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Dendritic Cells Expressing Transgenic Galectin-1 Delay Onset of Autoimmune Diabetes in Mice[1]

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Type 1 diabetes (T1D) is a disease caused by the destruction of the β cells of the pancreas by activated T cells. Dendritic cells (DC) are the APC that initiate the T cell response that triggers T1D. However, DC also participate in T cell tolerance, and genetic engineering of DC to modulate T cell immunity is an area of active research. Galectin-1 (gal-1) is an endogenous lectin with regulatory effects on activated T cells including induction of apoptosis and down-regulation of the Th1 response, characteristics that make gal-1 an ideal transgene to transduce DC to treat T1D. We engineered bone marrow-derived DC to synthesize transgenic gal-1 (gal-1-DC) and tested their potential to prevent T1D through their regulatory effects on activated T cells. NOD-derived gal-1-DC triggered rapid apoptosis of diabetogenic BDC2.5 TCR-transgenic $CD4^+$ T cells by TCR-dependent and -independent mechanisms. Intravenously administered gal-1-DC trafficked to pancreatic lymph nodes and spleen and delayed onset of diabetes and insulitis in the NOD*rag1*$^{-/-}$ lymphocyte adoptive transfer model. The therapeutic effect of gal-1-DC was accompanied by increased percentage of apoptotic T cells and reduced number of IFN-γ-secreting $CD4^+$ T cells in pancreatic lymph nodes. Treatment with gal-1-DC inhibited proliferation and secretion of IFN-γ of T cells in response to β cell Ag. Unlike other DC-based approaches to modulate T cell immunity, the use of the regulatory properties of gal-1-DC on activated T cells might help to delete β cell-reactive T cells at early stages of the disease when the diabetogenic T cells are already activated. ***The Journal of Immunology,*** **2006, 177: 5278–5289.**

Type 1 diabetes (T1D)[4] is a T cell-mediated autoimmune disease that destroys the insulin-secreting β cells of the pancreas (reviewed in Ref. 1). At present, it is possible to predict with great accuracy those candidates that will progress to T1D much before the full onset of the disease. Therefore, a prophylactic approach directed to inhibiting the function of self-reactive T cell clones as soon as they become activated would be beneficial for prevention of T1D or potential recurrence of the specific anti-β-cell autoimmunity in transplanted islets.

Galectin (gal)-1 is an endogenous lectin highly conserved throughout evolution (reviewed in Ref. 2) which recognizes as its ligands preferentially the β galactosides Galβ 1,3GlcNAc (type I lactosamine) and Galβ 1,4GlcNAc (type II lactosamine) oligosaccharides present on glycoproteins and glycolipids. These ligands include CD45, CD43, CD7, CD4, CD3, CD2, lysosome-associated membrane protein-1 and -2, the lipid GM1, and glycoproteins of the extracellular matrix (reviewed in Refs. 2 and 3). There is increasing evidence that gal-1 participates in T cell homeostasis maintenance. Perillo et al. (4) have demonstrated that gal-1 induces rapid death of activated T cells through a mechanism that is Fas-Fas ligand independent. The finding that thymic epithelial cells express gal-1 and trigger apoptosis of thymocytes through a gal-1-dependent mechanism suggests that this lectin functions in central T cell tolerance (5). The idea that gal-1 may play an important role in peripheral T cell suppression and/or tolerance is supported by previous studies showing that this lectin: 1) triggers apoptosis of activated T cells (4), 2) down-modulates Th1 responses (6), 3) is present in immune-privileged tissues (i.e., brain, placenta, testis) (7–9), 4) is used as a mechanism of immunoescape by melanomas (10), and 5) is expressed by regulatory T cells (Treg) (11). In this regard, it has been demonstrated that administration of gal-1 prevents/ameliorates T cell-mediated autoimmune disorders including experimental autoimmune encephalomyelitis (EAE) (12), collagen-induced arthritis (6), and experimental colitis (13) in rodents. To our knowledge, there is no information on the potential use of gal-1 or cells expressing transgenic (tg) gal-1 to prevent autoimmunity in T1D.

Dendritic cells (DC) are the most potent APC for the activation of naive and memory T cells (reviewed in Refs. 14 and 15) and, together with B cells, are intimately involved in the initiation of the T cell response that triggers β cell destruction (16). However, DC also participate in induction/maintenance of central and peripheral T cell tolerance (reviewed in Refs. 17 and 18) and a failure of DC to present tolerogenic peptides to thymocytes during central deletion may be the cause of T1D (19). Different laboratories have attempted to enhance the ability of DC to down-regulate the T cell

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[4] Abbreviations used in this paper: T1D, type 1 diabetes; gal-1, galectin-1; Treg, regulatory T cell; tg, transgenic; DC, dendritic cell; BM, bone marrow; PLN, pancreatic lymph node; TDG, thiodigalactoside; RAd, recombinant adenovirus; CMVp, CMV promoter; eGFP, enhanced GFP; $GAD65_{206-220}$, glutamic acid decarboxylase 65 peptide 206–220; EAE, experimental autoimmune encephalomyelitis; IRES, internal ribosomal entry site; CyC, CyChrome; MOI, multiplicity of infection.

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response by pharmacological methods or gene therapy with the objective of generating regulatory DC for treatment of T cell-mediated autoimmune disorders, transplant rejection, or graft vs host disease (reviewed in Refs. 20 and 21). Although, most current DC-based approaches to treat autoimmune disorders are focused at interfering with the ability of DC to activate naive T cells (22, 23), once T cells become activated, most conventional DC-based therapies are unable to inhibit the function of effector T cells. As occurs in T1D and other autoimmune disorders, the signs/symptoms that lead to diagnosis are detected after the onset of disease, when self-reactive T cells are fully activated. However, we can now predict with great accuracy, before individuals becoming symptomatic, who will progress to T1D. Therefore, strategies that target the deletion of activated self-reactive T cells early in the progression of the disease are warranted, because they would allow for the preservation of residual β cell mass. The potent regulatory effects of gal-1 on activated T cells, including induction of apoptosis (4) and down-modulation of the Th1 response (6, 13, 24, 25), makes this endogenous lectin a potentially useful transgene for engineering DC to treat T1D.

In this study, we engineered bone marrow (BM)-derived DC to synthesize tg gal-1 and tested whether these genetically engineered APC prevent development of T1D through their regulatory effects on activated T cells. We demonstrated that NOD-derived BM DC expressing high levels of tg gal-1 induce rapid apoptosis of NOD.BDC.2.5-TCRtg $CD4^+$ T lymphocytes. In vivo, administration of NOD BM DC expressing tg gal-1 delayed the onset of diabetes and insulitis in NOD*rag1*$^{-/-}$ mice reconstituted with splenocytes from overtly diabetic NOD mice. The beneficial effect of administration of NOD BM DC expressing tg gal-1 was accompanied by an increased number of apoptotic T cells and decreased number of IFN-γ-secreting $CD4^+$ T cells in pancreatic lymph nodes (PLN) and substantial reduction of T cell proliferation and IFN-γ secretion in response to β cell-derived Ag.

Materials and Methods

Animals and reagents

NOD/LtJ, NOD*rag1*$^{-/-}$, NOD.CB17-*Prkdc*^{scid}/J (NOD*scid*) (all IA^{g7}), and BDC2.5 TCRtg female mice 8–12 wk of age were purchased from The Jackson Laboratory. They were kept in our Association for Assessment and Accreditation of Laboratory Animal Care-certified animal facility of the Rangos Research Center and housed in microinsulator caging in a pathogen-free environment. Studies were approved by the Children's Hospital of Pittsburgh Animal Research and Care Committee. Mouse GM-CSF was purchased from PeproTech and mouse IL-4 from R&D Systems. DTT, thiodigalactoside (TDG), and OVA were obtained from Sigma-Aldrich. Recombinant human gal-1 was produced as previously described (26).

Generation and purification of NOD BM DC

NOD DC were generated from BM cell precursors of NOD*scid* mice. Cells were cultured in complete medium (RPMI 1640; Invitrogen Life Technologies), 10% FCS, glutamine, nonessential amino acids, sodium pyruvate, HEPES, 2-ME, and antibiotics) supplemented with GM-CSF and IL-4 (1000 U/ml of each), as described previously (27). At day 5, BM DC were purified by two steps of magnetic sorting. First, nonadherent cells were incubated with CD86 mAb (BD Pharmingen) and goat anti-rat IgG microbeads followed by negative selection by passage through paramagnetic columns (Miltenyi Biotec). As a second step, cells were incubated with anti-CD11c mAb microbeads and positively selected by magnetic-sorting (Miltenyi Biotec). $CD11c^+CD86^-$ DC purity was 94–96%.

Generation of recombinant adenovirus (RAd) encoding human galectin-1

The human gal-1 cDNA from pT7IML-1 (26) was subcloned into pIRES2-eGFP (BD Clontech) to construct pgal-1-IRES2-eGFP which was driven by the early human CMV promoter (CMVp) and contained the gal-1 cDNA followed by an internal ribosome entry site (IRES), the enhanced GFP (eGFP) cDNA, and the SV40 poly(A) signal. CMVp-gal-1-IRES-eGFP was subcloned into pDC311 (AdMax; Microbix Biosystems) to generate pDC311-gal-1-eGFP. RAd-gal-1-eGFP was generated by cotransfection of 293 cells with pDC311-gal-1-eGFP and BHGloxDE1,3Cre (Microbix). For generation of pcDNA3.1/human gal-1, the human gal-1 cDNA from pT7IML-1 was subcloned into pcDNA3.1$^+$ (Promega).

Analysis by flow cytometry

Noninfected or RAd-transduced NOD BM DC were blocked with normal goat serum and incubated (30 min, 4°C) with CyChrome (CyC) anti-CD11c mAb in combination with each of the following PE-conjugated mAb: anti-monomorphic MHC-I (clone MCA2189; Serotec), anti-OX6 (that cross-reacts with IA^{g7}), anti-CD40, anti-CD80 or anti-CD86, or with PE-annexin-V (BD Pharmingen). Appropriate fluorochrome-conjugated species and isotype-matched, irrelevant mAb were used as negative controls. Analysis was performed using an EPICS Elite FACS (Coulter).

SDS-PAGE and Western blot analysis

Cell pellets of purified NOD BM DC (noninfected or RAd-transduced) were suspended in 50 mM sodium phosphate/1% v/v SDS/40 mM 2-ME/2 mM EDTA. Cell extracts were diluted in 0.125 M Tris-HCl, 4% v/v SDS, 20% v/v glycerol, 0.2 M DTT, 0.02% v/v bromphenol blue (pH 6.8), boiled, and loaded in 15% w/v acrylamide SDS-PAGE. Gels were electroblotted on polyvinylidene difluoride membranes. The membranes were labeled with anti-human gal-1 mAb (1/250; Novocastra) followed by peroxidase anti-mouse IgG (1/10,000; Jackson ImmunoResearch Laboratories). Staining was developed by chemiluminescence (NEN Life Science Products).

In vitro assay of T cell apoptosis

In vitro assays of T cell apoptosis were performed using purified naive or activated splenic BDC2.5 $\alpha\beta$ TCRtg $CD4^+$ T cells as targets. BDC2.5 TCRtg $CD4^+$ T cells bear the Vα1 Vβ4 TCR of a $CD4^+$ T cell clone that recognizes a peptide derived from the β cell granules (28). Peripheral BDC2.5 TCRtg T cells exhibit a pronounced skewing into the $CD4^+$ T cell compartment and >95% of $CD4^+$ T cells bear the Vβ4 TCRtg (28). Therefore, naive BDC2.5 TCRtg $CD4^+$ T cells were purified by negative selection from splenocytes of BDC2.5 mice using the following mAb: OX6 (that cross-reacts with IA^{g7}), CD11b, CD11c, and CD8 (BD Pharmingen) followed by incubation with magnetic beads (Dynabeads) and immunomagnetic sorting (purity of $CD4^+$ T cells >92%). Activated BDC2.5 T cells were generated by incubating (3–4 days) splenocytes from BDC2.5 mice with 1 μM of the mimotope peptide EKAHRPIWARMDAKK (HRPI-RM) (29, 30) in 10% FCS medium supplemented 50 U/ml IL-2 (30). T cell activation was confirmed by up-regulation of CD25, CD44, and CD69 by flow cytometric analysis. Activated BDC2.5 TCRtg $CD4^+$ T cells were purified by negative selection by immunomagnetic sorting, as aforementioned for naive BDC2.5 $CD4^+$ T cells. Naive and activated splenic BDC2.5 $\alpha\beta$ TCRtg $CD4^+$ T cells (5×10^4) were cocultured (1 h, 37°C) with different numbers of immunobead-sorted ($CD11c^+$) NOD BM DC transduced with control RAd-eGFP or RAd-gal-1-eGFP. The apoptosis assay was performed in DMEM, 1% FCS, and 1.2 mM DTT. DC were pulsed or not (control) with 1 μM of the HRPI-RM peptide for 3 h at 37°C. The assay was stopped by addition of 0.1 M β-lactose. Assessment of apoptosis of BDC2.5 T cells was performed by TUNEL assay using the In Situ Cell Death Detection kit, TMR red (Roche) followed by flow cytometric analysis.

Transfer of diabetes to NODrag1$^{-/-}$ *mice*

Splenocytes from diabetic (glycemia >300 mg/dl) female NOD mice were depleted of erythrocytes by lysis with NH_4Cl. A cell suspension of 20×10^6 diabetogenic splenocytes was adoptively transferred (i.p.) into female NOD*rag1*$^{-/-}$ mice to induce diabetes (32–34). After that, mice were injected i.v. (tail vein) with 100 μl of PBS (control), or with cell suspensions of NOD BM DC noninfected (control), or transduced (multiplicity of infection (MOI) = 100) with either RAd-gal-1-eGFP or control RAd-eGFP (5×10^6 DC in 100 μl PBS/per dose/per mouse). Mice received two injections of PBS or DC per week through 3 consecutive weeks. Diabetes was diagnosed when glycemia reached 300 mg/dl or higher in two consecutive readings.

Immunofluorescence staining

Cytospins prepared from immunobead-sorted NOD BM DC (untreated or RAd-transduced) were air-dried, fixed in cold 96% ethanol (10 min), blocked with 5% normal goat serum and treated with the avidin/biotin blocking kit (Vector Laboratories). After that, cells were labeled with mouse anti-human gal-1 mAb (1/200; Novocastra) and biotin hamster

anti-mouse CD11c (1/100; BD Pharmingen) followed by Cy3 F(ab′)$_2$ anti-mouse IgG (1/400; Jackson ImmunoResearch Laboratories) and streptavidin Alexa Fluor 647 (1/400; Molecular Probes). Nuclei were counterstained with 4′6-diamidino-2-phenylindole 2HCl (DAPI; Molecular Probes).

Fragments of pancreas (3–5 mm) were fixed in 4% formaldehyde, embedded in paraffin, and processed for staining with H&E. For detection of insulin, tissue sections were blocked with 5% goat serum, treated with the avidin/biotin blocking kit (Vector Laboratories) and incubated with anti-human insulin mAb (1/100; BioGenex). As second step, sections were incubated with biotin goat anti-mouse Igs (1/100; BD Pharmingen) and avidin-biotin complex/peroxidase (Vector Laboratories). Endogenous peroxidase was blocked by successive passages in 70% ethanol, 1% H_2O_2 in methanol and 70% ethanol. Peroxidase activity was developed with 3,3′-diaminobenzidine (Sigma-Aldrich).

For labeling of frozen sections, fragments of pancreas and PLN were embedded in Tissue-Tek OCT (Miles Laboratories), snap-frozen in prechilled isopentane and stored at −80°C. Cryostat sections (8 μm) were mounted on slides pretreated with Vectabond (Vector Laboratories), air-dried, and fixed in cold 96% ethanol (10 min). Tissue sections were blocked with 5% goat serum and the avidin/biotin blocking kit (Vector Laboratories). For codetection of CD4 and CD8 T cells in pancreas, tissue sections were incubated with Alexa Fluor 488-CD4 mAb (Caltag Laboratories) and biotin-CD8α mAb (eBioscience) followed by Cy3-streptavidin (Jackson ImmunoResearch Laboratories). For detection of cytokines or FoxP3 in CD4$^+$ T cells, sections of PLN were labeled with Alexa Fluor 488-CD4 mAb and one of the following reagents: 1) biotin- IFN-γ mAb or biotin-IL-4 mAb (BD Pharmingen) followed by Cy3-streptavidin or 2) FoxP3 mAb (MF333F; Alexis Biochemicals) followed by Cy3-conjugated F(ab′)$_2$ donkey anti-rat IgG (Jackson ImmunoResearch Laboratories). For analysis of T cell apoptosis in situ, sections of PLN were fixed in paraformaldehyde and labeled with Alexa Fluor 488-CD3 mAb (Caltag Laboratories) followed by TUNEL staining using the In Situ Death Detection kit, TMR red (Roche).

For detection of DC expressing eGFP in spleen, PLN, thymus, and pancreas, tissues were fixed in paraformaldehyde (to preserve the localization of eGFP) and then snap-frozen, as described above. Tissue sections were postfixed with paraformaldehyde (10 min) and then incubated with either biotin-CD11c or biotin-human gal-1 mAb (16 h, 4°C). As a second step, sections were incubated with Cy3-streptavidin.

Insulitis was scored by examining at least 30 pancreatic islets per mouse from two to three different sections and given a 0–3 scale score as follows: 0, no insulitis; 1, peri-insulitis; 2, insulitis in <50% of the islets; and 3, insulitis in >50% of the islets (35). Percentage of insulitis was calculated by dividing the number of islets in each category by the total number of islets examined.

Assays of Ag-specific T cell proliferation and ELISPOT

Spleen cells form NOD*rag1*$^{-/-}$ mice reconstituted with splenocytes from overtly diabetic NOD and treated with PBS (control) or with NOD BM DC transduced with RAd-gal-1-eGFP or control RAd-eGFP were cultured in complete medium in bottom-rounded 96-well plates (2.5×10^5 cells/well) alone or with different concentrations of glutamic acid decarboxylase 65 peptide 206–220 ($GAD65_{206-220}$), insulin, NOD islet lysate (obtained by sonication of NOD pancreatic islets), or the control protein chicken OVA. The $GAD65_{206-220}$ peptide (TYEIAPVFVLLEYVT) was synthesized, purified by HPLC, and confirmed by mass spectroscopy. Splenocytes were culture for 3 days and 1 μCi/well [^{3}H]TdR was added for the last 18 h. Cells were harvested and [^{3}H]TdR uptake was measured using a beta counter. Proliferation was expressed as stimulation index ((Ag-specific proliferation − OVA-stimulated proliferation)/OVA-stimulated proliferation).

For ELISPOT assay, spleen cells were isolated from NOD*rag1*$^{-/-}$ mice reconstituted with splenocytes from overtly diabetic NOD mice and treated with PBS (control) or NOD BM DC transduced with RAd-gal-1-eGFP or control RAd-eGFP. Splenocytes (50×10^4 per well) were stimulated with $GAD65_{206-220}$ (50 μg/ml), NOD islet lysate (20 μg/ml), insulin (50 μg/ml), or OVA (50 μg/ml) in 96-well ELISPOT plates (BD Biosciences) precoated with anti-IFN-γ or anti-IL-4 mAb. ELISPOT plates were cultured for 36 h followed by incubation with biotin-IFN-γ or biotin-IL-4 mAb, streptavidin-peroxidase and 3-amino-9-ethylcarbazole. The spots were counted with an ImmunoSpot counter (Cellular Technology).

Statistical analysis

Results are expressed as means ± SD. Comparisons between different means were performed by ANOVA, followed by the Student Newman Keuls test. Comparison between two means was performed by the Student *t* test. Incidence of diabetes between groups was compared by Kaplan-Meier analysis and the log-rank test. A $p < 0.05$ was considered significant.

Results

Generation of NOD BM DC expressing tg galectin-1

Myeloid DC were generated from BM cell precursors of NOD*scid* mice (day 6) and then transduced with RAd-gal-1-eGFP (gal-1-DC) (MOI = 100). The RAd-gal-1-eGFP, encoding for the human gal-1 cDNA and the reporter gene eGFP under control of the CMVp, was used to engineer DC to express high levels of human gal-1. It has been previously shown that human gal-1 is functional on murine cells (13, 24, 25, 36) and that the amino acid sequence of gal-1 is highly conserved among mammals (reviewed in Ref. 2). Control NOD BM DC were left untreated or infected with an MOI of 100 of RAd-eGFP (eGFP-DC). Analysis of cytospins by immunofluorescence microscopy showed that gal-1-DC coexpressed intracellular gal-1, the reporter protein eGFP and the murine DC marker CD11c (Fig. 1*A*). Flow cytometric analysis showed that CD11c$^+$ gal-1-DC also expressed tg gal-1 on the cell surface (Fig. 2*B*) In marked contrast, control DC (untreated or transduced with RAd-eGFP) did not show tg gal-1 expression (Fig. 1, *A* and *B*). Following overnight culture, the efficiency of RAd transfection (MOI = 100) in gal-1-DC and in (control) eGFP-DC ranged between 75 and 98% based on their level of eGFP expression, as determined by flow cytometry (Fig. 1*B*). The presence of tg (human) gal-1 (14 kDa) was confirmed by Western blot analysis (reducing conditions) in whole cell extracts of NOD BM DC transduced with RAd-gal-1-eGFP (Fig. 1*C*). The lack of dimeric gal-1 (28 kDa) was due to the fact that gal-1 forms noncovalent homodimers that run as monomers during electrophoresis performed under reducing conditions. Cell extracts from control noninfected DC or eGFP-DC did not show reactivity with the anti-human gal-1 mAb in Western blots (Fig. 1*C*). Cell extracts from COS-7 cells transfected with pcDNA3/human gal-1 were included as positive control. By Western blot analysis, we did not detect soluble tg (human) gal-1 in ultrafiltered (100,000 kDa; Millipore Ultrafree-15 capsule filter) supernatants of gal-1-DC or control DC (untreated or infected with RAd-eGFP) (data not shown). These results indicate that soluble tg gal-1 is not released at detectable levels to the extracellular milieu by DC transduced with RAd-gal-1-eGFP but rather attaches to the APC surface after being secreted (36), as has been reported in other cell types (37).

Because gal-1 induces apoptosis of activated T cells, thymocytes, and certain T cell lines (4, 5), we investigated whether synthesis of tg gal-1 affects the viability of RAd-transduced NOD BM DC. Control DC (untreated DC or eGFP-DC) and gal-1-DC exhibited similar percentages of viable cells assessed by annexin-V staining when maintained in culture for 24 h after RAd infection (Fig. 1*D*).

NOD BM DC expressing tg galectin-1 induce apoptosis of activated T cells

We and others have demonstrated that RAd transduction affects DC maturation (38) and that gal-1 induces activation of BM DC prepared from C57BL/10 and BALB/c mice (36) and of monocyte-derived DC in humans (39, 40). Therefore, we tested whether transduction with RAd-gal-1-eGFP alters the phenotype of NOD BM DC. As expected, transduction with RAd (MOI = 100) increased the levels of MHC class I, MHC class II, CD40, CD80, and CD86 expressed by NOD BM DC (Fig. 2*A*). Compared with control eGFP-DC, gal-1-DC exhibited a slight augment in the percentage of positive cells or intensity of expression of MHC-I, MHC-II, CD40, and T cell costimulatory molecules (Fig. 2*A*).

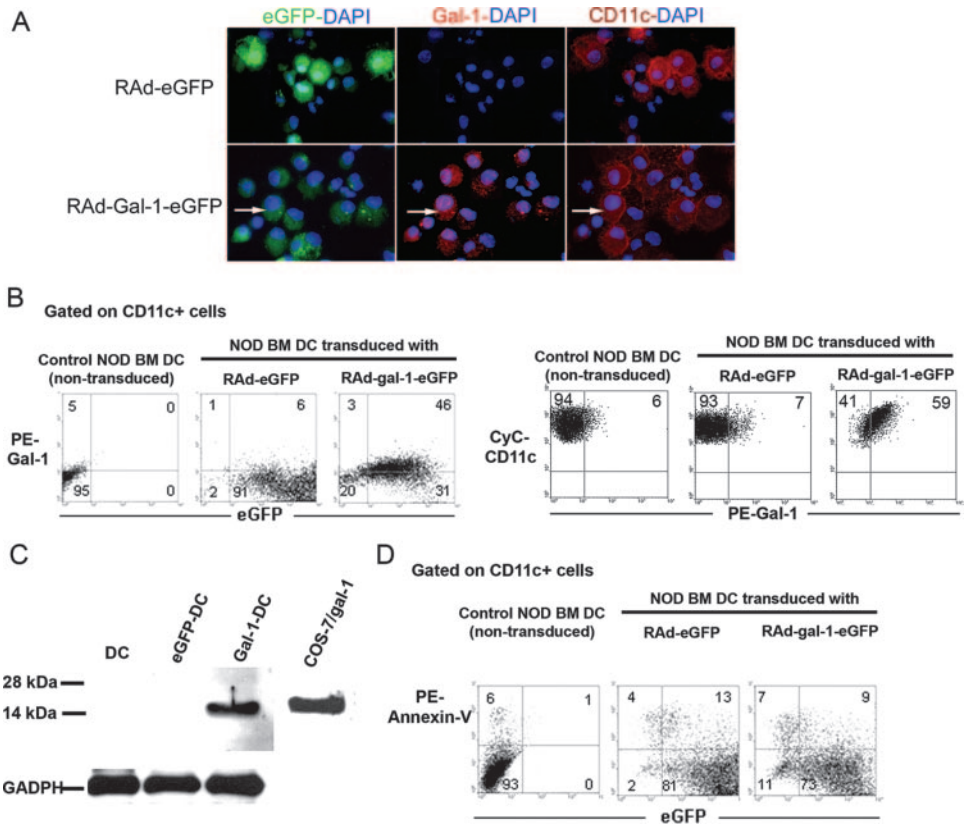

FIGURE 1. NOD BM DC transduced with RAd-gal-1-eGFP synthesize high levels of tg gal-1. *A*, Cytospins of NOD myeloid BM DC transduced with RAd-eGFP (control) or RAd-gal-1-eGFP (MOI = 100) and labeled with anti-human gal-1 and anti-CD11c mAb (CD11c is a murine DC marker). tg gal-1 was not detected in control eGFP-DC (*top panels*). Gal-1-DC coexpressed intracellular eGFP (green), tg gal-1 (red), and CD11c (far red) (*bottom panels*, arrows). Nuclei were counterstained with DAPI (blue). Fluorescence microscopy, original magnification, ×400. *B*, On the *left*, flow cytometric analysis of expression of eGFP and gal-1 (the latter on the cell surface) by NOD BM DC transduced with RAd-gal-1-eGFP and control NOD BM DC (noninfected or RAd-eGFP-transduced). On the *right*, expression of tg gal-1 on the surface membrane of $CD11c^+$ NOD BMDC. In both cases, cells were gated on $CD11c^+$ events. *C*, Western blot analysis of cell extracts prepared from NOD BM DC left untreated (DC) or transduced with either RAd-eGFP (eGFP-DC) or RAd-gal-1-eGFP (Gal-1-DC). As a positive control, COS-7 cells transfected with pcDNA3.1/human gal-1 were included. *D*, Cell viability of NOD BM DC left untreated or transduced with either RAd-eGFP (eGFP-DC) or RAd-gal-1-eGFP (Gal-1-DC) assessed by labeling with PE-annexin V followed by flow cytometric analysis. Results are representative of four (*A*, *B*, and *D*) and three independent experiments.

Because gal-1-DC express tg gal-1 on their surface (Fig. 1*B*) and it has been shown previously that the immunoregulatory activity of soluble gal-1 depends on its ability to trigger apoptosis of activated T cells (4), we tested whether NOD BM DC transduced with RAd-gal-1-eGFP induce T cell apoptosis. We used as targets BDC2.5 TCRtg $CD4^+$ T lymphocytes that bear the TCR of a $CD4^+$ T cell clone isolated from a diabetic NOD mouse (41). BDC2.5 $CD4^+$ T cells are β cell-specific, H-2^{g7}-restricted, and diabetogenic (41). The peptide(s) recognized by BDC2.5 cells is localized within the β-granules, has not been yet identified, and is not among the described β cell-derived Ag (42). Therefore, for the in vitro assays, we used the mimotope peptide HRPI-RM that functions as a stimulating Ag for BDC2.5 T cells (29, 30).

It has been shown that soluble gal-1 triggers externalization of phosphatidylserine in human T leukemic MOLT-4 cells, HL-60 cells, and fMLP-activated neutrophils without inducing apoptosis (43, 44). Because detection of apoptosis by annexin-V staining depends on its binding to phosphatidylserine exposed to the cell surface, we assessed the ability of gal-1-DC to induce apoptosis of BDC2.5 T cells by TUNEL assay, a method that is based on detection of fragmented genomic DNA as an indicator of apoptosis. Control eGFP-DC or gal-1-DC pulsed or control (nonpulsed) with the HRPI-RM peptide were mixed with naive ($CD25^-CD44^{low}CD69^-$) or preactivated ($CD25^+CD44^{high}CD69^+$) BDC2.5 $CD4^+$ T cells purified by immunomagnetic sorting (purity >92%) and cultured at different DC:target T cell ratios. After 1-h incubation, cells were stained with CyC-CD4 mAb and TMR-TUNEL and analyzed by flow cytometry. The in vitro assay of apoptosis by flow cytometry evaluated TUNEL positivity exclusively on target BDC2.5 $CD4^+$ T cells because eGFP-DC and gal-1-DC were gated out from the target BDC2.5 cells based on their eGFP expression and lack of CD4 labeling.

Unlike control eGFP-DC, gal-1-DC pulsed with the peptide-initiated apoptosis of preactivated, but not naive, BDC2.5 T cells following 1 h incubation at 37°C (Fig. 2*B*). The phenomenon was more pronounced at high DC:BDC2.5 T cell ratios and after normalizing the results of the experiment illustrated in Fig. 2*B* (representative of three independent experiments) to the background levels of apoptosis induced by control eGFP-DC on target BDC2.5 T cells, gal-1-DC induced apoptosis of 55, 14, and 9% of T cells at DC:T cell ratios of 10:1, 4:1, and 1:1, respectively (Fig. 2*B*). Induction of apoptosis of BDC2.5 T cells by gal-1-DC depended on galactose-specific interactions because it was blocked by addition of the gal-1 competitor TDG (0.1M) to the culture medium (Fig. 2*B*). At a DC:T cell ratio 10:1, addition of TDG decreased the percentage of apoptotic BDC2.5 T cells from 53 ± 7 to 14 ± 4% (after recalculation of the data by normalizing to eGFP-DC background, average of three independent experiments). Gal-1-DC preloaded with HRPI-RM induced higher percentage of apoptotic BDC2.5 T cells

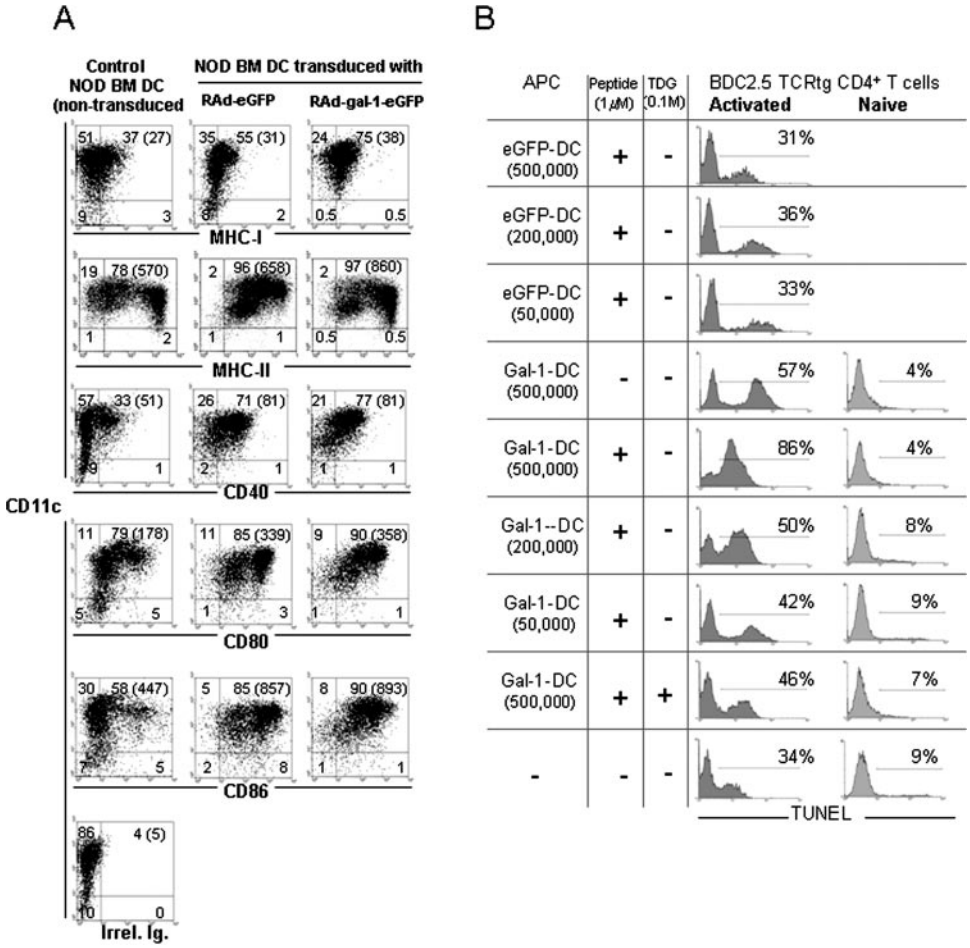

FIGURE 2. NOD BM DC transduced with RAd-gal-1-eGFP induce apoptosis of Ag-specific T cells. *A*, Surface phenotype of control NOD BM DC (noninfected or transduced with RAd-eGFP) and NOD BM DC transduced with RAd-gal-1-eGFP (MOI = 100, 16 h). DC were labeled with CyC-CD11c mAb (CD11c is a DC marker in mice) in combination with PE-labeled mAb against MHC-I, MHC-II, CD40, CD80, and CD86 followed by flow cytometric analysis. Numbers indicate the percentage of cells in each quadrant and the values between parenthesis represent the arithmetic mean fluorescence intensity. *B*, Control eGFP-DC and gal-1-DC pulsed or not (control) with the mimotope peptide HRPI-RM were incubated at different ratios with target BDC2.5 tg TCR $CD4^+$ T cells for 1 h at 37°C. Apoptosis of BDC2.5 was evaluated by TUNEL staining followed by flow cytometric analysis. Apoptosis was evaluated exclusively on target BDC2.5 $CD4^+$ T cells because DC were excluded based on their eGFP expression and lack of staining for CD4. Results in *A* and *B* are representative of three independent experiments.

than gal-1-DC not exposed to the peptide (Fig. 2*B*). However, these latter gal-1-DC still induced higher percentages of apoptotic BDC.2.5 T cells than control eGFP-DC pulsed with the peptide (Fig. 2*B*). These results indicate that NOD BM DC transduced with RAd-gal-1-eGFP induce apoptosis of activated T cells by TCR complex-dependent and -independent mechanisms.

Administration of NOD BM DC expressing tg galectin-1 delays onset of T1D

We assessed the potential of gal-1-DC to ameliorate type-1 diabetes in the NOD*rag1*$^{-/-}$ lymphocyte adoptive transfer model detailed in Fig. 3*A*. Young adult female NOD*rag1*$^{-/-}$ mice were adoptively transferred (i.p) on day 1 with 20×10^6 splenocytes from overtly diabetic female NOD mice. In this model, the onset of diabetes is more rapid (between 4 and 5 wk), aggressive, and controllable than the natural progression of the disease in NOD mice and triggered by polyclonal diabetogenic T cells (31–33). Multiple i.v. injections of 5×10^6 NOD BM DC transduced in vitro with RAd-gal-1-eGFP (two weekly injections, 3 consecutive weeks, Fig. 3*A*) delayed significantly ($p = 0.0014$) the onset of diabetes in reconstituted NOD*rag1*$^{-/-}$ mice. These mice develop hyperglycemia on day 60 ± 10. By contrast, control reconstituted NOD*rag1*$^{-/-}$ mice that received PBS (i.v.) developed diabetes on day 29 ± 4. Reconstituted NOD*rag1*$^{-/-}$ mice treated with multiple injections of control NOD BM DC untreated or transduced with RAd-eGFP developed diabetes on day 32 ± 5 and 31 ± 3, respectively, results that did not differ significantly from those of PBS-treated controls (Fig. 3*B*). Treatment with gal-1-DC was significantly ($p < 0.05$) more efficient that recombinant soluble human gal-1 administered under similar conditions (100 μg/mouse, i.p., two weekly injections, 3 consecutive weeks) where the onset of diabetes was on day 41 ± 4 (data not shown). In summary, i.v. administration of syngeneic DC that synthesize high levels of tg gal-1 induced a significant delay in the onset of T1D in mice, an effect that was significantly more pronounced than that achieved by control DC injected i.v. or soluble gal-1 administered i.p. under a similar regimen.

NOD BM DC expressing tg galectin-1 delay histopathological changes of T1D

Lymphocytic infiltration of pancreatic islets (insulitis) leads to destruction of β-cells (45) and eventually diabetes. To examine the effect of administration of gal-1-DC (i.v. transfer on days 1, 3, 9, 11, 16, and 18) on pancreata insulitis from normoglycemic

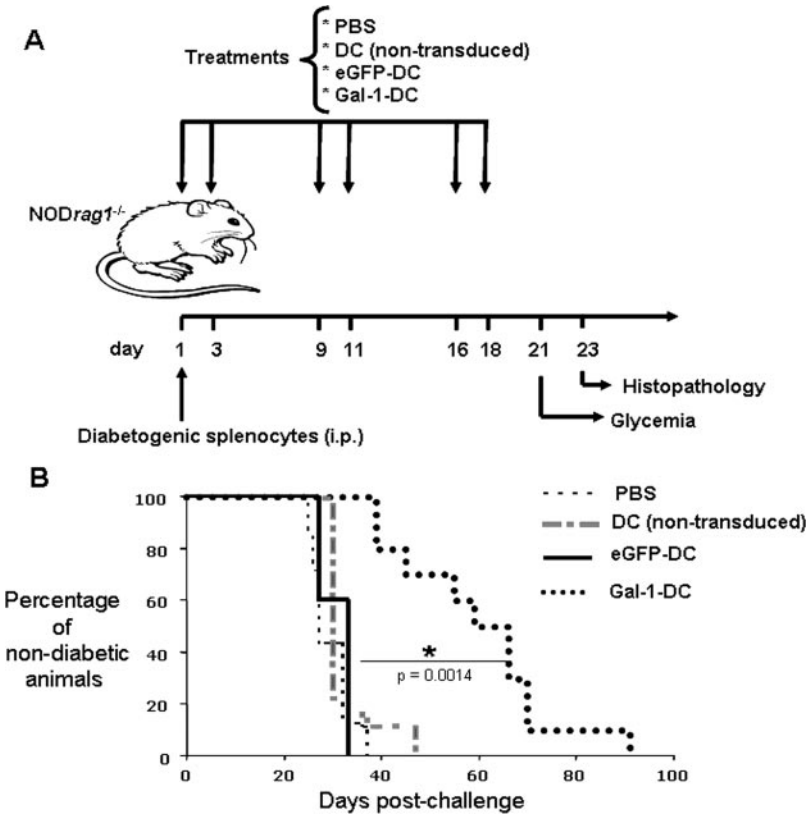

FIGURE 3. Administration of NOD BM DC expressing tg gal-1 delays onset of T1D in mice. *A*, Diagram of the experimental model. Female NOD*rag1*$^{-/-}$ mice were adoptively reconstituted (day 1) with splenocytes (i.p., 20×10^6 cells/per mouse) from overtly diabetic female NOD mice to induce autoimmune diabetes through diabetogenic polyclonal T cells. Mice were injected i.v. (tail vein) with PBS (100 μl, i.v.) ($n = 7$), control NOD BM DC (noninfected ($n = 9$) or transduced with RAd-eGFP ($n = 5$)) or NOD BM DC transduced with RAd-gal-1-eGFP ($n = 10$) (i.v., 5×10^6 DC in 100 μl of PBS, twice a week for 3 wk). Blood glucose levels were measured three times a week as of day 21. *B*, Treatment with NOD BM DC expressing tg gal-1 delayed onset of T1D compared with control groups.

NOD*rag1*$^{-/-}$ mice reconstituted with diabetogenic splenocytes (day 1), islets were harvested on day 23 for histological analysis (Fig. 3*A*). Tissue sections were stained with H&E, anti-insulin mAb and with anti-CD4 or anti-CD8 mAb. Pancreatic islets of reconstituted NOD*rag1*$^{-/-}$ mice treated with PBS exhibited significant leukocyte infiltration that correlated with a reduction of insulin staining indicative of specific β cell destruction (Fig. 4, *A–C*). The cellular infiltrate consisted mainly of both inter- and intraislet polyclonal CD4$^+$ and CD8$^+$ T cells. Islets of control NOD*rag1*$^{-/-}$ mice pretreated with eGFP-DC under the same conditions also showed a reduced content of insulin and numerous infiltrating CD4$^+$ and CD8$^+$ T cells (Fig. 4, *A–C*). However, the damage of the islets and the magnitude of the infiltrate in mice treated with eGFP-DC were less severe than in controls injected with PBS. By contrast, islets from reconstituted NOD*rag1*$^{-/-}$ mice pretreated with gal-1-DC exhibited normal histology, were mostly free of leukocyte infiltrate and had a content of insulin that resembled that found in normal islets (Fig. 4, *A–C*). The degree of insulitis was quantified as percentage of infiltrated islets. In reconstituted NOD*rag1*$^{-/-}$ mice treated with gal-1-DC, there was a significant ($p < 0.05$) decrease in the percentage of infiltrated islets ($10.3 \pm 5.5\%$) as compared with PBS or control DC-injected NOD*rag1*$^{-/-}$ mice (Fig. 4*D*). These results indicate that administration of gal-1-DC prevents the insulitis that causes islet destruction of prediabetic NOD*rag1*$^{-/-}$ mice reconstituted with diabetogenic splenocytes.

Expression of tg galectin-1 and traffic of NOD BM DC in vivo

Galectins have been shown to mediate cell-to-cell adhesion and cell-to-matrix adhesions (reviewed in Ref. 2). However, by cross-linking of oligosaccharide-binding sites on the cell surface, gal-1 blocks binding sites of surface glycoproteins that participate in adhesive interactions with the extracellular matrix (reviewed in Ref. 2). In fact, overproduction of gal-1 by myoblasts inhibits adhesion to laminin by sterically hindering the binding of integrins to laminin (46). Because migration of blood-borne DC to secondary lymphoid organs requires interaction with integrins and the extracellular matrix, we tested whether expression of tg gal-1 by NOD BM DC administered i.v. affects the ability of DC to traffic to PLN, spleen, and pancreas, tissues where T cells become activated and therefore likely targets of gal-1-induced apoptosis in diabetic mice. We also analyzed the traffic of gal-1-DC to the thymus in view of the previous observation that soluble gal-1 induces apoptosis of thymocytes that may play a role in central T tolerance (5). To address these questions, NOD BM DC were transduced with RAd-gal-1-eGFP or control RAd-eGFP (MOI = 100, overnight), purified by immunomagnetic sorting with anti-CD11c beads and injected (i.v.) into NOD*rag1*$^{-/-}$ mice. Eighteen hours later, tissues were removed, fixed in paraformaldehyde, snap-frozen, and the presence of DC expressing the reporter protein eGFP was examined in tissue sections by fluorescence microscopy. Both eGFP-DC and gal-1-DC migrated to a similar extent to the PLN (35 ± 5 vs 31 ± 3 cells, respectively, 10 high power fields, Fig. 5, *A* and *B*) and spleen (12 ± 3 vs 10 ± 4 cells, respectively, 10 high power fields, data not shown) of host mice. By means of labeling with PE mAb, we demonstrated that all eGFP$^+$ cells detected in PLN and spleens of mice injected with gal-1-DC coexpressed the murine DC marker CD11c (Fig. 5*C*) and tg (human) gal-1 (Fig. 5*D*). We did not detect gal-1-DC or eGFP-DC in the thymus or pancreas of DC-injected mice (not shown).

Cytokine content and T cell apoptosis in pancreatic lymph nodes of mice treated with gal-1-DC

It is known that in other models of Th1-mediated autoimmune disorders the therapeutic effect of gal-1 is associated with its ability to trigger apoptosis of activated T cells and decrease the Th1 response (6, 13, 24). Although still unknown for gal-1, generation/

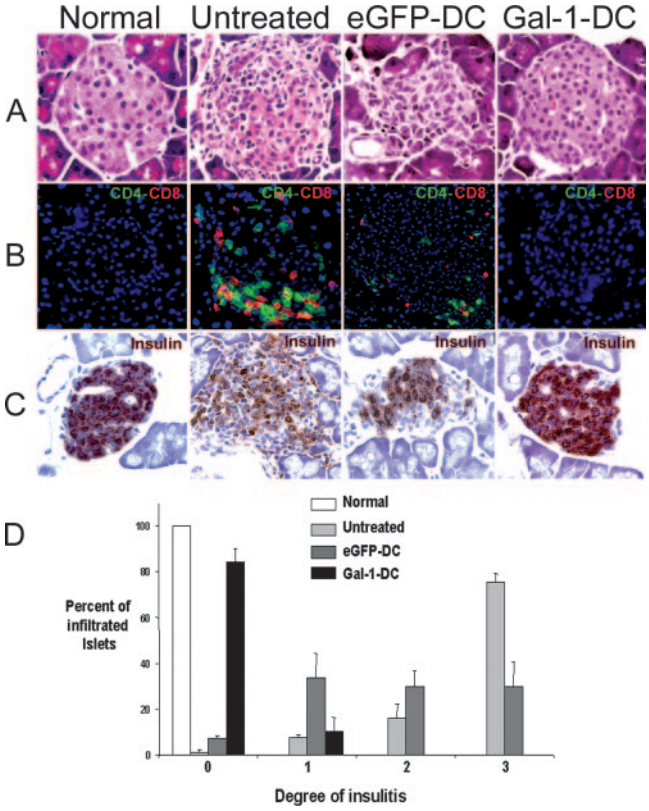

FIGURE 4. Treatment with NOD BM DC expressing tg gal-1 prevented lymphocyte infiltration in pancreatic islets. *A–C*, Pancreases of control NOD*rag1*$^{-/-}$ mice (Normal) and NOD*rag1*$^{-/-}$ mice adoptively transferred with diabetogenic splenocytes and left untreated (Untreated) or treated with six i.v. injections of NOD BM DC transduced with either control RAd-eGFP (eGFP-DC) or RAd-gal-1-eGFP (Gal-1-DC) were removed 23 days after splenocyte transfer for histopathological analysis. Tissue sections were stained with H&E (*A*), labeled with CD4$^-$ and CD8- mAb by double immunofluorescence (*B*) or with anti-insulin mAb by immunoperoxidase staining (*C*). Polyclonal CD4$^+$ and CD8$^+$ T cells infiltrated islets of control untreated and eGFP-DC-treated mice (*A* and *B*) but were absent in islets from gal-1-DC-treated mice. The number of insulin-producing cells and intensity of insulin staining of islets from mice treated with gal-1-DC resembled that of normal islets (*C*). By contrast, islets from control untreated and eGFP-DC-treated mice showed substantial loss of insulin-producing β cells (*C*). *D*, The degree of insulitis was quantified in 30 pancreatic islets per mouse in tissue sections stained with H&E.

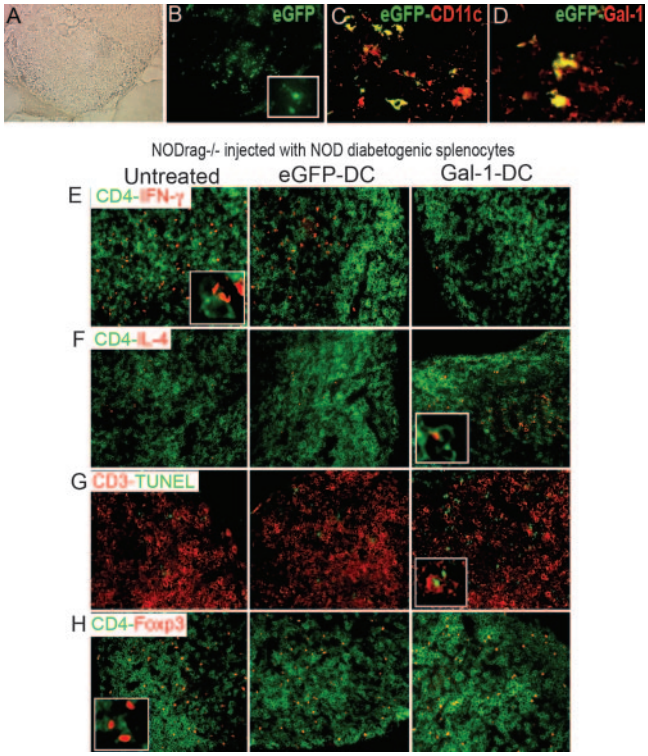

FIGURE 5. Effect of administration of NOD BM DC expressing tg gal-1 in PLN. *A–D*, Traffic of NOD BM DC expressing tg gal-1 to PLN of NOD*rag1*$^{-/-}$ mice. Eighteen hours after administration of NOD BM DC transduced with RAd-gal-1-eGFP, the localization of eGFP$^+$ DC in PLN was evaluated in tissue sections labeled by immunofluorescence. DC expressing eGFP were detected in PLN (*B*) and spleen (data not shown) of host mice. All eGFP$^+$ (green) cells detected in PLN of mice injected with DC transduced with RAd-gal-1-eGFP coexpressed the murine DC marker CD11c (in red, *C*) and the transgene (human) gal-1 (in red, *D*). Yellow is the result of overlapping between green and red. *E* and *F*, Production of IFN-γ and IL-4 by CD4$^+$ T cells from PLN of NOD*rag1*$^{-/-}$ mice adoptively transferred with splenocytes from overtly diabetic female NOD mice (to induce autoimmune diabetes through diabetogenic polyclonal T cells) or left untreated (Untreated) or treated with NOD BM DC transduced with either control RAd-eGFP (eGFP-DC) or RAd-gal-1-eGFP (Gal-1-DC). Treatment with gal-1-DC decreased the number of polyclonal IFN-γ-secreting CD4$^+$ T cells and increased the quantity of CD4$^+$ T cells with intracytoplasmic IL-4 in PLN. *G*, Mice treated with gal-1-DC augmented the percentage of TUNEL$^+$ (apoptotic) polyclonal T cells in PLN compared with control groups. *H*, The percentage of CD4$^+$ T cells expressing intranuclear FoxP3 in PLN of gal-1-DC-treated mice was similar to that found in control animals. *Insets*, In detail the intracytoplasmic concentration of IFN-γ (in *E*) and IL-4 (in *F*) and the nuclear localization of TUNEL (in *G*) and FoxP3 (in *H*) in T cells. *A*, Bright field; *B–H*, immunofluorescence. Original magnifications, $\times$200 (*A* and *B*), $\times$400 (*C–H*), and $\times$1000 (*insets*).

amplification of Treg by DC is another mechanism that may reduce the severity of autoimmune diabetes (47). Because PLN are the site where presentation of β cell-derived Ag takes place during the initial stages of T1D (48), we investigated the impact that administration of gal-1-DC exerts on the Th1/Th2 balance, level of apoptosis and percentage of Treg cells in the T cell population that resides in PLN. PLN were harvested from control (injected with PBS or eGFP-DC) and gal-1-DC-treated NOD*rag1*$^{-/-}$ mice sacrificed 23 days after reconstitution (5 days after the last injection of DC), when mice were still normoglycemic. Cryostat sections of PLN were labeled with CD4 or CD3 mAb in combination with: 1) IFN-γ or IL-4 mAb; 2) TUNEL technique for detection of apoptotic cells; or 3) Foxp3 mAb for identification of Treg. PLN from control groups (injected with PBS or eGFP-DC) exhibited an increased percentage of CD4$^+$ T cells with intracellular IFN-γ as compared with PLN from gal-1-DC-treated mice (160 $\pm$ 50 positive cells in eGFP-DC-treated mice vs 30 $\pm$ 9 positive cells in the gal-1-DC-treated group, 10 high power fields, Fig. 5*E*). In contrast, treatment with gal-1-DC increased the percentage of polyclonal CD4$^+$ cells synthesizing IL-4 in PLN (20 $\pm$ 5 positive cells in eGFP-DC-treated mice vs 78 $\pm$ 15 positive cells in the gal-1-DC-treated group, 10 high power fields, Fig. 5*F*). A higher percentage of apoptotic (TUNEL$^+$) polyclonal CD3$^+$ cells was present in PLN of gal-1-DC-treated animals (160 $\pm$ 31 positive cells, 10 higher power fields) compared with PLN of controls (35 $\pm$ 7 positive cells, 10 high power fields, Fig. 5*G*). No differences in the number of polyclonal CD4$^+$ T cells expressing intranuclear Foxp3 were detected between gal-1-treated mice and control groups (Fig.

5*H*), suggesting that in our model gal-1-DC do not mediate their beneficial effect through a local increase in the number of Treg. Taken together, these data suggest that administration of gal-1-DC is associated with a reduction in the number of Th1-biased cells and an increased percentage of apoptotic T lymphocytes in PLN of prediabetic NOD*rag1*$^{-/-}$ mice reconstituted with diabetogenic splenocytes.

Gal-1-DC inhibit proliferation and IFN-γ secretion of islet Ag-specific T cells

We hypothesize that local delivery of high levels of tg gal-1 by NOD BM DC to secondary lymphoid tissues may impair the expansion of β cell-specific T cells. Therefore, we investigated the ability of T cells from reconstituted NOD*rag1*$^{-/-}$ mice treated with gal-1-DC to proliferate ex vivo in response to islet cell-derived Ag. Mice were sacrificed 23 days after reconstitution (5 days after last injection with gal-1-DC, eGFP-DC, or PBS) and splenocytes were restimulated for 72 h with different concentrations of $GAD65_{206-220}$, insulin (whole protein), NOD pancreatic islet lysate, or the control protein OVA. The $GAD65_{206-220}$ was chosen because it is an immunodominant self-Ag peptide in NOD mice that binds with high affinity to IA^{g7} molecules (49, 50). Spleen cells from mice injected with PBS or eGFP-DC proliferated in response to $GAD65_{206-220}$, insulin, or NOD islet lysate (Fig. 6*A*). By contrast, splenocytes from mice treated with gal-1-DC showed a significant reduction in the level of proliferation in response to the same β cell-derived Ag (Fig. 6*A*), a result compatible with specific deletion of activated T cells specific for β cell-derived Ag as a result of the treatment with gal-1-DC. No cell proliferation was detected in response to OVA (data not shown).

The balance of cytokines secreted by Th1 and Th2 cells plays an important role in several autoimmune diseases and T1D is characterized by a Th1-driven response (51). In this regard, it has been shown that soluble gal-1 may alter the Th1/Th2 balance in autoimmune disorders (6, 13, 24). Therefore, the frequency of splenic T cells secreting Th1 (IFN-γ) and Th2 (IL-4) cytokines in response to islet-derived Ag was determined by ELISPOT assays in reconstituted NOD*rag1*$^{-/-}$ mice treated with gal-1-DC and in control groups. Mice were sacrificed 23 days after reconstitution and splenocytes were restimulated for 36 h with $GAD65_{206-220}$, insulin (whole protein), NOD pancreatic islet lysate, and OVA, as a control. Administration of gal-1-DC resulted in a significant reduction in the number of splenic T cells that secrete IFN-γ or IL-4 in response to $GAD65_{206-220}$, insulin, or NOD islet lysate when compared with T cells harvested from spleens of reconstituted NOD*rag1*$^{-/-}$ mice injected with PBS or with multiple injections of eGFP-DC (Fig. 6*B*). As control, splenic T cells from mice treated with gal-1-DC, eGFP-DC, or PBS generated a much lower number of spots for IFN-γ (< 10 spots/10^6 splenocytes) or IL-4 (< 100 spots/10^6 splenocytes) in response to the model Ag OVA (data not shown), without significant differences between groups. Importantly, the ratio between the number of IL-4- vs IFN-γ-secreting T cells (assessed by ELISPOT) in response to $GAD65_{206-220}$, insulin, or NOD islet lysate was higher after treatment with gal-1-DC than following administration of PBS or eGFP-DC (Table I). In particular, the Th2/Th1 cell ratio of insulin-specific T cells exhibited a 12- and 4-fold increase compared with the ratio of T cells from mice treated with PBS or eGFP-DC, respectively. Taken together, our results indicate

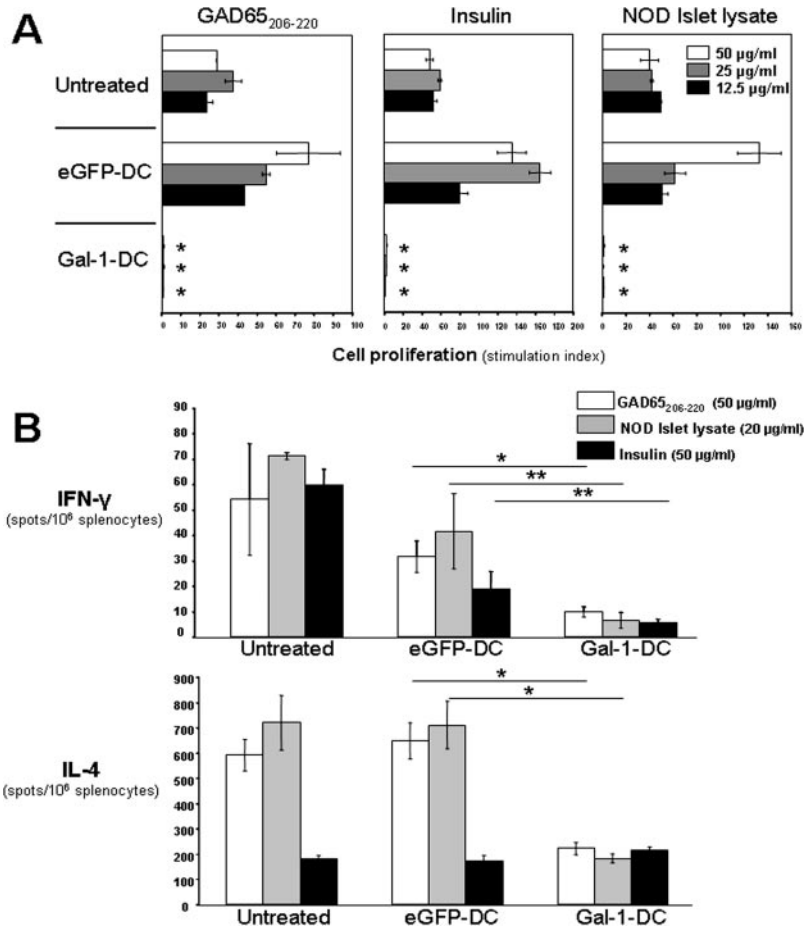

FIGURE 6. Ag-specific T cell proliferation and cytokine secretion following treatment with NOD BM DC expressing tg gal-1. *A*, Systemic administration of NOD BM DC expressing tg gal-1 (Gal-1-DC) inhibited ex vivo proliferation of splenic T cells in response to the peptide $GAD65_{206-220}$, insulin or NOD islet lysate. There was no differences in the response to OVA (control) between groups. Splenocytes from NOD*rag1*$^{-/-}$ mice adoptively transferred with diabetogenic splenocytes and left untreated (Untreated) or treated with NOD BM DC transduced with either control RAd-eGFP (eGFP-DC) or RAd-gal-1-eGFP (Gal-1-DC) were stimulated ex vivo for 3 days with different concentrations of $GAD65_{206-220}$, insulin, NOD islet lysate, or OVA (control). Cell proliferation is expressed as stimulation index ((Ag-specific proliferation − OVA-stimulated pro liferation/OVA-stimulated proliferation). *, $p < 0.001$. *B*, Assessment by ELISPOT analysis of the frequency of splenic T cells secreting IFN-γ and IL-4 in response to $GAD65_{206-220}$, insulin, and NOD islet lysate. Splenocytes were obtained from NOD*rag1*$^{-/-}$ mice adoptively transferred with diabetogenic splenocytes and left untreated (Untreated) or treated with NOD BM DC transduced with either control RAd-eGFP (eGFP-DC) or RAd-gal-1-eGFP (Gal-1-DC). *, $p < 0.01$; **, $p < 0.05$. Results are representative of four mice in each group.

Table I. *Th2 (IL-4)/Th1 (IFN-γ) cell ratios following administration of gal-1 DC*

Ag	Untreated (PBS)	eGFP-DC	Gal-1 DC
$GAD65_{206-220}$	10.9[a]	20.5	22.3
NOD pancreatic lysate	10.1	17.1	27.5
Insulin	3.0	9.1	37.4

[a] Ratios were calculated based on the number of spots assessed by ELISPOT assay in response to each Ag.

that administration of gal-1-DC interferes with the expansion of β cell-reactive Th1 cells.

Discussion

Several observations in humans and experimental models in rodents indicate that activated T cells are critical for the destruction of β cells in T1D: 1) T cells infiltrate the pancreatic islets at disease onset (45, 52–54); 2) T cell-directed immunosuppressants delay the onset of T1D (55, 56); 3) T1D can be transferred from a diabetic patient to an immunosuppressed nondiabetic recipient by BM transplantation (57); 4) T1D can be adoptively transferred to nondiabetic NOD or NOD*scid* mice using splenocytes, T lymphocytes (31, 58, 59) or T cell clones isolated from diabetic NOD mice (41, 60); 5) neonatal thymectomy prevents diabetes in NOD mice (61); 6) NOD*nu/nu* mice do not develop diabetes (62); and 7) β cell-specific T cells have been detected in prediabetic or recent-onset T1D patients (63, 64). Therefore, effective treatment of T1D will require the elimination of those diabetogenic T lymphocytes that initiate and perpetuate the destruction of the pancreatic β cells. The use of the potent immunoregulatory effects of gal-1 on activated T cells is a novel approach to control the T cell autoimmune response that triggers T1D.

Soluble gal-1 induces rapid death of T leukemic cell lines, $CD4^+$ $CD8^+$ thymocytes and activated, but not naive, T cells in humans (4, 5) and mice (24, 36). The mechanism by which gal-1 induces T cell apoptosis is not entirely understood. Gal-1 interferes with the assembly of the immunological synapse by obstructing the recruitment of kinases and the exclusion of phosphatases like CD45 in proximity to the TCR/CD3 complex. By this mechanism, gal-1 antagonizes signals that require sustained signal transduction (T cell proliferation, Th1 bias) but permits those that need partial TCR signaling (apoptosis and likely anergy) (65, 66). Activation of the transcription factor AP-1 and decrease levels of the anti-apoptotic protein BcL-2 are other mechanisms implicated in gal-1-mediated T cell apoptosis (67).

In this study, we demonstrated that administration of NOD BM DC genetically engineered to synthesize tg gal-1 delayed significantly the onset of diabetes in mice. Because only dimeric gal-1 bearing two β-galactoside binding sites has the ability to cross-link its ligands and the dissociation constant of the gal-1 homodimer is within the micromolar range (K_d ~7 μM) (68), the use of soluble gal-1 in vivo requires very high doses of the lectin with the potential side effect of agglutinating RBC and leukocytes due to the cross-linking ability of gal-1. Therefore, we avoided this limitation by delivering high levels of tg gal-1 to the site of APC:T cell interaction by means of genetically engineered NOD BM DC. To obtain high efficiency of transgene expression, DC were transduced with RAd, the most efficient method to deliver cDNA to BM DC (69).

Interestingly, the cellular viability of gal-1-DC was not affected by the presence of high levels of tg gal-1 indicating that, unlike activated T cells or thymocytes, NOD BM DC are resistant to gal-1-induced apoptosis. Our analysis by fluorescence microscopy and flow cytometry demonstrated that gal-1-DC accumulated most tg gal-1 in the cytosol and on the cell surface. The observation that gal-1-DC, and not control eGFP-DC, triggered apoptosis of activated BDC2.5 T cells in vitro indicates that gal-1-DC concentrate the functional (homodimeric) form of the lectin on the cell surface. The fact that the gal-1 competitor TDG reduced drastically the ability of gal-1-DC to trigger apoptosis of BDC2.5 T cells suggests that this effect was due to the galactose-binding capability of gal-1 and did not require cognate interactions (4). However, preloading of gal-1-DC with the mimotope peptide HRPI-RM enhanced the ability of gal-1-DC from NOD mice to induce apoptosis of activated BDC2.5 tgTCR $CD4^+$ T cells, indicating that TCR activation exerts a synergistic effect on induction of apoptosis by gal-1 in our model. These results agree with previous observations showing that gal-1 stimulation cooperates with TCR engagement via anti-CD3ε mAb to induce apoptosis of thymocytes (5) and T-T cell hybrids (70). Similarly in our system, gal-1-DC may increase their potential to induce gal-1-mediated apoptosis of activated T cells in vivo by presenting peptides derived from islet Ag drained constitutively to the PLN (48). The fact that previously reported DC-based approaches for therapy of diabetes in NOD mice did not require preincubation of DC with β cell-derived Ag is indicative that exogenous BM DC, once administered to the host, are able to internalize, process and present β cell-derived Ag to T cells (22, 71–74). Our results suggest that alternatively, in the absence of β cell-derived peptides, gal-1 DC are still able to trigger bystander apoptosis of activated T cells in PLN or spleen, the latter during T cell recirculation of self-reactive T cells at more advanced stages of the disease.

Previous studies have shown the feasibility of using myeloid BM DC precursors (75), DC-derived cell lines (76) and PLN, splenic or BM-derived DC untreated (73, 77), incubated with cytokines (22, 71, 74, 78) or genetically engineered (23, 72, 79) to prevent/delay the onset of T1D in NOD mice. These DC-based therapies have been focused to generating immature DC (75, 79), semimature DC (22) or DC with ability to induce Th2 differentiation by secreting tg IL-4 (72) or by down-regulating their synthesis of IL-12p70 (74). These therapeutic approaches are based on the concept that the "regulatory/tolerogenic" DC will induce differentiation of naive T lymphocytes into Th2 cells or Treg (72). However, once autoreactive T cells become activated at later stages of autoimmune diabetes, these DC-based methods are unable to down-regulate the T cell response (72). Therefore, the dual capability of gal-1 to trigger rapid apoptosis of activated $CD4^+$ and $CD8^+$ T cells (4, 36) and to reduce the Th1 response (6, 13, 24) make this lectin an ideal transgene to down-modulate the T cell response for therapy of T1D.

In the present study, we tested the therapeutic potential of NOD BM DC expressing tg gal-1 in a model of adoptive transfer of diabetes using immunodeficient NOD mice reconstituted with splenocytes from overtly diabetic NOD mice. In this experimental model, 1) the onset of diabetes occurs faster and takes place in a narrow window of time (4–5 wk) facilitating the experimental design (32, 33); 2) the disease is more aggressive than its natural variant in NOD mice, and more importantly, 3) the regulatory DC expressing tg gal-1 interact with preactivated polyclonal diabetogenic T cells resembling what occurs in patients with T1D where autoimmunity against β cells is mediated by multiple T cell clones (63, 64). Our results demonstrated that administration of NOD BM DC transfected with a RAd encoding for gal-1 delayed significantly the onset of diabetes in this model. This phenomenon cannot be ascribed to changes in the stage of DC maturation triggered by the RAd infection (38), because administration of NOD BM DC transduced with control RAd did not exert an effect (Fig. 3*B*).

Interestingly, injection of control nontransduced NOD BM DC generated with GM-CSF plus IL-4 did not delay the onset of diabetes, a result that agrees with the observation made by Morin et al. (74) but seems to contradict a previous study by Feili-Hariri et al. (22), showing that a protective Th2 response (22, 71) can be induced in young NOD mice by treatment with NOD BM DC generated in vitro with GM-CSF and IL-4. However, this latter DC-based approach reduced the incidence of diabetes when BM DC were administered to young (5–8 wk old) NOD mice, but it did not confer protection against T1D when DC were injected in NOD mice older than 10 wk (72), probably due to the consequent increase in the number and/or pathogenicity of β cell-reactive T cells (32, 33). The fact that in our model of adoptive transfer of diabetes in NOD*rag1*$^{-/-}$ mice, nontransduced NOD BM DC must interact with preactivated β cell-reactive T cells (derived from overtly diabetic NOD mice older than 10 wk) explains the reduced therapeutic efficacy of untreated NOD BM DC compared with previous studies (22).

Our results support the idea that DC expressing tg gal-1 protected from T1D through deletion of β cell-reactive (activated) T cells and by down-regulating the Th1 response. Administration of gal-1-DC was associated with lack of infiltration of $CD4^+$ and $CD8^+$ T cells within the islets and preservation of the content of insulin in β cells compared with control mice. The fact that i.v. administered gal-1-DC trafficked to the PLN and spleen and synthesized tg gal-1 locally indicate that gal-1-DC are fully capable of down-regulating the anti-β cell T cell response in peripheral lymphoid tissues. Our histological analysis of PLN of mice treated with gal-1-DC demonstrated an increased number of apoptotic T cells, a decreased percentage of $CD4^+$IFN-γ^+ cells and augmented population of $CD4^+$IL-4^+ cells compared with PLN of controls. Importantly, the fact that T cells from gal-1-DC-treated mice exhibited a significantly lower level of proliferation and synthesis of IFN-γ in response to $GAD65_{206-220}$, insulin, and NOD islet lysate compared with T lymphocytes from controls, demonstrated that gal-1-DC exert a profound down-regulatory effect on activated T cells recognizing β cell-derived Ag in vivo. In this regard, the immunodominant peptides $GAD65_{206-220}$ (49, 50) and insulin (B9-23) (80) are key autoantigen in NOD mice.

Although it is known that soluble gal-1 triggers apoptosis of double-positive thymocytes (5), central deletion of autoreactive thymocytes does not seem to be a mechanism used by gal-1 DC to delay diabetes in our model because gal-1-DC were not found in the thymus of treated mice. The absence of $CD4^+$ T cells expressing the Treg marker FoxP3 in the islets (data not shown) and lack of increase in numbers of $CD4^+$ $FoxP3^+$ lymphocytes in PLN and spleen of mice injected with gal-DC indicate that the effect of DC expressing tg gal-1 was not likely due to generation/expansion of Treg.

It has been previously shown that administration of soluble gal-1 or cells engineered to secrete gal-1 prevented and/or ameliorated cell-mediated autoimmune disorders in rodents including EAE (12), collagen-induced arthritis (6), experimental colitis (13), and Con A-induced hepatitis (24). This lectin has also proved to have a therapeutic effect on graft vs host disease in mice (25). As reported here for T1D, the therapeutic effect of gal-1 in these other models of autoimmune disorders seems to be related to the selective elimination of self-reactive cells by apoptosis (6) without inducing generalized immune suppression. In this regard, we have recently demonstrated that administration of gal-1-DC induces a drastic inhibition of the elicitation phase of the contact hypersensitivity reaction in a hapten-specific manner (36). In EAE, treatment with soluble gal-1 prevented the development of myelin basic protein-specific T cells, without affecting the immune response to other Ag (12).

In humans, once the β cell-reactive T cell become activated, there is often a quite long asymptomatic period of time until development of T1D (81). The protraction of complete β cell loss can now be exploited, since prediction in the early prediabetic stages or in Ab positive high-risk first-degree relatives and in young children has become remarkably more precise, agents that have a well-documented safety profile or strategies that take advantage of Ag-specific induction would be the most advantageous. A therapy based on administration of autologous DC expanded and genetically engineered in vitro to synthesize tg gal-1 will be focused, unlike most other DC-based approaches, to selectively down-regulating the deleterious effects of already activated T cells against β cells. Administration of gal-1-DC to T1D patients during the preclinical stage or following transplantation of allogeneic islets might help to eliminate activated β cell-reactive T cells at early disease onset and prevent/ameliorate the recurrence of β cell autoimmunity against transplanted islets.

Disclosures

The authors have no financial conflict of interest.

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