very intense infiltration with perivascular cuffing. Values obtained in groups of animals were expressed as means ± SEM.

CNS cell isolation

Mice were killed by asphyxiation in CO_2 and then perfused through the left cardiac ventricle with 20 ml of cold PBS buffer. Mouse brains were dissected and spinal cords flushed out with PBS by hydrostatic pressure using a syringe attached to an 18-gauge needle. CNS tissue was then cut into small pieces and digested with collagenase A (2 mg/ml, Roche Diagnostics) and DNase (1 mg/ml, Sigma-Aldrich) at 37°C for 40 min. Mononuclear cells were isolated by passing the tissue through a cell strainer (100 mm), followed by a Percoll gradient (70/30%) centrifugation. Mononuclear cells were removed from the interphase, washed, and resuspended in culture medium for further analysis.

Abs and flow cytometry analysis

Abs and apoptosis detection kit were purchased from BD Pharmingen unless mentioned otherwise. To examine the leukocyte phenotype in the draining lymph node (DLN) and spleen, or infiltrated in the CNS, we stained the collected single cells with the following anti-mouse Abs: anti-CD45-PE (clone 30-F11), anti-CD4-PerCP (clone RM4-5), anti-CD8α-PerCP (clone 53-6.7), anti-CD19-PerCP (clone 1D3), anti-CD11b-FITC (clone M1/70), anti-CD11c-FITC (clone HL3), and anti-F4/80 (clone BM8, eBioscience). Ab against Foxp3 (clone FJK-16s Set) was from eBioscience. Matched isotypes were used as negative control.

TUNEL

Spinal cord tissues were embedded in wax and sectioned at 5 μm. After de-waxing and Ag retrieval, the sections were incubated with a TUNEL mixture (TUNEL apoptosis detection kit, Millipore) for 60 min at 37°C with subsequent mounting and inspection under a microscope. For negative control, sections were incubated with the labeling solution only.

DLN and spleen cell cytokine measurement

On day 18 postimmunization, mouse DLN and spleen were collected and pooled within groups. The single-cell suspension was cultured in 24-well plates at 4×10^6 cells per well and stimulated with medium alone, 50 μg/ml MOG peptide, or precoated CD3 Ab (clone 2C11, BD Pharmingen). After 72 h, supernatants were collected and selected cytokine concentrations measured by ELISA using paired Abs (BD Pharmingen).

Measurement of cytokines in the serum

Individual mouse serum was collected and assayed for cytokine concentrations using a 20-plex mouse cytokine assay (for FGF, GM-CSF, IFN-γ, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p40/70, IL-13, IL-17, IP-10, KC, MCP-1, MIG, MIP-1α, TNF-α, and VEGF) according to the manufacturer's instructions (Luminex kit, BioSource products from Invitrogen).

RT-PCR and quantitative PCR

For RT-PCR, single-cell suspensions of DLN from each group were seeded in 96-well flat-bottom culture plates at 5×10^5 cells per 100 ml per well in triplicates with or without Con A (2.5 mg/ml) or MOG peptide (10 mg/ml). After 48 h of incubation, cells were collected and lysed with SV RNA lysis buffer (Promega). mRNA was isolated using an SV Total RNA Isolation System (Promega). cDNA synthesis and subsequent RT-PCR were performed using the Access RT-PCR system kit (Promega). For CNS studies, the spinal cords of mice were homogenized in lysis buffer solution and treated thereafter as the cells from the lymph nodes. All primer sequences are available upon request.

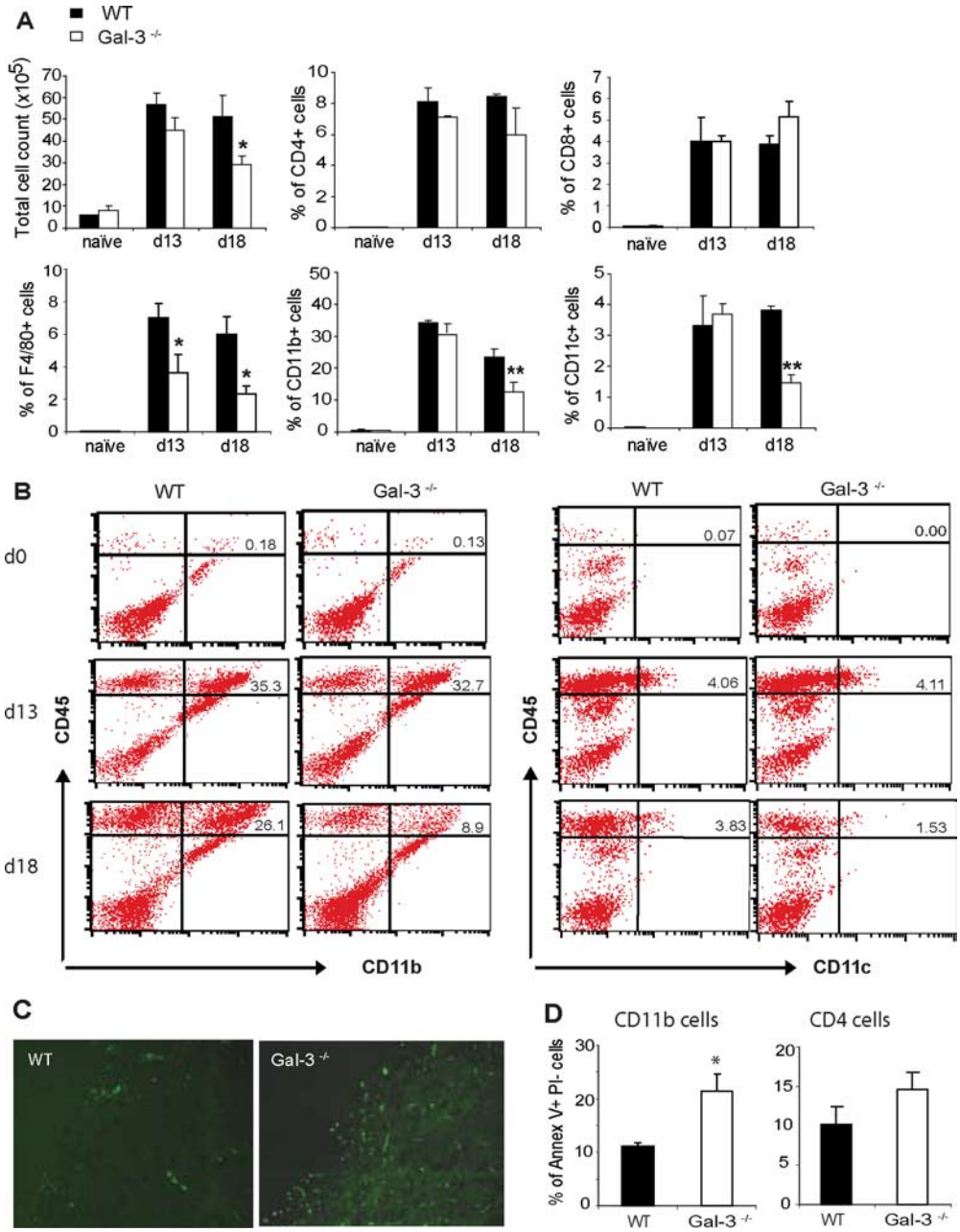

FIGURE 2. Gal-3$^{-/-}$ mice had reduced leukocyte infiltration in the CNS compared with WT mice. Brains and spinal cords from naive or immunized (day 13 or 18) WT and Gal-3$^{-/-}$ mice were collected and mononuclear cells analyzed by FACS. *A*, CNS of the Gal-3$^{-/-}$ mice exhibited significantly fewer total numbers of infiltrating cells and CD11c^{+}, CD11b^{+}, and F4/80^{+} cells than did that of the WT mice. *B*, Representative FACS data of CD11b and CD11c staining. The results are means ± SEM ($n = 4$). *, $p < 0.05$ and **, $p < 0.01$. *C*, TUNEL staining of the spinal cords of the immunized mice at day 15 postimmunization. There were fewer TUNEL-positive cells in the WT mice tissue than in that of the Gal-3$^{-/-}$ mice. *D*, The CNS cells were also assayed for apoptosis by annexin V staining. Only CD11b^{+} cells from Gal-3$^{-/-}$ mice show increased levels of apoptosis. The figures show percentage of annexin V^{+}propidium iodide (PI)$^{-}$ cells in the total CNS cell population. Data are representative of five mice per group and are representative of two experiments.

Generation of bone marrow-derived dendritic cells (BMDC)

BMDC were prepared by a modification (25) of the procedure described by Inaba et al. (26). In brief, a single-cell suspension of bone marrow cells from femurs and tibias of normal WT or Gal-3$^{-/-}$ mice was depleted of RBC. Cells were cultured in 12-well plates (7.5×10^5 cells/ml) in complete RPMI 1640 medium supplemented with 7.5% GM-CSF supernatant. The GM-CSF-producing cell line was a gift from Dr. J. V. Forrester (Aberdeen University, U.K.). Cultures were re-fed with fresh medium on day 2, and immature DC were harvested on day 6. Cells were seeded (1×10^6) in a new 24-well plate with medium with and without 100 ng/ml bacterial lipoprotein (BLP) or 200 ng/ml LPS. Supernatants were harvested after 24 h, and IL-10 and IL-12 were analyzed by ELISA. Spleens cells were also harvested from WT or Gal-3$^{-/-}$ mice immunized 16 days previously with MOG plus CFA. CD11c^{+} DC were purified and cultured with LPS or BLP for 24 h, and cytokines in the supernatant were analyzed by ELISA as for BMDC.

DC function test in vitro

BMDC derived as above were cultured overnight in 24-well plates (1×10^6 cells per well) with medium or OVA (30 mM) in the presence of LPS (100 ng/ml). DC were then collected and thoroughly washed in PBS. Meanwhile, CD4^{+} T cells were purified from the spleen and lymph nodes of DO.11.10 mice by autoMACS (Miltenyi Biotec). For cell proliferation, DC and T cells were cocultured in 96-well U-bottom plates at the ratio of 1:50 with 2×10^5 T cells per well for 96 h in complete RPMI 1640 medium. For cytokine production, DC and CD4^{+} T cells were cultured

together in 24-well plates at the ratio of 1:10 with 2×10^6 T cells per well. Supernatants were collected at 72 h for cytokine detection by ELISA.

Statistical analysis

Statistical evaluations of cell frequency, proliferation, cytokine production, and histological analysis were performed using two-tailed, unpaired Student's *t* tests. ANOVA were applied to analyze the EAE clinical and histological grading. A p value of <0.05 was considered to be statistically significant. All experiments were repeated at least twice.

Results

Gal-3$^{-/-}$ mice developed attenuated EAE

Gal-3$^{-/-}$ and WT mice were immunized s.c. with MOG_{35-55} peptide in CFA on day 0, and then injected i.v. with pertussis toxin on day 0 and day 2. WT mice started to develop EAE from day 9 and reached the peak around day 14. Gal-3$^{-/-}$ mice had a slight delay in the EAE onset and reached the peak of the disease also around day 14. However Gal-3$^{-/-}$ mice had significantly less severe EAE compared with WT mice (Fig. 1*A*). There was no difference in the incidence of the disease, as all mice developed EAE (Fig. 1*B*). The difference in disease scores was confirmed by histopathological analysis of the spinal cord, which showed significantly less severe inflammation in the Gal-3$^{-/-}$ mice than that in the WT mice (Fig. 1*C*). This was confirmed by quantitative measurement of the histological scores (Fig. 1*D*).

Gal-3$^{-/-}$ mice had reduced leukocyte infiltration in the CNS

To characterize the infiltrating cell populations in the CNS, we collected the brain and spinal cord tissues from WT and Gal-3$^{-/-}$ mice and analyzed their phenotype by flow cytometry. As expected, naive mice had very few mononuclear cells in the CNS. After immunization, there was a marked increase of infiltrating cells in the CNS tissue in both Gal-3$^{-/-}$ and WT mice. At the earlier time point (day 13), there was no significant difference in the total cellular infiltrates in the CNS between WT and Gal-3$^{-/-}$ mice. However, Gal-3$^{-/-}$ mice had consistently fewer total leukocyte infiltrates and lower percentages of F4/80$^+$, CD11b$^+$ CD45^{high}, and CD11C$^+$ cells at day 18 (Fig. 2, *A* and *B*). These data suggest that there was no significant difference in the initial migration of cells into the CNS between the two groups of mice. There was also no difference in the percentage of CD4$^+$ or CD8$^+$ T cells between the WT and Gal-3$^{-/-}$ mice. Gal-3 has been shown to prevent cell apoptosis (27, 28). We therefore examined the frequency of apoptotic cells in the spinal cord at day 15 after immunization. There were significantly more TUNEL-positive cells in the CNS of the Gal-3$^{-/-}$ mice than that of the WT mice (Fig. 2*C*). The increased apoptosis may contribute to the decreased cellular infiltration in the CNS of the Gal-3$^{-/-}$ mice. We further investigated the cell types that showed a difference in apoptosis between the WT and Gal-3$^{-/-}$ mice. Cells from the spinal cord were collected 15 days after immunization, and single-cell suspensions were analyzed by FACS. Only CD11b$^+$ cells from Gal-3$^{-/-}$ mice showed significantly higher frequency of apoptosis compared with those of the WT mice (Fig. 2*D*). Since we observed a significant reduction of the infiltrating CD11b$^+$CD45^{high} but not the CNS resident CD11b$^+$CD45^{low} population in the Gal-3$^{-/-}$ CNS at day 18 postimmunization, it is likely that the apoptotic CD11b$^+$ cells are the infiltrating inflammatory cells.

Gal-3$^{-/-}$ mice produced fewer proinflammatory cytokines but more inhibitory cytokines

We then investigated the immune mechanism associated with the reduced severity of EAE in the Gal-3$^{-/-}$ mice. DLN cells from Gal-3$^{-/-}$ and WT mice were harvested 18 days postimmunization. Single-cell suspensions were cultured in the presence or absence of

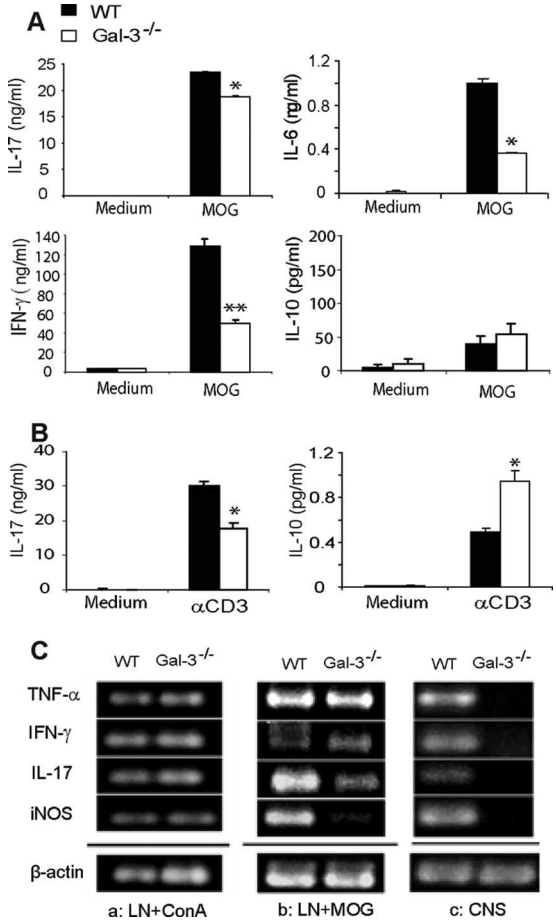

FIGURE 3. Gal-3$^{-/-}$ mice had biased IL-10 and Th2 cytokine production. DLN and spleen of WT and Gal-3$^{-/-}$ mice were collected on day 18 postimmunization and stimulated in vitro with MOG_{35-55} or anti-CD3 Ab for 72 h, and cytokine concentrations in the culture supernatant were determined by ELISA. *A*, Cells from Gal-3$^{-/-}$ mice produced less IL-17, IFN-γ, and IL-6 than did those of the WT mice in response to MOG_{35-55}. *B*, Cells from Gal-3$^{-/-}$ mice produced more IL-10 but less IL-17 in response to anti-CD3 Ab. Results are means ± SEM. *, $p < 0.05$ and **, $p < 0.01$ ($n = 5$), representative of three experiments. *C*, RT-PCR analysis. DLN cells from Gal-3$^{-/-}$ mice (10 days after immunization) expressed similar levels of proinflammatory mediators as did WT mice after Con A stimulation in vitro (*a*), but less of these mediators after MOG_{35-55} stimulation (*b*); *c* shows that the expression of the proinflammatory mediators was clearly detectable in the spinal cords of the WT mice 10 days after immunization, but was not detectable in the spinal cords of similarly immunized Gal-3$^{-/-}$ mice, except for a weak expression of IFN-γ. Data are representative of five mice per group.

MOG Ag or the polyclonal T cell-activating anti-CD3 Ab. LN cells from Gal-3$^{-/-}$ mice produced significantly less IL-17, IFN-γ, and IL-6 in response to MOG compared with cells from the WT mice (Fig. 3*A*). IL-10 concentration was low, and IL-4 and IL-5 levels were not detected. When cultured with anti-CD3 Ab, cells from Gal-3$^{-/-}$ mice also produced less IL-17, but more IL-10, than did those from the WT mice (Fig. 3*B*). These results were further confirmed by RT-PCR analyses of relevant proinflammatory mediators in the DLN at day 10 after EAE induction. There was no significant difference between WT and Gal-3$^{-/-}$ mice in the expression of TNF-α, IFN-γ, IL-17, or inducible NO synthase (iNOS) in the LN cells after a T cell mitogen (Con A) stimulation in vitro (Fig. 3*Ca*). However, when the same cell populations were restimulated with MOG_{35-55}, there was significant attenuation of the expression of IL-17 and iNOS but not the expression of TNF-α

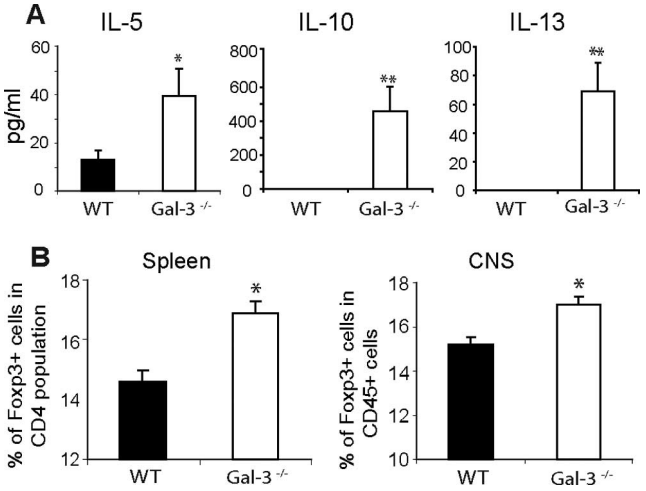

FIGURE 4. Gal-3$^{-/-}$ mice produced elevated levels of IL-5, IL-10, IL-13, and increased frequency of Treg cells. *A*, Serum cytokine from mice (18 days after immunization) were measured by Luminex. Serum from Gal-3$^{-/-}$ mice contained significantly more IL-5, IL-10, and IL-13 than did that of the WT mice. IFN-γ and IL-4 were not detectable. *B*, FACS analysis of Foxp3$^+$ cells in the CD4$^+$ populations of spleen and CNS of WT or Gal-3$^{-/-}$ mice 18 days after immunization. The frequency of Foxp3$^+$ cells in the spleen and CNS was increased in the Gal-3$^{-/-}$ mice compared with WT mice. Results are means $\pm$ SEM ($n = 16$–18). $*$, $p < 0.05$ and $**$, $p < 0.01$, pooled from three experiments.

and IFN-γ in the Gal-3$^{-/-}$ mice (Fig. 3*Cb*). Importantly, whereas the expression of TNF-α, IFN-γ, IL-17, or iNOS was clearly expressed in the CNS of the WT mice, these transcripts were not detectable in the CNS of the Gal-3$^{-/-}$ mice (Fig. 3*Cc*). The mechanism by which Gal-3 influences the expression of iNOS is at present unclear. Gal-3 could induce iNOS indirectly via the promotion of IFN-γ and TNF-α, as both are potent activators of iNOS.

Th2 cytokines and Treg cells are elevated in Gal-3$^{-/-}$ mice

We then examined the cytokines in the sera of mice using 20-plex Luminex kit. Serum from Gal-3$^{-/-}$ mice contained significantly higher levels of IL-10, IL-5, and IL-13 than did that of the WT mice following disease induction (Fig. 4*A*). There was no significant difference in the levels of serum IL-12, IL-17, or IL-6 between the Gal-3$^{-/-}$ and WT mice. Both IFN-γ and IL-4 were very low and had similar levels (data not shown). Since IL-10 is a major functional mediator of Treg cells, the elevated concentrations of IL-10 in immunized Gal-3$^{-/-}$ mice prompted us to investigate the frequency of Foxp3$^+$ Treg cells in the immunized mice. Both spleen and CNS of Gal-3$^{-/-}$ mice contained significantly higher frequency of Foxp3$^+$ Treg cells compared with those of the WT mice (Fig. 4*B*). Taken together, these results are concordant with the disease score and demonstrate that Gal-3$^{-/-}$ mice produce decreased proinflammatory cytokines but increased antiinflammatory cytokines and elevated Treg cells following EAE induction.

DC from Gal-3$^{-/-}$ mice produce more IL-10 and enhance Th2 polarization

The preponderance of IL-10 production could be the driving force in the reduced proinflammatory response in the Gal-3$^{-/-}$ mice. We therefore investigated the potential source of IL-10 by examining the relative ability of the DC from WT and Gal-3$^{-/-}$ mice to produce IL-10. BMDC were generated from bone marrow cells after 7 days of culture with GM-CSF as previously described (25). Cells were then restimulated with or without BLP or LPS. Culture supernatants were collected 24 h later, and IL-10 and IL-12p70

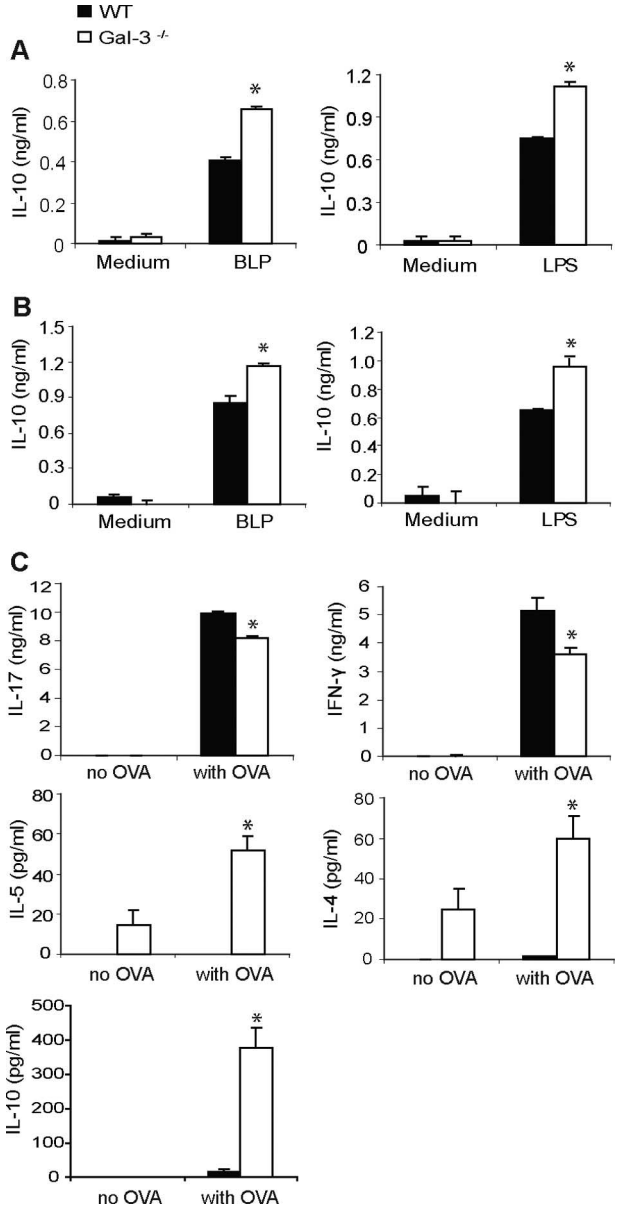

FIGURE 5. BMDC from Gal-3$^{-/-}$ mice produced increased IL-10 and polarized Th1 and Th17 cells. *A*, BMDC from WT or Gal-3$^{-/-}$ mice were cultured overnight with LPS or BLP, and IL-10 concentration in the supernatant was measured by ELISA. *B*, Splenic DC were purified from WT or Gal-3$^{-/-}$ mice immunized 16 days previously (with MOG_{35-55} peptide plus CFA) and cultured with BLP or LPS and cytokines in the supernatant measured by ELISA. *C*, BMDC from naive WT or Gal-3$^{-/-}$ mice were pulsed with OVA overnight in the presence of LPS, and then cells were washed and cocultured with purified DO11.10 CD4$^+$ T cells for 4 days. Cell proliferation was determined by [^{3}H]thymidine incorporation, and the cytokine concentrations in the culture supernatant were assayed by ELISA. There was no difference in cell proliferation between the two groups (data not shown). Results are means $\pm$ SEM. $*$, $p < 0.05$ ($n = 5$), representative of two experiments.

concentrations were assayed by ELISA. BMDC from Gal-3$^{-/-}$ mice produced significantly more IL-10 compared with those of the WT mice (Fig. 5*A*). Both groups of DC produced similar levels of IL-12 (data not shown). BMDC from both groups of mice expressed similarly elevated levels of the surface costimulatory molecules CD86 and CD40 24 h after BLP or LPS stimulation as determined by FACS analysis (data not shown). We also examined the cytokine production profile of splenic DC from WT and

Gal-3$^{-/-}$ mice (16 days after immunization) after BLP or LPS stimulation in vitro. The results were similar to those of BMDC. Splenic DC from Gal-3$^{-/-}$ mice produced significantly more IL-10 than did those from the WT mice (Fig. 5*B*). There was again no difference in IL-12 synthesis (data not shown). Both BMDC and splenic DC (as above) produced similar levels of IL-23 following 24 h of BLP or LPS stimulation in vitro (data not shown). To further examine the Ag presentation capability of the BMDC, we pulsed the BMDC from nonimmunized mice with 30 μM of OVA in the presence of 100 ng/ml LPS. Then the cells were washed thoroughly and cocultured with purified DO.11.10 CD4$^+$ T cells. T cells cultured with DC from both groups of mice produced comparable proliferative responses (data not shown). However, T cells stimulated with BMDC from Gal-3$^{-/-}$ mice produced significantly less IL-17 and IFN-γ, but more IL-10, IL-4, and IL-5, than did T cells cultured with BMDC from WT mice (Fig. 5*C*). These results indicate that Gal-3$^{-/-}$ DC have similar Ag presentation capacity as do the WT DC. However, the Gal-3$^{-/-}$ DC are more likely to polarize T cells toward Th2 cells, and possibly Treg cell phenotypes via enhanced IL-10 synthesis, than are the WT DC.

Discussion

Data presented herein indicate that Gal-3 deficiency reduces EAE development by decreasing IL-17 and IFN-γ production but expanding populations of Th2 cells and inducible Treg cells. Furthermore, this phenotype is probably secondary to the effect of Gal-3 on DC, since DC from Gal-3$^{-/-}$ mice produced elevated IL-10 and preferentially polarized T cells producing higher levels of Th2 cytokines but less IL-17 and IFN-γ. This is in partial agreement with the data from our in vitro polarization assay. Purified CD4$^+$ T cells from Gal-3$^{-/-}$ mice cultured in CD3 precoated plates under a Th1-polarizing condition (plus anti-IL-4 and IL-12) produced a modest reduction of IFN-γ but a similar level of IL-17 under a Th17-driving condition (in the presence of TGF-β and IL-6 and absence of DC) compared with the cells from WT mice (data not shown).

The role of Gal-3 in inflammatory disease is controversial. Gal-3 deficiency led to reduced inflammation during pneumococcal pneumonia (29), atherosclerosis (30), and dextran sulfate sodium-induced colitis (31) in mice. However, Gal-3$^{-/-}$ mice were reported to develop higher inflammatory response in the lung following infection with *Toxoplasma gondii* (10). Thus, the effect of Gal-3 on inflammation may be organ- and disease-specific. Herein we show that Gal-3$^{-/-}$ mice developed markedly reduced EAE, suggesting that Gal-3 promotes inflammation in the CNS in EAE. This is in apparent contrast with an earlier report (32) that indicated that reduction of Gal-3 functions through the deletion of Mgat5 (β1,6*N*-acetylglucosaminyltransferase V) in mice led to heightened susceptibility to EAE. However, the glycosylation deficiency in Mgat5$^{-/-}$ mice affects other pathways and cell types that may also contribute to the observed autoimmunity. Mgat5-modified glycans also reduce clusters of fibronectin receptors, causing accelerated focal adhesion turnover in fibroblasts and tumor cells, a functionality that may affect leukocyte motility (32).

Gal-3 expression is increased when monocytes differentiate into macrophages (33). Gal-3 and F4/80 expression are both enhanced in microglia and macrophage in the spinal cords and the optic nerves of mice with EAE (34). Furthermore, Gal-3 plays an important role in the recruitment of leukocytes to the lungs of mice infected with *Streptococcus pneumoniae* through β_2 integrin-independent migration (29). Moreover, Gal-3 induces chemotaxis of monocytes and macrophages in vitro and in vivo (8, 35). Consistent with these observations, we found that Gal-3$^{-/-}$ mice exhibited markedly reduced macrophage infiltration in the CNS during EAE, supporting a key role of Gal-3 in promoting inflammation via local recruitment of leukocytes. Our finding that the CNS of Gal-3$^{-/-}$ mice consisted of markedly elevated TUNEL-positive apoptotic cells is also consistent with the reported antiapoptotic role of Gal-3 (36) and may further explain the significantly reduced number of infiltrating leukocytes in the Gal-3$^{-/-}$ CNS compared with the WT CNS. Analysis of the apoptotic cells in the CNS shows that the predominant difference in the frequency of apoptotic cells in the CNS between the immunized WT and Gal-3$^{-/-}$ mice is in the CD11b$^+$ population of monocytic cells rather than in T cells. Given the ubiquitous expression of Gal-3 and the reported antiapoptotic role of Gal-3, the selective apoptosis of CD11b$^+$ cells in the immunized Gal-3$^{-/-}$ mice is counterintuitive and it may be subsequent to the production of an as yet unidentified proapoptotic mediator or mediators.

Both Th17 and Th1 are now thought to play an important role in EAE (17, 37). Our finding that Gal-3$^{-/-}$ mice produced less IL-17 and IFN-γ is consistent with the reduced severity of EAE in the Gal-3$^{-/-}$ mice. These results also indicate a hitherto unrecognized role of Gal-3 in optimizing Th17 polarization in vivo.

Both Th2 and Tregs are reported to contribute to the resolution of EAE (20, 22, 38, 39). Our finding that IL-4, IL-5, IL-10, and Treg cells are all elevated in Gal-3$^{-/-}$ mice during EAE is consistent with the protective role of Th2 and Treg cells in this disease model. Since IL-10 is a key effector molecule of Treg cells, and since IL-10 is a major driver of inducible Treg cells such as Tr1 (40), our data suggest that Gal-3 could play an important role in limiting Treg cell development, hence contributing to the pathogenesis of autoimmune diseases.

The role of Gal-3 in Th1/Th2 polarization is controversial. Gal-3$^{-/-}$ mice were reported to be Th2 defective in an OVA-induced asthma model (23). During parasitic infection, a reduced inflammation was also related with an elevated Th1 response in Gal-3$^{-/-}$ mice (10). In contrast, Gal-3 was shown to down-regulate IL-5 gene expression (41, 42). Gal-3$^{-/-}$ mice had a normal Th2 response and an elevated serum IgG1 following intestinal nematode and schistosome infection (43). We found here that Gal-3$^{-/-}$ mice developed enhanced Th2 response during EAE. Thus, the effect of Gal-3 is likely disease- and model-dependent.

Interestingly, Gal-3 is proinflammatory in EAE and other inflammatory disease models, while other members of the galectin family, including Gal-1 (44, 45), Gal-2 (46), and Gal-4 (47), have antiinflammatory effects, despite the fact that all members of the galectin family have similar carbohydrate specificity. The question as to how subtle structural differences in Gal-3 with regard to other members of the family could lead to a completely opposite effect in immune regulation remains unclear and merits further in-depth investigation.

The effect of Gal-3 on Th1, Th2, Th17, and Treg polarization may be secondary to its effect on DC functions. DC express a high density of Gal-3 (48). In our hands, BMDC from naive Gal-3$^{-/-}$ mice and splenic DC from immunized Gal-3$^{-/-}$ mice produced markedly elevated IL-10 (but not IL-12) in response to LPS and BLP (TLR4 and TLR2 ligand, respectively) compared with WT BMDC. Furthermore, BMDC from Gal-3$^{-/-}$ mice preferentially induced the development of IL-4-, IL-5-, and IL-10-producing T cells in vitro, suggesting an association of Gal-3 with the promotion of Th1 cells and limiting the differentiation of Th2 cells and Treg cells.

Taken together, our results demonstrate that Gal-3 plays an influential role in promoting EAE. This pathogenic role is likely associated with the ability of Gal-3 to preferentially limit IL-10 synthesis by DC, which in turn biases the production of IL-17 and

IFN-γ and inhibits the development of Th2 and Treg cells. Our data therefore reveal a hitherto unrecognized influence of Gal-3 in T cell regulation, as well as the role of Gal-3 in the pathogenesis of a key neurological disease.

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Disclosures

The authors have no financial conflicts of interest.

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