Glioblastoma-Derived IL6 Induces Immunosuppressive Peripheral Myeloid Cell PD-L1 and Promotes Tumor Growth

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Abstract

Purpose: Upregulation of programmed death-ligand 1 (PD-L1) on circulating and tumor-infiltrating myeloid cells is a critical component of GBM-mediated immunosuppression that has been associated with diminished response to vaccine immunotherapy and poor survival. Although GBM-derived soluble factors have been implicated in myeloid PD-L1 expression, the identity of such factors has remained unknown. This study aimed to identify factors responsible for myeloid PD-L1 upregulation as potential targets for immune modulation.

Experimental Design: Conditioned media from patient-derived GBM explant cell cultures was assessed for cytokine expression and utilized to stimulate naïve myeloid cells. Myeloid PD-L1 induction was quantified by flow cytometry. Candidate cytokines correlated with PD-L1 induction were evaluated in tumor sections and plasma for relationships with survival and myeloid PD-L1 expression. The role of identified cytokines on immunosuppression and survival was investigated in vivo utilizing immunocompetent C57BL/6 mice bearing syngeneic GL261 and CT-2A tumors.

Results: GBM-derived IL6 was identified as a cytokine that is necessary and sufficient for myeloid PD-L1 induction in GBM through a STAT3-dependent mechanism. Inhibition of IL6 signaling in orthotopic murine glioma models was associated with reduced myeloid PD-L1 expression, diminished tumor growth, and increased survival. The therapeutic benefit of anti-IL6 therapy proved to be CD8+ T-cell dependent, and the antitumor activity was additive with that provided by programmed death-1 (PD-1)-targeted immunotherapy.

Conclusions: Our findings suggest that disruption of IL6 signaling in GBM reduces local and systemic myeloid-driven immunosuppression and enhances immune-mediated antitumor responses against GBM.

Introduction

Glioblastoma (GBM) is the most prevalent primary central nervous system malignancy diagnosed in adults (1). In spite of surgical (2), chemoradiotherapeutic (3), and antiangiogenic (4, 5) treatment, the median overall survival for patients receiving the standard of care remains poor at approximately 15–16 months (6, 7). Immunotherapy has emerged as a promising approach for cancer, focused on generating durable, tumor-specific immune responses (8, 9). Yet, while immunotherapy has achieved unprecedented success in malignancies such as melanoma (10–12) and non–small cell lung cancer (11, 13, 14), its efficacy in GBM has been limited.

One factor limiting the success of GBM immunotherapy is tumor-mediated immunosuppression. While GBM employs multiple mechanisms to suppress antitumor immune responses, including expression of immune checkpoint molecules (15–18), release of anti-inflammatory cytokines (19), and expansion of regulatory T cells (20, 21), it has become apparent that myeloid-driven immunosuppression (22–25) is a significant contributor to the lack of immunotherapeutic success in GBM. Myeloid cells extensively infiltrate tumors and represent the most common nonmalignant cell within the GBM microenvironment (26, 27). Immunosuppressive myeloid cells in GBM include alternatively activated (M2) macrophages (28–30), myeloid-derived suppressor cells (MDSC; refs. 23, 24, 31), and immature monocytes (22, 25, 32). Multiple mechanisms of myeloid-promoted immunosuppression have been characterized, including immune checkpoint molecule expression (22, 31), reactive nitrogen and oxygen species release (33), anti-inflammatory
**Translational Relevance**

Patients with glioblastoma (GBM) exhibit profound intratumoral and systemic immunosuppression that reduce the efficacy of immunotherapy. Myeloid cell programmed death-ligand 1 (PD-L1) expression induces the apoptosis and anergy of antitumor T cells that is associated with worse overall survival in patients with GBM treated with vaccine immunotherapy. In this study, we identify GBM-derived IL6 as a significant contributor to myeloid cell PD-L1 induction. Utilizing murine glioma models, we demonstrate that therapeutic blockade of IL6 signaling results in decreased myeloid PD-L1 expression, suppressed tumor growth, and increased survival. Moreover, the therapeutic benefit of anti-IL6 therapy can be combined with programmed death-1 (PD-1)-targeted immunotherapy to further increase survival. As antibodies blocking the IL6 receptor (tocilizumab) and neutralizing soluble IL6 (siltuximab) are clinically available, we believe that further investigation into IL6 inhibition combined with other immunotherapeutic strategies, radiotherapy, and chemotheraphy is warranted to reduce immunosuppression and improve efficacy of interventions for GBM.

**Materials and Methods**

**Cell culture**

Tumor explant cell cultures were generated from patients undergoing resection for GBM. Informed consent was obtained prior to surgery and approved by the Northwestern University Institutional Review Board. All studies were conducted in accordance with recognized ethical guidelines (Declaration of Helsinki, CIOMS, Belmont Report, U.S. Common Rule). Tumor samples were dissociated before collagenase digestion (20 μg/mL, Sigma-Aldrich) to achieve single-cell suspensions. Tumor cells were cultured in RPMI1640 supplemented with 10% FBS, 1% sodium pyruvate, 1% nonessential amino acids, and 1% penicillin-streptomycin (Corning). GL261 cells were obtained from Dr. C. David James (Northwestern University, Chicago, IL) and were cultured under identical conditions. Normal human and mouse astrocytes were obtained from ScienCell and cultured using ScienCell media. Cells were not tested for Mycoplasma prior to use.

**Contextual Relevance**

Cytokine secretion (34), arginase-1 production (35), and indoleamine 2,3-dioxygenase (IDO) activity (36).

Myeloid-based immunosuppression is not limited to the tumor microenvironment, but extends to the systemic circulation of patients with GBM (22, 25). Previously, we demonstrated that circulating myeloid cells in patients with GBM exhibit elevated expression of the immune checkpoint molecule programmed death-ligand 1 (PD-L1), relative to that observed in healthy individuals (22). While normally involved in immune homeostasis, in the setting of malignancy, PD-L1 can interact with its receptor, programmed death-1 (PD-1), expressed on activated T cells, to induce T-cell anergy or apoptosis (37–39). Although PD-L1 is expressed on tumor cells (15, 16), evidence suggests that myeloid PD-L1 may contribute more to the suppression of antitumor T cells (40). We have previously reported that elevated peripheral myeloid PD-L1 was associated with reduced response to vaccine immunotherapy and worse survival in patients with GBM (25). Thus, an increased understanding of the mechanisms driving myeloid PD-L1 upregulation is important to achieve improved outcomes for patients with GBM treated with immunotherapy.

While GBM-derived soluble factors (22, 31) have been shown to upregulate PD-L1 expression on myeloid cells, the identity of specific factors mediating PD-L1 induction remain unknown. Here, we have sought to identify factors responsible for myeloid PD-L1 induction as potential therapeutic targets for reducing myeloid-driven immunosuppression in GBM. To this end, we identified a relationship between GBM-derived IL6 and myeloid PD-L1 that is accessible to therapeutic intervention.

**Conditioned media**

Conditioned media (CM) was collected from 90% confluent cultures after 72 hours of conditioning. To remove cellular debris, CM underwent differential centrifugation prior to concentration (20×, 20 kDa filter, Millipore).

**Cytokine measurement**

Assessment of cytokines present in CM was performed via multiplexed cytokine array (Quantibody 3000, RayBiotech). Quantification of murine IL6 (Abcam) and SAA (Phase) in CM and plasma was accomplished via ELISA. Analysis of murine IL6 (Cell Signaling Technology, D5W4V 1:500) in cell lysates and CM was conducted through Western blot analysis and normalized to GAPDH (Sigma-Aldrich, GA1R 1:10,000). All Western blots were imaged using the Bio-Rad ChemiDoc MP system.

**Myeloid cell stimulation**

Peripheral blood mononuclear cells (PBMC) were isolated from healthy donor blood through Ficoll density gradient separation (GE Healthcare) before CD14+ selection (Stemcell). Myeloid cell stimulation experiments were conducted utilizing RPMI1640 supplemented with 2.5% FBS. Myeloid cells were stimulated with CM (1×), IL6 (1–1,000 μg/mL, R&D Systems), or IL8 (1–1,000 μg/mL, R&D Systems) for 24 hours. Stimulations were also performed in the presence of tocilizumab (1 μg/mL, Genentech), siltuximab (10 μg/mL, Jansen Biotech), or IgG1 isotype control (1–10 μg/mL, QA16A12 BioLegend). Human myeloid cells were stained for CD45 (eBioscience, HI30 HTC), CD11b (Abcam, DCIS1/18 PerCP), CD163 (eBioscience, GHI/61 APC), and PD-L1 (eBioscience, MH11 PE) prior to analysis by flow cytometry (FACSAnCanto II). Murine myeloid cells were isolated from spleens of C57BL/6 mice (7–8 weeks, Jackson Laboratories) through CD11b+ selection (Stemcell). Murine myeloid cells were stimulated with CM or IL6 (1–1,000 μg/mL, Abcam) in the presence of anti-IL6 (10 μg/mL, BioXcell, MP5-20F3) or isotype control (10 μg/mL, BioXcell, HRPN). Murine myeloid cells were stained with CD115 (eBioscience, AF598 AF488) and PD-L1 (BioLegend, 10F.9G2 PE) before flow cytometry analysis (Attune NxT). Representative flow cytometry gating schemes are presented in Supplementary Fig. S1. A list of all antibodies utilized is presented in Supplementary Tables S1 and S2.

**STAT3 phosphorylation and inhibition**

Human myeloid cells were stimulated with GBM CM in the presence of tocilizumab or siltuximab and harvested 20 minutes after exposure to STAT3 phosphorylation (Cell Signaling Technology, Tyr705 3E2 1:1,000) and total STAT3 (Cell Signaling Technology, 7D7 1:2000) via Western blot analysis. Murine
myeloid cells were similarly examined for STAT3 phosphorylation (Cell Signaling Technology, Tyr705 M9C6 1:2,000) and total STAT3 (Cell Signaling Technology, 124H6 1:1,000) by Western blot analysis after stimulation with GL261 CM and treatment with anti-IL6 (10 μg/mL) or anti-IL6R (5 μg/mL, BioXCell, 15A7). In addition, myeloid cells were stimulated with IL6 and CM in the presence of STAT3 (20 μmol/L, EMD Millipore) or DMSO vehicle control (0.04%, Sigma-Aldrich) for 24 hours before assessment of PD-L1 by flow cytometry.

T-cell apoptosis and anergy

CD8+ T cells were harvested from healthy donor PBMCs through negative selection (Stemcell). To assess T-cell apoptosis, T cells were first activated with CD3/CD28 costimulation (Stemcell) for 48 hours. Activated T cells were cocultured with myeloid cells stimulated with IL6 or CM in a 1:1 ratio. After 24 hours of coincubation, cells were stained for CD8 (eBioscience, SK1 APC), Annexin V (eBioscience, FITC), and propidium iodide (eBioscience) to identify apoptotic cells via flow cytometry. Similarly, CD8+ cells were isolated for assessment of T-cell anergy. T cells were stained with CellTrace (Molecular Probes, CellTrace) and CD8 (eBioscience, SK1 PE) before proliferation analysis by flow cytometry. Identical experiments were conducted in the presence of PD-1 inhibitor, nivolumab (300 ng/mL, Bristol–Myers Squibb).

Tumor cell proliferation

Human tumor cells were treated with tocilizumab, silutimub, or IgG1 control as described previously. Murine tumor cells were treated with anti-IL6R (5 μg/mL) or isotype control (5 μg/mL, BioXCell, LTF-2). Proliferation was assessed through MTT viability assay (Sigma-Aldrich) at 0 and 48 hours and BrdU proliferation assay (Cell Signaling Technology) at 0 and 48 hours.

IHC

Tumor samples were fixed in 10% neutral buffered formalin for 24 hours before paraffin embedding. Antigen retrieval was performed at 95°C for 10 minutes with sodium citrate (pH 6.5). Sections were incubated in 3% hydrogen peroxide for 30 minutes at room temperature to allow formation of CAS9−/− and determined upon manifestation of neurologic de
cits. All mice were cared for in adherence to the NIH Guide for the Care and Use of Laboratory Animals and studies were approved by the Northwestern University Institutional Animal Care and Use Committee.

Statistical analysis

Statistical analyses and plot generation were performed using GraphPad Prism 7 (GraphPad) and MATLAB (Mathworks). To determine cytokine expression across high and low PD-L1–inducing GBM CM (GCM) samples, raw data were quantile

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IHC

Tumor samples were fixed in 10% neutral buffered formalin for 24 hours before paraffin embedding. Antigen retrieval was performed at 95°C for 10 minutes with sodium citrate (pH 6.5). Sections were incubated in 3% hydrogen peroxide for 30 minutes at room temperature, followed by 15-minute room temperature incubation in 0.1% Triton X-100. Sections were blocked in 3% BSA and 10% goat serum for 1.5 hours at room temperature. Staining for IL6 (Abcam, ab6672 1:400), CD68 (Abcam, ab19900 3 μg/mL), and GFAP (Abcam, ab10062 1:100) was performed overnight at 4°C. PD-L1 (Spring Bioscience, prediluted) staining was performed for 15 minutes at room temperature. IL6 staining was developed with tyramide amplification (Thermo Fisher Scientific, Tyramide SuperBoost), whereas CD68, GFAP, and PD-L1 were detected with anti-mouse (Thermo Fisher Scientific, AF488, 10 μg/mL) or anti-rabbit (Thermo Fisher Scientific, AF594, 10 μg/mL) secondary antibodies. Counter staining was done with DAPI (Abcam). Quantification was performed using ImageJ (NIH, Bethesda, MD) on 20× magnification images (EVOS FL) over three representative fields.

Murine staining was performed similarly, with the exception of antigen retrieval for CD8 staining. For CD8, sections were treated with proteinase K (20 μg/mL, Abcam) for 5 minutes at room temperature prior to blocking. Staining for CD8 (Novus Biologicals, AP-MAB0708 1:20) and CD68 (Abcam, FA-11 1:50) was performed overnight at 4°C. Sections were incubated for 1 hour at room temperature with secondary antibody (R&D Systems, rat IgG2a, H+L, pretiluted). CD8 and CD68 were developed using DAB substrate (Vector Laboratories) for 5 minutes. Counterstaining was performed with Mayer hematoxylin (Abcam). Quantification was performed using ImageJ on 20× magnification images (Zeiss Axioskop).

Patient plasma IL6 and myeloid PD-L1

Blood was collected on day of surgery in EDTA and sodium heparin tubes for plasma and PBMC isolation, respectively. Plasma IL6 was quantified through ELISA (Abcam) and myeloid PD-L1 expression was determined by flow cytometry.

GL261 IL6 knockout

To generate GL261 IL6 knockout (KO) cells, we transiently transfected equimolar amounts of CAS9 and gRNA (IL6-Exon1 TGGAGAGGAGCACTTACAG) at 30 nmol/L concentration. CAS9 and gRNA complexes (CAS9 and CRISPR crRNA, Integrated DNA Technologies) were reconstituted in 15 μL of PBS (3 μmol/L) to allow formation of CAS9–gRNA complexes for 10 minutes at room temperature. CAS9–gRNA complexes were supplemented with 30 μL DMEM and 4 μL RNai-Max Transfection Reagent (Thermo Fisher Scientific), and incubated for 20 minutes at room temperature. Thirty percent confluent cell cultures were exposed to CAS9–gRNA complexes with RNai-Max overnight. Confluent cell cultures were flow sorted to establish single-cell clones and analyzed by Western blot analysis. gRNA design was performed using public engines for CRISPR design (http://crispr.mit.edu; ref. 41) and CHOPCHOP (http://chopchop.cbu.uib.no; refs. 42, 43). Only top ranked gRNA with no off-target effects were selected.

In vivo murine studies

To model GBM in vivo, C57Bl/6 mice were intracranially implanted with syngeneic GL261, GL261 CAS9 control, or GL261 IL6 KO cells. A total of 3 × 10^5 cells in 2 μL of PBS were injected via Hamilton syringe 1 mm caudal to the central suture and 2 mm lateral to the bregma, at a depth of 3 mm. PBS sham injections served as controls and were evaluated 7 days postinjection. For treatment, intraarterial injections of either anti-IL6, anti-PD-1 (BioXcell, RMP1-14), combination anti-IL6/anti-PD-1, or isotype control (BioXcell, HRPN or ZA3) at a dose of 250 μg/mouse were started 7 days after tumor implantation and readministered every 3 days for 8 maximum treatments.

Brains and blood were collected on post-implantation day 14 for tumor analysis, IHC staining, and immune cell phenotyping. After Percoll or Ficoll density gradient centrifugation, cells were stained with CD11b (eBioscience, M1/70 PerCP-Cy5.5), CD115, and PD-L1 to assess myeloid PD-L1. Survival endpoints were determined upon manifestation of neurologic deficits. All mice were cared for in adherence to the NIH Guide for the Care and Use of Laboratory Animals and studies were approved by the Northwestern University Institutional Animal Care and Use Committee.
normalized and filtered on the basis of sample variance to exclude the lowest 10% of candidate cytokines. Unpaired two-sample t tests were performed on each candidate across high and low PD-L1–inducing samples, with P values calculated using permutation tests (1,000 permutations). Differences between groups were identified via one-way ANOVA with post hoc multiple comparisons tests or unpaired t tests. Correlations were assessed utilizing Pearson coefficient. Differences in survival were identified through log-rank test. For all analyses, \( P < 0.05 \) was considered significant.

### Results

**Increasing GBM IL6 expression results in increasing PD-L1 expression by stimulated myeloid cells**

To determine tumor-derived cytokines driving PD-L1 induction in myeloid cells, GCM was collected from tumor explant cell cultures originating from patients undergoing resection for GBM. Tumor origin of explant cultures was confirmed by comparison with clinical tumor samples using short tandem repeat analysis (Supplementary Table S3), as well as by morphology and vimentin staining (44, 45), with negative CD45 and CD31 expression (Supplementary Fig. S2A–S2C). GCM was utilized to stimulate myeloid cells isolated from healthy donors, with the resulting PD-L1 induction quantified by flow cytometry (Fig. 1A). All GCM samples induced greater PD-L1 than normal human astrocyte CM (NHAC; \( P < 0.0001 \)). GCM samples were then classified as high or low PD-L1 inducing, defined in relation to median PD-L1 induction (\( P = 0.001 \)). Utilizing a multiplexed cytokine array, we characterized cytokine expression within GCM samples and found that samples in the high PD-L1–inducing group possessed elevated IL6 (\( P = 0.020 \)) and IL8 (\( P = 0.049 \)), compared with samples in the low PD-L1–inducing group (Fig. 1B).

Samples in the high PD-L1 group exhibited a 3.1-fold mean increase in IL6 expression compared with samples in the low PD-L1 group (Fig. 1C). Myeloid cells stimulated with IL6 demonstrated a dose-dependent increase in both PD-L1 and CD163 expression (Fig. 1D; \( P < 0.05 \)), with CD163 expression utilized as a marker of an M2 phenotype (28). In contrast, there was no dose-responsive increase in either PD-L1 or CD163 when myeloid cells were stimulated with IL8 (Fig. 1E and F). Moreover, exposing myeloid cells to both IL6 and IL8 simultaneously did not produce an additive increase in PD-L1 or CD163 expression over IL6 alone (Supplementary Fig. S3A).

We then stimulated myeloid cells with GCM samples in the presence of the clinical-grade IL6-targeting antibodies tocilizumab and siltuximab (Fig. 1G and H). Functionally, tocilizumab inhibits IL6 receptor signaling, whereas siltuximab neutralizes soluble IL6. When myeloid cells were treated with clinically relevant concentrations of tocilizumab (46–48) or siltuximab (49), there was a reduction in both PD-L1 and CD163 expression compared with cells treated with isotype control (\( P < 0.01 \)). Moreover, treatment with tocilizumab or siltuximab did not impact myeloid cell viability or proliferative capacity (Supplementary Fig. S3B and S3C). Overall, these findings support IL6 as being both sufficient and necessary for induction of myeloid cell PD-L1.

**IL6-induced myeloid PD-L1 expression is STAT3 dependent**

We next investigated the role of STAT3 during GCM stimulation of myeloid PD-L1 expression. Exposing myeloid cells to IL6 resulted in increased phosphorylated STAT3 (pSTAT3), which could be reduced through treatment with STAT3i, an irreversible STAT3 inhibitor (Supplementary Fig. S3D; ref. 50). Treatment with STAT3i or DMSO vehicle control did not influence myeloid cell viability (Supplementary Fig. S3E). Stimulation of myeloid cells with GCM also resulted in increased pSTAT3 that was inhibited by treatment with tocilizumab or siltuximab (Fig. 2A). To determine the dependence of PD-L1 induction on STAT3 signaling, myeloid cells were stimulated with either IL6 or GCM in the presence of STAT3i or vehicle control (Fig. 2B). Inhibition of STAT3 prevented the PD-L1 upregulation typically observed with IL6 (\( P = 0.0001 \)) and GCM stimulation (\( P < 0.01 \)). Similar results were observed regarding CD163 expression (Supplementary Fig. S3F; \( P < 0.05 \)).

**GBM-derived IL6 promotes functionally immunosuppressive myeloid cells**

We then examined whether the immunosuppressive function of GCM stimulated myeloid cells could be modulated by agents targeting IL6 signaling. Myeloid cells were stimulated with IL6 or GCM, while being treated with tocilizumab, siltuximab, or isotype control. These cells were then cocultured with activated CD8+ T cells, which were analyzed for apoptosis and proliferation. When T cells were exposed to myeloid cells stimulated with IL6 or GCM, they underwent increased apoptosis compared with T cells cocultured with unstimulated myeloid cells (Fig. 2C; Supplementary Fig. S4A–S4C; \( P < 0.05 \)). This increase in apoptosis could be blocked through myeloid cell treatment with tocilizumab or siltuximab (\( P < 0.05 \)). Moreover, T-cell apoptosis could also be blocked by treatment of cocultures with nivolumab, a clinical-grade anti-PD-1 antibody (ref. 51; Fig. 2D; Supplementary Fig. S4D–S4F; \( P < 0.05 \)). Coculture of GCM-stimulated myeloid cells with activated T cells reduced T-cell proliferation compared with coculture with unstimulated myeloid cells (Fig. 2E; \( P = 0.03 \)). T-cell proliferation was restored to baseline levels when myeloid cells were treated with siltuximab (\( P = 0.04 \)). As with apoptosis, the anergic effect of GCM-stimulated myeloid cells was PD-L1/PD-1 dependent and could be prevented by treatment with nivolumab (Fig. 2F; \( P = 0.005 \)).

**GBM-derived IL6 stimulates tumor cell proliferation**

In addition to the immunomodulatory effects of IL6, evidence indicates that IL6 exerts a direct proliferative effect on GBM (52). We investigated the effect of IL6 on proliferation in GBM explant cell cultures with high and low endogenous IL6 expression utilizing MTT viability (Fig. 2G) and BrdU proliferation (Fig. 2H) assays. High IL6-expressing cells possessed greater basal proliferative capacity compared with low IL6-expressing cells (Supplementary Fig. S4G; \( P < 0.05 \)) and inhibition of IL6 signaling with tocilizumab or siltuximab significantly reduced the viability and proliferative capacity of high IL6-expressing cells (\( P < 0.05 \)), but had no effect on low IL6-expressing cells.

**IL6 expression in GBM correlates with survival**

We surveyed The Cancer Genome Atlas (TCGA) data to examine relationships between IL6, overall survival, and immunosuppressive markers in patients with GBM. Survival analysis of RNA-Seq and microarray data (Supplementary Fig. S5A) demonstrated that patients with high tumor expression of IL6 had worse survival.
outcomes than patients with low IL6 expression \((P < 0.05)\). Moreover, this correlation with survival remained significant in a multivariate model accounting for age, IDH1 status, and IL6 expression (Supplementary Table S4). High IL6-expressing tumors also demonstrated elevated levels of PD-L1 (Supplementary Fig. S5B; \(P = 0.0005\)) and CD163 (Supplementary Fig. S5C; \(P < 0.0001\)).

IL6 expression correlates with intratumoral and peripheral myeloid PD-L1 expression in patients with GBM

GBM patient specimens were examined to investigate the relationship between IL6 and myeloid PD-L1 expression within the tumor microenvironment and peripheral circulation. Immunofluorescent staining for IL6 and GFAP demonstrated differential GBM cell IL6 expression, ranging from 17.7% to

Figure 1.
GBM-derived IL6 induces an immunosuppressive myeloid cell phenotype. A, Stimulation with GCM induced differential expression of PD-L1 on myeloid cells, allowing identification of high \((N = 10)\) and low \((N = 9)\) PD-L1-inducing samples, defined relative to median PD-L1 expression. B, GCM samples associated with high PD-L1 induction exhibited increased expression of IL6 \((P = 0.020)\) and IL8 \((P = 0.019)\). C, 3.1-fold increased expression of IL6 \((P = 0.020)\) was observed in high PD-L1-inducing GCM samples. D, Moreover, stimulation of myeloid cells with IL6 resulted in a dose-dependent increase in PD-L1 and CD163 expression \((P < 0.05; N = 3\) replicates per dose). E, There was a 1.8-fold increased expression of IL8 in high PD-L1-inducing samples \((P = 0.049)\), although stimulation with IL8 did not result in increased myeloid PD-L1 or CD163 expression \((N = 3\) replicates per dose). F, G and H, Treatment of myeloid cells with tocilizumab (TCZ) or siltuximab (SIL) prevented the induction of PD-L1 \((P < 0.01)\) and CD163 \((P < 0.01)\) caused by exposure to GCM (each data point represents the mean of 3 replicates per sample; \(N = 9\) samples). One-way ANOVA with post hoc multiple comparisons test was performed for comparisons across experiments with \(\geq 3\) conditions. Unpaired t tests were performed for comparisons across two conditions. Bars represent the mean ± SEM. *, \(P < 0.05\); **, \(P < 0.01\); ***, ***, ***, ***, \(P < 0.0001\).
97.8% of GFAP⁺ GBM cells (Fig. 3A). Tumors were also stained for CD68 and PD-L1 to identify PD-L1-expressing myeloid cells (Fig. 3B). Intratumoral myeloid PD-L1 expression varied from 9.8% to 95.9% of CD68⁺ cells. GBM IL6 and myeloid PD-L1 expression within the tumor microenvironment were positively correlated (P = 0.045). High IL6-expressing tumors demonstrated a greater amount of infiltrating (Fig. 3C; P = 0.048) and PD-L1-expressing myeloid cells (Fig. 3D; P = 0.049). Patients with high IL6-expressing tumors also demonstrated elevated concentrations of plasma IL6 (Fig. 3E; P = 0.008). Analysis of peripheral myeloid PD-L1 expression by flow cytometry revealed PD-L1 positivity ranging from 9.5% to 24.0% of myeloid cells (Fig. 3F and G). Plasma IL6 concentration and peripheral myeloid PD-L1 expression exhibited a positive correlation (P = 0.027), as did peripheral and intratumoral myeloid PD-L1 expression (Fig. 3H; P = 0.044).

IL6 induces myeloid PD-L1 and increases tumor growth in the GL261 and CT-2A glioma models

To characterize the effects of tumor-derived IL6 in vivo, we employed the murine GL261-C57Bl/6 glioma model. In culture, GL261 cells demonstrated elevated expression of IL6...
compared with normal mouse astrocytes (NMA; Fig. 4A and B; \( P < 0.0001 \)). Similar to human, murine myeloid cells demonstrated a dose-dependent increase in PD-L1 in response to IL6 (Fig. 4C; \( P < 0.05 \)). In addition, stimulation of murine myeloid cells with GL261 CM resulted in increased PD-L1 expression compared with NMA CM or unstimulated controls (Fig. 4D; \( P < 0.0001 \)) that could be reduced by treatment with IL6 neutralizing antibodies (Fig. 4E, \( P = 0.02 \)). Moreover, GL261 CM

Figure 3.
Intratumoral and peripheral IL6 correlates with myeloid PD-L1 expression. A, Immunofluorescent staining of GBM sections (\( N = 12 \)) identified GFAP \(^{+} \) IL6 \(^{+} \) tumor cells with variable IL6 expression across samples (percentages represent the mean of three representative high powered fields; high/low cutoff = 50\% IL6 expression). B, Staining for CD68 \(^{+} \) PD-L1 \(^{+} \) myeloid cells also identified variable myeloid PD-L1 expression across samples that positively correlated with intratumoral IL6 expression (\( P = 0.045 \)). High IL6-expressing tumors demonstrated a greater overall frequency of infiltrating CD68 \(^{+} \) myeloid cells (\( P = 0.048 \); C) and immunosuppressive CD68 \(^{+} \) PD-L1 \(^{+} \) myeloid cells (\( P = 0.049 \); D). E, Moreover, patients with high IL6-expressing tumors also demonstrated elevated plasma IL6 (\( P = 0.008 \)) that correlated with peripheral myeloid cell PD-L1 expression (\( P = 0.027 \); F and G). H, Overall, intratumoral and peripheral myeloid cell PD-L1 expression was positively correlated (\( P = 0.044 \)). Correlations were determined by Pearson correlation coefficient. Unpaired t tests were performed for comparisons across conditions. Individual data points represent the mean of three replicates per sample. Bars represent the mean ± SEM. Image scale bars represent 200 \( \mu \)m. Image inset scale bars represent 50 \( \mu \)m (\( ^{*} P < 0.05; ^{**} P < 0.01 \)).
stimulation was associated with STAT3 phosphorylation that could be abrogated through treatment with IL6 or IL6R-targeting antibodies (Fig. 4F and G). STAT3 inhibition with STATTIC prevented myeloid PD-L1 induction by IL6 (P = 0.02) and GL261 CM (Fig. 4H, P = 0.0001) without affecting viability (Supplementary Fig. S3C). Furthermore, IL6 signaling blockade exerted an antiproliferative effect on GL261 cells as noted by MTT viability (Fig. 4I, P < 0.001) and BrdU proliferation (Fig. 4J, P = 0.03) assays.

Similar studies were conducted utilizing the CT-2A murine glioma model. Of note, CT-2A cells produced less IL6 than GL261 cells (Supplementary Fig. S6A, P < 0.0001). Like GL261, CT-2A CM was sufficient to induce PD-L1 expression on myeloid cells (Supplementary Fig. S6B, P < 0.0001), which could be partially inhibited through IL6 blockade (Supplementary Fig. S6C, P = 0.0001). Moreover, treatment of CT-2A cells with anti-IL6R reduced tumor cell proliferation measured through MTT viability (Supplementary Fig. S6D, P < 0.001) and BrdU proliferation assays (Supplementary Fig. S6E, P = 0.03).

Given the increased IL6 expression by GL261 cells, they were selected as the model system to pursue in vivo. To characterize the
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The role of tumor-derived IL6, GL261 cells were intracranially implanted into syngeneic C57BL/6 mice. Tumor-bearing mice demonstrated increasing tumor burden (Fig 4K; \( P < 0.0001 \)) that was associated with elevations in plasma IL6 (Fig 4L; \( P < 0.05 \)) and peripheral myeloid cell PD-L1 expression (Fig 4M; \( P < 0.05 \)). Significant increases in plasma IL6 (\( P = 0.03 \)) and myeloid PD-L1 (\( P = 0.03 \)) were observed as early as 14 days post-tumor implantation compared with mice receiving sham intracranial injections. Plasma IL6 correlated strongly with myeloid PD-L1 expression (Fig 4N; \( P < 0.01 \)).

IL6 knockout reduces myeloid PD-L1 expression, tumor cell proliferation, and increases murine survival in the GL261 model

To explore the effects of IL6 knockout (KO) on myeloid PD-L1 induction, tumor progression, and survival, a C57BL/6 mice and peripheral myeloid cell PD-L1 expression (Fig 4M; \( P < 0.05 \)). Peripheral myeloid cell PD-L1 was also higher in mice bearing control tumors compared with mice implanted with IL6 KO tumors (Fig 5F; \( P = 0.048 \)). Peripheral myeloid cell PD-L1 was also higher in mice bearing control tumors compared with mice implanted with IL6 KO tumors (Fig 5G; \( P = 0.002 \)), as was tumor-infiltrating myeloid cell PD-L1 (Fig 5H; \( P = 0.01 \)). Tumors established in mice implanted with IL6 KO cells were smaller than tumors arising from control cells (Fig 5I; \( P = 0.045 \)). Overall, there was a 77% increase in survival for mice bearing IL6 KO compared with control tumors (Fig 5J; 39 vs. 22 day median survival, \( P < 0.0001 \)).

Anti-IL6 therapy reduces myeloid PD-L1, intracranial tumor growth, and improves survival in the GL261 model

We next proceeded to determine whether treatment with IL6 neutralizing antibodies would provide therapeutic benefit in the GL261 model. To recreate treatment of an established tumor, anti-IL6 therapy was initiated in mice bearing GL261 tumors at 7 days post-tumor implantation (Fig 6A). Anti-IL6 treatment reduced plasma IL6 levels compared with isotype control (Supplementary Fig. S6J). Compared with myeloid cells stimulated with CM from control cells, myeloid cells stimulated with CM from IL6 KO cells demonstrated reduced PD-L1 induction (Fig 5C; \( P = 0.006 \)). IL6 KO cells also displayed reduced growth compared with control cells, as indicated by MTT viability (Fig 5D; \( P < 0.05 \)) and BrdII proliferation (Fig 5E; \( P = 0.001 \)). When intracranially implanted into C57BL/6 mice, tumors from control GL261 cells resulted in elevated plasma IL6 compared with mice bearing IL6 KO tumors (Fig 5F; \( P = 0.048 \)). Peripheral myeloid cell PD-L1 was also higher in mice bearing control tumors compared with mice implanted with IL6 KO tumors (Fig 5G; \( P = 0.002 \)), as was tumor-infiltrating myeloid cell PD-L1 (Fig 5H; \( P = 0.01 \)). Tumors established in mice implanted with IL6 KO cells were smaller than tumors arising from control cells (Fig 5I; \( P = 0.045 \)). Overall, there was a 77% increase in survival for mice bearing IL6 KO compared with control tumors (Fig 5J; 39 vs. 22 day median survival, \( P < 0.0001 \)).

Anti-IL6 therapy reduced plasma IL6 levels, increased CD8+ T-cell activation, and extended survival with long-term survivors that were not observed with either monotherapy (Fig 6K). No significant interaction between anti-IL6 and anti-PD-1 treatment was observed (\( P = 0.11 \)), suggesting that the effect of combinatorial therapy was additive, rather than synergistic.

Discussion

With 5-year survival rates less than 5%, GBM is a uniformly fatal disease (1). Despite extensive research, outcomes have only modestly improved beyond the 14.5-month median overall survival observed with the introduction of standard-of-care surgical resection, radiation, and temozolomide (6, 7). Given that immunotherapy has demonstrated durable survival benefits in other malignancies, its value in GBM is currently being explored (8); however, efficacy to date has been modest, in
part, due to immunosuppression. One critical mediator of immunosuppression in GBM is PD-L1 (37, 38). While only a fraction of GBM cells express PD-L1 (15, 16), myeloid cells in the tumor microenvironment and circulation abundantly express PD-L1 (40). Immunosuppressive myeloid cells are of particular importance in GBM as they extensively infiltrate tumors (26, 27) and correlate with decreased survival (28, 54). Previously, we demonstrated that patients with GBM exhibit increased PD-L1 on
Figure 6.
Anti-IL6 therapy decreases myeloid PD-L1 expression, inhibits tumor growth, improves survival, and can be combined with anti-PD-1 immunotherapy. A, To model treatment of an established tumor, GL261 tumor-bearing mice received anti-IL6 or isotype control beginning 7 days posttumor implantation. Efficacy of anti-IL6 treatment was validated by plasma IL6 (*P* < 0.0001; *N* = 5 replicates per condition; B) and serum amyloid A (SAA; *P* < 0.0001; *N* = 5 replicates per condition; C) ELISAs. D, Mice receiving anti-IL6 exhibited reduced peripheral myeloid PD-L1 (*P* = 0.0004; *N* = 5 replicates per condition). E, A modest decrease in tumor-infiltrating myeloid PD-L1 was also observed with anti-IL6 compared with isotype control (*P* = 0.01; *N* = 5 replicates per condition). F, Tumors of mice receiving anti-IL6 therapy exhibited decreased CD68^+^ myeloid cell infiltration (*P* = 0.02; *N* = 4 replicates per condition). G, In addition, tumors of anti-IL6-treated mice demonstrated increased CD8^+^ T-cell infiltration (*P* = 0.02; *N* = 4 replicates per condition). H, Tumors isolated from anti-IL6-treated mice were smaller than tumors isolated from isotype control mice (*P* = 0.048; *N* = 5 replicates per condition). I, Increased survival was seen in mice receiving anti-IL6 therapy compared with controls (*P* = 0.004; *N* = 8 isotype control mice, 10 anti-IL6 mice). J, When combined with anti-PD-1 therapy, anti-IL6 therapy resulted in decreased tumor volumes (*P* = 0.02; *N* = 3 replicates per condition). K, Moreover, combination therapy resulted in an improved survival benefit with 43% long-term survivors (*P* < 0.05; *N* = 6 mice per condition), compared with treatment with anti-IL6 or anti-PD-1 therapy alone. One-way ANOVA with post hoc multiple comparisons test was performed for comparisons across experiments with ≥3 conditions. Unpaired *t* tests were performed for comparisons across two conditions. Log-rank test was performed to determine survival differences. HR reported with 95% confidence interval. Bars represent the mean ± SEM. Scale bars represent 30 μm. *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001; ****, *P* < 0.0001.
their circulating myeloid cells (22) and that peripheral myeloid PD-L1 expression could serve as a prognostic marker of response to vaccine therapy (25). In this study, we sought to determine the GBM-derived factors capable of inducing myeloid PD-L1 to identify therapeutic targets to enhance antitumor immunity.

Proceding investigations have shown that GBM-derived soluble factors are sufficient to induce myeloid PD-L1 expression, but did not identify the specific cytokines responsible (22, 31). We, therefore, conducted a cytokine screen across high and low PD-L1–inducing GCM samples and identified IL6 as a GBM-derived cytokine that is necessary and sufficient for PD-L1 induction on myeloid cells. IL6 is a pleiotropic cytokine that has been associated with both proinflammatory and anti-inflammatory effects (55, 56). Across multiple malignancies, IL6 has been shown to recruit MDSCs (57, 58) and polarize myeloid cells toward an M2 phenotype (59–61). IL6 from glioma-initiating cells has been associated with expression of the immune checkpoint molecule B7-H4 on tumor-infiltrating and circulating myeloid cells (32). Moreover, vascular endothelial cells within the GBM microenvironment have recently been identified as sources of IL6 that can induce alternative activation of tumor-infiltrating macrophages (30).

Clinically, increased IL6 has been observed in GBM patient serum (62) and cerebrospinal fluid (63). Moreover, tumor IL6 expression has been associated with poor survival (30, 62, 64–66), which we recapitulated by TCGA analysis. Immunologically, patients with GBM with high IL6-expressing tumors demonstrated elevated PD-L1 and CD163 expression, in accordance with the relationship between IL6 and immunosuppression identified in vivo. Interestingly, evidence in the literature indicates that IL6, PDDL1, and CD163 expression are enriched in the mesenchymal GBM subtype (67), which is characterized by elevated immune infiltrates and immunosuppressive markers (15, 67–69). In patient samples, we correlated IL6 and myeloid PD-L1 expression within the tumor microenvironment and in the peripheral circulation. Patients with high IL6 tumor expression demonstrated elevated plasma IL6 and greater myeloid infiltration, consistent with the role of IL6 as a myeloid chemokine (70) and supporting the hypothesis that GBM-derived IL6 can direct systemic and local immunosuppression.

To study GBM-derived IL6 in vitro, we utilized murine glioma models. Similar to patients with GBM, we found that mice with intracranial GL261 and CT-2A tumors exhibited increased plasma IL6 and peripheral myeloid PD-L1 expression. Through CRISPR/Cas9 IL6 knockout in GL261 cells and the use of IL6 neutralizing antibodies in GL261 and CT-2A tumor-bearing mice, we demonstrated that IL6 suppression resulted in decreased myeloid PD-L1 within the tumor microenvironment and peripherally. However, this correlated with a significant decrease in tumor growth and improvement in survival in the GL261 model only. Compared with GL261 cells, IL6 expression by CT-2A cells is significantly lower. Moreover, the CT-2A model is characterized by highly immunosuppressed (71) and resistant to single-agent checkpoint inhibition (72). It is, therefore, not surprising that single-agent IL6 blockade was insufficient to improve survival in this model. Regardless, IL6 targeted therapy was successful in reducing myeloid cell PD-L1 induction across both models.

Mechanistically, we determined that GCM-driven PD-L1 induction is STAT3-dependent, with IL6 acting as the primary STAT3 activator. STAT3 directly binds to the PD-L1 promoter (73) and has been implicated in myeloid anti-inflammatory effects (74–76), such as upregulation of immunosuppressive cytokines (73, 77) and GBM exosome induction of myeloid PD-L1 (78). The induction of myeloid B7-H4 was similarly shown to be IL6/STAT3 dependent (32), supporting the notion that IL6 can activate redundant immunosuppressive mechanisms (79). Apart from mediating immunosuppression, GBM-derived IL6/STAT3 signaling has also been implicated in tumor proliferation (52, 80), invasion (81, 82), angiogenesis (82), autophagy (83), and glioma stem cell maintenance (66). In GBM explant, GL261, and CT-2A cells, we observed decreased proliferation with IL6 blockade. To distinguish the effects of anti-IL6 therapy on immunosuppression and proliferation in vivo, we conducted T-cell depletions studies and found the benefit of anti-IL6 therapy in GL261 to be CD8+ T-cell dependent. This is consistent with recent evidence indicating that CD8+ T cells undergo preferential functional suppression in the GBM microenvironment (71) and suggests that IL6 may be a contributor factor.

Given that the benefit of anti-IL6 therapy was immunologically dependent, we sought to determine whether it could be combined with other immunotherapeutic strategies (84, 85). In melanoma, pancreatic cancer, and hepatocellular carcinoma models, anti-IL6 therapy combined with PD-1/PD-L1–targeted treatment resulted in reduced tumor growth and increased survival (86–88). In our study, we treated GL261 tumor-bearing mice with a combination of anti-IL6 and anti-PD-1 therapy that resulted in suppressed tumor growth and increased survival with 43% long-term survivors. Improved survival was likely mediated by the additional blockade of tumor cell PD-L1/PD-1 signaling, reduced intratumoral immunosuppressive myeloid cell burden, and inhibition of PD-1 mediated myeloid IL6 release (88). Given the modest survival benefit of single-agent IL6 inhibition and the emerging consensus that successful GBM immunotherapy will likely require combinatorial strategies (84, 89), our findings support further investigation into the role of IL6 suppression in combination with other immunotherapy strategies such as vaccine, CAR T cell, and oncolytic viral therapy. Moreover, the interaction between IL6 inhibition and chemoradiation in immunocompetent GBM models will be required, especially given that IL6 has been implicated in mediating resistance to both chemo (90) and radiotherapy (91–93).

The limitations of our study include a low number of patient samples, use of murine models that differ from human GBM, and limited investigation into nontumor sources of IL6 such as reactive astrocytes (94). GL261, while the most prevalent murine glioma model, exhibits greater immunogenicity than its human counterpart, overestimating potential translational effects. Moreover, the survival benefit observed with single-agent IL6 inhibition was relatively modest and will require additional investigation in combination with other therapeutic strategies. Clinically, GBM is a heterogeneous disease and we have demonstrated that IL6 is not universally elevated, indicating that targeting IL6 may not be beneficial to all patients. Therapeutically, IL6 inhibition is well-tolerated with potential adverse effects similar to other immunosuppressants including increased risk for infection, skin pathologic, and gastrointestinal exacerbations (95). Of note, IL6 neutralization has already been used as a treatment for immune-related adverse effects encountered with immune checkpoint inhibition (96–99). Thus, addition of anti-IL6 treatment to immunotherapy regimens may be doubly efficacious in limiting immunosuppression, while also preventing adverse proinflammatory events.
In conclusion, we have identified IL6 as a GBM-derived cytokine that is necessary and sufficient for myeloid cell PD-L1 induction through a STAT3-dependent mechanism. Moreover, we demonstrated that IL6-targeted therapy is associated with reduced intratumoral and peripheral myeloid PD-L1 expression, delayed tumor progression, and improved survival outcomes. Importantly, combining anti-IL6 treatment with anti-PD-1 immunotherapy generated effective peripheral and intratumoral antitumor immune responses. Given that IL6-targeting agents (tocilizumab and siltuximab) are clinically available, our results suggest that further study of IL6 neutralization may prove beneficial in reducing immunosuppression, enhancing immunotherapeutic efficacy, and improving outcomes for patients with GBM.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Acknowledgments
The authors would like to thank the following core facilities at Northwestern University, without which the current study would not be possible: the Nervous System Tumor Bank (supported by P50CA221747 SPORE for Translational Approaches to Brain Cancer), the Interdepartmental Immunology Flow Cytometry Core Facility, the DNA/RNA Delivery Core of the Skin Disease Research Center for providing us the service of knocking down murine IL6 protein in GL261 cell lines by CRISPR-Cas9 gene editing, Center for Advanced Microscopy/Nikon Imaging Center (supported by NCI CCSG P30 CA60553 awarded to the Robert H Lurie Comprehensive Cancer Center), the Mouse Histology and Phenotyping Laboratory, the Center for Comparative Medicine, and the Quantitative Data Sciences Core (supported by NCI CCSG P30 CA60553). The authors would also like to thank Dr. Stephen D. Miller for his guidance in study design and interpretation of results and Lisa P. Magnusson for her assistance in animal studies. This work was supported by the NIH/National Cancer Institute (NCI) Ruth L. Kirschstein National Research Service Award F30 (CA206413; to Jonathan B. Lamano), NIH/NCI R01 (CA164714; to O. Bloch), and NIH/National Institute of Neurological Disorders and Stroke (NINDS) R00 [NS078055; to O. Bloch]. J.D. DiDomenico was supported by the Alpha Omega Alpha Honor Medical Society (AOA) Carolyn L. Kuckle Student Research Fellowship and the American Medical Association Seed Fellowship. Y.D. Li and L. Ampie were supported by individual student fellowships from the Howard Hughes Medical Institute. G. Kaur was supported by NIH/NINDS F32 (NS101884).

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Received July 26, 2018; revised January 2, 2019; accepted February 25, 2019; published first March 1, 2019.
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Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-18-2402

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