Cancer Therapy: Preclinical

A Short Treatment with Galactomannan GM-CT-01 Corrects the Functions of Freshly Isolated Human Tumor-Infiltrating Lymphocytes

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Abstract

**Purpose:** Several galectins are released by tumor cells and macrophages and accumulate in the tumor microenvironment. Galectin-1 and -3 were found to bind to glycosylated receptors at the surface of tumor-infiltrating lymphocytes (TIL), forming glycoprotein–galectin lattices that could reduce the motility and therefore the functionality of surface molecules. In contrast to blood T cells, human TIL show defective IFN-γ secretion upon ex vivo stimulation. We have previously shown that extracellular galectin-3 participates in the impairment of TIL functions. Indeed, disruption of glycoprotein–galectin-3 lattices using anti-galectin-3 antibodies, or N-acetyllactosamine as a competing sugar, boosted cytokine secretion by TIL. Here we have tested a clinical grade galectin antagonist: GM-CT-01, a galactomannan obtained from guar gum reported to be safe in more than 50 patients with cancer.

**Experimental Design:** TIL were isolated from human tumor ascites, treated for 2 to 20 hours with galectin antagonists and tested for function.

**Results:** We found that GM-CT-01 boosts cytotoxicity of CD8+ TIL and their IFN-γ secretion in a dose-dependent manner. Treating TIL obtained from patients with various cancers, during a few hours, resulted in an increased IFN-γ secretion in up to 80% of the samples.

**Conclusions:** These observations pave the way for investigating the potential benefit of this galectin antagonist in patients with cancer, alone or combined with cancer vaccination, in order to correct in vivo impaired functions of TIL. Clin Clin Cancer Res; 20(7); 1823–33. ©2014 AACR.

Introduction

Accumulation of human tumor–infiltrating lymphocytes (TIL) is considered as a good prognostic factor (reviewed in Wieers; ref. 1). Moreover, when gene expression was analyzed in tumor samples of patients from 3 active immunization clinical trials, a gene signature, including T-cell markers, correlated with clinical responses (2–4). However, TIL freshly isolated—without in vitro expansion—from various human tumor samples (melanomas, renal cell carcinomas, ovarian, and pancreatic carcinomas) often proved defective in lyzing relevant target cells and producing IFN-γ upon stimulation. This contrasted with blood T cells isolated from the same patients, which were readily cytotoxic and showed robust IFN-γ secretion (3, 5–9).

Several immunosuppressive mechanisms have been proposed to explain the impaired functions of freshly isolated TIL. First, TIL can express inhibitory receptors that down-modulate T-cell activation upon antigen recognition, for example PD1, KIR, BTLA, CTLA-4, Tim-3 (10–13). Blocking these receptors with antibodies has also shown to prolong survival of T cells and to boost their proliferation upon activation in vitro. Such antibodies have also shown their efficacy in vivo, as an objective-response rate of 40% was observed in a trial where 53 patients with advanced melanoma received combined injections of ipilimumab and nivolumab targeting CTLA-4 and PD-1, respectively (14). Second, soluble molecules present at the tumor site such as TGF-β and PGE2 can block T-cell function or activation (15, 16). Third, enzymes such as IDO can deplete the tumor microenvironment in tryptophane (17). Fourth, regulatory
This study was prompted by the need for a galectin antagonist approved for clinical use and able to correct the functions of human tumor-infiltrating lymphocytes (TIL). We describe data obtained with GM-CT-01, a clinical grade galactomannan extracted from guar gum and reported to be safe in more than 50 patients with cancer. Treating TIL obtained from patients with various cancers boosted IFN-γ secretion upon ex vivo stimulation in ~80% of the CD8+ TIL samples and ~50% of the CD4+ TIL samples. Increased IFN-γ secretion by CD8+ TIL induced by GM-CT-01 was concentration-dependent and correlated with cytotoxicity. It is remarkable, and so far unique to galectin antagonists, that treating human T cells ex vivo for only a few hours was sufficient to strongly boost TIL functions. The responsiveness of the vast majority of samples suggests that treatment with galectin antagonists, in particular GM-CT-01, could be effective in patients with cancer to correct impaired TIL functions.

**Materials and Methods**

**Cells, TIL, and reagents**

Samples of patient-derived solid tumors, tumor ascites, and blood were collected after approval by the institutional review boards of all collaborating institutions. Ascites cells were concentrated by centrifugation and either frozen at −80°C or resuspended in Iscove’s modified Dulbecco medium (IMDM; Life Technologies) supplemented with 0.24 mmol/L 1- asparagine, 0.55 mmol/L L-arginine, 1.5 mmol/L L-glutamine (AAG), 100 μg/mL streptomycin, 100 U/mL penicillin, and 30 μg/mL gentamycin (Sigma-Aldrich; culture medium), enriched by 2% human serum. Mononuclear cells were isolated from blood of donors without cancer, more precisely patients with hemochromatosis, or from some ascites samples by Lymphoprep (Axis-Shield PoCAS) and usually frozen at −80°C or in liquid nitrogen, in culture medium containing 10% DMSO/45% human serum. Cells were thawed for 2 hours in culture medium supplemented with 2% to 10% human serum and 5 U/mL DNase I (Sigma-Aldrich). T cells were isolated from blood mononuclear cells or ascites cells, by rosetting with sheep erythrocytes (BioMerieux) previously treated with 2(2-aminoethyl) isothiourea dihydrobromide (Sigma-Aldrich). CD8+ and CD4+ T cells were isolated by a positive selection strategy using immunomagnetic beads (AutoMACS system, Miltenyi Biotech). Purity of CD4+ or CD8+ T-cell preparations was between 90% and 95%. Autologous tumor cell lines were established in our laboratory from total cells, or a CD2− fraction, isolated from tumor samples. Cell lines were cultured in IMDM supplemented with AAG, 100 μg/mL streptomycin, 100 U/mL penicillin, 1/100 fungizone (Invitrogen), and 30 μg/mL gentamycin (Sigma-Aldrich), and with 10% FBS and ACL-4. Human recombinant interleukin (IL)-2 was from Chiron Healthcare SAS, IL-7 from R&D Systems, and LacNac from Carbosynth. GM-CT-01 was kindly provided by Anatole Klyosov from Galectin Therapeutics.

**T-cell functional assays**

Non-specific T-cell stimulation was performed in round-bottomed microwells with 10,000 T cells in 200 μL culture medium containing IL-2 (6 IU/mL) as appropriate, with

**Translational Relevance**

This study was prompted by the need for a galectin antagonist approved for clinical use and able to correct the functions of human tumor-infiltrating lymphocytes (TIL). We describe data obtained with GM-CT-01, a clinical grade galactomannan extracted from guar gum and reported to be safe in more than 50 patients with cancer. Treating TIL obtained from patients with various cancers boosted IFN-γ secretion upon ex vivo stimulation in ~80% of the CD8+ TIL samples and ~50% of the CD4+ TIL samples. Increased IFN-γ secretion by CD8+ TIL induced by GM-CT-01 was concentration-dependent and correlated with cytotoxicity. It is remarkable, and so far unique to galectin antagonists, that treating human T cells ex vivo for only a few hours was sufficient to strongly boost TIL functions. The responsiveness of the vast majority of samples suggests that treatment with galectin antagonists, in particular GM-CT-01, could be effective in patients with cancer to correct impaired TIL functions.
3,000 beads coated with anti-CD3 and anti-CD28 antibodies (Dynabeads, Invitrogen) or with B-EBV cells previously incubated at 37°C for 60 minutes with a cocktail of 1 µg/mL of superantigens SEA, SEB, and TSST-1 (Sigma), as indicated. IFN-γ secreted after overnight coculture was measured by ELISA using Biosource Cytoset reagents (Invitrogen). In the cytotoxicity assay, the target cells were either murine P1.aza' cells derived from mastocytoma cell line P815 (referred to as P815). P1.aza' cells were obtained in our institute (41). Target cells were labeled for 1 hour with 50 µCi of Na35CrO4 (Perkin Elmer LifeSciences), washed and incubated at room temperature (RT) for 15 minutes with 1 µg/mL anti-CD3 mAb (OKT3, Mabtech). For the degranulation assay, CD8+ T cells (75,000) were stimulated for 5 hours in IMDM 2% human serum AAG, with 150,000 P815 cells incubated with anti-CD3 antibody or 75,000 CD3/CD28 beads. Brefeldin-A (GolgiPlug, BD) and FITC-coupled anti-CD107a mAb (OKT3, Mabtech). For the degranulation assay, CD8+ T cells were isolated from human carcinoma ascites (UCHT-4, mouse IgG2a; Sigma-Aldrich) or anti-rabbit Ig secondary antibody coupled to neutravidin R-phycoerythrin conjugate (IP-26, mouse IgG1; e-Bioscience) followed by fluorescein-labeled lectin of Lycopersicon esculentum (LEL; 2 µg/mL; Vector). GalectinhighLELhigh cells and galectinlowLELlow cells were sorted using a BD FACSAriaIII.

Detachment of galectins

CD8+ T cells, isolated from the ovarian carcinoma ascites obtained from patient LB3122, were incubated at 37°C for 2 hours with LacNac or GM-CT-01, washed, incubated at 4°C for 15 minutes with FeR Blocking Reagent (1/5; Miltenyi Biotec) diluted in PBS/BSA 0.2% (BSA, Sigma-Aldrich), washed again and incubated with 5 µg/mL of either biotinylated rat anti-galectin-3 antibody M3/38 or polyclonal rabbit anti-galectin-1 IgG (5 µg/mL; Abcam). Cells were washed and incubated at 4°C for 15 minutes with either neutravidin R-phycocerythrin conjugate (1.25 µg/mL; Invitrogen) combined with fluorescein-labeled lectin of Lycopersicon esculentum (LEL; 2 µg/mL; Vector). GalectinhighLELhigh cells and galectinlowLELlow cells were sorted using a BD FACSAriaIII.

Fluorescence resonance energy transfer microscopy

Cells were plated at 1 to 2 × 105/cm² on poly-L-lysine (Sigma-Aldrich)-coated glass coverslips and allowed to bind at RT for 7 minutes, then labeled for 30 minutes on ice with anti-CD68 (UCHT-1, mouse IgG2a; Sigma-Aldrich) and anti-TCRβ (IP-26, mouse IgG1; e-Bioscience) diluted in PBS/BSA 0.2%. After 3 washes in the same cold buffer, cells were fixed at RT for 20 minutes with 4% formaldehyde and 0.1% glutaraldehyde (Sigma-Aldrich) in 0.1 mol/L phosphate buffer, washed twice in PBS/BSA and incubated with 10 mmol/L glycine in PBS for 10 minutes, then incubated for 30 minutes on ice with an anti-IgG2a-Alexa Fluor 488 antibody [fluorescence resonance energy transfer (FRET) donor, green; Invitrogen], and an anti-IgG1-Alexa Fluor 568 antibody (FRET acceptor, red; Invitrogen) diluted in PBS/BSA. After 3 washes, cells were fixed again and coverslips were mounted onto glass slides using Prolong Gold (Invitrogen). Images were acquired with an LSM 510 laser scanning microscope and analyzed by AIm Software (Zeiss). Imaging was performed with a 488 nm line generated by an Ar laser (30 mW) and a 561 nm line generated by a DPSS laser (10 mW), both used at 1%. Acceptor photobleaching was achieved using the DPSS laser at 100% with 100 iterations. Three images were recorded before and after bleaching. To calculate the increase in donor emission (indicative of FRET efficiency), 3 regions of interest for each cell were chosen, bleached, and compared with 3 control regions in the nonbleached area. Increase in donor emission was calculated as follows: %FRET efficiency = [1 − (donor intensity before bleaching/donor intensity after bleaching)] × 100.

Results

GM-CT-01 boosts IFN-γ secretion by CD8+ TIL in a concentration-dependent manner

CD8+ T cells were isolated from human carcinoma ascites and incubated for 2 hours with increasing concentrations of GM-CT-01 or with 5 mmol/L LacNac as a positive control. T cells were next stimulated nonspecifically by beads coated with anti-CD3 and anti-CD28 antibodies (CD3/CD28 beads) and, after 20 hours, IFN-γ secretion was measured in the culture supernatant. In this representative patient, preincubation of TIL with 0.3 mmol/L (~15 µg/mL) of GM-CT-01 boosted IFN-γ secretion by more than 3-fold. Half-maximal effect was obtained at 0.4 µmol/L (~20 µg/mL; Fig. 1). For the experiments described below, GM-CT-01 was used at concentrations in the micromolar range (either 0.6 or 1.8 µmol/L ~30 or 100 µg/mL), which yielded a maximal response equivalent to that of 5 mmol/L LacNac, as we observed previously (8). GM-CT-01 was thus at least 1,000 times more potent than LacNac on molar basis.

No growth inhibition was observed when 2 T-cell clones were cultured for 7 weeks in the presence of up to 2.6 µmol/L of GM-CT-01 (Supplementary Fig. S1). Two melanoma cell lines were also cultured for 25 days with GM-CT-01. Growth inhibition was observed starting at day 7 for only one of the melanoma cell lines at the concentration of 2.6 µmol/L. We concluded that GM-CT-01 effects reported below are not affected by toxicity.

GM-CT-01 boosts IFN-γ secretion by CD8+ and CD4+ TIL from patients with various cancers

CD8+ TIL were isolated from ascites obtained from 27 patients bearing tumors of different histologic origins.
GM-CT-01 boosts IFN-γ secretion by CD8^+ TIL obtained from tumor ascites and solid tumor from a patient with ovarian carcinoma

To further test this hypothesis, we searched for the opportunity to compare the effect of GM-CT-01 and LacNAc on CD8^+ and CD4^+ T cells isolated from samples collected on the same day in the same patient: the solid tumor, blood, and tumor ascites (Fig. 2D for IFN-γ and Supplementary Table S1 for other cytokines). IFN-γ secretion by untreated CD8^+ and CD4^+ TIL, whether from the solid tumor or ascites, was low compared with IFN-γ secretion by blood T cells. The presence of GM-CT-01 boosted IFN-γ secretion by CD8^+ and CD4^+ TIL by more than 4-fold, whether TIL were isolated from the solid tumor or from the ascites. LacNAc had a similar effect. CD8^+ but not CD4^+ blood T cells secreted slightly more IFN-γ in the presence of GM-CT-01. In patients with ovarian carcinoma, as in patients with other types of cancer, elevated level of galectin-3 can be found in the serum (42, 43). But serum galectins are possibly bound to serum glycoproteins and probably not capable to be loaded on circulating blood T cells in sufficient amount to block their function.

GM-CT-01 boosts cytotoxicity of CD8^+ TIL

Because the specificity of CD8^+ TIL was unknown, P815 cells coated with an anti-CD3 antibody were used as targets in a cytotoxicity assay. For some TIL samples, we had autologous tumor cell lines and used them also as targets. We tested CD8^+ TIL isolated from 4 different ascites and CD8^+ T cells isolated from the blood (PBL) of 3 different donors without cancer. Although the cytotoxicity of untreated CD8^+ TIL was minimal, treatment with GM-CT-01 or LacNAc triggered a significant cytotoxicity against both P815 bearing anti-CD3 and autologous tumor cell targets (Fig. 3A). The cytotoxicity of CD8^+ blood T cells, obtained from donors without cancer, was ~10-fold more potent than that of TIL and was similar for treated and untreated cells. Degranulation, a prerequisite for cytolysis, was also measured. The percentage of degranulating cells was estimated by surface expression of CD107a and CD107b, which reside at rest in cytolytic granules and, upon activation, are mobilized to the cell surface by exocytosis. After stimulation by P815 cells covered with anti-CD3 antibodies or anti-CD3/CD28 beads, the percentage of CD107^+ cells was low in untreated TIL and strongly increased in TIL treated by GM-CT-01 or LacNAc, but high and equivalent for treated and untreated blood CD8^+ T cells (Fig. 3B and Supplementary Fig. S2). We concluded that GM-CT-01 was as potent as LacNAc to restore cytotoxicity in fresh CD8^+ TIL.

The CD8^+ TIL responding to galectin antagonists harbor poly-LacNAc motifs and galectin-3

To explain how galectin antagonists boost TIL function, we hypothesized that (i) TIL had been recently activated by contact with tumor antigen so that the N-glycans include larger LacNAc oligomers—the natural galectin-3 ligands—on surface glycoproteins as compared with resting T cells.
Figure 2. Survey of the effect of GM-CT-01 on IFN-γ secretion by TIL obtained from patients with different types of cancer. CD8^+ (A) or CD4^+ TIL (B) were isolated from tumor ascites. (C) CD8^+ or CD4^+ blood T cells (PBL) were isolated from blood samples obtained from donors without cancer. (D) CD8^+ or CD4^+ T cells were isolated from a solid tumor, tumor ascites, or blood collected on the same day from patient with ovarian carcinoma LB3191. T cells (10,000 per microwell) were first incubated for 2 hours (patient code in bold) or 20 hours (patient code in italics) in culture medium supplemented with either GM-CT-01 (0.6–1.8 μmol/L, black bars) or 5 mmol/L LacNAc (gray bars) or left untreated (white bars). T cells were then stimulated with 3,000 CD3/CD28 beads in overnight culture as in Figure 1. IFN-γ was measured in the supernatant by ELISA. Values are means ± SD of triplicate microwells. Secretion of IFN-γ by nonstimulated CD8^+ or CD4^+ blood T cells, whether treated or not, was <15 pg/mL of IFN-γ.
(44), and (ii) binding of extracellular galectin-3 on surface glycoproteins favors galectin-3-glycoprotein lattices, thereby reducing the motility of surface glycoproteins and consequently impairing TIL functions. This extracellular galectin-3 could be secreted, for example, by the activated T cells or captured by contact with tumor cells or macrophages covered by galectin-3.

We decided to test if TIL responding to galectin antagonists were those harboring more LacNAc motifs and more galectin-3. CD8 TIL were isolated from ascites obtained from a patient with an ovarian carcinoma (LB2991, LB3015, LB3059, LB3023, LB3082) or a pancreatic carcinoma (LB3033). CD8 T cells were isolated from blood samples obtained from donors without cancer (PBL). CD8 T cells were treated for 20 hours with 1.8 μmol/L GM-CT-01 or 5 mmol/L LacNAc, and washed. A, CD8 T cells were incubated with 51Cr-labeled target cells at the indicated effector-to-target (E:T) ratios. Target cells were P815 cells coated with an anti-CD3 mAb (1 μg/mL) or autologous tumor cell lines for patients LB3023, LB3082, and LB3033. Chromium release was measured after 4 hours. B, CD8 T cells (75,000, treated or not) were stimulated with either 150,000 P815 cells previously incubated for 15 minutes with an anti-CD3 antibody, or with 75,000 CD3/CD28 beads. Brefeldin-A and FITC-coupled anti-CD107a and anti-CD107b antibodies were added simultaneously to the stimulators. After 5 hours of stimulation, cells were labeled for CD3 and CD8, and the percentage of CD3 CD8 T cells with detectable surface CD107 was determined.

**GM-CT-01 does not detach galectins from cells but disorganizes galectin–glycoprotein lattices**

Because we previously observed that LacNAc detaches galectin-3 from cells (8), we tested if GM-CT-01 has the same effect in TIL, which usually express low levels of galectin-3 surface expression is abundant (Fig. 3 and Supplementary Fig. S3). Cells were incubated for 2 hours with GM-CT-01 or LacNAc, labeled with specific anti-galectin antibodies, and analyzed by flow cytometry for surface expression of galectins. As previously reported, labeling of TIL for galectin-3 or galectin-1 decreased after LacNAc treatment (8), but we did not observe galectin detachment after GM-CT-01 treatment (Fig. 3). This difference was even more striking for melanoma cells (Supplementary Fig. S3).

To explain that GM-CT-01 treatment, which failed to detach galectins, nevertheless increased the ability of TIL to secrete other cytokines. The next 4% of the TIL secreted intermediate levels of IFN-γ in the absence of a treatment with a galectin antagonist. We tentatively concluded that galectin antagonists boosted cytokine secretion specifically in galectin-3 high LacNAc high TIL.

**Figure 3.** Effect of GM-CT-01 on CD8 TIL cytotoxicity. CD8 TIL were isolated from ascites from patients with an ovarian carcinoma (LB2991, LB3015, LB3059, LB3023, LB3082) or a pancreatic carcinoma (LB3033). CD8 T cells were isolated from blood samples obtained from donors without cancer (PBL). CD8 T cells were treated for 20 hours with 1.8 μmol/L GM-CT-01 or 5 mmol/L LacNAc, and washed. A, CD8 T cells were incubated with 51Cr-labeled target cells at the indicated effector-to-target (E:T) ratios. Target cells were P815 cells coated with an anti-CD3 mAb (1 μg/mL) or autologous tumor cell lines for patients LB3023, LB3082, and LB3033. Chromium release was measured after 4 hours. B, CD8 T cells (75,000, treated or not) were stimulated with either 150,000 P815 cells previously incubated for 15 minutes with an anti-CD3 antibody, or with 75,000 CD3/CD28 beads. Brefeldin-A and FITC-coupled anti-CD107a and anti-CD107b antibodies were added simultaneously to the stimulators. After 5 hours of stimulation, cells were labeled for CD3 and CD8, and the percentage of CD3 CD8 T cells with detectable surface CD107 was determined.
to secrete IFN-γ we examined if GM-CT-01 treatment could disorganize galectin–glycoprotein lattices enough to improve TIL function but not to result into galectin detachment. To this aim, we used a microscopy-based FRET approach that probes close contact between TCR and CD8 molecules at the surface of CD8⁺ TIL, hereafter referred to as colocalization. Indeed, we had previously observed a poor colocalization of TCR and CD8 at the surface of CD8⁺ TIL, as compared with CD8⁺ blood T cells. Moreover, treating CD8⁺ TIL with LacNAc increased both IFN-γ secretion and the colocalization of TCR with coreceptor CD8 (8, 24).

Here, CD8⁺ TIL freshly isolated from ascites were treated overnight with GM-CT-01 or LacNAc, attached to cover-slips, and double-labeled with an anti-TCR-β antibody coupled to an acceptor fluorochrome and with an anti-CD8-α antibody coupled to a donor fluorochrome. Upon excitation at donor wavelength, energy can be transferred from the donor to the acceptor if the 2 fluorochromes are closer than ~10 nm. In these conditions, full acceptor bleaching abrogates the energy loss, thus increasing donor emission. Data are shown in Table 1 for 3 CD8⁺ TIL samples.

In untreated CD8⁺ TIL, no increase in donor emission was detected upon acceptor photobleaching, indicating poor TCR:CD8 colocalization (Table 1). Negative values
Table 1. FRET colocalization of TCR and coreceptor CD8

<table>
<thead>
<tr>
<th></th>
<th>TIL CD8⁺ VUB155&lt;sup&gt;a&lt;/sup&gt;</th>
<th>TIL CD8⁺ GHC9</th>
<th>TIL CD8⁺ GHC8</th>
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<tr>
<td></td>
<td>Untreated</td>
<td>GM-CT-01</td>
<td>LacNAc</td>
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<td><strong>Increase in donor emission (%)</strong></td>
<td></td>
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<tr>
<td>Bleached region</td>
<td>Cell 1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Cell 2</td>
<td>Cell 1</td>
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<tr>
<td></td>
<td>–18</td>
<td>–1</td>
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<tr>
<td>Mean&lt;sup&gt;c&lt;/sup&gt;</td>
<td>–5 ± 7</td>
<td>12 ± 2</td>
<td>9 ± 2</td>
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<td><strong>Secretion of IFN-γ by 10⁴ TIL (pg/mL)&lt;sup&gt;d&lt;/sup&gt;</strong></td>
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<td></td>
<td>308 ± 31</td>
<td>913 ± 133</td>
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<sup>a</sup>CD8⁺ TIL were isolated from ascites of patients with carcinoma and frozen. Cells were thawed, washed, and incubated overnight at 37°C in medium containing 10% ascitic fluid in the presence of 1.8 μmol/L GM-CT-01. After washing, some cells were tested for IFN-γ secretion and the others were attached to coverslips, labeled on ice with anti-TCR antibodies (FRET acceptor), and anti-CD8 antibodies (FRET donor), fixed and stained with secondary antibodies.

<sup>b</sup>For each of the 2 cells that were analyzed, in 3 regions of interest, 5 images were recorded before bleaching and 5 others after bleaching of the acceptor fluorochrome. Three control regions were not bleached. Increase in donor emission was calculated as follows: \[ (\frac{\text{donor intensity before bleaching}}{\text{donor intensity after bleaching}}) \times 100. \]

<sup>c</sup>Mean increase in donor emission (in percentage) was –4.6 ± 5.1 for nontreated TIL, 9.1 ± 4.9 (P < 0.0001 by Mann–Whitney test) for GM-CT-01-treated TIL, and 7.3 ± 4.1 (P < 0.0001) for LacNAc-treated TIL.

<sup>d</sup>10⁴ TIL were distributed in microwells, treated overnight with 1.8 μmol/L GM-CT-01, and cultured overnight with 3,000 CD3/CD28 beads. The presence of IFN-γ in the supernatant was estimated by ELISA in triplicates.
indicate partial bleaching of the donor fluorochrome during the procedure. For 2 of the treated TIL, VUB155, and GHC9, there was a consistent strong increase in donor emission after photobleaching, indicating TCR:CD8 colocalization upon LacNAc and GM-CT-01 treatment. The response to treatments was mild with TIL GHC8. Although modest at first sight, these increases are within the range reported in the literature (8, 24). We conclude that the increased TCR:CD8 colocalization observed after GM-CT-01 treatment is in agreement with the hypothesis that this treatment disorganizes galectin–glycoprotein lattices without detaching galectins from the cell surface.

**Efficacy of different galactomannans for boosting TIL functions**

GM-CT-01 is obtained by hydrolysis of guar gum, a galactomannan extracted from guar beans (*Cyamopsis tetragonoloba*). The guar gum backbone is a linear chain of β 1.4-linked mannose residues to which galactose residues are 1.6-linked to mannose, forming short side-branches. The guar gum used to obtain GM-CT-01 has a mannose-to-galactose ratio of about 1.7. Considering that a large number of plant-derived galactomannans have the same mannose backbone but different mannose-to-galactose ratios, we wondered if different galactomannans would be able to boost TIL function. The nonhydrolyzed guar gum was inefficient for boosting TIL function (Supplementary Fig. S4A). We tested partially hydrolyzed guar gum generously provided by Galectin Therapeutics, the company producing GM-CT-01, and also tested a guar gum that we hydrolyzed using a protocol set up in the laboratory (Supplementary Fig. S4B). It seemed that hydrolyzed guar gum with fragments of about 44 to 50 kDa were able to boost the function of TIL (Supplementary Fig. S4A).

We subsequently tested a number of galactomannans obtained from various plants, either before or after partial hydrolyzation. None of them, except guar gum in its hydrolyzed version, was able to boost TIL function (Supplementary Fig. S4C). We tentatively concluded that both the size and the structure of the galactomannan are important for its ability to boost TIL function.

**Discussion**

This study was prompted by the need for a galectin antagonist approved for clinical use and able to boost TIL function *ex vivo*. We here reported that treating TIL obtained from patients with various cancers with GM-CT-01, a galactomannan extracted from guar gum, boosted IFN-γ secretion upon *ex vivo* stimulation in ~80% of the CD8⁺ TIL samples and ~50% of the CD4⁺ TIL samples. Increased IFN-γ secretion by CD8⁺ TIL induced by GM-CT-01 was concentration-dependent and correlated with cytotoxicity. The efficacy of GM-CT-01 for boosting IFN-γ secretion seems to be equivalent to that of 2 other galectin antagonists, LacNAc and modified citrus pectin GCS-100 (8). It remains remarkable, and so far unique to galectin antagonists, that treating human T cells *ex vivo* for only a few hours was sufficient to strongly increase IFN-γ secretion. In contrast, treating T cells with antibodies specific for inhibitory receptors or with IDO inhibitors does not provide an immediate functional correction but instead results in an enhanced proliferation of T cells, yielding a few days later a higher number of functional T cells (10, 45, 46).

The response to galectin antagonists in only half of the CD4⁺ TIL samples could be explained by the contamination by regulatory T cells that participate in blocking the function of effector CD4⁺ T cells. Testing this hypothesis would require Treg depletion from CD4⁺ TIL samples, but no specific surface marker is available for full removal of Treg. Moreover, eliminating the CD25-positive cells would also eliminate activated TIL, which could be abundant in ascites.

How does GM-CT-01 trigger IFN-γ secretion? Galectin-1 and -3 are abundantly released by tumor cells and macrophages, so as to reach nanomolar concentrations in ascites and readily bind to TIL. Our working hypothesis is that a high percentage of isolated TIL have been recently activated, and therefore harbor a glycome with many LacNAc motifs, the natural ligands of galectin-1 and galectin-3 (44). The abundance of LacNAc motifs and galectins would favor the formation of galectin–glycoprotein lattices at the TIL surface and result in a decreased surface motility of molecular actors of T cell activation. This hypothesis is supported by the lower secretion of IFN-γ by galectin-3highLELhigh as compared with galectin-3lowLELlow TIL, and by the selective response of the galectin-3highLELhigh TIL to LacNAc or GM-CT-01. This indicates that upon activation, more galectin-3 binds to TIL expressing glycoproteins bearing more LacNAc motifs and suggests that the dysfunction is related to the presence of galectin-3. LacNAc is able to detach both galectin-1 and galectin-3. Our previous observation that a galectin-3 antibody, which also detached galectin-3 from TIL surface, was able to boost IFN-γ secretion by TIL as efficiently as LacNAc, indicated that detaching galectin-3 from TIL is sufficient to restore function, while not excluding a contribution of other galectins, in particular galectin-1 (8).

How to reconcile this scenario with the failure of GM-CT-01 to detach galectin-1 and galectin-3 from the cell surface? First, LacNAc and GCS-100 interact with galectins by binding to their carbohydrate recognition domains. They are true competitors, whereas GM-CT-01 interacts with a site of galectin-1 opposite to the carbohydrate recognition domain and thus acts as an allosteric antagonist (47). The galectin-1 site interacting with GM-CT-01 is conserved in the galectin-3 protein (48). In line with these reports, 2 other studies have shown that the binding of oligomannan to galectin-3 cannot be completed by lactose (49, 50). Second, acute treatment of TIL with GM-CT-01 resulted in increased TCR:CD8⁺ colocalization based on FRET, such as LacNAc and GCS-100. We thus tentatively conclude that GM-CT-01 interacts with galectin-3 without causing its detachment from the TIL surface, but that this interaction results in disruption of glycoprotein:galectin lattices, therefore restoring motility of surface receptors implicated in T-cell activation.

In tumor-bearing mice vaccinated with a tumor antigen, we have previously shown as preliminary data that
injections of modified citrus pectin GCS-100 led to tumor rejection in half of the mice (8). In the same experimental setting, GM-CT-01 failed to confer any tumor rejection (data not shown). However, the experiments with GCS-100 were not extended to test whether T cells from tumor-bearing mice were dysfunctional because of glycoprotein–galexin lattices and whether GCS-100 treatments disrupted these lattices. Noteworthy murine T cells, as compared with human T cells, seem to be poor in tri- and tetra-antennary poly-LacNac glyans and therefore less susceptible to galexin-mediated dysfunction (44, 51, 52). GCS-100 could as well have increased apoptosis of tumor cells or inhibited neoangiogenesis and metastases formation, independently of an effect on the immune system as it was reported for modified citrus pectin in other experiments (53–55).

It remains remarkable that a short treatment of but a few hours with GM-CT-01 was sufficient to boost, if not fully restore, human TIL functional capacities. Moreover, responsiveness of the vast majority of samples suggests that treatment with galexin antagonists, in particular GM-CT-01, could be effective in patients with cancer to correct impaired TIL functions.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: N. Demotte, K. Thielemans, P. van der Bruggen Development of methodology: N. Demotte, R. Bigirimana, V. Stroobant, P.J. Courtoy, P. van der Bruggen

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