FGL2 promotes tumor progression in the CNS by suppressing CD103⁺ dendritic cell differentiation

Jun Yan¹23, Qingnan Zhao³, Konrad Gabrusiewicz⁴, Ling-Yuan Kong⁴, Xueqing Xia³, Jian Wang⁵, Martina Ott⁴, Jingda Xu⁶, R. Eric Davis⁶, Longfei Huo³, Ganesh Rao⁴, Shao-Cong Sun⁷, Stephanie S. Watowich⁷, Amy B. Heimberger⁴ & Shulin Li³

Few studies implicate immunoregulatory gene expression in tumor cells in arbitrating brain tumor progression. Here we show that fibrinogen-like protein 2 (FGL2) is highly expressed in glioma stem cells and primary glioblastoma (GBM) cells. FGL2 knockout in tumor cells did not affect tumor-cell proliferation in vitro or tumor progression in immunodeficient mice but completely impaired GBM progression in immune-competent mice. This impairment was reversed in mice with a defect in dendritic cells (DCs) or CD103⁺ DC differentiation in the brain and in tumor-draining lymph nodes. The presence of FGL2 in tumor cells inhibited granulocyte-macrophage colony-stimulating factor (GM-CSF)-induced CD103⁺ DC differentiation by suppressing NF-κB, STAT1/5, and p38 activation. These findings are relevant to GBM patients because a low level of FGL2 expression with concurrent high GM-CSF expression is associated with higher CD8B expression and longer survival. These data provide a rationale for therapeutic inhibition of FGL2 in brain tumors.

¹ Center for Brain Disorders Research, Capital Medical University, Beijing 100069, China. ² Beijing Institute for Brain Disorders, Beijing 100069, China. ³ Department of Pediatrics–Research, The University of Texas MD Anderson Cancer Center, Houston, TX 77030, USA. ⁴ Department of Neurosurgery, The University of Texas MD Anderson Cancer Center, Houston, TX 77030, USA. ⁵ Department of Biostatistics, The University of Texas MD Anderson Cancer Center, Houston, TX 77030, USA. ⁶ Department of Lymphoma and Myeloma, The University of Texas MD Anderson Cancer Center, Houston, TX 77030, USA. ⁷ Department of Immunology, The University of Texas MD Anderson Cancer Center, Houston, TX 77030, USA. Correspondence and requests for materials should be addressed to A.B.H. (email: aheimber@mdanderson.org) or to S.L. (email: sli4@mdanderson.org)
Brain tumor progression, relapse, and invasion are driven by abnormal transcriptional profiles resulting from either intrinsic genetic or epigenetic changes\textsuperscript{1,2}. The impact of intrinsic immune-associated genes on brain tumor progression in the presence of a host immune system is much less well understood. Thus far, only the gene for indoleamine 2,3-dioxygenase has been shown to have a role in glioma progression\textsuperscript{3}. Over 80% of mice implanted with GL261 gliomas in which this gene was knocked out had long-term survival that was associated with decreased T-regulatory cell (Treg) recruitment by tumors and enhanced T cell-mediated tumor rejection\textsuperscript{3}. This result suggests that immune regulatory genes within tumor cells may be the arbiters of tumor-cell fate in the central nervous system (CNS). Antigen-presenting cells (APCs) are essential for the induction of adaptive T cell responses\textsuperscript{4}. Tumor-associated dendritic cells (DCs) take up, process, and transport tumor antigens to draining lymph nodes for priming and activation of T cells\textsuperscript{4}. The transcriptional programs within DCs can influence their immunological role. Batf3-dependent CD103\textsuperscript{+}/CD8\textsuperscript{α} DCs are essential for inducing effector T cell recruitment to the tumor and priming T cells in tumor-draining lymph nodes (TDLNs)\textsuperscript{5}. It is unknown whether Batf3-dependent DCs have a role in CNS tumors.

Fibrinogen-like protein 2 (FGL2) is a membrane-bound or secreted protein expressed by macrophages, T cells, and tumor cells that has coagulation activity or immune-suppressive functions\textsuperscript{6–10}. FGL2 promotes mammary tumor progression by promoting tumor angiogenesis or inducing epithelial-mesenchymal transition\textsuperscript{10}. We previously showed, using an engineered gene expression system in mouse glioma cells, that FGL2 is a key hub of tumor-mediated immune suppression in glioblastoma multiforme (GBM) by regulating expression of immune checkpoints and augmenting intratumoral skewing of Tregs, myeloid-derived suppressor cells (MDSCs), and M2 cells\textsuperscript{8}. However, the exact functional role of FGL2 at both the molecular and cellular levels remains largely unknown. Likewise, the connection between FGL2 and CD103\textsuperscript{+} DCs is totally unknown. To determine the effect of tumor-cell intrinsic FGL2 on tumor progression, we used complete FGL2 knockout (KO) tumor-cell lines and FGL2-deficient (FGL2\textsuperscript{−/−}) mice to reveal a novel molecular immune mechanism for FGL2’s regulation of tumor progression in the CNS. We discovered that FGL2 secreted from tumor cells is the primary suppressor of differentiation of antigen-presenting CD103\textsuperscript{+} DCs via blocking of granulocyte-macrophage colony-stimulating factor (GM-CSF) signal transduction and subsequent T cell functions in the brain. By contrast, an FGL2KO host (FGL2\textsuperscript{−/−}) has no impact on inhibition of FGL2-positive tumors. Cumulatively, the data indicate that tumor cell-expressed FGL2 serves as a critical onco-immune target.

Results

Heterogeneous expression of FGL2 in GBM. FGL2 is primarily expressed in immune cells, including T cells, macrophages, and natural killer (NK) cells\textsuperscript{6,9}. Our previous data show that FGL2 is highly expressed in glioma and is associated with poor prognosis in glioma patients\textsuperscript{8}. To further characterize the cellular sources of FGL2 expression in GBMs, quantitative western blotting was performed among different cell types, including normal cells and glioma stem cells (GSCs). FGL2 was minimally expressed in normal neuron cells (HCN-1A), endothelial cells (HMVEC-L), and primary human peripheral blood monocytes (PBMCs) from healthy donors relative to the GSCs (GSC7-2, GSC11, GSC20, and GSC28) (Fig. 1a). Flow cytometric analysis of FGL2 expression showed 1.7–11.5% FGL2 positive-staining glioma cells among disaggregated GSC neurospheres (Fig. 1b; Supplementary Figure 1). Single-cell suspensions derived from fresh ex vivo GBM specimens demonstrated that FGL2 expression occurred primarily in the glioma subpopulation that expressed glia-specific marker GFAP (43.3 ± 14.3% of GFAP\textsuperscript{+} cells). Portions of CD45\textsuperscript{+} immune cells (37.3 ± 18.3% of CD45\textsuperscript{+} cells) and CD31\textsuperscript{+} endothelial cells (47.7 ± 14.7% of CD31\textsuperscript{+} cells) were also FGL2 positive, but endothelial cells (1.0 ± 0.6%) and leukocytes (5.7 ± 3.0%) accounted for only small fractions of total cells from these tissues (Fig. 1c). Immunohistochemical analysis of GBM specimens demonstrated abundant co-localization of FGL2 expression with brain tumor cells using the GFAP marker (15.8 ± 4.3%), moderate co-localization with immune cells (based on CD45 co-expression [6.5 ± 1.6%]), and occasional association with endothelial cells (denoted as CD31\textsuperscript{+} [3.3 ± 1.4%]) (Fig. 1d), consistent with the flow cytometry data. Cumulatively, these data indicate that the predominant cellular sources of FGL2 within the GBM are the tumor cells and GSCs.

Intrinsic FGL2 in tumor cells promotes tumor progression. Given the heterogeneous but typically high levels of FGL2 expression in GBM cells, we next wanted to analyze the specific role of FGL2 within tumor cells in brain tumor progression. Stable FGL2KO tumor-cell lines were generated utilizing CRISPR/Cas9 technology. Deletion of the DNA fragment in FGL2 exon 1 in each clone was confirmed by gene sequencing (Fig. 2a). Western blotting analysis showed complete knockout of FGL2 expression in glioma (GL261-FGL2KO) and Lewis lung cancer cells (LLC-FGL2KO) and downregulated FGL2 expression in FGL2-knockdown astrocytoma cells (DBT-FGL2KD) compared with the cognate cells in which the non-targeting control sequence was introduced via the same technology (Fig. 2b). As expected, mice bearing tumors generated by implantation of control GL261 tumor cells (GL261-Ctrl) experienced rapid disease progression and died. In contrast, immune competent mice, including wild-type (WT) and FGL2\textsuperscript{−/−}, survived after implantation of GL261-FGL2KO tumor cells (Fig. 2c,d). Similar results were obtained in mice implanted with a high (5-fold) or a maximal (20-fold) number of GL261-FGL2KO tumor cells (Supplementary Figure 2a-d). LLC was selected for this experiment because lung cancers are the most common source of brain metastasis, with 30–60% of lung tumor patients developing brain metastasis, a major cause of death\textsuperscript{1,12}. Like GL261-FGL2KO tumor cells, LLC-FGL2KO and DBT-FGL2KD tumor cells showed no progression in immunocompetent mice (Fig. 2e, i). Therefore, FGL2 expression in the tumor cell but not in the host is required for tumor progression in immune-competent mice (Supplementary Figure 2e). Notably, this was not secondary to FGL2’s impact on the cell growth rate, because both FGL2KO and Ctrl glioma cells proliferated equally in vitro (Supplementary Figure 2f). The tumor cell-specific FGL2KO-mediated impairment of tumor progression was also not due to differences in implantation, because bioluminescence imaging showed similar chemoluminescence signals in both the Ctrl (3.44E + 07) and FGL2KO (3.90E + 07) tumors on day 1. The luciferase signal was rapidly reduced in the FGL2KO tumor cell-implanted mice (5.149e + 06) while increasing markedly in Ctrl tumor cell-implanted mice (9.52e + 09) by day 7, illustrating the marked difference in tumor progression between Ctrl and FGL2KO tumor-bearing mice (Fig. 2g).
Fig. 1 FGL2 is expressed by glioma cells. a FGL2 protein levels were detected by western blot in human peripheral blood monocytes (PBMCs) from healthy donors, normal endothelial cells (HMVECs), normal neurons (HCN-1A), and four glioma stem cell lines (GSC7-2, GSC11, GSC20, and GSC28). Western blots shown are representative, and quantities are summarized as ratio changes from three independent experiments. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a protein-loading control. b The increase of FGL2 protein levels was detected by western blot in human peripheral blood monocytes (PBMCs) from healthy donors, normal endothelial cells (HMVECs), normal neurons (HCN-1A), and four glioma stem cell lines (GSC7-2, GSC11, GSC20, and GSC28). Western blots shown are representative, and quantities are summarized as ratio changes from three independent experiments. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a protein-loading control. c Representative flow cytometry plots of FGL2 expression on CD45+ cells from tumors of patients with newly diagnosed GBM. Data (n = 5) are presented as the mean ± S.D. and analyzed by one-way ANOVA. d Sections of clinical GBM samples were double stained for co-expression of FGL2 (red) and GFAP (green), or of FGL2 (red) and CD31 (green). Nuclei were counterstained with DAPI (blue). White arrows point to co-localization. Scale bars, 50 μm. Data are presented as the mean ± S.D., and representative images are shown (n = 5). A one-way ANOVA was used to calculate the two-sided P values. Significant results were presented as **P < 0.01, ***P < 0.001.

or FGL2KO tumor cells resulted in equivalent survival times and tumor progression-associated death (Fig. 3a), indicating that the immune system is required for tumor cell-specific FGL2KO-mediated tumor regression. To clarify which immune cell populations participate in the regression of FGL2KO tumors, we used immune cell depletion and T cell-deficient mice. Depletion of CD4+ T cells had no effect on the survival of mice implanted with FGL2KO tumors (Fig. 3b; Supplementary Figure 3). Whereas NK depletion had a modest negative effect on survival (P = 0.14, Fig. 3c), depletion of CD8+ T cells completely reversed the survival benefit to mice implanted with GL261-FGL2KO cells (Fig. 3d; Supplementary Figure 3). Consistent with the results of CD8+ T cell depletion, tumor regression was re-established in 70–80% of mice with a CD8− background implanted with either GL261-FGL2KO or LLC-FGL2KO tumor cells (Fig. 3e–g). The number of CD8+ T and CD4+ T cells significantly greater in the brains of mice implanted with FGL2KO tumor cells than in those implanted with Ctrl tumor cells, but this was not the case with NK or NKT cells (Supplementary Figure 4c). Furthermore, lymphocytes from tumor-draining lymph nodes (TDLNs) and spleens of FGL2KO tumor-bearing mice showed higher cytolytic activity against tumor cells than those from Ctrl tumor-bearing mice, indicating a heightened cytotoxic function of CD8+ T cells in mice with FGL2-deficient tumors (Fig. 3h). These data strongly support the idea that CD8+ T cells were responsible for suppression of FGL2KO tumors.

The increased cytolytic activity of cells derived from TDLNs and spleens after implantation of FGL2KO tumor cells suggests the induction of systemic protective adaptive immune responses (Fig. 3h). To validate this assumption, we rechallenged mice with GL261-Ctrl cells 45 days after FGL2KO tumor implantation to test the presence or absence of CD8+ T cell-dependent recall responses. All mice survived tumor rechallenge (Fig. 3i). The same results were obtained in FGL2−/− mice prechallenged with GL261-FGL2KO cells (Fig. 3j), showing that tumor cell-specific
FGL2KO is crucial to induce this systemic adaptive immune response. We rechallenged the same mice with GL261-Ctrl cells a second and third time, with a gap of 45 days between each challenge. At the second rechallenge, no mice developed tumors, but on the third rechallenge, 5 of 7 mice developed a tumor (Fig. 3i). To ensure that the GL261-Ctrl cells used in the challenges were tumorigenic, the same cells were injected into naïve WT mice and, as expected, these mice developed tumors (Fig. 3i, j). These results collectively suggest that depletion of FGL2 from tumor cells induced CD8+ T effector cells and adaptive immune memory against the parental Ctrl tumor cells (GL261-Ctrl).

CD103+ DCs accumulation augmenting CD8+ T cell response. Several steps are necessary to generate a systemic adaptive anti-tumor immune response that leads to effective tumor elimination. First, antigens released by tumor cells are taken up and processed by APCs to present to T cells, inducing T cell priming and activation; second, activated T cells must traffic to the tumor and infiltrate; last, tumor cells are recognized and killed by the antigen-specific T cells. We found no difference in the migration and survival of activated CD8+ T cells treated with conditioned medium (CM) from either FGL2KO tumor cells (FGL2KO-Ctrl) or Ctrl tumor cells (Ctrl-CM) in vitro (Supplementary Figure 5a, b). CM was used in vitro because FGL2 is a secreted protein.
protein and the secreted FGL2 is crucial to causing immune suppression\(^\text{14}\). However, this secretion has no immediate impact on T cell killing activity, since both ovalbumin (OVA)-expressing FGL2KO and control tumor cells had the same sensitivity to cell killing by OVA peptide-activated CD8\(^+\) T cells (OT-I) in vitro (Supplementary Figure 5c). Instead, this secreted FGL2 suppressed T cell expansion (CFSE dilution), but not activation (CD25 expression) in vitro (Supplementary Figure 5d). These results suggest that an initial step in the antitumor immune responses, such as antigen presentation, is affected by FGL2KO in tumor cells, which ultimately affects the capacity to trigger the CD8\(^+\) T cell antitumor immune response.

To successfully trigger the initial steps of priming/activation and expansion of functional T cells, multiple signals must be
Carefully orchestrated by the APCs, recent publications have shown that the Batf3-lineage CD103+/CD8α− DCs are the most effective APCs in cross-presentation of tumor antigens to CD8+ T cells for peripheral tumors. Notably, the population of Batf3-dependent cross-presenting DCs−migratory CD103+ DCs (CD45+CD11b+MHCIi−/Lin−CD11c+CD103−) was greater in the brains and TDLNs of mice implanted with FGL2KO tumor cells than in those of mice implanted with Ctrl tumor cells (Fig. 4a), and the population of CD8α+ DCs (CD45+CD11b+MHCIi−/Lin−CD11c+CD8α+) was significantly different between the two groups, showing that numbers of migratory antigen-presenting DCs was increased by intracerebral implantation of FGL2KO tumor cells. Furthermore, Batf3-deficient (Batf3−/−) mice implanted with FGL2KO tumor cells lacked both CD103+ DCs and CD8α− DCs in their brains and TDLNs compared with WT C57BL/6 mice (Fig. 4a), suggesting that FGL2KO tumor cell-mediated CD103+ DCs development depends on Batf3, which is known as a crucial regulator of CD103+/CD8α− DCs in other tumor model systems. We then asked whether DCs, especially CD103+ DCs, controlled the antitumor effect induced by FGL2KO. CD11c−DTR mice were used to investigate the interaction between DCs and T cells. DT toxin, injected once, resulted in rapid ablation of DCs and complete restoration of DCs at day 6 (Supplementary Figure 6a). As expected, FGL2KO tumors progressed when DCs were depleted, and regress after DCs were restored (Fig. 4b). To further confirm that Batf3-lineage CD103+ DCs are required for the antitumor response (for an effector T cell response against tumors), FGL2KO tumors were implanted in Batf3−/− mice (Fig. 4c). As expected, FGL2KO tumors were not eradicated in these mice as they were in the WT mice, showing that CD103+ DCs are required for inducing an effective CD8+ T cell response against FGL2KO tumor cells. Together, these results suggest that FGL2KO tumor cells are more potent than the parental tumor cells at inducing development of Batf3-lineage DCs, which are necessary for initiating an antitumor immune response.

To investigate the capacity of DCs to present endogenously loaded antigens, DCs were isolated from OVA tumor-bearing mice for direct ex vivo analysis of antigen presentation. Co-culture of naive OT-I cells with DCs found a greater induction of cross-presentation to OT-I cells from DCs of FGL2KO tumors than from DCs of Ctrl-OVA tumors (Fig. 4d). To determine the in vivo antigen-presentation capacity of DCs, exogenous OT-I cells were labeled with CFSE and adoptively transferred into OVA tumor-bearing CD45.1 mice. Mice implanted with FGL2KO-OVA tumor cells drew a greater accumulation of OT-I cells in their brains, with an associated higher proliferation of CFSE-labeled OT-I cells in the TDLNs than those of Ctrl-OVA tumor-implanted mice (Fig. 4e). This result shows that DCs from FGL2KO-OVA tumors have a more efficient antigen-presentation capacity than those from Ctrl-OVA tumors to promote antigen-specific priming and expansion of T cells in the TDLNs and their subsequent accumulation in the brain. To address whether CD8+ T cells are primed more efficiently in the FGL2KO tumor-bearing mice than in Ctrl tumor-bearing mice, T-bet expression was measured because it is required for differentiation and function of cytotoxic CD8+ T cell effectors. Greater numbers of CD8+T-bet+ Eomes− T cells were detected in both the brains and TDLNs at 7 days post implantation in mice implanted with FGL2KO tumor cells than in mice implanted with Ctrl tumor cells (Fig. 4f). TDLNs are the primary sites for cross-priming of CD8+ T cells by CD103+/CD8α− DCs during tumor growth. Immune modulator phosphorylated fingolimod (FTY720) acts as an agonist on sphingosine-1-phosphate receptors in vivo, inducing subsequent internalization of S1P1 and thereby inhibiting DC migration from the periphery to lymph nodes and preventing egress of primed T cells from lymph nodes to target sites. Upon administration of FTY720 in FGL2KO tumor-bearing WT mice, the tumors progressed (Fig. 4g), suggesting that T cells primed by CD103+ DCs in the TDLNs are required for inducing the adoptive antitumor T cell immune response in mice with FGL2KO tumor. FGL2KO in tumor cells resulted in increased T cell frequency in tumors and promoted T cell proliferation in TDLNs. These results suggest that T cell priming in the TDLNs is required to promote the antitumor response to FGL2KO tumors. Thus, expression of FGL2 in tumor cells prevents T cell priming by reducing the development of Batf3-dependent CD103+ DCs. FGL2 suppresses CD103 induction by blocking GM-CSF signaling. To verify the effect of FGL2KO and Ctrl tumor cells on generation of CD103+ DCs in vitro, bone marrow cells were cultured with medium conditioned by FGL2KO or Ctrl tumor cells with/without hFLT3L (50 ng/mL), and CD103+ DC differentiation was measured. In accordance with the in vivo results, the expression level of CD103 was significantly higher on the CD11c+ B220− DCs generated through culture with FGL2KO-CM than on those cultured with Ctrl-CM on both day 5 and day 15 (Fig. 5a, Supplementary Figure 6b). Clec9A, a specific and universal marker of mouse and human Batf3-dependent DCs which promotes DC cross-priming of cytotoxic T lymphocytes, was expressed on the cultured DCs at day 15 but not day 5 (Fig. 5a). These data indicate that more CD103+ DCs were differentiated from bone marrow cells in the absence of FGL2 at day 15 of culture, but CD103 expression on cultured bone marrow DCs was detected as early as day 5. We then examined the capacity of 15 days-cultured bone marrow DCs to stimulate OT-I cells when given OVA protein. Bone marrow DCs

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**Fig. 3** CD8+ T cell-dependent clearance of FGL2KO tumor. a Survival analysis of immunodeficient NSG mice implanted with GL261-Ctrl or GL261-FGL2KO tumor cells (5 × 104 cells per mouse; n = 6 per group). b–d Survival analysis of WT immunocompetent C57BL/6 mice implanted with GL261-FGL2KO tumor cells and treated with anti-CD4 (GK1.5), anti-NK1.1 (PK136), or anti-CD8 (2.43) depletion antibody or IgG as control (Ctrl) (n = 6 per group). e Survival analysis of WT mice and CD8−/− mice implanted with GL261-FGL2KO tumor cells (n = 6–8 per group). f H&E-stained histopathological sections of brain tissues from representative mice from each group shown in e. Scale bar = 1 mm. g Survival analysis of WT mice and CD8−/− mice implanted with LLC-FGL2KO tumor cells (n = 6 per group). h Cytolytic activity of CD8+ T cells from tumor-bearing mice against GL261 tumor cells. Lymphocytes from tumor-draining lymph nodes (TDLNs) or spleens of mice implanted with FGL2KO tumor cells caused significantly more tumor lysis than those from mice implanted with Ctrl tumor cells (n = 3 mice per group). Two-way ANOVA was used to calculate the two-sided P values. E, T, the ratio of effector cells to target cells. *P < 0.05, **P < 0.01, ***P < 0.001 compared with GL261-Ctrl tumor-bearing mice. i Tumor rechallenge by subcutaneous implantation of GL261-Ctrl cells at days 45, 90, and 135 days after primary challenge of GL261-FGL2KO by intracerebral implantation in WT mice (n = 7). Subcutaneous growth of GL261-Ctrl tumors in age-matched naive WT mice challenged with GL261-Ctrl cells at the same times but without primary challenge is also shown. ** The experiment shown in i was repeated in FGL2−/− mice (n = 6). All data are representative of at least two independent experiments. The survival curves were analyzed by Kaplan–Meier analysis and the log-rank test was used to compare overall survival between groups.
cultured with FGL2KO-CM were more efficient than those cultured with Ctrl-CM at inducing expression of CD69 on OT-I cells (Fig. 5b), indicating that DCs cultured with FGL2KO-CM had greater cross-presentation capacity than DCs cultured with Ctrl-CM.

It is well known that FLT3L and GM-CSF promote development of CD103+ DCs. To discern whether either factor was differentially expressed in FGL2KO-CM, we evaluated the abundance of FLT3L and GM-CSF in the CMs. To our surprise, levels of GM-CSF and FLT3L were almost identical in media.
**Fig. 4** Batf3-dependent CD103+/ dendritic cells are required for CD8+ T cells priming. a Brain-infiltrating leukocytes (BIL) and tumor-draining lymph nodes (TDLNs) of Ctrl or FGL2KO tumor-bearing wild-type (WT) mice and FGL2KO tumor-bearing Batf3−/− mice were analyzed for CD103+/CD8α+ dendritic cell (DCs) populations on day 7 after tumor implantation. Data are presented as the mean ± S.D. and were analyzed by one-way ANOVA (n = 5-7 per group). b FGL2KO tumor progression in CD11c-DTR mice treated with diphtheria toxin (DT) or PBS. FGL2KO tumor cells were implanted subcutaneously into CD11c-DTR mice on day 1 after injection of DT or PBS. Data are presented as the mean ± S.D. (n = 8 per group). c Survival analysis of Batf3−/− mice implanted with GL261-FGL2KO tumor cells (n = 7 per group). d FACS assay of CD69 on naive OT-I CD8+ T cells co-cultured with DCs isolated from tumors at a ratio of 1:2. Data are presented as the mean ± S.D. (n = 3 mice per group) and were analyzed by t-test. e Abundance and proliferation of CFSE-labeled OT-I cells in brains and TDLNs of GL261-Ctrl-OVA or GL261-FGL2KO-OVA tumor-bearing mice. CFSE-labeled OT-I cells were adoptively transferred to tumor-bearing CD45.1 mice on day 2 after tumor implantation. Brains and TDLNs were collected 5 days later for analyzing the presence and proliferation of the CD45.2+CD8+ T cell population. Data are presented as the mean ± S.D. (n = 5-6 per group) and were analyzed by t-test. f Representative cytometric analysis of CD8+ T cell priming (CD8+ T-bet+Eomes−) in BIL and TDLNs at 7 days after tumor implantation. Data are presented as the mean ± S.D. (n = 5 per group) and were analyzed by t-test. g Survival analysis of WT mice implanted with GL261-FGL2KO tumor cells treated with FTY720. Mice were treated with 1 mg/kg FTY720 1 h before tumor-cell implantation and were maintained with drinking water containing 2 μg/mL FTY720 for the duration of the experiment (n = 6 per group). All data are representative of at least two independent experiments. The survival curves were analyzed by Kaplan-Meier analysis and the log-rank test was used to compare overall survival between groups. Significant results were presented as *P < 0.05, **P < 0.01, ***P < 0.001

![Graphs and plots](image1)

**Fig. 5** FGL2 suppresses GM-CSF-dependent CD103 induction on cDCs in vitro. a Expression of CD103 on CD11c+B220− DCs cultured with conditioned medium (CM) for 5 or 15 days. Bone marrow cells were isolated from C57BL/6 mice and then cultured with Ctrl-CM (CM from Ctrl tumor cells) or FGL2KO-CM (CM from FGL2KO tumor cells) for 5 days or 15 days. Non-adherent cells were recovered, stained, and analyzed by FACS to determine the expression levels of CD103, Clec9A, and MHCII on the CD11c+B220− DCs. b OT-I T cell activation by OVA-pulsed CM-cultured bone marrow DCs. Non-adherent bone marrow DCs were recovered from CM-cultured bone marrow DCs at 15 days. The bone marrow DCs (2 × 10^5) were incubated with soluble OVA protein (100 μg/mL) for 2 h and then washed twice before co-culturing with the CFSE-labeled OT-I cells (5 × 10^5). OT-I activation was measured by CD69 expression level, which was determined by flow cytometry. c Protein levels of GM-CSF and FLT3L in medium conditioned by tumor cells were detected by ELISA. Data were summarized from five independent experiments and were analyzed by paired t-test. d Representative FACS assay of CD103 expression on CD11c+B220− DCs cultured with FGL2KO-CM in the presence of neutralization antibody anti-GM-CSF (5 μg/mL) or anti-FLT3L (5 μg/mL) for 5 days. Data were summarized from four independent experiments and were analyzed by paired t-test. **P < 0.01 comparing with IgG. e Representative FACS assay of CD103 expression on wild-type (WT) and FGL2KO bone marrow (BM) cells cultured with rmGM-CSF (10 ng/mL) for 7 days with or without rFGL2 (200 ng/mL). All plots are representative of three independent experiments.
conditioned by GL261-FGL2KO or GL261-Ctrl tumor cells (Fig. 5c). While neutralization of GM-CSF in FGL2KO-CM markedly decreased CD103 expression on CD11c+ B220− DCs, however, neutralization of FLT3L had no effect on CD103 expression (Fig. 5d). In this regard, we investigated the effect of FGL2 deficiency in bone marrow cells on CD103 induction by GM-CSF. Interestingly, CD103 expression was detected on CD11c+ B220− DCs derived from both WT and FGL2−/− bone marrow cells cultured with recombinant murine GM-CSF (rGM-CSF). However, rGM-CSF induced a higher level of CD103 expression on FGL2−/− DCs than on WT DCs (Fig. 5e). Moreover, the expression of CD103 on FGL2−/− DCs induced by rGM-CSF was suppressed by rFGL2 (Fig. 5e). These data indicate that FGL2KO tumor cells promote CD103 expression on DCs by a GM-CSF-dependent mechanism, which is suppressed by FGL2 secreted from Ctrl tumor cells.

FGL2 inhibits p65 and STAT1/5 signals for CD103 induction. GM-CSF regulates DCs development and differentiation by signal transduction through the PI3K/AKT, JAK2/STAT1/3/5, MAPK, and NF-κB pathways. To investigate the specific pathway through which FGL2 suppresses GM-CSF-induced CD103 expression, we examined the changes in these signal pathways in CM-cultured bone marrow cells. The results show that FGL2KO-CM did increase TRAF6-NF-κB signaling, JAK2/STAT1/5 signaling, and p38 activation (Fig. 6a, Supplementary Figure 7). Blocking FGL2 using an FGL2-neutralized monoclonal antibody in Ctrl-CM also increased activation of these three signaling pathways. Neutralizing GM-CSF in FGL2KO-CM reversed the activation of these signaling pathways (Fig. 6a). Thus, FGL2 suppresses GM-CSF-induced TRAF6-NF-κB signaling, JAK2/STAT1/5 signaling, and p38 activation in bone marrow cells.

Next, we asked whether these signaling pathways regulate CD103 expression on DCs. To address this question, we treated bone marrow cells with NF-κB activation inhibitor 6-amino-4-(4-phenoxyphenylethylamino) quinazoline (QNZ; EVP4593), IkB kinase activation inhibitor BAY11-7082, JAK2 activation inhibitor JSI-104, or p38 inhibitor PH797804. All of these inhibitors suppressed CD103 expression on DCs induced by FGL2KO-CM in a dose-dependent manner (Fig. 6b), showing that NF-κB signaling, JAK2 signaling, and p38 activation can indeed increase induction of CD103 expression in bone marrow cell culture. Therefore, these data indicate the underlying molecular mechanism by which FGL2 suppresses GM-CSF-induced CD103 expression on DCs or DC progenitors is mediated via inhibition of TRAF6-NF-κB, JAK2/STAT1/5, and p38 signal transduction (Fig. 6c).

Concurrent GM-CSF and FGL2 in GBM predicts outcome. The results presented in Figs. 5 and 6 collectively imply that the expression of GM-CSF in the absence of FGL2 may trigger an effective CD8+ T cell response in the brain against GBM. To ascertain whether this observation holds in GBM patients, we...
analyzed the expression of these two genes and CD8\(^+\) T cell-associated genes in the GBM dataset of The Cancer Genome Atlas (TCGA; dataset: Glioblastoma Multiforme, Provisional) to assess prognostic associations. As expected, increased expression levels of GM-CSF (hazard ratio [HR]: 0.854, \(P = 0.008\)) or CD88 (HR: 0.906, \(P = 0.078\)) in GBM was associated with longer overall survival (OS) (Supplementary Table 1), while increased FGL2 expression was associated with poorer OS, though the difference was not statistically significant (HR: 1.113, \(P = 0.071\)) (Supplementary Table 1). There is a statistically significant positive correlation between GM-CSF and CD88 expression (Fig. 7b), and a strong negative correlation between FGL2 and CD88 expression (Fig. 7c), suggesting that a low level of FGL2 levels and a high level of GM-CSF may be associated with high CD8\(^+\) T cell infiltration in tumors. When GBM samples were clustered by GM-CSF and FGL2 expression levels (FGL2\(^{2\text{high}}\)GM-CSF\(^{2\text{low}}\), FGL2\(^{2\text{high}}\)GM-CSF\(^{2\text{low}}\), and FGL2\(^{2\text{low}}\)GM-CSF\(^{2\text{low}}\)), OS differed significantly among the four clusters (\(P = 0.019\)) (Fig. 7c). Notably, patients with a concurrent decreased FGL2 level and increased GM-CSF had a longer median OS than other patients (median survival duration 15.4 vs 13.8 months, respectively, \(P = 0.0028\)) (Fig. 7f). Furthermore, patients with a concurrent decreased FGL2 level and increased effector CD8\(^+\) T cell signature (CD88\(^{2\text{high}}\)IFN\(_{\gamma}\)\(^{2\text{high}}\)) had a longer median OS than those with a concurrent increased FGL2 level and decreased effector CD8\(^+\) T cell signature (CD88\(^{2\text{low}}\)IFN\(_{\gamma}\)\(^{2\text{low}}\)) (median survival duration 15.08 vs 10.45 months, respectively, \(P = 0.0016\)) (Fig. 7g). Overall, these results validated our mouse study results showing that concurrent high GM-CSF and low FGL2 levels in tumors carry a more positive prognosis for GBM patients.

**Discussion**

GBM is a deadly disease because of its aggressive growth, invasion of surrounding tissues, and poor immunogenicity. Here we show that knockout of FGL2 in tumor cells induces adaptive immune responses, resulting in 100% tumor suppression in GL261, DBT, and LLC brain tumor model systems. This adaptive antitumor immune response is characterized by increased differentiation of innate tumor antigen-presenting CD103\(^+\)/CD8a\(^+\) DCs, which subsequently primes and induces tumor-specific CD8\(^+\) cytotoxic T cells. This induced adaptive CD8\(^+\) T cell response was also present in the periphery in the form of tumor-specific memory cells, although the FGL2KO tumor cells were implanted directly in the brain. These unexpected results suggest that FGL2 in tumor cells could serve as a potential therapeutic target for treating brain tumors and preventing tumor relapse. Supporting this conclusion, our previously published data showed that blocking FGL2 prolonged survival and reduced immune checkpoint CD39 and PD-1 expression and infiltration of CD39\(^+\) Tregs and M2-type macrophages in the brain. The current study puts forth the novel premise that tumor-elaborated FGL2 has a critical role in regulating CNS APCs.

APCs express major histocompatibility class II (MHCII) molecules and include DCs, macrophages, monocytes, B lymphocytes, and microglia in the brain. Microglia and macrophages are the first innate immune cells that are recruited to brain tumors, but they express low levels of MHCII molecules. Moreover, monocyte-derived macrophages in GBM assume an undifferentiated M0 state. Monocytes exposed to malignant glioma cells adopt an M0 macrophage phenotype. Therefore, both GBM-associated microglia/macrophages...
It has been challenging to define the optimal DC population that may have an impact on clinical outcome. Analyses of tumour-bearing brains by us and others have shown that the infiltrating CD45+CD11b+ population includes CD11b+ conventional DCs (cDCs), monocytes, monocyte-derived DCs induced by inflammation (moDCs), and macrophages; CD45+CD11c+CD11b– cells include plasmacytoid DCs (pDCs) and CD11c+ cDCs; and CD45+CD11b+CD11c– MHCII+ cells comprise B cells primarily. Among the three major categories of DCs in brain tumors, pDCs are poor APCs because of low levels of MHCII expression, but they are beneficial in enhancing cross-priming when activated38,39; moDCs exhibit less T cell priming functions than cDCs, yet they accounted for 30% of the total DC population and thus may influence the adaptive antitumor immune response40,41; and cDCs are highly effective professional APCs located in the meninges and peritumor area41. However, the specific subpopulation of cDCs that induce tumor-specific CD8+ T cells has not been defined until now. DCs exist as phenotypically and functionally distinct subpopulations within tissues, distinguished by characteristic patterns of surface marker expression29,42. Mounting evidence from peripheral tumors shows that Batf3-dependent cDCs are the most critical DCs in cross-presentation of tumor cell-associated antigens and priming tumor-specific T cell immunity in both mice and humans18–20,22,43. Indeed, tumor-isolated CD103+ DCs, which were induced in the presence of Batf3, have a markedly higher efficiency in cross-presenting tumor antigens than other DC subsets, macrophages, monocytes, and neutrophils19. Intratumoral CD103+ DCs traffic to TDLNs by expressing CCR7 to prime naive CD8+ T cells, causing tumor-specific CD8+ T cells to increase in number and infiltrate tumors5. Tumor-residing CD103+ DCs are necessary for CD8+ effector T cell recruitment by producing CXCL9/10 chemokines18. Moreover, CD103+ DCs, after activation, produce high levels of Th1-differentiating cytokines, such as IL-12, to provide essential signals for generation of memory CD8+ T cells19,22. Thus, CD103+ DCs have a crucial role in the initial antigen presentation and priming, effector, and memory phases required to induce an antitumor adaptive T cell response.

CD103+ cDCs have been characterized in peripheral tumors but not previously in brain tumors. Our findings in this study show the presence of both CD103+ and CD8α+ DC populations in brains and TDLNs of brain tumor-bearing mice, and total DC populations with higher proportions of CD103+CD8α+ cells have activated OT-I T cells in vitro and induced OT-I T cell proliferation in vivo, showing that CD103+/CD8α+ DC populations in the brain have roles in antigen-presenting and cross-priming effects, as observed in peripheral tumors.

Batf3 is a key transcription factor in regulation of CD103+/CD8α+ DC differentiation29. Indeed, Batf3−/− mice showed impaired rejection of FGL2KO tumors because of significantly reduced CD103+/CD8α+ DCs in the brain and TDLNs, showing impairment in initiating the CD8+ T cell response against tumor cells in the absence of Batf3. Supporting our results are findings by Quitana et al.44 that, although CD45+CD11c+CD11b+ileClec9A+ DC populations (Clec9A is specifically expressed by CD8α+/CD103+ DCs in mice) were increased in noninjured brains when treated with FLT3L, this effect was abolished in the brains of Batf3+/− mice. Therefore, the Batf3-dependent CD103+ DC population in the brain is critical for CD8+ T cell-dependent elimination of brain tumors.

Both GM-CSF and Flt3L are essential growth factors for DC development in vitro and in vivo. Culture of bone marrow cells with FLT3L in vitro yields cDCs and pDCs45. Flt3L treatment in vivo promoted the differentiation of CD103+ DC progenitors in the bone marrow and their expansion at the tumor site by enforcing IRF8 signaling, resulting in accumulation of immature CD103+ DCs and accumulation of Treg cells at the tumor site. Thus, Flt3L on its own has limited ability to improve antitumor immune responses in vivo22. GM-CSF promotes the differentiation of myeloid cells and has an important role in differentiation, proliferation, and activation of DCs46. Culture of bone marrow cells with GM-CSF yields macrophages and DCs47. GM-CSF is essential for tumor DC development. GM-CSF receptor-depleted bone marrow cells failed to reconstitute cDCs in tumors when transferred to tumor-bearing mice19. Knockout of GM-CSF in mice yields strong reduction of tissue-resident CD103+ DCs and migratory CD103+ DCs19. Likewise, absence of the GM-CSF receptor on lung DCs abrogated the induction of CD8+ T cell immunity after immunization with particulate antigens48.

The direct linkage of GM-CSF expression and CD103+ DC generation suggests that a GM-CSF vaccine should be effective in achieving an antitumor response. However, after over two decades of effort, clinical trials of different types of GM-CSF cancer vaccines have had mixed results49–51, and most human clinical trials have been disappointing52–54. Our study suggests that one possible reason for this is the presence of FGL2 in tumor cells, which inhibits CD103+ DC differentiation and has not been previously accounted for. Our results clearly show that FGL2 expressed by tumor cells reduces CD103+ DC differentiation. Therefore, future GM-CSF-based cancer vaccines may require the use of FGL2 depletion or blockade or patient stratification based on the absence of tumor-expressed FGL2. Of course, this view requires further validation because we have tested only GBM cells and a lung cancer cell line thus far. However, knockout or depletion of FGL2 may not only increase GM-CSF-mediated CD103+ DC differentiation but probably also reduces MDSC and TAM levels at the same time. Indeed, our previous data show that overexpression of FGL2 increased MDSC and M2 cell levels in GBM8. This increase in MDSCs and M2s may be another factor that negatively affects the development of a GM-CSF vaccine. Supporting this view, others have found that the GM-CSF-mediated bone marrow response is biphasic, with a low level reducing MDSC and macrophage numbers and activity and a high level having the opposite effect55,56.

It is well known that regulation of DC differentiation by GM-CSF depends on the activation and signaling of PI3K/PKB, JAK/STAT, MAPK, and canonical NF-κB. GM-CSF induces proliferation and prevents cell death, mainly by activating PCK/PKB and MAPK signaling, and induces differentiation and inflammatory responses by MAPK, NF-κB, and STAT5 signaling56. However, the signaling pathways underlying the induction of CD103+ cDC differentiation by GM-CSF are unclear. STAT5, Batf3, and IRF8 are known to facilitate GM-CSF-induced CD103+ DC development57,58, but no other signaling pathways are clearly linked yet. Here we show that GM-CSF induces TAK1/NF-κB, p38, and JAK2/STAT5/STAT1 signal transduction in order to induce CD103 expression. Adding to these novel discoveries is the crucial finding that the presence of subtle FGL2 levels from tumor cells can significantly reduce the intensity of these CD103+ DC-inducing signals. However, PI3K/AKT and ERK signaling did not participate in suppression of GM-CSF signaling by FGL2. Thus, TAK1/NF-κB signaling, JAK2/STAT1/
5 signaling, and p38 signaling are three pathways regulated by

**Methods**

**Human samples.** Sample collection was conducted under protocol #LAMB03-0687, which was approved by the Institutional Review Board of The University of Texas MD Anderson Cancer Center, after written informed consent was obtained. Patients’ tumors were graded by a neuropathologist according to the World Health Organization classification^18^.

**Rescued tumor samples from newly diagnosed primary GBM patients were used in this study.**

**Animals.** C57BL/6, BALB/c, CD4.5 (B6-SJL-Ptpcr^−^Pepck^−^Boyll/B6), 2004.1 OT-1 (C57BL/6-Tg(TcraTcr)110MjIfj), 003811, NRG (Nod.Cg-Prkdc^csd^Il2rg^−^Tg(CAG.WPRE-tdTomato)22^Staj^), 005575), CD11c-DTR (B6-FVB-Tg(Thy1.2-DTR)7Rki/Fgals1-TsR Tup/Gpp/CD2d2^Staj^), 004509, and B6.Fas^−^/− (B6.SJL-Cg-Kit^−/−^Ifngr1^−/−^Jcl^−/−^Ut^−/−^/)), 013755) mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). FGL2^−/−^ mice were a gift from Dr. Gary Levy^39^ (Toronto General Hospital/Research Institute, Toronto, ON, Canada). The RCAS/Env−a system was used to generate CD8a^−/−^ mice^111^.

**Bone marrow dendritic cell culture.** Femurs and tibiae from C57BL/6 mice were harvested under sterile conditions. Bone marrow was collected by flushing. The red blood cells were removed by lysis. Bone marrow cells (2 × 10^6^/mL) were cultured in RPMI1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS; Biochrom, Hellston, MA, USA), penicillin/streptomycin, 1 mM sodium pyruvate, and 50 μM β-mercaptoethanol. Conditioned medium (CM) produced by FGL2KO or control tumor cells was added at a volume ratio of 1:1. On day 5, equal volumes of pre-warmed fresh medium and CM were added, and cultures continued for another 4 days. Non-adherent cells in the supernatants were re-plated with fresh medium supplemented with 50 μg/mL hIL37L (Peprotech, Rocky Hill, NJ, USA) and CM for 6 more days. Bone marrow DCs were collected at day 5 or 15 for analyzing the expression of CD103, Clec9A, and CD25/γδ+ cell population^22^.

**FGL2 knockout.** Mouse FGL2 guide RNA (gRNA) sequences GCTGCCGCACTGCAAGGATG and GTGCTCCTCCACCGCTCGGC were cloned into the px458 vector for CRISPR-associated protein 9 and gRNA expression (provided by Dr. Feng Zhang and colleagues at the Massachusetts Institute of Technology, Cambridge, MA, USA). Mouse glial GL261 cells, mouse astrocytoma-derived D1T cells, and lung cancer LLC cells were transfected with both guide plasmids by electroporation. The cells were grown for 2 weeks, and green fluorescent protein (GFP)-positive cells were isolated from cultures of each cell type and seeded at 1 cell per well to get single-cell clones. The knockdown/knockout of FGL2 expression in clonal cells was confirmed by western blotting and sequencing of PCR fragment cloning of stable clones. The scramble control gRNA sequence was GCGTCGCGGACCGCTGCGCC.

**Mouse model systems.** For the orthotopic GBM mouse models, GL261 cells, both controls (GL261-CD63) and FGL2 knockout (GL261-FGL2KO), D1T cells (D1T-CD63), FGL2KO (FGL2KO-CD63), LLC cells (LLC-CD63), and LLC-FGL2KO (LLC-FGL2KO-CD63) were treated with mycoplasma removal agent and collected in logarithmic growth phase, washed twice with phosphate-buffered saline solution (PBS), and mixed with an equal volume of 3% methylene blue dye in PBS. Cells (5 × 10^4, 2.5 × 10^5, or 1 × 10^5 in total of 5 μL) were injected intracerebrally into the mice^61^.

**For in vitro cross-presentation assay, CM-cultured DCs were collected and resuspended at 2.5 × 10^6/mL with 100 μg/mL soluble OVA protein to pre-incubate for 2 h. After two washes, 5 × 10^5 CFSE-labeled OT-I cells were added to the culture for 24 h. The OT-I cells were collected and analyzed for CD69 expression by flow cytometry.**

**OT-I killing assay.** OT-I cells were isolated from the spleen and lymph nodes of OT-I mice and purified with a Mojosort Mouse CD8^+^ T cell Isolation Kit (485008; BioLegend). D1T cells isolated from FGL2KO-OVA or Ctrl-OVA tumor-bearing mice with an EasySep Mouse Pan DC Enrichment Kit Immunomagnetic negative selection cell isolation kit (17653; StemCell Technologies, Vancouver, BC, Canada). Isolated DCs (5 × 10^6) were co-cultured with naive CFSE-labeled OT-I cells (5 μM CFSE at 5 min) at a 2:1 ratio in 96-well round-bottom tissue culture plates^67_. After 24 h of co-culture, the OT-I cells were collected and analyzed for CD25 expression and CFSE dilution in OT-I cells^67_.

**In vitro cytotoxic T lymphocyte assay.** Target GL261 tumor cells were labeled with 1.5 μM CFSE (2 × 10^6) for 10 min. Effector CD8^+^ T cells were isolated from tumor-bearing mice. Target cells and effector CD8^+^ T cells were cocultured at ratios of 1:5, 1:20, and 1:80 for 6 h. CFSE^−^/propidium iodide^+^ positive tumor cells were detected by flow cytometry.

**For the OT-I killing assay, naive OT-I cells were pre-activated with 2 μg/mL ovalbumin peptide SINFEKL (OVA257-264) for 24 h. Tumor cells (10^6 cells/mL) were incubated with calcine AM in a final concentration of 2 μM for 30 min. Target cells and effector CD8^+^ T cells were cocultured at ratios of 1:1, 1:3, 1:12, and 1:80 for 6 h. The supernatants were collected and fluorescence was quantified at excitation filter 485 nm and emission filter 530 nm.**

**CD4, CD8, and NK cell depletion.** Anti-CD8a (clone 2.43), anti-NK1.1 (clone PK136), anti-CD4 (clone GK1.5) antibody, or their matched IgG isotype antibody was injected intraperitoneally in a dose of 200 μg per mouse, 2 days before and every 4 days thereafter. These antibodies were purchased from BioXCell (West Lebanon, NH, USA).
CD8+ T-cell survival assay. Naive CD8+ T cells (1 x 10^6/mL) were activated with plate-bound CD3 and CD28 antibodies for 2 days. Recombinant murine IL-2 (500 U/mL) was added, and the cells were cultured for 5 more days. Cells were collected, washed, and plated in CM from GL261-Cont or GL261-FGL2KO tumor cells and devoid of IL-2 and cultured for 5 more days. The cells were then stained with PI to isolate surviving cells.

Migration assay. OT-1 CD8+ T cells in suspension were stained with 2.5 µM Cell Tracker Blue CMV Qdot 655 Dye (C10994, Invitrogen) for 30 min. CM from tumor cells (0.75 mL of medium containing 5% FBS) were placed in the lower wells of a chamber assay plate, and OT-1 cells (2.5 x 10^5 cells in 0.1 mL of medium containing 1% FBS) were placed in the upper wells of the insert. After co-incubation for 1, 2, 4, or 6 h, cells were collected from the bottom wells and subjected to flow cytometry to identify BMQC cells.

Flow cytometry. Patient tumor tissues were minced and enzymatically digested to obtain single-cell suspensions for staining. Myelin debris was removed by using Myelin Isolation Beads (130-104-262; Miltenyi Biotec, Bergisch Gladbach, Germany). Mouse brain tissues and tumor-draining lymph nodes (TDLN)s were minced and enzymatically digested to obtain single-cell suspensions. Brain-infiltrating leukocytes were isolated according to a previously published protocol.

Briefly, each single-cell suspension was subjected to centrifugation through a 30% Percoll gradient at 7800 x g for 30 min. The leukocyte layer was collected and subjected to centrifugation on a discontinuous Ficoll-Paque Plus gradient to select and purify leukocytes. Fc receptors were blocked using anti-CD16/32 (2.4G2, BD Biosciences, Franklin Lakes, NJ, USA). The antibodies were purchased and assayed, eBioscience (Thermo Fisher Scientific, Waltham, MA, USA), BD Biosciences, CA, USA. The antibodies were purchased and assayed, BioLegend, CA, USA. The antibodies were purchased and assayed, BD Biosciences, CA, USA. The antibodies were purchased and assayed, BD Biosciences, CA, USA.

Western blotting. To quantify expression of specific proteins, cells were subjected to lysis in RIPA buffer (50 mM Tris-HCl, pH 7.4; 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA) supplemented with 50 mM NaF, 20 mM β-glycerophosphate, and a complete protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN, USA). Protein concentrations were determined using the bicinchoninic acid reagent (Thermo Fisher Scientific). Proteins were separated by sodium dodecyl sulfate 10% polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. Membranes were incubated overnight at 4°C with primary antibodies. After washing, the blots were incubated with the appropriate horseradish peroxidase-conjugated secondary antibody and then incubated in chemiluminescence signals. The anti-FGAL2 antibody was then applied for immunoblotting (monoclonal anti-FGAL2 antibody produced in mouse, 1:1000). The antibodies were purchased from Cell Signaling Technology (Danvers, MA) or Santa Cruz Biotechnology (Dallas, TX); dTRAPEF (D21G3), p-TPK1 (Ser21), CTAK1 (D21G7), p-IRAK1 (D4A3), p-ERK1/2 (Thr202/Tyr204), p- p38 (Thr180/Tyr182), p-AKT (Ser473), and p-GAPDH (D16H11). ImageJ software was used to quantify bands. GAPDH was used as a control, and results were quantified by normalizing the band intensity of protein to GAPDH. Relative quantities are summarized as ratio changes from at least two independent experiments.

Immunohistochemistry and immunofluorescence. Mouse brains were formalin-fixed and paraffin-embedded, and 4-µm sections were cut for immunohistochemistry (H&E staining). For immunofluorescence, sequential staining was used. First, GEM tissue sections were stained with mouse anti-FGAL2 antibody (1:100, H0900187S; Novus Biologicals, Littleton, CO, USA) overnight at 4°C, followed by 1 h incubation with anti-mouse Alexa Fluor 647-conjugated antibody. The sections were rinsed in wash buffer, and Alexa Fluor 647-conjugated secondary antibody was used for immunofluorescence signals. The anti-FGAL2 antibody was then applied for immunofluorescence (monoclonal anti-FGAL2 antibody produced in mouse, 1:1000). The antibodies were purchased from Cell Signaling Technology (Danvers, MA) or Santa Cruz Biotechnology (Dallas, TX); dTRAPEF (D21G3), p-TPK1 (Ser21), CTAK1 (D21G7), p-IRAK1 (D4A3), p-ERK1/2 (Thr202/Tyr204), p- p38 (Thr180/Tyr182), p-AKT (Ser473), and p-GAPDH (D16H11). ImageJ software was used to quantify bands. GAPDH was used as a control, and results were quantified by normalizing the band intensity of protein to GAPDH. Relative quantities are summarized as ratio changes from at least three independent experiments.

The expression levels of GM-CSF and FLT3L in the CM were determined by using ELISA kits, the Mouse GM-CSF Quantikine ELISA Kit (MGM80b; R&D Systems) and the Mouse FLT-3 ligand PicoKine ELISA Kit (EK0355; Boster Biological Technology, Pleasanton, CA, USA), according to the manufacturer’s instructions.

The Cancer Genome Atlas data analysis. The Cancer Genome Atlas (TCGA) mRNA expression data were accessed via the cbioPortal for the Cancer Genomics Web site [http://www.cbioportal.org/public-portal/]. The provisional mRNA expression z-scores (microarray) for FGL2, GM-CSF, CD8β, and IFNG genes with matched outcome data (OS) were used. The z-score (positive or negative) represents a normalized expression level relative to non-neoplastic tissue control. By recognized practice, the median gene expression z-score of the complete cohort (401 patients) was utilized as a cutoff value for classification of patients into high and low risk groups for each individual gene of interest, including FGL2 (cutoff value 0.33), GM-CSF (cutoff value ~0.22), CD8β (cutoff value 0.01), and IFNG (cutoff value ~0.24). According to the cutoff value of each gene, the patients with expression z-scores higher than the median were classified into the “high-expression” group (denoted as FGL2high, GM-CSFhigh, CD8βhigh, and IFNGhigh), and the patients with expression z-scores less than the median were classified into the “low-expression” group (denoted as FGL2low, GM-CSFlow, CD8βlow, and IFNGlow). A univariate Cox proportional hazards model was used to assess the association between gene expression levels (both individual genes and combined genes) and OS. The hazard ratio (HR) and 95% confidence interval (CI) were calculated and reported. Pearson correlation coefficients were calculated between CD8β, GM-CSF, IFNG, and FGL2 gene expressions. The statistical analyses were conducted using R package software (R Development Core Team, Version 3.3.2). A P value < 0.05 was considered statistically significant. Data were accessed from the cbioPortal [http://www.cbioportal.org/public-portal/] between January 1, 2018, and March 15, 2018.

Statistical analysis. All quantified data are presented as mean ± standard deviation (s.d.) as indicated. In the experimental means for flow cytometric and cytokine values were considered significant if P was < 0.05 as determined by one-way analysis of variance (ANOVA) or t-test. A two-way ANOVA was used to analyze tumor volume differences between groups. Normality of data was assessed using the Shapiro–Wilk test of normality. Assumption for homogeneity of variances was assessed using the Levene’s test. For the data for which the underlying assumptions were not met, we removed outliers, used a test accounting for unequal variances (e.g., Welch’s t-test, Welch’s ANOVA), or used a non-parametric test such as the Wilcoxon rank-sum test. Tukey’s multiple comparison test was used for pairwise comparisons in the ANOVA analysis. Differences in survival curves were analyzed by Kaplan–Meier analysis and the log-rank test, and differences were considered significant at P < 0.05. All data shown are representative of three independent experiments acquired in triplicate (in vitro) or at least two independent experiments (in vivo). Unless otherwise noted, statistical tests were two-sided and statistical analyses were conducted using GraphPad Prism 7 software (GraphPad Software, La Jolla, CA, USA) and R package software (R Development Core Team, Version 3.3.2).

Reporting summary. Further information on experimental design is available in the Nature Research Reporting Summary linked to this article.

Data availability. The authors declare that all data supporting the findings of this study are available within the paper and its Supplementary Information files or from the corresponding author upon reasonable request. The TCGA data referenced during the study are available in a public repository from the cbioPortal website [https://www.cbioportal.org/public-portal/].

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Author contributions

S.L. and A.B.H. initiated the original idea for this work, J.Y. and S.L. planned the experiments, J.Y., Q.Z., L.-Y.K., X.X., J.X., and L.H. performed the experiments. K.G. and M.O. collected clinical samples. J.Y. collected data and performed analyses. J.W. analyzed TCGA data. J.Y., A.B.H., and S.L. prepared the manuscript draft with input from all authors. S.S.W., S.C.S., R.E.D., and G.R. provided optimized protocol and experiment materials. All authors provided critical feedback and helped shape the research, analysis, and manuscript.

Additional information

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