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Suppression of Autoimmune Diabetes by Soluble Galectin-1

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Type 1 diabetes (T1D) is a T cell-mediated autoimmune disease that targets the β-cells of the pancreas. We investigated the ability of soluble galectin-1 (gal-1), an endogenous lectin that promotes T cell apoptosis, to down-regulate the T cell response that destroys the pancreatic β-cells. We demonstrated that in nonobese diabetic (NOD) mice, gal-1 therapy reduces significantly the amount of Th1 cells, augments the number of T cells secreting IL-4 or IL-10 specific for islet cell Ag, and causes peripheral deletion of β-cell-reactive T cells. Administration of gal-1 prevented the onset of hyperglycemia in NOD mice at early and subclinical stages of T1D. Preventive gal-1 therapy shifted the composition of the insulin infiltrate into an infiltrate that did not invade the islets and that contained a significantly reduced number of Th1 cells and a higher percentage of CD4+ T cells with content of IL-4, IL-5, or IL-10. The beneficial effects of gal-1 correlated with the ability of the lectin to trigger apoptosis of the T cell subsets that cause β-cell damage while sparing naive T cells, Th2 lymphocytes, and regulatory T cells in NOD mice. Importantly, gal-1 reversed β-cell autoimmunity and hyperglycemia in NOD mice with ongoing T1D. Because gal-1 therapy did not cause major side effects or β-cell toxicity in NOD mice, the use of gal-1 to control β-cell autoimmunity represents a novel alternative for treatment of subclinical or ongoing T1D. The Journal of Immunology, 2009, 182: 2641–2653.

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due to the short life and limited migration of the genetically engineered DC and the fact that gal-1 induces the maturation of DC with a consequent increase in their immunogenicity (27, 28). In addition, in vitro generation of genetically engineered DC is a complex, time-consuming, and difficult to standardize technique with still limited applicability in humans compared with the simple administration of soluble gal-1.

In the past, several strategies have been used to prevent TID in NOD mice, but only a very limited number of them have cured existing TID (reviewed in Ref. 29). Therefore, the ability of gal-1 to target activated T cells makes this lectin a promising tool to treat TID, which is diagnosed in patients at the onset of diabetes or at late subclinical stages when the diabetogenic T cells are already activated. In this study, we show that therapy with soluble recombinant gal-1 prevented the onset of TID in NOD mice, which developed a nonpathogenic leukocyte infiltrate around the pancreatic islets. The preventive effect of gal-1 on T1D was significantly associated with the following: 1) reduction in the Th1 immunity and an increase in the number of CD4+ T cells secreting IL-4 or IL-10 in response to β-cell Ag; and 2) peripheral deletion of β-cell reactive T cells. This latter effect was caused by the ability of the lectin to induce apoptosis of the T cell subsets that promote β-cell damage while sparing those subpopulations with a more protective role against T1D. Importantly, administration of gal-1 to NOD mice also prevented T1D at subclinical stages of the disease and reversed β-cell autoimmunity in mice with ongoing TID. Our findings suggest that the use of gal-1, an endogenous lectin with no apparent β-cell toxicity or major side effects, constitutes an efficient and novel approach to preventing diabetes and, more importantly, to suppressing ongoing β-cell autoimmunity in subclinical or overt T1D.

Materials and Methods

Animals and reagents

NOD/LtJ, NOD.CB17-Prkdcked, and BALB/c mice were from The Jackson Laboratory. Studies were approved by the University of Pittsburgh Animal Research and Care Committees. DTT, OVA, and 2,4,6-trinitrochlorobenzene (TNCB), n-glucose, β-lactose, PMA, ionomycin, and brefeldin A were from Sigma-Aldrich. Recombinant human gal-1 and brefeldin gal-1 were produced as described (30). The BW514Phα2.1 (Phα2.1) cell line was a gift from Dr. M. Pierce (University of Georgia, Athens, GA). Neutralizing mAb against IL-4 (clone 11B11) or IL-10 (clone JES5-2A5) and purified rat IgG for in vivo studies were purchased from BioXcell. The rest of the mAb were from BD Pharmingen unless specified.

Gal-1 treatment and glucose tolerance test

Mice were injected (i.p.) with 100 μg of gal-1 in PBS and 8 mM DTT (200 μl) or with PBS and 8 mM DTT alone three times a week for different lengths of time. In some experiments, NOD mice received gal-1 therapy (from the 6th to the 24th wk of age) in combination with neutralizing IL-4 or IL-10 mAb (from the 14th to 20th wk of age), the latter at doses of 500 μg (i.p.) three times a week during the first week and at 300 μg twice a week thereafter. Control mice received purified rat IgG at the same doses. Diabetes was diagnosed when glycemia reached ≥300 mg/dl in two consecutive readings. Glucose tolerance tests were performed i.p. as described (31). Before glucose challenge, mice fasted for 16 h. Blood samples were taken from the tail vein before fasting and 0, 15, 30, 60, 90, and 120 min after glucose injection.

Contact hypersensitivity (CH) and detection of serum Ab

NOD mice were sensitized (day 0) by the application of 50 μl of TNCB (5% in acetone/olive oil; 4:1) or vehicle on the abdominal skin and were challenged (day 6) by the application of TNCB (1%) on the right ear (control). The thickness of the right (challenged) and left ear (control) was measured with an electronic caliper 24, 48, and 72 h after challenge. CH was determined as the swelling on the hapten-challenged ear compared with that of the vehicle-treated ear and was expressed as the percentage increase in ear thickness (mean ± SD). Each group consisted of at least three mice and each experiment was performed twice.

For the detection of OVA-specific Ab in serum, NOD mice were immunized (tail base) with OVA (one priming dose plus one boosting dose 7 days apart). Seven days after the last immunization, the titers of IgG against OVA were assessed by ELISA in serum samples using OVA-coated ELISA plates.

Histopathological analysis

Fragments of pancreas, skin, liver, kidney, gut, spleen, heart, lung, thymus, and lymph node were fixed in 4% formaldehyde and processed for H&E staining. Insulitis was scored by examining at least 30 islets of Langerhans or three different organs and given the following score: 0, no insulitis; 1, peri-insulitis; 2, insulitis in <50% of the islet; and 3, insulitis in >50% of the islet. The percentage of insulitis was calculated by dividing the number of islets in each category by the total number of islets examined. For the detection of insulin, sections were blocked with 5% normal goat serum and the avidin/biotin blocking kit (Vector Laboratories), incubated with human insulin mAb (BioGenex), biotin anti-mouse IgG, and avidin-biotin/peroxidase (Vector Laboratories). Endogenous peroxidase was blocked by passages in 70% ethanol, 1% H2O2 in methanol, and 70% ethanol. Peroxidase activity was developed with 3,3′-diaminobenzidine (Sigma-Aldrich).

Fluorescence microscopy

Fragments of pancreas and pancreatic lymph nodes (PLN) were embedded in Tissue-Tek OCT (Miles Laboratories), snap frozen, and stored at −80°C. Cryostat sections (8 μm) were mounted on slides treated with Vectorshield (Vector Laboratories) and fixed in cold 96% ethanol. Sections were blocked with normal goat serum and the avidin/biotin blocking kit. For detection of cytokines or FoxP3 in CD4 T cells, sections were labeled with Alexa Fluor 488-CD4 mAb (Caltag Laboratories) and one of the following reagents: 1) biotin-IFN-γ, biotin-IL-4, biotin-IL-5, biotin-IL-10, or biotin-IL-17 mAb followed by Cy3-streptavidin; or 2) Fox3 mAb (MF333F; Alexis Biochemicals) followed by Cy3-conjugated F(ab′)2, donkey-antirat IgG (Jackson ImmunoResearch Laboratories). For labeling of CD11c and F4/80, sections were incubated with CD11c mAb plus biotin F4/80 mAb followed by Cy3-anti-hamster IgG and Cy2-streptavidin. For detection of IFN-γ in CD8 T cells, sections were labeled with biotin-IFN-γ and Cy3-streptavidin plus Alexa Fluor 488-CD8 mAb. For codetection of CD4 and CD8 T cells, sections were incubated with Alexa Fluor 488-CD4 mAb and biotin-CD8α mAb (eBioscience) followed by Cy3-streptavidin (Jackson ImmunoResearch Laboratories). For assessment of T cell apoptosis, sections were fixed in paraformaldehyde and labeled with Alexa Fluor 488-CD3 mAb followed by TUNEL staining (Roche). Sections of pancreas were labeled with anti-Ki67 mAb (GeneTex) plus Cy3-anti-rabbit IgG in combination with mouse anti-human insulin mAb, biotin-anti-mouse IgG, and Cy2-streptavidin. Nuclear were counterstained with DAPI (Molecular Probes). Tissue sections were analyzed using a fluorescence microscope (Zeiss) equipped with epifluorescence and a digital camera (AxioCam MRc; Zeiss). The percentages of infiltrating cells with cytokine content in the islets were calculated following image acquisition of 30 islets per experimental group with the image analyzing software AxioVision (Carl Zeiss Vision Imaging Systems).

ELISPOT assays

Spleen cells from NOD mice treated with vehicle or gal-1 (50 × 10^6 cells/well) were stimulated with the 65-kDa glutamic acid decarboxylase (GAD) peptide 206–220 (GAD65206–220, TSYEIPVFVLYETV; 100 μg/ml), mouse islet lysate (freeze-thaw; 50 μg/ml), insulin (100 μg/ml), or OVA (100 μg/ml) in 96-well ELISPOT plates coated with anti-IFN-γ, -IL-4, -IL-10, or -IL-17 mAb (BD-Biosciences). ELISPOT plates were cultured for 36 h followed by incubation with biotin-IFN-γ, biotin-IL-4, biotin-IL-10, or biotin-IL-17 mAb (BD-Biosciences). The spots were counted with an ImmunoSpot counter (Cellular Technology).

Adaptive transfer model of TID

For evaluation of the diabetogenic capability of in vitro polarized (polyclonal) NOD Th1 and Th2 cytokotic (Tc) 1 (Tc1) lymphocytes, 2 × 10^6 Th1 and 10^6 Thc1 cells were adoptively transferred (i.v.) into young adult (5–8 wk old) female NOD Scid mice. For evaluation of β-cell-specific regulatory T (Treg) cells, female NOD Scid mice were left untreated or injected i.v. with CD4 T cells (5 × 10^5 or 5 × 10^6 Tc cells/mouse) selected negatively with CD4 T cell enrichment columns (R&D Systems) from the spleens and PLN of NOD female mice that had been treated with gal-1 or vehicle from the 5th to the 15th wk of age. Twenty-four
hours later, the NOD<sup>scid</sup> mice were challenged i.v. with 20 × 10<sup>6</sup> splenocytes from overtly diabetic NOD mice to trigger T1D (32, 33). Diabetes was diagnosed in the NOD<sup>scid</sup> mice when glycemia reached ≥300 mg/dl in two consecutive readings.

**MHC class II tetramer staining**

For tetramer formation, biotinylated I-A<sup>g7</sup>-BDC-13 (AAVRPLWVRMEAA) or CD8<sup>+</sup> T cells with artificial APC and IL-2 (10 U/ml). After 5 days of culture, T cells were isolated from the spleens of young (5 wk old) NOD mice and cultured for 3 days in complete medium in 96-well plates (2.5 × 10<sup>5</sup> T cells/well) and IL-2 (10 U/ml) in the absence or with increasing concentrations of soluble gal-1. As control, similar assays were conducted in the presence of the gal-1 inhibitor β-lactose (30 mM).

**Assays of T cell proliferation**

The ability of gal-1 to bias the differentiation of T cells was studied using splenic T cells purified with T cell enrichment columns (R&D Systems) from young NOD mice. T cells were cultured in complete medium (with 0.5 × 10<sup>6</sup> T cells/ml in 24-well plates) and IL-2 (10 U/ml) in the absence of or with increasing concentrations of soluble gal-1. As control, similar assays were conducted in the presence of the gal-1 inhibitor β-lactose (30 mM). For analysis of Ag-specific T cell proliferation ex vivo, spleen cells from NOD mice untreated or injected with vehicle or gal-1 were cultured for 3 days in complete medium in 96-well plates (2.5 × 10<sup>5</sup> cells/well) alone or with GAD6<sup>592-620</sup> (100 µg/ml). One µCi of [<sup>3</sup>H]TdR per well was added 18 h before harvesting the culture. Cell proliferation was assessed based on the uptake of [<sup>3</sup>H]TdR measured using a beta counter. The levels of IFN-γ, IL-4, IL-10, IL-17, and TGF-β1 were evaluated in culture supernatants by ELISA.

**Statistical analysis**

Results are expressed as means ± SD. Comparisons between means were performed by ANOVA followed by the Student-Newman-Keuls test. Comparison between two means was performed by Student’s t test. The incidence of T1D between groups was compared by Kaplan-Meier analysis and the log-rank test. P ≤ 0.05 was considered significant.
FIGURE 2. Therapy with gal-1 alters the cellular composition of insulitis. A, Analysis of islets (H&E) and insulin content (peroxidase; brown) in the pancreata of NOD mice before and after the administration of vehicle or soluble gal-1 from the 5th to 36th wk of age and analyzed 1 wk later. Islets of mice treated with gal-1 were surrounded by an infiltrate that did not invade or damage the islets. B, Analysis of the cellular composition of insulitis by fluorescence microscopy. Mice treated with gal-1 contained fewer F4/80+ macrophages and CD11c+ DC in the islets compared with controls. Vehicle-treated mice exhibited an infiltrate enriched in CD4+ T cells secreting IFN-γ that invaded the islets in diabetic mice and respected the limits of the islets in prediabetic animals. By contrast, mice treated with gal-1 exhibited an infiltrate with a high percentage of CD4+ T cells with intracellular IL-4, IL-5, or IL-10 and very few CD4+ T cells with IFN-γ and CD8+ T cells with IFN-γ (the latter not shown). Insets show intracellular localization of cytokines. C, Assessment of the percentages of CD4+ T cells with intracellular cytokines analyzed on tissue sections by fluorescence microscopy with the image-analyzing software AxioVision. Results represent values pooled from 30 islets quantified per group with eight mice per group. Differences between means were analyzed by ANOVA, followed by the Student-Newman-Keuls test. Original magnification of main panels, ×200; magnification of insets, ×400. In B, nuclei were stained with DAPI.
Results
Administration of soluble gal-1 prevents T1D

In the absence of treatment, NOD mice develop peri-insulitis by ∼5 wk of age, and by ∼8 wk lymphocytes begin to invade the islets (20). Destruction of β-cells and hyperglycemia occurs between 13 and 15 wk, and by 30 wk of age ∼80% of female NOD mice are overtly diabetic (20). Therefore, to test the capability of soluble gal-1 to prevent T1D, 5-wk-old female NOD mice were injected i.p. with soluble gal-1, vehicle (alone or supplemented with the irrelevant protein OVA), or left untreated. We used recombinant human gal-1 because the lectin is highly conserved through evolution and human gal-1 is functional in mice (12, 15, 16, 26, 27). All mice that received gal-1 (from the 5th to 36th wk) remained normoglycemic during the 37-wk follow-up (n = 10). Conversely, 81% of untreated mice (n = 16) and 78% of control animals injected with vehicle alone (n = 10) or with OVA (n = 8; data not shown) developed hyperglycemia (Fig. 1A). The preventive effect of gal-1 therapy on T1D was still significant (p < 0.01) when administration of the lectin was discontinued after the 15th wk (n = 8) (Fig. 1A). Euglycemic mice treated with gal-1 exhibited levels of glucose clearance similar to those of control age-matched normoglycemic NOD mice, as assessed by a glucose tolerance test performed in five mice per group 1 wk after completing gal-1 therapy (Fig. 1B).

Administration of gal-1 did not cause apparent toxicity in NOD mice. NOD mice injected with gal-1, 5-wk-old female NOD mice were injected i.p. with soluble gal-1, vehicle (alone or supplemented with the irrelevant protein OVA), or left untreated. We used recombinant human gal-1 because the lectin is highly conserved through evolution and human gal-1 is functional in mice (12, 15, 16, 26, 27). All mice that received gal-1 (from the 5th to 36th wk) remained normoglycemic during the 37-wk follow-up (n = 10). Conversely, 81% of untreated mice (n = 16) and 78% of control animals injected with vehicle alone (n = 10) or with OVA (n = 8; data not shown) developed hyperglycemia (Fig. 1A). The preventive effect of gal-1 therapy on T1D was still significant (p < 0.01) when administration of the lectin was discontinued after the 15th wk (n = 8) (Fig. 1A). Euglycemic mice treated with gal-1 exhibited levels of glucose clearance similar to those of control age-matched normoglycemic NOD mice, as assessed by a glucose tolerance test performed in five mice per group 1 wk after completing gal-1 therapy (Fig. 1B).

Administration of gal-1 did not cause apparent toxicity in NOD mice. NOD mice injected with gal-1 did not exhibit an increase in mortality or morbidity compared with vehicle-treated animals. Likewise, therapy with gal-1 did not cause histopathological changes in exocrine or endocrine pancreas, parenchymal organs, peritoneum (the lectin was injected i.p.), thymus, spleen, or lymph nodes and did not affect the count or composition of PBMC (not shown). The ability of gal-1 (injected i.p. at the dose and the timing that prevented T1D) to cause generalized immunosuppression was determined by analyzing the B cell response against the model Ag OVA and in a CH assay in response to the hapten TNCB. Gal-1-treated NOD mice developed titers of total IgG against...
OVA similar to those of control normoglycemic NOD mice injected with vehicle alone (Fig. 1C). In addition, NOD mice treated with gal-1 and skin sensitized with TNCB responded to the challenge by TNCB to the same extent as control (vehicle-treated) normoglycemic NOD mice of similar age (Fig. 1D). Thus, administration of gal-1 prevents the onset of T1D without causing major side effects.

Preventive therapy with gal-1 modifies the composition of insulitis

We then investigated the mechanisms involved in the preventive effect of exogenous gal-1 on T1D by analyzing the severity and composition of the insulitis in the pancreata of female NOD mice injected with soluble gal-1 or vehicle. Pancreatic islets from vehicle-treated mice that developed T1D during the follow up period of 37 wk (n = 8 mice) exhibited abundant leukocyte infiltration within the islets, which correlated with the substantial reduction/absence of insulin indicative of β-cell damage (Figs. 1E and 2A). By contrast, all islets examined from the gal-1-treated mice (n = 10 animals) exhibited a normal insulin content at the end of the 37 wk of follow up, and 88% of islets were surrounded by leukocyte infiltrates that did not invade the islets (Figs. 1E and 2A).

Because female NOD mice treated with gal-1 did not develop T1D despite the presence of peri-insulitis (Figs. 1E and 2A), we investigated whether the administration of gal-1 affects the leukocyte composition of the infiltrate that surrounds the islets. Gal-1 therapy decreased the number of F4/80+ macrophages and CD11c+ DCs infiltrating the islets (Fig. 2B). Administration of the lectin did not alter the CD4+CD25+ FoxP3+ Treg cell ratio or the percentage of apoptotic (TUNEL+) CD3+ T cells in the insulitis compared
We next investigated the mechanisms by which the administration of exogenous gal-1 down-regulates autoimmunity against β-cells in the NOD model. First, we determined the effect of gal-1 treatment on the frequency and pattern of cytokines of β-cell-reactive T cells. Young (prediabetic) female NOD mice treated with gal-1 or vehicle (from the 5th to the 36th wk) were euthanized 1 wk after completing gal-1 therapy, and splenocytes were stimulated with GAD65, insulin, and OVA (irrelevant control) in cytokine ELISPOT assays. Administration of gal-1 reduced (p < 0.01) the frequency of T cells secreting IFN-γ in response to the β-cell Ag compared with vehicle-treated controls (Fig. 3A). By contrast, the amount of T cells releasing IL-4 or IL-10 in response to GAD65, insulin, and islet lysate increased significantly (p < 0.01) in mice injected with gal-1 (Fig. 3A). No differences were detected between groups in the low frequencies of islet cell-reactive Th17 cells assessed by ELISPOT (Fig. 3A). Similarly, PLN of mice treated with gal-1 (from the 5th to the 36th wk) contained lower percentages of CD4+ IFN-γ+ cells and higher numbers of CD4+ cells with intracellular IL-4 or IL-10 than those of vehicle-treated controls (Fig. 3, B and C).

Together, our findings indicate that gal-1 therapy decreases the number of T cells secreting IFN-γ and promotes the generation of T lymphocytes that release IL-4 and IL-10 in response to islet cell Ag. We investigated whether the cytokine shift promoted by gal-1 therapy in our model was caused by gal-1-mediated T cell polarization or by differential susceptibility of T cell subsets to gal-1-mediated apoptosis. We first tested whether gal-1 polarizes NOD T cell differentiation at concentrations that do not trigger apoptosis (based on labeling with annexin V and analysis by FACS). Results are representative of three mice per group. B. Incidence of T1D in NOD scid mice reconstituted i.v. with in vitro polarized NOD Th1 and Tc1 cells (2 × 10^6 Th1 plus 10^6 Tc1 per mouse) and then treated with vehicle alone (n = 4) or gal-1 (n = 6). Kaplan-Meier analysis and the log-rank test were used.

Gal-1 shifts the balance of cytokines secreted by β-cell-specific T cells

With controls (not shown). By contrast, the infiltrate surrounding the islets of the gal-1-treated mice contained a significantly reduced number of CD4+ and CD8+ T cells secreting the type I cytokine IFN-γ (p < 0.01) and an increased percentage of CD4+ T cells with intracellular content of the Th2 cytokines IL-4, IL-5, and IL-10 (p < 0.01) compared with the pancreatic infiltrates of vehicle-injected mice, overtly diabetic or not (Fig. 2, B and C). Although the role of CD4+ T cells secreting IL-17 (Th17) in T1D is still unknown, a recent study suggested that Th17 cells might participate in the pathogenesis of the disease (25). However, under our experimental conditions very few CD4+ T cells containing IL-17 were detected in the islet infiltrates, with no differences in their percentages between groups (not shown). In addition, gal-1 therapy did not alter the number of CD4+FoxP3+ Treg in the islet infiltrates (not shown). Together, our findings indicate that the preventive effect of gal-1 on T1D was associated with changes in the cellular composition of the insulitis, which switched from the pathogenic Th1 and Tc1 cell infiltrate present in untreated prediabetic/diabetic NOD mice into a Th2-like infiltrate that did not invade or damage the islets.

Gal-1 causes apoptosis of pathogenic T cell subsets but spares Th2 and Treg in NOD mice

We investigated whether the cytokine shift promoted by gal-1 therapy in our model was caused by gal-1-mediated T cell polarization or by differential susceptibility of T cell subsets to gal-1-mediated apoptosis. We first tested whether gal-1 polarizes NOD T cell differentiation at concentrations that do not trigger apoptosis (<7 μM). Naive T cells from NOD mice were stimulated with artificial
Incidence of T1D in NOD mice were exposed to different concentrations of biotin-gal-1 (6, 3, and 1.5 μM for 45 min at 37°C) followed by labeling with PE-streptavidin and analysis by FACS. Cells from age-matched BALB/c mice were used as controls. One representative experiment of four is shown (n = 3 mice per group). B, Quantification by fluorescence microscopy and the image analysis software AxioVision of the percentages of apoptotic (TUNEL+) CD3+ T cells in sections of PLN from 5-wk-old female NOD mice treated with gal-1 or vehicle for 10 consecutive weeks. Results represent means ± SD of values pooled from six mice per group. C, Detection of β-cell-specific CD4+ Treg by means of the NOD-scid lympocyte transfer model of T1D. Host NOD-scid mice were injected i.v. or not (control) with CD4+ T cells obtained from spleen and PLN of female NOD mice that had been pretreated (from the 5th to the 15th wk of age) with gal-1 or vehicle. The following day, the NOD-scid mice were challenged with 20 × 10^6 splenocytes from overtly diabetic NOD mice. D, Incidence of T1D in NOD-scid mice reconstituted with CD4+ T cells from NOD mice pretreated with gal-1 or vehicle and then challenged with diabetogenic splenocytes (n = 8 mice per group).

Pha-B2.1 (93 ± 3%, a reliable target for gal-1-induced cell death) at concentrations of the lectin >7 μM, the Kd of the gal-1 homodimers (Fig. 4C). Apoptosis was drastically reduced by addition of β-lactose, confirming that it was dependent on the galactose-specific binding of the lectin (Fig. 4D). By contrast, naive CD4 or CD8 T cells and Th2 cells were not targets of gal-1-induced cell death (Fig. 4C) (5). Interestingly, although CD4+ Treg (resting or activated) bound to biotin-gal-1 (Fig. 4B), they exhibited very low susceptibility to gal-1-induced apoptosis (<15% increase in cell death; Fig. 4C).

To evaluate the relevance of our findings in vivo, we studied the effect of gal-1 therapy on CFSE-labeled, in vitro polarized NOD Th1 and Tc1 cells adoptively transferred (i.v.) into NOD-scid/mice (2 × 10^6 Th1 plus 10^6 Tc1 per mouse). The host NOD-scid mice were then treated with vehicle or gal-1 the following 3 days, and 1 day later the traffic, proliferation, and viability of the injected T cells were analyzed by flow cytometry. Similar percentages of T cells were detected in PLN, peripheral lymph nodes (cervical, inguinal, axillary, and mesenteric), and spleens from mice treated with gal-1 or vehicle, indicating that gal-1 did not affect the traffic of the injected T cells in our model. Four days after transfer, we were unable to detect T lymphocytes in the pancreas by immunofluorescence microscopy on tissue sections (not shown).
CFSE-labeled CD4\(^+\) Th1 and CD8\(^+\) Tc1 cells proliferated vigorously only in PLN, and both subsets were highly susceptible to gal-1-mediated apoptosis as assessed by labeling with annexin V (Fig. 5A). These in vitro polarized Th1 and Tc1 cells were functionally diabetogenic in vivo and susceptible to gal-1 therapy as demonstrated by the fact that 75% of the NOD\(^{SCID}\) mice (\(n = 4\)) reconstituted with NOD Th1 and Tc1 cells developed T1D within 12 wk, and the onset of hyperglycemia was prevented by gal-1 therapy (\(n = 6\)) (Fig. 5B). These results indicate that the effect of gal-1 in T1D is associated with its ability to induce the apoptosis of pathogenic T cell subpopulations without promoting cell death in CD4 Treg and Th2 cells.

Deletion of \(\beta\)-cell-specific T cells by administration of gal-1

Because gal-1 promotes the apoptosis of mature T cells (5), we investigated in the NOD model in vivo whether the administration of gal-1 results in the deletion of T cell clones that recognize \(\beta\)-cell Ag. We used the IA\(^\beta\)-BDC-13 tetramer that detects \(\beta\)-cell-reactive CD4\(^+\) T cells in NOD mice (34). As controls, we used IA\(^\beta\) tetramers with the CLIP peptide (IA\(^\beta\)-CLIP). Five-week-old female NOD mice were treated for 10 wk with gal-1 (100 \(\mu\)g 3 times a week i.p.) or vehicle, euthanized along with control age-matched BALB/c mice, and single cell suspensions from PLN were labeled with PE-tetramers in combination with a mixture of mAb and 7AAD (the latter to exclude dead cells). As expected, the I-A\(^\beta\)-BDC-13 tetramer labeled a small population of CD4\(^+\) T cells (Fig. 6A) from the PLN of NOD mice treated with vehicle. All samples labeled with control IA\(^\beta\)-CLIP and BALB/c cells incubated with the IA\(^\beta\)-BDC-13 tetramer resulted only in background levels of staining (Fig. 6A). Administration of gal-1 was associated with a significant reduction in the percentage of CD4\(^+\) T cells labeled by IA\(^\beta\)-BDC-13 in PLN (from 0.52 \(\pm\) 0.16 to 0.17 \(\pm\) 0.05\%, \(p < 0.01\); values were pooled from four independent experiments with three mice per group) (Fig. 6A). This result was indicative of the peripheral deletion of \(\beta\)-cell-reactive CD4\(^+\) T cells in the gal-1-treated animals and correlated directly with the higher percentages of apoptotic (TUNEL\(^+\)) T cells found in the PLN when compared with those of vehicle-treated mice (Fig. 6B).

Gal-1 and generation of \(\beta\)-cell-specific Treg

Our results suggest that the preventive effect of gal-1 in T1D depends on down-regulation of Th1 immunity and/or expansion of IL-10-secreting CD4\(^+\) T cells, the latter compatible with the generation of CD4\(^+\) Treg. Thus, we assessed the relative contribution of these potential \(\beta\)-cell-specific CD4\(^+\) Treg to the preventive effect of gal-1 therapy in the absence of the down-regulation of the Th1 response caused by the lectin. We addressed this point using a lymphocyte adoptive transfer model of T1D, where young adult NOD\(^{SCID}\) mice were challenged with 20 \(\times\) 10\(^6\) splenocytes from overtly diabetic NOD mice (32, 33). The day before, the NOD\(^{SCID}\) mice were injected i.v. (or not; control) with increasing numbers of CD4\(^+\) T cells from spleens and PLN of female NOD mice pretreated (from the 5th to 15th wk of age) with gal-1 or vehicle (control; normoglycemic) (Fig. 6C). Transference of

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FIGURE 7. Gal-1 therapy in subclinical T1D. A, Percentage of euglycemic animals in female NOD mice treated from the 16th to the 31st wk of age with gal-1 or vehicle (\(n = 10\) mice per group). B, Comparison of blood glucose clearances between gal-1-treated (from the 16th to the 31st wk) mice and age-matched controls as assessed by a glucose tolerance test performed during the last week of follow up (31st wk). C, Assessment of insulitis in female NOD mice treated with gal-1 or vehicle (from the 16th to the 31st wk). Results represent values pooled from 10 mice per group.
CD4+ T cells from NOD mice treated with gal-1 did not suppress/ delay the onset of T1D in reconstituted NOD<sup>SCID</sup> mice (Fig. 6D). These results suggest that, in the absence of the down-regulation of the Th1 response against β-cells caused by the lectin, the generation of potential tissue-specific CD4+ Treg by gal-1 therapy is not sufficient to prevent the onset of T1D in our model.

**Therapeutic effect of gal-1 on ongoing T1D**

Next, we investigated the efficacy of gal-1 therapy in the subclinical stages of T1D by administering the lectin to normoglycemic NOD mice 16 wk of age (100 μg i.p. three times a week), a time when extensive insulitis is already established but is still associated with normal metabolic control of glycemia. By the end of the 15-wk follow-up (31st wk of age), gal-1 therapy prevented the onset of hyperglycemia in nine of the 10 female NOD mice (p < 0.01), whereas six of 10 vehicle-injected animals became diabetic (Fig. 7A). At the end of the experiment (31st wk), those mice treated with gal-1 exhibited levels of blood glucose clearance similar to those of control normoglycemic NOD mice of similar age (Fig. 7B), and 95 ± 4% of the pancreatic islets exhibited a peri-insulitis (Fig. 7C) of similar characteristics to that found in NOD mice that had received preventive therapy with gal-1 (data not shown).

We then tested whether the gal-1 therapy is effective in ongoing T1D. Diabetic female NOD mice (10 consecutive days of hyperglycemia) were treated for 12 wk with gal-1 or vehicle alone (100 μg three times a week i.p.). All of the mice injected with vehicle remained hyperglycemic (n = 6). By contrast, 60% of gal-1-treated mice (n = 10) became normoglycemic and independent of exogenous insulin at the end of the 13th wk of follow-up (Fig. 8A) with normal levels of blood glucose clearance (Fig. 8B). In those mice that did not reverse hyperglycemia following gal-1 therapy, we investigated whether the problem relied on a failure of the lectin to control β-cell autoimmunity or the incapacity of the

**FIGURE 8.** Effect of gal-1 in ongoing T1D. **A**, Percentage of remission of T1D in diabetic NOD mice treated for 12 wk with gal-1 (n = 10 mice) or vehicle (n = 6 mice). Kaplan-Meier analysis and the log-rank test were used. **B**, Comparison of blood glucose clearance between NOD mice that reversed T1D after gal-1 therapy and untreated (euglycemic) age-matched controls. **C** and **D**, Proliferation (C) and cytokine secretion (D) in response to the GAD65<sub>206–220</sub> peptide of splenic T cells obtained from control (normoglycemic) and diabetic NOD mice treated with vehicle (control) or gal-1 for 12 wk, the latter with T1D remission or not. Results represent values pooled from four animals per group. Differences between means were analyzed by ANOVA, followed by the Student-Newman-Keuls test. **E**, Analysis by fluorescence microscopy of islets from diabetic NOD mice that reversed T1D after gal-1 therapy. The islets were populated with numerous Ki67<sup>+</sup> cells with insulin content and were surrounded or infiltrated by CD4<sup>+</sup> T cells with intracellular IL-4 and no IFN-γ (detailed in the insets; original magnification, ×400) (n = 6 mice). Original magnification for fluorescence microscopy was ×200. **F**, Assessment of insulitis in female NOD with ongoing T1D that received gal-1 or vehicle for 12 wk. Results represent values pooled from 10 mice treated with gal-1 and six animals injected with vehicle.
islets to regenerate β-cells. To compare the effect of gal-1 on β-cell autoimmunity in cured vs noncured mice, we analyzed the proliferation and pattern of the cytokines of splenic T cells in response to GAD65<sub>206–220</sub> in vitro. We observed that the pancreatic infiltrates of gal-1-treated group showed a similar incidence of insulitis, scored simply as the histopathologic examination of pancreata from mice treated with gal-1, compared to vehicle. However, the incidence of insulitis in the gal-1-treated group did not match that of the control group.

Discussion

Effective therapy of T1D requires elimination and/or regulation of the T cell clonal responses that initiate and perpetuate the damage of the pancreatic β-cells. In this study, we demonstrated that the regulatory effects of gal-1 on the immune system can be used to prevent diabetes in NOD mice, and importantly, to reverse ongoing β-cell autoimmunity at late stages of the disease in subclinical and overt T1D.

Previous studies have shown that gal-1 down-regulates T cell survival, activation, and proliferation (36, 37), suppresses Th1 responses (14, 17, 18, 26, 27), and triggers apoptosis of thymocytes and activated T cells in humans (5, 6, 38) and mice (26, 27). Gal-1 is expressed by Treg (39) and immune-privileged sites (40 – 42), and is used as a mechanism of immunosuppression by neoplasms (43), and plays an important role in fetomaternal tolerance (44). Due to its regulatory effects on T cells, gal-1 has been used to treat human autoimmune diseases in murine models (12–18, 26). Although the mechanisms of the pleiotropic effects of gal-1 have not been elucidated, there is evidence that the homodimeric (bivalent) form of gal-1 is critical to crosslink its ligands on the T cell surface (9) and to transduce the death signal (5). T cell apoptosis triggered by gal-1 is caspase independent and involves rapid nuclear translocation of endonuclease G from mitochondria without release of cytochrome c (45). Gal-1 has numerous anti-inflammatory effects, some of which are at concentrations below its apoptotic threshold, including the induction of IL-10 release (46), the down-regulation of the secretion of TNF-α and IFN-γ (15, 47), and the inhibition of adhesion to the endothelium (48) and the trans-endothelial migration of leukocytes (49, 50).

We showed in this study that the administration of soluble gal-1 prevents the onset of T1D in female NOD mice. Interestingly, histopathologic examination of pancreas from mice treated with gal-1 showed a similar incidence of insulinis, scored simply as the percentage of islets with associated leukocytes, as that of the vehicle-treated group. However, the pancreatic infiltrates of gal-1-treated mice did not invade the islets and did not damage the β-cells. The nonpathogenic nature of the insulitis of the gal-1-treated mice was due likely to the qualitative difference in its cellular composition. Therapy with gal-1 shifted the balance of the ratio between Th1- and Th2-like-cells in the islet infiltrate and PLN, increasing the percentage of CD4<sup>+</sup> T cells with IL-4, IL-5, or IL-10 and substantially reducing the number of IFN-γ-secreting T cells. This Th2-like bias in the T cell response was specific to β-cell Ag as demonstrated in ELISPOT assays and agrees with previous studies showing that pancreatic infiltrates with an inverted Th1/Th2 cell ratio cause a nondestructive peri-insulitis in NOD mice (51, 52). Although the critical factors that trigger leukocyte mobilization into the islets (insulitis) are unknown, the ability of gal-1 to reduce the number of IFN-γ-secreting T cells around the islets and/or to inhibit the migration of T cells (and possibly DC and macrophages) through the extracellular matrix (47, 50) could account for the noninvasive nature of the infiltrates in gal-1-treated mice.

The cytokine shift in the T cell response against β-cell Ag in gal-1-treated mice could be ascribed to the ability of the lectin to drive T cell polarization at concentrations below its apoptotic threshold and/or to trigger the cell death of specific subsets of effector T cells in NOD mice. We showed that at concentrations that do not induce T cell apoptosis, the addition of gal-1 did not alter significantly polarization of the T cells. However, in our system gal-1 bound and triggered rapid apoptosis of effector Th1, Th17, and Tc1 cells, but spared Th2 cells and Treg, both subsets that have been proved to protect against the development of T1D in NOD mice (35, 53–56). Our findings extend the results of a recent study showing that differential glycosylation of Th1, Th2, and Th17 cells regulates their resistance to gal-1-induced cell death. The susceptibility of Th1 and Th17 cells to gal-1-mediated cell death correlated directly with their high levels of core 2 O-glycan epitopes generated by the enzyme core 2 β(1,6)-N-acetylgalcosaminyltransferase on cell surface glycoproteins, which are ligands for gal-1 (18). In contrast, addition of α(2,6)-linked sialic acid to N-glycans on Th2 cells protects these cells from gal-1-induced cell death (18). Interestingly, although we detected that gal-1 binds to resting and (with more avidity) activated CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> T cells of NOD mice, these cells were resistant to gal-1-mediated apoptosis. This finding suggests that CD4<sup>+</sup>CD25<sup>+</sup>Treg must have a mechanism of protection from gal-1-mediated cellular suicide, as they constitutively express gal-1 (39).

In T1D, presentation of β-cell Ag and activation of diabetogenic T cells takes place initially in PLN (57). Thus, following therapy with gal-1, suppression of β-cell-reactive T cells could occur in the PLN or when the activated T cells infiltrate the pancreas. We found that gal-1 therapy increased the percentage of apoptotic T cells in PLN, a finding that correlated with a reduction in the percentage of β-cell-reactive CD4<sup>+</sup> T cells specific for the IA<sup>β7</sup>-BDC-13 complex. Although we were unable to detect an increased number of apoptotic T cells in the islet infiltrates, we cannot completely rule out the possibility that gal-1 therapy promotes T cell death locally within the pancreas, because it is well established that apoptotic cells are removed rapidly from peripheral tissues by phagocytes (58).

There is evidence that the administration of in vitro expanded CD4<sup>+</sup>FoxP3<sup>+</sup> β-cell-specific Treg suppresses T1D in NOD mice (35, 55, 56) and that the transfer of CD4<sup>+</sup> T cells from gal-1-treated mice prevents the onset of autoimmune uveitis in untreated recipients (17), the latter suggesting that gal-1 therapy promotes generation/expansion of tissue-specific Treg. However, in our system the transfer of CD4<sup>+</sup> T cells from gal-1-treated mice did not prevent/delay the onset of hyperglycemia in a transfer model of...
T1D. This finding suggests that, in the absence of deletion and the Th2-bias of β-cell-specific T cells and probably other gal-1-mediated anti-inflammatory effects, the generation of CD4+ Treg is not the main mechanism by which gal-1 prevents T1D.

Gal-1 therapy was also effective in the subclinical stages of T1D in NOD mice. These results have clinical relevance, because the prediction of early prediabetic stages or Ab-positive, high-risk, first-degree relatives has become more precise in humans (2). Once the β-cell-reactive T cells become activated, there is often a quite long asymptomatic period of time until the development of T1D, known as “honeymoon period,” when therapy with the lectin could be beneficial.

One of the most important findings of this study is that gal-1 therapy was capable of restraining T cell autoimmunity against β-cells even in NOD mice with declared T1D. This is of clinical importance, because most patients are diagnosed at late stages of the disease when they are overtly diabetic. Besides, the remission of declared β-cell autoimmunity at a time early enough to preserve the remaining β-cell mass/precursors within the islets is still the best therapeutic approach for patients with T1D. The results indicate that once the T cell-mediated aggression against the β-cells was abrogated by the administration of gal-1, the remaining islets recuperated their capacity to maintain normoglycemia in a considerable number of the lectin-treated animals. Gal-1 therapy down-regulated β-cell autoimmunity even in those mice that remained hyperglycemic, demonstrating that the failure to restore normoglycemia was likely due to the incapacity of the β-cells to regain their function and/or recuperate the β-cell mass, leading to metabolic diabetes. This may be ascribed to the absence of viable β-cell precursors at the time of initiation of the treatment.

Because gal-1 is an endogenous lectin, it is expected to be non-toxic and nonimmunogenic when injected repeatedly. In fact, administration of the lectin did not cause major side effects in NOD mice. More importantly, unlike several immunosuppressive drugs used to control the recurrence of T1D and the rejection of islet cell allografts, gal-1 did not seem to be toxic for the β-cells in the mouse. The fact that gal-1 does not trigger apoptosis of Th2 cells could explain why, in our model, exogenous gal-1 did not affect the systemic B cell response against a model Ag. Interestingly, although gal-1 therapy prevented T cell autoimmunity against β-cells, it did not affect the skin CH response elicited by haptons. Because cutaneous CH is susceptible to down-modulation by local injection of cells expressing gal-1 (27), our findings indicate that, at the dose and route used, the concentration of exogenous gal-1 in periphery did not reach enough levels to induce generalized immunosuppression.

Gal-1 is a soluble protein with no posttranslational modifications; therefore, large batches of the lectin can be produced in bacterial expression systems for therapeutic applications. However, because the subunits of the gal-1 homodimer are not covalently linked and the affinity for each other is rather low, the in vivo efficacy of gal-1 still depends on the administration of relatively large amounts of the lectin. To overcome this problem, Batig et al. (59) have generated, by genetic engineering, covalently linked gal-1 homodimers 10-fold more effective than the wild-type molecule that could be used therapeutically at much lower doses. To our knowledge, this is the first study on the use of a soluble lectin to treat T1D. Optimization of the variables for therapy with gal-1, including dose and timing of administration, the use of genetically improved variants of gal-1, and its combination with other Th1 immune-regulatory galectins like gal-9 (60), could open new possibilities for the treatment of T1D.


