Galectin-9 promotes TGF-β1-dependent induction of regulatory T cells via the TGF-β/Smad signaling pathway

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Abstract. TGF-β1 induces the conversion of $CD4^+CD25^-$ T cells into $CD4^+CD25^+Foxp3^+$ regulatory T cells (Tregs). Galectin-9, one of the β-galactoside-binding animal lectins belonging to the galectin family, induces apoptosis of eosinophils, cancer cells and T cells. In this study, we report the effects of galectin-9 on the conversion of $CD4^+CD25^-$ T cells into Foxp3-expressing induced Tregs (iTregs). Galectin-9 together with TGF-β1 had synergistic effects on the rate of conversion to iTregs *in vitro*. TGF-β1 signaling appears to be essential for the effects of galectin-9 on the generation of iTregs, as galectin-9 promotes TGF-β1-induced phosphorylation of Smad2/3, ERK1/2 and formation of the Smad2/3-Smad4 complex. These data suggest a role for galectin-9 as a promoter of the TGF-β1-dependent conversion of $CD4^+CD25^-$ T cells into iTregs *in vitro*. Therefore, in addition to inducing apoptosis, galectin-9 may contribute to the regulation of inflammation via the expansion of peripheral Tregs.

Introduction

The maintenance of peripheral immune tolerance and prevention of chronic inflammation and autoimmune disease require $CD4^+CD25^+Foxp3^+$ regulatory T cells (Tregs) (1). The transcription factor Foxp3 is not only a marker for Tregs, but also programs the regulatory function of Tregs (2,3). Tregs were originally found to be generated in the thymus (4). However, it was recently demonstrated that peripheral $CD4^+CD25^-$ T cells could be converted into Foxp3-expressing Tregs *in vitro* (5,6) and *in vivo* (7-10). This conversion appears to depend on TGF-β1 (5,6,10) and the presence of IL-2 (8,11). Accordingly, Tregs may be divided into two subpopulations depending on their generation: i) naturally occurring Tregs, which are generated in the thymus and may represent a stable population of Tregs, and ii) induced Tregs (iTregs), which are generated in peripheral lymphoid tissues and may rapidly lose regulatory properties following their generation (4,12). TGF-β1 is essential for immune homeostasis, as deletion of TGF-β1 or targeted disruption of TGF-β1 or TGF-β signaling in T cells leads to multifocal inflammation with premature death (13-17), which at least in part appears to be a consequence of reduced Treg numbers or function (17-19). Galectin-9, one of the β-galactoside-binding animal lectins belonging to the galectin family, induces apoptosis of eosinophils, cancer cells and T cells (20-23). Galectin-9 preferentially induces apoptosis of activated $CD4^+$ T cells through the Ca^+ influx-calpain-caspase 1 pathway (24). Kashio *et al* and Zhu *et al* have recently demonstrated that galectin-9 is a ligand of T cell immunoglobulin- and mucin domain-containing molecule 3 (TIM-3) and was expressed selectively in terminally differentiated Th1 cells, Th17 cells and Tregs, and that galectin-9 induces apoptosis of TIM-3-expressing cells *in vitro* and *in vivo* (24,25). In fact, exogenous administration of galectin-9 ameliorates experimental allergic encephalitis, an autoimmune disease of the central nervous system (25). Furthermore, galectin-9 exhibits an anti-inflammatory role in lipopolysaccharide (LPS)-induced inflammation (26) and experimental allergic conjunctivitis (EAC) in mice (27). More recently it has been shown that galectin-9 ameliorates a mouse collagen-induced arthritis (CIA) model and herpes simplex virus (HSV)-induced lesions by regulating the T-cell response (28,29). Our previous study has indicated that galectin-9 administration effectively ameliorates CVB3-induced myocarditis by promoting the proliferation of T regulatory cells and the activation of Th2 cells (30). Due to its inhibitory effects on these inflammatory processes, an immunosuppressive role of galectin-9 has been suggested. However, the exact role of galectin-9 during inflammation remains to be elucidated.

In this study, we aimed to analyze the contribution of galectin-9 to the regulation of Treg numbers. Our data demonstrate a new role for galectin-9 for the TGF-β1-induced conversion of $CD4^+CD25^-$ T cells into Foxp3-expressing Tregs *in vitro* and *in vivo*.

Materials and methods

Ethics. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of

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Medical Laboratory Animals (Ministry of Health, China, 1998). The protocol was approved by the Medical Laboratory Animal Care and Use Committee of Anhui Province (Permit no. 2009-0132) as well as the Ethical Committee of Yijishan Hospital of Wannan Medical College (Permit no. 20090015).

Antibodies and reagents. Rabbit anti-Smad2/3, p-Smad2 and p-Smad3 antibodies were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). Mouse anti-p-ERK1/2, mouse anti-Smad4, mouse anti-glyceraldehyde-3-phosphate dehydrogenase (anti-GAPDH) antibody (MAB374) and rabbit anti-hemagglutinin (anti-HA) antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The $CD4^+CD25^+$ T cell isolation kit was obtained from Miltenyi Biotec (Bergisch Gladbach, Germany).

Lymphocyte separation. Spleens were isolated from 8- to 10-week-old Balb/c mice. $CD4^+CD25^-$ naive T cells were isolated from splenocytes using a $CD4^+CD25^+$ T cell isolation kit (Miltenyi Biotec) according to the manufacturer's instructions. The purity of $CD4^+CD25^-$ T cells was >90%.

In vitro conversion assay. The isolated naive $CD4^+$ T cells in RPMI-1640 (Sigma-Aldrich, St. Louis, MO, USA) with 10% heat-inactivated fetal bovine serum, IL-2 (20 ng/ml, R&D Systems, Minneapolis, MN, USA) and anti-CD28 (2 μg/ml, Becton-Dickinson, Bedford, MA, USA) were distributed into anti-CD3 coated 96-well plates at $2x10^5$ cells/0.1 ml/well in the presence or absence of human TGF-β1 (5 ng/ml, R&D Systems) and human galectin-9 (0.3, 1 and 3 μg/ml, ProSpec-Tany TechnoGene, Ltd., Israel), and maintained for 4 days at 37˚C in an atmosphere containing 5% CO_2. Then the percentage of $CD4^+CD25^+Foxp3^+$ cells was detected by fluorescence-activated cell sorting (FACS). For generation of Tr1 cells, FACS-sorted naive $CD4^+CD25^-$ T cells and irradiated splenic APCs were cultured $1x10^6$ cells/ml/well in 24-well plates in the presence of suboptimal IL-10 (10 ng/ml) or IL-10 and galectin-9 (1 μg/ml). The cultured T cells were then restimulated with freshly prepared irradiated splenic APCs in the presence of IL-10 at intervals of 10-14 days. The stimulation under the same conditions was repeated weekly for 3 consecutive weeks. The percentage of Tr1 was then detected by FACS.

Cell suppressor assay. For the analysis of suppressor function, various numbers of $CD4^+CD25^+$ cells or the whole cell suspension obtained from the conversion assay were added to $1x10^5$ $CD4^+CD25^-$ carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled wild-type T cells. Cells were stimulated with CD3mAb and CD28mAb in 96-well flat-bottomed plates for 96 h in serum-free medium. Proliferation was analyzed by FACS assessing the CFSE dilution.

RT-PCR. Total RNA was extracted by TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and reverse transcribed into cDNA. PCR was performed to evaluate the relative expression of Foxp3. The forward and reverse primers for Foxp3 (5'-GCTCAGGCTCCGTTTCAC-3' and 5'-AGGATCGTC TAAGTCCAG-3') and the GAPDH house-keeping gene (5'-GAAGGTGAAGGTCGGAGTCA-3' and 5'-GAAGAT GGTGATGGGATTTC-3') were used. The expression of Foxp3 was normalized using GAPDH expression.

T cell proliferation assay. Purified $CD4^+CD25^-$ T cells from Balb/c mice were cultured with IL-2 (20 ng/ml, R&D Systems), TGF-β1 (5 ng/ml, R&D Systems) and/or galectin-9 (1 μg/ml), as indicated, under activating conditions for 4 days. 5-Bromo-2-deoxyuridine (BrdU; Upstate, Lake Placid, NY, USA) was added to the cultured cells at a final concentration of 50 μM, 16 h prior to the end of the 4-day incubation period. The cells were stained with conjugated anti-mouse CD4, CD25 antibody (eBioscience, Inc., San Diego, CA, USA), fixed in IC fixation buffer (eBioscience, Inc.) for 30 min and permeabilized in permeabilization buffer (eBioscience, Inc.) for 20 min. Cells were then incubated at room temperature for 30 min in 0.15 M NaCl, 4.2 mM $MgCl_2$ and 10 mM HCl in the presence of 2 units DNaseI (Invitrogen), followed by staining with conjugated anti-BrdU antibody (BD Biosciences) for 30 min, and were finally analyzed by FACS.

Western blot analysis. Cells were lysed in RIPA extraction solution [(15 mM Tris, pH 7.5, 120 mM NaCl, 25 mM KCl, 2 mM EGTA, 2 mM EDTA, 0.1 mM dithiothreitol, 0.5% Triton X-100 and protease inhibitor cocktail (Sigma-Aldrich)]. Lysates were resolved by SDS-polyacrylamide gel electrophoresis on a 10% polyacrylamide gel, transferred to a nitrocellulose membrane, and probed sequentially with specific polyclonal antibodies against p-Smad2, p-Smad3 and Smad2/3 and monoclonal antibodies against Smad4 and GAPDH. Membranes were then incubated with secondary goat anti-mouse or goat anti-rabbit horseradish peroxidase-conjugated antibodies (1:10,000; Jackson Immunoresearch Laboratories Inc., West Grove, PA, USA) and developed using an ECL kit (Amersham Biosciences, Uppsala, Sweden).

Coimmunoprecipitation. The isolated naive T cells in RPMI-1640 (Sigma-Aldrich) with 10% heat-inactivated fetal bovine serum, IL-2 (20 ng/ml, R&D Systems) and anti-CD28 (2 μg/ml, Becton-Dickinson) were distributed into anti-CD3 coated 96-well plates at $2x10^5$ cells/0.1 ml/well. They were then serum-starved and treated with TGF-β1 (5 ng/ml, R&D Systems) or TGF-β1 and galectin-9 (1 μg/ml) for 30 min. Cells were then scraped into ice-cold PBS and lysed with lysis buffer containing 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.5% nonidet P-40, 150 mM NaCl, 1 mM dithiothreitol, 10% glycerol and protease inhibitors (Sigma-Aldrich). After being spun at top speed in a microcentrifuge to remove cellular debris, the supernatant was transferred to a clean test tube and precleared for 1 h with 50 μl of protein A. Following preclearing, rabbit anti-Smad2/3 antibody and control rabbit anti-HA antibody, preincubated with 30 μl of protein A, were added to the lysate. Immune complexes were precipitated overnight at 4˚C. The immunoprecipitated material was washed three times with 1 ml of washing buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.25% gelatin and 0.1% NP-40. The pellet was then resuspended in SDS sample buffer and analyzed by western blotting.

Statistical analysis. Data are shown as the means ± SEM. Statistical analysis of the data was performed with the

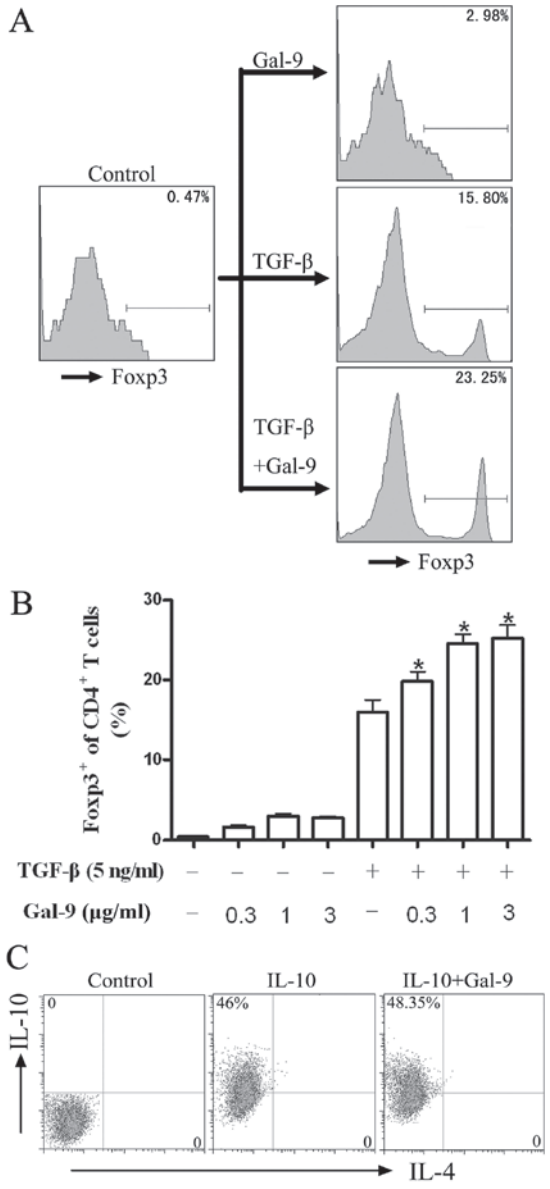

Figure 1. Effect of galectin-9 on the differentiation of Tregs *in vitro*. Spleens were isolated from 8- to 10-week-old Balb/c mice. $CD4^+CD25^-$ naive T cells were isolated from splenocytes by a $CD4^+CD25^+$ T cell isolation kit according to the manufacturer's instructions. Purity of $CD4^+CD25^-$ was >90%. (A and B) The isolated naive T cells in RPMI-1640 with 10% heat-inactivated fetal bovine serum, IL-2 (20 ng/ml) and anti-CD28 (2 μg/ml) were distributed into anti-CD3 coated 96-well plate at $2x10^5$ cells/0.1 ml/well in the presence or absence of human TGF-β1 (5 ng/ml) and human galectin-9 (0.3, 1 and 3 μg/ml) and maintained for 4 days at 37˚C in an atmosphere containing 5% CO_2. The percentage of $CD4^+CD25^+Foxp3^+$ cells was detected by FACS (gated on $CD4^+CD25^+$ T cells). (C) For generation of Tr1 cells, FACS-sorted naive $CD4^+CD25^-$ T cells and irradiated splenic APCs were cultured $1x10^6$ cells/ml/well in 24-well plates in the presence of suboptimal IL-10 (10 ng/ml) or IL-10 and galectin-9 (1 μg/ml). The cultured T cells were then restimulated with freshly prepared irradiated splenic APCs in the presence of IL-10 at intervals of 10-14 days. The stimulation under the same conditions was repeated weekly for three consecutive weeks. The percentage of Tr1 was detected by FACS. Similar results were obtained in 3 separate experiments and the representative results are indicated. Gal-9, galectin-9; *P<0.05 vs. TGF-β treatment alone; ANOVA analysis.

two-tailed independent Student's t-test and ANOVA analysis using SPSS, version 12.0 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

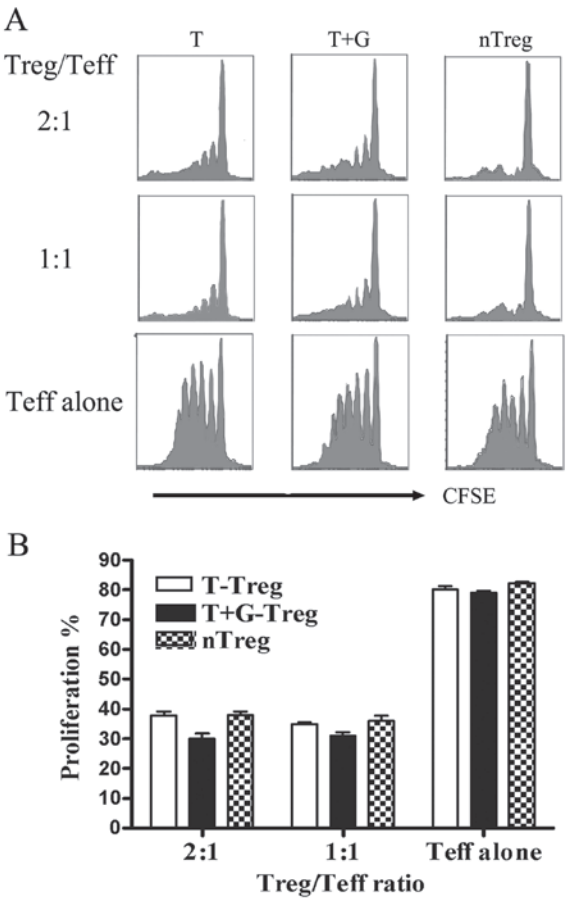

Figure 2. The induced Tregs are suppressive *in vitro*. To compare the suppressive capacity of these Tregs, T-Tregs, T+G-Tregs and nTregs were sorted to >90% purity. These Treg cell subsets were cultured with CFSE-labeled $CD4^+CD25^-$ effector T cells at the indicated ratios under activating conditions. (A) Representative CFSE plots of the effector T cells are shown, (B) with the percentage of dividing effector T cells quantified. Similar results were obtained in 3 separate experiments. Data show the means ± SEM. T, Tregs induced by TGF-β; T+G, Tregs induced by TGF-β and galectin-9; nTregs, natural Tregs; Teff, effector T cells; ANOVA analysis in each group.

Results

Galectin-9 synergizes with TGF-β1 to convert $CD4^+CD25^-$ T cells into Tregs. TGF-β1 is essential for the conversion of $CD4^+CD25^-$ T cells into iTregs (5,6). As this may represent an important physiological process to regulate and restrict inflammation, we aimed to investigate the roles for galectin-9 regarding the conversion of naive T cells into iTregs. We stimulated $CD4^+CD25^-$ T cells with plate-bound CD3mAb and soluble CD28mAb and cultured the cells for 4 days in the presence of TGF-β1 and/or galectin-9. We found that galectin-9 increased the conversion to $Foxp3^+$ cells induced by a concentration (5 ng/ml) of TGF-β1 (15.80 vs. 23.25%; Fig. 1), but not in a dose-dependent manner. However, in the absence of TGF-β1, galectin-9 (3 μg/ml) could not induce Foxp3 expression (Fig. 1B). In addition, we explored the role of galectin-9 in the differentiation of Tr1 regulatory T cells (Tr1) *in vitro*. We found that galectin-9 could not increase the conversion to Tr1 in the presence of suboptimal IL-10 (10 ng/ml) *in vitro*. These data show that galectin-9 potently enhances TGF-β1-dependent Treg induction.

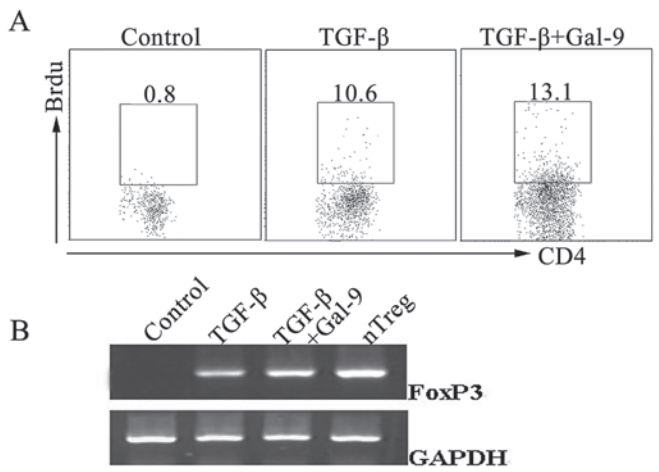

Figure 3. Galectin-9 enhances Foxp3 expression without impeding T cell proliferation. Purified $CD4^+CD25^-$ T cells from Balb/c mice were distributed into anti-CD3 coated 6-well plate at $1x10^6$ cells/0.1 ml/well in the presence or absence of anti-CD28 (2 μg/ml), IL-2 (20 ng/ml), TGF-β1 (5 ng/ml) and/or galectin-9 (1 μg/ml) and maintained for 4 days at 37˚C in an atmosphere containing 5% CO_2. 5-Bromo-2-deoxyuridine (BrdU) was added to the cultured cells at a final concentration of 50 μM, 16 h prior to the end of the 4-day incubation period. Cells were stained with conjugated anti-mouse CD4 antibody, fixed in IC fixation buffer for 30 min and permeabilized in permeabilization buffer for 20 min. Cells were then incubated at RT for 30 min in 0.15 M NaCl, 4.2 mM $MgCl_2$ and 10 mM HCl in the presence of 2 units DNaseI, followed by staining with conjugated anti-BrdU antibody for 30 min and were finally analyzed by FACS. (A) The mRNA levels of Foxp3 were analyzed by RT-PCR. (B) The proliferation of $CD4^+$ T cells was determined by BrdU incorporation assay. Similar results were obtained in 3 separate experiments and the representative results are indicated.

The induced Tregs are suppressive in vitro. The suppressive activity of Tregs induced by galectin-9 and TGF-β1 was measured relative to that of TGF-β1-induced Tregs and freshly harvested natural Tregs (nTregs) in a suppressor assay *in vitro*. The data show that Tregs induced by galectin-9 and TGF-β1 are potent suppressors of effector T cells *in vitro* (Fig. 2).

Galectin-9 enhances Foxp3 expression without impeding T cell proliferation. The observation that Treg cells can be induced by galectin-9 in the presence of TGF-β1 led us to question whether this occurs by galectin-9 functioning to interrupt T cell proliferation. To visualize this, purified $CD4^+CD25^-$ T cells were stimulated in the presence of TGF-β1 (5 ng/ml) alone or with galectin-9 (1 μg/ml), with T cell proliferation determined by BrdU incorporation assay. The addition of TGF-β1 to these cultures generated Tregs at expected frequencies (data not shown), with no impediment in cell proliferation observed due to the presence of galectin-9 (Fig. 3A). Notably, galectin-9 enhances TGF-β1-mediated Foxp3 expression (Fig. 3B). Collectively, these data show that galectin-9 enhances Foxp3 induction through a mechanism independent of dampening T cell proliferation.

Galectin-9 promotes the TGF-β1-induced phosphorylation of Smad2/3, ERK1/2 and formation of the Smad2/3-Smad4 complex. TGF-β1-induced transcriptional regulation is mediated mainly through phosphorylation of Smad2/3 proteins, followed by their translocation into the cell nucleus to further activate specific genes (28). Accordingly, it was speculated whether the increase of TGF-β1-induced gene expression mediated by galectin-9 involves alterations in the status of Smad2/3 following treatment. To test this possibility, $CD4^+CD25^-$ naive T cells were isolated from splenocytes. The isolated naive T cells in RPMI-1640 with 10% heat-inactivated fetal bovine serum, IL-2 and anti-CD28 were distributed into anti-CD3-coated 96-well plates at $2x10^5$ cells/0.1 ml/well. They were then serum-starved and treated with TGF-β1 or TGF-β1 and galectin-9 for 30 min and a western blot analysis was performed using antibodies against the phosphorylated form of Smad2 and Smad3. As demonstrated in Fig. 4A, a significant induction in Smad3 phosphorylation was already observed 30 min after treatment with TGF-β1. In contrast to TGF-β1, galectin-9 and TGF-β1-treated cells exhibited a significant increase in the phosphorylation of Smad3. By analyzing the status of the Smad2 protein, we noticed that

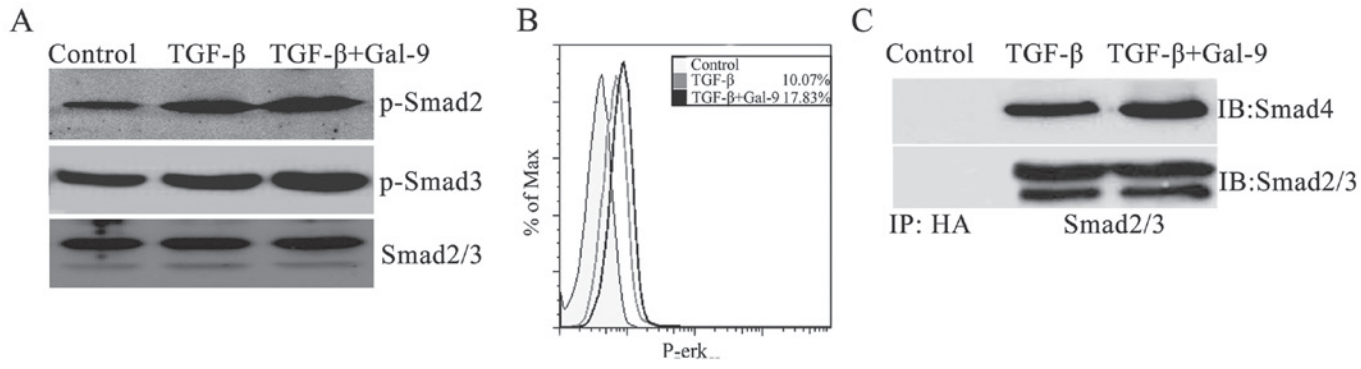

Figure 4. The effect of galectin-9 on the TGF-β1-induced phosphorylation of Smad2/3, ERK1/2 and formation of the Smad2/3-Smad4 complex. Naive $CD4^+CD25^-$ T cells were isolated from splenocytes by a $CD4^+CD25^+$ T cell isolation kit according to the manufacturer's instructions. Purity of $CD4^+CD25^-$ was >90%. The isolated naive T cells in RPMI-1640 with 10% heat-inactivated fetal bovine serum, IL-2 and anti-CD28 were distributed into anti-CD3 coated 96-well plates at $2x10^5$ cells/0.1 ml/well. They were then serum-starved and treated with TGF-β1 (5 ng/ml) or TGF-β1 and galectin-9 (1 μg/ml) for 30 min. (A) The phosphorylated form of Smad2/3 was analyzed by western blotting, using either rabbit anti-p-Smad2 or rabbit anti-p-Smad3 and total rabbit anti-Smad2/3 antibody. (B) The phosphorylation of ERK1/2 was monitored using mouse anti-p-ERK1/2 by FACS. (C) Cell lysates were immunoprecipitated with rabbit anti-Smad2/3 antibody and the precipitates were analyzed with mouse anti-Smad4 antibodies. Similar results were obtained in 3 separate experiments and the representative results are indicated. IP, immunoprecipitation; IB, immunoblotting.

similar to the case for Smad3, galectin-9 enhanced the phosphorylation of Smad2 following treatment of the cells with TGF-β1 for 30 min (Fig. 4A). It was already demonstrated that in addition to the Smad-dependent signaling pathway, TGF-β1 also has the ability to induce MAPK or phosphatidylinositol 3-kinase signaling pathways (31). Accordingly, we examined whether, as with Smad2/3, galectin-9 also promotes other TGF-β1-induced signaling pathways. To that end, ERK1/2 phosphorylation was analyzed by using specific antibodies against the phosphorylated form of the proteins. As depicted in Fig. 4B, treating cells with galectin-9 and TGF-β1 for 30 min significantly induced the phosphorylation of ERK1/2 proteins compared with TGF-β1 treatment alone. We also examined the involvement of galectin-9 in the formation of a complex between Smad2 and Smad4 upon TGF-β1 and galectin-9 treatment. As shown in Fig. 4C, TGF-β1-treated cells induced an association between Smad2 and Smad4, as detected by coimmunoprecipitation followed by western blot analysis. By contrast, treating cells with galectin-9 and TGF-β1 yielded more of the complex. These findings suggest that galectin-9, which promotes TGF-β1-induced activation of Smad2/3, eventually enhances the association between Smad2 and Smad4 following TGF-β1 treatment.

Discussion

The peripheral generation of iTregs is believed to be of physiological relevance for the control of inflammation (10,32). However, malignant or infected cells also appear to use similar mechanisms for conversion of $CD4^+CD25^+$ T cells into iTregs to suppress host immunity and to secure their own survival (7,33). Therefore, elucidation of the mechanisms that are operative in the conversion of T cells into iTregs may open new therapeutic strategies for manipulating peripheral Treg numbers and function. In this study, we report a novel role for the galectin family member galectin-9 as a promoter of the TGF-β1-induced conversion of $CD4^+CD25^-$ T cells into Foxp3-expressing iTregs *in vitro*. TGF-β1 has been shown to induce the conversion of $CD4^+CD25^-$ T cells into Foxp3-expressing iTregs *in vitro* (5,6) and *in vivo* (7-10). These newly generated iTregs were able to suppress T cell proliferation *in vitro* and to exert immunosuppressive functions *in vivo* when generated from endogenous cells (7-10) or upon adoptive transfer (34). There is also some indirect evidence that galectin-9 could be involved in the induction of $Foxp3^+$ cells, since such cells were reduced in numbers in galectin-9 knockout animals (28). We hypothesized that galectin-9 administration had an effect on the Treg response. We therefore investigated galectin-9 for its potential to substitute or enhance the previously described function of TGF-β1 for the conversion of $CD4^+CD25^-$ T cells into iTregs. We found that galectin-9 increased, but not in a dose-dependent manner, the conversion to $Foxp3^+$ cells induced by a concentration (5 ng/ml) of TGF-β1. However, in the absence of TGF-β1, galectin-9 (3 μg/ml) did not induce Foxp3 expression. In addition, we explored the role of galectin-9 on the differentiation of Tr1 regulatory T cells *in vitro*. We found that galectin-9 did not increase the conversion to Tr1 in the presence of suboptimal IL-10 (10 ng/ml) *in vitro*. These results suggest that galectin-9 mainly serves the promotion of the TGF-β1-induced conversion of non-Tregs into iTregs. The suppressive activity of iTreg cells was then measured relative to that of galectin-9-induced Tregs and freshly harvested nTregs in an *in vitro* suppressor assay. The data show that galectin-9-induced Tregs are potent suppressors of T effector cells *in vitro,* as well as freshly harvested nTregs. The observation that galectin-9-induced Tregs can be induced in the presence of high levels of co-stimulation and TGF-β1 led us to question whether this occurs by galectin-9 functioning to interrupt T cell activation and proliferation. Data show that galectin-9 enhances Foxp3 induction in the presence of TGF-β1 through a mechanism independent of dampening T cell proliferation. In searching for the mechanism underlying the increase of TGF-β1-induced transcriptional activity, we found that galectin-9 caused an increase in the phosphorylation of Smad2 and Smad3 following treatment with TGF-β1. It was previously shown that phosphorylated Smad2/3 oligomerizes with common Smad4 and that, together, they translocate to the cell nucleus to further activate specific target genes (35-38). By analyzing the effect of galectin-9 on the TGF-β1-induced Ras-MAPK pathway, we showed that galectin-9 is capable of promoting this pathway as well. This was observed by a significant increase in ERK1/2 phosphorylation upon galectin-9 and TGF-β1 treatment. Furthermore, we showed that galectin-9 promotes the formation of the complex commonly formed between Smad2 and Smad4 upon TGF-β1 treatment. Taken together, our findings indicate that the entire TGF-β1 signaling pathway is significantly affected by the presence of galectin-9. This observation may mean that galectin-9 may be increasing TGF-β receptor expression or inducing TGF-β in the cultures, which in turn could enhance Smad signaling pathway activation, although this needs to be formally confirmed.

In conclusion, our findings suggest a novel role for galectin-9 as a promoter of the TGF-β1-induced conversion of $CD4^+CD25^-$ T cells into Foxp3-expressing iTregs. As it is secreted early following inflammatory stimulation, galectin-9 could thus play a role in the downregulation of inflammatory reactions.

Acknowledgements

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