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Transgenic Galectin-1 Induces Maturation of Dendritic Cells That Elicit Contrasting Responses in Naive and Activated T Cells

Marcelo J. Perone,* Adriana T. Larregina,† ‡ William J. Shufesky,* Glenn D. Papworth,§ Mara L. G. Sullivan,§ Alan F. Zahorchak,* Donna Beer Stolz,§ Linda G. Baum,¶ Simon C. Watkins,§§ Angus W. Thomson,§§ and Adrian E. Morelli²*

Dendritic cells (DC) are professional APC that control the balance between T cell immunity and tolerance. Genetic engineering of DC to regulate the outcome of the immune response is an area of intense research. Galectin (gal)-1 is an endogenous lectin that binds to glycoproteins and exerts potent regulatory effects on T cells. Consequently, gal-1 participates in central deletion of thymocytes and exerts therapeutic effects on experimental models of T cell-mediated autoimmune disorders and graft-vs-host disease. Together, these observations strongly indicate that engineering DC to express transgenic (tg) gal-1 may be beneficial to treat T cell-mediated disorders. In this study, we have investigated the impact of the expression of high levels of tg gal-1 on maturation/activation of DC and on their T cell stimulatory function. Murine DC were transduced with a recombinant adenovirus encoding hu gal-1 (gal-1-DC). Tg gal-1 was expressed by a nonclassical pathway through exosomes and was retained on the DC surface inducing segregation of its ligand CD43. Expression of tg gal-1 triggered activation of DC determined by induction of a more mature phenotype, increased levels of mRNA for proinflammatory cytokines, and enhanced ability to stimulate naive T cells. Conversely, gal-1-DC induced rapid apoptosis of activated T cells. In vivo, gal-1-DC increased significantly the sensitization phase of contact hypersensitivity assays while inducing a drastic inhibition of the elicitation phase by triggering apoptosis of activated T cells in the dermis. Gal-1-DC represent a novel tool to control differentially the afferent and efferent arms of the T cell response. The Journal of Immunology, 2006, 176: 7207–7220.

Dendritic cells (DC)³ are professional APC that activate naive and memory T cells (1, 2). Peripheral tissue-resident DC take up, process, and transport Ag to secondary lymphoid organs, where they trigger differentiation of helper and CTLs (1, 2). Besides their function as initiators of T cell immunity, DC participate in negative selection of immature thymocytes in central T cell tolerance (2). In addition, DC play a critical role in induction/maintenance of peripheral T cell homeostasis by inducing T cell apoptosis/anergy and differentiation/amplification of regulatory T cells (3–6). Failure of DC to maintain T cell tolerance may contribute to the pathogenesis of autoimmune disorders (7). During the past decade, different groups have explored the possibility of down-regulating the T cell response by using in vitro-generated DC rendered “regulatory” by pharmacologic or genetic techniques (reviewed in Refs. 5, 8). In vitro generation of regulatory DC would be an invaluable tool for therapy of allograft rejection and autoimmune diseases (reviewed in Refs. 5 and 8). The potent regulatory effects of galectin (gal)-1 on T cells, including induction of apoptosis (9) and down-modulation of the Th1 response (10–13), make this endogenous lectin a potentially useful transgene for engineering DC to treat T cell-mediated disorders.

Gal-1 binds to lactosamines present on glycoproteins and glycolipids such as CD45, CD43, CD7, CD4, CD3, CD2, the lysosomal-associated membrane protein (LAMP)-1 and -2, the lipid GM1, and extracellular matrix glycoproteins (14–21). The observation that gal-1 is expressed by the thymic epithelium and triggers apoptosis of thymocytes suggests that gal-1 participates in central tolerance (22). The beneficial effect of gal-1 administration in experimental models of T cell-mediated autoimmune disorders (10–12, 23) and graft-vs-host disease (13) indicates that this lectin may be critical for T cell homeostasis and peripheral tolerance. In this regard, gal-1 is up-regulated by CD4⁴CD25⁺ regulatory T cells (24), is expressed by immune-privileged tissues (placenta, testis, brain) (25–27), and is used in mechanisms of immune escape by tumors (28). The basis of the regulatory effects of gal-1 on T cells is not well understood. Gal-1 might function as a T cell “counterstimulator” by cross-linking ligands on the T cell surface and, therefore, obstructing the organization of the immunological synapse (29, 30). Down-regulation of Bcl-2 and activation of the transcription factor AP-1 have been identified as other mechanisms by which gal-1 triggers T cell apoptosis (31).
Although the down-regulatory effects of gal-1 on T cells have been documented using soluble gal-1 in vitro or administered systemically in vivo (9–13), the effects of this lectin on APC have not yet been addressed. It is not known whether 1) DC can be genetically engineered to secrete soluble transgenic (tg) gal-1; 2) release of tg gal-1 by DC would have an impact on the function of DC; and 3) DC expressing high levels of tg gal-1 may be used to control the T cell response. In this study, we genetically engineered DC to produce high levels of tg gal-1. We demonstrate that DC release tg gal-1 through a nonclassical pathway of secretion via exosomes and that, once secreted, gal-1 is retained on the DC surface and induces segregation of its ligand CD43. We further analyzed the effects that expression of tg gal-1 by DC induced on the phenotypic maturation and pattern of cytokine mRNA transcription of DC and on their ability to interact with different T cell subsets. The in vivo ability of DC expressing tg gal-1 to modify the T cell response was investigated using a model of contact hypersensitivity (CH).

Materials and Methods
Animals, cell lines, and reagents
Ten- to 12-wk-old BALB/c (H2d), C3H (H2k), and C57BL/10 (B10, H2b) mice were purchased from The Jackson Laboratory. 1H3.1 TCR-H9251/H9252 mice were provided by Drs. C. Janeway and C. Viret (Yale University, New Haven, CT). Studies were approved by the Institutional Animal Care and Use Committee. Mouse (m) rGM-CSF was a gift from the Schering-Plough Research Institute (Kenilworth, NJ), and the BW514Pahα2.1 cell line (Pahα2.1) was a gift from Dr. M. Pierce (University of Georgia, Athens, GA). mrl-4 and IL-2 were purchased from R&D Systems, and DTT, thiodigalactoside (TDG), PHA, 2,4 dinitro-1-fluorobenzene (DNFB), and 2,4,6-trinitrobenzenesulfonic acid (TNBS) were obtained from Sigma-Aldrich. Protein A-Sepharose CL-4B was purchased from Amersham Biosciences. Soluble human (hu) gal-1 was obtained from PeproTech.

Generation of plasmids and recombinant adenovirus (RAd)
The hu gal-1 cDNA from pT7IML-1 was subcloned into pIREs2-eGFP (enhanced GFP) (Clontech Laboratories) to construct pgal-1-IREs2-eGFP, which was driven by the early hu CMV promoter (CMVp) and contained the gal-1 cDNA, followed by an internal ribosome entry site (IRES), the eGFP cDNA, and the SV40 poly(A) signal (Fig. 1A). 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 Biosystems). For generation of pcDNA3.1/gal-1, the gal-1 cDNA from pT7IML-1 was subcloned into pcDNA3.1+ (Promega).

Generation of bone marrow-derived DC (BMDC)
DC were generated in vitro from bone marrow precursors cultured with GM-CSF and IL-4 (each 1000 U/ml) as described previously (32). At day 5, nonadherent cells were plated into 24-well plates (10⁶ cells/well) and infected (day 6) with RAd (multiplicity of infection (MOI) = 100). DC were purified by positive selection using bead anti-CD11c mAb labeling and magnetic sorting (Miltenyi Biotec) or, alternatively, by anti-CD11c staining followed by sorting using an EPICS Elite FACS (Beckman Coulter) (DC purity 92–95%).

Purification of BMDC-derived exosomes
Exosomes were isolated from BMDC culture supernatants as described previously (33, 34). On day 4, the medium of BMDC cultures was replaced with fresh medium with cytokines and 10% v/v exosome-free FCS obtained by overnight ultracentrifugation (100,000 × g). DC supernatants were collected on days 6 and 8 and centrifuged at 4°C at 100,000 × g (20 min), 10,000 × g (20 min), 10,000 × g (30 min), and 100,000 × g (60 min) (35). To further purify the exosomes from protein aggregates, exosomes were underlaid with 500 μl of a 30% sucrose/D2O density cushion and ultracentrifuged (100,000 × g, 4°C, 60 min) (35). The 500 μl of sucrose/D2O solution were harvested, diluted in 14 ml of PBS, and concentrated by centrifugation (1,000 × g, 4°C, 30–60 min) in a prerinsed 100-kDa MWCO Millipore Ultrafree-15 capsule filter (Millipore) to a final volume of 200 μl (35). The amount of protein in the exosome preparation was assessed by Bradford assay (Bio-Rad).

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Cell pellets of immune-bead-sorted (CD11c+), noninfected or RAd-transduced DC were suspended in 50 mM Na-phosphate/1% v/v SDS/40 mM 2 ME/2 mM EDTA, boiled, and centrifuged at 15,000 g (10 min, 4°C). Supernatants (18 h) from noninfected or RAd-transduced DC were centrifuged (10,000 g) and treated with 1 U/L PMFSF, 10 mM pepstatin A, and 20 mM EDTA. Cell extracts and supernatants were diluted in 0.25 M Tris-HCl, 4% v/v SDS, 20% v/v glycerol, 0.2 M DTT, 0.02% v/v bromophenol blue (pH 6.8), boiled, and loaded in 15% w/v acrylamide SDS-PAGE. Gels were electrophoresed on polyvinylidiene difluoride membranes. The membranes were labeled with anti-hu gal-1 mAb (1:250; NovoCastra) followed by peroxidase anti-mouse IgG (1:10,000; Jackson Immunoresearch Laboratories). Staining was developed by chemiluminesence (NEN Life Science Products).

For immunoprecipitation of gal-1, medium containing 20 μM soluble hu gal-1 was 1) incubated with anti-hu gal-1 mAb (NovoCastra; 1:20, 16 h, 4°C, rotation), 2) treated with protein A-Sepharose (1:50, 4°C, 4 h, rotation), and centrifuged. The gal-1-free supernatant was used as control medium to culture BMDC.

**Fluorescence, confocal, and transmission electron-microscopy**

Immune-bead-sorted (CD11c+), noninfected or RAd-transduced DC were attached to poly-l-lysine-treated slides and fixed with 4% paraformaldehyde (PF). For surface staining, cells were blocked with 10% normal goat serum, labeled with mouse anti-hu gal-1 mAb, and then examined using a JEM1210 electron microscope (JEOL) at 80 kv.

**Analysis by flow cytometry**

Noninfected or RAd-transduced DC were blocked with 10% goat serum, incubated with mouse anti-hu gal-1 mAb alone or in combination with rat anti-CD3 mAb (BD Pharmingen), followed by goat anti-mouse IgG, Cy3-streptavidin, and Cy5 anti-rat IgG (Jackson Immunoresearch Laboratories). For intracellular staining, cells were fixed in 1% PF, incubated in 3% gelatin, suspended in 2.3 M sucrose, and then labeled with rat anti-hu gal-1 mAb followed by Dynabeads M-450 (Dynal Biotech) and negative selection by magnetic sorting. Five 10^6 naive CD44lowCD62Lhigh CD4+ T cells purified from spleens and lymph nodes of C3H/He mice were labeled with JC9 and cultured (1 h, 37°C) with 5 μg/ml of PSTA-M (Cedarlane Laboratories) to purify viable T cell blasts from dead cells. CD4+ and CD8+ T cells were purified by negative selection using a combination of CD8, CD4, B220, F4/80, and NK1.1 mAb, followed by Dynabeads M450 (Dynal Biotech) and negative selection by magnetic sorting. Five 10^6 naive or RAd/L2-activated T cells were preincubated with IC-9 (10 μg/ml, 20 min, 37°C) (Molecular Probes). Splenic naive (CD62LlowCD44high) T cells were purified by enrichment column R&D Systems. Activated T cells were generated by culturing splenocytes with PHA (10 μg/ml) + IL-2 (3 U/ml) for 5 days. The splenocytes were then centrifuged on a gradient of Lympholyte-M (Cedarlane Laboratories) to purify viable T cell blasts from dead cells. CD4+ and CD8+ T cells were purified by negative selection using a combination of CD8, CD4, B220, F4/80, and NK1.1 mAb, followed by Dynabeads M450 (Dynal Biotech) and negative selection by magnetic sorting. Five 10^5 naive or PHA/L2-activated T cells were preincubated with IC-9 and cultured (1 h, 37°C) with 5 μg/ml of noninfected or RAd-transfected DC. The assay was stopped by addition of 5 μl of anti-CD3 and anti-CD25 antibodies and the percentage of apoptotic cells was determined by the reduction of mi tochondrial membrane potential assessed by flow cytometry.

**Contact hypersensitivity**

To analyze the effect of DC expressing tg gal-1 on the sensitization phase of CH, B10 mice were sensitized (day 0) by passive transfer of immune-bead-sorted (CD11c+) B10 DC (noninfected or RAd-transduced) haptenized with preincubation (15 min) in 1 mM TNBS (3 × 10^6 BMDC/100 μl of PBS/footpad). As a specificity control, one group was injected with DC transfused with RAd-gal-eGFP and not exposed to TNBS. Mice were challenged (day 6) with 20 μl of DNFB (0.2% in acetone/olive oil, 4:1; Sigma-Aldrich) on the right ear pinna. The vehicle was applied to the left ear, as control.

To test the influence of DC expressing tg gal-1 on the elicitation phase of CH, mice were sensitized (day 0) by topical application of 50 μl of DNFB (0.5%) on the skin of the shaved abdomen. They were challenged (day 6) by passive transfer of immune-bead-sorted (CD11c+) B10 DC (noninfected or RAd-transduced) haptenized with TNBS (3 × 10^6 BMDC/50 μl of PBS/injected intradermally in the right ear). One experimental group was challenged with DC transfused with RAd-gal-eGFP in the absence of TNBS. The thickness of the right (challenged) and left ear pinna (control) was measured with a spring-loaded caliper after challenge. CH was determined as the amount of swelling on the hapten-challenged ear compared with the thickness of the vehicle-treated ear in sensitized animals and was expressed as percentage increase in ear thickness (mean ± SD). Each group consisted of at least three mice. Each experiment was performed twice.

**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 Student’s t test. A p < 0.05 was considered significant.
Results

Generation of DC expressing tg gal-1

The maximal efficiency of transfection of DC infected with RAd-gal-1-eGFP (gal-1-DC) was 85 ± 5% (MOI = 100–200), as demonstrated by the percentage of DC expressing eGFP assessed by flow cytometry (Fig. 1B). All gal-1-DC coexpressed eGFP and tg gal-1 in cytosols analyzed by fluorescence microscopy (data not shown). The viability of gal-1-DC was not affected by expression of tg gal-1 (or RAd infection) as assessed by annexin-V staining and flow cytometry (Fig. 1B). Similar RAd transfection efficiency and cell viability were detected in DC transduced with RAd-eGFP (eGFP-DC) (data not shown). Based on these results, all subsequent experiments were performed with RAd at a MOI of 100 unless stated.

High levels of (hu) gal-1 (14 kDa) were detected in gal-1-DC by Western blot analysis performed under reducing conditions (Fig. 1C). The absence of dimeric gal-1 (28 kDa) can be ascribed to the fact that gal-1 forms noncovalent homodimers that run electrophoretically as monomers under reducing conditions. By contrast, in control DC (untreated or RAd-eGFP-transduced) we were unable to detect endogenous (murine) gal-1 by using a mAb against hu gal-1 (Fig. 1C). This result may be due to expression of low levels of endogenous gal-1 by DC, or to low-affinity recognition of murine gal-1 by the mAb used. We did not detect soluble tg gal-1 in ultrafiltered supernatants of gal-1-DC (100,000 kDa MWCO Millipore Ultrafree-15 capsule filter; Millipore) (Fig. 1C), indicating that secreted soluble tg gal-1 may be mostly attached to the surface of DC as documented in other cell types (21), or that DC release low quantities of soluble tg gal-1 in culture supernatants.

Localization and intracellular distribution of tg gal-1 in DC

In our system tg gal-1 must be externalized by gal-1-DC to interact physically with T cells. Because gal-1 was not detected in filtered supernatants of gal-1-DC, we wondered whether the tg gal-1 produced by gal-1-DC might bind to oligosaccharide ligands on the DC surface. In fact, previous reports have demonstrated that extracellular gal-1 binds to counterreceptors present on the surface of T cells (9) and cell lines (21). By confocal microscopy we found that gal-1-DC concentrated tg gal-1 in small patches on the cell surface (Fig. 2A). By contrast, control (noninfected or eGFP-) DC were negative for tg (hu) gal-1 on the cell surface (Fig. 2B). Because tg gal-1 was retained in small patches on gal-1-DC and DC express the gal-1 ligand CD43 on their surface (36, 37), we investigated whether the membrane distribution of tg gal-1 was due to cosegregation of gal-1 and CD43 on the DC surface. We detected that in gal-1-DC, CD43, and gal-1 colocalized within the same areas of the cell surface (Fig. 2A). By contrast, control noninfected and eGFP-DC exhibited a diffuse pattern of CD43 labeling (Fig. 2B). A quantitative assessment of 200 cells analyzed demonstrated that 86% of gal-1-DC expressed CD43 in one or two small domains on the surface membrane (Fig. 2C, arrows), whereas 95% of control DC (untreated or RAd-eGFP-transduced) exhibited a diffuse pattern of CD43 staining (Fig. 2D). Because DC can transfer molecules between themselves (38) and certain cell lines can mobilize dimeric gal-1 from their surface to saccharide ligands present on extracellular matrix glycoproteins (21), we then investigated whether gal-1-DC were able to transfer tg gal-1 to the surface of other DC. To answer this question, gal-1-DC (or eGFP-DC, as a control) were mixed with nontransduced DC (1:1 DC ratio, 4 h; 37°C), surface labeled with CyChrome anti-CD11c and PE anti-gal-1 mAb, and analyzed by FACS. Following 4-h coculture, gal-1-DC (eGFP+ gal-1+) were able to transfer gal-1 to nontransduced DC (eGFP− gal-1−), a phenomenon that was not observed when nontransduced DC were mixed with control eGFP-DC (Fig. 2E).

In addition to cell surface localization, we asked whether tg gal-1 also localized to exosomes (65- to 100-nm vesicles formed...
within multivesicular bodies; MVB) as has been found for gal-3 (39). Intracellular tg gal-1 concentrated in LAMP-1^ vesicles in the cytoplasm of gal-1-DC (Fig. 2F). LAMP-1 is a ligand for gal-1 (40) that is present in MVB and lysosomes of DC (41). MVB are MHC class II-enriched compartments (41) involved in Ag processing and generation of exosomes (42). Ultrastructural analysis of gal-1-DC stained for LAMP-1 and gal-1 demonstrated that gal-1 was localized on the limiting membrane of MVB and inside exosomes (Fig. 3, A–E). Quantitative analysis showed that 40 ± 7% of the exosomes of gal-1-DC contained gal-1, compared with 2 ± 0.5% of exosomes in control eGFP-DC. To confirm that tg gal-1 was present within exosomes, we purified exosomes from 24-h

![Ultrastructural association of gal-1 with exosomes in gal-1-DC.](image)

**FIGURE 3.** Ultrastructural association of gal-1 with exosomes in gal-1-DC. A, Cryosections were labeled with anti-hu gal-1 (5-nm gold) and LAMP-1 (15-nm gold) mAb. Gal-1 was detected mainly in the lumen of exosomes (arrows) and LAMP-1 on the membrane of MVB (scale bar, 100 nm; magnification, ×100,000). B and C, Exosomes with intraluminal gal-1 (arrow; scale bar, 100 nm; magnification, ×150,000). D, Localization of gal-1 (arrows) on the limiting membrane of a MVB labeled with LAMP-1 (arrowheads), a site where exosomes are generated (Lu, MVB lumen; scale bar, 100 nm; magnification, ×100,000). E, Assessment of the percentage of gal-1 labeled with 5-nm gold mAb associated with MVB and exosomes in gal-1-DC. Quantification was performed on 50 cells analyzed by immunoelectronmicroscopy. F, Whole mount preparation of exosomes purified from supernatants of gal-1-DC. Exosomes are nanovesicles of 65–100 nm in diameter with cup-shaped morphology (scale bar, 100 nm; magnification, ×100,000). G, Detection of gal-1 by SDS-PAGE on extracts of exosomes purified from supernatants of DC left untreated or transduced with RAd-eGFP (eGFP-DC) or RAd-gal-1-eGFP (gal-1-DC). Soluble hu gal-1 (sGal-1) was used as control. Equivalent loading of exosome extracts was confirmed by detection of the exosome marker HSP73; NS, nonspecific band. In A–E, data are representative of four independent experiments. F and G illustrate one of two independent experiments.
culture supernatants of gal-1-DC and control DC by sequential ultracentrifugation, followed by a 30% sucrose/D2O gradient and ultrafiltration (Fig. 3F). By Western blot analysis (reducing conditions), we detected monomeric (14 kDa) gal-1 in exosomes released by gal-1-DC (Fig. 3G). We did not find detectable gal-1 in exosomes produced by control DC under similar conditions. Loading of equivalent amounts of exosome lysates between samples for Western blot was confirmed by detection of heat shock protein 73 (HSP73), a molecule expressed constitutively by DC-derived exosomes (Fig. 3G) (33). These results indicate that gal-1 DC secrete gal-1, at least in part, through exosomes and that externalized gal-1 binds back to the surface of DC.

Expression of tg gal-1 induces activation of DC

Because the binding of gal-1 to counterreceptors on the DC surface may affect the function of DC, we analyzed the activation phenotype, cytokine production, and T cell stimulatory function of gal-1-DC. Gal-1-DC were cultured for 24 h, surface labeled with mAb, and analyzed by flow cytometry. Noninfected DC and eGFP-DC were included as controls. Compared with eGFP-DC, gal-1-DC had enhanced levels of IAa, H2Kd, CD40, CD54, CD80, and CD86 expression (Fig. 4A). To confirm that the effect of gal-1 on the DC phenotype requires interaction of extracellular tg gal-1 with ligands on the DC, we 1) incubated DC transduced with RAd-gal-1 with the gal-1 competitor TDG and 2) added soluble hu gal-1 to nontransduced DC. After RAd-gal-1 transduction, incubation with 0.2 M TDG prevented maturation of gal-1-DC (Fig. 4B). The levels of expression of IAa and CD86 by gal-1-DC treated with TDG were similar to those of control DC infected with RAd-eGFP (Fig. 4B). Incubation of nontransduced DC for 18 h with medium supplemented with 10 μM soluble hu gal-1 induced an increase of IAa and CD86 expression similar to that triggered by RAd-gal-1 infection at a MOI of 50 (Fig. 4B). The gal-1-dependent DC maturation was not due to contaminating LPS or other DC-activation factors in the hu gal-1 added to the medium, because it decreased drastically when hu gal-1 was removed from the medium by immunoprecipitation (with anti-hu gal-1 mAb plus protein A-Sepharose) previous to the incubation with DC (Fig. 4B).

Activation of cytokine gene transcripts in relation to expression of tg gal-1 by DC was analyzed by RPA 16 h after infection with RAd-gal-1. mRNA was extracted from CD11c+ DC isolated by magnetic sorting. The comparative analysis of cytokine mRNA in gal-1-DC and in control DC (noninfected or transduced with RAd-eGFP) is illustrated in Fig. 4C. As we described previously (43),
infection of DC with control RAd-eGFP increased levels of IL-6, IL-12p40, and TNF-α transcripts and decreased TGF-β1 mRNA expression due to the maturation of DC that follows RAd-infection (Fig. 4C). Gal-1-DC increased the levels of the proinflammatory IL-1α, IL-1β, IFN-α, IL-6, TNF-α, and IL-12p40 and IL-12p35 mRNA drastically, and further decreased the extent of transcription of the anti-inflammatory cytokine TGF-β1 compared with untreated or RAd-eGFP-transduced control DC (Fig. 4C). These changes in the cytokine mRNA repertoire in response to tg gal-1 expression are indicative of DC maturation (32, 43).

Expression of tg gal-1 enhances the stimulatory ability of DC

We then investigated whether expression of tg gal-1 affects the capability of DC to stimulate syngeneic and allogeneic naive T cells. FACS-sorted (purity ≥94%) gal-1-DC, eGFP-DC, and untreated DC were prepared from B10 mice, pulsed or not (control) with the IEα52–68 peptide and used as stimulators of syngeneic naive CD4⁺ T cells. IEα52–68 peptides induce proliferation of 1H3.1 cells in the presence of IEα52–68 peptide to naive 1H3.1 T cells significantly compared with controls. The phenomenon was Ag-specific because gal-1-DC did not induce proliferation of IH3.1 cells in the absence of IEα52–68 (Fig. 5A). The enhanced stimulatory effect of gal-1-DC on IH3.1 cells was also dependent on the concentration of the IEα52–68 peptide (Fig. 5B). As expected within control DC, eGFP-DC exhibited a higher capability than nontransduced DC to stimulate T cells (Fig. 5A) due to the ability of RAd to induce partial DC activation (43). In this regard, it has been demonstrated that the ability of RAd to induce DC maturation is 1) not associated to RAd transcription (43), 2) is independent from the synthesis of the most commonly used reporter genes (43), and 3) even occurs following transfection of DC with RAd encoding no tranngenes (43).

Next, we determined whether the augmented stimulatory capability of gal-1-DC was due to increased DC maturation (induced by tg gal-1) or to a direct effect of extracellular tg gal-1 on T cell activation. To distinguish between these possibilities, we used two different sets of experiments. First, we compared the level of IH3.1 cell activation induced by untreated, eGFP- and gal-1-DC pretreated for 24 h with 100 ng of LPS (to induce full APC maturation) and pulsed with 10 μg/ml IEα52–68 peptide. Following LPS-induced maturation, gal-1- and control-DC (nontransduced and RAd-eGFP-infected) exhibited similar enhanced ability to stimulate IH3.1 cells (Fig. 5C). This result indicates that, in the presence of fully mature DC, tg gal-1 does not exert an additional effect on T cell activation. Next, we compared the level of IH3.1 cell proliferation in response to eGFP-DC pretreated or not with soluble gal-1. eGFP-DC pre-exposed to 10 μM soluble hu gal-1 (for 24 h and then washed three times) increased significantly their stimulatory function compared with control eGFP-DC (Fig. 5D).

In the absence of DC, incubation of naive IH3.1 T cells with 10 μM soluble hu gal-1 did not induce cell proliferation (Fig. 5D). These results indicate that tg gal-1 augments T cell proliferation through their ability to promote DC maturation.

The T cell allostimulatory activity of FACS-purified, gal-1-DC, eGFP-DC, and untreated DC (controls) prepared from B10 mice was also tested in 3-day MLR using allogeneic (C3H) and syngeneic naive T cells (CD62LhighCD44low, purity ≥95%) as responders. Gal-1-DC induced higher proliferation of allogeneic naive T cells that secreted predominantly IFN-γ (Fig. 5, E and G). The effect was allospecific because gal-1-DC did not stimulate division of syngeneic T cells (Fig. 5F).

DC expressing tg gal-1 induce rapid apoptosis of activated CD4⁺ and CD8⁺ T cells

Our results demonstrate that gal-1-DC induce proliferation of naive T cells. However, the immune-modulatory activity documented for soluble gal-1 is based on its ability to delete thymocytes and activated T cells (9). Because these studies have used soluble gal-1 in the absence of DC (9–13), we investigated whether, under our experimental conditions, gal-1-DC were still able to induce apoptosis of T cells.

In vitro, gal-1-DC triggered apoptosis of the T cell line PhaR².1, a reliable target for gal-1-induced apoptosis (21), at a DC:T cell ratio ≥2.5 (Fig. 6 A and B) and following a minimal incubation time of 1 h. PhaR².1 cells did not increase their basal percentage of apoptotic cells (<20%) following incubation with control DC (untreated or infected with RAd-eGFP) (Fig. 6A).

It has been documented that gal-1 induces exposure of phosphatidylserine in hu T leukemic MOLT-4 cells, HL60 cells, and fMLP-activated neutrophils without triggering apoptosis (45, 46). Because detection of apoptosis by annexin-V depends on its binding to externalized phosphatidylserine, we evaluated the ability of gal-1-DC to induce apoptosis by TUNEL assay, a technique that relies on detection of genomic DNA fragmentation as an indicator of apoptosis. Following 1 h coculture of DC infected with RAd-gal-1-eGFP with target PhaR².1 cells, we detected eGFP⁺ DC surrounded by apopotic PhaR².1 cells labeled by TUNEL (Fig. 6C). As controls, PhaR².1 cells cocultured with DC infected with RAd-eGFP were not labeled by TUNEL (Fig. 6C).

Conditioned medium (ultrafiltered) from gal-1-DC did not induce apoptosis of PhaR².1 cells (Fig. 6D), suggesting that gal-1-DC supernatants contained low amounts of soluble gal-1 likely because most of the released tg gal-1 bound to the DC surface (Fig. 2A) (21). Addition of the gal-1 competitor TG (0.2M) or anti-hu gal-1 serum (1:100) to cocultures of gal-1-DC and PhaR².1 cells decreased the percentage of apoptotic PhaR².1 cells significantly (Fig. 6E). These results indicate that induction of T cell apoptosis was gal-1-specific and dependent on galactose-specific interactions.

Next, we tested whether DC that take up soluble gal-1 from the medium are able to promote T cell apoptosis, as an alternative strategy for T cell suppressive therapy without the need for a tg intervention. Unlike RAd-gal-1-transduced DC, control DC (noninfected or RAd-eGFP-transduced) pre-exposed to 10 μM soluble hu gal-1 (1 h, 37°C) and washed extensively did not induce apoptosis of PhaR².1 cells (Fig. 6E). This result may be due to the fact that the soluble gal-1 attached to the surface of DC was rapidly internalized and/or that the levels of soluble gal-1 on the DC surface were insufficient to reach the concentration required to induce T cell death.

Unlike control DC, gal-1-DC (BALB/c) induced rapid apoptosis of allogeneic (B10) and syngeneic PHA/IL-2-activated splenic T cells, but not naive T cells (Fig. 7A). In these experiments, apoptosis was evaluated by preincubating the target T cells with the mitochondrial probe JC-9, followed by assessment of the reduction in the mitochondrial membrane potential (an early indicator of apoptosis) by flow cytometry. This approach allowed us to measure apoptosis exclusively in T cells, excluding unlabeled DC that may eventually become apoptotic during the assay. Next, we tested whether gal-1-DC induce preferential apoptosis of activated CD4⁺ and CD8⁺ T cells. Purified PHA/IL-2-activated CD4⁺ or CD8⁺ T cells were incubated with gal-1-DC (1 h, 37°C), and the percentage of apoptotic T cells was assessed according to the changes in the mitochondrial membrane potential. Both PHA/IL-2-activated CD4⁺ and CD8⁺ T cells were susceptible to apoptosis induced by gal-1-DC (Fig. 7B).
FIGURE 5. Expression of tg gal-1 enhances the ability of DC to stimulate naive T cells. A, B, The stimulatory activity of B10 DC (noninfected or transduced with RAd-eGFP or RAd-gal-1-eGFP) pulsed or not with the BALB/c allopeptide IEd52-68 was assessed at various concentrations of (A) DC or (B) peptide with 10^5 naive CD4^+ 1H3.1 TCRtg T cells during 48-h culture. C, Stimulatory function of B10 DC (noninfected or infected with RAd-eGFP or RAd-gal-1-eGFP), pretreated with 100 ng of LPS, pulsed with 10 μg of IEd52-68 and incubated with 10^5 naive CD4^+ 1H3.1 T cells (48 h) as responders. D, The stimulatory capability of DC transduced with RAd-eGFP, pulsed with IEd52-68, and either pretreated or not with soluble hu gal-1 (10 μM), was assessed using 10^5 naive CD4^+ 1H3.1 T cells (48 h) as responders. As control, 10^5 naive CD4^+ 1H3.1 T cells were cultured alone with soluble hu gal-1 (10 μM) (∆) or without addition of the exogenous lectin (▲). E and F, Allostimulatory ability of B10 DC, noninfected or transduced with RAd-eGFP or RAd-gal-1-eGFP for 2 × 10^5 naive (CD62LhighCD44low) T cells from C3H or B10 mice (syngeneic control). The allostimulatory activity of freshly isolated splenocytes is shown as control. Proliferation of responder cells was quantified by incorporation of [3H]Tdr added 18 h before harvesting. In A–F, the results are expressed as mean cpm ± SD and are representative of three to five separate experiments. G, Detection by ELISA of IFN-γ, IL-4, and IL-10 in 3-day MLR supernatants of naive C3H T cells maintained alone (control) or stimulated with allogeneic (B10) DC, noninfected or transduced with RAd-eGFP (eGFP-DC) or RAd-gal-1-eGFP (Gal-1-DC). Results are representative of four independent experiments.
Having demonstrated that expression of tg gal-1 matured DC, enhancing their capability to stimulate naive T cells and, at the same time, enabled DC to induce rapid apoptosis of activated T cells, we asked whether these observations had in vivo relevance. We used a model of CH that allow us to discriminate between the effects of gal-1-DC on naive and activated/effector T cells while testing whether the outcome was hapten-specific. We analyzed the influence that gal-1-DC exerted on 1) naive T cells during the sensitization phase and on 2) activated/effector T cells during the elicitation phase of CH.

To test the influence of gal-1-DC on the sensitization phase of CH, B10 mice were sensitized (day 0) by injection (footpad) of DC (noninfected or RAd-transduced) preincubated with TNBS. As a specificity control, one group was sensitized with gal-1-DC without the hapten. Six days later, the right ear pinna was challenged with topical DNFB and the thickness of swelling measured 24, 48, and 72 h later on the right (challenged) and left (control) ears. A significant \( p < 0.01 \) increase in swelling was detected 48 h post-challenge in mice sensitized with TNBS-gal-1-DC compared with animals sensitized with TNBS-eGFP-DC (Fig. 8A). Gal-1-DC without TNBS did not sensitize the mice, indicating that the reaction was hapten-specific (Fig. 8A).

Next, we investigated the influence of gal-1-DC on the elicitation phase of CH. Mice were sensitized with DNFB applied epicutaneously, followed 6 days later, by challenge with TNBS-treated DC (noninfected or transduced with RAd) injected in the base of the right ear. As a specificity control, one group was challenged with gal-1-DC not exposed to TNBS. Challenge TNBS-gal-1-DC suppressed CH (Fig. 8B). By contrast, challenge with control TNBS-eGFP-DC did not affect the outcome of CH (Fig. 8B). To analyze the mechanism by which gal-1-DC markedly suppressed the elicitation phase of CH, treated and nontreated ears were subjected to microscopic analysis 48 h after challenge. The ears challenged with control TNBS-eGFP-DC showed severe interstitial edema and infiltration with mononuclear cells (Fig. 9A), mainly CD3\(^+\) T cells and eGFP\(^+\) cells with DC morphology (Fig. 9C and E). By contrast, those ears injected with TNBS-gal-1-DC showed no signs of edema (Fig. 9B), few CD3\(^+\) T cells (Fig. 9D), and a high number TUNEL\(^+\)-infiltrating cells surrounding eGFP\(^+\) cells with DC morphology (Fig. 9F). By four-color fluorescence microscopy, we detected the expression of CD3 on TUNEL\(^+\) cells attached to eGFP\(^+\) cells with DC morphology (Fig. 9G). This suggests that induction of activated T cell apoptosis at the effector site is one of the mechanisms by which gal-1-DC inhibit the elicitation phase of CH. Nontreated ears showed normal histology (data not shown).
cross-link its ligands, gal-1 must form a homodimer bearing two β-galactoside binding sites. Because the dissociation constant of the gal-1 dimer is within the micromolar range ($K_d \sim 7 \mu M$) (47), the use of gal-1 in vivo requires the administration of very high doses of the soluble lectin (10–13, 23) with the potential complication of inducing agglutination of circulating erythrocytes and leukocytes due to the cross-linking ability of gal-1. Different methods have been used to achieve high levels of functional gal-1 dimers, including 1) fibroblasts engineered to synthesize gal-1 (10) and 2) a covalently linked gal-1 homodimer, 10-fold more effective at inducing T cell apoptosis than wild-type gal-1 (48). In this study, we circumvented this limitation by delivering high concentrations of gal-1 at the site where Ag is presented to T cells, using genetically engineered DC. To achieve high levels of transgene expression regardless of the DC maturation state, DC were transduced with Rad (43, 49).

The immunoregulatory effects of gal-1 require physical contact between dimeric gal-1 and the T cell surface (9), thus in our system, functional tg gal-1 also has to be secreted by DC as a dimer. However, the mechanism of externalization of gal-1 is not entirely understood. It is known that gal-1 mRNA is translated by free ribosomes and that the protein does not traffic via the classic pathway through the endoplasmic reticulum and Golgi (50). This alternative pathway prevents gal-1 from prematurely associating with glycan ligands secreted via the classical pathway and protects it from the oxidizing conditions of the endoplasmic reticulum/Golgi that affect its carbohydrate-binding capacity (20). In myoblasts, gal-1 is released in vesicles shed by “reverse budding” from the cell membrane by a mechanism known as ectocytosis (51). Our results are the first demonstration that tg gal-1 is secreted within the lumen of exosomes by DC. This observation confirms one of the models of secretion of gal-1 proposed by Hughes (50) and agrees with the fact that another member of this lectin family, gal-3, has been detected in exosomes produced by DC (39). Because exosomes contain cytosol, gal-1 is transported in a milieu of reducing conditions that preserves the integrity of its binding activity (20).

Once externalized, tg Gal-1 and its ligand CD43 cosegregate in microdomains on the DC surface. CD43 is a glycoprotein, highly expressed by DC, that possesses numerous lactosamine-bearing side chains that are recognized by gal-1. Cross-linking of multivalent CD43 with bivalent gal-1 generates the molecular lattice required to segregate CD43 in microdomains on the DC surface (52, 53). This latter observation, plus the fact that gal-1-DC induces rapid apoptosis of activated T cells, indicates that gal-1-DC concentrate dimeric gal-1, the functional form of the lectin, on their surface. Previous results are the first demonstration that tg gal-1 is secreted within exosomes produced by DC (39). Because exosomes contain cytosol, gal-1 is transported in a milieu of reducing conditions that preserves the integrity of its binding activity (20).

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In agreement with our observations, suspensions of rabbit lymph node leukocytes (containing naive lymphocytes and APC) proliferate when incubated with soluble gal-1 (56), and

**FIGURE 7.** Gal-1-DC trigger apoptosis of activated, but not naive, CD4$^+$ and CD8$^+$ T cells. A. Purified naive or PHA/IL-2-activated T cells from B10 (allogeneic) and BALB/c (syngeneic) mice were prelabeled with the mitochondrial probe JC-9 and incubated alone (control) or with BALB/c DC transduced with RAd-gal-1-eGFP or RAd-eGFP. One hour later, the percentage of apoptotic T cells was assessed by flow cytometry, according to the decrease in mitochondrial membrane potential, an early indicator of apoptosis. B. Gal-1-DC induced apoptosis of both PHA/IL-2-activated CD4$^+$ and CD8$^+$ T cells. Results are representative of three independent experiments.
gal-1 supports survival of lymph node T cells (57). By contrast, Rabinovich et al. (58) have shown that soluble gal-1 decreases in vitro proliferation of hu alloreactive T cells. Whether this discrepancy is related to species differences, the presence of activated T cells in hu MLR, or distinct mechanisms of delivery of gal-1 (soluble vs tg gal-1 released by DC) remains unclear. Our results indicate that the increased proliferation of Ag-specific naive T cells induced by tg gal-1 in mice is caused mainly by the maturation/activation of DC induced by the lectin and not by a direct effect of gal-1 on naive T cells. In this regard, control eGFP-DC pre-exposed to soluble gal-1 enhanced their T cell stimulatory potential (Fig. 5D) as occurs with DC expressing tg gal-1. In the absence of DC, naive T cells did not proliferate significantly in the presence of soluble gal-1 (Fig. 5D).

DC expressing tg gal-1 induced rapid apoptosis of activated CD4+ and CD8+ T cells, as has been reported for soluble gal-1 or gal-1 expressed by endothelial (9) and thymic epithelial and stromal cells (21, 22). Gal-1 induces apoptosis of thymocytes and activated T cells through cross-linking of glycosylated ligands on the cell surface (9, 22). By contrast, naive and memory T cells are not targets of gal-1-induced apoptosis. This differential susceptibility to gal-1 is regulated by the presentation of specific saccharide ligands created by glycosyltransferases expressed differentially by distinct subsets of thymocytes and T cells (59, 60). In our system, the tg gal-1 retained on the surface of DC was responsible for induction of apoptosis of activated T cells because the cell death was blocked by the gal-1 competitor TDG and an anti-hu gal-1 serum. Although DC did not release detectable levels of soluble tg gal-1 in the medium, they transferred the lectin to other DC, as a possible mechanism of amplification of the effects of gal-1 on target cells.

Our in vivo results are also consistent with the finding that gal-1-DC induced activation of naive T cells while killing activated T cells efficiently. Indeed, when gal-1-DC were used to sensitize mice to DNFB we observed a significantly enhanced CH response. This indicates that the presence of tg gal-1 induces a stronger cellular immune response than control DC, a fact that correlates with the higher T cell stimulatory function of gal-1-DC we found in vitro. Conversely, when gal-1-DC were used in the elicitation phase of CH, they induced apoptosis of effector T cells recruited to the skin, which resulted in abrogation of CH. The anti-inflammatory effect of gal-1 in the periphery (61) may have also contributed to the reduced inflammatory infiltrate in the dermis of TNBS-sensitized mice that were challenged with gal-1-DC. The fact that DC were not susceptible to tg gal-1-induced apoptosis, indicates the absence of the saccharide ligands or polypeptide backbones required for gal-1-mediated cell death. In this regard, DC do not express CD7, the surface glycoprotein responsible for delivering the gal-1 death signal to T cells (62).

Current efforts to manipulate the immune response are focused on modification of DC functions either to stimulate a strong T cell immunity for vaccine purposes, or to generate regulatory DC to

**FIGURE 8.** Effect of DC expressing tg gal-1 on CH. A, DC expressing tg gal-1 (gal-1-DC) enhanced sensitization of CH. Mice were sensitized by injection (footpad) of DC (noninfected or transduced with RAd-gal-1-eGFP or RAd-eGFP) and haptenized with TNBS. One group was injected with gal-1-DC not exposed to the hapten (control, □). Six days later, the right ear was challenged with topical DNFB. The thickness of swelling was measured on the right ear (challenged) and left (control) at different time points. B, Injection (base of the ear) of gal-1-DC haptenized with TNBS inhibited the elicitation phase of CH in mice previously sensitized (6 days earlier) by epicutaneous application of DNFB. One group was challenged with gal-1-DC not exposed to the hapten (control, □). The inhibitory effect of gal-1-DC was maximal between 48 and 72 h after challenge. In A and B, data are representative of two independent experiments, with three animals per group.
induce/maintain tolerance or to alleviate autoimmune disorders. Most current approaches are aimed at favor/interfere with the ability of DC to activate naive T cells (5, 8). However, once T cells become activated, most conventional DC-based therapies may be unable to inhibit the function of activated/effector T cells efficiently. As occurs in type-1 diabetes and autoimmune arthritis, the signs/symptoms that lead to diagnosis are detected after the onset of disease, when autoreactive T cells are fully activated.

In summary, our data contribute to elucidate the function of DC expressing tg gal-1. Our results demonstrate that expression of tg gal-1 by DC induces activation of DC, proliferation of naive T cells, and increased cellular immunity that may be relevant for vaccine development. In contrast, the ability of gal-1 DC to kill activated T cells can be used to eliminate auto- or alloreactive T cells in peripheral tissues. This may prove beneficial for the treatment of ongoing autoimmune disorders, graft-vs-host disease, and

**FIGURE 9.** Gal-1-DC trigger apoptosis of infiltrating T cells during elicitation of CH. Mice were sensitized with DNFB applied topically on the abdominal skin. Six days later, the animals were challenged by injection (intradermally) of 3 × 10⁶ DC transduced with RAd-eGFP (eGFP-DC; A, C, and E) or DC infected with RAd-gal-1-eGFP (gal-1-DC; B, D, F, and G) and haptenized with TNBS. Mice challenged with TNBS-gal-1-DC exhibited fewer infiltrating leukocytes (B, arrows), a reduced number of CD3⁺ T cells (D), and a higher percentage of apoptotic (TUNEL⁺) cells (F) expressing the T cell marker CD3 (G) compared with control mice challenged with TNBS-eGFP-DC (A, C, and E). Arrow in F indicates an eGFP⁺ cell with DC morphology surrounded by TUNEL⁺ cells (detailed in the inset). In G, CD3-Cy5 (far-red) was converted into red for better visualization. Dotted lines delineate the approximate trajectory of the needle. (A and B, H&E; C–G, immunofluorescence ×200; inset, ×400.) Nuclei were stained with DAPI. Data are representative of three independent experiments.
transplant rejection mediated by activated/memory T cells. Therefore, administration of DC expressing tg gal-1 represents a promising tool for manipulation of both afferent and efferent limbs of the immune response.

Disclosures
The authors have no financial conflict of interest.

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