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Myeloid-derived suppressor cell subsets drive glioblastoma growth in a sex-specific manner

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Abstract

Myeloid-derived suppressor cells (MDSCs) that block anti-tumor immunity are elevated in glioblastoma (GBM) patient blood and tumors. However, the distinct contributions of monocytic (mMDSC) versus granulocytic (gMDSC) subsets have yet to be determined. In mouse models of GBM, we observed that mMDSCs were enriched in the male tumors, while gMDSCs were elevated in blood of females. Depletion of gMDSCs extended survival only in female mice. Using

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Contributions

D.B., D.J.S., A.L., G.R., D.C.W., A.L., T.J.A. and B.O. carried out in vitro and in vivo animal experiments. Y.Z., C.P., C.H., D.V., A.M.K., T.H.H. and F.C. analyzed the sequencing data and performed bioinformatics analysis. D.B., M.M. and M.G. obtained and/or analyzed human data. M.S. and B.W.K. provided immunofluorescence analyses of human tissue. C.H. provided immunohistochemistry analyses of human tissue. M.A.V. C.H. B.W.K. A.M.K., T.H.H., M.S.A., F.C. and J.D.L. supervised the study. D.B. and J.D.L. conceptualized the study and wrote the manuscript. All authors read, revised, and approved the final manuscript.

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gene expression signatures coupled with network medicine analysis, we demonstrated in pre-clinical models that mMDSCs could be targeted with anti-proliferative agents in males, whereas gMDSC function could be inhibited by IL-1 β blockade in females. Analysis of patient data confirmed that proliferating mMDSCs were predominant in male tumors and that a high gMDSC/IL-1 β gene signature correlated with poor prognosis of female patients. These findings demonstrate that MDSC subsets differentially drive immune suppression in a sex-specific manner and can be leveraged for therapeutic intervention in GBM.

Keywords

glioblastoma; myeloid-derived suppressor cells; sex differences; immunotherapy

Introduction

Glioblastoma (GBM) is the most common primary malignant brain tumor with a median survival of 20 months post-diagnosis(1). Epidemiological studies further point to a 1.6-fold higher incidence among men, suggesting a male-dominant sexual dimorphism in GBM(2). Additionally, male patients have a worse prognosis than females, underscoring the clinical relevance of studying biological sex in GBM(3). This sex bias in part is dictated by tumor cell-intrinsic mechanisms. In particular, genome-wide association studies (GWAS) led to identification of distinct risk variants associated with GBM in males versus female patients(2). Moreover, differences in tumor mutational profile driving oncogenic transformation and cellular metabolism were linked to the differences observed in GBM manifestation, outcome and susceptibility to treatment(4–7). In mouse models of pediatric low-grade glioma, differential activation status of tumor-infiltrating myeloid cells and altered cytokine profile were shown to drive female dominance(8). However, the contribution of tumor-extrinsic host factors to sexual dimorphism in GBM is yet to be investigated. Notably, sex differences in host immunity, regulated by the combination of sex chromosomes, hormones and environmental factors, have been linked to efficacy of vaccine response, susceptibility to autoimmune diseases and protection against infectious agents(9). Overall a more active immune response in females, which is driven by a number of innate and adaptive elements, including increased type I interferon signaling, pro-inflammatory cytokine production and T cell activation, is a major factor leading to female predisposition to autoimmune diseases(9). In the context of tumor immunity, this could translate to more robust immunosurveillance and is thus likely to be an important contributor to the increased cancer mortality in males. On the other hand, studies in patients treated with immune checkpoint inhibitors suggested that males might benefit more from these treatment modalities compared to females(10). Nevertheless, it is unclear how sex-dependent immunological variation affects tumorigenesis or differential immunotherapy response.

Myeloid cells are a major component of the GBM microenvironment and serve as a therapeutic target to reverse the immunosuppression that drives tumor progression(11). Correspondingly, myeloid-derived suppressor cells (MDSCs) are more abundant in the peripheral circulation of patients with GBM compared to those with low-grade central nervous system (CNS) tumors, and increased tumor infiltration of MDSCs associates with

poor GBM outcome(12–15). Initially recognized for their ability to suppress anti-tumor immune response, MDSCs are a heterogeneous population of bone marrow-derived immature myeloid cells subclassified into monocytic (mMDSC) and granulocytic (gMDSC) subsets. While both MDSC subsets hinder the activity of T and natural killer (NK) cells, mMDSCs and gMDSCs can undertake additional roles in the maintenance of primary tumors and promotion of metastasis, respectively(16,17). Despite growing evidence implicating the distinct roles of MDSC subsets in cancer progression, little is known about their differential activity in GBM. Thus, we focused on the sex-specific roles of MDSC subsets as an underlying factor contributing to sexual dimorphism in GBM progression. Our data demonstrate that mMDSCs localize to the tumor microenvironment and support GBM progression in males. In contrast, systemic gMDSC accumulation is the dominant factor regulating the anti-tumor immune response in females. We further show that unique characteristics of MDSC subsets determine their susceptibility to distinct drug candidates, whose therapeutic efficacy was governed by the sex of the host.

Results

mMDSCs primarily localize to GBM tumors, while gMDSCs accumulate in the peripheral circulation in a sex-dependent manner

To determine the dynamics of MDSC subset accumulation and sex-dependent immunological changes in GBM, we orthotopically implanted GL261 or SB28 cells into immunocompetent male and female C57BL/6 mice via intracranial injection (Fig. 1A). Analysis of the immune profile of the tumor-bearing hemisphere with flow cytometry (Supplementary Fig. 1A) indicated an overall increase in CD45+ cells in both male and female mice 14 days after implantation with SB28 and 21 days after implantation with GL261 cells compared to the contralateral hemisphere and sham-injected controls (Fig. 1B, Supplementary Fig. 1B). Specifically, there was an overall increase in the frequency of mMDSCs with a concomitant decrease in the percentage of gMDSCs in the tumor microenvironment (Fig. 1C, Supplementary Fig. 1C), skewing the mMDSC:gMDSC ratio compared to sham-injected controls (Fig. 1D–E) as a result of accumulation of mMDSCs in the tumor tissue (Supplementary Fig. 1D–E). Based on recent studies demonstrating sex differences in GBM incidence rates and prognosis(2,3), we separately assessed MDSC subsets in male and female tumor models and found that mMDSCs accumulated particularly in male tumors leading to a ~5.5–6.5 fold increase in the mMDSC/gMDSC ratio, while no significant change was observed in females (Fig. 1F–H, Supplementary Fig. 1F–H). We also analyzed the peripheral immune response as it has been demonstrated that systemic immunity also plays a critical role in immunotherapy response(18). gMDSCs were the predominant population in the blood of sham-injected and tumor-bearing animals (Fig. 1I, Supplementary Fig. 1I). In only female mice, there was a 2-fold increase in the peripheral gMDSC frequency post-tumor implantation that was consistent across both GBM models, while mMDSCs remained unchanged in the blood of these animals (Fig. 1J–K, Supplementary Fig. 1J–K). To determine whether immunological sex differences were limited to MDSCs, we assessed other immune cell populations, including macrophages, dendritic cells, natural killer cells and T lymphocytes from tumors and peripheral blood of GL261 and SB28-bearing animals compared to matched sham-injected controls. The

abundance of other tumor-infiltrating immune cell populations was not significantly altered between male and female tumor-bearing mice (Supplementary Fig. 2A–L). Furthermore, peripheral differences were limited to the higher frequency of overall T cells and CD4⁺ T cells in female mice compared to male GL261-bearing mice (Supplementary Fig. 3A–L). The observed sexual dimorphism in immune profile of the two mouse GBM models associated with survival differences. As recently reported for patients with GBM(3), female mice experienced longer survival compared to male mice (Supplementary Fig. 4A–B). To further evaluate the immunological basis of this survival difference, we performed bone marrow transplantation with female hosts 4 weeks prior to the implantation of GL261 or SB28 tumors and demonstrated that donor marrows effectively reconstituted the host, generating both MDSC subsets (Supplementary Fig. 4C–D). Under these conditions, female hosts reconstituted with male donor bone marrow had decreased survival compared to female-to-female transplant controls (Supplementary Fig. 4E–F), demonstrating that the male immune system has a more tumor-promoting role. Collectively, these findings suggest that sex differences in the regulation of anti-tumor immunity can be driven by the distinct MDSC subset abundance and compartmentalization in GBM, with males having enhanced mMDSC accumulation in the tumor microenvironment, and females having elevated gMDSCs in the peripheral circulation.

gMDSC depletion provides survival benefit to females, while tumor-infiltrating mMDSCs could not be depleted effectively

Given these differences in MDSC subset accumulation pattern, we sought to determine the functional contribution of MDSC subsets by utilizing neutralizing antibodies for peripheral depletion. We established GL261 tumors in male and female mice as previously described and then intraperitoneally injected anti-Gr-1, anti-Ly6G or anti-Ly6C antibodies every other day starting 7 days post-tumor implantation (Fig. 2A). Bulk MDSC depletion with anti-Gr-1 antibody resulted in a survival extension that was exclusive to females (Fig. 2B, C). MDSC subset-specific depletion confirmed that the increase in survival of female mice with GL261 tumors was due to gMDSC depletion with anti-Ly6G antibody (Fig. 2D–E). Similarly, depletion of gMDSCs provided an 8-day survival advantage to only female SB28-implanted mice (Supplementary Fig 5A–B). However, there was no extension of survival with mMDSC targeting in either sex (Fig. 2F–G, Supplementary Fig 5C–D). To confirm the efficacy of this strategy, we assessed the frequency of MDSC subsets in the blood and tumors of GL261-bearing animals at humane endpoints using flow cytometry. While gMDSCs could be peripherally reduced with anti-Ly6G treatment, mMDSCs remained unaltered in the tumor microenvironment despite anti-Ly6C antibody injections (Supplementary Fig. 5E). We hypothesized that the lack of systemic mMDSC reduction was due to mMDSCs proliferating and thus repopulating. We directly assessed this by performing intracellular Ki-67 staining of MDSC subsets from tumor-bearing mice on day 21 and observed that mMDSCs, particularly those from blood, highly expressed Ki-67 compared to gMDSCs regardless of sex of the host (Fig. 2H–I; Supplementary Fig. 5F). These findings established that targeting systemic gMDSCs in females could be a relevant therapeutic target to curtail GBM progression, while strategies aiming to reduce mMDSC abundance by antibody neutralization might be ineffective due to cellular proliferation (Supplementary Fig. 5G).

Distinct biologically active pathways in mMDSCs and gMDSCs determine their drug susceptibility

To gain mechanistic insight into the differential roles of MDSC subsets, we generated mMDSCs and gMDSCs from the bone marrow of C57BL/6 mice by adopting previously described polarization methods involving GM-CSF and IL-4R α stimulation by IL-13(19,20). Both male and female MDSCs generated via this method were functionally suppressive as assessed by their ability to prevent the proliferation of T cells stimulated with anti-CD3/CD28 and IL-2 (Fig. 3A–C; Supplementary Fig. 6A). Expression profiling of these flow-sorted MDSC subsets revealed unique gene signatures between mMDSCs and gMDSCs, which translated to the upregulation of distinct biological pathways (Supplementary Fig. 6B–C). While cell proliferation pathways were active in mMDSCs, immune-modulatory pathways were elevated in gMDSCs (Fig. 3D–E; Supplementary Fig. 6D). To identify putative drug targets for these subsets, we leveraged a network medicine approach that takes advantage of reported drug-target interactions(21,22) using the differentially expressed gene profiles of mMDSCs and gMDSCs. This strategy identified fludarabine, a purine analog, as a potential drug candidate to target mMDSCs (Fig. 3F). Previous studies have demonstrated conflicting results regarding the effect of chemotherapies on MDSCs. Doxorubicin-cyclophosphamide treatment was shown to increase the frequency of MDSCs in breast cancer patients(23,24). In contrast, nucleoside analogs were shown to reduce MDSC frequencies in various tumor types via a mechanism that was not well defined(25–28). Our studies in mouse models and patients with GBM, indicated that 5-fluorouracil (5-FU) and its oral formulation capecitabine could be promising candidates to target innate immunosuppression(29,30), of which fludarabine was predicted to be more effective (Supplementary Fig. 6E). For gMDSC targeting, IL-1 pathway inhibitors were enriched among the top targets (Fig. 3F, Supplementary Fig. 6F). Consistent with the predictions of the network medicine analysis and the observation that gMDSC targeting provides survival benefit to females, we detected ~3-fold higher IL-1 β expression in in vitro polarized gMDSCs of female origin compared to those derived from male bone marrows (Supplementary Fig. 6G). To test the therapeutic utility of these predicted drugs, we assessed their efficacy in pre-clinical models of GL261 and SB28. Fludarabine was intraperitoneally injected for five consecutive days, while anti-IL-1 β antibody was given every other day in two cycles, starting 7 days after tumor implantation (Fig. 4A). We observed that fludarabine significantly extended survival in male mice, with no significant benefit for female mice as their survival duration was not improved (Fig. 4B–C, Supplementary Fig. 7A–B). In contrast, anti-IL-1 β treatment significantly prolonged the survival of female mice (Fig. 4D–E, Supplementary Fig. 7C–D). To identify immunological changes associated with treatment response, we analyzed the blood and tumors post-drug treatment. Given the differences in the tumor growth dynamics between the GBM models, we used SB28 to assess earlier immunological changes after 1 cycle of drug administration, while mice with GL261 were treated for two cycles to analyze the advanced disease scenario. In both GL261 and SB28-bearing male mice, we observe an increase in the frequency of circulating CD8 $^{+}$ T cells with fludarabine treatment, pointing to activation of anti-tumor immune response (Fig. 4F–G). In SB28-bearing male mice, one cycle of fludarabine was sufficient to decrease mMDSC abundance in tumors by 4-fold (Fig. 4H). Although a ~2-fold reduction in mMDSCs was observed in GL261-bearing male mice after

two cycles, the effect of the drug was more limited (Supplementary Fig. 7E). Instead, we observed that tumor proliferation rate was reduced in these animals (Fig. 4I), suggesting that inhibition of mMDSC infiltration into the tumors at an early stage can alter the growth dynamics. A similar increase in peripheral CD8⁺ T cells was also observed in female GL261-bearing animals, but frequency of the proliferating tumor cells or tumor mMDSCs were unaffected by the drug (Supplementary Fig. 7F–H). In contrast, completion of two cycles of anti-IL-1 β reduced systemic gMDSCs specifically in GL261-bearing females (Fig. 4J, Supplementary Fig. 7I). Anti-IL-1 β also delayed the tumor growth in female mice, as early as after completion of the 1st cycle, as evidenced by the reduction in the proliferating cell rates based on Ki-67 expression in non-immune tumor tissue (Fig. 4K–L, Supplementary Fig. 7J). Importantly, anti-IL-1 β had no direct impact on the proliferation of GL261 and SB28 cell *in vitro* (Supplementary Fig. 7K–L), suggesting that it functions by modulating the immune response. These data support that implementation of a sex-specific therapeutic strategy against GBM by targeting MDSC subsets with unique inhibitors to provide a survival advantage.

Male patients have an enhancement in tumor-infiltrating mMDSCs, while a gMDSC signature associates with poor prognosis in female patients

To validate our preclinical observations in patients, we re-analyzed the immune-inhibitory myeloid cell staining pattern in 188 IDH-wild type GBM specimens, while accounting for patient sex(31). Male patients had more IBA1⁺ and CD204⁺ cells in their tumors based on immunofluorescence staining (Fig. 5A), suggesting an increase in the immunosuppressive myeloid cell populations. Analysis of fresh tumor tissue with multicolor flow cytometry from a separate cohort of male patients with IDH-wild type GBM revealed that mMDSCs were present at significantly higher percentages in human GBM tissue compared to gMDSCs (Fig. 5B–D; Supplementary Fig. 8A). Moreover, these tumor-infiltrating mMDSCs were positive for Ki-67, confirming that they are actively proliferating in patient tumors (Fig. 5E–F). To evaluate the prognostic value of gMDSC abundance, we analyzed The Cancer Genome Atlas (TCGA) GBM Database for mRNA levels of OLR1 (LOX-1), a gMDSC-specific marker(32), and IL-1 β and found that there were no sex differences in the expression levels of these markers between male and female patients (Supplementary Fig. 8B, C). Further investigation of IL-1 β protein levels by immunohistochemistry of tumor tissue from patients with IDH-wild type GBM established that this cytokine was abundant in the tumor microenvironment regardless of patient sex (Fig. 6A–B). Notably, the expression levels of OLR1 and IL-1 β were associated with patient survival only when stratified based on sex. In both TCGA and the Chinese Glioma Genome Atlas (CGGA), high OLR1 expression inversely correlated with the survival of females, but had no effect in male patients with GBM (Fig. 6C; Supplementary Fig. 8D). A similar trend was observed with IL-1 β levels; high IL-1 β mRNA portended poor prognosis only in female patients (Fig. 6D; Supplementary Fig. 8E). Importantly, OLR1 expression positively correlated with IL-1 β expression, suggesting that these genes are part of the same signaling network (Fig. 6E). Collectively, these studies indicated that male tumors have enhanced immunosuppressive myeloid cell infiltration, which includes proliferating mMDSCs, while a gMDSC-IL-1 β axis associates with female disease activity and represents a therapeutic target for female patients (Fig. 6F).

Discussion

Immunotherapy options remain limited for GBM, despite the success of these treatment modalities in pre-clinical models and other solid tumors(33–35). The immunosuppressive tumor microenvironment mainly consisting of myeloid cells is one of the major factors limiting the efficacy of existing treatment options(11). Thus, understanding the variations in myeloid cell-driven immunosuppression and the associated molecular mechanisms can provide insight into the sexual dimorphism in GBM outcome. Our results demonstrated that MDSC subset heterogeneity could be leveraged for improved immunotherapy response in a sex-specific manner and highlight the importance of assessment of sex as a biological variable in pre-clinical and clinical immunotherapy studies.

In addition to their suppressive function, MDSC subsets can execute distinct roles during the course of tumorigenesis. It was recently demonstrated in breast cancer models that mMDSCs localize to the primary tumor, where they support cancer stem cells (CSCs), while gMDSCs promote metastatic spread to distant sites(16). Correspondingly, our group previously reported that MDSC recruitment in GBM is driven by CSCs via the secretion of macrophage-migration inhibitory factor (MIF), suggesting a possible cross-talk between these cell populations in multiple cancers(29). However, this study was conducted only in females and did not distinguish between the MDSC subsets. Consistent with these earlier reports, we observed that mMDSCs were the dominant subset localizing to GBM tissue both in mouse models and patients and that the frequency of tumor-infiltrating mMDSCs was significantly higher in males compared to females. We previously reported that increased MDSC accumulation at the time of recurrence predicts poor GBM outcome in patients(13). In line with this observation, higher mMDSC levels in pre-clinical models associated with shortened survival duration, and male mice succumbed to disease earlier than female mice. The lack of additional sex differences in the abundance of other tumor-infiltrating immune populations suggested that males have a more immunosuppressive tumor microenvironment, which is reinforced by mMDSC accumulation. Our bone marrow transplantation studies provided an additional level of evidence that sexual dimorphism in immune response drives survival differences in GBM. Future studies will focus on revealing the mechanisms through which these sex differences emerge.

In contrast to the tissue-dominant localization of mMDSCs, we observed that gMDSCs poorly infiltrated into the tumors in pre-clinical models and patients with GBM. It is noteworthy that gMDSCs constitute the main subpopulation elevated in the circulating blood of patients with GBM(12,14,15), an observation recapitulated in murine models. Despite the observation that systemic gMDSC levels were not altered in tumor-bearing male mice compared to the sham-injected controls, we observed that males had overall higher levels of circulating MDSCs at baseline compared to females. In the context of lupus, a higher MDSC frequency was previously linked to the resistance of male (NZB × NZW)F1 mice(36), further suggesting that the differences in systemic MDSC abundance could provide a mechanistic explanation for the increased immunosuppression in males. These findings are supported by the epidemiological evidence indicating that males are predisposed to malignancies as opposed to inflammatory diseases, which are more prevalent in females(9). However, in the context of GBM prognosis, there was a significant systemic expansion of

gMDSCs in female mice implanted with GL261 or SB28 cells. Consistent with earlier reports, depletion of gMDSCs by neutralizing antibody treatment reduced the peripheral gMDSCs without affecting the tumor-infiltrating population in these animals(37). Extension in the survival span of tumor-bearing female animals under these experimental conditions further highlights the importance of the systemic immune response in anti-tumor immunity in females(18) and establishes that gMDSC expansion in the peripheral blood might serve as a potential therapeutic target.

We also observed MDSC subsets had unique gene expression signatures and biologically active pathways that could account for the differences in their frequency and compartmentalization. Importantly, along with the modulation of immune response, the top cellular pathways upregulated in mMDSCs were related to cell cycle regulation. Although low-dose chemotherapies (5-FU, capecitabine and gemcitabine) were used to deplete MDSCs in pre-clinical models and patients with various cancers, including GBM, the mechanism by which these drugs have shown efficiency remained unknown(27,29,30). Here, we demonstrate that mMDSCs are actively proliferating in vivo and propose that the inhibition of cellular proliferation emerges as a likely explanation for the selective targeting of systemic mMDSCs by chemotherapies, thereby reducing their tissue levels(25). In contrast, gMDSCs were predicted to be more effective modulators of immune response based on pathway analysis. We leveraged these distinct gene expression signatures associated with this function to identify drug candidates that could be repurposed for cancer immunotherapy. Although IL-1 is a prototypical pro-inflammatory cytokine, recent studies suggest that IL-1 β can drive tumorigenesis by promoting a CSC phenotype, modulating the immune response and inducing tumorigenic edema(38–41). Importantly, randomized clinical testing of canakinumab, an anti-IL-1 β antibody, pointed to a reduction in lung cancer incidence of patients with atherosclerosis(42). We observed that IL-1 β was significantly expressed in the gMDSC subset as opposed to the mMDSC subset, with particular elevation in female gMDSCs. Consistently, blockade of IL-1 β significantly extended the survival of female mice by delaying tumor growth and subsequently reducing gMDSC frequencies in the circulation. It is conceivable that gMDSCs are not the only cell type that produces IL-1 β at high levels, and tumor-associated macrophages/microglia comprise additional sources of IL-1 β (8,41). Although this line of research warrants further investigation, a lack of sex-specific response to anti-CSF-1R antibodies and an absence of changes in macrophage abundance in mice treated with anti-IL-1 β point to a gMDSC-dominant role in females and suggest that MDSCs are the likely regulators of the sexual dimorphism in anti-GBM immune response (Supplementary Fig. 9A–C).

In summary, our findings identify a differential MDSC subset signature and candidate mechanisms that could comprise a therapeutic opportunity for improved immunotherapy of GBM by accounting for patient sex. These results implicate that blockade of mMDSC proliferation with chemotherapies can reprogram the immunosuppressive tumor microenvironment in males. Finally, our study unveils the role of a gMDSC-IL-1 β axis in systemic GBM immunity in females and further provide the rationale for clinical testing of IL-1 β inhibitors in patients with GBM.

Materials and Methods

Reagents

Fluorophore-conjugated anti-Ly6C (HK1.4, 128024), anti-Ly6G (1A8, 27618 or from BD Biosciences 551460), anti-CD11b (M1/70, 101212), anti-CD68 (FA-11, 137024), anti-I-A/I-E (M5/114.15.2, 107606), anti-CD11c (N418, 117330), anti-CD3 (145-2C11, 100330), anti-CD4 (GK1.5, 100422), anti-CD8 (53-6.7, 100712), anti-NK1.1 (PK136, 108741), anti-F4/80 (BM8, 123118), anti-Ki-67 (16A8, 652404 or 652428), anti-CD45 (30-F11, 103132), anti-CD45.1 (A20, 110728), and anti-CD45.2 (104, 109808) antibodies were obtained from Biolegend (San Diego, CA) for analysis of mouse immune profiles.

*In Vivo*MAb anti-mouse Ly6C (Monts1, BE0203), *In Vivo*MAb anti-mouse Ly6G (1A8, BE0075-1), *In Vivo*MAb anti-mouse Gr-1 (RB6-8C5, BE0075), *In Vivo*MAb anti-mouse anti-CSF1R (AFS98, BE0213), *In Vivo*MAb anti-mouse/rat IL-1 β (B122, BE0246), *In Vivo*MAb rat IgG2a, anti-trinitrophenol (2A3, BE0089), *In Vivo*MAb rat IgGb, anti-keyhole limpet hemocyanin (LTF-2, BE0090), and *In Vivo*MAb polyclonal Armenian hamster IgG (BE0091) were purchased from BioXCell (West Lebanon, NH) for depletion/targeting of MDSCs.

Fludarabine (Sagent Pharmaceuticals, Schaumburg, IL), Sulfatrim (Pharmaceutical Associates Inc., Greenville, SC), isoflurane (Piramal Critical Care Inc., Bethlehem, PA), xylazine (Akorn Inc., Lake Forest, IL) and ketamine (Zoetis Inc., Kalamazoo, MI) were obtained from the Department of Pharmacy, Cleveland Clinic.

For analysis of human GBM immune profile, anti-CD3 (SP34-2, 557757, BD Biosciences), anti-CD11b (ICRF44, 557743, BD Biosciences), anti-CD33 (WM53, 562492, BD Biosciences), anti-CD66b (G10F5, 561650, BD Biosciences), anti-CD14 (M5E2, 558121, BD Biosciences), anti-HLA-DR (L243, 307638, Biolegend), anti-CD68 (Y1/82A, 333814, Biolegend), anti-LOX-1 (15C4, 358606, Biolegend), anti-CD45 (HI30, 560777, BD Biosciences), anti-Ki67 (B56, 563755, BD Biosciences) and BV711 Mouse IgG1, k isotype control (X40, 563044, BD Biosciences) antibodies were used.

Cell lines

The GL261 cell line was obtained from the Developmental Therapeutics Program, National Cancer Institute. SB28 cells were gifted by Dr. Hideho Okada (University of California San Francisco). Both cell lines were karyotyped upon receipt and determined to be de-sexualized, consistent with previous reports(43). All cell lines were treated with 1:100 MycoRemoval Agent (MP Biomedicals, Solon, OH) upon thawing, and routinely tested for *Mycoplasma spp.* (Lonza, Walkersville, MD). Cells were maintained in RPMI 1640 (Media Preparation Core, Cleveland Clinic) supplemented with 10% FBS (ThermoFisher Scientific, Waltham, MA) and 1 % Pen/Strep (Media Preparation Core). Cells were not grown for more than 10 passages.

For proliferation, 500 cells GL261 and SB28 cells were cultured in white-walled 96-well plates and treated with 10 μ l/ml anti-IL-1 β or isotype control. Proliferation was measured over the course of 5 days with CellTiter-Glo.

Mice

All experiments were approved by the Cleveland Clinic Institutional Animal Care and Use Committee and performed in accordance with the established guidelines. Four-week-old C57BL/6 (JAX Stock #000664) or B6 CD45.1 (B6.SJL-Ptprc^a Pepc^b/BoyJ; JAX Stock #002014) mice were purchased from the Jackson Laboratory as required and housed in the Cleveland Clinic Biological Research Unit Facility under a 12 h:12 h light/dark cycle.

Four- to eight-week-old C57BL/6 mice were intracranially injected with 10,000–25,000 GL261 cells or 5,000–20,000 SB28 cells in 5 μ l RPMI null media into the left hemisphere 2 mm caudal to the coronal suture, 3 mm lateral to the sagittal suture at a 90° angle with the murine skull to a depth of 2.5 mm. A fraction of age- and sex-matched animals were injected with 5 μ l RPMI null media to be used as sham controls. Mice were monitored daily for neurological symptoms, lethargy and hunched posture that would qualify as signs of tumor burden.

Seven days post-tumor implantation, mice were randomly assigned to control or treatment groups. For the depletion studies, mice were intraperitoneally injected with 10mg/kg neutralizing antibodies or isotype control in 100 μ l PBS every other day until experimental endpoint, not more than 20 times. For targeting of IL-1 β , mice were intraperitoneally injected with 10mg/kg targeting antibody or isotype control three times a week for two cycles. Fludarabine (50 mg/kg) was administered intraperitoneally daily with a regimen of 5 days treatment and 2 days break for two consecutive cycles.

For immune profiling experiments, GL261-bearing mice were treated with anti-IL-1 β , isotype control, fludarabine or vehicle for two cycles as described above. The mice were euthanized within 72 hours of treatment completion and only asymptomatic animals that were not at humane endpoints were included for analysis. Samples from vehicle and drug treated mice were processed simultaneously. The treatment of SB28-implanted animals was started 3 days after the implantation of tumors. Male mice were injected with fludarabine or vehicle for 5 consecutive days, and mice were euthanized the day after the completion of the treatment regimen. Female mice were administered anti-IL-1 β or isotype every other day following the same experimental design. Tissue harvesting and flow cytometry were performed as detailed below.

Murine tissue harvest

At pre-defined time points or humane endpoints, tumor-bearing or sham-injected mice were euthanized and samples were processed concurrently. Blood was collected directly into EDTA-coated Safe-T-Fill® micro capillary blood collection tubes (RAM Scientific, Nashville, TN) via cardiac puncture. Tubes were centrifuged at 1000 *g* for 10 minutes to isolate serum, and cell pellets were stained for flow cytometry. Femurs and tibia were flushed with 10ml of PBS for isolation of bone marrow cells. Single-cell suspensions were prepared from organs by mincing in 10% RPMI, strained on a 40 μ m filter (FisherBrand), washed with PBS, counted and stained for flow cytometry analysis.

Flow cytometry

Harvested mouse tissue and blood were transferred into 96-well round-bottom plates (ThermoFisher Scientific) and washed twice with 200 μ l PBS. Samples were stained with LIVE/DEAD Fixable Stains (ThermoFisher Scientific) diluted 1:1000 for 10 minutes on ice. Following a wash step, cells were resuspended in FcR Blocking Reagent (Miltenyi Biotec) at a 1:25 dilution in PBS/2% BSA (Sigma-Aldrich, St. Louis, MO) for 10 minutes on ice. Fluorophore-conjugated antibodies diluted 1:50 were added at a 1:2 ratio, and cells were further incubated for 20 minutes on ice. Samples were washed with PBS/BSA and fixed overnight in eBioscience™ Fc γ R3/Transcription Factor Fixation Buffer. For intracellular staining, antibodies were diluted at a ratio of 1:100 in Fc γ R3/Transcription Factor Permeabilization Buffer, and cells were incubated at room temperature for 20 minutes. Samples were acquired with a BD LSR Fortessa (BD Biosciences), and FlowJo (Version 10.5.0, FlowJo LLC, Ashland, OR) was used for analysis of the staining data. Mouse MDSC subsets were phenotypically defined based on established guidelines(44).

Bone marrow transplantation

Four-week-old C57BL/6 female mice were subjected to whole-body irradiation. Radiation (12 Gy) was given in two fractions 3–4 hours apart. Reconstitution was achieved by retro-orbital injection of 2×10^6 bone marrow cells from B6 CD45.1 mice. Drinking water was supplemented with Sulfatrim during the first 10 days, and mice were monitored for an additional 3 weeks for weight loss and infection symptoms before tumor implantation. Survival analysis was performed as described above.

MDSC co-culture

Bone marrow was isolated from the femur and tibia of 8–12-week-old mice. Two million bone marrow cells were co-cultured with 1×10^5 GL261 cells in 6-well plates in 2 ml RPMI/10% FBS supplemented with 40 ng/ml GM-CSF and 80 ng/ml IL-13 (PeproTech, Rocky Hill, NJ) for 3–4 days. For validation experiments, bone marrow cells were incubated with the cytokine combination alone. Cells were stained for viability, blocked with Fc receptor inhibitor and stained with a combination of CD11b, Ly6C and Ly6G for sorting of MDSC subsets (mMDSCs: CD11b⁺Ly6C⁺Ly6G⁻ versus gMDSCs: CD11b⁺Ly6C⁻Ly6G⁺) and control (CD11b⁺Ly6C⁻Ly6G⁻) population using a BD FACS Aria II (BD Biosciences).

T cell proliferation assay

T cells were isolated from splenocytes of 8–12-week-old mice using a Pan T Cell Isolation Kit II (Miltenyi Biotec) with >90% purity. T cells were stained with 1 μ M carboxyfluorescein succinimidyl ester (CFSE, Biolegend) for 5 minutes at 37°C and washed with ice cold 10% RPMI twice. A total of 100,000 T cells were co-cultured with 50,000 sorted myeloid cells in the presence of 30 IU recombinant hIL-2 plus anti-CD3/CD28 Dynabeads (ThermoFisher Scientific) for 3–4 days in round-bottom plates. Samples were stained with anti-CD11b and anti-CD3. CFSE dilution was analyzed from CD3⁺CD11b⁻ cells using a BD LSR Fortessa.

RNA Sequencing

RNA was isolated from a minimum of 2×10^6 sorted MDSCs or nonMDSCs using an RNeasy Mini Kit (Qiagen, Germantown, MD). RNA sequencing was performed by GENEWIZ (South Plainfield, NJ) using an Illumina HiSeq, 2×150 bp configuration and 350 M raw paired-end reads. An average 40.8 M paired-end reads were sequenced across 18 samples. After Illumina universal adapters were trimmed, the average read length was shortened to 136 bp, and 40.6 M reads were kept for the downstream analysis. The RNA-seq reads were converted to transcriptome abundant matrix in the format of TPM (transcripts per kilobase million)(45) via kallisto v0.44.0(46) in default parameters. The kallisto reference genome sequence index was built on a mouse reference transcript sequences (GRCm38.p5) with the gene model annotation, gencode.vM16.annotation.gtf. The differential gene expression analysis was conducted with DESeq2 v1.18.1(47) in a default setting as suggested. Then, we selected genes for which the absolute log2 fold change value was higher than or equal to 1 and Benjamini and Hochberg FDR (false discovery rate) < 0.001 and queried them into the DAVID website for a functional enrichment analysis(48). The biological processes in both the Gene Ontology and KEGG pathway (P-value < 0.001) were used for functional annotation. GEO accession number: [GSE148467](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE148467)

Network Medicine Analysis

Gene sets for network analysis were generated by determining the overlapping differentially expressed genes between MDSC subsets and for each MDSC subset, in comparison to the nonMDSC fraction from three samples. Reads were quantified using Salmon (v0.9.1)(49) Quasi-Mapping with an index created from ENSEMBL reference non-coding RNA and cDNA transcriptomes (GRCm38 release 95). Differential expression was calculated using DESeq2 (v1.20.0)(47) after collapsing transcript level quantifications to gene level quantifications. Secondary elimination was done for mMDSCs based on read count > 100 and for gMDSCs using the top 1500 highly expressed genes based on average read count. The full gene list is provided in Supplementary Table 1. Lineage markers and macrophage and dendritic cell markers were used as negative controls. Positive controls were determined based on previously published profiles(16,17).

We collected physical drug-target interactions as described in our previous studies(21,22). We used the human interactome including 351,444 unique protein-protein interactions (edges or links) connecting 17,706 proteins (nodes)(21). The network proximity of the drug targets and differentially expressed genes was computed using the closest method based on the human interactome:

$$d_{AB} = \frac{1}{\|A\| + \|B\|} \left(\sum_{a \in A} \min_{b \in B} d(a, b) + \sum_{b \in B} \min_{a \in A} d(a, b) \right)$$

where $d(a, b)$ is the shortest path between gene a and b from gene list A and B , respectively. The proximity was then converted to Z-score using the following method:

$$Z_{dAB} = \frac{d_{AB} - \overline{d_r}}{\sigma_r}$$

where $\overline{d_r}$ and σ_r are the mean and standard deviation of a permutation test repeated 1,000 times, each time using two randomly selected gene lists that have similar degree distributions to the differentially expressed genes and drug targets. A cutoff of -1 was used. Drugs were ranked based on their Z-scores. Networks were visualized using Cytoscape v3.7.1 (<https://cytoscape.org/>).

Quantitative reverse-transcriptase PCR

RNA from nonMDSC, mMDSC and gMDSC fractions was isolated using an RNeasy mini kit, and cDNA was synthesized with qSCRIPT cDNA Super-mix (Quanta Biosciences). qPCR reactions were performed using an Applied Biosystems™ StepOnePlus™ Real-Time PCR System and Fast SYBR-Green Mastermix (ThermoFisher Scientific). For qPCR analysis, the threshold cycle (CT) values for IL-1 β were normalized to the expression levels of CycloA by nonMDSCs. The following primers (Integrated DNA Technologies) were used:

IL-1 β :	Forward: 5'-ACGGACCCCAAAAGATGAAG-3'
	Reverse: 5'-TTCTCCACAGCCACAATGAG-3'
CycloA:	Forward: 5'-GCGGCAGGTCCATCTACG-3'
	Reverse: 5'-GCCATCCAGCCATTGAGTC-3'

Immunofluorescence

Specimens from 188 patients with a diagnosis of primary isocitrate dehydrogenase (IDH)-wild type GBM were stained with ionized calcium-binding adaptor molecule-1 (IBA-1) and cluster of differentiation 204 (CD204)(31). The published dataset was reanalyzed by accounting for patient sex, and the IBA-1 and CD204 staining intensities were graphed separately for male versus female patients.

Flow analysis of GBM tumors

IDH-wild type GBM specimens were collected by the Rose Ella Burkhardt Brain Tumor and Neuro-Oncology Center in accordance with the Institutional Review Board (IRB2559) of Cleveland Clinic. Patient demographic information is provided in Table 1. Tumors were cut into small pieces with a razor blade and incubated with collagenase IV (STEMCELL Technologies, Kent, WA) on a rotator at 37°C for 1 hour. Cells were strained over a 40- μ m filter and further minced with a plunger to obtain single cell suspensions. Samples were washed with 30 ml PBS twice and treated with RBC Lysis Buffer (Biolegend). Samples were stained with LIVE/DEAD Fixable Stains for 10 minutes on ice and incubated with FcR Blocking Reagent for 15 minutes on ice. Staining with fluorophore-conjugated antibodies was performed in Brilliant Stain Buffer (BD Biosciences) for 20 minutes on ice. Cells were fixed overnight in eBioscience™ Foxp3/Transcription Factor Fixation Buffer. Isotype and Ki-67 staining was performed in eBioscience™ Foxp3/Transcription Factor Permeabilization Buffer with 20 minutes of incubation at room temperature. Samples were

acquired with a BD LSR Fortessa. Human MDSC subsets were phenotypically defined based on established guidelines(44).

Immunohistochemistry

All samples were collected and processed at the Northwestern University Feinberg School of Medicine in accordance with approved IRB guidelines. Specimens from 10 male and 10 female patients diagnosed with IDH-wild type primary GBM were stained with anti-IL-1 β antibody (Clone ab156791, Abcam, Cambridge, MA) diluted 1:250. Patient demographic information and molecular characteristics of the tumors are provided in Table 1.

To quantify IL-1 β staining, slides were scanned with a Leica SCN400 (Leica, Buffalo Grove, IL) at 40x magnification. Four random 5000 $\times$ 5000 micron sections were extracted from each tissue with Aperio ImageScope (Leica). The number of IL-1 β -positive cells per visual field was counted with Fiji-ImageJ software and normalized to the total number of cells (<https://imagej.net/Fiji>).

Bioinformatic Analysis

The Cancer Genome Atlas (TCGA) GBM dataset was accessed via <https://xenabrowser.net/heatmap/> on 6/24/2019 and 8/5/2019 for extraction of patient sex, overall survival, and IL-1 β and OLR1 (LOX-1) expression level. Survival duration was graphed for patients with highest (1st quartile) versus lowest (4th quartile) IL-1 β and OLR1 expression. Correlation between IL-1 β and OLR1 expression was determined using the GlioVis database (<https://gliovis.shinyapps.io/GlioVis/>)(50). The Chinese Glioma Genome Atlas (CGGA) was accessed via <http://www.cgga.org.cn/> on 1/29/2020 for extraction of patient sex, overall survival, and IL-1 β and OLR1 (LOX-1) expression level information from patients with IDH-wild type primary GBM. Survival duration was graphed for patients separated based on median expression values.

Statistical analysis

GraphPad PRISM (Version 6, GraphPad Software Inc., San Diego, CA) software was used for data presentation and statistical analysis. Unpaired t-test, paired t-test, and two-way ANOVA were used for comparison of differences among sample groups. The Gehan–Breslow–Wilcoxon test was used to analyze survival data. The specific statistical method employed for individual data sets is listed in the figure legends.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Statement of Significance

Sexual dimorphism at the level of MDSC subset prevalence, localization and gene expression profile comprises a therapeutic opportunity. Our results indicate that chemotherapy can be used to target mMDSCs in males, while IL-1 pathway inhibitors can provide benefit to females via inhibition of gMDSCs.

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Figure 1. mMDSCs accumulate in the tumors of male mice, while gMDSCs accumulate systemically in female mice. **A**, Timeline of immune infiltration analysis. **B**, Frequency of CD45^{high} bone marrow-derived immune cells in resected tumors versus the contralateral hemisphere of sham-injected or 25,000 GL261-implanted mice on day 14 and day 21. Data shown as mean $\pm$ s.d. of $n = 10$ /group from one of independently repeated experiments. * $p < 0.05$, ** $p < 0.01$ as determined by two-way ANOVA. **C**, Percentage of mMDSCs (CD11b⁺CD68⁻Ly6C⁺Ly6G⁻I-A/I-E⁻) and gMDSCs (CD11b⁺CD68⁻Ly6C⁻Ly6G⁺) in CD45⁺ cells of the left hemisphere from $n = 17$ sham-injected and $n = 26$ GL261-bearing animals. Data shown for individual animals, and ** $p < 0.01$ as calculated by unpaired t-test. **D**, Ratio of mMDSCs to gMDSCs in the left hemisphere from $n = 16$ sham-injected and $n = 26$ GL261-bearing animals 21 days post tumor-implantation or sham injection. Data shown for individual mice combined from three independent experiments and *** $p < 0.001$ as calculated by unpaired t-test.

test. **E**, Ratio of mMDSC-to-gMDSC in the left hemisphere from $n = 15$ sham-injected and $n = 19$ SB28-bearing animals, 14 days post tumor-implantation or sham injection. Data shown for individual mice combined from two independent experiments, and *** $p < 0.001$ as calculated by unpaired t-test. **F**, Percentage of mMDSCs in the $CD45^+$ immune cells infiltrating the left hemisphere 21 days post-GL261 implantation or sham injection. Data shown as mean $\pm$ s.d. from three independent experiments. $n = 8-9$ sham-injected and $n = 13$ GL261-bearing mice per sex, and * $p < 0.05$ as determined by unpaired t-test. **G**, Percentage of gMDSCs in the $CD45^+$ immune cells infiltrating the left hemisphere 21 days post-GL261 implantation or sham injection. Data shown as mean $\pm$ s.d. from three independent experiments. $n = 8-9$ sham-injected and $n = 13$ GL261-bearing mice per sex. **H**, Ratio of mMDSCs to gMDSCs in the left hemisphere of sham-injected or tumor-bearing mice 21 days after the procedure. Data shown as mean $\pm$ s.d. from three independent experiments. $n = 8-9$ sham-injected and $n = 13$ GL261-bearing mice per sex, and ** $p < 0.01$ as calculated by unpaired t-test. **I**, Frequency of mMDSCs and gMDSCs as a fraction of $CD45^+$ cells in the systemic circulation of sham-injected or GL261-bearing mice. Data shown for individual mice combined from three independent experiments. $n = 16$ sham-injected and $n = 26$ GL261-bearing mice, and *** $p < 0.001$ as determined by unpaired t-test. **J**, Percentage of mMDSCs in the circulation of sham-injected and GL261-bearing mice on day 21. Data shown as mean $\pm$ s.d. from three independent experiments. $n = 9$ sham-injected and $n = 13$ GL261-bearing mice per sex and * $p < 0.05$ as determined by unpaired t-test. **K**, Percentage of gMDSCs in the circulation of sham-injected and GL261-bearing mice on Day 21. Data shown as mean $\pm$ s.d. from three independent experiments. $n = 9$ sham-injected and $n = 13$ GL261-bearing mice per sex and * $p < 0.05$ as determined by unpaired t-test.

Figure 2. gMDSCs depletion extends the survival span of female mice. **A**, Schematics of MDSC depletion regimen. **B-G**, Kaplan-Meier curves depicting survival of female and male mice treated with (**B-C**) anti-Gr-1, (**D-E**) anti-Ly6G, and (**F-G**) anti-Ly6C neutralizing antibodies every other day starting 7 days post-GL261 implantation with respect to the isotype-treated mice. For **B**, $n = 9$ for isotype- and $n = 13$ for anti-Gr-1-treated female mice; for **C**, $n = 9$ for isotype- and $n = 11$ for anti-Gr-1-treated male mice; for **D**, $n = 13$ for isotype- and $n = 14$ for anti-Ly6G-treated female mice; for **E**, $n = 10$ for isotype- and $n = 10$ for anti-Ly6G-treated male mice; for **F**, $n = 9$ for isotype- and $n = 9$ for anti-Ly6C-treated female mice; for **G**, $n = 9$ for isotype- and $n = 10$ for anti-Ly6C-treated male mice. Data combined from two-to-three independent experiments. Significance was determined by Gehan-Breslow-Wilcoxon test with $p < 0.05$ being considered a significant difference. **H**, Representative histograms depicting ex vivo intracellular Ki-67 staining of mMDSCs and gMDSCs from blood and

tumor. **I**, Quantification of Ki-67 staining as mean fluorescence intensity from $n = 4$ (2 male and 2 female) animals euthanized on day 21 post-GL261 implantation. Data corrected for background based on fluorescence-minus-one staining and shown as mean $\pm$ s.d. ***
 $p < 0.001$ as determined by unpaired Student's t-test.

Figure 3.

Proliferation of mMDSCs can be targeted by fludarabine, while IL-1 pathway inhibition is predicted to counteract gMDSC-mediated immunosuppression. **A**, Schematics of in vitro MDSC polarization approach. Bone marrow cells from C57BL/6 mice were co-cultured with GL261 cells in the presence of GM-CSF/IL-13 for 3 days. **B**, mMDSC, gMDSC and nonMDSC populations were phenotypically discriminated based on CD11b, Ly6C and Ly6G expression. **C**, Proliferation rates of activated T cells co-cultured with nonMDSCs, gMDSCs and mMDSCs generated by cytokine polarization, compared to unstimulated T cells. Data analyzed separately for $n = 3$ male and female mice and shown as mean $\pm$ s.d. *** $p < 0.001$ as determined by two-way ANOVA. **D-E**, GeneOntology Enrichment Analysis using differentially expressed genes between (**D**) mMDSCs versus nonMDSCs and (**E**) gMDSCs versus nonMDSCs from $n = 6$ biological replicates based on $\log(\text{fold change}) - 1$ and adjusted p -value < 0.001 . **F**, Potential mechanism-of-action of fludarabine and rilonacept by

network inference. Differentially expressed genes of mMDSCs for fludarabine and gMDSCs for rilonacept were directly connected to the drug targets and/or through one or more common neighbors. For fludarabine, a maximum distance of 2 between drug targets and differentially expressed genes was used to visualize the network. For rilonacept, the distance was set to 4. Node size indicates $\log_2FC$, and interaction types are color coded.

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Figure 4. MDSC subset targeting with predicted drugs confers sex-specific survival advantage. **A**, Treatment regimen for testing the predicted drug candidates fludarabine and anti-IL-1 β neutralizing antibody. Kaplan-Meier curves depicting survival of **B**, male and **C**, female GL261-bearing mice treated with fludarabine. Data presented from two independent experiments with **(B)** $n = 10$ vehicle-treated males and $n = 10$ fludarabine-treated males and **(C)** $n = 10$ vehicle-treated females and $n = 10$ fludarabine-treated females. $p < 0.01$ as determined by Gehan–Breslow–Wilcoxon test. Kaplan-Meier curves depicting survival of **D**, male and **E**, female GL261-bearing mice treated with anti-IL-1 β neutralizing or isotype control antibody. Data presented from three independent experiments with **(D)** $n = 15$ isotype control-treated males and $n = 15$ anti-IL-1 β antibody-treated males, **(E)** $n = 15$ isotype control-treated females and $n = 15$ anti-IL-1 β antibody-treated females. $p < 0.05$ as determined by Gehan–Breslow–Wilcoxon test. **F**, Frequency of CD8+ T cells in the

circulation of $n = 5$ vehicle- and $n = 4$ fludarabine-treated SB28-bearing male mice. Data shown as mean $\pm$ s.d., and *** $p < 0.001$ as calculated by unpaired t-test. **G**, Frequency of CD8+ T cells in the circulation of $n = 8$ vehicle- and $n = 7$ fludarabine-treated GL261-bearing male mice. Data shown from two independent experiments as mean $\pm$ s.d., and *** $p < 0.001$ as calculated by unpaired t-test. **H**, Relative percentage of tumor-infiltrating mMDSCs as a fraction of live cells in $n = 5$ vehicle and $n = 5$ fludarabine-treated SB28-bearing male mice after one cycle. Data shown as mean $\pm$ s.d. and * $p < 0.05$ as calculated by unpaired t-test. **I**, Relative percentage of Ki-67+ cells in the nonimmune cells of the left hemisphere from $n = 8$ vehicle- and $n = 7$ fludarabine-treated GL261-bearing male mice after two cycles. Data shown from two independent experiments as mean $\pm$ s.d. **J**, Frequency of gMDSCs in the circulation of $n = 8$ isotype- and $n = 8$ anti-IL-1 β -treated GL261-bearing female mice. Data shown as mean $\pm$ s.d. from two independent experiments and * $p < 0.05$ as calculated by unpaired t-test. **K**, Relative percentage of Ki-67+ cells in the nonimmune cells of the left hemisphere from $n = 5$ isotype- and $n = 5$ anti-IL-1 β -treated SB28-bearing female mice after one cycle. Data shown as mean $\pm$ s.d., and ** $p < 0.01$ as calculated by unpaired t-test. **L**, Relative percentage of Ki-67+ cells in the nonimmune cells of the left hemisphere from $n = 8$ isotype- and $n = 8$ anti-IL-1 β -treated GL261-bearing female mice after two cycles. Data shown from two independent experiments as mean $\pm$ s.d. and * $p < 0.05$ as calculated by unpaired t-test.

Figure 5.

IDH-wild type GBM tumors from male patients have more immunosuppressive myeloid cells, which include proliferating mMDSCs. **A**, Fraction of IBA1⁺ and CD204⁺ area of $n = 188$ patients with IDH-wild type GBM from Sorensen et al., *Neuropathology and Applied Neurobiology*, 2018, was re-analyzed by accounting for biological sex. Data shown from $n = 108$ males and $n = 80$ females as their median. ** $p < 0.01$ as determined by unpaired t-test. **B**, Percentage of CD45^{high} cells of the viable single cells isolated from GBM tumors. Data shown for $n = 8$ individual patients. **C**, The frequency of mMDSCs (CD11b⁺CD33⁺CD14⁺HLA-DR⁻CD68⁻) and gMDSCs (CD11b⁺CD33⁺CD66b⁺LOX-1⁺) in tumor-infiltrating leukocytes from $n = 8$ male patients with IDH-wild type GBM. Data shown as mean $\pm$ s.d. * $p < 0.05$ as determined by unpaired Student's t-test. **D**, Ratio of mMDSCs to gMDSCs in tumors of $n = 8$ patients with IDH-wild type GBM. **E**, Representative histograms showing Ki-67 expression levels in matched mMDSCs and gMDSCs from the

same patient, in comparison to the isotype control staining. **F**, Mean fluorescence intensity of Ki-67 in tumor-infiltrating mMDSCs and gMDSCs from $n = 8$ patients with IDH-wild type GBM. Data shown as mean $\pm$ s.d. * $p < 0.05$ as determined by unpaired Student's t-test.

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Figure 6. gMDSC prevalence predicts poor prognosis of female patients with GBM. **A**, Representative IL-1 β immunohistochemistry tumor sections from male and female patients confirming IL-1 β protein expression in IDH-wild type GBM. **B**, Quantification of IL-1 β + cells in tumors from four visual fields of n = 10 male and n = 10 female patients with IDH-wild type GBM. **C**, Correlation among OLR1 expression levels, patient sex and survival duration was analyzed via TCGA GBM dataset. High and low expression levels were determined based on quartiles. Data shown for n = 14 female patients with high OLR1 expression and n = 16 female patients with low OLR1 expression (left) and for n = 29 male patients with high OLR1 expression and n = 25 male patients with low OLR1 expression (right). p<0.01 as determined by Gehan–Breslow–Wilcoxon test. **D**, Correlation among IL-1 β expression levels, patient sex and survival duration was analyzed via TCGA GBM dataset. High and low expression levels were determined based on quartiles. Data shown for n = 15 female

patients with high IL-1 β expression and n = 14 female patients with low IL-1 β expression (left) and for n = 37 male patients with high IL-1 β expression and n = 27 male patients with low IL-1 β expression (right). p<0.05 as determined by Gehan–Breslow–Wilcoxon test. **E**, Correlation between OLR1 and IL-1 β expression levels from n = 538 patients. p<0.01 based on two-sided t-test. **F**, Proposed model of relative mMDSC abundance and IL-1 β presence in patients with GBM.

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Table 1:

Patient demographic information. Sex, age, race, ethnicity, diagnosis, recurrence, IDH1/ATRX expression, p53/Ki-67 staining and MGMT status of patient specimens used to assess MDSC accumulation (CCF cohort) and IL-1 β expression (NU cohort).

CCF ID	Sex	Age	Race	Ethnicity	Final Diagnosis	New diagnosis vs. recurrent diagnosis	IDH1 Status	ATRX Expression	P53 Stain	Ki-67 Stain	MGMT Promotor Methylation
Patient 1	Male	38	White	Non-hispanic	GBM	New	Wild Type	Retained	5%	70%	unmethylated
Patient 2	Male	44	White	Non-hispanic	GBM	New	Wild Type	Retained	5%	70%	methylated
Patient 3	Male	60	White	Non-hispanic	GBM	Recurrent	Wild Type	Retained	>80%	> 50%	unmethylated
Patient 4	Male	72	White	Non-hispanic	GBM	New	Wild Type	Retained	80%	60%	unmethylated
Patient 5	Male	67	White	Non-hispanic	GBM	Recurrent	Wild Type	Retained	10%	30%	unmethylated
Patient 6	Male	63	White	Non-hispanic	GBM	New	Wild Type	Retained	20%	40%	unmethylated
Patient 7	Male	63	White	N/A	GBM	New	Wild Type	Retained	10%	50%	unmethylated
Patient 8	Male	59	White	Non-hispanic	GBM	Recurrent	Wild Type	Retained	>90%	60%	unmethylated
NU ID	Sex	Age	Race	Ethnicity	Final Diagnosis	New diagnosis vs. recurrent diagnosis	IDH1 Status	ATRX Expression	P53 Stain	Ki-67 Stain	MGMT Promotor Methylation
Patient 1	Female	74	White	Non-hispanic	GBM	New	Wild Type		-	25%	unmethylated
Patient 2	Female	85	White	Non-hispanic	GBM	New	Wild Type				unmethylated
Patient 3	Female	59	White	Non-hispanic	GBM	New	Wild Type	Retained	10%	70%	unmethylated
Patient 4	Female	67	White	Non-hispanic	GBM	New	Wild Type	Retained		20%	unmethylated
Patient 5	Female	74	White	Non-hispanic	GBM	New	Wild Type				unmethylated
Patient 6	Female	53	White	Non-hispanic	GBM	New	Wild Type	Retained			unmethylated
Patient 7	Female	81	White	Non-hispanic	GBM	New	Wild Type	Retained	5-7%	50%	unmethylated
Patient 8	Female	68	White	Non-hispanic	GBM	New	Wild Type				unmethylated
Patient 9	Female	30	White	Non-hispanic	GBM	New	Wild Type	Retained	10%	40%	unmethylated
Patient 10	Female	63	White	Non-hispanic	GBM	New	Wild Type				unmethylated
Patient 11	Male	69	White	Non-hispanic	GBM	New	Wild Type			25%-30%	unmethylated
Patient 12	Male	56	White	Non-hispanic	GBM	New	Wild Type	Retained	<10%	40%	unmethylated
Patient 13	Male	63	White	Non-hispanic	GBM	New	Wild Type			30%	unmethylated
Patient 14	Male	77	White	Non-hispanic	GBM	New	Wild Type				unmethylated
Patient 15	Male	53	White	Non-hispanic	GBM	New	Wild Type				unmethylated

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Patient 16	Male	62	White	Non-hispanic	GBM	New	Wild Type	Retained	70%	40%	unmethylated
Patient 17	Male	72	White	Non-hispanic	GBM	New	Wild Type				unmethylated
Patient 18	Male	80	White	Non-hispanic	GBM	New	Wild Type	Retained	<5%	40%	unmethylated
Patient 19	Male	61	White	Non-hispanic	GBM	New	Wild Type	Retained	5%	25%	unmethylated
Patient 20	Male	54	White	Non-hispanic	GBM	New	Wild Type	Retained	10%	20%	unmethylated