An Orally Active Galectin-3 Antagonist Inhibits Lung Adenocarcinoma Growth and Augments Response to PD-L1 Blockade

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Abstract

A combination therapy approach is required to improve tumor immune infiltration and patient response to immune checkpoint inhibitors that target negative regulatory receptors. Galectin-3 is a β-galactoside-binding lectin that is highly expressed within the tumor microenvironment of aggressive cancers and whose expression correlates with poor survival particularly in patients with non–small cell lung cancer (NSCLC). To examine the role of galectin-3 inhibition in NSCLC, we tested the effects of galectin-3 depletion using genetic and pharmacologic approaches on syngeneic mouse lung adenocarcinoma and human lung adenocarcinoma xenografts. Galectin-3−/− mice developed significantly smaller and fewer tumors and metastases than syngeneic C57/B6 wild-type mice. Macrophage ablation retarded tumor growth, whereas reconstitution with galectin-3-positive bone marrow restored tumor growth in galectin-3−/− mice, indicating that macrophages were a major driver of the antitumor response. Oral administration of a novel small molecule galectin-3 inhibitor GB1107 reduced human and mouse lung adenocarcinoma growth and blocked metastasis in the syngeneic model. Treatment with GB1107 increased tumor M1 macrophage polarization and CD8+ T-cell infiltration. Moreover, GB1107 potentiated the effects of a PD-L1 immune checkpoint inhibitor to increase expression of cytotoxic (IFNγ, granzyme B, perforin-1, Fas ligand) and apoptotic (cleaved caspase-3) effector molecules. In summary, galectin-3 is an important regulator of lung adenocarcinoma progression. The novel galectin-3 inhibitor presented could provide an effective, nontoxic monotherapy or be used in combination with immune checkpoint inhibitors to boost immune infiltration and responses in lung adenocarcinoma and potentially other aggressive cancers.


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Introduction

Globally, lung cancer is the leading cause of cancer-related mortality (1). Non–small cell lung carcinoma (NSCLC) comprises 80% of total lung cancer cases, with lung adenocarcinoma being the major subtype (1). In recent years immune checkpoint therapies targeting various negative regulatory receptors on tumor infiltrating cytotoxic T lymphocytes (CTL) such as programmed death-1 (PD-1), programmed death-ligand 1 (PD-L1), cytotoxic T-lymphocyte associated protein 4 (CTLA-4), and others, have shown unprecedented efficacy in NSCLC patients even against late-stage disease (2). However, patient response is limited, thus driving intensive research toward combining immune checkpoint inhibition with other targeted agents to overcome resistance (2).

Tumor-associated macrophages (TAM) are present in the stroma of many tumors including NSCLC (3). TAMs acquire an alternative (M2)-like macrophage phenotype and secrete angiogenic and anti-inflammatory cytokines, which contribute to the immunosuppressive milieu of the tumor microenvironment (4). TAMs can also be important direct targets of PD-1/PD-L1 blockade improved T-cell infiltration and antitumor activity of PD-1 antagonists in preclinical models of melanoma and breast cancer (7, 8), suggesting that strategies aimed at inhibiting macrophage responses are necessary to permit effective immune checkpoint therapy.

One possible target for such combination treatment is galectin-3, a member of a protein family defined by affinity for β-galactoside-containing glycoconjugates and a conserved carbohydrate-recognition-binding domain (9). Galectin-3 is widely expressed in several cell types such as macrophages, fibroblasts, activated T-lymphocytes and epithelial cells (10–12) and is highly expressed in high fatality cancers such as NSCLC (13). In NSCLC particularly in adenocarcinoma, increased galectin-3 expression in tumors, lymph nodes and serum correlates with metastases and is a negative prognostic indicator (13–18). The galectin-3 genetic polymorphism rs4652 associated with impaired galectin-3 secretion, has been linked to increased survival and response to chemotherapy in NSCLC (18). Galectin-3 can directly enhance cell proliferation (19), apoptosis resistance (20), metastatic potential (19, 21), as well as lung cancer stemness (22). It is also an important constituent of the tumor microenvironment acting on endothelial cells to promote angiogenesis (23). Furthermore, many studies have revealed the inhibitory effects of galectin-3 on activated cytotoxic T lymphocytes CTLs (24–27) and we have shown it to be essential for M2 macrophage differentiation (28, 29). Hence, galectin-3 forms an ideal candidate target for combining with checkpoint blockade.

We examined the role of galectin-3 in NSCLC by utilizing the syngeneic mouse Lewis Lung Carcinoma (LLC1) model, comparing tumor growth in wild-type (WT) and galectin-3-deficient mice showing an essential non-redundant tumor-promoting role for galectin-3. Bone marrow (BM) transfer and macrophage depletion experiments show that macrophages are a major source of tumor-promoting galectin-3. A newly developed, selective small molecule galectin-3 inhibitor inhibited mouse and human NSCLC tumor growth and metastasis and significantly potentiated response to an immune checkpoint inhibitor.

Materials and Methods

Cell lines, culture, and transfections

LLC1 cells and A549 cells were purchased from the European Cell Culture Collection (ECACC 90020104) and were cultured at 37°C in 5% CO2 (95% air) in DMEM (Sigma D5671) supplemented with 10% FCS, 1% l-glutamine, and 1% penicillin/streptomycin. Human cell line A549 was authenticated using short tandem repeat (STR) DNA profiling by ECACC. Cells were routinely tested for Mycoplasma every 6 months. Vector pCMV-KDEL-Gluc-1, expressing G.princeps luciferase (Lux Biotechnologies) was transfected by electroporation (Lonza electroporation kit VCO-1001). Stably transfected cells were selected with G418.

Animals

All animal experimental work was carried out under a project license approved by the local Animal Welfare and Ethical Review body (AWERB) and issued in accordance with the Animals (Scientific Procedures) Act 1986. C57Bl/6 mice and female CD1 nude mice were purchased from Harlan Laboratories. Generation of galectin-3−/− mice by gene-targeting technology has been described previously (30). CD11b-DTR (Diphtheria Toxin Receptor) mice were derived from FVB mice as described (31) and backcrossed over 10 generations onto the C57Bl/6 background. Human galectin-3 knockin mice were generated by Cyagen Biosciences using the TurboKnockout (conditional Knockin) approach by inserting the entire human LGALS3 sequence into exon 1 of mouse IgαlS3 so that the expression of human galectin-3 is under control of the mouse gene regulatory element.

Orthotopic LLC1 model

Mice were anaesthetized with isofluorane. A 1 mm skin incision was made below the right shoulder blade. A total of 103 LLC1-luciferase cells were injected through the intercostal muscles into the lung parenchyma prior to the incision being stapled.

Subcutaneous LLC1 model

LLC1 (2.5 × 105) cells were injected subcutaneously into the flanks of age-matched male WT and galectin-3−/− C57Bl/6 mice. Each animal received an injection of 2.5 × 105 cells suspended in 100 μL PBS in both flanks. Tumor volumes were measured with calipers every 1 to 3 days [tumor volume = π/6 × (L × W)3/2].

LLC1 metastasis model

LLC1 cells were administered via the tail vein (1 × 106 cells) and lung lobes were harvested at 7 days. RNA was extracted from lung lobes using a Qiagen RNeasy Kit, converted into cDNA (Quantitect cDNA Synthesis Kit; Qiagen), and luciferase expression was measured by qPCR using primers against G.princeps luciferase (5′-TCCTGCTTGGCCACATCAG-3′ forward and 5′-GGCTGGCAGACTTCTTG-5′ reverse; Primer Design) and SYBR Green (ThermoFisher Scientific).

Human adenocarcinoma xenograft model

CD-1 nude female mice received 3 × 105 human lung adenocarcinoma cells (A549) in 100 μL 1:1 Matrigel-serum-free DMEM in both flanks. Tumor volumes were measured every 2 to 3 days using digital calipers.
Macrophage ablation

Macrophages were ablated in C57BL/6 CD11b-DTR mice (or WT littermates) by administration of 10 ng/g diphtheria toxin (DT) intraperitoneally (i.p.) prior to subcutaneous tumor cell injections.

BM transplant

Mice were injected with 400 μL liposomal clodronate (Liposoma). After 36 hours, mice were irradiated with 10.5 Gy delivered from an IBL637 gamma irradiator (Gamma Services Ltd.) at a dose rate of 0.64 Gy/min. Following irradiation mice received a single tail-vein infusion of 107 BM cells obtained by flushing the femurs of WT and galectin-3−/− donor mice. Transplanted mice were used 8 weeks posttransplant.

Drug preparation

Galectin-3 inhibitor GB1107 (3,4-dichlorophenyl 3-deoxy-3-[4(3,4,5-trifluorophenyl)-1H-1,2,3-triazol-1-yl]-1-thio-β-D-galactopyranoside; Galecto Biotech; ref. 32) was prepared at a concentration of 1 mg/mL in 1% polyethylene glycol, 0.5% galactopyranoside; Galecto Biotech (Liposoma). After 36 hours, mice were injected (1×10^7) BM cells obtained by flushing the femurs of WT and galectin-3−/− donor mice. Transplanted mice were used 8 weeks posttransplant injection.

Luciferase assays

LLC1 cells stably transfected with pCMV-KDEL-Gluc-1 were assessed for luciferase expression upon addition of n-coleterazene (n-CTZ; Lux Biotechnologies 20001) substrate to a final concentration of 10 μmol/L to live cells in 96-well plates. Lymph nodes were disaggregated by passing through 40 μm cell strainers and suspended in PBS. N-CTZ was added at a final concentration of 10 μmol/L. Luciferase activity was assessed with a BioTek SynergyHT Luminometer.

Immunofluorescence

Tumor sections were incubated with rat anti-mouse F4/80 followed by horseradish peroxidase (HRP)-labeled goat anti-rat IgG (Dako) and tyramide green (Invitrogen). Sections were microwaved in 0.01M sodium citrate (pH 6) for 5 minutes, reblocked, and probed with rabbit anti galectin-3 (R&D) or rabbit anti Ym1 followed by HRP-labeled goat anti-rabbit IgG (Dako) and tyramide red (Invitrogen) and mounted in fluoromount-G with DAPI (eBioscience). Images were captured on a Nikon Eclipse Ti-e600 microscope.

Tumor RNA extraction and RT-PCR

Total RNA from LLC1 tumors and lung tissue was prepared using RNeasy kits (Qiagen) and reverse transcribed into cDNA using Quantitect RT kits (Qiagen). cDNA was analyzed using either a SYBR green-based quantitative fluorescence method (Invitrogen) and Kigstart primers (Sigma Aldrich) or Taqman primer probe sets (Life Technologies).

SDS PAGE and Western blotting

Cells were lysed in NP-40 (Invitrogen) and separated by 10% SDS-PAGE. Proteins were transferred to nitrocellulose membrane and probed using antibodies against galectin-3, 1:500 (BioM3;38; eBioscience) and GAPDH, 1:3,000 (14C10; Cell Signaling Technology), followed by species specific HRP-conjugated secondary antibodies (Dako). Bound antibodies were detected using the Enhanced Chemiluminescence 2 Detection Kit (Pierce).

Tumor dissociation and flow cytometry

Tumors were minced in serum-free DMEM and digested with Liberase (2 mg/mL; Sigma-Aldrich) and DNase I (Sigma-Aldrich) at 37°C for 30 minutes. Disaggregated tissue was filtered through a 35-μm nylon mesh, washed, and resuspended in FACS buffer (PBS with 0.1% BSA). Fc receptors were blocked with anti-mouse CD16/32 (Biolegend). Antibody cocktails (anti-mouse Ly6G-Pacific blue, CD11b-BV605, Galectin-3-FTTC, CD45-PerCP and CD45-APCcy7, MHC-II-PE, CD206-PerC35, PD-1-APC, F4/80-AF700, CD4-Pacific blue, PD-L1 BV605, CD3-PerCPCy5.5, IFNγ-PE, CD8-AF700, all from Biolegend) were added to cells and incubated for 20 minutes at room temperature. Samples were fixed and RBCs were simultaneously lysed in RBC lysis/Fixation solution (Biolegend). For intracellular staining, cells were permeabilized with intracellular staining permeabilization wash buffer (Biolegend) and incubated with anti-CD206 or anti-IFNγ (Biolegend). Cells were analyzed using an LSR-Fortessa cell analyzer (Beckton Dickenson).

Statistical analysis

Statistical analyses were performed using Graphpad Prism 7.0 software. Results are represented as mean ± SEM and statistical tests are described in the figure legends.

Results

Galectin-3−/− mice do not support the growth and metastasis of LLC1 tumors

To examine lung cancer growth within the correct tissue compartment, LLC1 cells stably expressing G. princeps-luciferase were injected (1×10^5 cells) through the intercostal space directly into the lung parenchyma of control and galectin-3−/− mice.
Hematoxylin and eosin staining of lung tissue confirmed the presence of tumors in control but not galectin-3−/− mouse lungs (Fig. 1A). At 20 days post-injection, 4/10 of control mice had tumors, whereas none of the galectin-3−/− mice developed tumors (Table 1). In addition, 7/10 control animals displayed gross swelling of the mediastinal lymph nodes (MLN; Table 1), which were positive for metastatic cells as assessed by luciferase assay on homogenized MLNs. Only 1/11 of galectin-3−/− mice had luciferase positive MLNs (Table 1).

LLC1 cells expressing luciferase were injected subcutaneously in both flanks of WT and galectin-3−/− mice. After day 10, subcutaneous tumors from control animals were much larger than those of galectin-3−/− mice. This difference became statistically significant at day 12 (P = 0.0004). By the end of the study, tumors of controls had an average volume of 286 mm3 compared with a volume of 9 mm3 in galectin-3−/− animals (96.9% reduction, P < 0.0001; Fig. 1B and C). The weight of tumors from control mice was 98% heavier than that of galectin-3−/− mice, 153 ± 31 mg and 3 ± 2 mg, respectively (P < 0.0001; Fig. 1D). Of all the tumor cell injections received by each group, only 11/40 led to tumors in galectin-3−/− mice compared with 36/40 in controls (Table 1). A total of 5/12 control mice had luciferase positive metastases in their MLNs whereas galectin-3−/− mice had no metastases (Table 1). These results indicate that galectin-3−/− mice do not support tumor establishment and spread in a subcutaneous LLC1 model. Although LLC1 inoculation increased the serum concentrations of anti-galectin-3 IgG antibodies in galectin-3−/− mice, no correlation, either negative or positive, was noted between antibody production and tumor volume (Supplementary Fig. S1).

Table 1. Galectin-3−/− mice do not support tumor growth

<table>
<thead>
<tr>
<th>Orthotopic tumors</th>
<th>Genotype</th>
<th>No. of mice with primary tumors</th>
<th>No. of mice with LN metastases</th>
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<tr>
<td></td>
<td>WT</td>
<td>4/10 (40%)</td>
<td>0/11 (0%)</td>
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<tr>
<td></td>
<td>Gal-3−/−</td>
<td>7/10 (70%)</td>
<td>1/11 (9%)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Subcutaneous Tumors</th>
<th>Genotype</th>
<th>No. of mice with primary tumors</th>
<th>No. of mice with LN metastases</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
<td>36/40 (90%)</td>
<td>5/12 (41.7%)</td>
</tr>
<tr>
<td></td>
<td>Gal-3−/−</td>
<td>11/40 (27.5%)</td>
<td>0/12 (0%)</td>
</tr>
</tbody>
</table>

NOTE: Prevalence of mice with established primary tumors and inguinal lymph node metastases are summarized. Orthotopic and subcutaneous tumor data are representative of one or two independent experiments, respectively.

M2 macrophages are reduced in tumors from galectin-3−/− mice

Tumor stroma F4/80+ macrophages were significantly higher in galectin-3−/− animals compared with control (P = 0.0217). However, the ratio of Ym1+/F480− macrophages was significantly higher in controls (P = 0.0484, Fig. 2A and B), indicating that higher galectin-3 levels around the tumor environment can drive expansion of M2 macrophages (28). Transcript analysis from whole tumor RNA showed that control tumors had 2.5-, 3.3-, and 16.7-fold higher levels of IL4, IL10, and IL13 transcripts, respectively (P = 0.04, 0.024, 0.119, respectively), and displayed a 28.8-fold reduction in IFNγ mRNA when compared with galectin-3−/− tumors (P = 0.0066, Fig. 2C). These results indicate a cytokine environment that favors M2 macrophage activation in tumors of control but not galectin-3−/− hosts and suggests an important role for galectin-3 in the regulation of TAM phenotype.
Figure 2.
Bone marrow-derived macrophages support LLC1 tumor growth. A, Subcutaneous tumors from WT or galectin-3−/− mice were stained for F4/80 or Ym-1. B, Quantitation of Ym1 and F480 staining in tumor sections. C, RNA was extracted from these tumors and gene expression of various cytokines was assessed by qPCR. D, LLC1 cells were injected subcutaneously in both flanks of CD11b DTR mice (n = 12) and their WT siblings (n = 11). All mice were administered DT (10 ng/g) prior to cell injection. D and E, Tumor volume (D) and tumor weights (E) following DT administration. F, WT and galectin-3−/− mice were treated with clodronate, irradiated, and subsequently transplanted with BM cells from WT or galectin-3−/− mice. LLC1 cells were injected subcutaneously into both flanks of transplanted mice and tumor volumes (F) and weights (G) were measured. H, Double immunofluorescence staining for F4/80 (green) and galectin-3 (red) was performed in tumors from galectin-3−/− mice receiving either WT or galectin-3−/− BM transplants. Dotted line represents the boundary between tumor cells (T) and stroma (S). Data are representative of eight mice per irradiation/transplant control group and 12 mice per experimental group. Two-tailed t tests were used to assess statistical differences. *, P < 0.05; ***, P < 0.001.
Macrophage depletion impairs tumor initiation

We hypothesized that tumor macrophages may contribute to tumor growth in the LLC1 model. C57Bl/6 CD11b-DTR transgenic mice were used as a model of macrophage ablation (33). CD11b-DTR transgenic mice and WT siblings received a single diphtheria toxin (DT) injection immediately prior to cell implant. At day 12, 15/24 tumors developed in CD11b-DTR mice compared with 20/22 in controls. CD11b-DTR animals had significantly smaller tumor volumes compared with controls (29.4 ± 4.1 mm³ and 89.4 ± 0.9 mm³, respectively; P = 0.0005, Fig. 2D) and significantly reduced tumor weights (9.1 ± 1.0 and 23.4 ± 4.0 mg, respectively; P = 0.0011, Fig. 2E). To assess the efficiency of macrophage ablation in this model, DT was administered to mice with established tumors and F4/80 staining carried out 24 hours after DT administration. An 88% reduction in TAMs was observed in the tumors of DT transplanted animals (P < 0.0001; Supplementary Fig. S2A).

Galectin-3 phenotype of BM-derived cells in the tumor microenvironment determines LLC1 tumor growth

To determine which galectin-3–expressing cells are necessary to support tumor growth, we first altered galectin-3 expression in recruited cells. Control and galectin-3−/− mice were irradiated and transplanted with 10⁶ control or galectin-3−/− BM cells. Eight weeks post-BM transplant, LLC1 cells were injected subcutaneously. Transplantation of control BM cells into galectin-3−/− mice resulted in significantly increased average tumor volume and final tumor weight compared with mice transplanted with galectin-3−/− BM cells (final tumor volume 335 ± 41 mm³ and final weight of 297.6 mg compared with 163.9 and 124.4 mg, P < 0.0001 and P = 0.0007, respectively; Fig. 2F and G). Dual immunofluorescence staining showed that the stroma of tumors harvested from galectin-3−/− animals transplanted with control BM had F4/80 and galectin-3 dual positive cells (Fig. 2H), although the total number of infiltrating macrophages was not different between control or galectin-3−/− BM transplanted mice (Supplementary Fig. S2B), suggesting that galectin-3 positive macrophages are recruited to the tumor stroma and contribute to tumor growth. LLC1 cells in vitro display cell surface and cytoplasmic galectin-3 staining and release galectin-3 into the culture medium (Supplementary Fig. S3A). To determine whether tumor-derived galectin-3 also contributes to tumor growth, galectin-3 was stably knocked down (KD) in LLC1 cells prior to subcutaneous injection (Supplementary Fig. S3B). Although LLC1 proliferation was reduced by galectin-3 KD in vitro (Supplementary Fig. S3C and S3D), tumor growth and final tumor weights of LLC1-galectin-3-KD cells was similar to WT cells (Supplementary Fig. S3E–S3G).

High-affinity galectin-3 inhibitor prevents human lung adenocarcinoma growth in vitro

Recently, a series of monosaccharide galectin-3 inhibitors with low nmol/L affinities and good selectivity over other galectins have been described (32). From this series GB1107 has high affinity in man at 37 nmol/L but due to species differences in the galectin-3 carbohydrate binding domain (CBD), the mouse galectin-3 affinity is 38-fold lower. GB1107 has low clearance (1.2 ml/min/kg, t₁/₂ = 4.5 hours, i.v.) and good uptake upon oral administration, resulting in high oral availability (F = 75%, orally). As a consequence, dosing GB1107 at 10 mg/kg orally once daily results in a plasma concentration above mouse Kₚ over 24 hours (Supplementary Fig. S4). CD-1 nude mice bearing human lung A549 adenocarcinoma xenografts were treated from day 18 postimplantation once daily with 10 mg/kg GB1107. This resulted in significantly reduced tumor growth and final tumor weights (46.2% smaller compared with vehicle control tumors with final average weights of 117 ± 16 and 63 ± 11 mg, respectively; P = 0.0132; Fig. 3A). Treatment with GB1107 also inhibited LLC1 growth (tumor volumes decreased 48% compared with controls on day 18, P < 0.001) and reduced final tumor weights (47 ± 14 mg vs. 120 ± 29 mg controls, P = 0.0524) when administered daily from the outset (Fig. 3B). Transcript analysis of tumor RNA from the LLC1 tumors revealed reduced galectin-3 (48% less than vehicle, P = 0.018) and mesenchymal markers TGFβ (45% less than vehicle, P = 0.015) and trends for reductions of VEGF and αSMA expression (Fig. 3C). Although there was no change in the M1 marker Nos2, there was a trend towards a reduction in significant reduced tumor burden by 79.2%. These data suggest that inhibition of galectin-3 with an orally active selective galectin-3 inhibitor can significantly reduce lung adenocarcinoma growth and metastasis in vivo.

Mice were generated that express the human LGALS3 gene in place of the mouse gene (Hu-Gal-3-KI). Western blot analysis confirmed expression of only human galectin-3 in mouse liver lysates from Hu-Gal-3-KI mice (Fig. 3E). LLC1 tumor growth was inhibited by GB1107 in Hu-Gal-3-KI mice when administration was delayed until day 5 after inoculation (Fig. 3F).

Galectin-3 inhibitor blocks LLC1-induced alternative macrophage activation

Given the altered M1:M2 TAM ratio in LLC1 tumors from galectin-3−/− mice and inhibitor-treated mice, we next determined the role of LLC1-derived galectin-3 on macrophage polarization. Conditioned media from LLC1 cells in vitro increased IL4-stimulated arginase activity in BM-derived macrophages (BMDM) and increased gene expression of arginase-1 and fizz1 (Supplementary Fig. S5A and S5B). This increase was inhibited by GB1107, suggesting galectin-3 secreted by LLC1 cells induces macrophages to adopt an alternative M2-like phenotype (Supplementary Fig. S5A and S5B). GB1107 did not affect LPS-induced Nos2 expression or nitric oxide (NO) production by BMDMs (Supplementary Fig. S5A and S5C). Although our data show TAMs to be a vital determinant of tumor growth in vivo, treatment of LLC1 cells with inhibitor in vitro also impacted on cell migration and proliferation albeit at higher concentrations (Supplementary Fig. S5D–S5F), suggesting some direct effect on galectin-3-mediated tumor cell expansion and migration.

Galectin-3 depletion reduces M2-like macrophages and enhances infiltration of activated CD8 T cells

TAMs can contribute to T-cell immunosuppression (4). In particular, M2-like macrophages secrete more galectin-3 (34) and...
Galectin-3 inhibitor GB1107 inhibits lung adenocarcinoma growth and metastasis in vivo. A, Female CD-1 nude mice received two subcutaneous injections of $3 \times 10^6$ A549 cells in a 1:1 ratio of Matrigel and serum-free media. Tumors grew to an average of 166 mm$^3$ before commencement of single daily dosing of vehicle ($n = 6$) or 10 mg/kg GB1107 ($n = 6$) from day 18. A, Tumor volumes and weights, along with representative images are shown. B, C57Bl/6 mice were injected subcutaneously with LLC1 cells and orally dosed once daily with vehicle or 10 mg/kg GB1107 from day 1. Tumor volumes and weights are shown. C, RNA was extracted from tumors from B and expression of various genes was evaluated by qPCR. D, To test the effect of GB1107 on metastasis, mice were injected with $1 \times 10^6$ LLC1-luciferase cells via the tail vein, followed by daily oral gavage of vehicle ($n = 10$) or GB1107 (10 mg/kg, $n = 10$) from day 1. On day 7, tumor burden in whole lungs was determined by qPCR using luciferase-specific primers. E, Western blot analysis confirming expression of human galectin-3 in liver lysates from hu-Gal-3-KI mice. Two-tailed t-tests were used to determine statistical significance. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (compared with vehicle controls). F, Hu-Gal-3-KI mice bearing bilateral subcutaneous LLC1 tumors received vehicle or 10 mg/kg GB1107 once daily orally from day 5. Tumor volumes and weights on day 14 are shown. Results from E represent the mean ± SEM of two independent experiments of $n = 6$. 

Figure 3.
Galectin-3 directly impedes T-cell infiltration and activation (24–27, 35). We therefore investigated whether galectin-3–dependent M2 polarization is also associated with changes in T-cell infiltration and activation in vivo. Flow cytometric analysis of tumor digests from hu-Gal-3-KI mice treated with GB1107 showed no increase in macrophage infiltration, but showed a decrease in macrophage CD206 expression indicative of reduced M2 TAMs (Supplementary Fig. S6A; Fig. 4A). Similarly, although there was no significant change in the total number of CD3+ T cells, GB1107 caused an increase in CD8+ but not CD4+ T cells within tumors (Fig. 4A). This pattern of immune infiltration was also observed in tumor digests from galectin-3–/– mice compared with WT C57Bl/6 mice (Fig. 4B).

To assess whether other systemic changes in galectin-3–depleted mice may also influence CD8 T-cell activation, we assessed myeloid populations within the BM of WT and galectin-3–/– mice. We observed no differences in total CD11b+ cells, neutrophils, monocytes, or dendritic cells (DC) compared with WT tumors (Supplementary Fig. S6B).

To assess whether other systemic changes in galectin-3–depleted mice may also influence CD8 T-cell activation, we assessed myeloid populations within the BM of WT and galectin-3–/– mice. We observed no differences in total CD11b+ cells, neutrophils, monocytes, or dendritic cells (DC) compared with WT tumors (Supplementary Fig. S6B).

Galectin-3 inhibitor potentiates the antitumor effects of PD-L1 blockade

We next examined the effect of galectin-3 inhibition in combination with immune checkpoint inhibition. In this study, GB1107 treatment was delayed until day 6 postimplantation. Delayed administration of GB1107 alone did not reduce tumor burden and administration of an anti-PD-L1 antibody administered twice weekly intraperitoneally from day 6 also had no impact on tumor growth. However, a combination of GB1107 and anti-PD-L1 antibody treatment significantly potentiated the effect of the single agents (49.5% and 51.4% tumor growth in the combination group was associated with an increase in the number of CD3+ T cells, GB1107 caused an increase in CD8+ T cells compared with untreated controls; Fig. 5A and B). The reduced tumor growth in the combination group was associated with an increase in CD8+ T cells (Fig. 5C; Supplementary Fig. S8). We therefore investigated whether galectin-3 depletion influenced CD8 T-cell activation, we assessed tumor infiltration and activation (28). Our data show that macrophages in tumors from galectin-3–/– mice or mice treated with GB1107 have reduced CD206+ M2-like macrophages and we observe reduced M2-promoting cytokine transcripts and elevated IFNγ expression within galectin-3–/– tumors. In addition, conditioned media from LLC1 cells increases alternative activation of macrophages in vitro and this can be blocked by coculture with GB1107. This demonstrates that galectin-3 contributes to the M2 immunosuppressive function of TAMs.

TAMs promote many important features of tumor progression including angiogenesis, tumor cell invasion, motility, and metastasis and can also suppress T-cell responses (4). These data show that galectin-3–expressing macrophages are recruited to the tumor site, develop an M2 phenotype and induce downregulation of CD8+ CTL functions. Galectin-3 has been shown to induce M2 cell tolerance resulting in T-cell anergy, through various mechanisms including inhibiting CD8 and TCR clustering (39), destabilizing the immune synapse and promoting internalization of TCR and CD3ε chains (40). It can also restrict membrane movement and TCR-associated signaling functions of CD45 (41) and inhibit LFA-1 recruitment thus disrupting proper synapse formation and secretion of IFNγ (27).

Galectin-3 may also suppress CTL effector function by binding to Lag-3, a negative regulatory checkpoint, on CD8+ T cells (26) and by inducing apoptosis of CTLs (25) and impairs the antitumor functions of natural killer (NK) cells (42). CTLs activated in vitro show an alteration in the N-glycome with longer and more branched N-glycans resulting in the expression of surface glyco-proteins that exhibit high galectin-3 binding (43). The high concentration of galectin-3 found in tumor microenvironments could potentially explain the loss of CTL functions through reduced motility and signaling functions of surface molecules.

Galectin-3–/– mice have also been shown to have an increase in lymph node plasmacytoid DCs (pDC) compared with WT mice, which are superior in activating CD8+ CTLs compared with conventional DC (26). In addition, galectin-3 knockdown in monocyte-derived DCs increases the proliferation and IFNγ production from antigen-stimulated CD4+ T cells (44). Our profiling of BM from WT and gal-3 KO mice showed an increase in CD45+ MHC-II+/CD11b+ DCs in BM of gal-3 KO mice compared with WT. Although our study did not distinguish DC subsets, together the data suggest that galectin-3 may indirectly regulate CD8 function by promoting DC functions. This requires further study.
Figure 4.
Galectin-3 depletion reduces intratumor M2-like macrophages and CD8 T-cell activation. LLC1 tumors from human Hu-Gal-3-KI mice from Fig. 3E treated with vehicle or GB1107 (n = 6) were digested and processed by flow cytometry. A, Relative prevalence of total macrophages and CD206 expression in macrophages and CD3⁺, CD4⁺, and CD8⁺ T cells in tumor digests. B, Flow cytometry analysis of subcutaneous tumors from WT or galectin-3⁻/⁻ mice (n = 4). NS, nonsignificant.
Figure 5. Combination therapy with galectin-3 inhibitor GB1107 and PD-L1 blocking antibody promotes tumor cell apoptosis and cytotoxic CD8 T-cell activation. On day 6, mice bearing subcutaneous LLC1 tumors were randomized into four groups and received either no treatment (n = 8), α-PD-L1 neutralizing antibody (200 μg twice weekly i.p.; n = 8), or GB1107 (10 mg/kg once daily orally; n = 8) or α-PD-L1 plus GB1107 (n = 8). Tumor volumes (A) and weights (B) on day 16 are shown. C, Tumor-infiltrating immune populations were analyzed by flow cytometry (n = 4). D, Total RNA was extracted from tumors in each group and qPCR was used to assess relative gene expression. E and F, IHC staining of cleaved caspase-3 was quantified as described in Materials and Methods. Scale bars, 100 μm. Data represent mean ± SE from a single (A, B, and D–F) or two (C) independent experiments. Two-way ANOVA with Tukey’s post hoc test was used to test for differences in tumor volume and cleaved caspase-3 IHC scores. One-way ANOVA and Fisher LSD test were used to compare tumor weights, and qPCR and flow cytometry data were compared using two-tailed t tests. *, P < 0.05; **, P < 0.01; ***, P < 0.001. ns, nonsignificant.
We show that treatment with GB1107 alone from the outset inhibits LLC1 growth and delayed treatment inhibits LLC1 growth in human galectin-3-expressing mice. This reflects the increased affinity this inhibitor has on human versus mouse galectin-3. In addition, the galectin-3 inhibitor significantly potentiates the effect of immune checkpoint blockade with an anti-CD-L1 blocking antibody. It is believed that the limited patient responses to checkpoint inhibition is attributable to the lack of T-cell infiltration in so-called “cold” tumors (2). Gordon-Alonso and colleagues, show that galectin-3 binds to the extracellular matrix and to glycosylated IFNγ, preventing release of IFNγ-induced CXCL9, which acts as a T-cell chemo-attractant (35). Consistent with this, GB1107 both alone or in combination with anti-CD-L1 increases the number of tumor infiltrating CD8+ TILs. Therefore, galectin-3 inhibition might provide the critical means to turn a “cold” tumor “hot,” and thus responsive to immune checkpoint intervention. Furthermore, CD8+ TILs within the combination drug-treated tumors are more activated (express more surface PD-1), and the cytokine environment favors tumor rejection with increased expression of cytotoxic (IFNγ, perforin-1, and granzyme B) and apoptotic (fas ligand) genes with increased caspase activation.

Reduced galectin-3 expression within tumor cells has been shown to reduce tumor growth in many cancers (reviewed in ref. 45), suggested to be due to the anti-apoptotic effect of cytoplasmic galectin-3 binding to K-RAS and engaging anti-apoptotic pathways via its NWGR motif (20). However, we show that knockdown of galectin-3 in tumor cells with siRNA had only a partial effect on tumor growth in vivo but had no significant effect on LLC1 growth in vitro. We also show that treatment with the galectin-3 inhibitor alone could inhibit human adenocarcinoma growth in CD-1 nude mice, which lack a T-cell response but which display innate immunity. These suggest that either tumor derived or macrophage-derived galectin-3 can impact on tumor growth in this model, independent of the T-cell-mediated effects.

In conclusion, our results demonstrate that galectin-3 inhibition leads to a reduction in M2-like TAMs and increased infiltration and activity of CD8+ TILs within LLC1 tumors resulting in reduced tumor growth and metastasis. Several studies have used other approaches to inhibit galectin-3 in cancer including peptide inhibitors (46), lactulose amines (47), a glycopeptide isolated from cod (48) and large complex plant-derived polysaccharides including modified citrus pectin (49), GCS-100 (59), and galactomannans such as CM-CT-01 (50). GCS-100 is currently being developed for chronic lymphoid leukemia and multiple myeloma (51). However recent evidence suggests that these complex carbohydrates do not act as inhibitors of the canonical carbohydrate-binding site of galectin-3 and their physiologic effects may be due to unrelated actions (52). We show using a specific and high affinity inhibitor of the galectin-3 carbohydrate site that pharmacologic inhibition of galectin-3 inhibits lung adenocarcinoma growth and potentiates the effect of immune checkpoint inhibitors. Therefore, galectin-3 has a strong regulatory effect on cancer-related inflammation and could present a key target in the management of lung, and potentially other galectin-3-driven carcinomas, in combination with immune checkpoint blockade.

Disclosure of Potential Conflicts of Interest

L. Vuong reports receiving other commercial research support from Galecto Biotech. N.C. Henderson is a consultant/advisory board member of Galecto Biotech. U.J. Nilsson has ownership interest (including stock, patents, etc.) in Galecto Biotech AB. H. Leffler reports receiving a commercial research grant from Galecto Biotech AB, has ownership interest (including stock, patents, etc.) in Galecto Biotech AB and is a consultant/advisory board member of Galecto Biotech AB. H. Schambye is a CEO at Galecto Biotech and has ownership interest (including stock, patents, etc.) in Galecto Biotech. T. Sethi is a Chief Physician Scientist at AstraZeneca and has ownership interest (including stock, patents, etc.) in Galecto Biotech. No potential conflicts of interest were disclosed by the other authors.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): L. Vuong, E. Kouvierianou, C.M. Rooney, B.J. McHugh, C.D. Gregory, A. Pedersen, L. Gravelle, A.C. Mackinnon


Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S.J. Forbes

Study supervision: T. Sethi

Other (design and synthesis of GB1107): F.R. Zetterberg

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An Orally Active Galectin-3 Antagonist Inhibits Lung Adenocarcinoma Growth and Augments Response to PD-L1 Blockade

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