Tumor-Associated Galectin-3 Modulates the Function of Tumor-Reactive T Cells

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Abstract

T cells play an important role in cancer immunosurveillance and tumor destruction. However, tumor cells alter immune responses by modulating immune cells through antigen stimulation and immunoregulatory cytokines. A better understanding of the interplay between tumor cells and T cells might provide new strategies to enhance antitumor immunity. Through an antigen-screening approach using colorectal tumor–reactive T cells, we identified an HLA-DR11–restricted T-cell epitope encoded by *KIAA0040* as well as MHC-unrestricted human galectin-3 (Gal-3) expressed by tumor cells. Although the biological function of KIAA0040 remains to be determined, we found that Gal-3 functioned as an immune regulator for direct T-cell activation and function. T-cell activation induced by Gal-3 resulted in T-cell apoptosis. We showed that a high level of expression of Gal-3 promoted tumor growth *in vitro* and *in vivo*. Using a mouse tumor model, we showed that delivery of high doses of Gal-3 inhibited tumor-reactive T cells and promoted tumor growth in mice receiving tumor-reactive $CD8^+$ T cells. These findings suggest that Gal-3 may function as an immune regulator to inhibit T-cell immune responses and promote tumor growth, thus providing a new mechanism for tumor immune tolerance. [Cancer Res 2008;68(17):7228–36]

Introduction

Increasing evidence from both preclinical tumor models and human clinical trials indicates the importance of T cells in the control and destruction of tumor cells (1). The identification of many tumor antigens recognized by T cells has set the stage for the development of effective cancer vaccines (2). Many clinical trials with molecularly defined cancer antigens or in combination with dendritic cells (DC) show that antigen-specific immune responses can be readily induced, but the clinical response rate remains relatively low (3). Hence, these studies suggest that immune suppression in the tumor microenvironment is a major obstacle for the development of effective cancer immunotherapy.

Recent studies show that regulatory T (Treg) cells play a detrimental role in cancer immunotherapy because these cells accumulate in the tumor microenvironment and suppress immune responses (4, 5). Moreover, we recently showed the presence of tumor-specific $CD4^+$, $CD8^+$, and γδ Treg cells in several types of tumors, suggesting that they can induce antigen-specific, local immune tolerance at tumor sites (6–8). Tumor-associated macrophages and myeloid-derived suppressor cells (MSC) could also play an important role in inhibiting immune responses and chronic inflammation (9), which has been linked to cancer development and progression (10). Both tumor-associated macrophages/DCs and MSCs promote tumor growth either by secreting immunosuppressive cytokines, including interleukin (IL)-10, transforming growth factor-β (TGF-β), and IL-1β, or by inducing Treg cell differentiation (11). More importantly, tumor cells have been shown to express inhibitory factors (IL-10, TGF-β, and IDO) to alter T-cell function (4, 12). Immunosuppressive factors, such as FasL and TGF-β expressed by tumor cells, may directly inhibit tumor-reactive T-cell expansion or induce T-cell apoptosis (13). A recent study suggests that tumor-associated galectin-1 (Gal-1), a member of the animal lectin family, contributes to tumor immune escapes by inhibiting the function of tumor-reactive T cells (14). Therefore, tumor cells constantly modulate T-cell responses by presenting tumor antigens and secreting immunoregulatory cytokines. Understanding the interplay between tumor cells and immune cells in the tumor microenvironment is essential for the development of effective cancer immunotherapy.

In this study, we describe the identification of HLA-DR11–restricted T-cell epitope encoded by the *KIAA0040* gene, which can stimulate the cytokine production of a colorectal tumor–reactive T-cell line. Interestingly, we also identified human galectin-3 (Gal-3) expressed by tumor cells as an immune regulator of T cells using the same screening system. Although Gal-3 can activate antigen-experienced T cells, it induces T-cell apoptosis at a relatively high concentration. Using a mouse tumor model, we showed that a high dose of Gal-3 treatment abrogates the efficacy of tumor-reactive T cells and promotes tumor growth. Because Gal-3 is highly expressed in various types of tumor cells (15, 16), our study may provide a new mechanism by which tumor cells escape the attack of tumor-reactive T cells. These new findings provide a better understanding of interplay between immune cells and cancer cells and identify a new mechanism by which tumor cells induce immune tolerance.

Materials and Methods

Cell lines and antibodies. The tumor-reactive T-cell lines used in this study were previously generated in our laboratory as described (6). The CT28 T-cell line was established from a colon cancer patient; TIL586, TIL108, TIL162, TIL1333, and TIL102 T-cell lines came from five melanoma patients; TILBT16 and TILBT29 came from two breast cancer patients; and TIL194 came from a prostate cancer patient. The melanoma cell line 586mel and the colon carcinoma cell line CC28 were established from fresh tumor samples. 293IMDR11 cells were established by transfecting plasmid DNA encoding DRB1*1101 cDNA into 293 ECII cells as described (17). The expression of DR11 in 293 ECII cells was verified by fluorescence-activated cell sorting.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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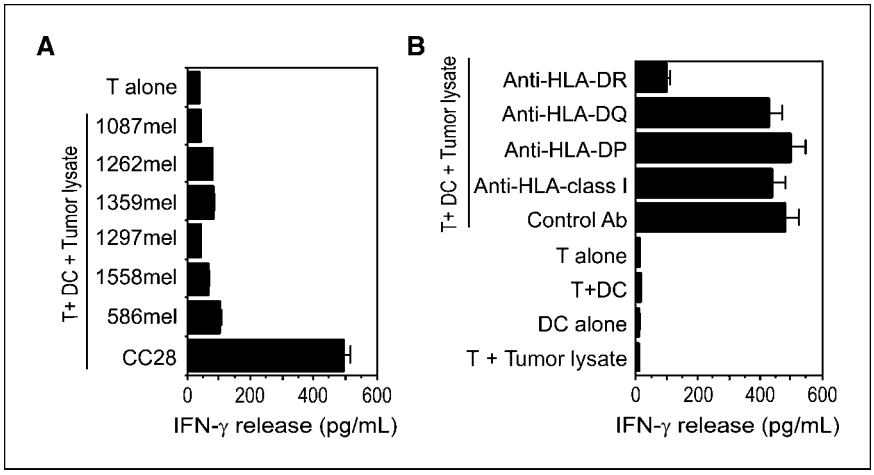

Figure 1. The tumor reactivity of colorectal tumor–reactive CD4$^+$ CT28 T cells. *A,* recognition of autologous tumor cells by CT28 T cells. CT28 T cells were tested for their ability to recognize cell lysates from various target cells. CT28 T cells specifically responded to autologous DC pulsed with autologous tumor cells (CC28) but did not recognize autologous DC pulsed with other cell lines tested. *B,* HLA restriction of CT28 T-cell recognition. Autologous DCs pulsed with CC28 tumor lysates were cocultured with CT28 T cells in the presence or absence of various anti-HLA antibodies.

To determine whether T-cell recognition could be blocked by specific antibodies, we measured T-cell activity in the presence or absence of various antibodies, as previously described (18).

Tumor cDNA library screening. The generation and screening of the cDNA library of CC28 tumor cells was similar to that described previously (19).

Peptide synthesis and T-cell epitopes. The candidates of antigenic peptides for CT28 T-cell epitopes were predicted by the SYFPEITHI T-cell epitope prediction tool.[4] The peptides were synthesized by a solid-phase method using a peptide synthesizer (model Apex396; AApptec, Inc.). The synthesized peptides were dissolved in DMSO in 10 mg/mL. HLA-DR11 expressing 1558 EBV-transformed B cells (1558 LCLs) were pulsed with these peptides at the concentration of 5 μg/mL for 3 h in RPMI 1640. The pulsed LCLs were cocultured with CT28 T cells overnight. To determine the reactivity of CT28 to these peptides, IFN-γ concentrations in the supernatants were measured by ELISA.

Cell proliferation assay. To evaluate T-cell proliferation, Gal-3 or a control protein was diluted to the indicated concentrations with PBS and coated at 100 μL/well to flat-bottomed 96-well plates at 4°C overnight. The plates were washed twice with PBS to remove unbound proteins. Cell proliferation assays were performed as described (6). All experiments were performed in triplicate.

Detection of Gal-3 binding on tumor-reactive T cells. According to the manufacturer's instructions, Gal-3 and a control protein, prostate epithelium–derived ets transcription factor (PDEF), were labeled with FITC (Invitrogen, Inc.). Cells (5×10^5) were stained for 30 min by FITC-labeled Gal-3 or labeled control protein (5 μg/mL) with or without 50 mmol/L lactose on ice and analyzed by flow cytometry (BD Biosciences). For immunofluorescence microscopy analysis, 1×10^6 cells were fixed with paraformaldehyde (2%) for 30 min and stained with FITC-labeled Gal-3 and PE-labeled cholera toxin subunit B (Invitrogen) for 30 min. The cells were washed, dropped on glass microscope slides, and observed under a fluorescence microscope.

Cell apoptosis analysis. Tumor-reactive T cells were stimulated with soluble Gal-3 or a control protein for the indicated time. Treated cells were washed twice using PBS and stained with propidium iodide (PI) and FITC-Annexin V (BD Biosciences) according to the manufacturer's instruction. Stained cells were analyzed on a FACSCalibur (BD Biosciences).

Measure of Gal-3 in cell culture supernatants. Cells (10×10^6) were cultured in 15 mL RPMI 1640 with 10% fetal bovine serum for 24 h. The concentrations of Gal-3 in the supernatants were determined by ELISA using standard methods. The paired antibodies for Gal-3 ELISA assay are anti-human Gal-3 (AF1154) and biotin-conjugated anti-human Gal-3 (BAF1154) from R&D Systems. Each reported value is the mean of triplicate assays.

Establishment of a tumor cell line expressing short hairpin RNA to Gal-3. The expression of Gal-3 in tumor cells was inhibited by green fluorescent protein (GFP)-expressing lentivirus-based short hairpin RNAs (shRNA) for human Gal-3 (20). Two Gal-3–specific shRNAs targeting 5′-GAGAGTCATTGTTTGCAATA and 5′-GCTCACTTGTTGCAGTACA, respectively, were constructed and tested for their knockdown efficiency. Irrelevant gene ORF3 shRNA served as a control with the target sequence of 5′-GCCCTTCATTGTAGATCTGA. The generation of tumor cell lines expressing Gal-3 shRNA or control shRNA was performed as described previously (21). Infected tumor cells were collected on day 10 and the GFP$^+$ expressing cells were sorted with FACSAria (BD Biosciences).

***In vivo* animal experiments.** For s.c. tumor challenge, 6- to 8-wk-old Rag2γC-deficient mice (Taconic, Inc.) were injected with 5×10^6 586mel tumor cells per mouse with or without Gal-3 expression. The growth of tumor cells was monitored every 3 d. For adoptive T-cell transfer, Rag2γC-deficient mice were injected with 5×10^6 cells per mouse (control shRNA expressing 586mel tumor cells or Gal-3 shRNA expressing 586mel tumor cells) and the tumors were allowed to grow for 2 d. The mice were given an i.v. injection of 20×10^6 TIL586 T cells per mouse. Treatment with Gal-3 (0.5 mg/mL) was delivered into the mice through i.v. injection.

Statistical analysis. Unless indicated otherwise, data are expressed as mean ± SD. The significance of difference between groups was determined by Student's *t* test or the two-way ANOVA.

Results

Establishment of tumor-reactive CD4$^+$ T cells and tumor library screening. Although many tumor antigens have been identified from melanoma, very few colorectal cancer antigens are identified thus far. We have recently established a colorectal cancer–reactive CD4$^+$ T-cell line, CT28, from a fresh colon carcinoma sample. This T-cell line recognized autologous DC pulsed with tumor cell lysate but not DC pulsed with lysates from other melanoma lines (Fig. 1*A*). Recognition of CC28 by CT28 could be specifically blocked by a monoclonal antibody against HLA-DR but not by anti-HLA-DP, anti-HLA-DQ, or anti-MHC class I molecules (Fig. 1*B*). HLA typing analysis indicated that the patient was homozygous for HLA-DR11, suggesting that CT28 T cells recognize a tumor antigen in the context of the HLA-DR11 molecule. Therefore, HLA-DR11 was selected as the restriction element for the initial cDNA library screening.

To isolate proteins that stimulate tumor-reactive CT28 T cells, we used a genetic targeting expression system developed in our laboratory that has been successfully used to identify

[4] http://www.syfpeithi.de/scripts/MHCServer.dll/home.htm

several MHC class II–restricted tumor antigens (6, 22, 23). After screening a total of 2×10^5 cDNA clones, we identified one positive cDNA pool that was recognized by CT28 cells when transfected into 293IMDR11 cells (Supplementary Fig. S1*A*). From this positive pool, two positive clones, clone 2C6 containing 700 bp insert and clone 1E5 including a 1 kb cDNA, were identified (Supplementary Fig. S1*B*). These two clones were used as representative clones for further experiments.

Two positive clones are capable of activating CD4$^+$ T cells in different manners. DNA sequence analysis revealed that these two clones contain two different genes. The cDNA insert of clone 2C6 shares 100% similarity with the human KIAA0040 clone (BC020789.1) in the gene database, which is a novel gene that has been mapped to chromosome 1 (Fig. 2*A*). The function of KIAA0040 is not known. T-cell reactivity of KIAA0040 by CT28 could be specifically blocked by a monoclonal antibody against HLA-DR but not by anti-HLA-DP, anti-HLA-DQ, or anti-MHC class I molecules (Fig. 2*B*). This result suggests that this novel gene encodes an HLA-DR11–restricted tumor antigen recognized by colon cancer–reactive T cells. To identify the DNA fragment of *KIAA0040* that encodes the T-cell epitope for CT28 T cells, we made several deletions of *KIAA0040* and tested their ability to stimulate T cells. We found that the first 158 bp fragment can stimulate CT28 to produce the same amount of cytokine as the full *KIAA0040* gene, whereas further deletions of *KIAA0040* either partially or fully lost their capacity to stimulate CT28 T cells (Fig. 2*C*). To identify the epitope from KIAA0040 recognized by CT28 T cells, eight overlapping peptides were synthesized based on the predicted amino acid sequence and were tested for T-cell recognition by pulsing these peptides onto HLA-DR11–expressing LCLs. CT28 T cells were found to respond to stimulation from the two overlapping peptides, indicating that the T-cell epitope of KIAA0040 for the recognition of CT28 T cells is AGSPSADR-KIVPHFGD (Fig. 2*D*).

In contrast, clone 1E5 encodes the full-length protein of human Gal-3, which belongs to a growing family of animal lectins defined by their high affinity for β-galactoside and the presence of at least one conserved carbohydrate recognition domain (CRD; ref. 15). No mutation was detected in clone 1E5 when compared with the Gal-3 sequence from the databases (data not shown). To our surprise, CT28 T cells could be activated only by the full-length form of Gal-3 but not by any of the truncated forms, suggesting that the full-length protein of Gal-3 is required for the activation of CT28 (Fig. 3*A*). Moreover, all anti-MHC molecule antibodies failed

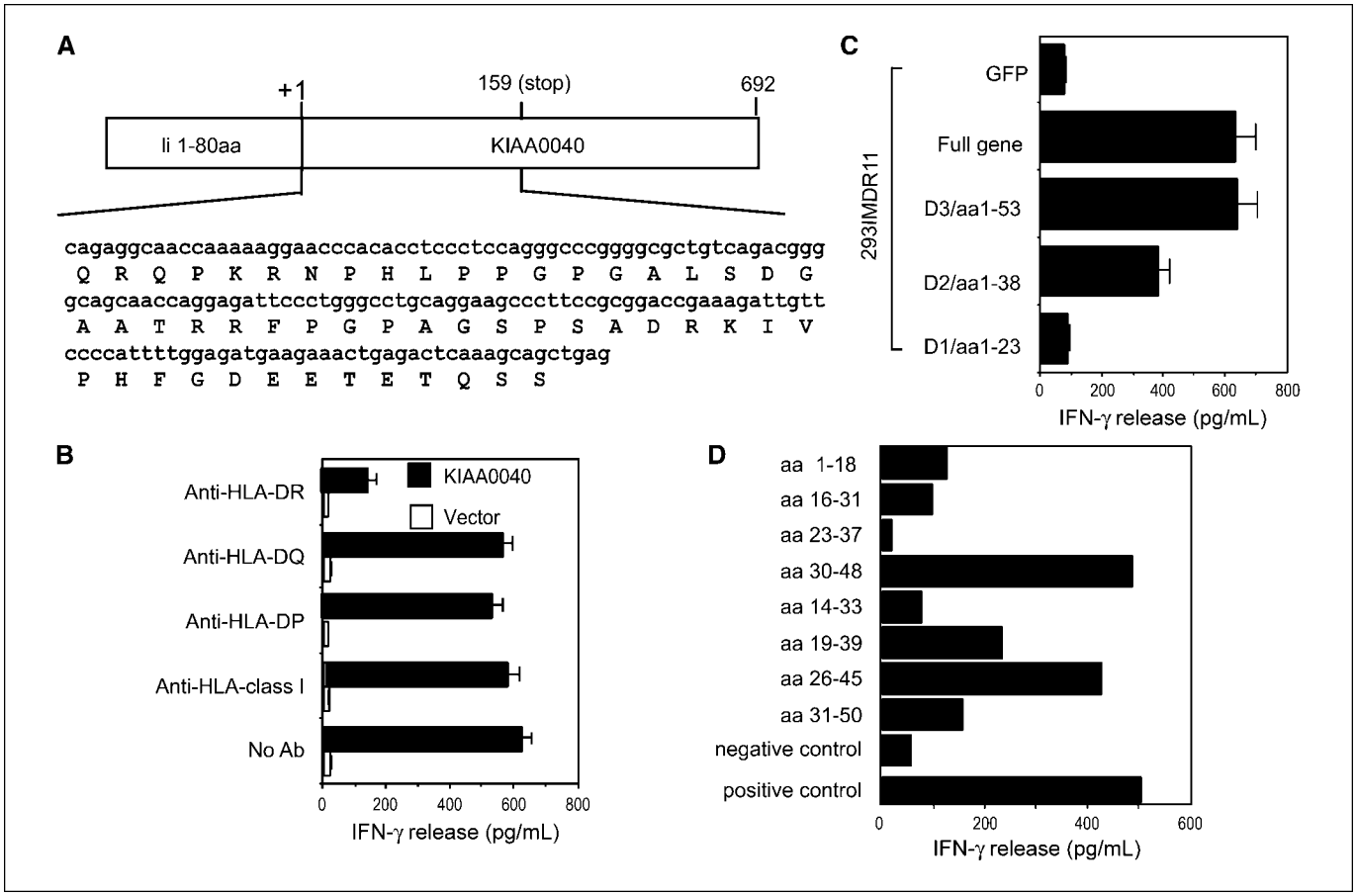

Figure 2. Identification of T-cell epitopes of KIAA0040 recognized by T cells in the context of HLA-DR11 molecules. *A,* schematic presentation of the DNA and the predicted amino acid sequence of human *KIAA0040* cDNA. *B,* determination of the requirement of the HLA restriction element for recognition of *KIAA0040* by CT28 T cells. 293IMDR11 cells were transfected with *KIAA0040* or vector only and then cocultured with T cells in the presence or absence of various anti-HLA antibodies. *C,* identification of the DNA fragment of *KIAA0040* with T-cell reactivity. A series of 3′ end deletions of *KIAA0040* were generated by PCR and cloned into the pTSX vector. Truncated fragments were transfected into 293IMDR11 cells and tested for their ability to stimulate CT28 T cells. *D,* specific recognition of two peptides by CD4$^+$ CT28 T cells. Eight overlapping synthetic peptides were pulsed onto HLA-DR11–expressing 1558 EBV-transformed B cells (1558 LCLs) for 3 h. After three washes, T cells were added and incubated for 18 h. T-cell reactivity was determined by ELISA. 293IMDR11 cells transfected with *KIAA0040* served as a positive control. Similar results in *B–D* were obtained in repeated experiments.

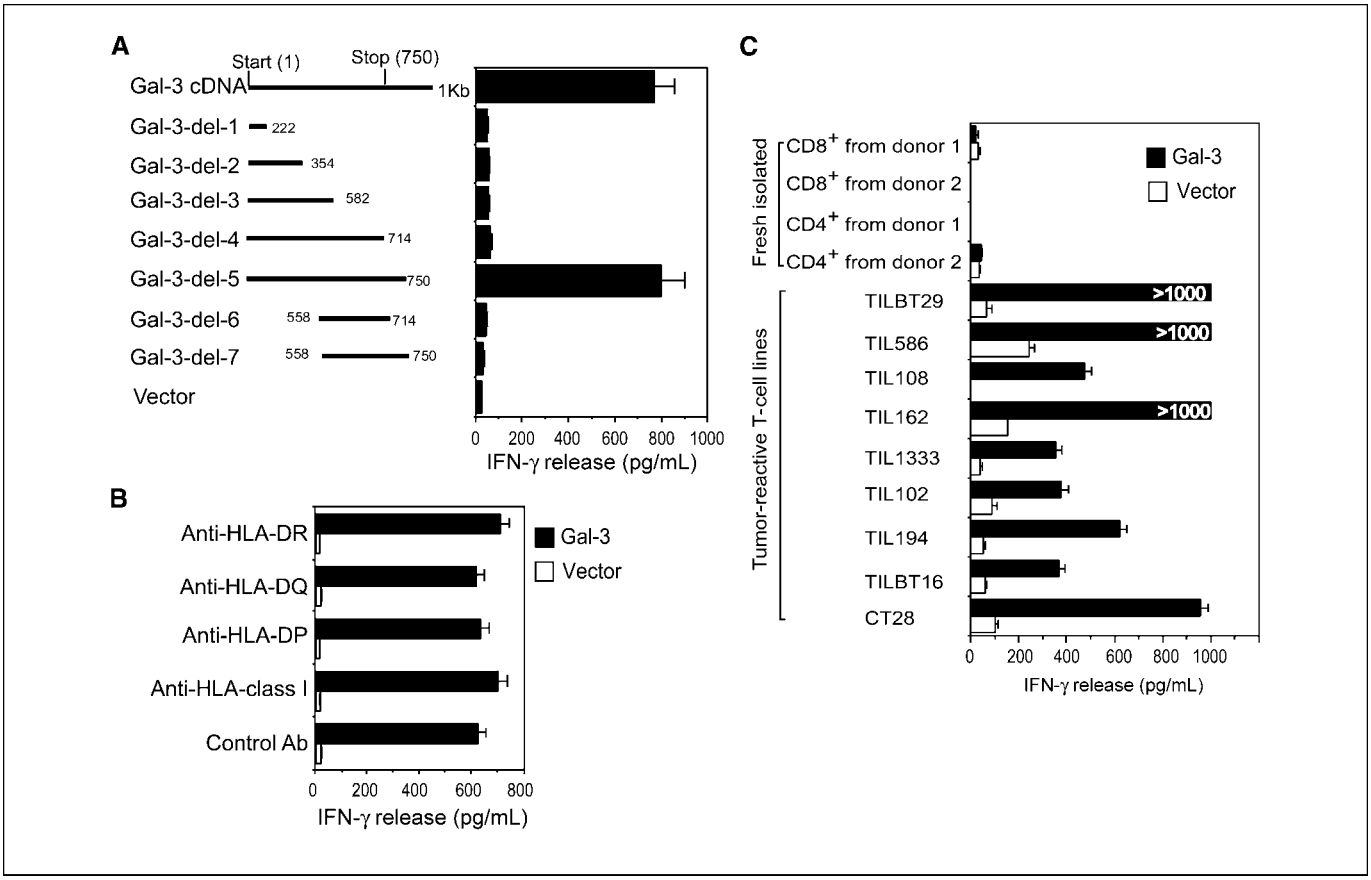

Figure 3. Activation of CT28 CD4$^+$ T cells and other antigen-experienced T cells by Gal-3 without the involvement of MHC class II molecules. *A,* requirement of full-length Gal-3 for CT28 T-cell reactivity. Truncated Gal-3 fragments were transfected into 293IMDR11 cells and tested for their ability to stimulate CT28 T cells based on IFN-γ release. *B,* failure of anti-HLA antibodies to inhibit the activity of CT28 T cells by Gal-3 presented by 293IMDR11 cells. *C,* the response of naive T cells and tumor-reactive T-cell lines to human Gal-3. 293 cells expressing Gal-3 were cocultured with various T cells, and IFN-γ release in the supernatant was measured by ELISA. TILBT29 and TILBT16 cell lines were generated from two breast cancer patients; TIL194 T-cell line was from a prostate cancer patient. TIL586, TIL108, TIL162, TIL1333, and TIL102 cell lines were obtained from different melanoma patients. Naive CD4$^+$ and CD8$^+$ T cells were purified from the PBMC of healthy donors. Similar results in (*A–C*) were obtained in three repeat experiments.

to block the reactivity of CT28 T cells to Gal-3 (Fig. 3*B*). Unlike the T-cell recognition of KIAA0040, CT28 T cells can produce IFN-γ when exposed to Gal-3 presented by 293 cells with or without matched MHC class II molecule expression (Supplementary Fig. S1*C*). These results indicate that Gal-3 can directly activate CT28 T cells without the requirement of MHC class II molecules.

We next tested whether Gal-3 could activate other tumor-reactive or antigen-experienced T cells. Five melanoma-reactive T-cell lines, one prostate cancer–derived T-cell line, and two breast cancer–derived T-cell lines were selected and cocultured with 293 cells expressing Gal-3 for 12 to 16 h. We found that Gal-3–expressing 293 cells activated all of these T-cell lines to secrete IFN-γ but failed to activate naive CD4$^+$ and CD8$^+$ T cells purified from peripheral blood mononuclear cells (PBMC) of healthy donors (Fig. 3*C*), suggesting that naive T-cell activation requires a strong T-cell receptor (TCR)-mediated activation, whereas tumor-reactive T cells can be readily activated by Gal-3. We also evaluated the cytokine profiles of CT28 T cells on Gal-3 stimulation. Gal-3 induced a high level of IFN-γ, granulocyte macrophage colony-stimulating factor, and low to middle levels of IL-4, which is similar to cytokine production induced by anti-CD3 (OKT3) stimulation (Supplementary Fig. S2). Although the cytokine profiles were different among the tumor-reactive T-cell lines tested, there was no difference between Gal-3 and anti-CD3 stimulation for each tumor-reactive T-cell line (data not shown).

Cytokine production and proliferation of tumor-reactive T cells by recombinant Gal-3. To further assess the stimulatory effect of soluble Gal-3 on T cells, we expressed and purified the full-length Gal-3 using a His-tag purification system. The purity of the recombinant Gal-3 was analyzed by SDS-PAGE and was >95% (data not shown). The identity of recombinant human Gal-3 was also confirmed by Western blotting analysis using anti-Gal-3 and anti-His antibodies, showing a band with 30 kDa molecular weight (Supplementary Fig. S3*A* and *B*). An irrelevant protein, PDEF, was also purified by the same way as a control protein for further experiments. We observed that tumor-reactive T cells developed homotypic aggregation as a sign of activation 8 h after culturing with 50 μg/mL of soluble Gal-3 (Supplementary Fig. S3*C*). However, tumor-reactive T cells treated with the control protein did not show such an aggregation, suggesting that the soluble Gal-3 protein can stimulate tumor-reactive T cells. Titration experiments revealed that Gal-3–induced cytokine production from tumor-reactive T cells was in a dose-dependent manner and that 25 μg/mL Gal-3 was required to stimulate detectable T-cell response (Fig. 4*A*). We next tested whether Gal-3 can enhance the proliferation of tumor-reactive T cells. Although tumor-reactive

T cells did not display appreciable proliferation at a 25 μg/mL concentration of soluble Gal-3 (data not shown), a Gal-3–coated plate of the same concentration induced potent proliferation of tumor-reactive T cells (Fig. 4*A*), suggesting that Gal-3 immobilization is necessary to induce the proliferation of tumor-reactive T cells.

Given that Gal-3–mediated T-cell activation does not require MHC class II molecules, we postulated that Gal-3 might directly interact with the immunologic synapse for T-cell activation. To test this possibility, we analyzed the colocalization of Gal-3 with lipid rafts, which locate in the center of the immunologic synapse, by fluorescence microscopy. Tumor-reactive CT28 T cells were stained with the nuclear staining dye 4′,6-diamidino-2-phenylindole (DAPI; violet), FITC-labeled Gal-3 (green), and PE-labeled cholera toxin (red), which recognizes gangliosides enriched in lipid rafts. We found that Gal-3 can bind to the lipid raft–accumulated regions on the surface of tumor-reactive T cells (Fig. 4*B*). These results indicate that Gal-3 has the ability to bind the immunologic synapse on tumor-reactive T cells, which might contribute to the activation of tumor-reactive T cells.

Lactose inhibits the activation of tumor-reactive T cells induced by Gal-3. Because Gal-3 is a carbohydrate-binding protein, it is important to determine whether its effect on tumor-reactive T cells is dependent on protein-protein interactions or protein-sugar interactions. Given the essential role of the CRD domain for the interaction of Gal-3 with sugars, we next investigated whether the effect of Gal-3 on tumor-effective T cells was dependent on the CRD domain. Lactose is a simple disaccharide with a high affinity to the CRD domain of Gal-3 and can specifically block the interaction of Gal-3 and glycosylated proteins. We found that Gal-3–induced cytokine production and proliferation of tumor-reactive T cells were inhibited by lactose in a dose-dependent manner (Fig. 4*C*). Lactose treatment alone had no effect on the cytokine production and proliferation of tumor-reactive T cells. The binding of Gal-3 on the surface of tumor-reactive T cells was also strongly inhibited by lactose at a 50 μmol/L concentration (Fig. 4*D*). Taken together, these results indicate that the activation of tumor-reactive T cells by Gal-3 requires the interaction between carbohydrate molecules in the immunologic synapse and the CRD domain of Gal-3.

Induction of tumor-reactive T-cell apoptosis by Gal-3. Several members of the galectin protein family have been shown to induce the apoptosis of T cells (24). We next tested whether Gal-3 has the ability to induce the apoptosis of tumor-reactive T cells and found that the proliferation of tumor-reactive T cells treated with both an anti-CD3 antibody and Gal-3 was lower than that of T cells treated with anti-CD3 alone (Fig. 5*A*), suggesting that Gal-3 can induce T-cell activation-induced apoptosis. To directly

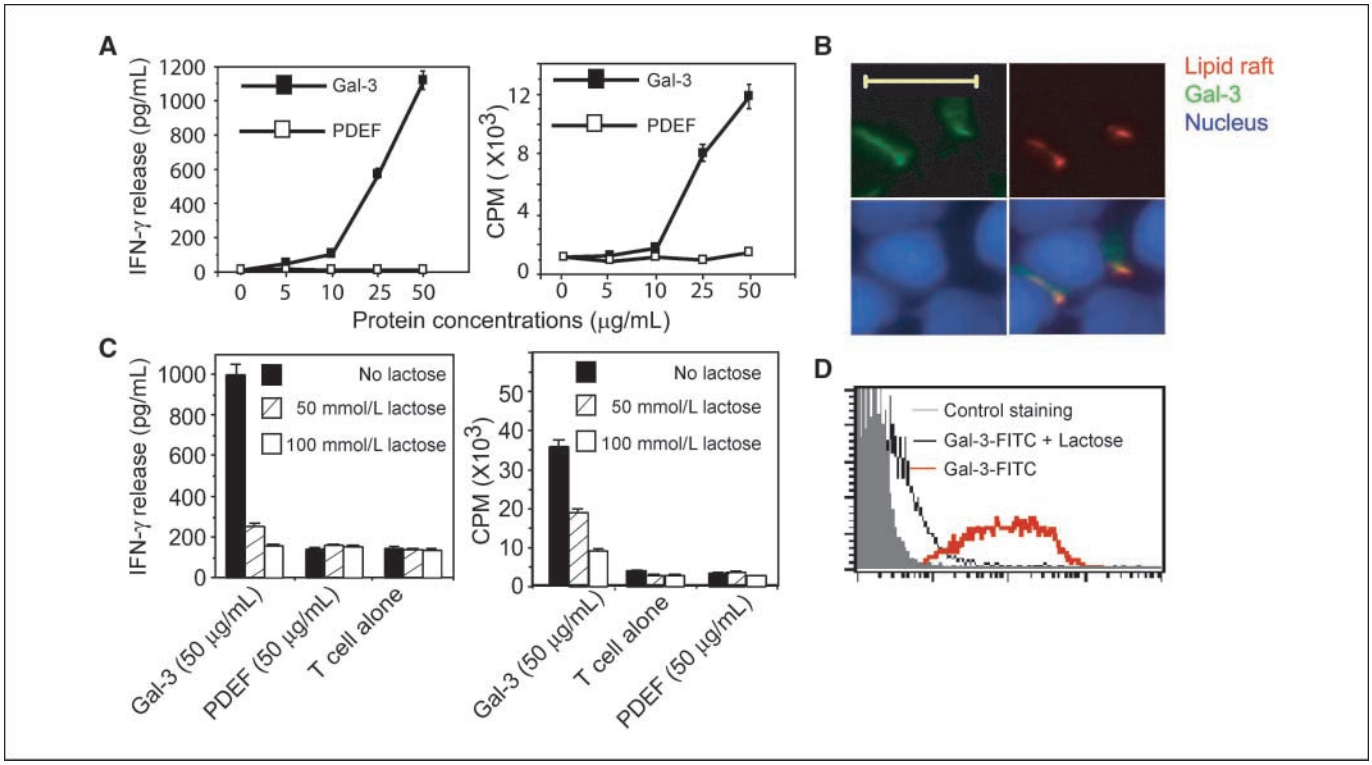

Figure 4. Gal-3 binds and activates tumor-reactive T cells through carbohydrate-specific interaction. *A,* dose-dependent responses of the cytokine production and proliferation of the tumor-reactive CT28 T cells to human Gal-3. For cytokine production (*left*), CT28 T cells were cultured in the presence of human Gal-3 or control protein at indicated concentrations for 18 h. The concentration of IFN-γ in the supernatant was measured by ELISA. For proliferation assay (*right*), 96-well plates were coated with Gal-3 or a control protein for 18 h and followed by three washes with PBS. CT28 cells were cultured in the presence of coated Gal-3 and the proliferation was measured by adding [^{3}H]thymidine during the last 12 to 16 h of culture. *B,* the interaction of Gal-3 and lipid rafts on tumor-reactive T cells. CT28 T cells were fixed and stained with nuclear dye, DAPI (*violet*), FITC-labeled Gal-3 (*green*), and PE-labeled cholera toxin subunit B (*red*), which recognizes ganglioside enriched in lipid rafts. The cells were subjected to fluorescence microscopic analysis. The colocalization of Gal-3 and lipid rafts was shown in the overlay image at bottom right. *Scale bar,* 10 μm. *C,* lactose inhibits Gal-3–induced cytokine production and proliferation of tumor-reactive T cells. For cytokine production (*left*), CT28 cells were cultured with 50 μg/mL recombinant Gal-3 in the presence or absence of lactose for 18 h at 37°C. The IFN-γ production of CT28 cells was measured by ELISA. For proliferation assay (*right*), CT28 T cells were cultured in the Gal-3–coated plates with or without lactose for 72 h and used to measure the uptaking of [^{3}H]thymidine. Each experiment was performed in triplicate. *D,* the binding of Gal-3 to CT28 cells is completely blocked by lactose. CT28 cells stained by FITC-conjugated Gal-3 were analyzed for fluorescence intensity using flow cytometry. The data are representative of three individual experiments.

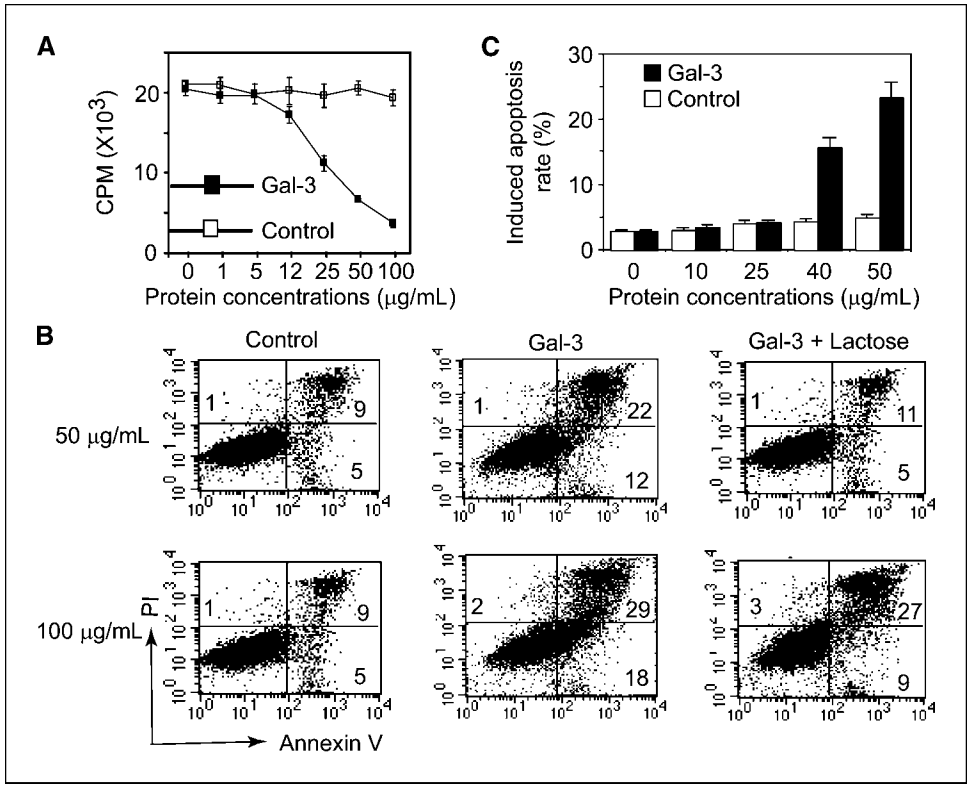

Figure 5. Gal-3 induces the apoptosis of tumor-reactive T cells. *A,* proliferation of tumor-reactive T cells after stimulation with anti-CD3 and Gal-3. Ninety-six–well plates were coated with CD3 (3 μg/mL) at 4°C overnight. CT28 cells were cultured in anti–CD3-coated plates with soluble Gal-3 or control protein for 72 h and used to measure the uptaking of [^{3}H]thymidine. *B,* CT28 T cells were treated with Gal-3 or a control protein with or without lactose for 24 h. Apoptosis was determined by Annexin V-FITC and PI staining. The numbers in the panels indicate percentages of cells in each quadrant. Similar results were obtained in repeated experiments. *C,* Gal-3 induces T-cell apoptosis in a dose-dependent way. Tumor-reactive T cells were cultured in T-cell growth medium in the presence of Gal-3 or a control protein with indicated concentrations for 16 h. Apoptosis was determined by Annexin V-FITC and PI staining. Shown are mean percentages of Annexin V$^+$ cells, which include the cells in early-stage (Annexin V$^+$/PI$^-$ cells) and late-stage (Annexin V$^+$/PI$^+$ cells) apoptosis. Similar results were obtained in repeated experiments.

determine the ability of Gal-3 to induce tumor-reactive T-cell apoptosis, Gal-3 was added to CT28 cells for 24 h and the rates of Annexin V$^+$ cells, which include cells in early stage of apoptosis (Annexin V$^+$/PI$^-$) and late stage of apoptosis (Annexin V$^+$/PI$^+$), were determined. Increased cell apoptosis rate of CT28 cells was observed in the presence of Gal-3 but not with a control protein (Fig. 5*B*). Furthermore, lactose could effectively block T-cell apoptosis induced by Gal-3. We also observed that Gal-3–induced T-cell apoptosis increased with increasing concentrations of Gal-3 (Fig. 5*C*), whereas Gal-3–induced T-cell apoptosis was negligible when the concentration of soluble Gal-3 was <25 μg/mL. These results suggested that the induction of tumor-reactive T-cell apoptosis by Gal-3 is dose dependent.

The role of Gal-3 expression in tumor growth. The expression of Gal-3 seems to be significantly up-regulated in tumor cells and in the serum of cancer patients (16, 25, 26). Moreover, our results showed that Gal-3 expressed by tumor cells can be secreted into extracellular compartments (Supplementary Fig. S4*B*). As shown in Fig. 6*A*, both melanoma tumor cells and colorectal tumor cells released ~100 ng of Gal-3 per million cells in 24-h *in vitro* culture. It is conceivable that the expression of Gal-3 by tumor cells might contribute to tumor progression through regulating tumor cell growth and T-cell–mediated antitumor immunity. In our previous study, we found that 586mel tumor cells can progressively grow in immunodeficient mice but were inhibited when cotransferred with autologous tumor-reactive TIL586 cells (21). To test the role of Gal-3 in tumor development both *in vitro* culture and in this mouse tumor model, we constructed Gal-3–specific lentiviral-based shRNA to knock down endogenous Gal-3 expression in 586mel tumor cells. 586mel cells transduced with Gal-3 shRNAs effectively reduced the levels of Gal-3 expression compared with 586mel cells transduced with a control shRNA (Supplementary Fig. S4*A*). Gal-3 could be detected in the cell medium of 586mel cells transduced with control shRNA but not from Gal-3–silencing 586mel cells (Supplementary Fig. S4*B*). We next determined whether there is any difference in the growth rate *in vitro* between Gal-3–silencing 586mel and control shRNA–expressing 586mel cells. As shown in Fig. 6*B*, the growth rate of Gal-3–silencing 586mel tumor cells was slower than that of control shRNA–expressing 586mel cells under *in vitro* culture condition. 586mel cells expressing another independent Gal-3–specific shRNA also showed decreased growth rate compared with control tumor cells (Supplementary Fig. S4*C*). To determine their growth properties *in vivo*, we inoculated Rag2γC-deficient mice with control shRNA–expressing 586mel and Gal-3–silencing 586mel tumor cells and found that Gal-3–silencing 586mel cells grew much slower than the control shRNA–expressing tumor cells (Fig. 6*C*). These results show that Gal-3 promotes tumor growth, whereas the knockdown of Gal-3 reduces tumor growth both *in vitro* and *in vivo*.

We next tested whether Gal-3 secreted by tumor cells can modulate the function or apoptosis of tumor-reactive T cells, thus inhibiting or promoting tumor growth *in vivo*. We first injected Rag2γC-deficient mice with control shRNA–expressing 586mel tumor cells and then i.v. injected tumor-reactive CD8$^+$ TIL586 T cells 2 days later. The formation and size of tumors in these mice was monitored every 3 days. As shown in Fig. 6*D* (*left*), tumor growth was inhibited by TIL586 cells compared with tumor cells alone, but no significant difference in tumor growth was observed in a group of mice treated with TIL586 plus a control protein or in a group of mice treated with TIL586 plus a low dose (50 μg/mouse) of Gal-3. However, treatment with Gal-3 at a high concentration (100 μg/mouse) completely abrogated the ability of TIL586 to inhibit tumor growth. Similar results were obtained from Rag2γC-deficient mice challenged with 586mel tumor cells expressing

Gal-3 shRNA (Fig. 6*D, right*). Notably, tumor cells with a high concentration of soluble Gal-3 alone did not change tumor growth. Taken together, these results show that soluble Gal-3 can induce T-cell activation and apoptosis, thus inhibiting the ability of T cells to kill tumor cells at a relatively high concentration. Inhibition of Gal-3 expression may serve as a therapeutic target for cancer therapy.

Discussion

Using the tumor-reactive T-cell line (CT28), we identified two tumor-associated antigens by screening a colorectal cancer–derived Ii fusion cDNA library. One of the positive clones is the gene product encoded by a nonmutated gene, *KIAA0040*, mapping to chromosome 1. Recognition of the *KIAA0040* gene product by CT28 cells is restricted by HLA-DR11 molecules, suggesting that the *KIAA0040* gene product may represent one of the tumor antigens expressed on colorectal cancer cells and recognized by CT28. Like many tumor antigens, such as NY-ESO-1, the biological function of this protein remains to be determined. The second gene we identified is human *Gal-3*. However, Gal-3 does not serve as a true antigen for T-cell recognition because T-cell activation does not require the involvement of MHC class II molecules. Similar observations have been reported in the earlier studies showing that MUC1 is one of the first tumor antigens recognized by human tumor-reactive T cells but is independent of MHC molecules. The tandem repeat of the extracellular domain of tumor MUC1 is required for the MHC-unrestricted T-cell recognition (27). Subsequently, MHC class I–restricted and MHC class II–restricted T-cell epitopes of MUC1 were identified (28). Compared with MUC1, our data showed that MHC-unrestricted recognition of Gal-3 is dependent on the CRD of Gal-3. Unlike MUC1, activation of tumor-reactive T cells by Gal-3 induces cell apoptosis in a dose-dependent fashion. Therefore, Gal-3 functions as an immune regulator of tumor-reactive T cells.

Galectins are a family of animal lectins, which includes 15 mammalian galectins. Based on the number of CRD, the members of this family are divided into three types. Gal-3 is a unique one-CRD galectin that contains nonlectin domain fused with the CRD (15). The overall homology of human intergalectins is ~20%, but their CRDs are relatively conserved (29). In the last few years, it has been shown that the members of the galectin family proteins are closely associated with immunoregulation in various diseases (24). The galectin protein family plays important roles in various biological processes, including modulation of the function of immune effector cells, such as T cells (15). For example, Gal-1 induces the apoptosis of thymocytes and activated peripheral T cells (30). Administration of human Gal-1 in mice contributes

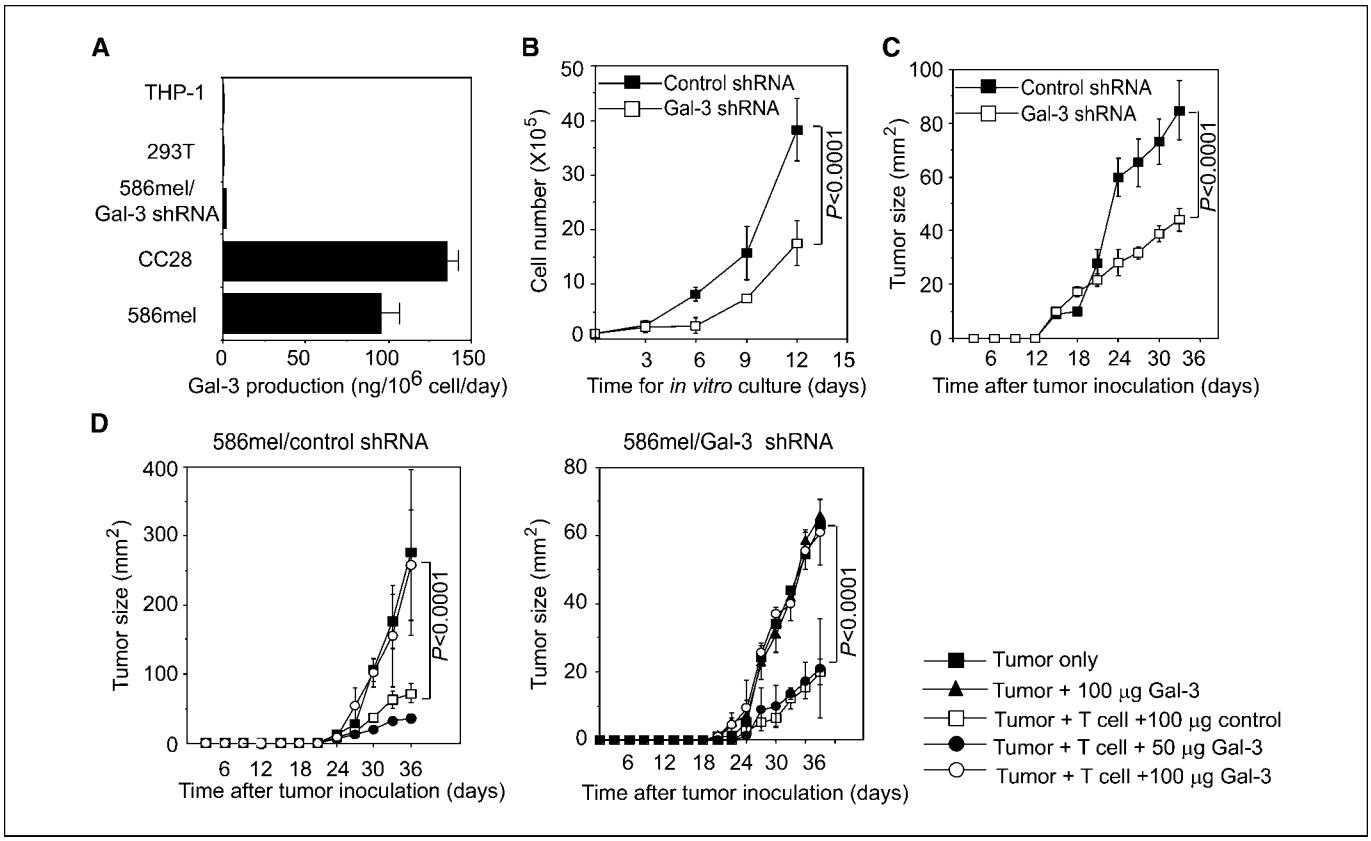

Figure 6. Effects of intracellular and extracellular Gal-3 on tumor growth. *A,* secretion of Gal-3 by tumor cells. Indicated cells (10×10^6) were cultured in 15 mL culture medium for 24 h. The concentrations of Gal-3 in the supernatants were determined by ELISA. Shown are converted amounts of Gal-3 released by the indicated cells (ng/10^6 cell/day). *B,* effect of Gal-3 knockdown on tumor growth *in vitro*. Control shRNA–expressing 586mel tumor cells and Gal-3–silencing 586mel tumor cells were cultured and their cell numbers were determined. *C,* Gal-3–silencing tumor cells reduce the growth rate in Rag2γC-deficient mice compared with control shRNA–expressing 586mel tumor cells. *D,* effect of Gal-3 treatment on T-cell–mediated antitumor immune response and tumor growth. Control shRNA–expressing (*left*) or Gal-3–silencing (*right*) 586mel tumor cells were s.c. injected into Rag2γC-deficient mice and followed by i.v. injecting tumor-reactive TIL586 T cells. Tumor growth rates were monitored every 3 d. *Points,* mean; *bars,* SD. *P* values in *B, C,* and *D,* were determined by two-way ANOVA.

to the suppression of intestinal inflammation induced by IFN-γ production from Th1 cells (31). Treatment of recombinant Gal-1 is also reported to suppress Th1-mediated experimental autoimmune retinal disease in mouse model by inducing $CD4^+$ Treg cells (32). The targeted inhibition of Gal-1 gene expression in tumor cells promotes T-cell–mediated tumor rejection *in vivo* (14). Gal-2 and Gal-9 have been reported to induce apoptosis of activated T-cell lines (29, 33). Gal-4 acts as a stimulator of mucosal $CD4^+$ T cells by specifically inducing IL-6 production in mice with inflammatory bowel disease and contributes to the exacerbation of intestinal inflammation (34).

Similar to other members of the galectin family, several studies show the role of Gal-3 in regulating the function and apoptosis of human Jurkat T cells (35–38). However, very little is known about the role of Gal-3 in the regulation of tumor-reactive T cells in the tumor microenvironment. Our studies show that Gal-3 directly interacts with the immune synapse on the surface of tumor-reactive T cells and activates T cells for apoptosis depending on the status of T cells and its protein concentrations. The activation of freshly isolated naive T cells by Gal-3 is very weak or negligible; however, T cells become responsive to Gal-3 stimulation once they are treated with anti-CD3 and then rested for 10 days (data not shown). Consistent with this observation, we also found that antigen-experienced effector T cells can be readily activated by Gal-3. These results are consistent with studies using resting leukemic T-cell lines (38). It seems that the initial TCR activation might change the glycosylation status of the surface of T cells and render these cells more sensitive to Gal-3 stimulation. An alternative explanation is that antigen-experienced T cells require a lower threshold for their activation by Gal-3. Moreover, recent studies show that the sensitivity of T cells to stimulation from galectin family members is influenced by different T-cell subsets generated at a special condition. For example, only $CD4^+$ T cells that are derived from inflammatory conditions can respond to Gal-4 stimulation (34). The susceptibility of Th1, Th2, and Th17 to Gal-1–induced apoptosis is different, suggesting that the glycosylation status of different subsets of T cells is different (39). Taken together, we believe that Gal-3 secreted by tumor cells may effectively or preferentially activate antigen-experienced or tumor-reactive T cells to produce cytokines and induce them for apoptosis at a high level of concentration, thus inducing immune tolerance at tumor sites.

Like other members of galectin protein family, Gal-3 is highly expressed in many types of cancer cells (40–42). Besides its regulatory role in T-cell activation and immune tolerance, Gal-3 may be associated with tumor growth as well as the aggressive phenotype of tumors (15, 16). Intracellular Gal-3 promotes tumor growth, survival, and metastasis. We show that the knockdown of Gal-3 by shRNA inhibits tumor growth *in vitro* and *in vivo*, which is consistent with the published data obtained from different tumor cells, such as breast cancer (43, 44). However, we found that the soluble form of Gal-3 did not affect the growth of tumor cells; instead, it modulated the tumor-reactive T-cell function by inducing T-cell activation and apoptosis *in vitro*. More importantly, we show that soluble Gal-3 inhibits T-cell responses and promotes tumor growth *in vivo*, but this effect requires a relatively high concentration of Gal-3. This agrees with the data published by other groups, showing that the minimal concentration of Gal-3 required for the induction of apoptosis of a human T-cell line is 3 μmol/L (~100 μg/mL; ref. 36). Given the elevated expression of Gal-3 in tumor cells as well as in the serum of cancer patients (16, 26, 45), we postulate that Gal-3 may accumulate in the local tumor environment, creating the high concentrations necessary for the above observed effects. The high concentrations of Gal-3 in the local tumor environments may eventually drive tumor-reactive T-cell apoptosis and thereby the loss of their antitumor effector functions. Supporting this premise, one study using immunohistochemical staining shows that the expression of Gal-3 in human melanoma biopsies correlates with T-cell apoptosis (46). Treatment of mice with Gal-3 inhibitor reduces the growth rate of human cancer cells *in vivo* (47–49). Taken together, our own findings along with those of other groups clearly indicate that Gal-3 inhibits antitumor immunity and promotes tumor growth through two distinct mechanisms. Inhibition or knockdown of Gal-3 not only reduces tumor growth but also improves the therapeutic potential of cancer immunotherapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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