Aberrant Glycosylation Promotes Lung Cancer Metastasis through Adhesion to Galectins in the Metastatic Niche

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Metastasis is the leading cause of cancer-associated deaths. Although dissemination of tumor cells likely occurs early in tumorigenesis, the constituents of the microenvironment play essential rate-limiting roles in determining whether these cells will form clinically relevant tumors. Recent studies have uncovered many molecular factors that contribute to the establishment of a protumorigenic metastatic niche. Here, we demonstrate that galectin-3, whose expression has clinical associations with advanced malignancy and poor outcome, contributes to metastatic niche formation by binding to carbohydrates on metastatic cells. We show that galectin-3 is expressed early during tumorigenesis by both CD11b^+Gr-1^ and CD11b^+Ly-6C^hi^ leukocytes. Tumors mobilize these myeloid populations through secretion of soluble factors, including IL6. We find that metastatic cancer cells exhibit elevated presentation of the oncofetal galectin-3 carbohydrate ligand, the Thomsen–Friedenreich antigen, on their surfaces as a result of altered C2GnT2 and St6GnAC4 glycosyltransferase activity that inhibits further glycosylation of this carbohydrate motif and promotes metastasis.

**SIGNIFICANCE:** Although clinical observations of elevated serum galectin-3 levels and altered glycosylation have been associated with malignancy, we identify novel roles for glycosyltransferases in promoting adhesion to galectins in the metastatic niche. This identification of a cytokine–leukocyte–glycosylation axis in metastasis provides mechanistic explanations for clinical associations between malignancy and aberrant glycosylation. Cancer Discov; 5(2); 1–14. © 2014 AACR.

See related commentary by Arnal-Estapé and Nguyen, p. 109.
galectins, however, as components of the early metastatic niche, remains to be investigated.

Alterations in gene expression, cytokine secretion, and ECM and soluble factor production have all been associated with improved interactions between metastatic cells and specific microenvironments (14–16, 18, 19, 22–26). To determine the involvement of galectin-3 within the metastatic niche and of aberrant glycosylation of cancer cell–associated surface glycoproteins, we used a mouse model of lung adenocarcinoma metastasis (27–29). Previously, we used our ECM microarray platform to identify adhesion ligands associated with metastasis and identified galectin-3 and galectin-8 as exhibiting preferential adhesion to metastatic cells (30). Here, we identify galectin-3 as a component of the metastatic niche through expression on myeloid cells. We find that changes in glycosylation by the cancer cells mediate their increased interactions with galectins, and that these alterations play essential roles in conferring metastatic potential. In particular, we find that the upregulation of the sialyltransferase St6GalNAc4 and downregulation of the core 2 N-acetylgalcosaminyltransferase C2GnT2 (GCNT3) are key mediators of the changes in glycosylation that potentiate metastatic proclivity by preserving presentation of the T-Antigen.

RESULTS

Tumor-Bearing Mice Exhibit Elevated Levels of Galectin-3 in the Early Metastatic Niche

To determine the role of galectin-3 in metastasis, we used a genetic mouse model of lung adenocarcinoma metastasis (KrasLSL-G12D/;p53^flox/flox) from which cell lines representing distinct stages of metastatic progression were derived (27–29). Following Cre-mediated recombination and the development of autochthonous lung tumors and metastases, lines were generated from primary tumors that did not give rise to metastases (TnonMet), primary tumors that did give rise to metastases (TMet); lymph node metastases (N), and liver metastases (M). Previously, we found that increasingly metastatic lines exhibited increased adhesion to galectin-3 in vitro (Fig. 1A; ref. 30). As ECM molecules and their modification by BMDCs play
important roles in the development of the metastatic niche, we asked whether galectin-3 has a role in its establishment. Livers were harvested from tumor-bearing mice before the detection of overt metastases, and examined for accumulation of galectin-3. Western blot analysis of liver protein lysates revealed an elevation in galectin-3 in the livers of tumor-bearing mice compared with naive mice (Fig. 1B). To identify the source of this galectin-3, we examined both the tumor cells and recruited leukocytes. Gene expression microarray and Western blot analyses of galectin-3 in the tumor cell lines revealed equivalent expression of the molecules across all four classes of lines, indicating that galectin-3 expression by tumors is not differentially regulated during tumor progression (Supplementary Fig. S1A and S1B). In light of recent reports of myeloid-derived populations supporting outgrowth of metastatic tumors, however, we asked whether such populations might also be presenting galectin-3 within the early metastatic niche. Staining for macrophages and galectin-3 in liver sections of mice bearing either T<sub>cm</sub> or M tumors but no detectable overt metastases revealed colocalization of galectin-3 with F4/80<sup>+</sup> macrophages (Fig. 1C). Although galectin-3<sup>+</sup> macrophages are present in the livers of tumor-free mice as well, their numbers are two to four times lower than those observed in tumor-bearing mice (Fig. 1C). Furthermore, immunostaining of the engrafted tumors also revealed a dense macrophage infiltration that colocalized with galectin-3 staining (Supplementary Fig. S1C). Although other sources of galectin-3 could include secretion directly by the tumors, these findings suggested the possibility that leukocytes might be expressing galectin-3 themselves.

**Tumor-Bearing Mice Exhibit Circulating CD11b<sup>+</sup> galectin-3<sup>+</sup> Myeloid Cells Early in Tumorigenesis**

Given our observation of galectin-3-expressing macrophages in the metastatic niche, we asked whether tumors induce the mobilization of leukocytes expressing galectin-3. We harvested peripheral blood from naive mice or mice bearing tumors generated by either T<sub>cm</sub> or M lines (Supplementary Fig. S2A–S2C) and analyzed it for the presence of galectin-3<sup>+</sup> and CD11b<sup>+</sup> myeloid cells. Analysis of peripheral blood cells by flow cytometry revealed a marked increase in galectin-3<sup>+</sup> and CD11b<sup>+</sup> leukocytes in the circulation of mice bearing both the M and T<sub>cm</sub> tumors (Fig. 2A–C and Supplementary Fig. S2D–S2F). Consistent with the findings of galectin-3<sup>+</sup> macrophages in the liver, there was an enrichment in galectin-3<sup>+</sup>CD11b<sup>+</sup> double-positive populations in the blood of tumor-bearing mice (Fig. 2A and D). In agreement with previous reports of increases in inflammatory myeloid cells in tumor-bearing mice, there was a significant increase in the frequency of CD11b<sup>+</sup>Gr-1<sup>+</sup> and CD11b<sup>+</sup>Ly-6C<sup>hi</sup> populations (Fig. 2E and F and Supplementary Fig. S2G and S2H), which may represent neutrophils or MDCs and inflammatory monocytes (IM), respectively, with the latter population being CD115<sup>+</sup> (Supplementary Fig. S2I). Comparisons of the galectin-3 levels on the CD11b<sup>+</sup>Ly-6C<sup>hi</sup> and CD11b<sup>+</sup>Ly-6C<sup>lo</sup> populations reveal 3- to 4-fold higher expression on the enriched population (Fig. 2G). In contrast, galectin-3 levels on the various subpopulations of CD11b<sup>+</sup> leukocytes were not affected by the presence of tumors (Supplementary Fig. S3A–S3C), suggesting that there is increased mobilization of multiple galectin-3<sup>+</sup> populations into the circulation rather than increased expression on individual leukocytes.

Galectin-3 has been reported to bind a variety of macrophage cell-surface glycoproteins (31). To determine whether the galectin-3 is bound to leukocytes through carbohydrate interactions or is presented in a manner that still permits interactions with the CRD, we isolated CD11b<sup>+</sup> cells from the blood of tumor-bearing mice and incubated them in the presence of a competitive galectin-3 ligand, lactose, or a control, sucrose. Subsequent analysis revealed no significant difference in galectin-3 binding, suggesting that galectin-3 is presented on the surfaces of these populations in a carbohydrate-independent manner, thus preserving the availability of the CRD to interact with other glycan ligands (Supplementary Fig. S4). Taken together, these data support the hypothesis that the presence of tumors induces the mobilization of CD11b<sup>+</sup>galectin-3<sup>+</sup> myeloid-derived cells to the blood and that these leukocytes present galectin-3 in a manner that permits additional carbohydrate interactions with the lectin.

**Tumor-Derived IL6 Mobilizes CD11b<sup>+</sup> galectin-3<sup>+</sup> Leukocytes into the Peripheral Blood**

Tumor-derived soluble factors frequently induce the mobilization and recruitment of prometastatic CD11b<sup>+</sup> populations (17, 19, 20, 25, 26, 32). To determine whether tumor-secreted factors were responsible for the observed mobilization of CD11b<sup>+</sup>galectin-3<sup>+</sup> populations, we collected conditioned media (CM) from the cell lines and injected it into the circulation of wild-type mice. Two hours following injection of the CM, we harvested peripheral blood and analyzed the populations by flow cytometry. This analysis revealed pronounced increases in circulating CD11b<sup>+</sup>galectin-3<sup>+</sup> leukocytes and relevant subpopulations (Fig. 3A and Supplementary Fig. S5A–S5D). Again, differences in the level of galectin-3 expression on individual cells were not detectable in the peripheral blood between the two conditions (Supplementary Fig. S5E), suggesting that the elevated galectin-3 levels were due to increased mobilization of galectin-3<sup>+</sup> populations rather than increased expression of galectin-3 by the mobilized leukocytes.

Soluble galectin-3 has been reported to act as a chemoattractant for macrophages, monocytes, and neutrophils (1, 33, 34). The galectin-3 that we have observed has primarily been presented on the surfaces of leukocytes (Figs. 1C and 2). As the tumors also secrete galectin-3, however, we asked whether galectin-3 itself is capable of inducing the rapid mobilization of CD11b<sup>+</sup>galectin-3<sup>+</sup> leukocytes. Injections of recombinant murine galectin-3 in control medium, however, failed to elicit any increased mobilization of the CD11b<sup>+</sup>galectin-3<sup>+</sup> populations (Fig. 3A and Supplementary Fig. S5). Nonetheless, it is possible that galectin-3 might act in combination with other secreted factors to mobilize these populations. To determine whether galectin-3 is necessary to induce mobilization, we knocked down galectin-3 in the M line using short hairpins (Supplementary Fig. S6A). Although this knockdown reduced the CM concentrations of galectin-3 by approximately 80%, there was no observable difference in the mobilization of any of the galectin-3<sup>+</sup> leukocytes or their surface levels of galectin-3 (Fig. 3B and Supplementary Fig. S6B–S6F). Furthermore, mice bearing tumors formed by the knockdown lines exhibited no
Figure 2. Tumor-derived soluble factors induce the mobilization of galectin-3+ myeloid cells into peripheral circulation early in tumorigenesis. Wild-type mice or mice bearing T_nonMet (802T4) or M (393M1) tumors were analyzed for the presence of galectin-3+ leukocytes in their peripheral blood by flow cytometry. A, analysis of staining for galectin-3 and CD11b on all leukocytes from mice with no tumors (NT), T_nonMet tumors, or M tumors. Numbers represent percentages of all leukocytes that were double positive. B, percentage of all leukocytes that were galectin-3+ (Gal-3+). C, percentage of all cells that were CD11b+. D, percentage of all cells that were CD11b+ galectin-3+. E, percentage of all leukocytes that were Gr-1+. F, ratio of CD11b+Ly-6Chi cells to CD11b+Ly-6Clo cells. G, galectin-3 expression on Ly-6Clo and Ly-6Chi cells. P values in B to F were determined by one-way ANOVA with the Tukey multiple comparison test. P values in G were determined by two-way ANOVA with the Bonferroni post-test. *, P < 0.05; **, P < 0.01; ***, P < 0.001.

differences in circulating galectin-3+ leukocytes in the peripheral blood or the number of galectin-3+F4/80+ macrophages in their livers (data not shown and Supplementary Fig. S6G). Taken together, these data suggest that tumor-derived galectin-3 has no effect on the mobilization of galectin-3+ leukocytes or their presentation of galectin-3, nor does it act in an autocrine fashion on tumor cells to induce secretion of other inflammatory cytokines.

Although galectin-3 does not affect mobilization of leukocytes, we asked whether its expression by metastatic cells is necessary for seeding or colonization. To determine whether tumor-derived galectin-3 acts in a cell-autonomous manner to support growth of disseminated tumor cells, we performed experimental metastasis assays with the galectin-3 knockdown lines. Two weeks following intrasplenic inoculation of tumor cells, we queried mice for hematogenous dissemination to the liver and subsequent colonization. Analysis of GFP+ tumor nodules on livers of mice injected with the M cell line containing the shRNAs revealed no differences in metastasis formation between the control hairpin (shLuc) and the galectin-3 hairpins (Supplementary Fig. S6H), suggesting that galectin-3 production by the metastases does not affect colonization. Thus, any interactions with galectin-3 that promote metastasis likely result from its expression by stromal populations.
On the basis of the observation that galectin-3-deficient tumors are still capable of inducing myeloid cell mobilization into circulation, we investigated whether other tumor-derived factors might elicit this mobilization. We next asked whether the tumors produce other cytokines or chemokines that may recruit the leukocytes. Analysis of the CM by Luminescent ELISA revealed elevated levels of IL6 compared with control medium (Fig. 3C). IL6 is a pleiotropic cytokine known to act upon multiple leukocyte subsets. As the T Moodle line also elicited myeloid cell mobilization, we examined its mobilization of CD11b+ leukocytes to peripheral blood following injections of control medium (white), M line conditioned medium (red), or recombinant murine galectin-3 conditioned medium (gray). We also supplemented control medium (gray).

In contrast with galectin-3, injections of recombinant IL6 induced the rapid mobilization of CD11b+galectin-3+ leukocytes. To determine the relevance of tumor-derived IL6 to the human disease, we examined genomic amplification of the IL6 gene in patients with lung adenocarcinomas. Compared with normal blood or lung, lung adenocarcinomas had significantly elevated copy numbers of the IL6 gene (Fig. 3E, P = 5.04 x 10^-7). Thus, we asked whether IL6 induces the mobilization of the galectin-3+ leukocytes in our system. In contrast with galectin-3, injections of recombinant IL6 induced the rapid mobilization of CD11b+galectin-3+ leukocytes and the relevant subpopulations into the circulation in a manner that mirrored CM injections (Fig. 3F and G and Supplementary Fig. S7A–S7D). To identify the set of leukocytes responsible for galectin-3 presentation, we analyzed the granulocytes, as they may represent a large portion of the mobilized CD11b+Gr-1+ cells. Thus, we repeated the IL6 experiments in Csf3r(-/-) mice, which exhibit impaired granulocyte production and maturation. Although IL6 was still capable of inducing CD11b+Ly-6Chi cell mobilization, Csf3(-/-) mice exhibited a profound reduction in CD11b+galectin-3+ and CD11b+Gr-1+ leukocytes compared with wild-type mice (Supplementary Fig. S8A–S8D). These findings suggest that tumor-derived IL6 is sufficient to induce mobilization of galectin-3+ leukocytes.

To determine whether galectin-3 expression by the myeloid cells or another nonparenchymal population is a prerequisite to their mobilization into circulation, we repeated the IL6 injections in Lgals3 (galectin-3) knockout mice and observed no differences in CD11b+ myeloid cell mobilization compared with wild-type mice (Fig. 3H and Supplementary Fig. S9A–S9E). The relevant subpopulations also demonstrated similar mobilization (Supplementary Fig. S9A–S9D). Taken together, these data suggest that IL6, and not galectin-3, acts systemically as a factor to induce the mobilization of CD11b+galectin-3+ leukocytes from the bone marrow and that display of galectin-3 by the BMDCs is not a prerequisite to their mobilization.

**Figure 3.** Secretion of IL6 by tumors induces the rapid mobilization of CD11b+galectin-3+ leukocytes. A, analysis of CD11b+galectin-3+ (CD11b+Gal-3+) myeloid cell mobilization to peripheral blood following injections of control medium (white), M line conditioned medium (red), or recombinant murine galectin-3-supplemented control medium (gray). B, analysis of CD11b+galectin-3+ myeloid cell mobilization following injections of CM from M lines containing either a control hairpin (shLuc, black) or galectin-3 hairpins (red). C, Luminescent cytokine levels of the conditioned medium. D, gene expression microarray analysis of IL6 expression in all cell lines from the four classes. E, IL6 exhibits a gain of copy number in human lung adenocarcinomas compared with normal lung tissue or blood (P = 5.04 x 10^-7) in the ‘lung adenocarcinoma’ dataset available from The Cancer Genome Atlas (TCGA) website (see Methods). F and G, analysis of CD11b+galectin-3+ cell mobilization to peripheral blood following injections of control medium or medium supplemented with recombinant murine IL6. H, mobilization of CD11b+leukocytes in wild-type (WT) and galectin-3 knockout (Lgals3(-/-)) mice following IL6 injections. Error bars in C are SEM. N.D., not detected. P values in A and H were calculated by one-way ANOVA with the Tukey multiple comparison test. P value in G determined by the Student t test. *** P < 0.001. n.s., not significant.
Increasingly Metastatic Cells Exhibit Elevated T-Antigen Surface Presentation That Mediates Galectin-3 Binding

Our previous studies demonstrated that cells increase their adhesion to galectin-3 as they gain metastatic potential (30). In light of the enrichment of galectin-3 leukemia in the peripheral blood (Fig. 2) and metastatic niche (Fig. 1D), we asked how the metastatic populations achieve enhanced adhesion to galectin-3. As galectins bind β-galactoside glycans through their CRD, we asked whether changes in glycan presentation by tumor cells confer their adhesion to galectin-3 on recruited leukocytes during metastasis. Analysis of glycan microarray datasets (35) and existing literature reveal a variety of carbohydrate ligands for these galectins (Fig. 4A). Although all galectins bind lactosamines, galectin-3 exhibits specific affinities for the T-Antigen (Galβ1-3GalNAcα1-S/T), a pan-carcinoma marker expressed on many glycoproteins (Fig. 4B; ref. 36). Galectin-3 is unable to interact with the T-Antigen if the disaccharide is further glycosylated. Thus, we asked whether metastatic cells exhibit elevated

**Figure 4.** Elevated T-Antigen presentation promotes increased galectin-3 adhesion in metastatic populations. **A**, potential carbohydrate ligands for the galectin-3 CRD. The T-Antigen (Galβ1-3GalNAcα1-O-S/T) is specific for the CRD of galectin-3 and galectin-8. **B**, A- and B-Antigens (Type 2)根本没有 binding of Gal-3 to T-Antigen. Normal O-Glycosylation Abberant T-Ag presentation. Glycoprotein backbone T-Antigen (core 1 disaccharide) Gal-3. **C**, peanut agglutinin (PNA) labeling of the T-Antigen on representative cell lines from the TnonMet (blue), TMet (green), and M (red) classes as determined by flow cytometry. **D**, binding of galectin-3 fluorophore conjugates in the presence of a glycan competitor for galectin-3, LacNAc, or control disaccharide (sucrose). **E** and **F**, human non–small cell lung cancer (NSCLC) tissue microarrays were analyzed for surface T-Antigen presentation by PNA staining. **E**, sample tissue staining in lung and lymph node tissue. Examples of tissues scored as PNA− and PNA+ are shown. **F**, quantification of tissue microarray spots for PNA staining: N, noncancerous tissue; C, cancer tissue. **G**, PNA labeling of human NSCLC cell lines as determined by flow cytometry. P values in **F** determined by the Fisher exact test. Scale bars in **E** are 400 μm (100 μm for insets).
T-Antigen levels on their surfaces that might mediate interactions with galectin-3. Staining of TnonMet, T Met, and M lines with a T-Antigen–specific lectin, peanut agglutinin (PNA), revealed that the levels of T-Antigen surface presentation (~20-fold increase) correlate with metastatic potential (Fig. 4C and Supplementary Fig. S10A). To determine whether metastatic cell adhesion to galectin-3 is indeed carbohydrate mediated, we incubated the cells with fluorescent galectin-3 in the presence of a competitive binder, N-Acetyllactosamine (LacNAc). Flow cytometry revealed a reduction in binding in the presence of LacNAc compared with control (sucrose), suggesting that galectin-3 adhesion to the metastatic cells is carbohydrate mediated (Fig. 4D).

To investigate whether these alterations are observed in the human disease, we stained human non–small cell lung cancer (NSCLC) tissue microarrays of 100 tissue samples with PNA to assess the presence of the T-Antigen. This staining revealed elevated levels of cell-surface T-Antigen presentation in cancerous tissue compared with noncancerous tissue, with a notable enrichment in the lymph node metastases (Fig. 4E and F and Supplementary Fig. S10B). In addition, we examined four common NSCLC cell lines for their relative expression of the T-Antigen. Flow cytometry revealed that, indeed, three of the four lines bound PNA at comparable levels to the M line, with the most invasive of the lines, A549 (37), exhibiting the highest degree of staining (Fig. 4G). Taken together, these data suggest that lung tumors bind galectin-3 through carbohydrate epitopes, such as the T-Antigen, and that this epitope is expressed more highly on metastatic cells.

### Differential Glycosyltransferase Expression Promotes Increased T-Antigen Presentation through Reduction of Glycan Chain Elongation and Increased Capping

A variety of O-linked glycoproteins, such as MUC1, can exhibit profound degrees of O-glycosylation, and differential regulation of such glycoproteins can significantly alter the overall glycan presentation of cells. Thus, we asked whether increased T-Antigen expression is a result of upregulation of individual glycoproteins or altered glycosyltransferase activity. We isolated cell membrane proteins from TnonMet, T Met, and M lines and performed PNA lectin blots. These revealed a global increase in T-Antigen expression across many glycoproteins in the increasingly metastatic cells, as opposed to PNA labeling of a specific glycoprotein (Fig. 5A).

In light of this broad distribution in glycosylation, we investigated alterations in the expression and activity of glycosyltransferases. Analysis of gene expression microarrays for 216 glycosyltransferase genes in the four human NSCLC cell lines that exhibited varying degrees of PNA staining. Although minimal differences in expression were observed for the transferases that give rise to the core disaccharide (CIGALTI and CIGALTIIC1), there were marked variations across the lines for the genes encoding transferases that produce the branched and capped structures (Supplementary Fig. S13B). These data suggest that alterations in glycosyltransferase activity that regulate capping or branching of the T-Antigen may also pertain to the human disease.

### Regulation of Gcnt3 and St6galnac4 Mediates Galectin-3 Adhesion In Vitro and Metastasis In Vivo

To test the functional role of these transferases on galectin-3 binding and metastasis, we transfected the metastatic cells with Gcnt3 or knocked down St6galnac4 with short hairpins. Transfection of Gcnt3 resulted in decreased T-Antigen presentation and decreased galectin-3 binding, and, in a complementary fashion, knockdown of St6galnac4 yielded a reduction in galectin-3 binding, while having no effect on nonsialylated T-Antigen presentation (as PNA does not recognize sialylated forms of the antigen; Fig. 6A and Supplementary Figs. S14A–S14E, S15A, and S15B). To determine the functional role of St6GalNAc4 activity on metastasis in vivo, we performed experimental metastasis assays with the St6galnac4 knockdown line. Cells expressing a hairpin for the sialyltransferase or a control hairpin against firefly luciferase were injected into the spleens of mice and monitored for liver tumor formation following hematogenous dissemination. We found that mice injected with the 393M1-shSt6galnac4 cells exhibited more than 95% fewer liver metastases than control mice 2 weeks following tumor cell injection (Fig. 6B and C and Supplementary Fig. S16). We observed no differences in proliferation rates between the knockdown and control hairpin cell lines in vitro (Supplementary Fig. S17). Taken together, these data suggest that alterations in glycosyltransferase activity observed in increasingly metastatic cells have a functional impact on the metastatic potential of the tumor cells and support the hypothesis that presentation of the T-Antigen, through the
prevention of further glycosylation, promotes adhesion of tumor cells to galectin-3 in the metastatic niche.

**DISCUSSION**

A variety of molecules have been demonstrated to play important roles in colonization of the early metastatic niche through their systemic secretion and by interacting with recruited CD11b⁺ leukocytes (14–19). The identification of direct interactions between these leukocytes and tumor cells within the blood vessels and colonized tissues suggests a role for a conserved adhesion receptor–ligand axis during metastatic progression. Here, we identify galectin-3 as a novel regulator of lung adenocarcinoma metastasis through its presentation within the metastatic niche and binding to the oncofetal disaccharide known as the T-Antigen (Fig. 7). On the basis of our findings, we propose that early in tumorigenesis, tumors secrete soluble factors, such as IL6, that induce the mobilization of CD11b⁺galectin-3⁺ BMDCs into circulation. In addition, galectin-3 is presented on macrophages in the early metastatic niche of tumor-bearing mice. As tumors gain metastatic potential, they increase their presentation of the T-Antigen on their surfaces. In the context of our lung adenocarcinoma mouse model, we show that this elevated presentation does not result from increased O-glycosylation through core 1 glycosyltransferases, but rather is due to the abrogation of core 1 chain extension conferred by downregulation of GCNT3 and capping of the motif by the α2–6 sialyltransferase St6GalNAc4. These changes in transferase activity and T-Antigen presentation result in increased carbohydrate-dependent binding of the tumor cells to galectin-3 and increased metastasis in vivo. As the presence of tumors induces elevated numbers of galectin-3⁺ myeloid cells in the circulation and metastatic niche, these alterations in transferase activity within the tumor cells may represent an essential mechanism by which metastatic cells interact with these prometastotic leukocytes at those sites. Furthermore, perturbations in expression of these transferases are correlated with humans with NSCLC (Fig. 5 and Supplementary Fig. S13B), and the T-Antigen is frequently overexpressed on tumor cells to galectin-3 in the metastatic niche.

**Figure 5.** Metastatic cells downregulate Gcnt3 and upregulate St6GalNAc4. A, lectin blot of surface proteins. Cell-surface proteins were isolated from representative TnonMet (802T4), TMet (393T5), and M (393M1) lines and run on SDS-PAGE gels. Membranes were blotted for the T-Antigen with PNA. B, gene expression microarray analysis of all glycosyltransferases. The average expression for all primary tumor-derived lines (ordinate) is plotted against the average expression for all metastatic lines (abscissa). Size of points represents the absolute difference in expression between the clonally related TMet and M lines (393T5 and 393M1, respectively). The color represents statistical significance of the differences between any two adjacent cell line classes (i.e., TnonMet vs. TMet, TMet vs. M, N vs. N, N vs. M) as determined by the Student’s t test. The dashed line represents equivalent expression between the metastatic and primary tumor-derived lines. C, qRT-PCR analysis of transferase gene expression. Top, gene expression of Gcnt3 and St6GalNAc4 in representative TnonMet, TMet, and M cell lines. Bottom, Gcnt3 can transfer a GalNAc to the core GalNAc of T-Antigen (T-Ag) by a β1–6 linkage. St6GalNAc4 can transfer a NeuAc to the core GalNAc of the sialyl-T-Antigen. D, GCNT3 exhibits a loss of copy number in human lung adenocarcinomas compared with normal lung tissue or blood (P = 1.44 × 10⁻¹¹) in the “lung adenocarcinoma” dataset available from the TCGA website (54). P values in C were determined by one-way ANOVA with the Tukey multiple comparison test: *, P < 0.05; **, P < 0.01; ****, P < 0.001.
local microenvironments in a manner that permits colonization (11). Additional studies have suggested that early, or even “pre-,” metastatic niches may exist wherein a protumorigenic microenvironment is generated (12). Such microenvironments harbor inflammatory leukocytes that act to promote metastatic colonization (11, 13, 40). Our findings on the mobilization and recruitment of these inflammatory populations by nonmetastatic primary tumors are consistent with the hypothesis that involvement of prometastatic stromal populations is, at least in part, an early event. Thus, adaptations by the metastatic tumor cells that potentiate interactions with these leukocytes may represent the rate-limiting events permitting colonization.

An abundance of clinical and experimental evidence has suggested a role for IL6 in cancer metastasis (19, 21, 42–44). Tumor-derived soluble factors, including a variety of ECM molecules such as laminins and versican (19), have been reported to act upon macrophages, through Toll-like receptors, to induce the production of IL6. Here, we find that lung tumors themselves produce IL6 independent of the presence of the myeloid cells. Our conditioned medium experiments suggest that soluble factors such as IL6 are capable of inducing the rapid mobilization of CD11b+Ly6C+ and CD11b+Gr-1+ myeloid cells into circulation, which are thought to promote metastasis. Other molecules traditionally considered to be produced by myeloid cells, such as CCL2,
can be produced by tumor cells to induce the recruitment of prometastatic CD11b+ populations (25). The production of cytokines, such as IL6, by tumors represents another means by which tumors can indirectly promote the development of inflammatory microenvironments at distant sites.

Alterations in glycosylation have long been associated with malignancy (45–47). Many clinical biomarkers, such as CA19-9, CA125, DUPAN-II, and AFP-L3, are carbohydrate antigens (48). Furthermore, overexpression of many O-linked glycoproteins, such as MUC1, has been correlated with malignancy (49). As a result, MUC1 has been the subject of numerous vaccine development initiatives, and these vaccines are typically more efficacious when the glycosylated antigen is used (49, 50). In addition to the expression of particular glycoproteins, aberrant glycosyltransferase activity is frequently observed in a variety of cancers. We show that upregulation of ST6GalNAc4 and downregulation of GCNT3 preserve presentation of the T-Antigen through prevention of O-glycan chain elongation. Together with previous findings of overexpression of ST6GALNAC5 in breast cancer metastasis to the brain (24) and general increases in sialylation on metastatic cell lines (51), our study suggests a conserved mechanism by which the T-Antigen is presented on metastasizing cells that appears to potentiate colonization through interactions with galectins in the metastatic niche. It is worth noting that a recent study found that another sialyltransferase, ST6GalNAc2, may influence metastatic potential (52). In this case, however, knockdown of ST6GalNAc2 in a breast cancer cell line led to an increase in metastasis. Although on the surface this finding appears to contradict those presented within this article, these data are consistent in that in both cases, glycosylation-dependent galectin-3 binding promotes metastasis formation. One possibility is that the inverse regulation of the two transferases may result in the same functional phenotype, with ST6GalNAc2 preventing presentation of the galectin-3 ligand LacNAc or similar ligands, and ST6GalNAc4 promoting expression of the T-Antigen. Previously, we found that the integrin α3β1 promotes metastasis through binding to combinations of fibronectin with either galectin-3 or galectin-8 (30). Integrins contain a variety of glycosylation sites, and these data may suggest that glycosylation of integrins could promote metastasis through synergistic interactions between galectins and other ECM molecules within the metastatic niche. Regardless of the particular proteins exhibiting these glycosylation patterns, our observations of alterations in transferase expression and T-Antigen presentation in humans with the disease suggest a role for a highly conserved mechanism of glycan-mediated lung cancer metastasis that warrants consideration for therapeutic intervention.

**METHODS**

**Cell Lines and Mouse Transplantation Assays**

Murine cell lines (a gift from Tyler Jacks and Monte Winslow, Massachusetts Institute of Technology, Cambridge, MA) were described previously (28) and were cultured in DMEM with 10% (vol/vol) FBS and 1% l-glutamine. The cell lines were used within five passages and were tested frequently for consistency in gene expression by qRT-PCR. No additional authentication was performed. All cell lines were tested for pathogens by PCR by the MIT Division of Comparative Medicine during the time period in which they were being used. The
human NSCLC lines were provided by the NCI through the Division of Cancer Treatment and Diagnosis Tumor Repository. The repository authenticates the lines and tests all cell lines for pathogens by PCR before distribution. Experiments were performed within five passages.

All animal procedures were performed in accordance with the MIT Institutional Animal Care and Use Committee under protocol 0211-014-14. Cell injection studies were performed in female B6129SF1/J mice 6 to 10 weeks of age (The Jackson Laboratory; Stock Number 101043). In addition, IL6 experiments were performed in B6129 × (Cg-Cd8+tg(129Pep)/a) J mice (The Jackson Laboratory; Stock Numbers 017838 and 006338) with C57BL/6J mice as controls (The Jackson Laboratory; Stock Number 000664). For CD11b mobilization experiments, 3 × 105 cells resuspended in 100 μL of PBS were injected into the subcutaneous region of flanks of mice anesthetized with isofluorane. Tumors and peripheral blood were harvested 5 to 8 weeks following injections. Approximately 1 mL of peripheral blood was harvested by cardiac puncture, while mice were anesthetized with isofluorane, and used for analysis by flow cytometry. Mice were immediately euthanized following recovery of the blood, and their tumors were harvested.

Intrasplenic transplantation assays were performed as previously described (30). Briefly, 5 × 105 cells were resuspended in 100 μL of PBS. Animals were anesthetized with 2.5% isofluorane and administered buprenorphine (150 μg/kg). Fur was removed with surgical clippers, and the incision site was sterilized with Betadine and 70% ethanol. The spleen was exposed through a small incision, and cells were injected into the tip of the spleen with a 27-gauge needle. The cells were allowed to travel through the circulation for two minutes before removal of the spleen. The splenic vessels were then cauterized and the entire spleen was removed. The muscle wall was closed with 5-0 dissolvable sutures, and the skin was closed with 7-0 wound clips (Roboz). Mice were euthanized 2 weeks following injection, and their livers were excised. GFP+ tumors were visualized and quantified using an epifluorescence dissection scope (Nikon). Tissues were fixed in 4% paraformaldehyde and stained with hematoxylin and eosin following paraffin embedding.

**Immunohistochemical and Immunocytochemical Analysis**

Following necropsy, tumors and livers were fixed overnight in 4% paraformaldehyde at 4°C. Tissues used for paraffin embedding were transferred to cassettes and placed in 70% ethanol. Those used for frozen sections were placed in 30% sucrose overnight at 4°C. Tissues were then transferred to optimal cutting temperature (OCT) compound (Tissue-Tek) for 4 hours at room temperature followed by freezing in isopentane (Sigma) placed in a liquid nitrogen bath. Costaining of galectin-3 and F4/80 was performed on frozen sections using antibody clones M3/38 (BioLegend) and CEA-3 (BioLegend), respectively. Nuclei were stained with Hoechst (Invitrogen). For studies of livers before the detection of overt metastases, excised tissues were examined grossly for the appearance of nodules, and microscopically, following staining, for the presence of tumors.

For fluorescent lectin analysis of T-Antigen presentation, cells were stained with PNA-AlexaFluor 647 (Invitrogen). All paraffin sections were developed using DAB and counterstained with hematoxylin. Carbo-Free Blocking Solution (Vector Laboratories) was used for all staining involving the use of PNA. Endogenous peroxidase activity was blocked using the Dual Endogenous Enzyme Block (Dako). PNA staining of human tissue microarrays was performed with PNA-HRP (Sigma, 4 μg/mL) following antigen retrieval in citrate buffer. Staining of the Human Lung Cancer and Normal Tissue Microarray (US Biomax, Inc.) was scored for the presence of surface T-Antigen staining in a blinded fashion.

All images were acquired using an inverted Nikon Ti-E epifluorescence microscope using Elements software (Nikon). Pseudocoloring was performed using Elements or Imagej (NIH). Quantification of histology was performed using Elements.

**Western and PNA Lectin Blot Analysis**

Cell lysates were harvested in RIPA buffer (Sigma) containing Complete Protease Inhibitor (Roche) and Phospho Stop (Roche) on ice and run on SDS-PAGE gels. Polyvinylidene difluoride (PVDF) membranes were stained for galectin-3 (Abcam, ab53082, 1:500; or BioLegend, M3/38, 1:1,000), galectin-8 (Abcam, ab69631, 1:500), and α-tubulin (Cell Signaling Technology, 2125, 1:1,000). Lectin blotting for glycans was performed using PNA-Biotin (Sigma) followed by detection using the ABC Elite Kit (Vector Labs; PK-6100) or PNA-Peroxidase conjugates (Sigma). Ponceau S solution (Thermo) was used for total protein detection following transfer to PVDF membranes.

For liver homogenate Western blots, mice bearing 393M1 flank tumors or no tumors were perfused by intracardiac injection of 20 mL of PBS. Livers were then excised and 50-500 mg portions were added to gentleMACS M tubes (Miltenyi Biotech) in 4.5 mL of RIPA (Sigma) with Complete Protease Inhibitors (Roche). Tissue was dissociated using the gentleMACS Octo Dissociator (Miltenyi Biotech) and run on polyacrylamide gels as described above.

**Galectin-3 ELISA**

Peripheral blood was harvested from mice without tumors or bearing contralateral flank tumors of both 802T4 and 393T5 cell lines by cardiac puncture as described above. Blood was collected in serum collection tubes (Cappiject) and allowed to clot for 30 minutes before centrifugation and removal of serum. The galectin-3 ELISA Kit (R&D Systems) was used according to the manufacturer’s instructions.

**Cell-Surface Protein Isolation**

Cell-surface proteins were isolated before Western blot analysis using Thermo Scientific kit 89881 following the manufacturer’s instructions. Briefly, cells were washed followed by biotinylation of the surface proteins by a Sulfo-NHS-Biotin through conjugation to free amines. The reaction was quenched and cells were harvested and lysed. Lysates were washed through a column containing a neutravidin resin to capture the biotinylated surface proteins. Finally, the disulfide linkage was reduced through incubation with 50 mmol/L DTT, and the proteins were captured.

**Flow Cytometric Analysis**

Peripheral blood was drawn by cardiac puncture and transferred to K2 EDTA 5.4-mg Plus Blood Collection Tubes (BD Vacutainer; BD Biosciences). Red blood cells were lysed using red blood cell (RBC) lysis buffer (eBiosciences). Cells were fixed in 4% paraformaldehyde and washed before staining. Cells were blocked using anti-mouse CD16/CD32 antibody clone 2.4G2 (BD Biosciences). Antibodies against mouse antigens were as follows: CD11b (M1/70; BD Biosciences), CD115 (A589; eBioscience), Mac-2 (M3/38; BioLegend), Ly-6C (HK1.4; BioLegend), and Gr-1 (RB6-8C6; BioLegend). Flow cytometry was performed on an LSR Fortessa (BD Biosciences) and analyzed using Flowjo (Tree Star).

Magnetic separation of CD11b+ cells was performed using CD11b MACS microbeads (Miltenyi Biotech). Following collection of peripheral blood by cardiac puncture and lysis of erythrocytes, cells were incubated with the MACS beads following the manufacturer’s instructions. CD11b+ cells were isolated using MACS MS Collection Columns (Miltenyi Biotech).

PNA staining was performed by incubating 3 × 10^6 cells in PBS with 10 μL PNA-AlexaFluor 647 (Invitrogen) on ice for 30 minutes. Fluorescent galectin-3 was produced by reacting recombinant murine galectin-3 (R&D Systems) with Dylight 650 NHS Ester (Thermo Pierce). Conjugated protein was purified by fast protein liquid chromatography and concentrated to 0.5 mg/mL using 3,000 MWCO (molecular weight cut-off) Amicon centrifugal filter units (Millipore). Cells were stained by incubating 3 × 10^6 cells in PBS with 10 μL of the fluorescent galectin-3 on ice for 30 minutes.
For galectin-3 binding inhibition experiments on tumor cells, cells were incubated with galectin-3, as described above, in the presence of 20 μmol/L N-Acetylated-D-lactosamine (Carbosynth) or sucrose (Sigma). For competition of galectin-3 presentation on leukocytes, peripheral blood was harvested from mice bearing 393M1 flank tumors, as described above. Cells were incubated with 200 μmol/L L-lactose (Sigma) or sucrose (Sigma) in 2% FBS on ice for 30 minutes. Cells were then fixed, stained, and analyzed by flow cytometry.

**Conditioned Media Experiments**

Twenty-five milliliters of conditioned medium was harvested from one T150 flask of 393M1 cells following 3 days of culture. This medium (or fresh control medium with serum) was filtered through 0.2-μm filters and concentrated 15-fold using 3,000 MWCO centrifugal filters (Amicon). Medium (200 μL) was injected into the lateral tail vein of mice. Two hours after injections, peripheral blood was harvested from the mice by cardiac puncture, as described above, followed by fixation, staining, and flow cytometric analysis. For experiments involving recombinant galectin-3, murine recombinant galectin-3 (R&D Systems) was supplemented to the medium at 1 μg per injection. For IL6 experiments, murine recombinant IL6 (R&D Systems) was supplemented to the medium to 50 ng per injection.

**Luminex Cytokine Profiling**

Cytokine profiles of 100 μL cell culture supernatant from control and CM were assessed using an EMD Millipore Milliplex custom magnetic bead panel using the manufacturer’s protocol: IL2, IFNγ, IL10, TNFα, IL1α, IL4, IL5, IL6. Luminex assays were analyzed using Bioplex 200 with Bioplex Manager 6.1.

**RNA Isolation and Expression Profiling**

Murine gene expression microarray analysis was described previously (28, 30) and is available from NCBI under accession numbers GSE40222 and GSE26874. Human NSCLC expression microarray data were accessed from NCBI accession number GSE32474. RNA was isolated using RNeasy mini kits (Qiagen) according to the manufacturer’s instructions. cDNA reactions were performed using the iScript cDNA Synthesis Kit (Bio-Rad). qPCR reactions were performed using 2 μL of cDNA, 12.5 μL of iQ SYBR Green Supermix (Bio-Rad), and 1 μmol of each primer. Fold change is reported following analysis using the ΔΔCt method, where genes were normalized to the Hprt housekeeping control. Primers were as follows: St6galnac4 forward 5′-GGTGTGTTTACCACTGATTTCG-3′, St6galnac4 reverse 5′-GGAGCGGGGACTCTTCTC-3′, Gent3 forward 5′-GCCAGAACAGATACCAAA-3′, Gent3 reverse 5′-ACAGGCCAGGACCACATCAAA-3′ or forward 5′-ATGAGAGCCATTGGCAACT-3′ reverse 5′-CTTGTGGCGCAAAGGTCTGAT-3′, Hprt forward 5′-GTACACCGGGGACATTAC-3′, Hprt reverse 5′-CACACATCAGAATCCTTTTCA-3′. Gene expression analysis and visualizations were performed using Spotfire (Tibco) and MATLAB (Mathworks).

**Human Copy-Number Analysis**

Copy-number determinations of GCNT3 and IL6 were performed using Oncomine (53) based on publicly available data from TCGA (54).

**Hairpins and Plasmids**

Knockdown of *St6galnac4* was performed using shRNA (5′-TTCT GCTCCTCAACTGTGATCTTGGACA-3′) or a control hairpin targeting firefly luciferase in the pGFP-V-RS vector (Oligene; TG502032 and TR30002). Plasmids were packaged into retroviruses using the Phoenix-Eco system. Viral supernatant was filtered using 0.45-μm syringe filters and cultured with 393M1 cells at no dilution. Transduced cells were selected with puromycin (Invitrogen), and knockdown efficiency was determined by qRT-PCR following 2 weeks of culture. Transfection of *Gcnt3* was performed using Lipofectamine (Invitrogen) according to the manufacturer’s instructions with plasmids purchased from Origene (MC215765). Knockdown of *Lgal3* was performed using two shRNAs (Sigma; shGal3-3: 5′-CCGGGCGGCTAACCTGCGAT GAATCTGGCTTATCCTGAGTTCTGCCTTTTGG-3′; shGal3-5: 5′-CCGGCGGCATGGTGACCACATCTCGGAGATGTGATG TGCAGATCGGTCTTTTG-3′) or a control hairpin targeting firefly luciferase (Sigma; SHC007V). In addition, a second hairpin system was used to knockdown *St6galnac4* from Sigma (shSt6galnac4-2: CCGGGTGACACCTCACTGAGCGACATCTCGAGATGTCAGAAAGGTA CTTTTTGG-3′).

**In Vitro Proliferation Assays**

Cells expressing hairpins against *St6galnac4* or control hairpins were seeded into 24-well plates. New wells were trypsinized and counted using a hemocytometer with Trypan Blue exclusion at 24-hour intervals.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

Conception and design: N.E. Reticker-Flynn, S.N. Bhatia

Development of methodology: N.E. Reticker-Flynn

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): N.E. Reticker-Flynn, S.N. Bhatia

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): N.E. Reticker-Flynn, S.N. Bhatia

Writing, review, and/or revision of the manuscript: N.E. Reticker-Flynn, S.N. Bhatia

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): N.E. Reticker-Flynn, S.N. Bhatia

Study supervision: S.N. Bhatia

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