

# Targeted inhibition of galectin-1 gene expression in tumor cells results in heightened T cell-mediated rejection: A potential mechanism of tumor-immune privilege

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## Summary

**Despite the existence of tumor-specific immune cells, most tumors have devised strategies to avoid immune attack. We demonstrate here that galectin-1 (Gal-1), a negative regulator of T cell activation and survival, plays a pivotal role in promoting escape from T cell-dependent immunity, thus conferring immune privilege to tumor cells. Blockade of immunosuppressive Gal-1 in vivo promotes tumor rejection and stimulates the generation of a tumor-specific T cell-mediated response in syngeneic mice, which are then able to resist subsequent challenge with wild-type Gal-1-sufficient tumors. Our data indicate that Gal-1 signaling in activated T cells constitutes an important mechanism of tumor-immune escape and that blockade of this inhibitory signal can allow for and potentiate effective immune responses against tumor cells, with profound implications for cancer immunotherapy.**

## Introduction

A long-standing paradox in tumor immunology is the growth of antigenic tumors in spite of demonstrable antitumor T cell responses (Pardoll, 2002; Smyth et al., 2001). Recent progress toward an improved understanding of the interactions between tumor cells and the host's immune system has led to the realization that tumor cells have devised multiple strategies to evade immune attack (Marincola et al., 2000; Trapani, 2002; Uyttenhove et al., 2003; Igney and Krammer, 2002). Evasion mechanisms can either be preexisting, arise through outgrowth of escape mutants, or take place during tumor-sculpting actions by the immune system, in accordance with the recently proposed "cancer immunoediting" hypothesis (Dunn et al., 2002).

One of the most studied strategies of tumor-immune escape is represented by the acquisition of FasL expression that may enable cancer cells to deliver death signals to activated Fas-

positive T lymphocytes (Hahne et al., 1996; O'Connell et al., 2001; Andreola et al., 2002; Whiteside, 2002). However, despite the wealth of data accumulated in support of the FasL counterattack hypothesis, many contradictory studies have been reported showing that FasL can also have proinflammatory and antitumoral effects in some contexts (Restifo, 2001; Simon et al., 2002). To reconcile these conflicting findings, it has been hypothesized that the maintenance of immune privilege in tumors relies not only on FasL itself but also on the collective production of as yet unidentified immunosuppressive factors (Green and Ferguson, 2001; O'Connell et al., 2001). These inhibitory signals may create an appropriate setting that will prevent the proinflammatory effects of FasL, thus favoring immune escape by eliminating antitumor effector cells. Accordingly, it has been suggested that the final outcome of an effective antitumor response is determined by a delicate interplay among activating and inhibitory regulatory pathways and that removal of inhibitory

## SIGNIFICANCE

In recent years, one of the most important insights into tumor immunity was provided by the identification of negative regulatory pathways and immune escape strategies that, by counteracting tumor effector mechanisms, greatly influence the magnitude of antitumor responses. Galectin-1, a  $\beta$ -galactoside binding protein with immune regulatory functions, is expressed by many different tumor types, and its expression correlates with the aggressiveness of these tumors and the acquisition of metastatic phenotypes. Here we provide evidence both in vitro and in vivo showing that Gal-1 contributes to immune privilege of tumors by modulating survival and polarization of effector T cells. More importantly, our data highlight a potential molecular target for manipulation of T cell tolerance and apoptosis with profound implications for cancer immunotherapy.

signals may be a particularly useful adjunct to other therapeutic approaches for cancer (Pardoll, 2002; Phan et al., 2003; Cheng et al., 2003).

Galectins, a growing family of carbohydrate binding proteins, have recently attracted the attention of immunologists as novel regulators of immune cell homeostasis (Liu, 2000). Despite extensive sequence homology and similar carbohydrate specificity, various members of this protein family behave as amplifiers of the inflammatory response, while others activate homeostatic signals that serve to shut off immune effector functions (Rabinovich et al., 2002a). Recently, it has become clear that galectin-1 (Gal-1), a prototype member of this protein family, has the potential to inhibit T cell effector functions by promoting growth arrest and apoptosis of activated T cells (Blaser et al., 1998; Perillo et al., 1995; Rabinovich et al., 1998, 2002b), inducing partial T cell activation (Chung et al., 2000) and/or blocking proinflammatory cytokine secretion (Rabinovich et al., 1999a, 1999b). In vivo, therapeutic administration of Gal-1 suppresses T helper (T<sub>H</sub>)-1-dependent chronic inflammation in experimental models of autoimmunity by increasing T cell susceptibility to activation-induced cell death and skewing the balance of the immune response toward a T<sub>H</sub>2 profile (Rabinovich et al., 1999a; Santucci et al., 2003).

Expression of Gal-1 has been well documented in immune privileged organs (Rabinovich, 1999) and in many different tumor types including astrocytoma (Camby et al., 2001) and prostate (van den Brule et al., 2001), thyroid (Xu et al., 1995), colon (Sanjuan et al., 1997), bladder (Cindolo et al., 1999), and ovary (van den Brule et al., 2003) carcinomas. Interestingly, such expression correlates with the aggressiveness of these tumors and the acquisition of metastatic phenotype.

On the basis of these data, we hypothesized that tumor cells may impair T cell effector functions by secretion of Gal-1 and that this mechanism may contribute in tilting the balance toward an immunosuppressive environment at the tumor site. Our data provide evidence both in vitro and in vivo of the relevance of Gal-1-mediated immunosuppression as a novel mechanism of tumor-immune escape, indicating that blockade of this pathway has strong therapeutic potential to shift the balance in favor of an efficient T cell-mediated antitumor immune response. To the best of our knowledge, this is the first in vivo study demonstrating a link between Gal-1-mediated immunoregulation and immune privilege in a setting relevant to cancer immunotherapy.

## Results

### Identification of Gal-1 as a major immunosuppressive factor secreted by human and murine melanoma cells

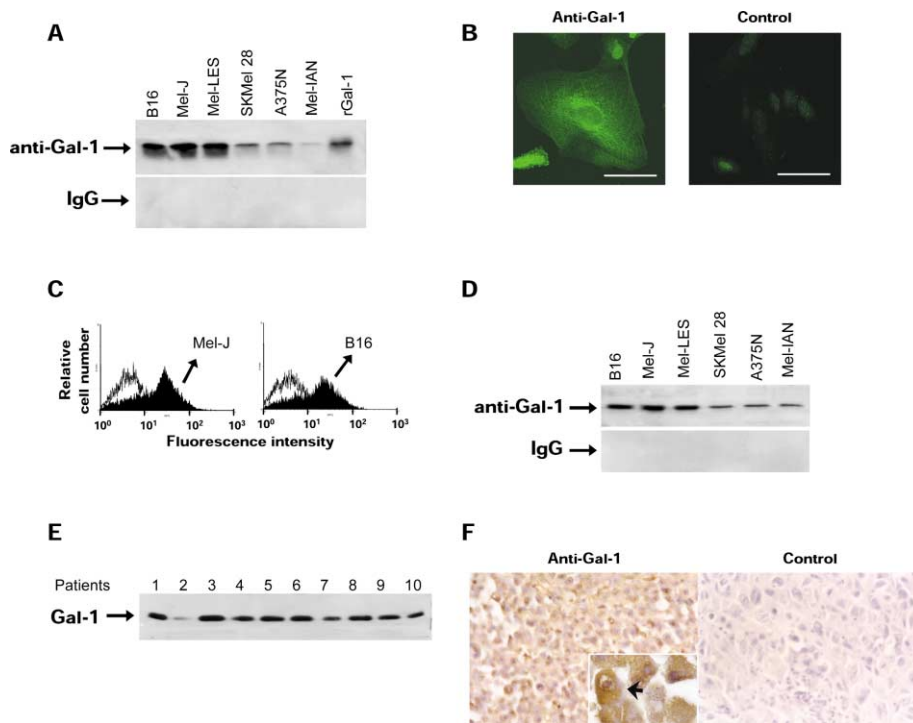
Using a specific anti-Gal-1 antibody (Ab), we screened a panel of six human and murine melanoma cell lines for Gal-1 expression. We selected melanoma as a tumor model on the basis of previous findings reporting the isolation of a low molecular weight (~14 kDa) immunosuppressive factor from human melanoma cell supernatants (Morvillo et al., 1996), whose biochemical and functional properties closely resembled those of Gal-1. Western blot analysis showed that all melanoma cell lines ex-

pressed Gal-1 (Figure 1A), although particularly high expression was detected in murine B16 and human Mel-J and Mel-LES cell lines. Immunofluorescence staining followed by confocal microscopy or flow cytometry revealed a predominant cytoplasmic and nuclear localization of this protein, although specific labeling was also observed at the cell surface of human and murine melanoma cells (Figures 1B and 1C). In addition, we detected the presence of Gal-1 in concentrated serum-free conditioned media (SFCM) from different tumor cell lines (Figure 1D). To confirm its identity, we purified this protein from SFCM of two different cell lines (Mel-J and Mel-LES) by size exclusion and affinity chromatography. Amino acid sequence analysis of selected trypsin-digested peptides revealed the complete identity of the isolated protein with human placental Gal-1 (see Supplemental Figure S1 at <http://www.cancer.org/cgi/content/full/5/3/241/DC1>). Because tumor cell lines grown in vitro may not reflect the real state of tumor cells in vivo, we also tested expression of Gal-1 in human tumor samples. Western blot analysis and immunohistochemistry demonstrated high expression of this protein in freshly isolated tumors from melanoma patients (Figures 1E and 1F).

To investigate the possibility that expression of Gal-1 may confer immune privilege to tumor cells, we transfected B16 melanoma cells with Gal-1 antisense cDNA and established 12 different knockdown clones with low or intermediate Gal-1 levels from the heterogeneous population of stably transfected melanoma cells. Based on Gal-1 expression, we then selected representative clones for in vitro and in vivo assays. Western blot analysis of supernatants collected from knockdown transfectants revealed decreased levels of Gal-1 secretion (Figure 2A). Clone one (B16/C1) showed the lowest Gal-1 levels, representing a relative decrease of ~93% compared to cells transfected with empty vector (B16/-), while clones two and three (B16/C2 and B16/C3) showed intermediate Gal-1 levels, representing relative decreases of ~65% and ~59%, respectively.

To evaluate the contribution of Gal-1 to the immunosuppressive and proapoptotic activity of these tumor cell supernatants, we next incubated activated splenocytes from C57BL/6 (B6) normal mice for 18 hr with SFCM obtained from control or Gal-1 knockdown transfectants and analyzed the apoptotic T cell population by double staining using FITC-annexin V and PE-conjugated anti-CD3 monoclonal antibody (mAb). Supernatants collected from control B16/- cells induced high levels of T cell apoptosis (71.0% ± 3.2%), whereas SFCM collected from knockdown transfectants expressing the lowest Gal-1 levels (B16/C1) induced only 27.3% ± 2.8% of annexin V staining. Remarkably, splenocytes incubated in the presence of SFCM obtained from B16/C2 and B16/C3 underwent apoptotic cell death at intermediate levels (40.1% ± 3.9% and 50.8% ± 3.3%, respectively) (Figure 2B). These results indicate a functional correlation between the levels of Gal-1 in B16 culture supernatants and the extent of tumor-induced T cell death.

To validate this finding in a human setting, we activated peripheral blood mononuclear cells (PBMCs) with phytohemagglutinin (PHA) and then cultured for 18 hr in the presence of different concentrations of SFCM from Mel-LES or Mel-J melanoma cell lines (Figure 2C). Activated T cells exposed to Mel-LES SFCM showed an average of 75.2% ± 4.1% annexin V staining. Remarkably, when SFCM was preincubated with a neutralizing anti-Gal-1 IgG (Fab)<sub>2</sub>, the percentage of apoptotic T cells decreased to 34.2% ± 5.6%, whereas addition of a



**Figure 1.** Expression and secretion of Gal-1 by human and murine melanoma cells

**A:** Western blot detection of Gal-1 (~14.5 kDa) in total cell lysates (15  $\mu$ g) from human and murine melanoma cell lines using an affinity-purified anti-Gal-1 Ab. Blots incubated with preimmune IgG were used as negative controls.

**B:** Indirect immunofluorescence staining and confocal microscopy after permeabilization of a representative human melanoma cell line (MEL-J) (Scale bar, 70  $\mu$ m).

**C:** Flow cytometry of nonpermeabilized Mel-J and B16 cell lines using an anti-Gal-1 Ab (dark histogram) or control rabbit IgG (empty histogram).

**D:** Western blot detection of concentrated serum-free conditioned medium (SFCM) (15  $\mu$ g) from different melanoma cell lines.

**E:** Western blot analysis of total cell lysates from representative samples obtained from human melanoma patients.

**F:** Immunoperoxidase staining of a paraffin-embedded tissue section of a representative human melanoma specimen using an anti-Gal-1 Ab or the same dilution of a control rabbit IgG (magnification, 40 $\times$ ; inset, 100 $\times$ ). Scarce amounts of Gal-1 were detected in normal human melanocytes (data not shown).

control rabbit IgG (Fab)<sub>2</sub> had no effect (data not shown). We also observed a similar reduction in the frequency of annexin V positive cells when thiodigalactoside (TDG, a galectin-specific sugar inhibitor) was incorporated to the culture medium. We confirmed these findings by propidium iodide staining of subdiploid nuclei (see Supplemental Figure S2 at <http://www.cancer.org/cgi/content/full/5/3/241/DC1>). In addition, human PBMCs simultaneously exposed to PHA and the indicated stimuli for 72 hr showed a similar outcome in a cell proliferation assay (Figure 2D).

Taken together, these results demonstrate that both murine and human melanoma cells secrete functional Gal-1, which substantially contributes to the immunosuppressive and proapoptotic activities of these tumor cells.

#### Targeted inhibition of Gal-1 gene expression renders mice resistant to tumor challenge

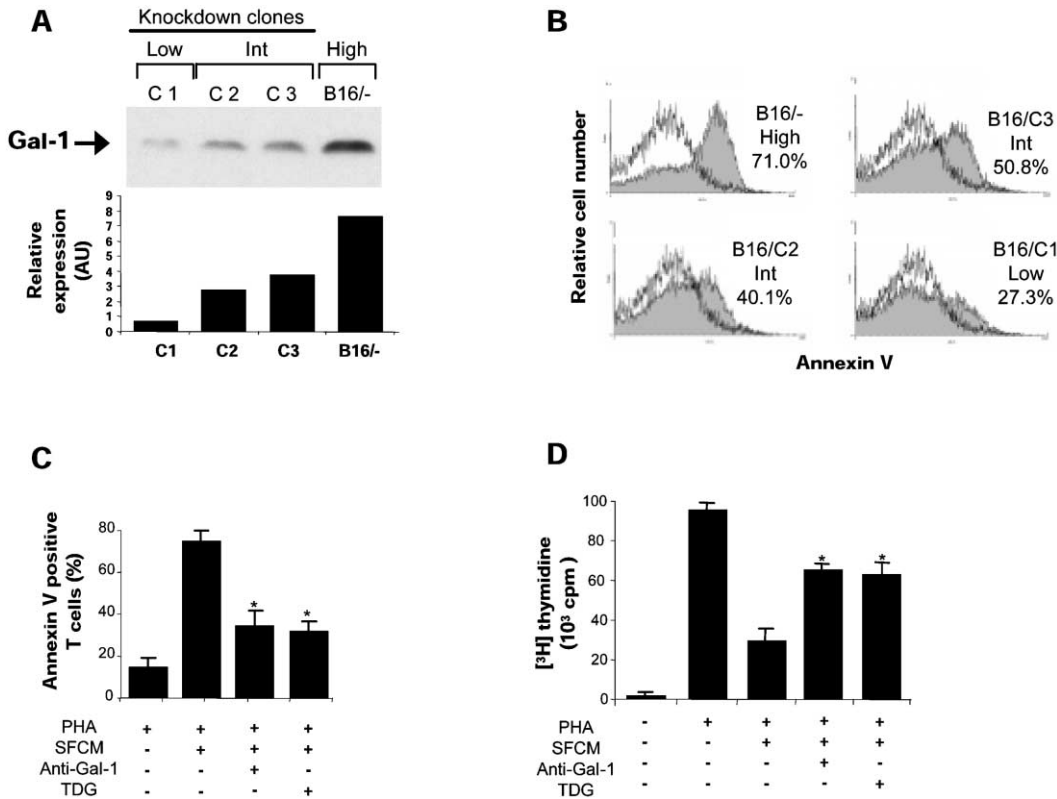
To investigate whether tumor cells may overwhelm the antitumor T cell responses *in vivo* through Gal-1-dependent mechanisms, we explored the impact of Gal-1 blockade on tumor progression by injecting B6 mice with representative B16 knockdown transfectants. All mice ( $n = 21$ ) injected subcutaneously with  $2 \times 10^5$  B16<sup>-</sup> transfectants developed progressively growing tumors at a rate similar to that of nontransfected B16 cells, leading to uniform terminal morbidity by about 20–25 days post-inoculation (Figures 3A and 3B). Remarkably, B16/C1 tumor transfectants, expressing the lowest Gal-1 levels, were rejected rapidly and almost completely, since only 2 of 27 mice showed tumor growth by day 40. Furthermore, administration of B16/C2 and B16/C3 transfectants resulted in substantially delayed tumor growth in all mice, in comparison with B16<sup>-</sup> cells (Figure 3A). As expected, mice inoculated with B16/C1 knockdown transfectants remained tumor free for about 35 days (Figure

3B). To rule out the possibility that stable transfection with anti-sense Gal-1 may directly affect tumor cell growth, we analyzed the *in vitro* cell proliferation of B16<sup>-</sup> and Gal-1 knockdown clones. All transfectants exhibited a similar growth rate (Figure 3C), indicating no growth advantage of B16<sup>-</sup> over antisense-transfected tumor cells. In addition, all tumor transfectants grew progressively at the same rate in immunodeficient nude mice (data not shown).

These results demonstrate that targeted inhibition of Gal-1 expression renders mice resistant to tumor challenge and indicate a functional correlation between Gal-1 levels in tumor cells and the extent of *in vivo* tumor rejection.

#### Tumor rejection induced by Gal-1 blockade requires intact CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses

In order to investigate the involvement of the immune system in the tumor rejection effect induced by Gal-1 blockade, we depleted CD4<sup>+</sup>, CD8<sup>+</sup>, or NK1.1<sup>+</sup> cells *in vivo* and challenged mice with knockdown transfectants. Depletion of either CD4<sup>+</sup> or CD8<sup>+</sup> T cells partially restored B16/C1 cell growth in B6 mice (Figures 4A and 4B). Remarkably, depletion of both T cell subsets completely abrogated resistance to tumor challenge induced by Gal-1 blockade and resulted in rapid tumor progression and similar growth rate as mice injected with B16<sup>-</sup> transfectants (Figures 4A and 4B). On the other hand, depletion of NK1.1<sup>+</sup> cells had only a marginal effect in 3 out of 15 mice tested (Figure 4A). Furthermore, pretreatment of mice with control mouse IgG did not alter the tumor rejection effect induced by Gal-1 blockade (data not shown). These results support the concept that the generation of antitumor activity in the absence of Gal-1 requires participation of both CD4<sup>+</sup> and CD8<sup>+</sup> T cell subsets.



**Figure 2.** Identification of Gal-1 as a major immunosuppressive and proapoptotic protein secreted by human and murine melanoma cells

**A:** Western blot and densitometric analysis of SFCM (15  $\mu$ g) from knockdown (B16/C1, C2, C3) or control (B16/-) transfectants using an anti-Gal-1 Ab.

**B:** Splenocytes from B6 mice were activated with Con A for 72 hr and then cultured for 18 hr in the presence of SFCM collected from control (B16/-) or knockdown transfectants expressing low (B16/C1) or intermediate (B16/C2, B16/C3) Gal-1 levels. T cell apoptosis was assessed by double staining with FITC-annexin V and PE-anti-CD3 mAb. Proportion of annexin V-positive cells is shown in histograms as percentage of a representative out of three independent experiments ( $p < 0.01$  B16/C1 versus B16/-;  $p < 0.05$  B16/C2 and B16/C3 versus B16/- Student's *t* test).

**C:** Human PBMCs were activated with PHA for 72 hr and then cultured for 18 hr with SFCM from Mel-J in the absence or presence of a neutralizing anti-Gal-1 (Fab)<sub>2</sub> (10  $\mu$ g/ml) or 30 mM thiodigalactoside (TDG). Positive controls of apoptosis were analyzed in parallel by incubating activated T cells with increasing doses of rGal-1. T cell apoptosis was assessed by double staining using FITC-annexin V and PE-anti-CD3 mAb (\* $p < 0.001$  Student's *t* test versus SFCM).

**D:** Human PBMCs were cultured for 72 hr with PHA in the presence of the indicated stimuli and cell proliferation was assessed by (<sup>3</sup>H)-thymidine uptake. Data are expressed as mean (cpm  $\pm$  SD) of triplicate determinations (\* $p < 0.001$  Student's *t* test versus SFCM).

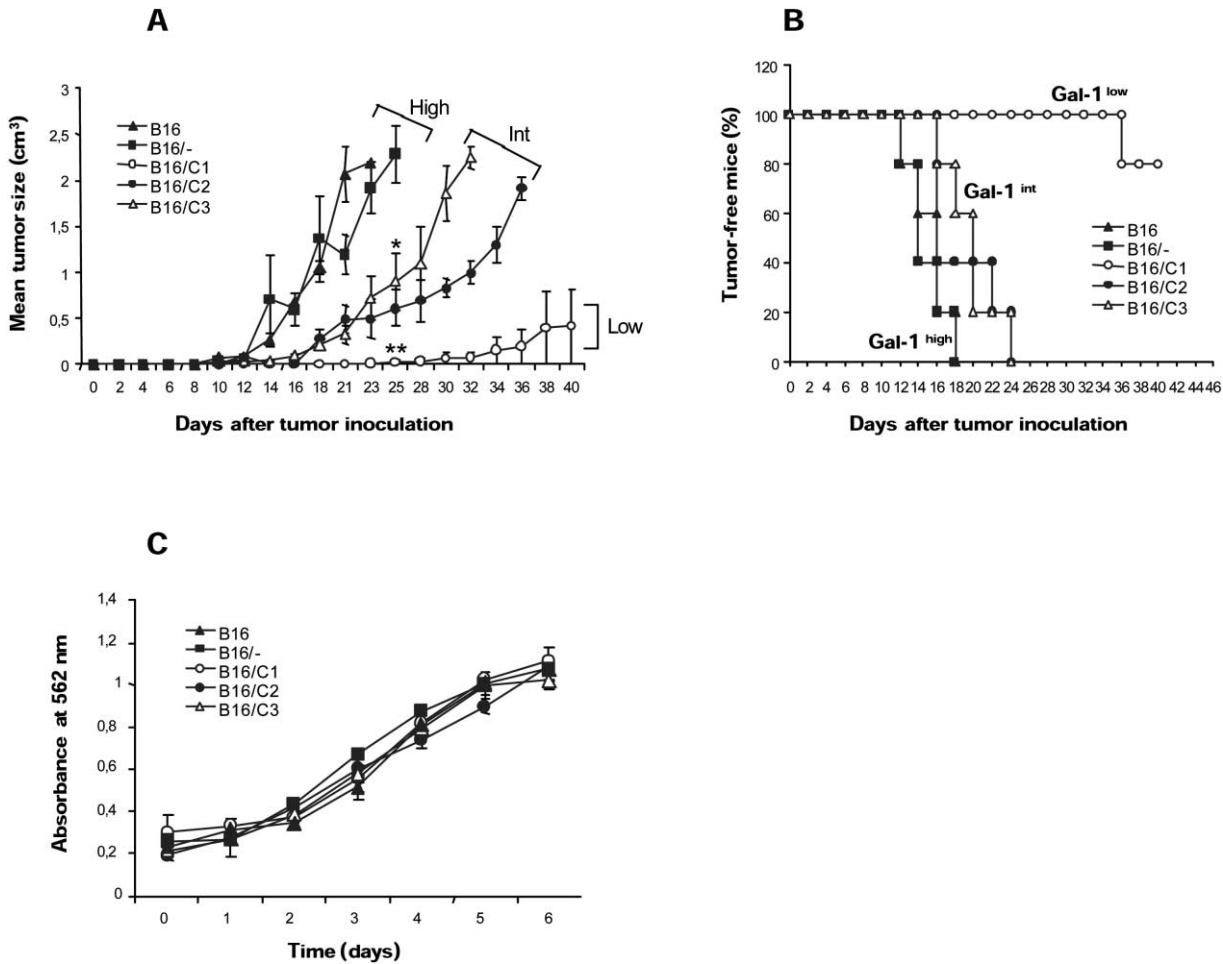
### Blockade of Gal-1 synthesis in tumor cells allows the generation of a tumor-specific T1-type immune response in vivo

The immune regulatory effects of Gal-1 and its ability to skew the balance of the immune response toward a T2-type profile (Rabinovich et al., 1999a) prompted us to investigate whether resistance to tumor challenge induced by selective Gal-1 blockade could be associated with an enhancement of a T1-type antitumor response in vivo. For this purpose, we obtained tumor-draining and distal lymph nodes from mice inoculated with B16 knockdown or control transfectants. Lymph node cells were restimulated ex vivo with mitomycin C-treated B16 parental cells and then analyzed for T cell proliferation, cytokine production, and susceptibility to apoptosis.

Consistent with a role for Gal-1 in tumor-induced immunosuppression, B16/C1 knockdown cells were more effective than B16/- tumor cells in stimulating an effective T cell-mediated response, as demonstrated by the greatly increased tumor-specific T cell proliferation (Figure 5A). In addition, while tumor-draining lymph nodes from mice inoculated with B16/-

transfectants produced low levels of IL-2 (Figure 5B) and IFN- $\gamma$  (Figure 5C), lymph node cells from mice inoculated with B16 knockdown transfectants had greatly enhanced B16-specific T1-type cytokine production. In contrast, no significant differences were found in IL-4 (Figure 5D) or IL-5 (Figure 5E) levels between both experimental groups. These effects were clearly observed in tumor-draining lymph nodes, but no significant changes could be detected in lymph nodes distal to tumor sites (Figure 5A and data not shown). Furthermore, we could not observe changes in T cell proliferation or cytokine secretion when lymph node cells obtained from mice that rejected B16/C1 transfectants were restimulated ex vivo with syngeneic EL-4 thymoma cells (Figures 5A-5E). Thus, selective blockade of Gal-1 in tumor cells can stimulate the generation of an otherwise repressed tumor-specific T1-type response in tumor-draining lymph nodes.

Since Gal-1 contributes substantially to the proapoptotic activity of melanoma cells (Figure 2), we next investigated whether tumor-secreted Gal-1 may sensitize T cells in vivo toward an apoptotic phenotype. Tumor-draining lymph nodes



**Figure 3.** Targeted inhibition of Gal-1 gene expression renders mice resistant to tumor challenge

**A:** B6 mice were challenged by subcutaneous inoculation of  $2 \times 10^5$  parental B16 cells (▲), control B16/- (■), B16/C1 (○), B16/C2 (●), or B16/C3 (△) transfectants. Tumor growth was monitored every second day (\*\* $p < 0.001$  B16/C1 versus B16/-; \* $p < 0.01$  B16/C2 and C3 versus B16/- Student's  $t$  test at day 25 postinoculation). Data represent the mean  $\pm$  SD of at least three different experiments.

**B:** Kaplan-Meier analysis of mice challenged with control (B16/-) or knockdown transfectants ( $p < 0.001$  B16/C1 versus B16/-;  $p < 0.05$  B16/C2 and C3 versus B16/-). Results in (A) and (B) summarize data from inoculated B16 clones expressing Gal-1 at low (B16/C1), intermediate (B16/C2, B16/C3), or high (B16/-) levels.

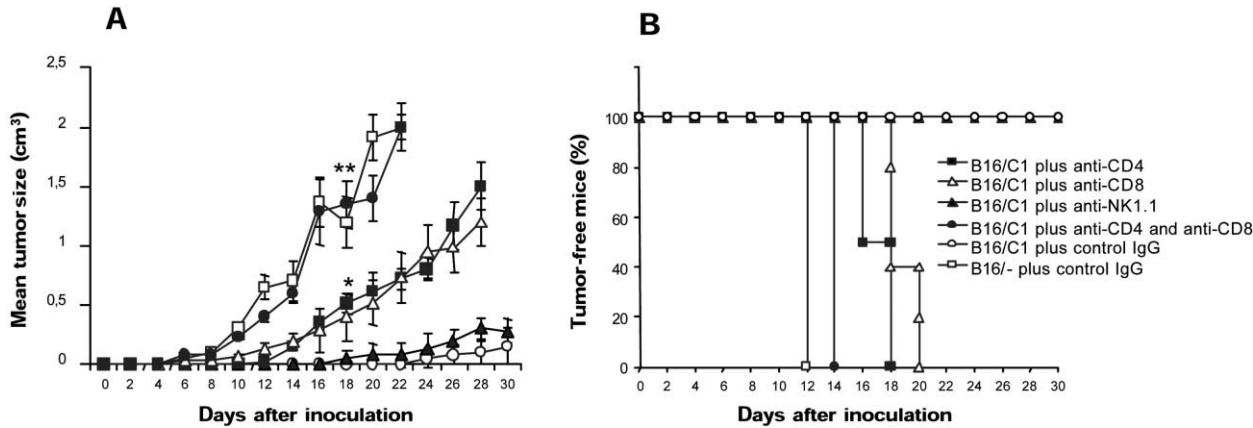
**C:** In vitro cell growth of parental B16 cells, control (B16/-), and knockdown transfectants by the MTT assay.

from mice treated with Gal-1 knockdown transfectants and further restimulated with parental B16 cells demonstrated a reduced T cell susceptibility to apoptosis compared to lymph node cells from mice inoculated with B16/- Gal-1 sufficient tumor cells (Figure 5F). In order to investigate whether the proliferating antitumor cells were capable of infiltrating tumor sites and promote tumor cell destruction, and to further explore the impact of Gal-1 blockade on local immune cell apoptosis, we performed histopathological analysis and TUNEL staining on paraffin-embedded tumor sections from B16/- and B16/C1-treated mice. Ten days after tumor challenge, most peritumoral mononuclear cells showed apoptotic morphology (4–7 TUNEL-reactive cells/field, 40 $\times$ ) in mice inoculated with B16/- cells, compared to mice injected with B16/C1 (0–1 TUNEL-reactive cells/field) (Figures 5G and 5H). In addition, blockade of Gal-1 was effective at promoting tumor cell destruction accompanied by extensive melanin release at sites of B16/C1 injection, whereas the integrity of the tumors was completely preserved

following treatment with B16/- cells (Figure 5H). Thus, tumor rejection induced by Gal-1 blockade is associated with tumor cell destruction along with an extensive nonapoptotic mononuclear cell infiltrate, suggesting an increased availability of survival signals influencing the antitumor activity at tumor sites.

To address whether the generation of a tumor-specific T cell response in the absence of Gal-1 is sufficient to stimulate protective immunity to a subsequent challenge with wild-type B16 tumor cells, we rechallenged mice that had previously rejected B16/C1 transfectants with the corresponding wild-type tumors 20 days after the first exposure. While B16 tumors grew progressively in naive B6 mice, we observed a significant delay in tumor growth in mice that had been previously exposed to Gal-1 knockdown transfectants (Figures 6A and 6B).

Taken together, these results demonstrate that selective inhibition of Gal-1 gene expression in melanoma cells can allow for and potentiate a tumor-specific T1-type response in syngene-



**Figure 4.** Tumor rejection induced by Gal-1 blockade requires intact CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses

**A and B:** Selective depletion of lymphocyte subsets was accomplished by intraperitoneal injection of B6 mice with the indicated mAbs at days -1, 1, 8, 15, and 22, relative to tumor inoculation. Mice inoculated with  $2 \times 10^5$  B16/C1 cells were treated with the following mAbs: anti-CD4 (■), anti-CD8 (△), anti-NK1.1 (▲), or a combination of anti-CD4 plus anti-CD8 (●). Control mice received the equivalent amounts of mouse normal IgG at the same days relative to inoculation with B16/C1 (○) or B16/- (□) transfectants (\*\* $p < 0.001$  depletion of CD4 plus CD8 versus B16/C1 control IgG; \* $p < 0.01$  depletion of either CD4 or CD8 versus B16/C1 control IgG; Student's *t* test at day 18 postinoculation).

**B:** Kaplan-Meier analysis of depleted mice challenged with B16/C1 cells. Results are compiled from two independent experiments ( $p < 0.001$  depletion of CD4 plus CD8 versus B16/C1 control IgG;  $p < 0.05$  depletion of CD4 or CD8 versus B16/C1 control IgG).

neic mice, which are then able to resist subsequent challenge with wild-type Gal-1-sufficient tumor cells.

## Discussion

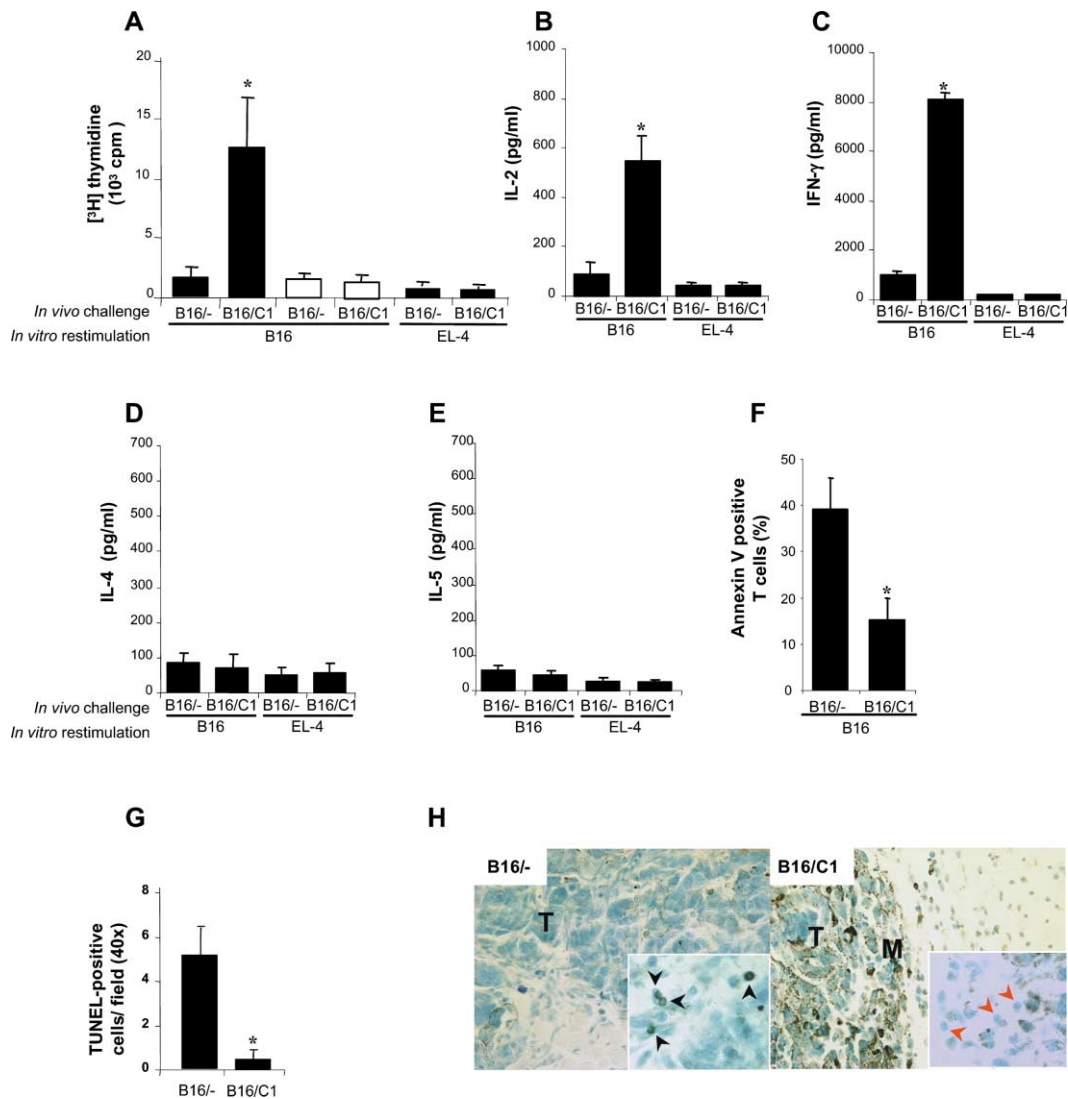
Here we demonstrate that tumors can overwhelm T cell effector functions through Gal-1-dependent mechanisms. By a combination of *in vitro* and *in vivo* experiments using knockdown transfectants, we established a link between Gal-1-mediated immunoregulation and its contribution to tumor-immune privilege. Blockade of the inhibitory effects of Gal-1 within tumor tissue resulted in reduced tumor mass and enhanced tumor rejection, stimulating the generation of a potent tumor-specific T1-type response in syngeneic mice.

During the past few years, there has been increasing appreciation for the impact of differential T cell glycosylation in the modulation of immune responses (Daniels et al., 2002; Feizi, 2000). Through interaction with poly-N-acetyllactosamine ligands, created by specific glycosyltransferases (Nguyen et al., 2001; Lowe, 2001), Gal-1 influences T cell homeostasis by inducing T cell apoptosis (Rabinovich et al., 1999a), blocking T cell activation (Chung et al., 2000), inhibiting proinflammatory cytokine secretion (Rabinovich et al., 1999b), and favoring turnover of activated leukocytes (Dias-Baruffi et al., 2003). Therefore, the stimulation of an effective antitumor response following downregulation of Gal-1 could be attributed to several nonexclusive mechanisms. While this effect may be a consequence of the removal of an inhibitory signal involved in T cell death, it is equally likely to result from the activation of a larger number of naive T cells due to a lowering of the threshold for activation. In this context, Gal-1 blockade may increase both the survival and frequency of helper and cytotoxic T cells, which could act in concert to orchestrate the host response against tumors. Accordingly, depletion experiments revealed the requirement for both CD4<sup>+</sup> and CD8<sup>+</sup> T cells to promote tumor rejection in our model, suggesting that both T cell subsets could be

responsible for the significant rise in IFN- $\gamma$  secretion following selective blockade of Gal-1 synthesis in tumors. Although CD8<sup>+</sup> T cells are the cytotoxic effectors in melanoma, CD4<sup>+</sup> T cells have been shown to mediate CD8<sup>+</sup>-independent antitumor effector functions (Hung et al., 1998). Interestingly, NK cells were not required to promote tumor rejection in our model. However, IFN- $\gamma$  production by NK cells was a critical factor in the development of antitumor immunity following different stimuli, including overexpression of CD70 (Kelly et al., 2002) or NKG2D ligands (Diefenbach et al., 2001). Thus, the combination of different strategies, stimulating both innate and adaptive immune responses (Duguay et al., 2002) but also targeting negative regulatory pathways (Pardoll, 2003), will be necessary to provide complete antitumor protection and allow selective amplification of immune responses for successful cancer immunotherapy.

An alternative mechanism of enhanced antitumor immunity following removal of the inhibitory effects of Gal-1 could be the emergence of a previously repressed T1-type specific immune response, as we found greatly increased IFN- $\gamma$  production by lymph node cells from mice inoculated with Gal-1 knockdown transfectants. This finding strengthens the hypothesis that an IFN- $\gamma$ -mediated response is critical to prevent tumor formation and shape tumor immunogenicity (Shankaran et al., 2001; Hu et al., 1998) and may counteract the gradual loss of T<sub>H</sub>1 populations observed during tumor progression (Ghosh et al., 1995). Interestingly, we have previously shown that recombinant Gal-1 and its genetic delivery suppress T<sub>H</sub>1-mediated inflammation in experimental models of collagen-induced arthritis and TNBS-induced colitis (Rabinovich et al., 1999a; Santucci et al., 2003). In these models, Gal-1 administration resulted in a selective elimination of antigen-activated T cells and in a T<sub>H</sub>2 shift that induced a remission state in the evolution of the ongoing inflammatory disease.

Since Gal-1 was effective at promoting apoptosis of effector T cells in autoimmune settings, it may be possible that blockade of this inhibitory pathway may allow the recruitment and activa-



**Figure 5.** Blockade of Gal-1 synthesis in tumor cells allows the generation of a tumor-specific T1-type immune response in vivo

Two weeks after tumor challenge, draining (filled bars) or distal (open bars) lymph nodes were taken from mice inoculated with knockdown (B16/C1) or control (B16/-) transfectants. Lymph node cells ( $5 \times 10^5$ /well) were subjected to restimulation ex vivo with mitomycin C-inactivated B16 tumor cells ( $5 \times 10^5$ /well) and analyzed for T cell proliferation (A), cytokine production (B-E), and susceptibility to apoptosis (F).

**A:** After 5 days of restimulation with mitomycin C-treated B16 or EL-4 tumor cells, lymph node cells were pulsed with 1 Ci/ml (methyl- $^3$ H) thymidine for the last 16 hr. Results are expressed as cpm (mean  $\pm$  SD) of triplicate determinations (\* $p < 0.001$  B16/C1 versus B16/-).

**B-E:** After 72 hr of restimulation with mitomycin C-treated B16 or EL-4 tumor cells, supernatants were collected from lymph node cells and analyzed for IL-2 (B), IFN- $\gamma$  (C), IL-4 (D), and IL-5 (E) production by capture ELISA. Mean values of different groups are indicated (mean  $\pm$  SD) as combination of three independent experiments (\* $p < 0.001$  B16/C1 versus B16/- for IL-2 and IFN- $\gamma$ ;  $p = \text{NS}$  B16/C1 versus B16/- for IL-4 and IL-5).

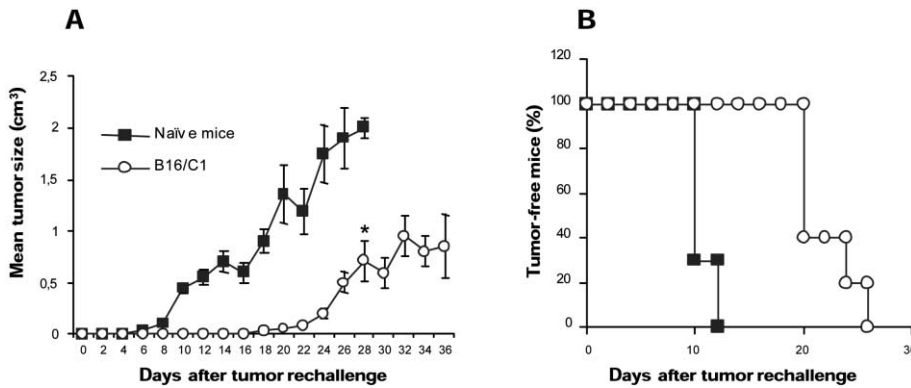
**F:** Lymph node cells from mice treated with B16/C1 or B16/- transfectants were cultured in the presence of mitomycin C-treated B16 cells for 72 hr. T cell apoptosis was analyzed by double staining with FITC-annexin V and PE-anti-CD3 mAb (\* $p < 0.05$  B16/C1 versus B16/-).

**G and H:** Examination of tumor histopathology and peritumoral mononuclear cell infiltrate in mice injected with B16/C1 or B16/- cells. Palpable tumors or sites of tumor injection were removed and processed for histological examination in a blinded fashion (Magnification, 40 $\times$ ; insets, 100 $\times$ ). The number of apoptotic mononuclear cells (mean  $\pm$  SD) per field (40 $\times$ ) was determined in situ by TUNEL assay (\* $p < 0.05$  B16/C1 versus B16/-;  $\chi^2$ ). Insets show apoptotic (black arrows) or viable (red arrows) mononuclear cells in representative fields. The nuclear staining profile of TUNEL-positive cells (black arrows) is clearly distinguishable from the irregular and granular aspect of the pigment melanin (M) released by tumors (T).

tion of low-affinity autoreactive T cells that might have escaped tolerance induction. Although we have not found signs of tissue-specific self reactivity in our model, a long-term follow up will be necessary to investigate whether mice that rejected challenge with melanoma transfectants will develop signs of autoimmune depigmentation, as described for other immunotherapy strategies (Ramirez-Montagut et al., 2003).

The data presented here demonstrate that melanoma cells may take advantage of Gal-1 expression for inducing apoptosis of tumor-specific effector T cells and that this mechanism may contribute in tilting the balance toward an immunosuppressive environment at the tumor site. However, we cannot rule out the possibility that alternative Gal-1-dependent mechanisms may contribute to tumor progression. In accordance, previous stud-





**Figure 6.** Blockade of Gal-1 synthesis in tumor cells promotes resistance to subsequent challenge with wild-type Gal-1-sufficient tumors

**A and B:** B6 mice that had previously rejected Gal-1 knockdown transfectants (B16/C1) were inoculated in the opposite flank 20 days after the first tumor challenge with wild-type B16 tumor cells ( $2 \times 10^5$ ). Age-matched naïve B6 mice were challenged in parallel with the same number of tumor cells. Tumor size was monitored every second day and mice were sacrificed when tumor size reached 2 cm<sup>3</sup>. \* $p < 0.001$  B16/C1 versus naïve mice; Student's *t* test at day 28 postinoculation (**A**);  $p < 0.002$  B16/C1 versus naïve mice; Kaplan-Meier analysis (**B**).

ies show that Gal-1 may affect homotypic cell aggregation (Tinari et al., 2001), adhesion (van den Brule et al., 1995), migration (Camby et al., 2001), and tumor cell proliferation (Kopitz et al., 2001) in vitro. Furthermore, since human tumors express a complex pattern of galectin expression (Lahm et al., 2001), future studies are warranted to elucidate the immunoregulatory properties of other members of the galectin family (Fukumori et al., 2003) and their contribution to tumor-immune privilege.

The hypothesis of “tumor counterattack” suggests that tumors may deliver death signals to Fas-sensitive effector cells, thus rendering the tumor an immunologically privileged site (O’Connell et al., 2001). Although this model has not been universally accepted, recent studies highlight the contribution of novel tumor-associated molecules to tumor-immune evasion, including B7-H1 (Dong et al., 2002) and the enzyme indoleamine 2,3 dioxigenase (IDO) (Uyttenhove et al., 2003; Friberg et al., 2002). Furthermore, accumulating evidence indicates that blocking different inhibitory signals such as CTLA-4 (Phan et al., 2003), Stat3 (Cheng et al., 2003), or TGF- $\beta$  (Gorelik and Flavell, 2001) may be effective alone or in combination with other immunotherapy strategies to break immunological tolerance and promote tumor regression (Boon and van den Eynde, 2003).

Taken together, our results provide evidence both in vitro and in vivo showing that Gal-1 contributes to immune privilege of tumors by modulating survival and/or polarization of effector T cells, providing a link between the immunoregulatory properties of this protein and its contribution to tumor progression. More importantly, our data highlight a novel molecular target for manipulation of T cell tolerance and apoptosis with profound implications for cancer immunotherapy.

## Experimental procedures

### Tumor cell lines

The human melanoma cell lines IIB-Mel-LES, IIB-Mel-J, and IIB-Mel-IAN were established from human metastatic melanomas as described (Guerra et al., 1989; Kariyama et al., 1995; Ledda et al., 1997), SKMel28 was obtained from ATCC, and the A375N cell line was a gift from Dr. E. Medrano. The murine B16 melanoma cell line was obtained from ATCC and the EL-4 thymoma was a gift from Dr. C. Milstein. Both murine cell lines are syngeneic to C57BL/6 (B6) background.

### Mice

Eight- to ten-week-old inbred female C57BL/6 (H-2b) mice were used in this study. Animals were maintained at the Animal Resource Facilities (Institute Leloir) in accordance with the experimental ethics committee guidelines.

### Generation of knockdown tumor clones

Murine Gal-1 cDNA (length 495 bp) was obtained from the IMAGE Consortium (MRC, Cambridge) and subcloned immediately downstream of the cytomegalovirus (CMV) promoter of a HindIII/BamHI-cut pcDNA3 expression vector (Invitrogen) as described (Rabinovich et al., 1999a). A clone in the antisense orientation was obtained from pcDNA3-Gal-1, excised with BamHI and EcoRV, and subcloned into the CMV-driven pcDNA6 expression vector (Invitrogen) to create the plasmid p6/G1-As.

Subconfluent B16 cells were transfected with p6/G1-As using Lipofectamine 2000 (Life Technologies). Blasticidine-resistant (5  $\mu$ g/ml, ICN) stable transfectants were cloned by limited dilution, and 12 clones were generated. Representative clones with low (B16/C1) or intermediate (B16/C2; C3) Gal-1 levels were selected for in vitro and in vivo experiments. B16 cell lines transfected with vector alone (B16/-) were used as controls. Stable transfectants and controls were screened for Gal-1 expression and secretion by flow cytometry and Western blot analysis.

### Western blot analysis

Human and murine melanoma cell lines and different knockdown clones ( $1.5 \times 10^6$  cells) were extensively washed and lysed in 100  $\mu$ l lysis buffer (5 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 50 mM Tris-HCl) in the presence of a mixture of protease inhibitors (Sigma). SFCM were collected from 18 h-semiconfluent melanoma cell cultures, centrifuged at 1000 g for 5 min to discard cell debris, and stored frozen at  $-70^\circ\text{C}$ . Proteins were precipitated with 9 vol of methanol overnight at  $-20^\circ\text{C}$ . Human melanoma tissue samples were obtained from the Pathology Department, Hospital Eva Perón, with approval of the Institutional Review Board, and total tissue extracts were prepared as described (Xu et al., 1995).

Protein concentration was measured using the Micro-BCA kit (Pierce). Equal amounts of protein of cell lysates and supernatants (15  $\mu$ g) were resolved on a 15% SDS-PAGE. After electrophoresis, the separated proteins were electroblotted onto polyvinylidene difluoride (PVDF) membranes (Amersham) and probed with a 1:4000 dilution of an affinity-purified rabbit anti-Gal-1 Ab (kindly provided by Drs. J. Hirabayashi and K.I. Kasai) or the same dilution of a control preimmune IgG. Blots were then incubated with a peroxidase-labeled anti-rabbit IgG (BioRad) and developed using an enhanced chemoluminescence detection kit (Amersham). Films were analyzed with the Scion Image Analysis software, and the intensity of each band was recorded and expressed as arbitrary units (AU). Recombinant Gal-1 (rGal-1) was used as a positive control of immunodetection. Equal loading was checked by Ponceau S staining or by incubation of the blots with an anti- $\alpha$  tubulin mAb (Sigma).

### Immunofluorescence staining and confocal microscopy

Tumor cells were washed twice in PBS and fixed in 2% paraformaldehyde for 15 min on ice. Cells were then stained with a 1:500 dilution of a rabbit anti-Gal-1 Ab (or control rabbit IgG) for 30 min on ice and then incubated with a FITC-conjugated goat anti-rabbit IgG (Cappel). In each experiment,  $10^4$  viable cells were processed for FACS analysis in a FACSCalibur flow cytometer (Becton Dickinson). For confocal microscopy, tumor cells were cultured overnight on glass coverslips washed with PBS, fixed with 4% paraformaldehyde for 10 min, permeabilized using PBS containing 0.2%



Triton X-100, and stained as described above, except that donkey anti-rabbit Cy2 (Jackson) was used as secondary Ab. Color confocal and transmitted light images were acquired with the use of a Zeiss LSM 510 microscope (Jena, Germany) with C-apochromat 63×/1.2 W objective. The image size was set to 1024 pixels with 8 bits pixel depth.

#### Protein purification and amino acid sequence analysis

Serum-free culture medium was collected from semiconfluent monolayers of Mel-J or Mel-LES cell lines, processed as described (Morvillo et al., 1996), and subjected to size-exclusion chromatography (Superose 12HR 10/30 column, Pharmacia). The eluted low molecular weight fraction was concentrated and applied to a lactosyl-Sepharose column (Sigma) to enrich for  $\beta$ -galactoside binding proteins. After elution with lactose, one major peak was obtained, which was subjected to trypsin digestion and amino acid sequence analysis, as described (Rabinovich et al., 1998). Tryptic peptides were separated by reverse phase HPLC on a Brownlee C18 column, applied to a Polybrene-coated glass filter, and sequenced in an Applied Biosystems model 477A automatic sequencer.

#### Lymphocyte preparation and activation

Splenocytes from B6 mice were prepared by mechanical disruption of spleens using standard protocols. For apoptosis assays, splenocytes ( $5 \times 10^6$  cells/ml) were activated with Con A ( $5 \mu\text{g/ml}$ ) for 72 hr and then cultured for 18 hr in the absence or presence of optimal dilutions (1:2) of SFCM derived from an equal cell number of knockdown or control transfectants.

Human PBMCs were isolated from healthy donors by Ficoll-Paque Plus (Amersham) gradient centrifugation, washed, and resuspended in complete RPMI 1640 medium. Cells ( $1 \times 10^6$  cells/ml) were then stimulated for 72 hr with  $1 \mu\text{g/ml}$  PHA (Sigma) and further cultured for 18 hr in the presence of optimal dilutions of SFCM (1:2) collected from human Mel-J and Mel-LES cell lines. In neutralization experiments, melanoma cell supernatants were added to PBMCs in the presence of 30 mM TDG (Sigma) or saturating amounts ( $10 \mu\text{g/ml}$ ) of a blocking anti-Gal-1 IgG (Fab')<sub>2</sub> prepared using the Immobilized Pepsin kit (Pierce) or (Fab')<sub>2</sub> fragment of a control rabbit IgG. After incubation with different stimuli, cells were washed with sterile PBS containing 30 mM lactose to dissociate cell clusters and processed for apoptosis detection. As control of Gal-1-induced apoptosis, activated PBMCs were exposed to different doses of rGal-1 as described (Rabinovich et al., 2002b). To analyze cell proliferation, PBMCs were cultured for 72 hr either with PHA ( $1 \mu\text{g/ml}$ ) or an anti-CD3 mAb (25 ng/ml; Becton Dickinson) in the presence of the indicated stimuli. Then cultures were pulsed with  $1 \mu\text{Ci/ml}$  (methyl-<sup>3</sup>H) thymidine (specific activity 50 Ci/mmol; New England Nuclear) for the last 16 hr. Results are expressed as cpm  $\pm$  SD of triplicate determinations.

#### Apoptosis assays

T cell apoptosis was evaluated by a double staining procedure using the FITC-annexin V binding assay (Immunotech) and PE-conjugated anti-CD3 mAb. The percentage of nuclei with subdiploid DNA content was determined by propidium iodide (PI) staining. Briefly, cells were gently resuspended in 1 ml hypotonic fluorochrome solution ( $50 \mu\text{g/ml}$  PI diluted in 0.1% sodium citrate plus 0.1% Triton X-100) and kept at 4°C for 3 hr in the dark. Ten thousand events were acquired in a FACSCalibur flow cytometer.

#### In vivo tumor challenge

Melanoma tumors were established in B6 mice by subcutaneous injection of  $2 \times 10^5$  cells in 50  $\mu\text{l}$  of sterile PBS into the right flank. Mice were challenged with B16/- transfectants ( $n = 21$ ; seven mice per experiment) or independent knockdown clones ( $n = 27$ ; nine mice per experiment). Representative clones used for challenge were selected on the basis of Gal-1 expression. Tumor development was monitored every second day by measuring tumor perpendicular diameters with a metric caliper. Tumor volume was estimated as ( $d^2 \times D \times 0.5$ ), where  $d$  and  $D$  are the minor and major diameters, respectively. For ethical reasons, animals were sacrificed when tumors reached a volume greater than 2 cm<sup>3</sup>. Mice with tumor volume less than 0.5 cm<sup>3</sup> were considered as tumor free for the Kaplan-Meier analysis. For rechallenge experiments, groups of five mice that had completely rejected the initial tumor (20 days after first inoculation) were injected in the opposite flank with wild-type B16 tumor cells. Age-matched naive mice injected with B16 tumor cells were used as controls.

#### In vivo lymphocyte subset depletion

CD4<sup>+</sup>, CD8<sup>+</sup>, or NK1.1<sup>+</sup> cells were depleted in vivo by intraperitoneal administration of 0.2 mg of mAbs against CD4 (clone YTS 191.1; ATCC), CD8 (clone YTS 169.4; ATCC), or NK1.1 (clone 4D11; ATCC) at days -1, 1, 8, 15, and 22, relative to tumor inoculation as described (Diefenbach et al., 2001). Control mice received equivalent amounts of normal mouse IgG at the same days. Depletions were confirmed in lymph nodes and spleen cells 7 days after tumor challenge by flow cytometry using non crossreactive Abs. In general, less than 1.7% of the depleted cell population could be detected in spleen and lymph nodes, whereas mice treated with control IgG demonstrated unchanged lymphocyte profiles. Kaplan-Meier analysis was performed as described above. Results are expressed as mean  $\pm$  SD from two independent experiments with  $n = 5$  mice in each group per experiment.

#### In situ TUNEL labeling, histopathology, and immunohistochemistry

Ten days after tumor challenge, palpable tumors of mice inoculated with B16/- or sites of tumor injection of mice inoculated with B16/C1 were removed, paraffin-embedded, and processed for histological examination and apoptosis detection by a peroxidase-conjugated in situ TUNEL assay (Intergen) according to the manufacturer's instructions. Representative tissue samples for each treatment were also stained with hematoxylin and eosin. Qualitative and quantitative studies of stained sections were performed in a blinded fashion by a medical pathologist on five or more samples from distinct mice, and the number of TUNEL-reactive mononuclear cells per field (40 $\times$ ) was recorded. Immunoperoxidase staining was performed on paraffin-embedded tissue sections from human melanoma samples using the anti-Gal-1 Ab (1:500) or the same dilution of a control rabbit IgG. The avidin-biotin peroxidase complex was used according to the manufacturer's recommended instructions (ABC Vectastatin, Vector).

#### MTT assay

Tumor cell growth in vitro was measured by the colorimetric MTT assay. Briefly, exponentially growing control or knockdown transfectants were thoroughly washed and incubated for 24 hr in serum-free medium to synchronize cell growth. Thereafter,  $10^3$  cells/well were cultured in 96-well plates in complete DMEM. Cell growth was determined at 24 hr intervals by replacing the culture medium with 0.1 ml of MTT solution (Sigma). Cell lines were then incubated at 37°C for 2 hr and exposed to 200  $\mu\text{l}$  of solubilizer solution (90% isopropanol, 20% DMSO, 4% Tween 20), and absorbance was determined at 562 nm.

#### Ex vivo analysis of T cell proliferation and apoptosis

Tumor-draining or distal lymph nodes were obtained from mice injected with knockdown or control transfectants 2 weeks after tumor challenge. Lymph node cells were prepared using standard protocols and analyzed for T cell proliferation, cytokine production, and susceptibility to apoptosis. Briefly, cells ( $5 \times 10^5$ /well) were stimulated in RPMI 1640 complete medium with mitomycin C-treated B16 parental cells ( $5 \times 10^5$ /well) or mitomycin C-treated EL-4 cells ( $5 \times 10^5$ /well). After 5 days, cultures were pulsed with  $1 \mu\text{Ci/ml}$  (methyl-<sup>3</sup>H) thymidine (specific activity 50 Ci/mmol; New England Nuclear) for the last 16 hr. Incorporation of radioactivity was measured in a liquid scintillation  $\beta$ -counter (Packard Instruments) and expressed as cpm  $\pm$  SD of triplicate determinations. Susceptibility to apoptosis was assessed by annexin V and PI staining, following restimulation of lymph node cells with mitomycin C-inactivated B16 cells for 72 hr.

#### Cytokine assays

Lymph node cells ( $5 \times 10^5$ /well) taken from mice inoculated with knockdown or control transfectants were restimulated with or without mitomycin C-inactivated B16 ( $5 \times 10^5$ /well) or EL-4 cells ( $5 \times 10^5$ /well). After 72 hr, cell-free supernatants were collected and subjected to cytokine determination (IFN- $\gamma$ , IL-2, IL-4 and IL-5) using specific capture ELISA kits (Pharmingen), according to the manufacturer's instructions. Data are given as the mean of triplicate measurements of three independent experiments.

#### Statistical analysis

Comparison of two groups was made using Student's *t* test for unpaired data when appropriate. Kaplan-Meier analysis was used to establish statistical

significance for in vivo experiments. The chi-square ( $\chi^2$ ) test was used for analysis of histological data.

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