Galectin-1 Mediates Radiation-Related Lymphopenia and Attenuates NSCLC Radiation Response

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Abstract

Purpose: Radiotherapy can result in lymphopenia, which has been linked to poorer survival. Here, we test the hypothesis that radiotherapy-induced lymphopenia is mediated by a tumor-secreted factor, Galectin-1 (Gal-1), which possesses T-cell proapoptotic activities.

Experimental Design: Matched Gal-1 wild-type (WT) or null mice were implanted with Lewis lung carcinoma (LLC-1) that either expressed Gal-1 or had Gal-1 stably downregulated. Tumors were irradiated locally and circulating Gal-1 and T cells were measured. Tumor growth, lung metastasis, intratumoral T-cell apoptosis, and microvessel density count were quantified. Thiodigalactoside (TDG), a Gal-1 inhibitor, was used to inhibit Gal-1 function in another group of mice to validate the observations noted with Gal-1 downregulation. Lymphocyte counts, survival, and plasma Gal-1 were analyzed in cohorts of radiotherapy-treated lung [non–small cell lung cancer (NSCLC)] and head and neck cancer patients.

Results: LLC irradiation increased Gal-1 secretion and decreased circulating T cells in mice, regardless of host Gal-1 expression. Inhibition of tumor Gal-1 with either shRNA or thiodigalactoside ablated radiotherapy-induced lymphopenia. Irradiated shGal-1 tumors showed significantly less intratumoral CD8+ T-cell apoptosis and microvessel density, which led to marked tumor growth delay and reduced lung metastasis compared with controls. Similar observations were made after thiodigalactoside treatment. Radiotherapy-induced lymphopenia was associated with poorer overall survival in patients with NSCLC treated with hypofractionated radiotherapy. Plasma Gal-1 increased whereas T-cell decreased after radiation in another group of patients.

Conclusions: Radiotherapy-related systemic lymphopenia appeared to be mediated by radiotherapy-induced tumor Gal-1 secretion that could lead to tumor progression through intratumoral immune suppression and enhanced angiogenesis. Clin Cancer Res; 20(21); 1–12. ©2014 AACR.

Introduction

Radiotherapy-related lymphopenia is a persistent occurrence that has been frequently observed with radiotherapy to different sites, including brain, thorax, abdomen, and pelvis (1–5). More importantly, radiotherapy-induced lymphopenia has been shown to be a negative prognostic factor in various cancers, including high-grade glioma, sarcomas, and carcinomas of the lung, breast, and pancreas (1–4). The causes of radiotherapy-induced lymphopenia and its association with poor prognosis are largely unknown.

Galectin-1 is the prototype member of the Galectin superfamily, characterized by high-affinity binding to β-galactosides through a well-conserved carbohydrate recognition domain (CRD). In cancer, Gal-1 also plays a critical role in altering the fate and phenotype of T cells to suppress their antitumor functions and create an immune-privileged tumor microenvironment, which are well documented in melanoma, lymphoma, lung, and breast cancer syngeneic models (7–10). Importantly, tumor rejection mediated by Gal-1 inhibition requires intact CD4+ and CD8+ T-cell immunity (7). We have previously shown that Gal-1 promotes tumor growth and metastasis, in part, through intratumoral T-cell modulation. Notably, tumor-derived Gal-1, as opposed to host, drives disease progression through mediating the apoptosis of CD4+ and CD8+ T cells within the tumor, stymieing antitumor immunity (9).

We hypothesized that Gal-1 may be a mediator of radiotherapy-induced lymphopenia and its poor prognosis. Using a syngeneic mouse tumor model, we show that tumor irradiation increases plasma Gal-1 and induces significant depletion of circulating T lymphocytes in a tumor-specific Gal-1 manner. This function was disrupted...
by Gal-1 knockdown or thiodigalactoside. Gal-1 secretion from the tumor was associated with large tumor growth and development of spontaneous pulmonary metastasis. Tumor irradiation resulted in a small delay in tumor regrowth but no impact on lung metastasis, which improved significantly with either Gal-1 downregulation or inhibition. This was associated with more infiltrating intratumoral CD8⁺ T cells, less intratumoral CD4⁺ and CD8⁺ T-cell apoptosis, and lower microvessel density. Clinically, we noted that lymphopenia occurred in a cohort of early-stage non–small cell lung cancer (NSCLC) patients treated with small-field, hypofractionated radiotherapy and is associated with reduced survival. In particular, patients receiving radiotherapy for non–small cell lung cancer (NSCLC) did significantly worse than their matched counterparts if they exhibited lymphopenia. Targeting the mechanisms underlying this phenomenon, currently elusive, could improve curability. This is the first study to not only show that radiation enhances Gal-1 secretion in NSCLC, but also that a tumor-secreted factor can significantly influence the level of circulating T lymphocyte after tumor irradiation while augmenting tumor growth and metastasis. Our results suggest that combining Gal-1 blockade with radiotherapy can enhance radiation effectiveness in NSCLC through the prevention of radiation-related lymphopenia and immune suppression within the tumor microenvironment.

Translational Relevance
Our identification of Gal-1 as a modulator of radiation-related systemic lymphopenia provides an exciting new path in cancer therapy with Gal-1 as a target to combine with radiation treatment. Radiation-related lymphopenia is frequently observed with fractionated external beam radiotherapy and is associated with reduced survival. In particular, patients receiving radiotherapy for non–small cell lung cancer (NSCLC) did significantly worse than their matched counterparts if they exhibited lymphopenia. Targeting the mechanisms underlying this phenomenon, currently elusive, could improve curability. This is the first study to not only show that radiation enhances Gal-1 secretion in NSCLC, but also that a tumor-secreted factor can significantly influence the level of circulating T lymphocyte after tumor irradiation while augmenting tumor growth and metastasis. Our results suggest that combining Gal-1 blockade with radiotherapy can enhance radiation effectiveness in NSCLC through the prevention of radiation-related lymphopenia and immune suppression within the tumor microenvironment.

Materials and Methods
Cell lines and generation of stable Gal-1 knockdown
Lewis lung carcinoma (LLC-1) was obtained from the American Type Culture Collection (ATCC). LKR13 murine lung adenocarcinoma cell line derived from KrasLA1 mice (11) was a generous gift from Alejandro Sweet-Cordero (Department of Pediatrics, Stanford University School of Medicine, Stanford, California). Cells were cultured in DMEM (LLC-1) or RPMI (LKR13) with 10% fetal calf serum, penicillin (100 U/mL), and streptomycin (0.1 mg/mL) at 37°C and 5% CO₂ in air. Cell lines were not independently validated in our laboratory. To generate stable Gal-1 knockdowns, LLC-1 and LKR13 cells were subject to lentivirus transduction with scrambled or Gal-1 shRNAs, CTTAAGGTTAAGCTGCCCTGCTTCGAGCGAGG GCCAGCTAACCTTGAGG or CGGCGCTACAATCCCTGCTTCTCGAGAACAGCTTAGTTITT (Thermo Scientific Open Biosystems) as previously described (9).

Western blotting
Lysates were probed with goat anti-Gal-1 primary (1:1,000; R&D Systems), secondary anti-goat horseradish peroxidase (HRP; 1:10,000; Zymed), mouse anti-β-actin primary (1:1,000; Sigma-Aldrich), and anti-mouse-HRP secondary (1:10,000; Invitrogen) antibodies. Secreted Gal-1 in serum-free media was collected from LLC-1, LKR13 Scr, and shGal-1 cells, concentrated (EMD Millipore), and thereafter volumes were normalized by cell number.

Mice and tumor models
C57BL/6 mice were obtained from The Jackson Laboratory and bred with Lgals1-null (Gal-1⁻/⁻) mice from the Consortium for Functional Glycomics (Scripps Research Institute, La Jolla, CA) to generate wild-type (WT) littermates (9). Genotyping by PCR WT forward primer: GACCCCATCCCTACACCCCAG; Gal-1-null forward primer: CTATCAGGACATAGCGTTGG; and common antisense primer: AAACCTCAGCCCCAGAGAGG (9). WT and Gal-1⁻/⁻ mice (8–10-weeks old) received subcutaneous inoculation of LLC scramble or shGal-1 cells (2 × 10⁵) on the flank. Once tumors reached approximately 150 mm³, mice were placed in lead shielding jigs and exposed tumors received 20 Gy irradiation using a 250 kVp orthovoltage therapeutic X-ray machine (Philips). Tumors were caliper measured and volume was calculated as [(width)² × length]/2.

Thiodigalactoside treatment
Thiodigalactoside was injected intratumorally in mice (120 mg/kg) 1 week before radiation and every 2 to 3 days thereafter.

Isolation of splenocyte and whole blood T cells
Splenocytes were isolated from spleen of 8- to 10-week-old C57BL/6 WT and Gal-1⁻/⁻ mice. After filtration and red blood cell lysis (Sigma), splenocytes underwent density gradient centrifugation (Cedarlane) and resuspension at 1 to 2 × 10⁶ cells/mL in RPMI containing 5 μg/mL concanavalin A (Sigma), 55 μmol/L betamercaptoethanol (Gibco), and dithiothreitol (1.1 mmol/L). Peripheral blood from retro-orbital eye blood was collected in EDTA tubes and RBCs were lysed (Sigma-Aldrich).

In vitro T-cell apoptosis and TUNEL staining
Lymphocytes were plated at 1 to 2 × 10⁶ cells/mL for 24 hours followed by treatment with recombinant rat Gal-1 (10 μg/mL; Sigma) for 24 to 48 hours. TUNEL (terminal deoxynucleotidyltransferase–mediated dUTP nick end labeling)
FTC staining was performed using the Apo-Direct Kit (BD Biosciences).

**Flow cytometry**

Lymphocyte subsets were stained with Pacific Blue anti-mouse CD3e rat IgG (clone 500A2), PE anti-mouse CD4 rat IgG (clone RM4–5), and Alexa Fluor-647 anti-mouse CD8a rat IgG (clone 53–6.7), at 4°C for 45 minutes in staining buffer (BD Biosciences). Cell acquisition was performed with the FACSDiva software on a LSRII flow cytometer (BD Biosciences) and analyzed with FlowJo software (TreeStar Inc.).

**Immunohistochemistry**

Lung metastasis was quantified by H&E staining (13). Two sections 100 μm apart were used for metastasis quantification, with at least 5 mice per group. Fixed sections were stained with goat anti-CD31 (1:100, clone M-20; Santa Cruz Biotechnology Inc.) followed by secondary anti-goat detection by DAB (Vector Laboratories; ref. 9).

**Immunofluorescence**

Frozen tissue sections were incubated with CD4 and CD8 primary (1:100; BD Biosciences) and Alexa Fluir-594 anti-rat secondary antibodies (1:200; Invitrogen; ref. 12). Slides were labeled with TUNEL-FITC (Roche Applied Science) and mounted in DAPI (Vector Laboratories). Immunofluorescence images were acquired using a Zeiss LSM510 laser scanning confocal microscope, using 40x/0.95 NA and 63x/1.4 NA Plan Apochromat objectives.

**Galectin-1 ELISA**

Whole blood from retro-orbital bloods were collected in BD Microtainer tubes with EDTA and centrifuged at 3,000 rpm for 10 minutes. Plasma was diluted and subject to mouse Galectin-1 ELISA per the manufacturer’s instructions (R&D Systems).

**Patient population**

Patients with early-stage NSCLC included in our analysis were diagnosed between 1999 and 2013 with inoperable disease, received only SABR (no chemotherapy), and had pre- and postradiation lymphocyte values up to 38 months after treatment. Patients with HNC in our study were diagnosed between 2007 and 2013 and received radiation with cetuximab or cisplatin. Because some patients had multiple postradiation lymphocyte counts, we calculated the maximum lymphocyte change as difference between the pre-radiation value and the lowest postradiation therapy value.

**Statistical analysis**

Patient survival analysis was performed using Cox proportional hazards model with the maximum drop in absolute lymphocyte counts between before and after radiotherapy as the only predictor. Four quartiles of the maximum drop in absolute lymphocyte counts before and after radiotherapy were plotted for the indicated endpoints. Overall survival (OS) was defined as the time from treatment date to death. Distant progression-free survival (DPFS) was defined as the time from treatment to documented first distant relapse or death, local progression-free survival (LPFS) as the time from treatment to documented first local relapse or death, and regional progression-free survival (RPFS) as the time from treatment to documented first regional nodal relapse or death. In all other cases, quantifications were subject to two-tailed, paired Student t test to analyze statistical differences between groups. Data are expressed as mean ± SE. A P value of <0.05 was considered significant.

**Results**

**Gal-1’s secretion is enhanced by radiation and induces T-cell apoptosis in vitro**

Gal-1 secretion increased significantly after radiotherapy in several different tumor cell lines, including two murine NSCLC cell lines. This increase of Gal-1 secretion was noted as early as 3 hours in LLC-1. Intracellular level of Gal-1 did not change, suggesting that the bulk of newly synthesized Gal-1 proteins were secreted after radiotherapy (Fig. 1A). Similar to others (7, 9, 10), we found that rGal-1 induced apoptosis of concanavalin A–activated lymphocytes isolated from mouse spleen in a dose-dependent manner (Fig. 1B). This effect was abrogated by a Gal-1–specific antibody (anti-Gal1) or thiodigalactoside, a Gal-1 competitive inhibitor (Fig. 1C) when T cells were exposed to Gal-1–containing conditioned media. In addition to T lymphocytes, rGal-1 can also cause apoptosis in CD19+ B cells (assessed by TUNEL) though to a lesser extent (data not shown). Gal-1 is expressed in memory B cells and has been shown to regulate apoptosis through Bcl2 signaling (14).

**Tumor-secreted Gal-1 reduces circulating helper and cytotoxic T cells after tumor irradiation**

On the basis that radiation enhances the secretion of Gal-1, which has proapoptotic function in T cells, we evaluated the role of Gal-1 in radiotherapy-related lymphopenia. To address this, we examined the levels of circulating Gal-1 and T lymphocytes in the following four host and LLC-1 tumor combinations to differentiate between host and tumor Gal-1 contribution to radiation-related lymphopenia: WT/Scr (both host- and tumor-expressed Gal-1), WT/shGal-1 (only host-expressed Gal-1), Gal-1+/–/Scr (only tumor-expressed Gal-1), and Gal-1+/–/shGal-1 (neither host- nor tumor-expressed Gal-1). Once tumors reached a volume of approximately 150 mm3, 20 Gy of radiation was delivered in a single fraction at the tumor site (Supplementary Fig. S1).

Local tumor irradiation significantly increased the level of circulating Gal-1 only in Gal-1–expressing tumors (∼1.5 in WT host and ∼0.9 in WT host and ∼2.9 in WT and in WT host and ∼2.9) but not in Gal-1 downregulated tumors (Fig. 2A). These data indicated that radiotherapy increased Gal-1 secretion from the tumor that could be measured in the plasma. In parallel to the increase of plasma Gal-1, there was a significant decrease in circulating T cells in WT mice bearing Gal-1–secreting tumors (WT/Scr), 32.9% (P = 0.026) for CD3+ mature T cells and 31.7% (P = 0.033) for CD3+ CD8+ cytotoxic T cells, 2 weeks after tumor irradiation. There was also a decrease in CD4+ T helper cells...
but the reduction did not reach statistical significance (28.2%; \(P = 0.09\); Fig. 2B–D). In contrast, CD4\(^+\) and CD8\(^+\) T lymphocyte levels did not decrease significantly after irradiation of shGal-1 tumors implanted in Gal-1 WT or Gal-1\(^{+/+}\)/C0 mice (WT/shGal-1 and Gal-1\(^{+/+}\)/C0/shGal-1). Furthermore, we observed a significant decrease in the CD3\(^+\) and CD8\(^+\) T cells (29.4%, \(P = 0.02\) and 34.4%, \(P = 0.02\), respectively) but not CD4\(^+\) T cells (12.9%; \(P = 0.48\)) in Gal-1\(^{+/+}\)/C0 mice bearing Gal-1–secreting tumors (Gal-1\(^{+/+}\)/Scr), suggesting that radiotherapy-related lymphopenia occurred independently of host Gal-1 status (Fig. 2B–D).

To ensure that the noted effect was due to Gal-1 secretion from radiotherapy rather than from Gal-1 secretion from growing tumors during the 2 weeks of observation, we also analyzed circulating T cells in WT mice, implanted with Gal-1–secreting tumors that were not irradiated and allowed to grow for 2 weeks after reaching approximately 150 mm\(^3\). Without tumor irradiation, there was a small decrease in the level of circulating CD3\(^+\), CD4\(^+\), and CD8\(^+\) T cells (18.1%, 18.05%, and 6.60%, respectively) but none of the changes reached statistical significance (Fig. 2E). Similarly, day 28 terminal blood from mice in Fig. 2E showed a 2-fold higher plasma Gal-1 level in the irradiated group compared with the non-irradiated mice, despite larger tumors in the non-irradiated group, suggesting that radiotherapy induced Gal-1 secretion into the blood (Fig. 2F). These data suggested that Gal-1–containing tumors did release a low basal level of Gal-1 into the blood that caused a small drop in circulating lymphocytes, and that this effect was significantly augmented by tumor irradiation.

We also evaluated the effect of Gal-1 secretion on common lymphoid progenitors or nesting mature T cells in the bone marrow. There was no change in these cell populations with radiation and there was no change in Gal-1 expression on the surface of these cells (Supplementary Fig. S2). To ensure that there was no difference in inherent sensitivity to radiation or Gal-1 between lymphocytes from WT and Gal-1\(^{+/+}\)/Scr mice, we also tested these effects in vitro; no difference was noted between the two T-cell populations (Supplementary Fig. S3).

To corroborate our previous findings with the shGal-1 inhibition, we also evaluated the effect of inhibiting Gal-1 pharmacologically with the competitive inhibitor, thiodigalactoside (Fig. 2G). Thiodigalactoside is a naturally occurring thioglycoside that is highly stable in vivo, and binds to the CRD of Gal-1, thereby preventing ligand interactions.
We performed intratumoral thiodigalactoside injections in Gal-1 WT mice implanted with LLC scorable tumors to inhibit Gal-1 and examined changes in the abundance of circulating CD3⁺, CD4⁺, and CD8⁺ lymphocytes before and after tumor irradiation. Thiodigalactoside treatment before radiotherapy resulted in a small, nonsignificant increase of total, CD4⁺, and CD8⁺ T cells, suggesting that basal Gal-1 release from the tumor caused some circulating T-cell death, and this was blocked by thiodigalactoside injection. More importantly, radiation resulted in a significant drop of circulating CD3⁺ (51.2%; \( P = 0.006 \)), CD4⁺ (42.2%; \( P = 0.005 \)), and CD8⁺ (43.5%; \( P = 0.008 \)) lymphocytes, which was rescued with thiodigalactoside treatment. Altogether, our data suggest that Gal-1 was secreted into the blood by the tumor after radiation, resulting in lymphopenia that could be abrogated by Gal-1 inhibition.

**Gal-1 modulates tumor radiation response through the modulation of tumor T-cell apoptosis and angiogenesis**

In light of our observation that Gal-1 mediates radiotherapy-related lymphopenia, which is associated with worse outcomes in the clinic, we hypothesized that Gal-1 reduced tumor response to radiotherapy and enhanced tumor metastasis and that these effects could be minimized with Gal-1 downregulation or inhibition. In vitro, Gal-1 downregulation in LKR-13 lung cancer cells moderately enhanced radiation cell killing under either normoxic or severe hypoxia, as assessed by clonogenic survival curves (Supplementary Fig. S4A and S4B). To investigate Gal-1 effect on tumor radiosensitivity in vivo, we implanted LLC scorable and shGal-1 tumors in Gal-1 WT C57Bl/6 mice and irradiated the tumors with 20 Gy in a single fraction, after allowing the tumors to reach a volume of approximately 150 mm³. Tumor growth was monitored and upon sacrifice, lung metastases, intratumoral T-cell infiltration, and apoptosis, as well as, microvessel density count, were quantified. As expected, either downregulation of Gal-1 alone or radiation alone resulted in similar decreases in tumor growth (Fig. 3A). However, the combination of radiation and Gal-1 downregulation nearly abolished tumor growth (mean, \( 1.16 \pm 0.97 \) fold volume increase when normalized to tumor volume on day of irradiation) during the 2 weeks of observation (\( P = 0.02 \); Fig. 3A). This observation was also made with LKR13 tumors in vivo (Supplementary Fig. S4C). Radiation alone did not affect the number of spontaneous lung metastasis (Fig. 3B). Downregulation of Gal-1 significantly decreased the number of lung metastases, which was further reduced with the addition of local field radiotherapy (\( P < 0.05 \)).

Similar results for tumor growth were observed when we inhibited Gal-1 with intratumoral thiodigalactoside injection. Thiodigalactoside injection alone or radiation alone reduced tumor growth to a similar extent and the combination of radiation and thiodigalactoside yielded the slowest growth rate (\( P = 0.05 \); Fig. 3C). Again, tumor irradiation alone did not reduce lung metastases compared with the nonirradiated control (Fig. 3D). Intratumoral thiodigalactoside injection resulted in a nonsignificant reduction in the number of lung metastasis. However, the combination of thiodigalactoside and radiation resulted in a significant decrease in lung metastases (\(-40\%\)) compared with control (\( P = 0.037 \)).

Mechanistically, downregulation of Gal-1 increased CD8⁺ but not CD4⁺ T-cell tumor infiltration (Fig. 4A and B). Gal-1 depletion combined with radiation increased tumor infiltration by CD8⁺ T cells even further (Fig. 4B). Notably, Gal-1 knockdown reduced apoptosis of both intratumoral CD4⁺ and CD8⁺ T-cell subsets (Fig. 4C and D). Gal-1 inhibition by knockdown and thiodigalactoside also reduced tumor angiogenesis, which was further attenuated when combined with radiation (Fig. 4E and F).

**Radiotherapy-related lymphopenia is associated with reduced survival in early-stage NSCLC treated with hypofractionated radiotherapy**

The study cohort consisted of 20 patients with stage I–II NSCLC treated definitively with small-field hypofractionated stereotactic ablative radiotherapy alone. Supplementary Table S1 summarizes the patient characteristics. The majority of patients, 15 out of 20 (75%), experienced a drop in absolute lymphocyte count during the first year and the mean maximum decrease for the entire group was \( 0.4 \pm 0.22 \times 10^7/\text{mL} \). Figure 5A shows the mean absolute lymphocyte counts before (pre-SABR) and after SABR (post-SABR), when the counts were at their lowest within the first year. The decrease was statistically significant (\( P = 0.025 \)). Univariate Cox proportional hazards analysis showed that the degree of drop in absolute lymphocyte count (split by quartile) was associated with worse OS (\( P = 0.037 \); HR, 1.148; Fig. 5B), disease-free survival (DFS; \( P = 0.033 \); HR, 1.134; Fig. 5C), DPFS (\( P = 0.023 \); HR, 1.159; Supplementary Fig. S5A), and LPFS (\( P = 0.049 \); HR, 1.129; Supplementary Fig. S5B). It was also associated with a trend for worse RPFS (\( P = 0.056 \); HR, 1.126; Supplementary Fig. S5C). These findings revealed that radiotherapy-related...
lymphopenia also occurred in SABR using very small fields in one to four fractions and was prognostic for tumor control and survival in early-stage NSCLC.

Because plasma samples from these 20 patients with NSCLC were not available for Gal-1 measurement, we measured circulating Gal-1 and T lymphocytes in a group of patients with HNC treated with radiotherapy with either concomitant cetuximab or cisplatin (N = 24). Patient characteristics for this group are shown in Supplementary Table S2. We observed a significant decrease in lymphocyte count (0.88 ± 0.18 × 10⁹/mL; P < 0.001) and increase in plasma Gal-1 (5.89 ± 4.3 ng/mL; P = 0.05) after treatment (Fig. 5D and E). The degree of radiotherapy-induced lymphopenia was not significantly different between HNC treated with radiotherapy combined with cisplatin or cetuximab (Supplementary Fig. S5D). To date, we only noted three failures in the patients with HNC with a median follow-up of 11.2 months; such few number of events precludes accurate survival time analysis. However, there was a trend for larger lymphocyte drop (1.2 vs. 0.8 × 10⁹/mL) and a higher rise in Gal-1 level (14.1 vs. 4.7 ng/mL) in patients who failed compared with those who did not, although the difference did not reach statistical significance due to the small number of patients (Supplementary Fig. S5E).

Our findings indicate that in addition to proangiogenic function, Gal-1–mediated intratumoral T-cell apoptosis and peripheral T-cell depletion, which increase after radiation, facilitate radiation resistance and poor prognosis with SABR-treated NSCLC. Figure 6 summarizes our proposed mechanism.

Discussion

Radiation, which is major treatment modality for solid tumors, is a modulator of the antitumor immune response; yet, its stimulatory activity can be counteracted by radiotherapy-induced lymphopenia, which is associated with worse treatment response. In this study, we identified Gal-1 as a mediator of radiotherapy-driven lymphopenia and attenuator of tumor radiation response. Targeting Gal-1 prevents intratumoral and systemic

Figure 3. Gal-1 mediates tumor radiation response in vitro and in vivo and promotes lung metastases. A, tumor growth curves for Gal-1 WT C57Bl/6 mice implanted with scramble (Scr) or Gal-1 knockdown LLC-1 tumor cells (shGal-1) with and without radiation. Twenty Gy of ionizing radiation was delivered in a single fraction (bolt). Tumor volumes were normalized to size on day of radiation (N = 7 to 10 mice/group; P = 0.02). B, spontaneous metastases quantified from hematoxylin and eosin–stained lungs from mice in A (P < 0.05). Representative light microscopy images at right (40 objective). C, tumor growth curves for Gal-1 WT mice implanted with scramble LLC-1 and treated with intratumoral thiodigalactoside (TDG) or PBS injection on the days indicated. Irradiated mice receiving PBS (PBS IR) and thiodigalactoside (TDG IR) received a single dose of 20 Gy ionizing radiation at the indicated time (bolt). Tumor volumes normalized to size on day of radiation (N = 5 mice/group; P = 0.05). D, lung metastases of mice from C is shown (P = 0.037). Data represent the mean ± SE.
immunosuppression, supporting antitumor immune responses after radiotherapy.

The clinical impact of radiotherapy-induced lymphopenia has only been systematically studied recently. One possible mechanism for radiotherapy-related lymphopenia is direct irradiation of T lymphocytes as they enter radiation fields. The proportion of irradiated circulating lymphocytes increased with greater fractions and slower dose rates (16). On the other hand, direct irradiation-induced lymphocyte killing is unlikely responsible for lymphopenia observed in SABR-treated early-stage NSCLC. These patients receive hypofractionated therapy, which encompasses high dose delivered in a few fractions, limiting lymphocyte exposure to radiation as they circulate through the small treatment fields. Instead, in SABR-treated early-stage NSCLC, alternative mechanisms such
as secreted cytokines (with Gal-1 being of such cytokines) may drive this phenomenon.

Radiation-related lymphopenia is also often persistent, extending from months to years after radiotherapy (1–3, 12). This cannot be explained by radiation destruction of the bone marrow when treatment sites do not encompass active marrow. The half-lives of CD4+ (87 days), CD8+ T cells (77 days; ref. 17), and Gal-1 (20 hours; ref. 13) are not long enough to explain persistent lymphopenia exceeding a year after radiation (1, 3, 12). Our assessment of the bone marrow niche also revealed that radiotherapy-induced tumor Gal-1 secretion did not affect common lymphoid progenitors or nesting mature T cells. Interestingly, a study conducted by Ellsworth and colleagues revealed that in patients with high-grade glioma treated with radiotherapy and temozolomide, patients with radiotherapy-related lymphopenia had lower levels of IL-7, a cytokine involved in T-cell homeostatic proliferation, and IL15, which is responsible for CD8+ T-cell survival. The reduction of these cytokines prevented a compensatory response to treatment-related lymphopenia, allowing the depletion of lymphocytes to endure (18). T-cell homeostatic factors can therefore contribute to the persistence of this phenomenon.

Although we first noted that hypoxia increased Gal-1 secretion, we also found that radiation exposure had the same effect in multiple cell lines, suggesting that Gal-1 secretion can be driven by different stressors (Supplementary Fig. S6). Although upregulation of Gal-1 by radiation...
has been observed in cultured cell lines by others (19–21), we are the first to show that localized tumor radiation resulted in a measurable rise in plasma Gal-1 in vivo. Because we did not have plasma samples from patients with NSCLC, we measured Gal-1 level in patients with HNC, who generally have larger tumor volumes, hence potentially more detectable change of Gal-1 with radiation. In addition, we have previously shown that HNC produced Gal-1 that could be detected in the tumor stroma (22). Indeed, we detected a significant increase in plasma Gal-1 after radiation in these patients with a concomitant drop in T lymphocytes. Therefore, radiotherapy-induced secretion of Gal-1 can be detected in both mouse and human plasma.

Most notably, we found that Gal-1 drives radiotherapy-induced depletion of circulating T cells. Gal-1 is also produced by tumor-associated endothelial cells (21); however, our tumor model, which controls for host Gal-1 with Gal1+/- mice, suggests that tumor Gal-1 alone is responsible for the drop in circulating T cells following radiotherapy. Gal-1–mediated T-cell suppression at the periphery has been shown in other contexts, including the maintenance of central T-cell tolerance (23). Here, we found that tumor-secreted Gal-1 also drives the depletion of peripheral T cells after radiotherapy.

In lung cancer, Gal-1 expression is associated with increased tumor size, nodal metastasis, and decreased survival (24, 25). Gal-1 promotes distant metastasis and down-regulation of Gal-1 substantially reduced the development of spontaneous lung metastasis, an effect exclusive to immunocompetent hosts (9). In addition, Gal-1–positive lung metastases showed more CD3+ T-cell apoptosis within the metastatic site (26). Although local field radiotherapy caused some shrinkage of the primary tumor, it did not affect the number of lung metastasis, which could be explained by radiotherapy stimulation of Gal-1 secretion. Interestingly, thiogalactoside treatment alone in our model did not significantly affect lung metastasis, contradictory to Gal-1 genetic inhibition and is potentially due to the fact that thiogalactoside is a less efficient inhibitor of Gal-1 than shRNA and we used local rather than systemic thiogalactoside administration. When Ito and Ralph (26) used intravenous administration of thiogalactoside in a breast cancer model, they showed that systemic thiogalactoside treatment reduced lung metastasis. Here, combined thiogalactoside and radiotherapy, however, significantly reduced the number of lung metastasis by 40%, indicating that combining a Gal-1 inhibitor and radiation can minimize both tumor growth and dissemination.

In this study, we provide evidence that tumors shed more Gal-1 into the blood after radiotherapy, causing greater systemic lymphopenia as a bystander phenomenon. Less circulating lymphocytes would result in fewer lymphocytes available to infiltrate the tumor, further suppressing the antitumor response, promoting more aggressive tumor growth and spread. This was supported by our observation that Gal-1 depletion alone increased intratumoral CD8+ T cells compared with scramble tumors (9, 27, 28) and that combined Gal-1 downregulation and radiation increased tumor CD8+ T-cell infiltration even further.

A complex relationship exists between radiotherapy and the immune system. Radiotherapy can enhance the antitumor immune response, acting indirectly on the tumor through the host immune system (29, 30). Radiotherapy increases the peptide repertoire, enhancing cytokines and components of the antigen presentation pathway to promote recruitment and tumor lysis by CD8+ T cells (31–33).
Despite its immune-stimulating effects, radiotherapy does not result in protective immunity in the clinic because relapse occurs. Our observations support that Gal-1 works in opposition of these cytokines, to disrupt accumulation of functional intratumoral effector T cells locally and from the periphery through radiotherapy-related immunosuppression. Combined, these functions, and Gal-1 involvement in tumor immune surveillance, can explain for poor prognosis related to Gal-1 mediated systemic lymphopenia.

Our identification of Gal-1 as a modulator of radiation-related lymphopenia provides an exciting new path in cancer immunotherapy with Gal-1 as a target to combine with radiation treatment. It also provides a rapid biologic read out in the clinic to track the success of anti-Gal-1 therapy. Future efforts devoted to improving radiotherapy require maximizing the antitumor immune response (34), and we are currently exploring Gal-1 immunomodulatory effects as they relate to immune checkpoint proteins and tumor immunity. Targeting the Gal-1–N-Glycan interaction has been shown to promote vascular normalization and T-cell recruitment to the tumor (35). This may be one of the mechanisms by which Gal-1 modulate tumor aggressiveness. Our continued investigation focuses on the molecular pathways involved in Gal-1 modulation of the tumor microenvironment, including its effect on angiogenesis, to improve the antitumor immune response with radiotherapy. Gal-1 blockade is currently achieved with neutralizing antibodies as well as peptide and synthetic small-module inhibitors (36–39). Altogether, our future work will focus on the best way to inhibit Gal-1 function in combination with radiotherapy to improve curability in NSCLC.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
Conception and design: P. Kuo, A.J. Giaccia, A.C. Koong, M. Diehn, Q.-T. Le
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