Natural killer cells require monocytic Gr-1\(^+\)/CD11b\(^+\) myeloid cells to eradicate orthotopically engrafted glioma cells

Gregory J. Baker\(^{a,b}\), Peter Chockley\(^c\), Daniel Zamler\(^{a,b}\), Maria G. Castro\(^{a,b}\), and Pedro R. Lowenstein\(^{a,b}\)

\(^a\)Department of Neurosurgery, University of Michigan Medical School, Ann Arbor, MI, USA; \(^b\)Department of Cell and Developmental Biology, University of Michigan Medical School, Ann Arbor, MI, USA; \(^c\)Graduate Program in Immunology, University of Michigan Medical School, Ann Arbor, MI, USA

**ABSTRACT**

Malignant gliomas are resistant to natural killer (NK) cell immune surveillance. However, the mechanisms used by these cancers to suppress antitumor NK cell activity remain poorly understood. We have recently reported on a novel mechanism of innate immune evasion characterized by the overexpression of the carbohydrate-binding protein galectin-1 by both mouse and rat malignant glioma. Here, we investigate the cytokine profile of galectin-1-deficient GL26 cells and describe the process by which these tumors are targeted by the early innate immune system in RAG1\(^{-/-}\) and C57BL/6J mice. Our data reveal that galectin-1 knockdown in GL26 cells heightens their inflammatory status leading to the rapid recruitment of Gr-1\(^+\)/CD11b\(^+\) myeloid cells and NK1.1\(^+\) NK cells into the brain tumor microenvironment, culminating in tumor clearance. We show that immunodepletion of Gr-1\(^+\) myeloid cells in RAG1\(^{-/-}\) mice permits the growth of galectin-1-deficient glioma despite the presence of NK cells, thus demonstrating an essential role for myeloid cells in the clearance of galectin-1-deficient glioma. Further characterization of tumor-infiltrating Gr-1\(^+\)/CD11b\(^+\) cells reveals that these cells also express CCR2 and Ly-6C, markers consistent with inflammatory monocytes. Our results demonstrate that Gr-1\(^+\)/CD11b\(^+\) myeloid cells, often referred to as myeloid-derived suppressor cells (MDSCs), are required for antitumor NK cell activity against galectin-1-deficient GL26 glioma. We conclude that glioma-derived galectin-1 represents an important factor in dictating the phenotypic behavior of monocytic Gr-1\(^+\)/CD11b\(^+\) myeloid cells. Galectin-1 suppression may be a valuable treatment approach for clinical glioma by promoting their innate immune-mediated recognition and clearance through the concerted effort of innate myeloid and lymphoid cell lineages.

**INTRODUCTION**

Glioblastoma (GBM) is the most common and aggressive primary astrocytoma in adults carrying a median survival of 15–21 mo post-diagnosis despite the current standard-of-care.\(^1\) GBM tumors are highly immunosuppressive due to secreted (i.e., TGF-β) and cell-surface bound factors (i.e., FasL, PD-L1, CD70, gangliosides, and certain HLAs) produced by their constituent cells.\(^2\) Tumor-derived immunosuppressive factors are a major hurdle to the achievement of successful immunotherapeutic intervention.\(^3,5\) Despite its immunosuppressive activities, GBM is highly infiltrated by immune cells of myeloid origin such as peripheral monocytes/macrophages and MDSCs.

MDSCs are a heterogeneous population of immature cells shown to accumulate in the blood and tumor microenvironment of humans and mice-bearing malignant tumors.\(^5,9\) These cells have the capacity to suppress T cell proliferation and function through factors that include arginase 1 (Arg-I),\(^10\) inducible nitric oxide synthase (iNOS),\(^6,10,11\) reactive oxygen species (ROS),\(^11\) indoleamine 2,3-dioxygenase (IDO) expression, accumulation of Foxp3\(^+\) T regulatory cells (T\(_{reg}\)),\(^12\) and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX2).\(^6,13\) MDSCs also influence innate immunity by altering macrophage cytokine production\(^14\) and suppressing NK cell effector function.\(^15,16\)

Two major MDSC subsets are recognized, granulocytic (Ly-6G\(^{hi}\)/CD11b\(^{hi}\)/Ly-6C\(^{low}\)) and monocytic, (Ly-6G\(^{low}\)/CD11b\(^{hi}\)/Ly-6C\(^{hi}\)).\(^5,17,18\) Mounting evidence supports a role for glioma-derived factors in influencing the function of immature myeloid precursor cells;\(^19-25\) however, the molecular mechanisms remain poorly understood.

Galectin-1 (gal-1) is a member of a family of β-galactoside-binding lectins characterized by the presence of one or more homologous carbohydrate recognition domains (CRDs) that mediate interactions with glycoproteins bearing the basic core disaccharide N-acetyllactosamine (LaCNAc) found in N- and O-linked glycans.\(^26\) Gal-1 induces apoptosis in activated antigen-specific T lymphocytes, a mechanism linked to tumor immune escape.\(^27,29\) It has been further proposed that gal-1 dampens inflammatory (M1) mono/macrophage activities, in turn favoring those that are anti-inflammatory (M2) and pro-tumor.\(^30,31\) Our own work has shown that mouse GL26 and rat CSN1S1 glioma suppress NK-mediated tumor killing by expressing high-levels of this lectin.\(^32\) Tumor-derived lactate dehydrogenase 5 (LDH5) represents an additional mechanism of innate immune suppression in human glioma causing the upregulation of NKGD2 ligands on monocytes and leading to NK exhaustion and tumor progression.\(^33\) Further work is...
required to fully elucidate the mechanistic details of innate immune escape in GBM.

Here, we demonstrate that intracranial GL26 glioma cells rendered gal-1-deficient through shRNA knockdown are marked by increased cytokine production and elimination through the concerted action of myeloid and lymphoid cells of the innate immune system. These tumors are rapidly infiltrated by a 7-fold higher number of Gr-1+/CD11b+ myeloid cells compared to gal-1-expressing GL26 glioma, which is followed by a 9-fold induction in the number of tumor-infiltrating NK1.1+ NK cells and ultimately tumor eradication. Further experiments reveal that the Gr-1+/CD11b+ myeloid cells shown to infiltrate early gal-1-deficient gliomas express CCR2 and Ly-6C, cell surface markers consistent with inflammatory monocytes. Together our findings show that (1) glioma-derived gal-1 is an important mechanism of tumor-induced innate immunosuppression through its ability to cloak tumor cells from innate immune recognition and (2) that gal-1 favors the conversion of immature myeloid cells into anti-inflammatory MDSCs known to support glioma progression.8

Results

Gal-1-deficient GL26 cells elicit innate immune rejection of co-implanted gal-1-expressing cells

We have previously demonstrated that GL26 cells grown in vitro secrete gal-1, an effect significantly diminished by shRNA-mediated gal-1 knockdown. Based on this observation, we examined whether NK-resistant gal-1-expressing GL26 cells could, through a bystander effect, protect co-implanted NK-sensitive gal-1-deficient GL26 cells from innate immune-mediated rejection. To assess this, we performed Kaplan–Meier survival analysis on RAG1-/- mice bearing mixtures of orthotopically implanted GL26-Cit-NT cells (GL26 mouse glioma cells expressing mCitrine fluorescent protein for visualization purposes and a non-targeting control shRNA32) and GL26-Cit-gal1i cells (GL26 mouse glioma cells also expressing mCitrine fluorescent protein, but with a gal-1-specific shRNA.32 These two cell lines will be referred to as GL26-NT and GL26-gal1i throughout the rest of this text.

A total of 2 \times 10^5 glioma cells were implanted into the brain of each mouse at the following NT-to-gal1i ratios: 100:0, 80:20, 20:80. Three reference groups were also included comprising 2 \times 10^5, 1 \times 10^5, and 4 \times 10^5 GL26-NT cells alone. Our analysis revealed that gal-1-expressing cells did not protect gal-1-deficient cells from innate immune clearance. On the contrary, gal-1-deficient cells caused the rejection of the gal-1-expressing cells. This was evident by the fact that mouse median survival was extended in response to an increased percentage of GL26-gal1i cells in the co-implants. In fact, all mice receiving 80% gal-1-deficient glioma cells achieved long-term survival with no evidence of brain tumor burden 100-days post-implantation despite having also received 20% GL26-NT cells (Fig. 1A). This result indicated that NK sensitive glioma cells are capable of eliciting an innate immune response, not only against themselves, but also against glioma cells that express normal levels of gal-1. The capacity of glioma cells to block innate immune killing therefore appears to be overcome under the right conditions of innate immune activation, as occurs when tumor-derived gal-1 is reduced.

Orthotopically implanted gal-1-deficient glioma drives NK cells into the tumor microenvironment, but does not influence their abundance in the blood

We next asked if intracranial gal-1-deficient glioma cells would cause an increase in the number of circulating NK cells available to enter the tumor microenvironment, or whether these tumors would merely provoke the recruitment of existing numbers of these cells into the tumor microenvironment. To distinguish between these two alternatives, we engrafted 3 \times 10^4 GL26-NT or GL26-gal1i cells into the striatum of RAG1-/- mice, and performed transcardial blood draws 5-days post-tumor implantation to assess the percentage of circulating NK cells in the blood stream. This time point corresponds both to tumors well vascularized by normal mouse brain blood vessels, and active tumor rejection as demonstrated by our previous work with GL26 cells. A cohort of mice was included in the experiment that underwent intracranial injection with vehicle alone to control for potential inflammatory reactions due to the implantation procedure. The results of this experiment showed that the percentage of circulating NK cells in all three groups were similar (14.95 ± 3.16% NT vs. 22.25 ± 3.95% gal1i vs. 17.50 ± 0.80% vehicle alone; n.s.; p > 0.05, one-way ANOVA followed by Tukey’s post-test) (Fig. 1B), suggesting that GL26-gal1i tumor rejection was not due to alterations in the profile of circulating NK cells, but rather due to a tropism of normal levels of NK cells toward the gal-1-deficient tumor microenvironment. Histologic analysis on the brains of these mice confirmed that GL26-gal1i tumors were indeed undergoing tumor rejection 5-days after tumor implantation, as the tumors were significantly smaller (3.53 ± 1.32 \times 10^3 pixels NT vs. 2.27 ± 1.48 \times 10^3 pixels gal1i; p < 0.05, one-way ANOVA followed by Tukey’s post-test) and more highly infiltrated with granzyme B (GzmB) positive cells compared to GL26-NT tumors (Figs. 1C and D). GzmB+ cells were completely absent from the brains of mice injected with vehicle alone, demonstrating the requirement of intracranial glioma cells to drive GzmB+ cells into the brain. Further experiments showed that 61.0% of circulating CD11b+/NK1.1+ NK cells in tumor-naive RAG1-/- mice expressed GzmB (53% of total NK cells) (Fig. 1E), and that FACS-purified circulating NK1.1+ NK cells lyse GL26-gal1i cells by nearly 30% after 4 h of co-culture at a 10:1 effector:target (E:T) ratio without requiring ex vivo stimulation (8.39 ± 1.21 \times 10^3 relative luminescence units (RLU) gal1i alone vs. 6.08 ± 9.51 \times 10^3 RLU gal1i + NK cells; **p < 0.0001, unpaired, two-tailed student’s t-test.) (Fig. 1F). Our previous work had already demonstrated that gal-1-deficient glioma cells are more sensitive to NK-mediated lysis compared to gal-1-expressing cells. These experimental results now indicated that circulating NK cells express cytopotxic granules and are active against gal-1-deficient glioma cells prior to tumor implantation.

Gal-1-deficient glioma cells exhibit enhanced chemokine production

Our results up to this point showed that (1) gal-1-deficient gliomas do not alter the percentage of circulating NK cells
Figure 1. Gal-1-deficient GL26 glioma cells are proinflammatory. (A) Kaplan–Meier survival analysis of RAG1−/− mice bearing GL26-NT cells alone (gray, blue and purple curves), or together with an increasing percentage of GL26-gal1i cells (green, orange and red curves). NT:gal1i ratios are indicated to the left of the three co-implant groups. Three alternative experimental outcomes are shown in the table below. The actual results are consistent with outcome number 3. Mantel–Cox log-rank test detected a significant survival difference between the indicated groups. *p < 0.05; **p < 0.005. (B) Percentage of circulating NK1.1+ NK cells in RAG1−/− mouse blood 5-d after intracranial engraftment of GL26-NT (n = 4), GL26-gal1i (n = 4), or injection with vehicle alone (n = 2). (C) Quantitative comparison of brain tumor size 5-days after implantation into RAG1−/− mice. GL26-NT (n = 4), GL26-gal1i (n = 4), and vehicle alone (n = 2) groups are shown. (D) Representative histology from the brain tumors represented in panel C showing tumor-derived mCitrine fluorescent protein (top micrographs) and granzyme B (GzmB) expression (bottom micrographs). (E) Circulating CD11b+NK1.1+ NK cells from RAG1−/− mouse blood demonstrating the expression of GzmB (open black histogram) above isotype control (closed gray histogram). Experiment performed in triplicate. Data from a representative experiment is shown. (F) NK-mediated cytotoxicity assessed using an ATP-dependent luminescence assay showing the level of viable GL26-gal1i cells (RLU) alone, or in the presence of a 10:1 E:T ratio of circulating NK1.1+ NK cells from RAG1−/− mice after 4 h of co-culture (n = 4 technical replicates per group, experiment repeated × 3).
compared to gal-1-expressing gliomas within 5-days of tumor implantation, (2) circulating NK cells in tumor-naive mice express GzmB, and (3) GzmB⁺ NK cells lyse GL26 cells in vitro without requiring exogenous stimulation. The sum of these results suggested that proinflammatory cues within the gal-1-deficient tumor microenvironment were likely driving normal levels of cytotoxic NK cells from the circulation into the tumor microenvironment. We therefore speculated that GL26-gal1i cells might exhibit a proinflammatory cytokine signature compared to their gal-1-expressing counterparts. To test this hypothesis, we incubated whole cell lysate from GL26-NT and GL26-gal1i cells cultured in vitro with commercially available cytokine arrays capable of simultaneously detecting the relative abundances of 40 different cytokines. We found that gal-1-deficient GL26 cells exhibited a 27.8-fold induction in CXCL10/IP-10 (7.99 ± 0.82 mean pixel density (MPD) NT vs. 213.88 ± 14.93 MPD gal1i; **p = 0.0052), a 1.6-fold induction in CXCL12/SDF-1 (113.83 ± 1.72 MPD NT vs. 179.02 ± 6.00 MPD gal1i; **p = 0.0090), a 39.5-fold induction in CCL5/RANTES (0.21 ± 0.02 MPD NT vs. 8.29 ± 0.71 MPD gal1i; **p = 0.0076), an 8.5-fold reduction in CXCL1/KC (120.85 ± 5.49 MPD NT vs. 14.21 ± 3.68 MPD gal1i; **p = 0.0038), and a 6.7-fold reduction in IL-1ra (34.31 ± 1.13 MPD NT vs. 5.11 ± 0.57 MPD gal1i; **p = 0.0019) compared to GL26-NT cells as determined by unpaired, two-tailed student’s t-tests. Additional experiments in which GL26 conditioned media was used as cytokine array input corroborated our findings with whole cell lysate by showing that GL26-gal1i cells secrete 12.2-fold more CXCL10/IP-10 (17.30 ± 1.02 MPD NT vs. 211.61 ± 6.32 MPD gal1i; **p = 0.0011), 1.7-fold more CXCL12/SDF-1 (28.80 ± 0.27 MPD NT vs. 49.77 ± 4.29 MPD gal1i; *p = 0.0396), 188.2-fold more CCL5/RANTES (0.49 ± 0.02 MPD NT vs. 92.20 ± 3.47 MPD gal1i; **p = 0.0014), and 1.4-fold less CXCL1/KC (247.34 ± 1.817 MPD NT vs. 180.17 ± 4.08 MPD gal1i; **p = 0.0044) compared to GL26-NT cells as determined by unpaired, two-tailed student’s t-tests (Fig. 2B).

We next examined whether intracranially implanted gal-1-deficient GL26 cells would also express the cytokines we observed in vitro, and whether differences in additional cytokines might also be revealed between intracranial gal-1-deficient and gal-1-expressing gliomas. To test this, we used homogenized brain tissue from C57BL/6J mice inoculated 72 h earlier with $3 \times 10^4$ GL26-NT or GL26-gal1i glioma cells. These experiments confirmed the increased production of CXCL10/IP-10 and CCL5/RANTES associated with gal-1-deficient GL26-gal1i cells by revealing a 3.1-fold induction in CXCL10/IP-10 (73.98 ± 31.83 MPD NT vs. 228.28 ± 5.79 MPD gal1i; *p = 0.0413) and a 5.6-fold induction in CCL5/RANTES (1.25 ± 1.16 MPD NT vs. 7.01 ± 0.50 MPD gal1i; *p = 0.0451) in the gal-1-deficient tumor microenvironment compared to that of gal-1-expressing tumors using unpaired, two-tailed student’s t-tests (Fig. 2C). We also observed statistically significant differences in the levels of alternative cytokines not detected in GL26 cells grown in vitro. We found a 5.4-fold induction in CCL2/MCP-1 (22.99 ± 9.06 MPD NT vs. 125.29 ± 3.32 MPD gal1i; **p = 0.0088), a 9.3-fold induction in

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CCL12/MCP-5 (5.81 ± 2.43 MPD NT vs. 54.15 ± 9.14 MPD gal1i; \( p = 0.0362 \)), and a 5.9-fold-induction in IL-1ra (0.49 ± 0.31 MPD NT vs. 2.89 ± 0.26 MPD gal1i; \( p = 0.0279 \)) in the GL26-gal1i tumor microenvironment compared to that of GL26-NT tumors. The fact that we could detect increased levels of prototypical monocyte-derived chemokines such as CCL2/MCP-1 and CCL12/MCP-5 in the gal-1-deficient glioma microenvironment suggested that monocytes/macrophages might play a role in the eradication of gal-1-deficient glioma.

**Gr-1\(^+\)/CD11b\(^+\) myeloid cells accumulate in the gal-1-deficient tumor microenvironment prior to the recruitment of NK1.1\(^+\) NK cells**

To comprehensively assess the different types of immune cells that penetrate the gal-1-deficient glioma microenvironment, we developed a protocol for the isolation and flow cytometric analysis of peripheral blood mononuclear cells (PBMCs) that infiltrate the early brain tumor microenvironment. This procedure was used to characterize, and temporally resolve, the immune infiltrates associated with gal-1-deficient glioma rejection. C57BL/6J mice were engrafted with 3 \( \times 10^6 \) GL26-NT or GL26-gal1i cells and euthanized 48 or 72 h post-tumor engraftment. GL26-gal1i tumors were infiltrated by 2.4-fold more CD45\(^+\) PBMCs at the 48-h time point (2,048 ± 212 NT vs. 4,941 ± 442 gal1i; \( p = 0.0011 \), unpaired, two-tailed, Student’s t-test). Of total CD45\(^+\) cells, Gr-1\(^+\)/CD11b\(^+\) myeloid cells were the most disparate between the two tumor types, with 7-fold more of these cells in GL26-gal1i tumors compared to GL26-NT (235 ± 65 NT vs. 1,649 ± 275 gal1i; \( p = 0.0024 \), unpaired, two-tailed, Student’s t-test). We also detected a population of Gr-1\(^{int}\)/CD11b\(^+\) myeloid cells that was 1.5-fold higher in gal-1-deficient tumors (1,222 ± 133 NT vs. 1,805 ± 136 gal1i; \( p = 0.022 \), unpaired, two-tailed, Student’s t-test) and a trend toward higher numbers of NK1.1\(^+\) NK cells in gal-1-deficient gliomas 48 h post-engraftment that failed to reach statistical significance (20 ± 7.2 NT vs. 141 ± 56 gal1i; n.s., \( p = 0.0759 \)) as determined by unpaired, two-tailed, Student’s t-tests (Figs. 3A and B).

By the 72-h time point, the total number of CD45\(^+\) tumor-infiltrating PBMCs had increased in both groups, although the difference was now no longer statistically significant (3,802 ± 1,050 NT vs. 8,328 ± 1,474 gal1i; n.s., \( p = 0.0667 \)). Total CD45\(^+\) cells, the number of Gr-1\(^+\)/CD11b\(^+\) myeloid cells also failed to reach statistical significance different between the two groups (1,008 ± 395 NT vs. 2,472 ± 65 gal1i; n.s., \( p = 0.1270 \)). Conversely, statistical significance persisted in the Gr-1\(^{int}\)/CD11b\(^+\) myeloid subset (1,411 ± 504 NT vs. 3,904 ± 576 gal1i, \( p = 0.0311 \)) and a significant 9.3-fold induction in the recruitment of NK1.1\(^+\) NK cells was now observed in the gal-1-deficient tumor microenvironment (142 ± 29 NT vs. 1,322 ± 298 gal1i; \( p = 0.0170 \)) as determined by unpaired, two-tailed, Student’s t-tests (Fig. 3C).

Although significantly more Gr-1\(^+\)/CD11b\(^+\) myeloid cells accumulated in the tumor microenvironment of gal-1-deficient gliomas 48 h post-engraftment, the fact remained that these cells also accumulated in GL26-NT tumors. Immunohistochemical analysis of gliomas 5-days post-engraftment revealed that the Gr-1\(^+\) cells that infiltrate GL26-NT tumors are relatively spherical and evenly distributed throughout the tumor mass, while those infiltrating GL26-gal1i tumors display an amorphous shape, suggestive of cellular activation (Fig. 3D).

**Immunodepletion of Gr-1\(^+\) cells permits gal-1-deficient glioma growth in RAG1\(^-/-\) mice**

The enhancement of NK cell activity by collateral cells such as neutrophils, monocytes, macrophages, and dendritic cells has been well documented. Many of these myeloid cells express Gr-1. We therefore examined whether the Gr-1\(^+\)/CD11b\(^+\) myeloid cells shown to infiltrate the early gal-1-deficient glioma microenvironment played a significant role in NK-dependent tumor lysis. To test our hypothesis, we immunodepleted Gr-1\(^+\) cells in RAG1\(^-/-\) mice using anti-Gr1 monoclonal antibodies (clone: RB6-8C5) beginning one day prior to GL26-gal1i tumor implantation. GL26-gal1i tumors in mice treated with anti-Gr1 antibodies were 9.4-times larger than those in mice treated with an equivalent regimen of control rat IgG antibodies after a 7-d growth period (1.94 ± 10\(^5\) ± 2.89 ± 10\(^5\) pixels rat IgG vs. 1.82 ± 10\(^6\) ± 1.91 ± 10\(^6\) pixels anti-Gr1; \( p = 0.0011 \), unpaired, two-tailed, Student’s t-test) (Fig. 4A). Further quantitative analysis revealed that GzmB expression in GL26-gal1i tumors treated with anti-Gr1 antibodies was substantially lower on a per unit tumor area basis compared to GL26-gal1i tumors treated with rat IgG control antibodies (3.75 ± 0.36 rat IgG control vs. 0.63 ± 0.14 anti-Gr1; \( p = 0.0012 \), unpaired, two-tailed, Student’s t-test) (Fig. 4B).

Control experiments showed that Gr-1\(^+\) myeloid cells are efficiently immunodepleted by 73 ± 1.3% (86 ± 2.06% rat IgG control vs. 12.85 ± 2.56% anti-Gr1; \( p < 0.0001 \), unpaired, two-tailed, Student’s t-test) 48-h after a single 500 \( \mu \)g dose of anti-Gr1 antibodies (Fig. 4C).

Two types of Gr-1\(^+\)/CD11b\(^+\) myeloid cells exist, monocytic and polymorphonuclear. To determine which of these two cells was responsible for aiding NK-dependent gal-1-deficient glioma rejection, we specifically immunodepleted polymorphonuclear cells using anti-Ly-6G monoclonal antibodies (clone: 1A8). Treatment with anti-Ly-6G antibodies permitted a small, yet statistically significant, increase in the size of GL26-gal1i tumors compared to rat IgG control antibodies over a 7-d growth period (2.92 ± 10\(^5\) ± 8.33 ± 10\(^5\) pixels rat IgG vs. 1.08 ± 10\(^6\) ± 2.74 ± 10\(^5\) pixels anti-Ly-6G; \( p = 0.0421 \), unpaired, two-tailed, Student’s t-test) (Fig. 4D). However, the average anti-Ly-6G treated tumor was still nearly twice as small as those which had grown in response to Gr1 immunodepletion (1.08 ± 10\(^6\) ± 2.74 ± 10\(^5\) pixels anti-Ly-6G vs. 1.82 ± 10\(^6\) ± 1.91 ± 10\(^6\) pixels anti-Gr1), which targets both Ly-6G\(^+\) and Ly-6C\(^+\) cells. Control experiments revealed that the 1A8 clone immunodepleted circulating polymorphonuclear Gr-1\(^+\)/CD11b\(^+\) myeloid cells by 96.6% after 24-h in response to a single 600 \( \mu \)g dose, while only reducing the monocytic subtype by 8.5% (Fig. 4E), thus demonstrating the specificity of anti-Ly-6G antibodies.

**Gr-1\(^+\)/CD11b\(^+\) myeloid cells that infiltrate early gal-1-deficient gliomas also express CCR2 and Ly-6C**

Our data up to this point suggested that Ly-6C\(^+\) monocytes might play a more central role in the aiding of gal-1-deficient glioma rejection because (1) Gr-1 (Ly-6G/Ly-6C)
immunodepletion had a greater effect on gal-1-deficient glioma growth compared to Ly-6G-specific immunodepletion and (2) our in vivo cytokine array analysis showed higher levels of monocyte chemoattractants in the gal-1-deficient glioma microenvironment. We therefore hypothesized that CCR2, the cognate receptor for CCL2/MCP-1 and CCL12/MCP-5, may be
responsible for the chemotraction of monocytic Gr-1<sup>+</sup>/CD11b<sup>+</sup> myeloid cells into the brain tumor microenvironment. To test this hypothesis, we engrafted $3 \times 10^4$ GL26-gal1i cells into the striatum of wild-type C57BL/6j mice or B6.CCR2<sup>rfp/rfp</sup> knock-out mice, a model in which cells that would otherwise be CCR2<sup>+</sup> express red fluorescent protein (RFP). Glioma growth in these two models was then compared. Quantitative histological analysis revealed that the tumors were equivalent in size 7-d post-engraftment ($4.76 \pm 2.77 \times 10^4$ pixels C57 vs. $5.32 \pm 1.76 \times 10^4$ pixels CCR2<sup>rfp/rfp</sup>; n.s., $p = 0.8708$, unpaired, two-tailed, Student’s t-test) (Fig. 5A). Scanning fluorescence confocal analysis further revealed that GL26-
gal1i tumors in the B6.CCR2\textsuperscript{rfp/rfp} mice were highly infiltrated by RFP\textsuperscript{+} cells (Fig. 5B), thus demonstrating that the CCR2 signaling axis is not required for the trafficking of these cells into the tumor microenvironment. Flow cytometric analysis of circulating leukocytes from B6.CCR2\textsuperscript{rfp/rfp} mice demonstrated that only monocytic Gr-1\textsuperscript{+}/CD11b\textsuperscript{+} myeloid cells (not the polymorphonuclear subtype) are RFP\textsuperscript{+} (Fig. 5C), implying that the RFP\textsuperscript{+} cells seen in confocal micrographs were likely monocytes. Immunohistochemical analysis with anti-Ly-6G (clone: 1A8) or anti-Ly-6C (clone: AL-21) antibodies on brain tissue sections from C57BL/6J mice bearing GL26-gal1i glioma revealed very few tumor-infiltrating Ly-6G\textsuperscript{+} cells, but numerous Ly-6C\textsuperscript{+} cells 7-d post-engraftment (Fig. 5D). Flow cytometric analysis of PBMCs infiltrating the early gal1-deficient tumor microenvironment in C57BL/6J mice confirmed that tumor infiltrating Gr-1\textsuperscript{+}/CD11b\textsuperscript{+} myeloid cells co-express Ly-6C, and showed that the Gr-1\textsuperscript{int.}/CD11b\textsuperscript{+} cells are Ly-6C\textsuperscript{−} (Fig. 5E).

Specific microenvironmental cues cause circulating monocytes to differentiate into either macrophages or conventional dendritic cells upon extravasating from the blood into inflamed tissue.\textsuperscript{49} We cultured FACS purified monocytic Gr-1\textsuperscript{+}/CD11b\textsuperscript{+} cells with GL26-NT or GL26-gal1i glioma cells for 20-h, then assessed the resultant status of the myeloid cells. Our experiments revealed that monocytic Gr-1\textsuperscript{+}/CD11b\textsuperscript{+} myeloid cells express more CD11c, a prototypical conventional dendritic cell marker, in the presence of gal1-deficient GL26-gal1i cells (653 NT geometric mean vs. 1,410 gal1i geometric mean, experiment repeated \times 2), and more F4/80, a prototypical macrophage marker, in the presence of gal1-expressing GL26-NT cells (1,594 NT geometric mean vs. 1,127 gal1i geometric mean, experiment repeated \times 2) showing that gal1-deficient

![Figure 5](image_url)

**Figure 5.** Gr-1\textsuperscript{+}/CD11b\textsuperscript{+} myeloid cells that infiltrate early gal1-deficient glioma express markers of inflammatory monocytes. (A) Quantification of GL26-gal1i tumor size 7-d after intracranial engraftment in wild-type C57BL/6J (n = 4) or B6.CCR2\textsuperscript{−/−} (n = 4) mice. (B) Scanning fluorescence confocal analysis of GL26-gal1i glioma (green) 48-h post-engraftment into the brain of a B6.CCR2\textsuperscript{−/−} mouse showing the presence of numerous RFP\textsuperscript{+} cells (red), a surrogate marker for cells that normally express CCR2. (C) Flow cytometric analysis of circulating leukocytes from tumor-naive B6.CCR2\textsuperscript{−/−} mice reveals that only the monocytic subtype of Gr-1\textsuperscript{−}/CD11b\textsuperscript{−} myeloid cell expresses RFP. (D) Fluorescence immunohistochemical analysis on brain tissue sections bearing GL26-gal1i 7-d post-engraftment into wild-type C57BL/6J mice (n = 4) using anti-Ly-6G (clone: 1A8) (left two panels) or anti-Ly-6C (clone: AL-21) (right two panels) antibodies, experiment repeated \times 2. The aspects of the low-magnification micrographs outlined by the white boxes within the respective micrographs are shown at higher-magnification in the micrographs to the right, demonstrating the paucity of Ly-6G\textsuperscript{−} cells, but high degree of Ly-6C\textsuperscript{−} cells within the gal1-deficient glioma microenvironment. Insets show examples of immunopositive cells whose nuclear morphology is consistent with Ly-6G\textsuperscript{−} polymorphonuclear cells (left panel) and Ly-6C\textsuperscript{−} monocytes (right panel). (E) Flow cytometric analysis of Gr-1\textsuperscript{−}/CD11b\textsuperscript{−} (blue gate) and Gr-1\textsuperscript{+} /CD11b\textsuperscript{+} myeloid cells (red gate) within the GL26-gal1i tumor microenvironment 6-d post-engraftment (left panel). Color-coded PBMC populations are further stratified based on Ly-6C expression in the histograms to the right, demonstrating that the Gr-1\textsuperscript{int.}/CD11b\textsuperscript{+} population is Ly-6C\textsuperscript{−} while the Gr-1\textsuperscript{−}/CD11b\textsuperscript{−} cells are Ly-6C\textsuperscript{−}. Isotype control is shown (white histogram); experiment repeated \times 2. (F) Flow cytometric analysis for CD11c (left) and F4/80 in FACS-purified Gr-1\textsuperscript{−}/CD11b\textsuperscript{−} mononuclear cells after 20-h of in vitro co-culture with GL26-NT or GL26-gal1i cells. The geometric mean of each color-coded histogram (bottom panels) is plotted as a bar graph above the respective histogram plots. The schematic between the two bar graphs shows the known fates of circulating monocytes toward either conventional myeloid dendritic cells (i.e., mDCs) or macrophages (MΦ) in response to different microenvironmental queues, experiment repeated \times 2.
Figure 6. Summary models. (A) Schematic summary of innate immune-mediated gal-1-deficient GL26 glioma rejection. Step 1: gal-1-knockdown causes GL26 cells to increase production of the chemokines CXCL10/IP-10, CXCL12/SDF-1, and CCL5/RANTES. Step 2: gal-1-deficient GL26 cells are engrafted into the brain of RAG1−/− or C57BL/6 mice. Step 3: gal-1-deficient glioma cells produce proinflammatory factors in the brain. Step 4: circulating Gr-1+/CD11b+/Ly-6C−/CCR2+ monocytederived inflammatory cells (MDICs). Immunosuppressive malignant glioma generated through natural selective pressures influence the inherently plastic Gr-1+/CD11b+ myeloid precursor cell population to act as MDSCs with immunosuppressive or pro-tumor functionality. Gr-1 immunodepletion in these cancer systems is expected to extend survival (left panel). Experimental or therapeutic interventions that enhance the inflammatory state of the glioma microenvironment (i.e. tumor-derived gal-1 suppression) influence the same population of Gr-1+/CD11b+ myeloid precursor cells to act as MDICs with antitumor functionality. Gr-1 immunodepletion in these cancer systems is expected to reduce survival.

Discussion

Although immunotherapy for high-grade glioma is an active area of investigation, tumor localization within the immunospecialized CNS and the production of immunosuppressive factors act as major impediments to immune-mediated targeting of the disease. While the literature is well annotated with studies pertaining to mechanisms of glioma-induced adaptive immunosuppression, mechanisms of innate immunosuppression lack an equivalent depth of knowledge. We have recently contributed to a better understanding of glioma-induced innate immunosuppression by showing that orthotypically engrafted mouse and rat glioma cells rendered gal-1-deficient through shRNA knockdown are sensitized to NK-mediated recognition and clearance. We now demonstrate that an unexpected population of Gr-1+/CD11b− myeloid cells is central to the ability of NK cells to exert immunosurveillance activity against gal-1-deficient GL26 glioma.

Our data showing that monocytic Gr-1+/CD11b+ myeloid cells act as antitumor cells against gal-1-deficient glioma opposes the prevailing view of these cells as tolerogenic and immunosuppressive in murine cancer models. Since we find that antitumor function in this myeloid cell subpopulation correlates with gal-1-deficiency in glioma cells, we propose that tumor-derived gal-1 may play an important role in the promotion of immunosuppressive MDSC expansion and activity. The work of others supports this hypothesis by demonstrating that gal-1 indeed favors the conversion of peripheral macrophages toward the M2 phenotype and deactivates M1 microglia within the CNS.

The view of tumor inflammatory status as a principle determinant of immature myeloid cell function helps explain why we and others investigating experimental glioma models with enhanced inflammatory characteristics ascribe antitumor activity to a population of myeloid cells conventionally thought to mediate immune regulatory effects in the context of cancer. Our experiments with gal-1-deficient glioma reveal the capability of Gr-1−/CD11b+ cells to have immunostimulatory effects. We therefore suggest a definition of mouse MDSCs that goes beyond the cell surface markers Gr-1−/CD11b+ should be required to determine if such cells are indeed immunosuppressive (i.e., MDSCs), or immune-stimulating, as demonstrated here by us (Fig. 6B). Such a functional definition may mitigate future controversies regarding disparate results obtained when Gr-1−/CD11b+ cells are immunodepleted in experimental murine cancer studies. A still outstanding question is whether immature human myeloid cells frequently defined as CD33+/CD11b+/HLA-DR− are influenced in a similar manner by human-specific gal-1 which shares 88.1% identity with the rodent protein (HomoloGene database, NCBI), or if these cells also exhibit modularity in their phenotype in response to microenvironmental cues.

We suspect that immunotherapeutic strategies designed to suppress the expression or function of glioma-derived gal-1 may be of significant clinical value. Reductions of gal-1 protein by as little as 50% in mouse and rat glioma cells are sufficient to elicit robust recognition and eradication of both glioma models by the innate immune system of the respective species. Despite this compelling observation, care must be taken in moving forward with early phase human clinical trials that implement anti-gal-1 immunotherapeutic strategies. We have
yet to fully understand how glioma-derived gal-1 exerts its immunosuppressive activity. Simple application of extracellular gal-1 inhibitors may fail to provide significant clinical improvements in humans with malignant glioma. Aside from its extracellular functions, gal-1 also appears to play an important role through a dampening of the uncoupled protein response (UPR) (i.e., ER stress response) as demonstrated by others.54 Our own work supports this view by showing that gal-1-deficient GL26 cells exhibit increased inflammatory cytokine production, a potential indicator of heightened cell stress. The observation of elevated levels of proinflammatory cytokines produced by gal-1-deficient glioma cells may therefore be explained by an associated increase in intracellular stress. Assuming this to be a correct assumption, one would reasonably expect that further reductions in tumor-derived gal-1 (greater than the ~50% decrease at the population level demonstrated by us53) would lead to corresponding increases in intracellular stress and further production of proinflammatory cytokines. A situation such as this would be expected to lead to stronger influx of inflammatory Gr-1+/CD11b+ myeloid cells and NK cells into the tumor microenvironment. Investigations are now underway in our laboratory to identify the inflammatory factors responsible for heightened innate immune recognition and clearance of gal-1-deficient glioma, and to understand the molecular interactions necessary for CCR2+/Ly-6C+/Gr-1+/CD11b+ myeloid cells to license antitumor NK activity.

Materials and methods

Animal strains

Eight to 10 week-old female C57BL/6J, B6.129S7-Rag1tm1Mom/J (i.e., RAG1-/-) and B6.129(Cg)-Ccr2tm2.1Icf/J (i.e., B6.C57CR2r/ri) mice were purchased from the Jackson Laboratory. RA/EGxdelCre mice were kindly provided by Angelika Bierhaus of the Department of Internal Medicine I, University of Heidelberg (Heidelberg, Germany). All animal experiments were conducted in accordance with procedures approved by the University Committee on Use and Care of Animals (UCUCA) and conformed to the policies and procedures of the Unit for Laboratory Animal Medicine (ULAM) at the University of Michigan.

Flow cytometry antibodies

The following fluorochrome-conjugated flow cytometric antibodies were used throughout this work (each used at a 1:100 dilution): Alexa Fluor 700-conjugated rat anti-mouse CD45 (clone: 30-F11), Cat#: 103128, Biolegend; PE-conjugated rat anti-mouse Gr-1 (clone:RB6-8C5), Cat#: 553128, BD Pharmingen; PerCP/Cy5.5-conjugated rat anti-mouse CD11b (clone: M1/70), Cat#: 101228, Biolegend; Pacific Blue-conjugated hamster anti-mouse CD3e (clone: 500A2), Cat#: 558214, BD Pharmingen; APC-conjugated mouse anti-Granzyme B (clone: GB11), Cat#: 553128, BD Pharmigen, conjugated to polyclonal rabbit anti-human/mouse granzyme B (1:1,000), Cat#: 515408, Biolegend, conjugated to polyclonal rabbit anti-mouse immunoglobulins/biotinylated secondary antibodies (1:1,000), Cat#: E0468, Dako; Pacific Blue-conjugated mouse anti-human/mouse granulocyte (clone: C11), Cat#: 515408, Biolegend, conjugated to polyclonal rabbit anti-mouse immunoglobulins/biotinylated secondary antibodies (1:1,000), Cat#: E0464, Dako; PerCP/Cy5.5-conjugated rat anti-mouse Ly-6G (clone: 1A8) (1:500), Cat#: 127616, Biolegend, conjugated to Alexa Fluor® 594-conjugated goat anti-rat IgG (H+L) secondary antibodies (1:1,000), Cat#: A-11007, Life Technologies; APC-conjugated rat anti-mouse Ly-6C (clone: AL-21) (1:500), Cat#: 560595, BD Pharmingen, conjugated to Alexa Fluor® 594-conjugated goat anti-rat IgG (H+L) secondary antibodies (1:1,000). No surfactants or antigen retrieval steps were used in any immunohistochemical immunolabeling procedures.

Immunohistochemistry antibodies

PE-conjugated rat anti-mouse Gr-1 (clone: RB6-8C5) (1:500), Cat#: 553128, BD Pharmagen, conjugated to polyclonal rabbit anti-rat immunoglobulins/biotinylated secondary antibodies (1:1,000), Cat#: E0468, Dako; Pacific Blue-conjugated mouse anti-human/mouse granulocyte (clone: C11), Cat#: 515408, Biolegend, conjugated to polyclonal rabbit anti-mouse immunoglobulins/biotinylated secondary antibodies (1:1,000), Cat#: E0464, Dako; PerCP/Cy5.5-conjugated rat anti-mouse Ly-6G (clone: 1A8) (1:500), Cat#: 127616, Biolegend, conjugated to Alexa Fluor® 594-conjugated goat anti-rat IgG (H+L) secondary antibodies (1:1,000), Cat#: A-11007, Life Technologies; APC-conjugated rat anti-mouse Ly-6C (clone: AL-21) (1:500), Cat#: 560595, BD Pharmingen, conjugated to Alexa Fluor® 594-conjugated goat anti-rat IgG (H+L) secondary antibodies (1:1,000). No surfactants or antigen retrieval steps were used in any immunohistochemical immunolabeling procedures.

Imaging modalities

Fluorescence and bright-field micrographs were taken with a Zeiss Axioplan-2 microscope equipped with a digital camera (Carl Zeiss MicroImaging, Inc.) and Axiovision Release 4.6 analysis software. Fluorescence scanning confocal micrographs were taken with a Leica DMIRE2 confocal microscope equipped with Leica Confocal Software version 2.61 (Leica Microsystems). Fluorescence channels were scanned sequentially to reduce inter-channel bleed.

Quantifying tumor size from PFA-fixed brain tissue sections

PFA fixed mouse brains were coronally sectioned 50 μm thick using a vibratome. Every sixth section was placed into the same well of a 12-well plate (only 6-wells of the plate were occupied per mouse brain). The contents of an entire well was then extracted and mounted on a glass microscope slide and coverslipped using Prolong Gold anti-fade reagent. Fluorescence images of all brain tissue sections containing an aspect of the mCit+ GL26 gliomas were taken using a 5× objective. Micrographs were then imported into ImageJ analytical software (National Institutes of Health, Bethesda, MD) and processed according to the following method: I. Image > Type > 8-bit. II. Image > Adjust > Threshold > Apply (an arbitrary threshold was chosen for each experiment; however, the threshold was not altered over the course of analyzing tissue from any one particular experiment). III. Process > Binary > Make Binary. IV. Analyze > Measure. The area output of each
measurement was then summed to afford an estimate of the overall tumor size.

**In vivo immunodepletion antibodies**

The following antibodies were administered intraperitoneally for immunodepletion in RAG1\(^{-/-}\) mice (per mouse): 500 \(\mu\)g of purified rat anti-mouse Gr-1 (Clone: RB6-8C5), Cat#: BE0075, Bio X Cell; 600 \(\mu\)g of rat anti-mouse Ly-6G (Clone:1A8), Cat#: BE0075-1, Bio X Cell. Both antibodies were diluted to a final volume of 200 \(\mu\)L in sterile DPBS and were administered one day before tumor implantation, then once every 4 d. Control mice received non-specific rat IgG immunoglobulins (Equitech-Bio Inc.) at an equivalent molar dose and volume.

**Flow cytometric analysis**

All analysis was performed using FlowJo analysis software v10.0.7 (Tree Star, Inc.).

**Statistical analysis**

Statistical analyses were performed using GraphPad Prism5 (GraphPad Software, Inc.). All data are reported as the mean \(\pm\) SEM and were examined with the statistical tests specified throughout the results section or associated figure legend. Biological replicates of each experiment are reported in the associated figure legend. Values were considered significant at the \(p \leq 0.05\) level.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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**Author contributions**

590 G.J.B. and P.R.L. designed research; G.J.B., P.C. and D.Z. performed research; G.J.B., P.C., M.G.C. and P.R.L. analyzed data; G.J.B., M.G.C. and P.R.L. wrote the paper.

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