



Galectin-9 suppresses Th17 cell development in an IL-2-dependent but Tim-3-independent manner

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Abstract Galectin-9 (Gal-9) ameliorates autoimmune reactions by suppressing Th17 cells while augmenting Foxp3⁺ regulatory T cells (Tregs). However, the exact mechanism of Gal-9-mediated immune modulation has been elusive. In a MOG-induced experimental allergic encephalomyelitis model using Gal-9^{-/-} mice, we observed exacerbated inflammation and an increase in IL-17-producing Th17 cells balanced by a decrease in Foxp3⁺ Tregs. During in vitro Th17 skewing using TGF-β1 and IL-6, exogenous Gal-9 suppressed Th17 cell development and expanded Foxp3⁺ Tregs from naïve CD4 T cells in an IL-2-dependent manner. Although Gal-9 induced cell death in Tim3-expressing differentiated Th17 cells, Gal-9 suppressed Th17 development in a Tim-3-independent. Benzyl-α-GalNAc (an O-glycan biosynthesis inhibitor), but not swainsonine (a complex-type N-glycan biosynthesis inhibitor) abrogated Gal-9-mediated inhibition of Th17 development indicating that there is a linkage between Gal-9 and an unidentified glycoprotein(s) with O-linked β-galactosides that suppress Th17 development.

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1. Introduction

Galectin-9 (Gal-9) is an apoptosis-inducing factor [1] and also a chemoattractant for eosinophils [2], neutrophils [3] and NK cells [4]. Gal-9 induces apoptosis of activated Th1 cells through Ca⁺⁺-calpain-caspase 1 pathway by binding

with T cell immunoglobulin and mucin domain containing molecule-3 (Tim-3) [5,6]. In contrast to Th1 cells, we have shown that Gal-9/Tim3 interaction is required for the up-regulation of CTL activity of CD8 T cells by Tim-3-expressing dendritic cells (DCs) [7]. Furthermore, Gal-9 can modulate DC function via Gal-9/Tim3 interaction [8], induce maturation of DCs [9] and augment anti-microbial activity of macrophages [10]. Collectively, it is suggested that Gal-9 can exhibit positive or negative effects on different cell types, or even the same cell type in different inflammatory

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settings [11]. Therefore, interaction between Gal-9 and Tim-3 has been a common focus in recent immunological studies.

Gal-9 exhibits therapeutic effects in a mouse collagen-induced arthritis model by decreasing the levels of IFN- γ and IL-17 in the joints, concomitant with a decreased number of Tim-3⁺ CD4 T cells in the peripheral blood [12]. The fact that Th17 cells also express Tim-3 [13] implies a suppressive function for Gal-9 on Th17 cells. However, an involvement of Tim-3/Gal-9 interaction in Th17 cell apoptosis has not been directly demonstrated. Although Gal-9/Tim-3 interactions are the most common focus in the regulation of immune responses by Gal-9, Gal-9 also binds a variety of other molecules, including IgE, CD44, and many integrins [14–16]. It has been conclusively shown that Gal-9 induces cell death in Tim-3-expressing, terminally differentiated Th1 cells through Tim-3 [6,12]. However, we have evidence that Gal-9 induces cell death of many Tim-3-negative cell lines [unpublished data]. It has also been shown recently that Gal-9 induces production of pro-inflammatory cytokines from Th cells independently of Tim-3 [17]. Thus, we sought to determine if Gal-9 regulation of Th17 cells was Tim-3-independent or otherwise.

In this study, we show that Gal-9 suppresses Th17 development independently of Tim-3, and that it induces cell death in Tim-3-expressing differentiated Th17 cells through binding Tim-3.

2. Materials and methods

2.1. Animals

Male C57BL/6J mice were obtained from Charles River Laboratories Japan (Yokohama, Japan). All animals were maintained under standard conditions with a 12-h day/night rhythm and with *ad libitum* access to food and water. Mice received humane care in accordance with international guidelines and national law. The study protocol was approved by the Animal Care and Use Committee of Kagawa University.

2.2. Induction of EAE

Generation of Gal-9-deficient (Gal-9^{-/-}) mice was described previously [12]. Female C57BL/6J mice (Charles River Laboratories Japan) and Gal-9^{-/-} mice were immunized with 100 μ g myelin oligodendrocyte glycoprotein (MOG) (35–55) peptide in Complete Freund's Adjuvant supplemented with 400 μ g *Mycobacterium tuberculosis* (Difco). Mice received 200 ng pertussis toxin intraperitoneally on days 0 and 2. For administration of Gal-9, expression and purification of recombinant human stable Gal-9 have been previously described [18]. Stable Gal-9 was used rather than WT Gal-9, because WT Gal-9 is quickly degraded and loses its activity. Gal-9 (3 μ g/mouse) or PBS was injected subcutaneously twice a week from 14 days after immunization.

Disease progression was monitored and assigned scores as follows: limp tail, 1; uneven gait and hindlimb weakness, 2; total hindlimb paralysis, 3; weak forelimb and hindlimb paralysis or forelimb and hindlimb paralysis, 4; and moribund, 5.

2.3. Isolation and culture of mouse CD4⁺ CD62L⁺ naive T cells

Spleens were isolated from 8 to 10-week-old mice. CD4⁺ CD62L⁺ naive T cells were isolated from splenocytes using a CD4⁺ CD62L⁺ T cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. CD4⁺ CD62L⁺ purity was >94%. Isolated naïve T cells were cultured on anti-CD3-coated plates (Becton Dickinson) in RPMI 1640 with 10% heat-inactivated fetal bovine serum, penicillin G (10 IU/ml, Sigma-Aldrich), and streptomycin (100 μ g/ml, Sigma-Aldrich) at 2×10^5 cells/well for 72–96 h with anti-CD28 (2 μ g/ml, Becton Dickinson) under the following conditions. (1) Th17 development: human TGF- β 1 (3 ng/ml, R&D Systems), mouse IL-2 (5 ng/ml, R&D Systems), and mouse IL-6 (20 ng/ml, R&D Systems); (2) Th1 development: mouse IL-12 (10 mg/ml, R&D Systems) and anti-IL-4 (10 μ g/ml, Becton Dickinson); and (3) Th2 development: mouse IL-4 (20 mg/ml, R&D Systems) and anti-IL-12 (10 μ g/ml, Becton Dickinson). For some experiments, human stable Gal-9 (30 nM), lactose (3, 10, 30 mM), sucrose (3, 10, 30 mM), Tim-3 mAb (10 μ g/ml, eBioscience), or rat IgG2a (10 μ g/ml, eBioscience) were included during culture.

2.4. ELISA

Murine IL-17 was detected using an IL-17 DuoSet (R&D Systems), and performed according to the manufacturer's instructions.

2.5. Flow cytometric analysis

CD4 T cells were evaluated by flow cytometry using the following antibodies: anti-mouse CD4-FITC (Becton Dickinson), anti-mouse Tim-3-PE (eBioscience), anti-mouse CD25-APC (BioLegend), anti-mouse Foxp3-APC (eBioscience). An Annexin V-EGFP Apoptosis Detection kit (Medical & Biological Laboratories, Nagoya, Japan) was used to examine apoptosis according to the manufacturer's instructions. All data were acquired with a FACSCalibur (Becton Dickinson) and analyzed with FlowJo software (Tree Star, Ashland, OR).

2.6. Real-time RT-PCR

mRNA levels were evaluated by SYBR Green I based real-time RT-PCR with an ABI PRISM 7000 sequence detector (Applied Biosystems, Foster City, CA) as previously described [12]. All gene primers were obtained from Takara Bio (Otsu, Japan). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels were used as an internal standard for calibration.

2.7. Statistical analysis

Results shown in the text and figures are means $\pm$ SEMs. Group comparisons were made by non-parametric two-tailed Mann–Whitney test, Log rank test, or 2-way ANOVA. A *p* value < 0.05 was considered significant.

3. Results

3.1. Th17 and Foxp3⁺ regulatory T cells in Gal-9^{-/-} mice

We first tested the effects of Gal-9 on disease severity and Th17 and Foxp3⁺ Treg frequency using Gal-9^{-/-} mice in an experimental model of allergic encephalomyelitis (EAE). Gal-9^{-/-} mice exhibited greater and more prolonged disease severity than wild type (WT) mice (Figs. 1A and B). Splenic IL-17⁺ CD25⁺ CD4⁺ T cells were increased in Gal-9^{-/-} mice, whereas Foxp3⁺ CD25⁺ CD4⁺ T cells were decreased (Fig. 1C). Furthermore, contraction of Foxp3⁺ cells and expansion of Th17 cells was observed only during inflammatory

conditions, because there was no significant difference in the frequency of Foxp3⁺ cells and IL-17⁺ cells between WT and healthy Gal-9^{-/-} mice (Fig. 1C). *In vitro*, Gal-9^{-/-} T cells skewed to Th17 exhibited increased IL-17 production over WT (Fig. 1D). As expected, subcutaneous treatment with stable Gal-9 (3 µg/mouse, twice weekly) significantly improved the clinical and histological signs of EAE (Fig. 1E).

3.2. Suppression of Th17 cell development by Gal-9

Two explanations can be attributed for Gal-9-mediated Th17 cell suppression. Gal-9 could induce cell death in Th17 cells, or Gal-9 could inhibit Th17 cell development. To clarify the effects of Gal-9 on Th17 development, naïve CD4⁺

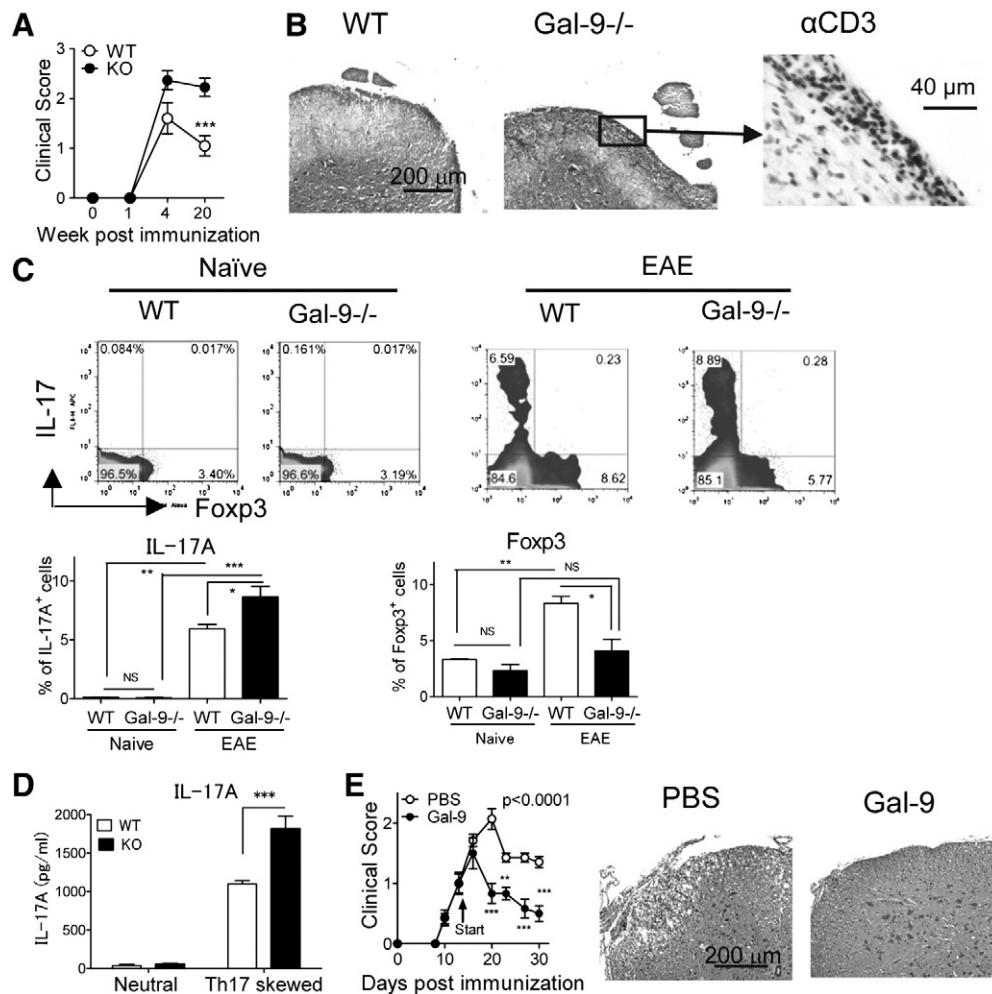


Figure 1 Gal-9 regulates Th17/Foxp3⁺ Treg development in MOG-induced EAE. Wild type (WT) or Gal-9^{-/-} mice (C57B6/J strain) were immunized with MOG (35–55) peptide. Mice were killed 2 weeks after immunization, and spleen and spinal cord were removed. (A) Clinical progression of MOG-induced EAE in Gal-9^{-/-} or WT mice. Data are represented as the means ± SEMs of 10 mice. ***, p < 0.001. (B) Histology of spinal cord sections at 20 weeks after immunization (WT: left, Gal-9^{-/-}: center). Sections were stained with H. & E.; Gal-9^{-/-} mice were stained for CD3 (right). (C) Intracellular IL-17 and Foxp3 staining of splenic CD4⁺ CD25⁺ cells cultured with TCR stimulation for 3 days and re-stimulated with PMA plus ionomycin. Data are represented as the means ± SEMs of 4 mice. *, p < 0.05; **, p < 0.01; and NS, not significant. (D) IL-17 production by Th17-skewed T cells from wild type and Gal-9^{-/-} mice. For Th17 skewing, naïve CD4⁺ T cells were cultured with anti-CD28 antibody, IL-2, and TGF-β1 together with IL-6 on anti-CD25-coated plates for 4 days. Data are represented as the means ± SEMs from quadruplicate samples. ***, p < 0.001. (E) The clinical score of MOG-induced EAE treated with Gal-9 and H.E. staining of spinal cord sections at 19 weeks after immunization. Subcutaneous treatment with Gal-9 was started on day 14. Data are represented as the means ± SEMs of 7 mice. **, p < 0.01, and ***, p < 0.001.

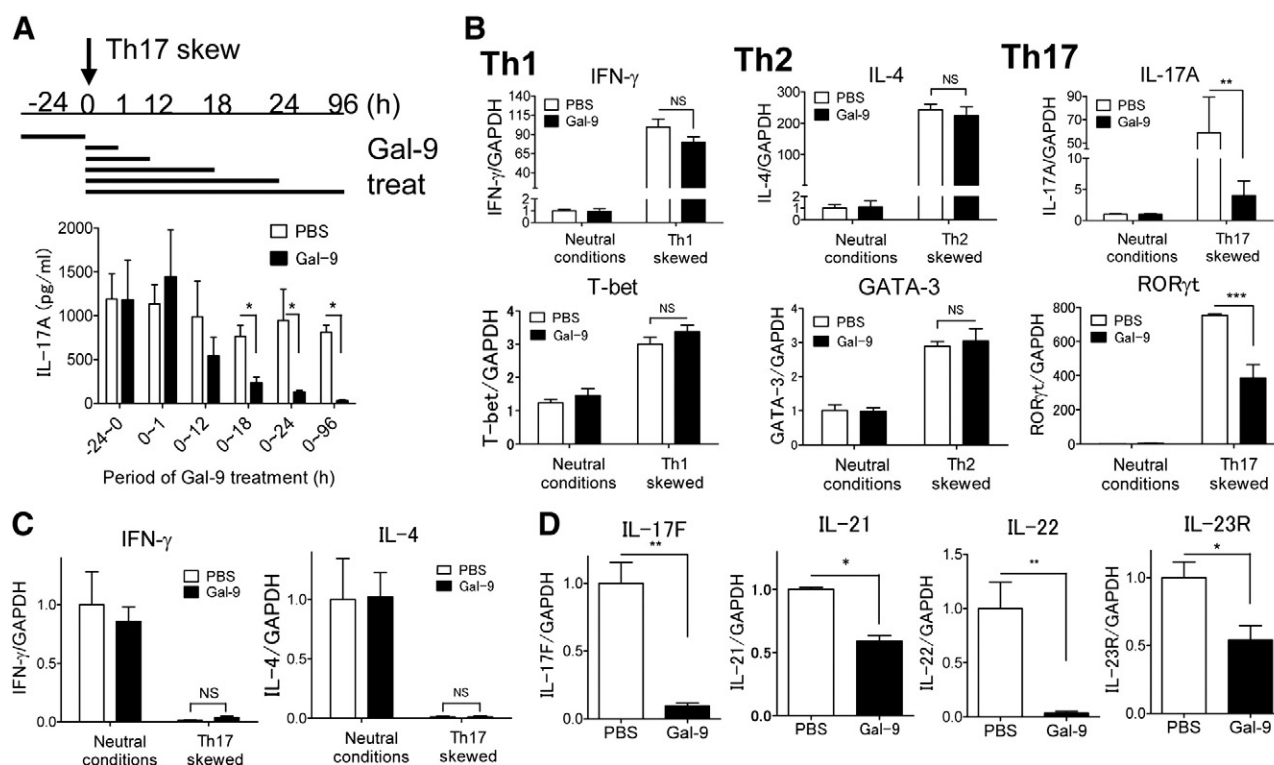


Figure 2 Gal-9 suppresses IL17 production. (A) Gal-9 suppresses IL-17A production under Th17-skewing conditions. Results are means $\pm$ SEMs of quadruplicates. * $p < 0.05$. (B) No involvement of Th1- and Th2-skewing in suppression. Th1- and Th2-skewing conditions are described in [Materials and methods](#). After 96-h culture, mRNA expression was determined by real-time RT-PCR. Results are means $\pm$ SEMs of quadruplicates. NS: not significant, ** $p < 0.01$ and *** $p < 0.001$. (C) Effect of Gal-9 on IFN- γ and IL-4 production under Th17-skewing conditions. mRNA expression was determined by real-time RT-PCR. Results are means $\pm$ SEMs of quadruplicates. NS: not significant. (D) mRNA levels for IL-17F, IL-21, IL-22, and IL-23R genes. mRNA levels were determined by real-time RT-PCR. Results are means $\pm$ SEMs of quadruplicates. * $p < 0.05$ and ** $p < 0.01$.

cells were treated with Gal-9 (30 nM) under Th17-skewing conditions for 96 h. Gal-9 treatment during the first 18 h dramatically inhibited IL-17A production. This Gal-9-mediated inhibition during the first 24 h was nearly equivalent to that by Gal-9 throughout the 4-day culture ([Fig. 2A](#)). In contrast, Gal-9 did not suppress IL-17A production when cells were pretreated with Gal-9 for 24 h prior to Th17 skewing or during the first 1 h after the skewing ([Fig. 2A](#)).

We next sought to determine how Gal-9 suppressed Th17 development. First, we addressed whether Gal-9 affected Th1 and Th2 cell development. Although Gal-9 significantly suppressed Th17 development, it did not affect either IL-12-induced Th1 development or IL-4-induced Th2 development ([Fig. 2B](#)). T-bet, GATA-3, and ROR γ t are critical transcription factors for Th1, Th2, and Th17 development, respectively [19]. ROR γ t expression was significantly suppressed by Gal-9, while T-bet and GATA-3 were not ([Fig. 2B](#)). Although Th1 and Th2 cell development suppresses Th17 development [20], IFN γ and IL-4 mRNA expression was not enhanced by Gal-9 under Th17-skewing conditions ([Fig. 2C](#)). This suggested that enhanced Th1 and Th2 development was not the direct cause of Gal-9-mediated suppression. We further found that Gal-9 treatment from 0 to 24 h after Th17-skewing also suppressed the expression of other Th17-related genes, such as IL-17F, IL-21, IL-22, and IL-23R ([Fig. 2D](#)).

3.3. Up-regulation of CD25 and Foxp3 expression by Gal-9

We next addressed the mechanisms of Gal-9-mediated decrease of Th17 cells and a reciprocal increase of Foxp3⁺ Tregs. About 6% of IL-17A⁺ Foxp3⁻ cells and 25% of IL-17A⁻ Foxp3⁺ among CD25⁺ CD4⁺ T cells were observed among untreated Th17-skewed cells ([Fig. 3A](#)). Conversely, Gal-9 treatment significantly reduced IL-17A⁺ Foxp3⁻ CD4⁺ T cells, and increased IL-17A⁻ Foxp3⁺ CD4⁺ T cells ([Fig. 3A](#)). Gal-9 treatment also increased IL-17A⁺ Foxp3⁺ CD4⁺ T cells weakly but significantly.

Next, we addressed IL-2 dependency for the suppression of IL-17 and up-regulation of Foxp3⁺ CD25⁺ cells. It has been shown that IL-2 signaling via STAT5 constrains Th17 generation [21]. Consistent with this report, IL-2 suppressed IL-17A production under Th17-skewing conditions in a dose-dependent manner ([Fig. 3B](#)). Gal-9 treatment significantly suppressed IL-17A production in the presence of IL-2 even at low concentrations (1 ng/ml), similar concentrations of IL-2 given alone did not suppress IL-17A production, though Gal-9 in the absence of exogenous IL-2 only weakly suppressed IL-17 production ([Fig. 3B](#)).

IL-2 also increased Foxp3⁺ cells weakly but significantly, and Gal-9 synergistically up-regulated IL-2 induced Foxp3⁺

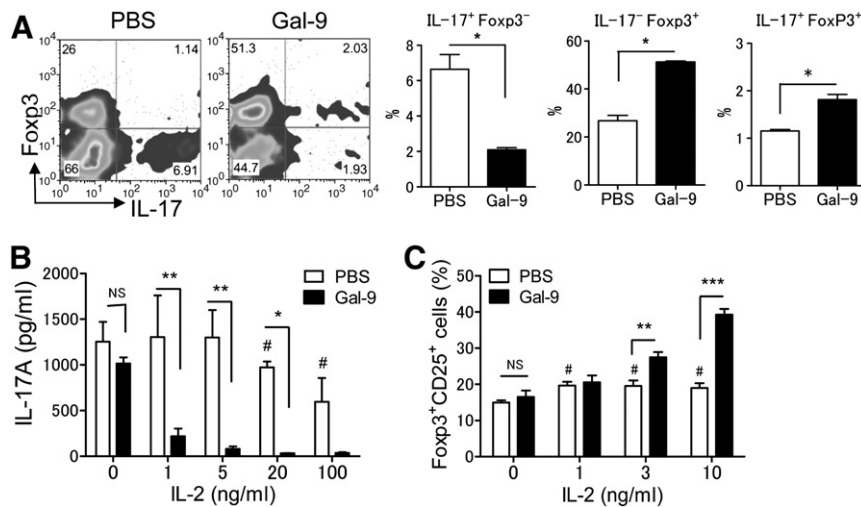


Figure 3 IL-2 dependency of Gal-9-induced suppression of IL-17 production. (A) Effect of Gal-9 on IL-17A and Foxp3 expression. Naïve CD4 T cells were cultured as in Th17 skewing condition for 4 days. Harvested CD4 T cells were re-stimulated with PMA and ionomycin in medium containing brefeldin A for an additional 6 h. IL-17A- and/or Foxp3-expressing cells were determined by flow cytometry. Results are means $\pm$ SEMs of quadruplicates. * $p < 0.05$. (B) Effect of IL-2 on the suppression of Th17 cell development by Gal-9. Naïve CD4 T cells were cultured under Th17-skewing conditions, with or without IL-2. Results are means $\pm$ SEMs of quadruplicates. * $p < 0.05$ and ** $p < 0.01$. #, $p < 0.05$ (vs IL-17A level in the culture supernatant from T cells stimulated without IL-2). (C) Effect of IL-2 on expansion of frequency of Foxp3⁺ CD25⁺ cells by Gal-9. Naïve CD4 T cells were cultured under Th17-skewing conditions, with or without IL-2. Results are means $\pm$ SEMs of quadruplicates. NS, not significant; * $p < 0.05$; ** $p < 0.01$; and ***, 0.001. #, $p < 0.05$ (vs T cells stimulated without IL-2).

cells (Fig. 3C). Furthermore, the up-regulation of Foxp3 expression by Gal-9 treatment was only induced in the presence of IL-2 (3 and 10 ng/ml; Fig. 3C). These results suggest that IL-2 is required for the Gal-9-mediated regulation of not only IL-17 production but also Foxp3 up-regulation.

3.4. Gal-9 suppresses Th17 cell development independently of Tim-3

Since differentiated Th17 cells express Tim-3 [13], we investigated whether Th17 development involved Gal-9/Tim-3 interactions. Tim-3 expression on Th17-skewed cells at 24 h of the culture was negligible (Fig. 4A). Naturally, addition of an anti-Tim-3 blocking antibody did not block Gal-9-mediated suppression of Th17 development (Fig. 4B). In contrast, 5 to 10% of Th17-skewed cells expressed Tim-3 4 days after culture (Fig. 4C), and blocking Tim-3 Ab inhibited Gal-9-induced Th17 cell death in a dose-dependent manner (Fig. 4D). This provides direct evidence that Gal-9 induces the death of Tim-3-expressing Th17 cells through Gal-9/Tim-3 interactions. Conversely, Gal-9-mediated suppression of Th17 development is likely induced independently of Tim-3.

Additional experiments were performed to clarify the mechanism of Gal-9-mediated suppression. First, we determined if Gal-9-mediated suppression of Th17 development was carbohydrate-dependent. Lactose (30 mM), a galectin inhibitor, but not sucrose, abrogated the suppression by Gal-9 in a dose-dependent manner (Fig. 4E). Experiments were performed to further determine whether N- or O-glycans were involved in Gal-9-mediated suppression. Benzyl- α -GalNAc, an inhibitor of O-glycan biosynthesis, significantly blocked the inhibitory effect of Gal-9, while swainsonine, an inhibitor of complex-type N-glycan biosynthesis, did not (Fig. 4F). These

data indicated that O-linked β -galactosides are important in Gal-9-mediated suppression, although N-glycans are critical for Gal-9-induced apoptosis [22].

4. Discussion

We have previously shown that Gal-9 ameliorates collagen-induced arthritis by decreasing Th1 and Th17 cells and inducing an increase in Foxp3⁺ Tregs [12]. We now show that MOG-induced EAE is exacerbated in Gal-9^{-/-} mice, and that increased IL-17 production and a concomitant decrease in the number of Foxp3⁺ Tregs is observed in these mice. We have also found that Gal-9 increases IL-17⁺ Foxp3⁺ CD25⁺ CD4 T cells in vitro. IL-17⁺ T cells co-expressing Foxp3 have been previously described in humans [23,24]. Those cells differentiate into Th17 cells upon TCR stimulation in the presence of IL-1, IL-2, IL-21 and IL-23. However, from the present results, we cannot exclude the possibility that IL17⁺ Foxp3⁺ cells differentiate into Foxp3 single-positive T cells in the presence of Gal-9. These lineage decisions should be the subject of further studies.

We further delineated two effects of Gal-9 on Th17 cells: Tim-3-independent suppression of Th17 development from TCR-stimulated naïve T cells and Tim-3-dependent induction of apoptosis in differentiated Th17 cells. Although suppression of Th1- or Th2-related cytokine production, such as IFN- γ or IL-4, has been shown to be critical for Th17 cell development [20], Gal-9-mediated suppression of Th17 development is likely not caused by increased production of IFN- γ and IL-4, because IFN- γ and IL-4 levels are not enhanced by Gal-9 under Th17-skewing conditions.

Gal-9 induces Foxp3⁺ Treg development in an IL-2-dependent manner: Gal-9 does not exhibit any induction in

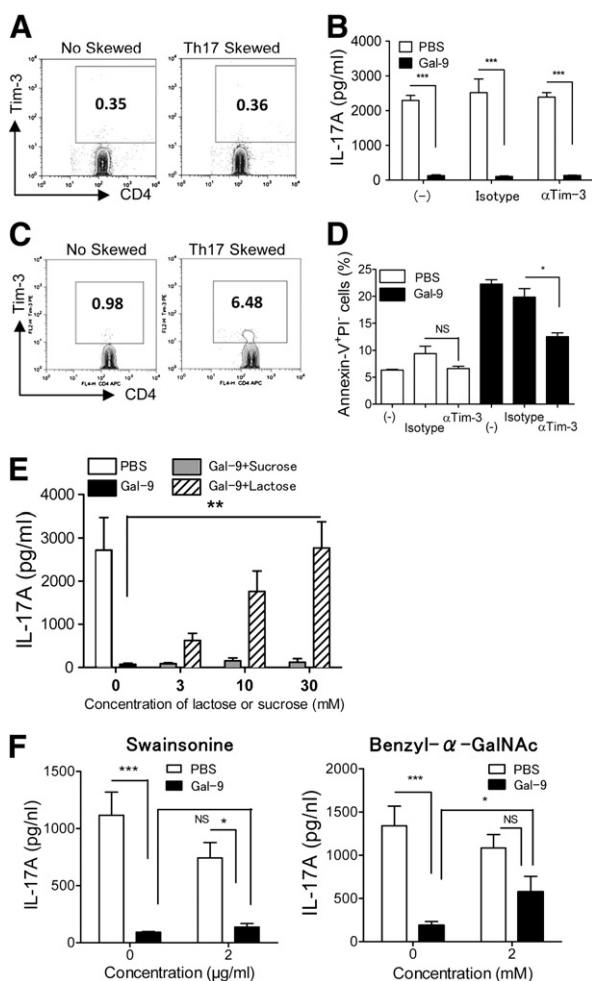


Figure 4 Gal-9 suppresses Th17 development independently of Tim-3. (A) Lack of Tim-3 expression on Th17-skewed T cells at 24 h after Th17 skewing. (B) Gal-9 suppresses Th17 cell development independently of Tim-3. Cells were incubated with Gal-9 and either anti-Tim-3 mAb or isotype control antibody for 24 h under Th17-skewing conditions. Results are means $\pm$ SEMs of quadruplicates. *** $p < 0.001$. (C) Th17-skewed cells on 4 days after culture express Tim-3. (D) Gal-9-induced apoptosis is dependent on Tim-3. Th17-skewed cells 4 days after skewing were incubated with Gal-9, with or without α Tim-3, for 4 h. Early apoptotic cells (Annexin-V⁺PI⁻ cells) were measured by flow cytometry. Results are means $\pm$ SEMs of quadruplicates. * $p < 0.05$. (E) The suppression of IL-17A production by Gal-9 is inhibited by lactose. Naive CD4 T cells were incubated with Gal-9 and either lactose or sucrose for 24 h in Th17-skewing conditions and cultured for additional 72 h. IL-17A in the culture supernatant was measured by ELISA. Data are represented as the means $\pm$ SEMs from quadruplicate experiments. **, $p < 0.01$. (F) An O-glycosylation inhibitor blocks the inhibitory effect of Gal-9 on IL-17A production. Naive CD4 T cells were incubated with Gal-9 and either an N-glycosylation inhibitor, swainsonine or an O-glycosylation inhibitor, Benzyl- α -GalNAc, for 24 h in Th17-skewing conditions and cultured for an additional 72 h. IL-17A levels in the culture supernatant were measured by ELISA. Data are represented as the means $\pm$ SEMs from quadruplicate experiments. NS, not significant; *, $p < 0.05$ and ***, $p < 0.001$.

the absence of IL-2. These results appear to be consistent with findings that IL-2 is required for the expansion and maintenance of Foxp3⁺ Tregs [25]. In contrast to expansion of Foxp3⁺ Tregs, IL-2 signaling inhibits Th17 development [21]. We have shown that IL-2 inhibits Th17 development in a dose-dependent manner. Although Gal-9 (30 nM) in the absence of IL-2 only weakly suppresses Th17 development, Gal-9 can dramatically suppress Th17 development in the presence of IL-2, even at very low concentrations, indicating that Gal-9 and IL-2 play a critical and synergistic role in the suppression of Th17 differentiation. Furthermore, since IL-21 is critical for Th17 generation [26–28], it seems reasonable that Gal-9 suppresses IL-21 together with IL-17 during Th17 differentiation.

After the discovery that apoptosis of terminally differentiated Tim-3⁺ Th1 cells is induced by Gal-9 via Gal-9/Tim-3 interaction [6,12], many studies have mainly focused on Gal-9/Tim-3 interaction in immunoregulatory activity by Gal-9 and Tim-3. These studies confirmed the importance of the Gal-9/Tim-3 interaction in Gal-9-mediated regulation of T cell-related immune responses [29,30]. Moreover, Gal-9 treatment reduces the number of cytotoxic CD8 T cells in transplantation or graft versus host disease models via Gal-9/Tim-3 interaction [31,32]. These results suggest that Gal-9 beneficially acts during autoimmune responses via Gal-9/Tim-3 interaction.

Conversely, Gal-9 can enhance immune responses in certain situations through Gal-9/Tim-3 interaction. Gal-9 stimulates conventional dendritic cells (cDCs) to produce TNF- α [8]. Gal-9 also enhances microbicidal activity of macrophages against *M. tuberculosis* [10]. Intriguingly, in MethA-bearing mice, expansion of Tim-3-expressing DCs and CD8 T cells is induced by Gal-9 treatment, and DCs enhance CTL activity of Tim-3-expressing CD8 T cells through Gal-9/Tim-3 interactions [7]. Thus, we propose that Gal-9 exhibits a “thermostat”-like function through Gal-9/Tim-3 interactions: Immunosuppression in autoimmune responses and immunopotentialization in immunocompromised conditions, although the precise mechanisms of these reciprocal functions of Gal-9 through Gal-9/Tim-3 interaction are yet unknown.

Because Gal-9 is a β -galactoside binding lectin, Gal-9 can exhibit its biological function by binding to glycoproteins with β -galactosides other than Tim-3. For example, Gal-9 binds nearly all integrins and inhibits tumor cell invasion and metastasis [16]. Gal-9 also binds to CD44 and inhibits binding of CD44 with hyaluronic acid, resulting in the suppression of activated T cell infiltration in allergic settings [15]. Furthermore, Gal-9 binds IgE and inhibits the allergen-induced release of chemical mediator from mast cells [14].

In addition, Gal-9 can exhibit a variety of biological activities by binding unidentified molecules. For instance, Gal-9 expands myeloid suppressor cells [33,34], as well as plasmacytoid DC-like macrophages [4,35]. Recently it has been shown that Gal-9 promotes effector Th cell function independently of Tim-3 [17]. The present experiment has shown that the expression of Tim-3 on CD4 T cells is negligible at 24 h after Th17 skewing. Taken together, the fact that Gal-9 suppresses Th17 development independently of Tim-3 is not surprising. Furthermore, Gal-9-mediated regulatory function is significantly inhibited by lactose but not anti-Tim-3 Ab, suggesting that the lectin nature of Gal-9 is critical for Gal-9-induced regulation of Th17/Treg development.

Glycans are divided into two subsets, N-glycans and O-glycans. It has been shown that Gal-9/Tim-3 interaction requires carbohydrate motifs on Tim-3IgV [6]. Further, in co-immunoprecipitation experiments with untreated or N-glycosidase F-treated (de-glycosylated) soluble Tim-3-Ig and Gal-9, only untreated soluble Tim-3-Ig is able to precipitate Gal-9, suggesting the importance of N-glycosylation in Tim-3 binding to Gal-9 [6]. We have also found that the expression of N-glycans in Jurkat T cell line cells is essentially required for Gal-9-mediated apoptosis [22]. In contrast, we have found in these experiments that O-glycans may be important for the suppression of Th17 cell development by Gal-9. This finding may support the notion that Gal-9-mediated suppression of Th17 cell development is induced independently of Tim-3.

In this study, we provide evidence that Gal-9 induces cell death of terminally differentiated Th17 cells via interaction with Tim-3, while it suppresses Th17 development in an IL-2-dependent but Tim-3-independent manner. Since Gal-9 is an attractive therapeutic candidate for various diseases, including autoimmune and infectious diseases [30], identification of Gal-9-binding proteins that are involved in these broad therapeutics is critical for better understanding Gal-9 biology, and may contribute novel pathways in immunological research.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgments

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