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Targeting Galectin-1 Overcomes Breast Cancer-Associated Immunosuppression and Prevents Metastatic Disease

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Abstract

Galectin-1 (Gal1), an evolutionarily conserved glycan-binding protein, contributes to the creation of an immunosuppressed microenvironment at sites of tumor growth. In spite of considerable progress in elucidating its role in tumor-immune escape, the mechanisms underlying the inhibitory functions of Gal1 remain obscure. Here, we investigated the contribution of tumor Gal1 to tumor growth, metastasis, and immunosuppression in breast cancer. We found that the frequency of Gal1⁺ cells in human breast cancer biopsies correlated positively with tumor grade, while specimens from patients with benign hyperplasia showed negative or limited Gal1 staining. To examine the pathophysiologic relevance of Gal1 in breast cancer, we used the metastatic mouse mammary tumor 4T1, which expresses and secretes substantial amounts of Gal1. Silencing Gal1 expression in this model induced a marked reduction in both tumor growth and the number of lung metastases. This effect was abrogated when mice were inoculated with wild-type 4T1 tumor cells in their contralateral flank, suggesting involvement of a systemic modulation of the immune response. Gal1 attenuation in 4T1 cells also reduced the frequency of CD4⁺CD25⁺ Foxp3⁺ regulatory T (T_{reg}) cells within the tumor, draining lymph nodes, spleen, and lung metastases. Further, it abrogated the immunosuppressive function of T_{reg} cells and selectively lowered the expression of the T-cell regulatory molecule LAT (linker for activation of T cells) on these cells, disarming their suppressive activity. Taken together, our results offer a preclinical proof of concept that therapeutic targeting of Gal1 can overcome breast cancer-associated immunosuppression and can prevent metastatic disease. *Cancer Res*; 73(3); 1107–17. ©2012 AACR.

Introduction

The breast cancer microenvironment is composed of innate and adaptive immune components that actively influence the antitumor response (1). The current view supports the idea that immune cells efficiently protect the host from arising tumors; however, once a primary tumor is generated, the

tumor-associated immune cell network can paradoxically promote tumor growth and metastasis (2).

Lectin–glycan interactions have emerged as critical determinants of tumor progression (3). Galectins, a family of endogenous lectins abundantly expressed in tumor microenvironments, can regulate transformation, angiogenesis, cell adhesion, and tumor-immune escape (4–7). Galectin-1 (Gal1), a 'prototype' member of this family, binds to multiple *N*-acetylglucosamine (Galβ1-4GlcNAc) units decorating complex *N*- and *O*-glycans on cell surface glycoproteins (8). Research from our laboratory has recognized an essential role for Gal1 as an immunoevasive mechanism in human and mouse melanoma (9). These observations were further confirmed in mouse lung adenocarcinoma (10, 11), and in human cancers including Hodgkin lymphoma (12, 13), neuroblastoma (14), head and neck squamous cell carcinoma (15), pancreatic carcinoma (16), glioma (17), and T-cell lymphoma (18), suggesting that targeting the Gal1–glycan axis might contribute to overcome immunosuppression and potentiate immunotherapeutic approaches. Interestingly, Gal1 was identified as a tumor-associated protein capable of delineating the metastatic potential of human breast carcinoma (19, 20). Importantly, stromal cell expression of Gal1 is upregulated in invasive breast

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carcinoma as compared with *in situ* carcinoma, showing a positive correlation with T or TNM progression stages (21).

Extracellularly, Gal1 acts by cross-linking glycosylated receptors on the surface of immune cells and modulating their survival, cytokine production, and trafficking (3, 22–25). This glycan-binding protein inhibits immune effector functions by shifting the balance toward a Th2 cytokine profile (9, 23), by selectively deleting Th1 and Th17 cells (22) and by promoting the differentiation of tolerogenic dendritic cells (DC; refs. 11, 14, 26). Moreover, Gal1 facilitates the expansion of IL-10-producing T regulatory type-1 (Tr1) cells (23, 26, 27) and contributes to the immunosuppressive activity of CD4⁺CD25⁺FoxP3⁺ T regulatory (T_{reg}) cells (28, 29).

With the ultimate goal of validating the Gal1–glycan axis as a novel therapeutic target, in the present study we examined the contribution of Gal1 to breast cancer-induced immunosuppression, tumor growth, and metastasis. We used the highly metastatic mouse mammary tumor model 4T1, which recapitulates several features of advanced human breast cancer including lack of hormone receptors and the ability to generate spontaneous lung and lymph nodes metastasis (30). Inhibition of Gal1 expression not only prevented tumor growth but also suppressed the development of lung metastasis and disarmed the suppressive activity of T_{reg} cells through mechanisms involving downregulation of the linker for activation of T cells (LAT), an adaptor protein involved in T-cell receptor (TCR) signaling. Thus, targeting Gal1–glycan interactions in the breast cancer microenvironment represents a novel therapeutic approach with significant translational potentiality.

Materials and Methods

Mice and human samples

Female BALB/c mice or C57BL/6 (8–12 weeks) were housed at the animal facility of the Institute of Biology and Experimental Medicine (IBYME) according to NIH guidelines. Formalin-fixed, paraffin-embedded tumor sections were obtained from the Department of Pathology, Centro Hospitalario Pereira Rossell, Montevideo, Uruguay. Protocols were approved by the Institutional Review Board of the IBYME.

Immunohistochemistry

Paraffin-embedded tissues were sectioned at 4-μm thickness and mounted. After deparaffinization, tissues were stained with rabbit anti-Gal1 antibody (Ab; ref. 9; 1:500 or 1:400 for human or mouse, respectively) or anti-Foxp3 (1:100; eBioscience) and revealed using the Vectastain Elite ABC kit (Vector). Staining was scored as percentage of Gal1⁺ cells.

Cells and knockdown clones

4T1 and B16 cells were acquired from the American Type Culture Collection and were maintained in Dulbecco's Modified Eagle Medium (DMEM) with 10% FCS (Gibco). Three Gal1-specific and 1 scramble short-hairpin RNA (shRNA) were cloned into the pSIREN-RetroQ vector (12). Viral production was carried out using RetroPack PT-67 packaging cell line (BD Clontech) according to the manufacturer's instructions. 4T1 cells were infected using viral supernatants plus 8 μg/mL

polybrene. Clones were selected with puromycin (5 μg/mL) by limited dilution.

Immunoblotting

Cells were lysed (NH₄Cl 150 mmol/L, KHCO₃ 10 mmol/L, EDTA 0.01 mmol/L) and proteins were resolved by SDS-PAGE, blotted, and probed with Ab against Gal1 (1:2,000), LAT (11B.12, 1:1,000; Abcam), Gal3 (B2-C10, 1:500; provided by Fu-Tong Liu), Foxp3 (eBio7979, 1:1,000; eBiosciences), or Actin (Pan Actin Ab-5, 1:2,000; Neomarker). Protein bands were analyzed with the ImageJ 1.440 analysis software (NIH).

In vivo tumor models

BALB/c mice were challenged subcutaneously (s.c.) in the abdominal mammary gland with 4T1 and/or 4T1 KD cells (15,000 cells/50 μL) in serum-free PBS (*n* = 5). Tumor diameter was calculated as $W^2 \times L/2$, where *W* = width and *L* = length. Mice were sacrificed at day 28. Lung metastases were counted after fixing lungs with Bouin reactive or quantified by the clonogenic assay (30). B16 cells (1 × 10⁶/50 μL) were inoculated s.c. in C57BL/6 or 4T1 tumor-bearing BALB/c mice (*n* = 4).

In vitro proliferation assays

In vitro tumor cell growth was measured by the MTS assay (9) and by a clonogenic assay. Briefly, cells were seeded (200 cells/P6 well). After 2 weeks, media was removed and colonies were stained with crystal violet. For T-cell proliferation, tumor-draining lymph nodes (TDLN) cells or spleen cells (20,000/well) were restimulated for 72 hours with anti-CD3 and anti-CD28 monoclonal antibodies (mAb; R&D) and 4T1 lysates. Proliferation was assessed by [³H]-thymidine incorporation in 96-well plates.

Flow cytometry and adoptive transfer

Frequency of T_{reg} cells was determined by using the mouse T_{reg} staining kit (eBioscience) and analyzed on a FACSARIA (BD Biosciences) using a FlowJO software. CD4⁺ T lymphocytes were isolated by sorting using a PE-labeled anti-CD4 Ab (eBioscience). T_{reg} cells were purified by sorting with anti-CD4, anti-CD25, and anti-FR4 mAb (all from eBioscience). For adoptive transfer, sorted T_{reg} cells (300,000 cells) from TDLN were labeled with CFSE (0.5 μmol/L) and intravenously injected in knockdown (KD) tumor-bearing mice (*n* = 4).

Suppression assay

Sorted CD4⁺CD25[−] responder T (T_{resp}) and CD4⁺CD25⁺FR4⁺ T_{reg} cells were stimulated with anti-CD3 and anti-CD28 mAb plus IL-2 and mixed at the indicated ratio as described (31). Proliferation was assessed by [³H]-thymidine incorporation in 96-well plates.

In vitro differentiation of T_{reg} cells

T_{reg} cells were differentiated for 4 days from sorted naïve T cells (CD62L⁺; eBiosciences) in serum-free RPMI in the presence of TGF-β₁ (3 ng/mL) and IL-2 (100 U/mL) with or without conditioned media (CM, 1:10, 1:50, and 1:100) collected from

4T1 WT or KD cells cultured in p60 plates in 2 mL serum-free RPMI medium for 18 hours.

ELISA

TDLN or splenic cells (200,000 cells/well) were restimulated *ex vivo* for 72 hours with anti-CD3 and anti-CD28 mAb and supernatants were analyzed by ELISA for detection of mouse IFN- γ and IL-10 (BD Biosciences) and IL-5 (R&D). Soluble Gal1 was determined using an in house-made ELISA as described previously (32).

Real-time PCR

RNA was obtained using TRIzol reagent according to the manufacturer's instructions. DNA contamination was removed using DNase I. cDNA was synthesized using Super Script III Reverse Transcriptase (Invitrogen). For real-time PCR, the SYBR Green PCR Master Mix was used with an ABI System 7500 (Applied Biosystem). Primers: Gal1 5'-TCAGCCTGGTCAAAGGTGAT-3' 5'-TGAACCTGGGAAAAGACAGC-3', Foxp3 5'-ACTGGGGTCTTCTCCCTCAA-3' 5'-CGTGGGAAGGTGCA-GAGTAG-3', LAT 5'-TGCCGTGAGTTGCCAGTCTCCT-3' 5'-AGCAGGTGTTCCGGGGGACGG-3', GAPDH 5'-CAGAACATCATCCCTGCAT-3' 5'-GTTTCAGCTCTGGGATGACCTT-3'. A

commercial kit was used to screen mouse genes implicated in T-cell anergy and tolerance RT2 Profiler PCR array (PAMM-074, SA Biosciences). Screening of the 84 pathway-specific genes was carried out in duplicate ($n = 4$).

Statistical analysis

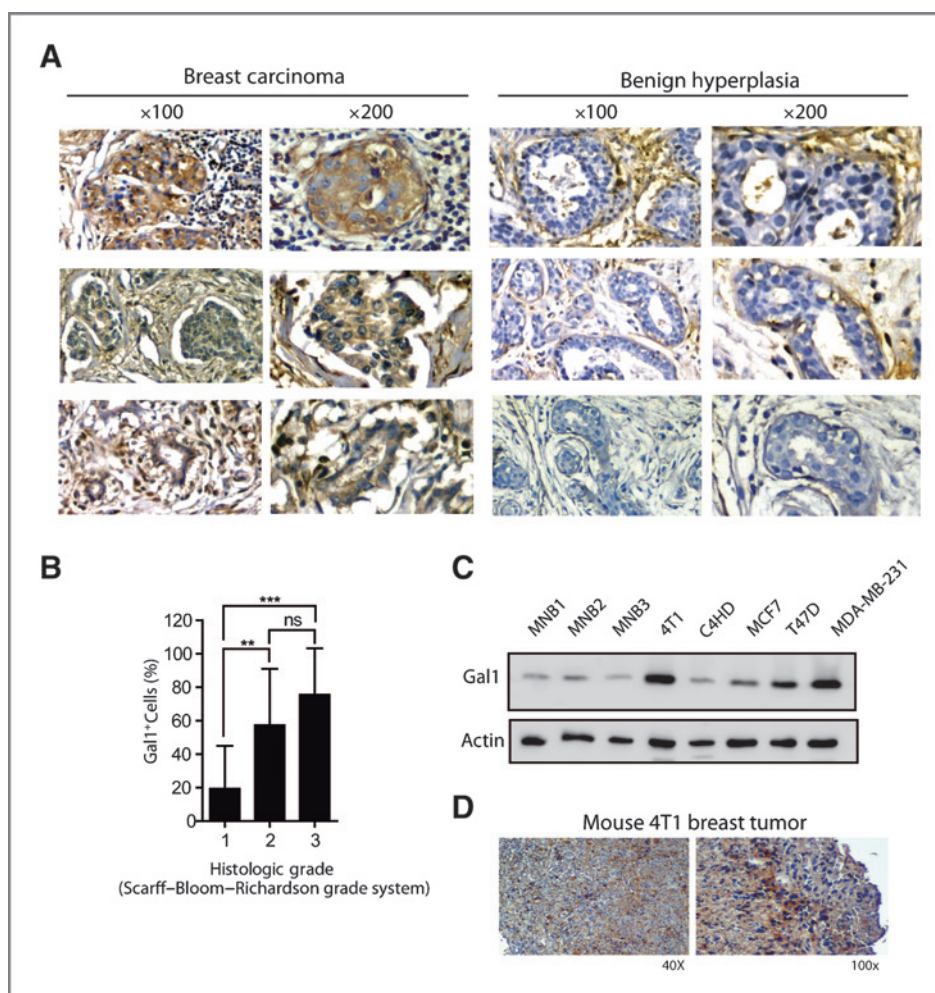
Prism software (GraphPad) was used. Two groups were compared with the Student *t* test for unpaired data. One-way ANOVA Tukey or Bonferroni *post hoc* tests were used for multiple comparisons. Nonparametric analysis was conducted using the Kruskal–Wallis test. *P* values of 0.05 or less were considered significant.

Results

Expression of Gal1 correlates with the aggressiveness of human breast tumors and is upregulated in the mouse metastatic 4T1 breast cancer model

We first examined the expression of Gal1 in human breast carcinoma tissues. Immunohistochemical analysis of a collection of human breast adenocarcinomas ($n = 55$ patients) revealed expression of Gal1 in both epithelial and stromal cells (Fig. 1A). Notably, a positive correlation was found between the number of Gal1⁺ cells and the Scarff–Bloom–Richardson scale

Figure 1. Gal1 expression in breast cancer. **A**, immunohistochemistry of human breast tumors ($n = 55$) histologically classified according to the Scarff–Bloom–Richardson grading system, Grade I ($n = 16$), Grade II ($n = 24$), and Grade III ($n = 15$) as well as benign mammary hyperplasia ($n = 7$). Tissue sections were stained with a polyclonal anti-Gal1 Ab or hematoxylin and eosin. Representative micrographs are shown. **B**, percentage of Gal1-positive cells in at least 10 fields ($\times 200$). **, $P < 0.01$ Grade I versus II and ***, $P < 0.001$ Grade I versus III, Kruskal–Wallis and Dunn multiple comparison test. **C**, immunoblotting of Gal1 and Actin in human (T47D, MCF-7, and MDA-MB-231) and mouse (4T1 and C4HD) breast cancer cell lines and mouse normal breast tissue (MNB). **A** representative experiment of a total of 4 experiments is shown. **D**, representative micrographs of mouse 4T1 tumors stained for Gal1 ($\times 40$ and $\times 100$).



(histopathological grades 2 and 3) (Fig. 1B). In contrast, Gal1 staining was barely detectable in epithelial cells from benign breast hyperplasia (Fig. 1A), suggesting upregulated expression of this lectin during mammary carcinogenesis. Similarly, we observed higher expression of Gal1 in the mouse 4T1 breast tumor compared with normal adjacent mammary gland, and other breast cancer cell lines including the hormone-dependent T47D and MCF-7 (human) and C4HD (mouse) cell lines (Fig. 1C and D). Moreover, the 4T1 cell line expressed Gal1 at similar levels than the highly metastatic MDA-MB-231 cell line, highlighting 4T1 as an optimal tumor model for studying the relevance of this lectin in breast cancer.

Knocking down Gal1 in breast cancer decreases tumor burden and prevents lung metastasis

To study the role of Gal1 in breast cancer, we knocked down Gal1 in 4T1 cells by transduction with a retroviral vector containing a mouse Gal1-specific shRNA sequence (4T1-KD). Clone shRNA3.7 was selected as the clone with the lowest Gal1 expression (Fig. 2A). Scrambled-transduced cells (Scr1) were used as controls. The introduced shRNA specifically targeted Gal1 as expression of galectin-3 (Gal3), another member of the galectin family, was not affected (Fig. 2B). Secretion of Gal1 to CM was also impaired (Fig. 2C and D). Mice inoculated with 4T1 KD cells (shRNA3.7) showed a considerable reduction of

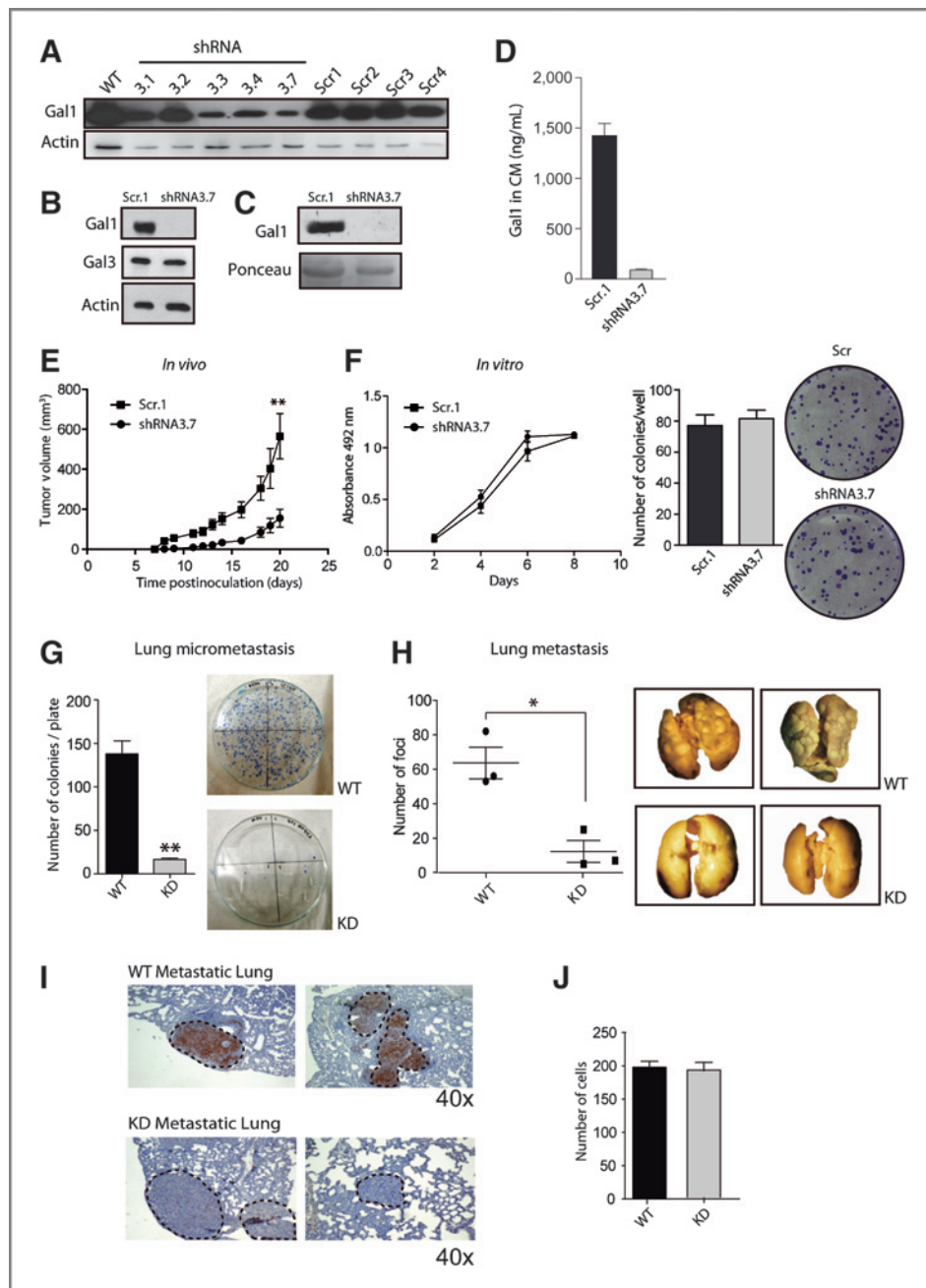


Figure 2. Targeting Gal1 inhibits tumor growth and prevents lung metastasis in breast cancer. **A** and **B**, immunoblot of Gal1 (**A** and **B**) and Gal3 (**B**) in 4T1 cells transduced with retroviral vectors expressing Gal1 shRNA or scrambled (Scr) shRNA. Results are representative of 5 independent experiments. **C** and **D**, detection of Gal1 secretion by 4T1 cells transduced with Gal1 or Scr shRNA by immunoblot (**C**) or ELISA (**D**). Equal loading was checked using Ponceau S staining. Data are representative (**C**) or are mean \pm SEM (**D**) of 3 experiments. **E**, kinetics of tumor growth in BALB/c mice inoculated in the abdominal mammary gland with 15,000 KD or Scr1 4T1 cells. Results are the mean \pm SEM of 4 experiments. **F**, *in vitro* cell proliferation by MTS assay and clonogenic assay ($n = 5$). Data are the mean \pm SEM (left and middle) or are representative (right) of 3 experiments. **G**, number of clonogenic lung metastasis. Results are the mean \pm SEM (left) or are representative (right) of 4 experiments. **H** and **G**, **, $P < 0.01$, one-way Anova and Tukey multiple comparisons test. **H**, number of macroscopic metastatic foci in the lung. Results are the mean \pm SEM (left) or are representative (right) of 4 experiments ($n = 3$ mice per group). *, $P < 0.05$; unpaired *t* test. Magnification, $\times 5$. **I**, immunohistochemistry of Gal1 in lung metastasis. Data are representative of 4 experiments ($n = 3$ mice per group). **J**, *in vitro* invasion assay. 4T1 cells were seeded in Matrigel (8 μ m-pore inserts) and, 24 hours later, stained and counted in the outer membrane. Data are the mean \pm SEM of 3 experiments.

tumor growth compared to mice receiving 4T1 Scr1 control cells (Fig. 2E). The observed antitumor effect could not be attributed to intrinsic differences in cell proliferation rates, as Gal1-sufficient (WT and Scr1) 4T1 cell lines showed no growth advantage *in vitro* over Gal1 KD clones (Fig. 2F). The Scr1 clone showed an *in vivo* behavior similar to that of the parental 4T1 cell line and was, therefore, used as control. Remarkably, Gal1 silencing almost completely suppressed lung metastasis, as reflected by a lower number of colonies (Fig. 2G), and a reduced tumor burden in the lungs (Fig. 2H). Interestingly, in lung tissue from mice bearing 4T1 WT tumors, Gal1 was selectively expressed in metastatic lesions (Fig. 2I) and was barely detected in the surrounding nontumoral parenchyma, suggesting that Gal1 could serve as a biomarker capable of detecting emerging metastatic foci. Notably, disruption of Gal1 expression did not impair the invasive capacity of the 4T1 cell line as all clones migrated equivalently in *in vitro* invasion assays (Fig. 2J). Thus, targeting Gal1 in the breast cancer microenvironment restrains tumor growth and prevents lung metastasis.

Tumor Gal1 promotes immunosuppression in breast cancer

As 4T1 breast cancer cells promote the expansion of T_{reg} cells, which sustain the development of lung metastasis (33), we used this model to investigate whether Gal1 blockade may influence antitumor responses by modulating the tumor-associated T_{reg} cell compartment. We found a diminished frequency of T_{reg} cells (CD4⁺CD25⁺ Foxp3⁺) in the spleen, TDLN, primary tumor, and lungs of mice bearing Gal1 KD tumors as compared with their WT counterpart (Fig. 3A–E). Moreover, we found reduced differentiation toward a T_{reg} cell phenotype when naïve T cells were cultured in the presence of CM from 4T1 KD cells as compared with naïve T cells exposed to CM from Gal1-sufficient cells (Fig. 3F), suggesting that a Gal1-enriched microenvironment favors the generation of inducible T_{reg} cells. Notably, this effect was observed when naïve T cells were exposed to CM from 4T1 cells either in the presence or absence of exogenous TGF- β (Fig. 3F).

It has been proposed that Th2-type associated inflammation contributes to breast cancer progression (34). As Gal1 promotes Th2 responses in autoimmune settings (22, 23), we asked whether impaired tumor growth and metastasis after Gal1 blockade were associated with a reduced Th2-cytokine profile. Cells isolated from the spleen or TDLN from mice bearing KD 4T1 tumors produced lower amounts of IL-5 and IL-10 and showed reduced IL-10/IFN- γ ratio than their WT counterpart (Fig. 3G). Moreover, *in vitro* culture of TDLN T cells isolated from mice bearing Gal1-deficient 4T1 tumors displayed more robust proliferation than T cells from mice bearing WT tumors (Fig. 3H). Thus, tumor-derived Gal1 contributes to delineate an immunosuppressive breast cancer microenvironment characterized by increased frequency of T_{reg} cells and a shift toward a Th2 cytokine profile.

Targeted disruption of Gal1 eliminates tumor-mediated immunosuppression *in vivo*

To further dissect the mechanisms underlying the protumoral effects of Gal1, we examined whether expression of this

lectin in the tumor microenvironment influences systemic immunosuppression. We hypothesized that if a Gal1-deficient tumor is inoculated simultaneously with a Gal1-sufficient tumor in the contralateral side, tumor-derived Gal1 will provide the tolerogenic milieu necessary to systemically blunt effector T-cell responses. As a result, the Gal1 KD tumor will behave as a WT tumor in terms of promotion of tumor growth and metastasis. Notably, inoculation of a WT tumor markedly influenced the behavior of a contralateral KD tumor, which grew progressively as its WT counterpart (Fig. 4A). On the contrary, when mice bearing a KD tumor were inoculated in the opposite flank with another KD tumor, both tumors grew slowly (Fig. 4A). The number of metastatic foci in lungs of mice bearing a WT/KD tumor combination was similar to that observed in the WT/WT group and was considerably enhanced when compared with the KD/KD group (Fig. 4B). Remarkably, splenocytes from mice bearing WT/WT or WT/KD tumors failed to proliferate *ex vivo* in response to tumor antigens, while splenocytes isolated from KD/KD tumors showed robust proliferation in response to 4T1 cell lysates (Fig. 4C). Notably, the frequency of T_{reg} cells present within the Gal1 KD TDLN was as elevated as the WT TDLN (Fig. 4D). Collectively, these results indicate that the presence of a WT tumor promotes a dominant systemic immunosuppression that thwarts the development of an effective antitumor response.

Previous studies showed that mice inoculated with 4T1 tumors exhibit impaired rejection of allogeneic tumors, suggesting a compromised effector T-cell function (35). We examined whether targeting Gal1 in 4T1 breast tumors could restore the ability of BALB/c mice to reject a contralateral allogeneic B16 melanoma. For this, BALB/c mice that had been inoculated with 4T1 WT or KD tumors were challenged 21 days later with B16 melanoma cells (background C57BL/6) in the contralateral flank. As previously reported (35), only 1 out of 4 B16 tumors was rejected in mice bearing 4T1 WT tumors. In sharp contrast, all B16 tumors were rejected in mice bearing Gal1 KD 4T1 tumors. As expected, B16 tumors grew progressively in C57BL/6 mice (Fig. 4E). Thus, targeting Gal1 locally in the tumor microenvironment successfully overcomes systemic immunosuppression displayed by 4T1 breast tumors.

Targeting tumoral gal1 disarms the immunosuppressive activity of T_{reg} cells

The contribution of Gal1 to the immunosuppressive activity of breast tumors prompted us to investigate whether targeting tumoral Gal1 might affect not only the frequency of T_{reg} cells, but also their suppressive capacity. T_{reg} cells sorted from TDLN of Gal1-deficient tumors showed reduced suppressive activity over CD4⁺CD25⁺ T_{Resp} cells as compared with T_{reg} cells isolated from a Gal1-enriched microenvironment (WT tumors; Fig. 5A). Thus, targeting Gal1 expression in the tumor microenvironment attenuates T_{reg} cell suppressive activity.

It has been shown that 4T1 tumors progress toward a metastatic phenotype by influencing the immune composition of the lung microenvironment (36). To test this possibility, we analyzed gene expression profiles of CD4⁺ T cells infiltrating lungs colonized with WT or Gal1 KD 4T1 metastasis. We used a qRT-PCR array for screening genes functionally associated

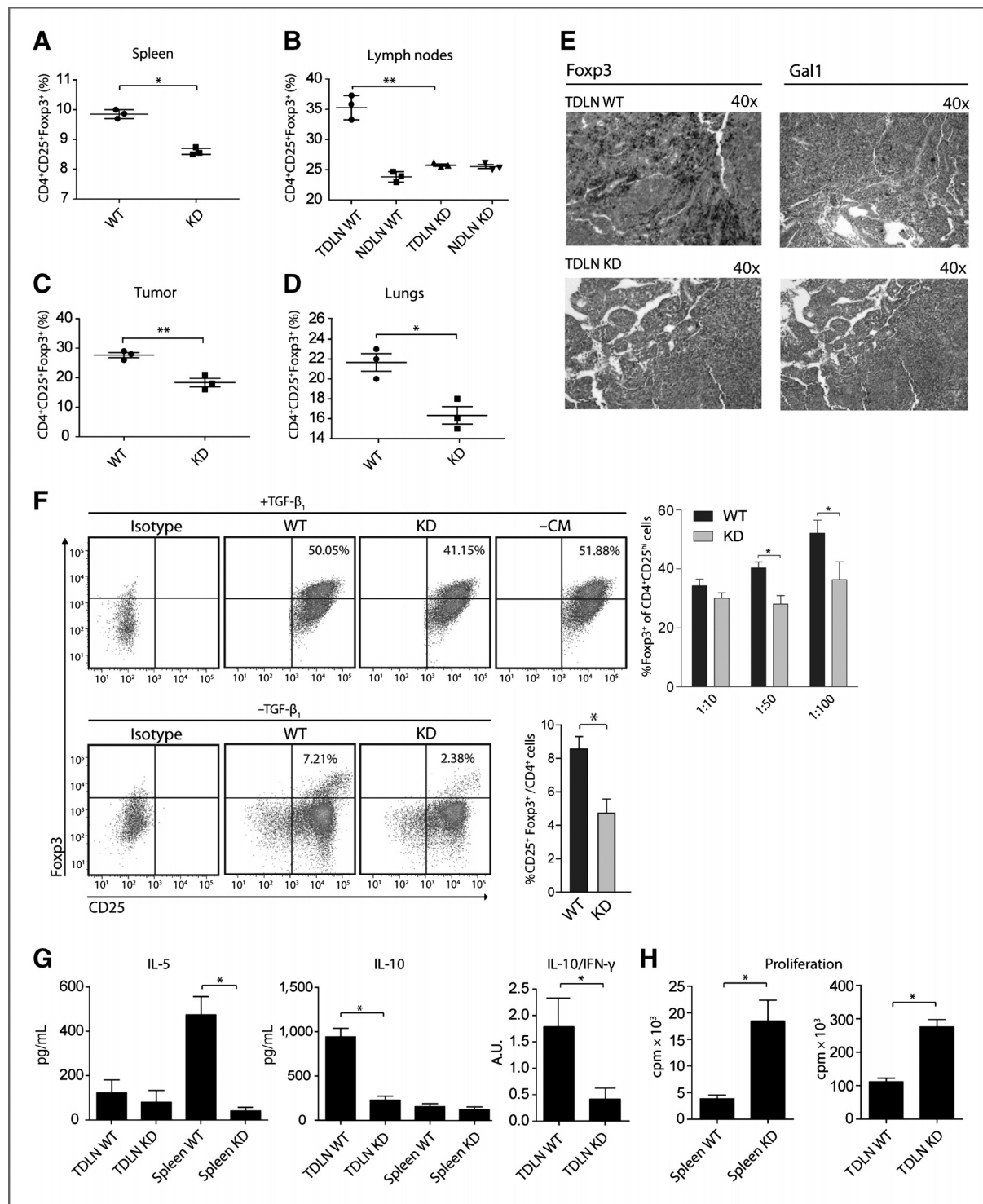


Figure 3. Targeting Gal1 prevents tumor-associated immunosuppression and T_{reg} cell expansion. A–D, frequency of CD4⁺CD25⁺Foxp3⁺ T_{reg} cells in the spleen, lymph nodes, primary tumor, and lungs of mice bearing WT or Gal1 KD 4T1 tumors. Results are representative of 5 independent experiments ($n = 3$ mice per group). *, $P < 0.05$ and **, $P < 0.01$. One-way Anova and Tukey multiple comparisons test. E, immunohistochemistry of FoxP3 and Gal1 in tumor-draining lymph nodes (TDLN). Data are representative of 3 experiments (magnification, $\times 40$). F, *in vitro* differentiation of T_{reg} cells in the absence or presence of TGF-β and conditioned medium (CM) from Gal1 KD or WT 4T1 cells. Dot plots depict the percentage of CD4⁺CD25⁺FoxP3⁺ cells within the CD4-gated T-cell population. Data are representative (left) or are the mean \pm SEM (right) of 3 independent experiments. *, $P < 0.05$; one-way Anova. G and H, ELISA for IL-5, IL-10, and IFN-γ (G) and proliferation (H) of lymphocytes purified from the spleen or TDLN from mice bearing Gal1 KD or WT tumors and cultured for 72 hours in the presence or absence of anti-CD3 and anti-CD28 mAb. The ratio of IL-10 and IFN-γ (G, right) is calculated as a measurement of Th2 polarization. Data are the mean \pm SEM of 3 experiments. *, $P < 0.05$, one-way Anova, and Tukey multiple comparisons test.

with immune tolerance and anergy (data not shown). Notably, among other discrete changes, we observed a consistent altered expression of *Lat*, a gene that encodes a tyrosine phosphorylated transmembrane adaptor protein whose expression is associated with the suppressive activity of T_{reg} cells (37, 38). Recent findings showed that inhibition of LAT in mice leads to impaired immune tolerance due to diminished suppressive activity of T_{reg} cells (37). Consistent with this observation, we observed substantial downregulation of LAT expression in CD4⁺ T cells isolated from pulmonary metastasis, primary tumor, and spleen of mice bearing 4T1 Gal1 KD tumors (Fig. 5B). Particularly, LAT expression was considerably lower in T_{reg} cells isolated from metastatic lungs (Fig. 5C) or from TDLN of mice bearing Gal1 KD tumors (Fig. 5D). Moreover, LAT expression was lower in T_{reg} cells differentiated *in vitro* in the presence of TGF- β and CM from 4T1 KD cells compared with those exposed to CM from 4T1 WT cells (Fig. 5E). However, LAT expression was unaltered during tumor growth in the CD4⁺CD25⁻ non-T_{reg} cell population (Fig. 5D).

To further confirm that T_{reg} cells mediate tumor-induced immunosuppression and metastasis in Gal1-sufficient 4T1 tumor-bearing mice, we conducted a series of *in vivo* adoptive transfer experiments. Sorted T_{reg} cells isolated from TDLN of WT or Gal1 KD 4T1 tumors were intravenously injected in KD or WT tumor-bearing mice. Before inoculation, sorted T_{reg} cells were labeled with CFSE for tracking purposes. We could find no differences in tumor growth rates in KD tumor-bearing mice transferred either with KD or WT Treg cells (Fig. 5F). As expected, WT tumors transferred with WT Treg cells grew progressively to a greater volume than their KD counterparts (Fig. 5F). However, metastatic foci were significantly increased when mice bearing Gal1 KD tumors were adoptively transferred with Treg cells obtained from WT TDLN (Fig. 5G). This increase was comparable to the metastatic number observed in mice bearing WT tumor that were transferred with WT Treg cells (Fig. 5G). At day 28, CFSE staining was positive on T_{reg} cells collected from the spleen, TDLN, primary tumor, and metastatic lungs (data not shown). Thus, targeting Gal1 expression in primary breast tumors abrogates lung metastasis through mechanisms involving, at least in part, a reduced suppressive activity of T_{reg} cells associated to downregulation of LAT.

Discussion

Gal1, an endogenous β -galactoside-binding lectin, has emerged as key a regulator of immune tolerance and homeostasis (3, 39). As Gal1 is overexpressed in a wide range of tumors and tumor-associated stroma, and its expression correlates with impaired antitumor responses, it has been hypothesized that Gal1 contributes to create an immunosuppressive micro-environment at sites of tumor growth (3). Here, we show that Gal1 favors the tumorigenic and metastatic potential of 4T1 breast tumors. Breast cancer cells producing low amounts of Gal1 were less tumorigenic and failed to metastasize to lungs through mechanisms involving inhibition of T_{reg} cell expansion and/or down-modulation of their suppressive activity. In addition, we found selective inhibition of LAT expression in T_{reg} cells from mice bearing Gal1 KD tumors, suggesting that

silencing Gal1 might contribute to interrupt the TCR/LAT signaling and impair T_{reg} cell function.

Previous studies indicated that breast cancer-derived Gal1 could control tumor progression (21, 40, 41). Using the mouse mammary adenocarcinoma LM3 and the human breast tumor line MCF-7, we previously found that Gal1 is under the control of TGF- β , suggesting that both mediators might act in concert to counteract antitumor responses (42). Importantly, in samples of human breast carcinoma tissues, higher Gal1 expression was observed in stromal cells of invasive compared with *in situ* carcinomas (21). The authors found that Gal1 expression in the tumor stroma positively correlated with T and TNM stages in a selected cohort of breast cancer patients (21). Moreover, Gal1 was identified as an abundant protein in the tumor interstitial fluid in breast cancer tissues as compared with normal interstitial fluid (43), suggesting that tumor-secreted Gal1 could serve as a potential biomarker for early detection of the disease.

Research over the past few years has revealed novel insights into the mechanisms underlying the immunoregulatory functions of Gal1. We previously found that Gal1-glycan interactions negatively regulate the survival of effector Th1 and Th17 cells (22). As Th1 and Th17 responses are often associated with antitumor immunity (2), Gal1 effects may contribute to enhance protumoral Th2 responses. In addition, Gal1 acts by promoting the differentiation of IL-27-producing DCs, which in turn trigger the expansion of IL-10-producing type-1 T regulatory (Tr1) cells (26). Here, we found that inhibition of Gal1 expression in 4T1 breast cancer cells targets the FoxP3⁺ T_{reg} cell compartment and leads to decreased IL-10 and IL-5 production, suggesting that tumor-derived Gal1 could influence the metastatic niche in the lung by inducing a Th2- and T_{reg} cell-dominant response, which facilitates metastasis formation.

Here, we found that Gal1-sufficient 4T1 breast tumor cells promote tumor growth and metastasis by increasing the frequency of tumor-associated and systemic CD4⁺CD25⁺Foxp3⁺ T_{reg} cells. Targeting tumoral Gal1 induced a reduction in the frequency of T_{reg} cells in the spleen, lymph nodes, primary tumor, and metastatic lungs. In this regard, we previously reported the ability of recombinant Gal1 to promote the differentiation of CD4⁺CD25⁺ T_{reg} cells *in vitro* (12). Here, we showed the capacity of tumor-derived Gal1 to increase the relative abundance and/or expansion of peripheral T_{reg} cells *in vivo* and to modulate their suppressive capacity. Our findings suggest a possible scenario in which elimination of Gal1-induced immunosuppression and T_{reg} cell expansion in the primary tumor could be reproduced at distance in the metastasis target organ. Alternatively, decreased number of lung metastasis, in addition to the lack of Gal1 expression in the few remaining metastatic foci, might prevent tumor-induced differentiation of T_{reg} cells in the lungs.

Interestingly, we found considerable downregulation of the protein adaptor LAT in T_{reg} cells isolated from TDLN, spleen, primary tumors, and lungs when Gal1 was suppressed. The transmembrane adaptor LAT binds Grb2, GADS, and phospholipase C- γ 1 (PLC- γ 1) (44) and is essential for T-cell

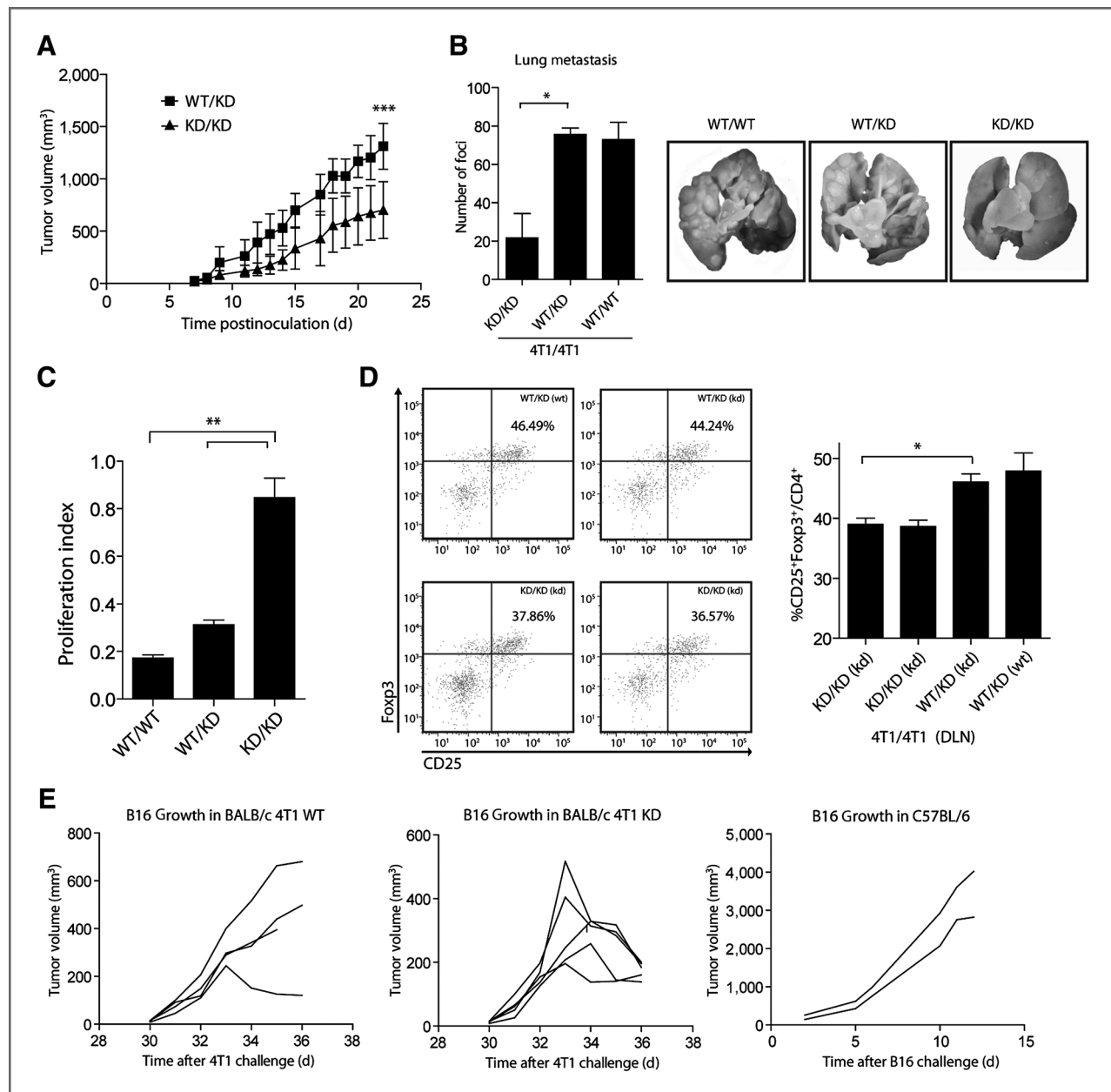
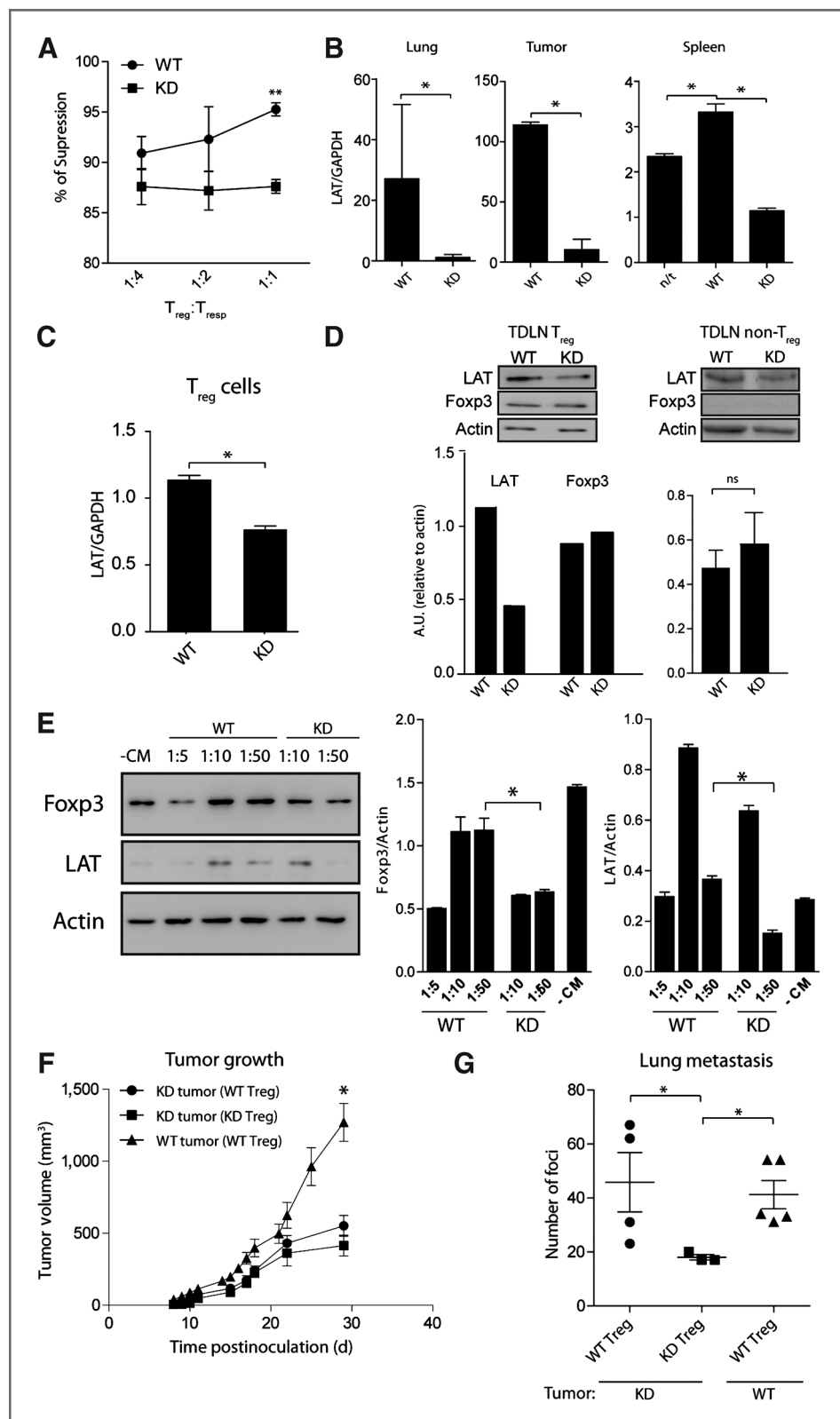


Figure 4. Breast cancer-derived Gal1 promotes systemic immunosuppression. A–D, mice ($n = 5$) were simultaneously inoculated with 15,000 KD 4T1 cells in the right flank and with 15,000 WT (WT/KD) or 4T1 KD cells (KD/KD) in the left flank. A, kinetics of tumor growth in the right flank. Results are the mean \pm SEM of 3 experiments. ***, $P < 0.001$; Student t test. B, number of lung metastasis foci. Data are the mean \pm SEM (left) or are representative (right) of 3 experiments. Magnification, $\times 5$. *, $P < 0.05$; unpaired t test. C, T-cell proliferation in TDNL from different mice groups. Proliferation index is defined as the ratio between proliferation of antigen-specific (anti-CD3 + anti-CD28 + 4T1 lysate)/nonspecific (anti-CD3 + anti-CD28). Data are the mean \pm SEM of 3 experiments. **, $P < 0.01$; one-way Anova and Tukey multiple comparisons tests. D, flow cytometry of CD25⁺Foxp3⁺ T_{reg} cells within the CD4-gated population in TDNL from different mice groups. Data are representative (left) or are the mean \pm SEM (right) of 3 experiments. *, $P < 0.05$; one-way Anova and Bonferroni multiple comparison tests. The corresponding tumor drained by the collected lymph node is shown in parenthesis (kd or wt). E, growth of B16 tumors in BALB/c mice bearing WT 4T1 (left) or Gal1 KD (middle) tumor. As control, B16 tumors inoculated in C57BL/6 mice are shown (right). Each line represents B16 tumor growth in an individual mouse. A representative set of tumor growth curves out of 3 independent experiments is shown.

activation, thymocyte development, and immune cell homeostasis. Mice harboring a knock-in mutation in LAT at the PLC- γ 1-binding site show severe autoimmune disorders (45, 46). In addition, it has been shown that proximal signals downstream of the TCR, specifically the LAT–PLC- γ 1 interaction, controls

FoxP3 expression and development of T_{reg} cells (47). More recently, Chuck and colleagues showed that binding of LAT to PLC- γ 1 is essential for sustaining the suppressive function of CD4⁺CD25⁺Foxp3⁺ T_{reg} cells (37), highlighting the importance of LAT as an inhibitory checkpoint during immune cell

Figure 5. Targeting breast cancer-derived Gal1 impairs T_{reg} -suppressive activity and LAT expression. Mice bearing WT or Gal1 KD tumors ($n = 5$ per group) were sacrificed and primary tumors, spleen, lungs, and TDLN were excised. **A**, suppression assay. $CD25^+CD4^+T_{reg}$ cells were sorted and cocultured with $CD4^+CD25^+FR4^+T_{reg}$ cells isolated from TD at the indicated ratios. Data are the mean \pm SEM of 3 experiments. **, $P < 0.01$; one-way Anova. **B** and **C**, real-time qRT-PCR of LAT expression relative to GAPDH in $CD4^+$ T cells sorted from lungs, primary tumor, and spleen (B) or in T_{reg} cells sorted from lung metastasis (C). n/t, no tumor. Data are the mean \pm SEM of 3 experiments. *, $P < 0.05$. **D** and **E**, immunoblot of LAT and FoxP3 in T_{reg} and non- T_{reg} cells sorted from TDLN (D) or differentiated *in vitro* in the presence of TGF- β and CM (1:10 or 1:50) of 4T1 WT or Gal1 KD cells (E). Data are the representative (D, top; E, left) or are the mean \pm SEM (D, bottom; E, right) of 3 experiments. *, $P < 0.05$; one-way Anova. ns, not significant. **F** and **G**, adoptive transfer of sorted T_{reg} cells isolated from TDLN (WT or KD) to BALB/c mice previously inoculated with a KD or a WT tumor ($n = 4$). Tumor growth (F) and metastasis (G) are shown. Data are the mean \pm SEM of 3 experiments. *, $P < 0.05$. Unpaired *t* test.



homeostasis. In agreement, our findings show that T_{reg} cells from mice bearing Gal1 KD tumors express lower LAT levels and have reduced suppressive activity. Thus, the more effective antitumor response observed in mice bearing Gal1 KD tumors

could be explained, at least in part, by the reduced expression of LAT in T_{reg} cells and their impaired suppressive activity. In line with our findings, LAT-deficient T_{reg} cells were unable to suppress the expansion of conventional effector T cells (37, 38),

an effect that recapitulates T_{reg} cell inhibition observed upon Gal1 blockade. The precise mechanisms underlying the cross-talk between Gal1 and LAT expression in T_{reg} cells remain to be investigated. As Gal1 can interact with the TCR complex and influence downstream signaling pathways on effector T cells (48), it would be interesting to examine whether tumoral Gal1 may affect TCR signalling in T_{reg} cells.

The identification of Gal1 as a pivotal mediator of cancer-induced immunosuppression and a critical factor responsible of thwarting immunotherapeutic strategies opens new avenues in cancer immunotherapy. Our study shows, in a breast cancer model, that blocking Gal1 in the primary tumor abrogates the development of distant lung metastasis through mechanisms involving reversal of local and systemic immunosuppression. Several therapeutic strategies have been proposed to block the detrimental effects of Gal1–glycan interactions; however, none of these strategies are fully specific for Gal1. For example, thiodigalactoside, a non-metabolizable small disaccharide that binds Gal1, prevents some tumor-promoting effects of this endogenous lectin, including angiogenesis and immune escape (40, 41, 49, 50). However, this compound also binds and inhibits the action of other members of the galectin family through blockade of the carbohydrate-recognition domain (49, 50). With the long-term goal of translating basic findings into potential clinical approaches, we envisage that specifically blocking Gal1 in the tumor microenvironment (e.g., using an anti-Gal1 neutralizing mAb; ref. 32), will be successful alone or in combination with other standard treatments to counteract breast cancer-induced immunosuppression, restrain tumor growth, and prevent lung metastasis.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Other: Tumors of our laboratory archive, in methods, protocols and immunohistochemical analysis and interpretation, besides conventional histopathology, D. Mazal, D.O. Croci

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