A Galectin-3 Ligand Corrects the Impaired Function of Human CD4 and CD8 Tumor-Infiltrating Lymphocytes and Favors Tumor Rejection in Mice

Nathalie Demotte¹, Grégoire Wieërs¹, Patrick Van Der Smissen², Muriel Moser⁶, Christopher Schmidt⁶, Kris Thielemans³, Jean-Luc Squifflet⁴, Birgit Weynand⁵, Javier Carrasco⁷, Christophe Lurquin¹, Pierre J. Courtoy², and Pierre van der Bruggen¹

Abstract

Human CD⁸⁺ tumor-infiltrating T lymphocytes (TIL), in contrast with CD⁸⁺ blood cells, show impaired IFN-γ secretion on ex vivo restimulation. We have attributed the impaired IFN-γ secretion to a decreased mobility of T-cell receptors on trapping in a lattice of glycoproteins clustered by extracellular galectin-3. Indeed, we have previously shown that treatment with N-acetyllactosamine, a galectin ligand, restored this secretion. We strengthened this hypothesis here by showing that CD⁸⁺ TIL treated with an anti-galectin-3 antibody had an increased IFN-γ secretion. Moreover, we found that GCS-100, a polysaccharide in clinical development, detached galectin-3 from TIL and boosted cytotoxicity and secretion of different cytokines. Importantly, we observed that not only CD⁸⁺ TIL but also CD⁴⁺ TIL treated with GCS-100 secreted more IFN-γ on ex vivo restimulation. In tumor-bearing mice vaccinated with a tumor antigen, injections of GCS-100 led to tumor rejection in half of the mice, whereas all control mice died. In nonvaccinated mice, GCS-100 had no effect by itself. These results suggest that a combination of galectin-3 ligands and therapeutic vaccination may induce more tumor regressions in cancer patients than vaccination alone.

Introduction

Most cancer patients mount a spontaneous T-cell response against their tumor, and antitumor T cells can accumulate at metastatic sites (1–4). In several tumor types, the presence of T-cell infiltrates, which may contain tumor-specific lymphocytes, seems to correlate with better prognosis (5–7). In patients with advanced cancer, tumors progress despite the presence of tumor-infiltrating lymphocytes (TIL). This suggests that the antitumoral T cells become ineffective either because tumor cells have become resistant to the immune attack or because TILs have become functionally impaired (8, 9).

Despite this potentially adverse tumoral environment, human TIL can easily be expanded in vitro and the resulting T-cell lines and clones can lyse autologous tumor cells (10, 11). However, reports on the ex vivo function of human TIL are scarce. CD⁸⁺ T lymphocytes isolated from renal cell carcinomas displayed no cytotoxicity in a redirected killing assay, in contrast with blood CD⁸⁺ T cells of healthy donors (12). CD⁸⁺ T cells, which were isolated from a metastatic lymph node of a melanoma patient and recognized the modified Melan-A⁴¹ peptide, failed to produce IFN-γ on short-term restimulation with peptide, as opposed to anticytomegalovirus CD⁸⁺ T cells from the same tumors (13). TILs were also shown to differ from blood T cells for gene expression patterns and for several markers such as granzyme B and perforin, suggesting an impaired function (5, 14–17).

We previously described a physical dissociation of T-cell receptors (TCR) from coreceptors CD8 on human TIL and CTL clones with impaired function (18). A galectin ligand, N-acetyllactosamine (LacNAc), restored the ability of nonfunctional T cells to secrete cytokines, and this increased secretion was associated with an increased proximity of surface TCRs with the coreceptor CD8. We therefore attributed the TCR-CD8 dissociation observed on nonfunctional CTL
clones to a reduced mobility of the TCRs, which are trapped in a lattice of glycoproteins clustered by extracellular galectin-3, a lectin abundantly secreted by macrophages and various types of tumor cells (19, 20). Our data suggested that intratumoral galectin-3 could impair T-cell function and that galectin-3 ligands could improve antitumor immunity in vivo.

Several galectin ligands, in particular galectin-3 ligands, have been identified in the past decade, such as polysaccharides extracted from plants or fungi (e.g., ref. 21; a complete list of references is available as Supplementary Data). Synthetic galectin-3 ligands were obtained by decorating lactose and galactose cores with various moieties or by creating branched structures such as glycodendrimers (reviewed in refs. 22, 23). A few of them were reported to reduce the number of tumor metastases in mice (24–29). Only GCS-100, a modified citrus pectin, and DAVANAT, a galactomannan extracted from guar beans, have previously been used in clinical trials (30–32).

We here compared the effect of GCS-100 with that of LacNAc on the cytotoxicity and cytokine secretion by CD8+ and CD4+ human TIL on ex vivo stimulation, and tested the therapeutic potential of GCS-100 in a murine tumor model.

Materials and Methods

Cells, TILs, and reagents

Patient-derived solid tumor samples, tumor ascites, and blood cells were collected after approval by institutional review boards of all collaborating institutions. Cells from ascites were concentrated by centrifugation and either frozen at −80°C or resuspended in culture medium made of Iscove’s modified Dulbecco’s medium (Life Technologies) supplemented with 0.24 mmol/L of L-asparagine, 0.55 mmol/L of L-arginine, 1.5 mmol/L of L-glutamine (AAg), 100 μg/mL of streptomycin, 100 units/mL of penicillin, 30 μg/mL of gentamicin (culture medium; Sigma-Aldrich), and 2% human serum (HS) or ascitic fluid (ascites without cells). Ascitic fluid was frozen at −80°C. Solid tumors were mechanically dissociated and either passed through a 0.1-mm sieve before cryopreservation in 10% DMSO/3.6% HS or incubated for 2 to 3 hours in culture medium supplemented with 0.14 units/mL Liberase Blendzymes (Roche). Mononuclear cells were isolated from the blood of hemochromatosis patients, ascites, or solid tumor samples using Lymphoprep (Axis-Shield PoCAS) and usually frozen at −80°C or −196°C. Cells from ascites were concentrated by centrifugation at 3000 × g for 10 minutes, washed in culture medium supplemented with 2% to 10% HS, 6 IU/mL of interleukin (IL)-2, with 3,000 (ELISA and Bioplex) or 10,000 (ELISpot) beads coated with anti-CD3/CD28 antibodies (Dynabeads, Invitrogen), IFN-γ secreted after overnight coculture was measured by ELISA using Biosource Cytoset reagents (Invitrogen) or by ELISpot (ELISpotPLUS kit ALP; Mabtech) using the AID-ELISpot classic reader software 5.0 (Autoimmun Diagnostika). In the cytoxicity assay, the target cells were murine P1.azα cells derived from mastocytoma cell line P815 (33). Target cells were labeled for 1 hour with 50 μCi of Na[51Cr]O4, washed, and incubated for 15 minutes at room temperature with anti-CD3 monoclonal antibody (OKT3, 1 μg/mL; Mabtech). CD8+ T cells were added to the targets, and chromium release was measured after 4 hours of incubation. For the degranulation assay, CD8+ T cells (75,000) were stimulated for 5 hours in Iscove’s modified Dulbecco’s medium 2% HS AAG with 150,000 mouse cells (P815) incubated with anti-CD3 antibody or 75,000 CD3/CD28 beads. Brefeldin-A (GolgiPlug, Becton Dickinson) and FITC-coupled anti-CD107a and anti-CD107b antibodies, were added after 5 hours of stimulation, cells were washed, labeled for 15 minutes at 4°C with peridinin chlorophyll protein (PerCP)-coupled anti-CD3 (1:40, SK1; Becton Dickinson) and allophycocyanin-coupled anti-CD8 (1:40, RPA-T8; Becton Dickinson) antibodies, washed, and fixed in PBS and 1% paraformaldehyde (PFA). Cells were analyzed on a FACScalibur (Becton Dickinson). The percentage of CD107+ cells was estimated for the CD3+CD8+ T cells.

Detachment of galectins

Cells from ascites were treated for 15 minutes at 4°C with Fc blocking reagent (Miltenyi Biotec) before treatment with anti–galectin-1 or anti–galectin-3 antibodies. Melanoma cell line MZ2-MEL43 and cells from ascites were incubated for 2 hours at 37°C under agitation with LacNAc (5 mmol/L), GCS-100 (25 or 5 μg/mL), or B2C10 (mouse IgG2a; Becton Dickinson); fixed for 5 minutes at room temperature in PBS with 0.5 mg/mL dithio-bis-succinimidyld propionate (Thermo Scientific); washed; incubated for 20 minutes at room temperature with 25 mmol/L of glycine; washed again; and incubated with 5 μg/mL of either anti–galectin-3 antibody M3/38 (rat IgG2a, BioLegend) or polyclonal anti–galectin-1 rabbit IgG (Abcam). Cells were further incubated at 4°C for 15 minutes, washed, and incubated for 15 minutes at 4°C with relevant anti-immunoglobulin secondary antibodies coupled to Alexa Fluor 488 (Invitrogen), together

recombinant IL-2 was from Chiron Healthcare SAS, IL-7 was from R&D Systems, LacNAc was from Carboxynth, and unmodified citrus pectin was from Sigma. GCS-100 was provided by Prospect Therapeutics, Inc. (now Solana Therapeutics). All the cell lines were obtained several years ago in our laboratory and described previously, but the cell lines were not authenticated according to guidelines described in http://cancerres.aacrjournals.org/site/misc/ifora.xhtml.

Functional assays

Polyclonal stimulations were performed in round-bottomed microwells with 10,000 T cells in 200 μL of culture medium, containing 0–6 IU/mL of IL-2, with 3,000 (ELISA and Bioplex) or 10,000 (ELISpot) beads coated with anti-CD3/CD28 antibodies (Dynabeads, Invitrogen). IFN-γ secreted after overnight coculture was measured by ELISA using Biosource Cytoset reagents (Invitrogen) or by ELISpot (ELISpotPLUS kit ALP; Mabtech) using the AID-ELISpot classic reader software 5.0 (Autoimmun Diagnostika). In the cytoxicity assay, the target cells were murine P1.azα cells derived from mastocytoma cell line P815 (33). Target cells were labeled for 1 hour with 50 μCi of Na[51Cr]O4, washed, and incubated for 15 minutes at room temperature with anti-CD3 monoclonal antibody (OKT3, 1 μg/mL; Mabtech). CD8+ T cells were added to the targets, and chromium release was measured after 4 hours of incubation. For the degranulation assay, CD8+ T cells (75,000) were stimulated for 5 hours in Iscove’s modified Dulbecco’s medium 2% HS AAG with 150,000 mouse cells (P815) incubated with anti-CD3 antibody or 75,000 CD3/CD28 beads. Brefeldin-A (GolgiPlug, Becton Dickinson) and FITC-coupled anti-CD107a and anti-CD107b antibodies, were added after 5 hours of stimulation, cells were washed, labeled for 15 minutes at 4°C with peridinin chlorophyll protein (PerCP)-coupled anti-CD3 (1:40, SK1; Becton Dickinson) and allophycocyanin-coupled anti-CD8 (1:40, RPA-T8; Becton Dickinson) antibodies, washed, and fixed in PBS and 1% paraformaldehyde (PFA). Cells were analyzed on a FACScalibur (Becton Dickinson). The percentage of CD107+ cells was estimated for the CD3+CD8+ T cells.

Galectin-3 Ligand Corrects the Impaired Function of TIL

Cancer Res; 70(19) October 1, 2010 www.aacrjournals.org

Published OnlineFirst August 18, 2010; DOI: 10.1158/0008-5472.CAN-10-0761
Figure 1. GCS-100 boosted IFN-γ secretion by CD8+ and CD4+ TIL. Columns, mean of triplicate measurements; bars, SD. Nonstimulated TIL or PBL, whether treated or not, secreted <15 pg/mL of IFN-γ. A, CD8+ TILs were isolated from ascites (ovary, colon, pancreas, and melanoma VUB88), breast carcinoma pleural effusion, and solid melanomas. B, CD8+ PBLs were isolated from blood samples from hemochromatosis patients. C, CD4+ TILs were isolated from ascites or solid melanomas. CD8+ and CD4+ T cells were treated for 2 h (bold) or 20 h with 5 mmol/L of LacNAc or 5 μg/mL of GCS-100, and then 10,000 cells were cultured overnight in 200 μL of culture medium with 3,000 CD3/CD28 beads. The accumulation of IFN-γ in the supernatant was measured by ELISA. D, the number of IFN-γ–secreting cells among 10,000 cells was estimated by ELISpot.
Figure 2. Efficacy of GCS-100 treatment in different culture conditions. A, CD8^+ TILs were isolated from ascites and treated for 2 h (bold) or 20 h with 2 to 5 mmol/L of LacNAc or 5 μg/mL of GCS-100 in culture medium containing 20% to 25% ascitic fluid, and 10,000 cells were stimulated with beads as described in Fig. 1. B, frozen cells collected from ascites were thawed and incubated for 2 h in culture medium and then resuspended in ascitic fluid or in culture medium containing 2% HS throughout the following procedures. Cells were incubated for 2 to 20 h in the presence of LacNAc (5 mmol/L), GCS-100 (5 μg/mL), or medium. CD8^+ cells were isolated by positive selection, resuspended at 10,000 cells per round-bottomed microwell with LacNAc or GCS-100, and stimulated as described in Fig. 1.
with anti–CD3/PerCP (1:40, SK7; Becton Dickinson) and anti–CD8-Alexa Fluor 647 (1:40, 38B; Invitrogen). After a final washing step, cells were fixed with 2% PFA in PBS and analyzed on a FACSCalibur. We attributed a molecular weight of 120 kDa (average) to GCS-100, according to the molecular weight described for another modified pectin by Miller and colleagues (34).

Fluorescence resonance energy transfer microscopy

A detailed protocol is available as Supplementary Data. The following antibodies were used: anti–TCRβ (1:40, IP-26, mouse IgG1: eBioscience), anti–CD8α (1:40, UCHT-4, mouse IgG2a; Sigma-Aldrich), anti–CD4 (1:40, RPA-T4, mouse IgG1; eBioscience), anti–IgG2a-Alexa Fluor 488 (1:400, donor, green; Invitrogen), and anti–IgG1-Alexa Fluor 568 secondary antibodies (1:400, acceptor, red; Invitrogen).

Mice vaccination and GCS-100

P815 clone 3 cells were expanded in DMEM supplemented with AAG, 10% FCS, glucose, and 75 μmol/L of mercaptoethanol (33). The recombinant adenovirus construct contains the coding sequence of a fusion protein combining the first 81 amino acids of the mouse invariant chain with the first 83 amino acids of P1A, a protein containing an antigenic peptide, which is a tumor rejection antigen of P815 (35).

Results

GCS-100 boosts IFN-γ secretion by CD8+ and CD4+ TILs

CD8+ TILs were isolated to >90% purity from 18 tumors: 5 solid melanomas and 1 melanoma ascites, 1 breast carcinoma pleural effusion, and 11 carcinoma ascites from different histologic origins. The lymphocytes were incubated with GCS-100 or LacNac for 2 to 20 hours and then stimulated by the addition of beads coated with anti-CD3 and anti-CD28 antibodies (CD3/CD28 beads). IFN-γ secretion by nontreated TIL isolated from four of five solid tumors was undetectable, whereas secretion by nontreated TIL isolated from ascites was more variable. The presence of GCS-100 boosts IFN-γ secretion by >3-fold in 13 of 18 cultures, and the average increase in IFN-γ secretion for all cultures was 4-fold (Fig. 1A). Treatment with LacNac instead of GCS-100 had a similar effect (Fig. 1A). When the same GCS-100 or LacNac treatment was applied to blood CD8+ T cells, the increase in IFN-γ secretion was marginal (Fig. 1B). We concluded that CD8+ TILs, compared with normal blood CD8+ T cells, are often impaired in their ability to secrete IFN-γ on stimulation. However, this defect can be reverted, at least in part, with the addition of GCS-100 or LacNac.

In our previous report (18), CD4+ TILs were not analyzed. CD4+ TILs were therefore isolated from 13 of the 18 TIL samples shown in Fig. 1. They were also incubated with GCS-100 or LacNac and then stimulated by the addition of CD3/CD28 beads. In the presence of GCS-100, IFN-γ secretion was increased by >3-fold in 6 of 13 samples (Fig. 1C). We conclude that the impaired secretion of IFN-γ can be corrected by GCS-100 and LacNac not only for CD8+ TIL but also for CD4+ TIL.

To estimate the number of IFN-γ–secreting T cells, we performed ELISpot experiments with a few TIL and blood samples. Isolated CD8+ and CD4+ T cells were incubated overnight with GCS-100 or LacNac and then stimulated by the addition of CD3/CD28 beads. After sugar treatment, the number of IFN-γ–secreting cells increased by a factor of 2 for the TIL, whereas the sugar treatment had a marginal effect on the blood CD8 T cells (Fig. 1D).

To increase TIL function in patients, GCS-100 has to be effective in a tumor environment, despite the presence of suppressive factors found in ascitic fluid and cells that could inhibit TIL, such as tumor cells or regulatory T cells. To mimic these situations, two types of experiments were conducted. First, nine CD8+ TIL populations were incubated with galex-tin ligands as above and stimulated in medium supplemented with 20% to 25% of cell-free carcinomatous ascitic fluid. IFN-γ secretion increased >3-fold in five of nine samples (Fig. 2A). Second, the effect of LacNac and GCS-100 was tested on either CD2+ cells (including natural killer cells, CD8+, and CD4+ T cells) or on the total cell population, further containing macrophages and tumor cells. The cells were resuspended either in culture medium or in ascitic fluid. CD8+ TILs were purified after a first GCS-100 or LacNac treatment and then stimulated with CD3/CD28 beads in the presence of LacNac or GCS-100 (Fig. 2B). The results suggest that both LacNac and GCS-100 can boost IFN-γ secretion by CD8+ TIL even in the presence of either carcinomatous ascitic fluid or other cells present in ascites (Fig. 2B).

GCS-100 boosts other functions of CD8+ and CD4+ TILs

In addition to IFN-γ secretion, several other TIL functions were also tested (i.e., secretion of other cytokines, cytotoxicity, and degranulation). In comparison with untreated cells, GCS-100– and LacNac-treated cells also secreted more IL-2 and tumor necrosis factor-α, but no IL-4 (Supplementary Table S1). With some TIL samples, we observed slightly more secretion of IL-10 and IL-17 after treatment. Cytokine secretion was similar for treated and untreated blood CD8+ T cells.

We estimated the cytotoxicity of CD8+ TIL using mouse target cells coated with an anti-CD3 antibody (Fig. 3A). Only the CD8+ TIL treated with GCS-100 had a significant cytotoxicity, whereas the cytotoxicity of untreated CD8+ TIL was minimal. The cytotoxicity of CD8+ peripheral blood lymphocytes (PBL) was higher than that of TIL, but similar for treated and untreated cells. We also measured degranulation, a prerequisite for cytosis. The percentages of degranulating cells were identified by surface expression of CD107a and CD107b, which reside in cytolytic granule membranes within the cytoplasm and are mobilized to the cell surface following activation-induced granule exocytosis (Fig. 3B). The percentage of CD107+ cells was much higher in treated TIL compared with untreated TIL, whereas this percentage was equivalent for treated and untreated blood CD8+ cells. Finally, we...
measured the intracellular content of perforin and granzyme B as phenotypic markers of cells that might have a potential to kill. Both treated and untreated TILs have a similar content of the two enzymes, indicating that the boosting effect of GCS-100 and LacNAc treatment was not due to an effect on perforin or granzyme content (Supplementary Fig. S1).

The toxicity of GCS-100 was evaluated with human melanoma cells and T-cell clones. Their proliferation was measured for at least 4 weeks in the presence of various concentrations of GCS-100. Growth inhibition was observed starting at day 7 at a concentration of 125 μg/mL (Supplementary Fig. S2). At 5 μg/mL, which was efficient in augmenting cytokine secretion by TIL, GCS-100 had no effect on proliferation.
An anti–galectin-3 antibody promotes IFN-γ secretion by stimulated CD8+ TILs

Our working hypothesis for impaired IFN-γ secretion by CD8+ TIL on ex vivo stimulation is that galectin-3, which can be secreted by tumor cells and macrophages, binds to glycoproteins and oligomerizes on the T-cell surface, resulting in galectin–glycoprotein lattices that decrease TCR mobility (36). This hypothesis was also based on the dissociation of TCR from CD8 coreceptors observed on nonfunctional CTL clones and the increased TCR/CD8 colocalization after LacNAc treatment (18). However, because GCS-100 and LacNAc could compete with several galectins, we tested the effect of an anti–galectin-3 monoclonal antibody, B2C10, which binds to the NH2-terminal domain of galectin-3 and presumably affects the oligomerization of the lectin: blocking this process should therefore decrease the T-cell inhibitory activity of galectin-3 (37). CD8+ TILs were incubated and stimulated in the presence of either antibody B2C10, a control antibody as negative control, or GCS-100 as a positive control (Fig. 4A). B2C10 boosted IFN-γ secretion to a comparable extent as GCS-100.

Other galectins (i.e., galectin-1 and galectin-9) were detected in human and/or murine tumors and thought to play a role in antitumor immunity (reviewed in ref. 19). We failed to detect galectin-8, galectin-9, or MGL (a lectin implicated in the regulation of T-cell function) on TIL (38). However, in addition to galectin-3, we detected galectin-1 (Fig. 5). We compared GCS-100 and LacNAc for their ability to detach galectin-3 and galectin-1 from melanoma cells that carry both galectins (Supplementary Fig. S3). The concentrations of GCS-100 that were able to detach 50% (EC50) of the surface galectin-3 and galectin-1 were estimated at 1.3 μmol/L (159 μg/mL) and 2 μmol/L, respectively. EC50s were estimated for LacNAc at 9 and 18 μmol/L, respectively. Different modified citrus pectins and synthetic ligands were previously shown to detach galectin-3 in a solid-phase assay with EC50 as low as 1 mmol/L (22, 39–42). The most effective synthetic galectin-3 ligand seems to be a thiodigalactoside derivative that has a Kd of 33 nmol/L (39).

Incubation of TIL for 2 hours with 5 mmol/L of LacNAc, 25 μg/mL of GCS-100, or 5 μg/mL of GCS-100 resulted in a decrease of galectin-3 staining by 67%, 40%, and 24%, respectively (Fig. 5). Staining of galectin-1 did not decrease after GCS-100 treatment, whereas it decreased by 38% after LacNAc treatment. Treatment with anti–galectin-3 antibody B2C10 detached galectin-3 but not galectin-1 (Fig. 4B). We failed to detach galectin-1 by treating TIL with three different anti–galectin-1 antibodies, and these treatments did not boost IFN-γ secretion either (data not shown). Because the anti–galectin-3 antibody seemed unable to detach galectin-1 while boosting TIL function, we conclude that detaching galectin-3 from TIL is sufficient to restore function while not excluding the contribution of other galectins.

GCS-100 treatment promotes colocalization of TCR and coreceptors CD8 or CD4

We have previously used a flow cytometry–based fluorescence resonance energy transfer approach to show a loss of
colocalization of TCR and CD8 at the surface of CD8+ TIL compared with blood CD8+ T cells (18). We examined here the effect of GCS-100 or LacNAc on this colocalization in both CD8+ and CD4+ TIL using a microscopy-based fluorescence resonance energy transfer approach known as “acceptor photobleaching.” TILs were treated for 2 hours with GCS-100 or LacNAc, attached to coverslips, and labeled with an anti-TCRβ antibody coupled to an acceptor fluorochrome and anti-CD8α or anti-CD4 antibodies coupled to a donor fluorochrome. Upon excitation at donor wavelength, energy could be transferred from the donor to the acceptor if closer than ∼10 nm. In these conditions, full acceptor bleaching abrogates the energy loss, thus increasing donor emission. For each analyzed cell, three regions of the membrane were bleached and donor emission increases were measured. Data are shown in Table 1 for two CD8+ and two CD4+ TIL samples and for two cells in each condition.

When CD8+ TILs were not treated with LacNAc or GCS-100, no increase in donor emission was detected upon acceptor photobleaching, indicating poor TCR-CD8 colocalization (Table 1). For TIL VUB147, increases in donor emission were 20% and 14% after incubation for 2 hours with GCS-100 and LacNAc, respectively, thus indicating increased TCR-CD8 colocalization after treatment. Lower but significant increases were observed with TIL LB3022. Although modest at first sight, these increases are well within the reported range in the literature and similar to our previously reported value (26%) with a functional CTL clone (18, 43, 44).

For CD4+ TILs not treated with LacNAc or GCS-100, there was also no increase in donor emission detected upon acceptor photobleaching (Table 1). After 2 hours of treatment of TIL VUB129, increases were 11% with GCS-100 and 11% with LacNAc. Comparable values were obtained with TIL LB3022. For both CD4+ and CD8+ TILs treated with GCS-100 or LacNAc, increased TCR/CD8 or TCR/CD4 colocalization was paralleled by increased secretion of IFN-γ upon ex vivo restimulation (Table 1).

Vaccinated tumor-bearing mice that were also treated with GCS-100 reject their tumors

We tested the therapeutic effect of GCS-100 in tumor-bearing mice. Forty mice were injected s.c. in the flank with 2 × 10⁶ P815 mastocytoma cells. On day 4, half of the animals were vaccinated with an adenovirus encoding P815 tumor antigen P1A (Fig. 6A; ref. 35). On day 10, at which time all mice, vaccinated or not, bore a tumor of ∼70 mm², treatments with either PBS or GCS-100 (200 μg s.c. close to the tumor and 500 μg i.p.) were initiated. Mice were injected three times a week. Three weeks later, the tumor had become undetectable in 6 of the 10 vaccinated mice treated with GCS-100, of which 5 were still alive after another 3 months. Vaccine alone had no effect on tumor growth, but slightly increased survival, with a median life span 10 days longer than in nonvaccinated mice. In nonvaccinated mice, GCS-100 had no effect by itself, either on tumor size or on animal survival (Fig. 6B and C).

Discussion

Our previous observations that human CD8 TIL increased their secretion of IFN-γ after ex vivo treatment with disaccharide LacNAc prompted us to test GCS-100, a polymeric galectin ligand approved for clinical trials. The results reported here indicate that GCS-100 is as efficient as LacNAc in boosting TIL to be cytotoxic and to secrete different cytokines, not only CD8+ but also CD4+ TIL. This effect was obtained
at concentrations (5 μg/mL) much lower than those used to induce apoptosis of tumor cells (>100 μg/mL), the activity that prompted its administration to cancer patients (30).

Why do galectin-3 ligands improve human TIL function? Our working hypothesis is that TILs have been stimulated by antigen recently and that activation could modify the expression of enzymes of the N-glycosylation pathway and change the structure of N-glycans exposed at the cell surface, as shown for murine T cells (45). We surmise that the recently activated TILs, compared with resting T cells, harbor a set of glycans that are either more numerous or better ligands for galectin-3. Galectin-3 is abundant in many solid tumors and carcinomatous ascites and can form lattices with surface glycoproteins that would thereby reduce TCR mobility. This could explain the absence of colocalization of TCR and CD4/CD8 coreceptors, the impaired function of TIL, and the boosting effect of galectin ligands.

Several of our new results support this hypothesis. First, treatment with an anti–galectin-3 antibody that was able to detach galectin-3 from TIL is as efficient as GCS-100 in increasing IFN-γ secretion by TIL. Second, both GCS-100 and LacNAc can detach galectin-3 from the surface of cells. Third, treatment of TIL with GCS-100 or LacNAc increased both IFN-γ secretion and colocalization of TCR with coreceptors CD8 or CD4. In this respect, the increased TCR-CD4 colocalization after GCS-100 or LacNAc treatment suggests a common

### Table 1. Colocalization of TCR and coreceptors CD8 or CD4 on TIL

<table>
<thead>
<tr>
<th></th>
<th>TIL CD8 VUB147</th>
<th>LacNAc</th>
<th>TIL CD8 LB3022</th>
<th>LacNAc</th>
<th>TIL CD4 VUB129</th>
<th>LacNAc</th>
<th>TIL CD4 LB3022</th>
<th>LacNAc</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>/</td>
<td>GCS-100</td>
<td>/</td>
<td>GCS-100</td>
<td>/</td>
<td>GCS-100</td>
<td>/</td>
<td>GCS-100</td>
</tr>
<tr>
<td>Increase in donor emission (%)</td>
<td>Cell 1</td>
<td>Cell 2</td>
<td>Cell 1</td>
<td>Cell 2</td>
<td>Cell 1</td>
<td>Cell 2</td>
<td>Cell 1</td>
<td>Cell 2</td>
</tr>
<tr>
<td>1</td>
<td>−5</td>
<td>0</td>
<td>20</td>
<td>22</td>
<td>8</td>
<td>14</td>
<td>−1</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>−13</td>
<td>5</td>
<td>16</td>
<td>13</td>
<td>9</td>
<td>15</td>
<td>3</td>
<td>−6</td>
</tr>
<tr>
<td>3</td>
<td>−10</td>
<td>3</td>
<td>19</td>
<td>32</td>
<td>15</td>
<td>20</td>
<td>−4</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>−3</td>
<td>20</td>
<td>14</td>
<td>−1</td>
<td>8</td>
<td>15</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Secretion of IFN-γ by TIL population (pg/mL)

<table>
<thead>
<tr>
<th></th>
<th>TIL CD4 VUB129</th>
<th>LacNAc</th>
<th>TIL CD4 LB3022</th>
<th>LacNAc</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>/</td>
<td>GCS-100</td>
<td>/</td>
<td>GCS-100</td>
</tr>
<tr>
<td>Increase in donor emission (%)</td>
<td>Cell 1</td>
<td>Cell 2</td>
<td>Cell 1</td>
<td>Cell 2</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>1</td>
<td>8</td>
<td>11</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>1</td>
<td>20</td>
<td>9</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>1</td>
<td>11</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>11</td>
<td>11</td>
<td>−1</td>
</tr>
</tbody>
</table>

Secretion of IFN-γ by TIL population (pg/mL)

<table>
<thead>
<tr>
<th></th>
<th>TIL CD4 VUB129</th>
<th>LacNAc</th>
<th>TIL CD4 LB3022</th>
<th>LacNAc</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>/</td>
<td>GCS-100</td>
<td>/</td>
<td>GCS-100</td>
</tr>
<tr>
<td>Increase in donor emission (%)</td>
<td>Cell 1</td>
<td>Cell 2</td>
<td>Cell 1</td>
<td>Cell 2</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>102</td>
<td>153</td>
<td>12</td>
</tr>
</tbody>
</table>

NOTE: CD8⁺ or CD4⁺ TILs were isolated from ascites of carcinoma patients, frozen, and thawed 2 h before the experiment. Cells were subsequently incubated for 2 h at 37°C with medium containing 10% ascitic supernatant in the presence of 2 mmol/L LacNAc or 5 μg/mL GCS-100. After washing, some cells were tested for IFN-γ secretion, and the others were attached to coverslips, labeled on ice with anti-TCR antibodies (acceptor) and anti-CD8 or anti-CD4 antibodies (donor), fixed, and stained with secondary antibodies. For each of the two cells that were analyzed, in three regions of interest, five images were recorded before bleaching and five others after bleaching of the acceptor fluorochrome. Three control regions were not bleached. Increase in donor emission was calculated as follows: [(1 − (donor intensity before bleaching/donor intensity after bleaching)) × 100. For CD8⁺ TIL, mean increase in donor emission (in percentage) was −2.1 ± 1.6 for nontreated TIL, 14.5 ± 2.2 (P < 0.001, Mann-Whitney test) for GCS-100–treated TIL, and 14.4 ± 1.5 (P < 0.001) for LacNAc–treated TIL. For CD4⁺ TIL, mean increase in donor emission was 0.1 ± 0.7 for nontreated TIL, 13.8 ± 2.6 (P < 0.001, Mann-Whitney test) for GCS-100–treated TIL, and 9.3 ± 1.3 (P < 0.001) for LacNAc–treated TIL. Secretion of IFN-γ was analyzed as described in Fig. 1.
Figure 6. Tumor-bearing mice vaccinated against a tumor antigen and treated with GCS-100 survived longer and rejected tumors. A, forty 7-wk-old DBA2 mice were injected s.c. in the flank with $2 \times 10^6$ cells of mastocytoma P815 clone 3. Four days later, half of the mice were vaccinated with an adenovirus encoding P1A by i.d. injections, half a dose in each ear. In a double-blinded study, mice received, from days 10 to 62, 700 µg GCS-100 three times a week; 200 µg were injected s.c. in the periphery of the tumor and 500 µg i.p. B, Kaplan-Meier survival curves. The experiment was performed once ($n = 10$ per group; $P < 0.001$, log-rank test). C, tumor size indicated as the product of the longest and the shortest tumor lengths.
This hypothesis agrees well with data from two other groups. Demetriou and colleagues observed that T cells from mice deficient in N-acetylgalactosaminyl-transferase V, an enzyme of the N-glycosylation pathway, have a lower activation threshold than T cells from wild-type mice. The authors were the first to propose that reduced N-glycosylation, resulting in looser glycoprotein/galectin lattices, could improve TCR clustering at the immune synapse (46). In addition, Kuball and colleagues (47) observed an increased frequency of cells able to secrete cytokines upon stimulation, if they were expressing a transfected TCR lacking a glycosylation site. They proposed therefore that N-glycosylation modifies TCR avidity.

Other galectins (i.e., galectin-1 and galectin-9) were detected in human and/or murine tumors and thought to play a role in antitumor immunity (reviewed in ref. 19). In humans, exposure to galectin-1 induced a shift from Th1 to Th2 cytokine response, promoted the expansion of CD4+CD25highFOXP3+ T cells, and increased the tolerogenic potential of dendritic cells (41, 48–50). Galectin-1 was reported to induce T lymphocyte apoptosis (48). In mice, tumor growth and immune dysfunction correlated with the presence of galectin-1 (51). On the contrary, injections of galectin-9 were reported to improve the survival of tumor-bearing mice (52). Here, we show that, in addition to galectin-3, we also detected galectin-1 on TIL. At the concentration used for treating TIL, LacNAc but not GCS-100 was able to detach galectin-1 from treated TIL. Because the anti–galectin-3 antibody did not detach galectin-1 from TIL, we conclude that the mere detachment of galectin-3 could result in a boosted secretion of IFN-γ by stimulated TIL.

Impaired TIL function most probably results from a combination of mechanisms, all ultimately depending on tumor cells. It is therefore remarkable that a few hours of treatment with GCS-100 or LacNAc was sufficient to strongly increase IFN-γ secretion by stimulated TIL. However, the respective contribution of other galectins, transforming growth factor-β, IL-10, PD-1/PD-L1, or regulatory T cells, remains important to clarify in order to define whether different lymphocyte populations are impaired through different mechanisms and whether several inhibitory pathways add up in the same cells.

The identification of antigens recognized by T lymphocytes on human tumor cells has resulted in numerous clinical trials involving the vaccination of tumor-bearing patients with defined tumor antigens. Among vaccinated metastatic melanoma patients, ~5% show a complete or partial clinical response, whereas an additional 10% show some evidence of tumor regression without clear clinical benefit. Analyses of the T-cell responses of melanoma patients suggest the following scenario (1, 53). Spontaneous tumor-specific T-cell responses are frequent. Although they could control early tumors, they eventually fail to do so and become impaired in their function, most probably due to immunosuppressive factors present in the tumor. Galectin-3 could be one of them. Thus, the tumors of the patients about to receive the vaccine already contain functionally impaired T cells directed against tumor antigens. It is therefore possible that, in the few patients who show tumor regression following vaccination, some T cells generated by the vaccine reach the tumor and succeed in reversing the local immunosuppression.

The relevance of combining vaccination and galectin ligands is supported by the results obtained with GCS-100 in vaccinated mice bearing P815 tumors. Previous results indicated that prophylactic vaccination with an adenovirus encoding P815 tumor antigen P1A induced an anti-P1A T-cell response that conferred protection against tumor challenge in 57% of mice (54). However, therapeutic vaccination was ineffective at inducing tumor rejection in tumor-bearing mice.9 Injection in mice bearing a subcutaneous P815 tumor of a Semliki Forest virus encoding P1A was therapeutic for 4 of 10 animals, provided that tumors were small (<25 mm²) and that intratumoral injections were used (55). In the present report, injection of another P1A adenoviral construct conferred only a mild survival advantage. However, in combination with GCS-100 injections, half of the mice rejected their tumor and appeared free of disease on day 130. The vaccination of tumor-bearing mice with P1A was essential for tumor rejection, and we are tempted to believe that GCS-100 positively affected the anti-P1A T cells.

These experiments, however, do not provide any evidence that GCS-100 exerts its therapeutic effects in mice by acting on murine T cells or by disrupting galectin-glycan lattices. Galectins were shown to play pleiotropic roles in tumor metabolism. Myeloma cell growth was inhibited in vitro in the presence of 500 μg/mL of GCS-100, which also triggered apoptosis and decreased cell migration in response to vascular endothelial growth factor (30, 56). Modified citrus pectin inhibited the homoagglutination of melanoma cells, adhesion of tumor cells to extracellular matrix and endothelial cells, as well as the formation of capillary tubes in the presence of galectin-3 (reviewed in ref. 57). Although treatment of nonvaccinated, tumor-bearing mice with GCS-100 did not provide a survival benefit compared with control mice, GCS-100 could have increased the apoptosis of tumor cells and inhibited neoangiogenesis and metastasis formation and therefore participated in the therapeutic effect independently of an effect on the immune system.

It might seem risky to inject a galectin-3 ligand systemically, considering the pleiotropic role of galectin-3 (58, 59). However, no life-threatening side effect was observed in a clinical trial with GCS-100. GCS-100 has been administered to cancer patients based on its potential to induce apoptosis of myeloma cells in vitro (30). Twenty-four patients with chronic lymphocytic leukemia, who had relapsed after one or two therapies, received 160 mg/m² of GCS-100 i.v. for 5 days every 21 days. Six of them experienced a partial clinical response. The treatment was generally well tolerated with minimal hemato logic toxicity. Skin rash was observed on two patients that resolved with cessation of treatment or steroids.

9 C. Uyttenhove and G. Warnier, personal communication.
Taken together, our observations suggest that treatment of cancer patients with galectin ligands could correct an impaired function of TIL. As observed in mice, it is possible that vaccination combined with local and/or systemic injection of a galectin ligand would be more effective at producing tumor regressions than vaccination alone.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Prospect Therapeutics (now Solana Therapeutics), and in particular F. Tao, for providing us with GCS-100; D. Latinne and A. Cornet for ELISpot experiments; D. Godelaine, P. Coulie, T. Boon, and B. Van den Eynde for critical reading; C. Uyttenhove for helpful suggestions; G. Warnier for providing the recombinant adenovirus; E. Marbaix for help in statistical analysis; N. Krack for editorial assistance; and L. Vanbiervliet, S. Ottaviani, C. Wildmann, V. Ha Thi, and T. Lac for their assistance.

Grant Support

Fondation contre le Cancer (Belgium), Fonds de la Recherche Scientifique Médicale (Belgium), European Community Sixth Framework Programme, LIFE/HEALTH-5-IP (contract number 518234), and Delori family. P. van der Bruggen is a fellow du fonds Alland-Jansen for la recherche sur le cancer. G. Wiërs is supported by a grant from the Fonds National de la Recherche Scientifique (Belgium). The cell imaging platform was funded by FNRS, Région Bruxelloise, RégionWallonne, Loterie Nationale, Université Catholique de Louvain (Belgium).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

References

34. Miller NC, Nesmelova IV, Platt D, Klyosov A, Mayo KH. The carbo-
hydrate-binding domain on galectin-1 is more extensive for a complex glycan than for simple saccharides: implications for galectin-glycan interactions at the cell surface. Biochem J 2009; 421:211–21.
36. Ahmad N, Gabius HJ, Andre S, et al. Galectin-3 precipitates as a pentamer with synthetic multivalent carbohydrates and forms hetero-

38. van Vliet SJ, Gringhuis SI, Geijtenbeek TB, van Kooyk Y. Regulation
of effector T cells by antigen-presenting cells via interaction of the
41. Iurisci I, Cumashi A, Sherman AA, et al. Synthetic inhibitors of
43. Anikovsky M, Dale L, Ferguson S, Petersen N. Resonance energy
44. Elder AD, Domai A, Kaminski Schierle GS, et al. A quantitative pro-
tocol for dynamic measurements of protein interactions by Förster
46. Demetriou M, Granovsky M, Quaggin S, Dennis JW. Negative
regulation of T-cell activation and autoimmunity by Mgat5 N-
47. Kuball J, Hauproetz B, Malina V, et al. Increasing functional avidity of
TCR-directed T cells by remodeling defined N-glycosylation sites in
distinct cell surface glycoprotein receptors to induce T cell death.
49. Igarregui JM, Croci DO, Bianco GA, et al. Tolerogenic signals deliv-
ered by dendritic cells to T cells through a galectin-1-driven immu-
noregulatory circuit involving interleukin 27 and interleukin 10.
50. Motran CC, Molinder KM, Liu SD, Poirier F, Miceli MC. Galectin-
51. Rubinstein N, Alvarez M, Zwimer NW, et al. Targeted inhibition of
galectin-1 gene expression in tumor cells results in heightened T

cell-mediated rejection: a potential mechanism of tumor-immune
dendritic cells and CD8+ T cells and enhances antitumor immunity
T cells in the blood of melanoma patients before and after vaccina-
boost vaccinations using Semliki Forest virus, adenovirus, and
ALVAC vectors demonstrate differences in the generation of a
protective central memory CTL response against the P815 tumor. J
55. Colmenero P, Chen M, Castanos-Velez E, Liljestrom P, Jondal M.
Immunotherapy with recombinant SFV-replicons expressing the
P815A tumor antigen or IL-12 induces tumor regression. Int J Cancer
antagonist, modulates MCL-1, NOXA, cell cycle to induce myeloma
57. Gilszky GV, Raz A. Modified citrus pectin anti-metastatic properties:
59. Demetriou M, Granovsky M, Quaggin S, Dennis JW. Negative
regulation of T-cell activation and autoimmunity by Mgat5 N-
A Galectin-3 Ligand Corrects the Impaired Function of Human CD4 and CD8 Tumor-Infiltrating Lymphocytes and Favors Tumor Rejection in Mice

Nathalie Demotte, Grégoire Wieërs, Patrick Van Der Smissen, et al.

*Cancer Res* 2010;70:7476-7488. Published OnlineFirst August 18, 2010.

Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-10-0761

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2010/08/18/0008-5472.CAN-10-0761.DC1

Cited articles
This article cites 59 articles, 21 of which you can access for free at:
http://cancerres.aacrjournals.org/content/70/19/7476.full#ref-list-1

Citing articles
This article has been cited by 14 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/70/19/7476.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.