GM-CSF Promotes the Immunosuppressive Activity of Glioma-Infiltrating Myeloid Cells through Interleukin-4 Receptor-α

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Abstract
Malignant gliomas are lethal cancers in the brain and heavily infiltrated by myeloid cells. Interleukin-4 receptor-α (IL-4Rα) mediates the immunosuppressive functions of myeloid cells, and polymorphisms in the IL-4Rα gene are associated with altered glioma risk and prognosis. In this study, we sought to evaluate a hypothesized causal role for IL-4Rα and myeloid suppressor cells in glioma development. In both mouse de novo gliomas and human glioblastoma cases, IL-4Rα was upregulated on glioma-infiltrating myeloid cells but not in the periphery or in normal brain. Mice genetically deficient for IL-4Rα exhibited a slower growth of glioma associated with reduced production in the glioma microenvironment of arginase, a marker of myeloid suppressor cells, which is critical for their T-cell inhibitory function. Supporting this result, investigations using bone marrow-derived myeloid cells showed that IL-4Rα mediates IL-13–induced production of arginase. Furthermore, glioma-derived myeloid cells suppressed T-cell proliferation in an IL-4Rα–dependent manner, consistent with their identification as myeloid-derived suppressor cells (MDSC). Granulocyte macrophage colony-stimulating factor (GM-CSF) plays a central role for the induction of IL-4Rα expression on myeloid cells, and we found that GM-CSF is upregulated in both human and mouse glioma microenvironments compared with normal brain or peripheral blood samples. Together, our findings establish a GM-CSF–induced mechanism of immunosuppression in the glioma microenvironment via upregulation of IL-4Rα on MDSCs. Cancer Res; 73(21); 6413–23. ©2013 AACR

Introduction
Malignant gliomas are lethal cancers in the brain and heavily infiltrated by myeloid cells. Interleukin-4 receptor-α (IL-4Rα) mediates the immunosuppressive functions of myeloid cells, and polymorphisms in the IL-4Rα gene are associated with altered glioma risk and prognosis. In this study, we sought to evaluate a hypothesized causal role for IL-4Rα and myeloid suppressor cells in glioma development. In both mouse de novo gliomas and human glioblastoma cases, IL-4Rα was upregulated on glioma-infiltrating myeloid cells but not in the periphery or in normal brain. Mice genetically deficient for IL-4Rα exhibited a slower growth of glioma associated with reduced production in the glioma microenvironment of arginase, a marker of myeloid suppressor cells, which is critical for their T-cell inhibitory function. Supporting this result, investigations using bone marrow-derived myeloid cells showed that IL-4Rα mediates IL-13–induced production of arginase. Furthermore, glioma-derived myeloid cells suppressed T-cell proliferation in an IL-4Rα–dependent manner, consistent with their identification as myeloid-derived suppressor cells (MDSC). Granulocyte macrophage colony-stimulating factor (GM-CSF) plays a central role for the induction of IL-4Rα expression on myeloid cells, and we found that GM-CSF is upregulated in both human and mouse glioma microenvironments compared with normal brain or peripheral blood samples. Together, our findings establish a GM-CSF–induced mechanism of immunosuppression in the glioma microenvironment via upregulation of IL-4Rα on MDSCs. Cancer Res; 73(21); 6413–23. ©2013 AACR

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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MDSCs is known to play a role in their immunosuppressive functions (14–17).

With regard to the roles of myeloid cells in glioma environment (reviewed in ref. 18), although glioblastoma are highly infiltrated by microglia/macrophages (19), molecular mechanisms need to be elucidated as to how glioma-infiltrating myeloid cells influence the glioma growth. Recent epidemiology studies have reported that single-nucleotide polymorphisms in IL-4Rα are associated with altered glioma risk and prognosis (20, 21), suggesting a possibility that IL-4Rα expression on myeloid cells may impact the glioma development. We therefore sought to determine whether IL-4Rα expression on myeloid cells plays a role in glioma development. Here, we show, using a de novo glioma model and human malignant glioma tissues, granulocyte-macrophage colony-stimulating factor (GM-CSF), which is expressed at high levels in the glioma microenvironment, leads to upregulation of IL-4Rα in de novo myeloid cells plays a role in glioma development. Here, we show, using a de novo glioma model and human malignant glioma tissues, granulocyte-macrophage colony-stimulating factor (GM-CSF), which is expressed at high levels in the glioma microenvironment, leads to upregulation of IL-4Rα on CD11b+Gr1+ IMCs, thereby promoting the induction of arginase via IL-13. Our data show a novel immunosuppressive mechanism in malignant glioma.

Materials and Methods

Animals
BALB/c-background wild-type (WT) and Il4ra-deficient mice were obtained from The Jackson Laboratory. Animals were maintained in the Animal Facility at the University of Pittsburgh (Pittsburgh, PA) per an Institutional Animal Care and Use Committee–approved protocol.

Bone marrow MDSC generation
A similar procedure has been previously described (22, 23). Briefly, red blood cell-depleted bone marrow (BM) cells were isolated from WT or Il4ra−/− mice. Granulocyte colony-stimulating factor (G-CSF; 100 ng/mL) and GM-CSF (250 U/mL) were added on days 0, 4, and 9 with IL-13 added on days 4 and 9. All cytokines were purchased from Peprotech. CD11b+ (80 ng/mL) on days 4 and 9. The procedure has been described previously (24). Briefly, DNA transfection reagent (in vivo-jetPEI) was obtained from Polyplus Transfection. The following DNA plasmids were used for glioma induction: pT2/C-Luc//PGK-SB13, pT/CAGGS-NRASV12, pT2/shP53, and PT3.5/CMV-EGFRIII (0.125 μg for each). For immunologic evaluation of WT and Il4ra−/− tumors, we conducted bioluminescence imaging (BLI) using an IVIS200 (Caliper Life Sciences) and evaluated tumors of comparable size (BLI of 2 × 10⁶ luciferase units).

Arginase activity assay
The QuantiChrome arginase assay detection kit (DARG-200) was used according to the manufacturer’s instructions, optical density was determined at 430 nm using a multiscan RC plate reader (Thermo Scientific).

MDSC-mediated T-cell Inhibition
CD8+ T cells were isolated from WT BALB/c splenocytes (SPC) using magnetic bead negative separation (Miltenyi Biotec), labeled with 100 nmol/L carboxyfluorescein diacetate succinimidyl ester (Invitrogen), and incubated with varying amounts of day 10 cultured bone marrow– or glioma-derived MDSCs for 5 days in the presence of anti-CD3/anti-CD28 Dynabeads (Invitrogen) and 30 U/mL of hIL-2 (Peprotech). Cells were then analyzed by flow cytometry on an AccuriC6 (BD Biosciences).

Antibody-mediated immune cell depletion
The procedure has been described previously (8). Anti-Gr1 (RB6-8C5), anti-CD4 (GK1.5), and anti-CD8 (TIB105) monoclonal antibodies (mAb) were obtained from Taconic; control immunoglobulin G (IgG) was obtained from Sigma-Aldrich. Mice with developing gliomas received intraperitoneal injections of anti-Gr1 (0.25 mg/dose) 3 times per week or anti-CD4 and anti-CD8 (0.5 mg/dose) 2 times per week starting on day 21 after induction of de novo glioma.

Real-time PCR
The procedure has been described previously (7, 8). Primers and probes were obtained from Applied Biosystems. Human or mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. All reactions were done in triplicate and relative expressions of RNAs compared with control samples were calculated using the ΔΔCt method.

Induction of de novo gliomas by intraventricular transfection of sleeping beauty transposon-flanked proto-oncogenes
Bone marrow chimera experiments were carried out as previously described (25). Briefly, red blood cell-depleted bone marrow cells were isolated from donor WT or Il4ra−/− mice. Host BALB/c-background WT mice received 10 Gy of total body irradiation followed by tail vein injection of 1 × 10⁶ viable bone marrow cells. The efficiency of our bone marrow chimera protocol was confirmed to be more than 96% using donor bone marrow cells derived from enhanced GFP transgenic mice (Supplementary Fig. S1).

Isolation of murine brain-infiltrating leukocytes
Brain-infiltrating leukocytes (BIL) were isolated using the methods described previously (7, 26), using the Percoll (Sigma-Aldrich) isolation method. Because of the small number of BILs obtained per mouse, BILs obtained from all mice in a given group (5 mice/group) were pooled and then evaluated for the relative number and phenotype of the BILs between groups.

Isolation of human glioma-infiltrating leukocytes and peripheral blood mononuclear cells
De-identified fresh glioma tissues were obtained from the operating room per Institutional Review Board–approved protocol, mechanically minced, resuspended in 70% Percoll (Sigma-Aldrich), overlaid with 37% and 30% Percoll, and centrifuged for 20 minutes at 500 × g. Enriched leukocyte populations were recovered at the 70% to 37%. Peripheral blood mononuclear cells (PBMC) were isolated from whole blood using a standard Ficoll procedure (Stemcell Technologies).
Statistical analyses

Statistical significance of differences between two groups was determined by Student t test. The log-rank test was used to determine significant differences in survival curves on Kaplan–Meier plots among groups. All data were analyzed by GraphPad Prism (v5.0), P < 0.05 was considered to be statistically significant.

Results

Il4ra−/− mice exhibit delayed growth of de novo glioma compared with WT mice

To evaluate the role of IL-4Rα on glioma growth, we induced de novo gliomas by Sleeping Beauty transposon-mediated intraventricular transfection of the oncogenes, EGFRvIII, NRas, and short hairpin (sh)P53 in neonatal BALB/c-background WT and Il4ra−/− mice (hereby Sleeping Beauty glioma). Although the median symptom-free survival (SFS) for WT mice was 55.5 days, Il4ra−/− mice exhibited prolonged survival with a median SFS of 90 days (P < 0.001; Fig. 1A). As IL-4Rα is expressed on some MDSCs (15–17), we next evaluated whether the genetic deletion of Il4ra impacts the glioma infiltration of myeloid cells, such as CD11b+Gr1+ cells, which are likely MDSC (Fig. 1B and C). In WT mice, Sleeping Beauty glioma-bearing brains showed higher numbers of CD11b+Gr1+ cells compared with nontumor-bearing brains. Furthermore, in the presence of the Sleeping Beauty gliomas, WT CD11b+Gr1+ contained a higher percentage of IL-4Rα–expressing cells than those in nontumor-bearing animals. Compared with WT animals, nontumor-bearing brains of Il4ra−/− hosts had significantly fewer numbers of CD11b+Gr1+ cells that did not increase significantly in the presence of the Sleeping Beauty tumor (Fig. 1B and C).

To determine whether the different expression levels of IL-4Rα and numbers of CD11b+Gr1+ cells in tumor-bearing hosts are also seen in periphery, we analyzed SPCs derived from Sleeping Beauty glioma-bearing mice (Supplementary Fig. S2). Similar to our observation in the brain, WT but not Il4ra−/− hosts showed an increase of CD11b+Gr1+ cells in the spleen following induction of Sleeping Beauty glioma. However, unlike the brain, IL-4Rα expression levels on peripheral CD11b+Gr1+ cells remained low even in tumor-bearing animals. Thus IL-4Rα expression on CD11b+Gr1+ cells seems to be relatively limited to the cells infiltrating in the gliomas.

Figure 1. Effects of IL-4Rα on glioma development. De novo Sleeping Beauty gliomas were induced in neonatal mice. A, SFS following the induction of gliomas. SFS of Il4ra−/− mice was significantly longer than WT mice. B, brains with or without de novo gliomas were harvested from WT and Il4ra−/− mice. BILs were analyzed for the percentage of CD11b+Gr1+ populations in leukocyte-gated cells and IL-4Rα expression on CD11b+Gr1+ cells. C, absolute numbers of CD11b+Gr1+ BILs in tumor-bearing or nontumor-bearing mice. Bars represent the mean and the SD of results from three independent experiments. D, glioma-bearing WT and Il4ra−/− mice were depleted for CD4+ and CD8+ cells. Control mice received isotype control rat IgG. SFS was evaluated. MFI, mean fluorescence intensity.
T-cells deficient of IL-4R or its major signaling molecule STAT-6 are typically skewed toward type-I immune response, which is known to promote antitumor immunity (27–29). To exclude a possibility that the prolonged survival of $\text{Il4ra}^{-/-}$ mice is solely due to enhanced antitumor T-cell response, we induced Sleeping Beauty gliomas in WT and $\text{Il4ra}^{-/-}$ hosts in which CD4$^+$ and CD8$^+$ T cells were depleted (Fig. 1D and Supplementary Fig. S3). Although depletion of T cells significantly accelerated the growth of gliomas in both WT and $\text{Il4ra}^{-/-}$ mice, $\text{Il4ra}^{-/-}$ mice still showed improved SFS over WT mice when both were depleted of T cells. These data show that the improved survival of $\text{Il4ra}^{-/-}$ mice is at least partially independent of T cells.

**$\text{Il4ra}^{-/-}$ tumor tissue and tumor-derived CD11b$^+$Gr1$^+$ myeloid cells express decreased levels of inhibitory molecules**

To examine the impact of IL-4R$\alpha$ on the glioma microenvironment, total RNA was extracted from WT or $\text{Il4ra}^{-/-}$ de novo gliomas of similar size, and inflammation-associated genes were evaluated by real-time PCR (RT-PCR; Fig. 2A). The gliomas in WT mice showed significantly higher levels of immunosuppressive $\text{Tgf}b$ and $\text{Arg1}$ than those in $\text{Il4ra}^{-/-}$ mice. Notably, although similar levels of IL-13 were detected in WT and $\text{Il4ra}^{-/-}$ tumors, IL-4 expression was undetectable.

To better understand the significance of IL-4R$\alpha$ expression in CD11b$^+$Gr1$^+$ cells in the glioma, we isolated two subsets of CD11b$^+$Gr1$^+$ cells by fluorescence-activated cell sorting (FACS), CD11b$^+$Ly6C$^{hi}$ monocytic cells and CD11b$^+$Ly6C$^{hi}$ granulocytic cells, and analyzed MDSC-associated genes by RT-PCR (Fig. 2B). Although CD11b$^+$Ly6C$^{hi}$ cells expressed higher levels of both $\text{Tgf}b$ and $\text{Arg1}$ than CD11b$^+$Ly6C$^{lo}$ cells, $\text{Arg1}$ expression was significantly lower in $\text{Il4ra}^{-/-}$ CD11b$^+$ Ly6C$^{hi}$ cells than WT counterparts. These data suggest a significant role of IL-4R$\alpha$ expression on MDSCs in the glioma microenvironment, especially through Arg1.

**Depletion of CD11b$^+$Gr1$^+$ cells prolongs survival of mice bearing de novo gliomas**

We next examined whether depletion of CD11b$^+$Gr1$^+$ cells prolongs survival in mice bearing the de novo gliomas. Although there are multiple methods to deplete CD11b$^+$Gr1$^+$ cells, such as with chemotherapeutics sunitinib or gemcitabine (30, 31), these also could have direct antitumor activities. Thus, we used anti-Gr1 (RB6-8C5) monoclonal mAb, which efficiently depleted CD11b$^+$Gr1$^+$ cells in de novo glioma models in our research.

![Figure 2](image-url). Effects of IL-4R$\alpha$ on the glioma microenvironment and glioma-infiltrating myeloid cells. A, total RNA was isolated from brains of nontumor-bearing mice (normal brain), the contralateral (contra), or tumor-bearing (tumor) hemispheres of brains derived from WT and $\text{Il4ra}^{-/-}$ mice. mRNA expression levels of $\text{Tgf}b$, $\text{Arg1}$, $\text{Il13}$, and $\text{Il4ra}$ were analyzed by RT-PCR relative to normal brain. B, BILs from WT or $\text{Il4ra}^{-/-}$ mice were sorted for cell population double positive for CD11b and Ly6C or CD11b and Ly6G. $\text{Arg1}$ and $\text{Tgf}b$ mRNA levels were evaluated by RT-PCR in each of these populations. Samples were analyzed relative to WT Ly6G$^+$ sorted cells. Bars represent the mean and SD of results from triplicates.
previous studies (7, 8). To maintain complete depletion, we administered 50 mg/dose anti-Gr1 mAb 3 times per week starting at day 23 (Fig. 3A; refs. 7, 8). Mice depleted of CD11b<sup>+</sup>/Gr1<sup>+</sup> cells experienced significantly prolonged SFS (Fig. 3B) with 3 of 7 animals surviving past day 120 (median survival of 74 days), whereas all control mice treated with control isotype IgG died by day 68 (median survival of 55.5 days). BIL revealed that some (n = 3) mice treated with anti-Gr1 mAb even experienced tumor regression below the level of detection (Fig. 3C). These data show the importance of CD11b<sup>+</sup>/Gr1<sup>+</sup> cells in the development of <i>de novo</i> glioma.

**Bone marrow chimeric mice reveal that Il4ra on bone marrow cells is critical for accumulation of CD11b<sup>+</sup>/Gr1<sup>+</sup> cells in the brain**

As we showed in Fig. 1, gliomas in <i>Ih4ra</i><sup>−/−</sup> mice had fewer infiltrating CD11b<sup>+</sup>/Gr1<sup>+</sup> cells than in WT controls. To assess whether the difference was specifically due to intrinsic factors in bone marrow cells, we evaluated the impact of <i>Ih4ra</i> status on glioma infiltration of CD11b<sup>+</sup>/Gr1<sup>+</sup> cells using a bone marrow chimera system. Because induction of <i>de novo</i> gliomas is possible in neonatal mice only but not in adult mice (24), as an alternative glioma model, bone marrow chimeric mice received stereotactic injections of cultured glioma cells established from syngeneic <i>de novo</i> glioma. In both SPCs and BILs, mice with WT bone marrow displayed greater numbers of CD11b<sup>+</sup>/Gr1<sup>+</sup> cells but lower numbers of CD4<sup>+</sup> and CD8<sup>+</sup> T cells compared with the ones with <i>Ih4ra</i><sup>−/−</sup> bone marrow (Fig. 4). These data suggest that IL-4Ra expression on bone marrow-derived cells promotes the systemic distribution of CD11b<sup>+</sup>/Gr1<sup>+</sup> cells but may inhibit that of T cells.

**IL-4Ra signaling promotes the T-cell-suppressing function of bone marrow-derived and glioma-infiltrating CD11b<sup>+</sup>/Gr1<sup>+</sup> cells**

Using mouse CD11b<sup>+</sup>/Gr1<sup>+</sup> cells derived from bone marrow of WT or <i>Ih4ra</i><sup>−/−</sup> mice, we first confirmed the previously reported observations (6, 14) that the IL-13-IL-4Ra signaling mediates T-cell suppressing activities of IMCs via induction of arginase (Supplementary Fig. S4). Notably, WT CD11b<sup>+</sup>/Gr1<sup>+</sup> cells suppressed T-cell proliferation and IFN-γ levels at lower BM-CD11b<sup>+</sup>/Gr1<sup>+</sup>:T-cell ratios than <i>Ih4ra</i><sup>−/−</sup> CD11b<sup>+</sup>/Gr1<sup>+</sup> cells. Furthermore, supplementation with an arginase inhibitor Nω-hydroxy-nor-arginine or L-arginine inhibited the T-cell suppressing activity of WT CD11b<sup>+</sup>/Gr1<sup>+</sup> cells. When we isolated CD11b<sup>+</sup> cells from gliomas growing in the brain of WT or <i>Ih4ra</i><sup>−/−</sup> mice, WT mouse-derived myeloid cells showed more profound levels of inhibition on CD8<sup>+</sup> T-cell proliferation compared with the ones derived from <i>Ih4ra</i><sup>−/−</sup> mice (Supplementary Fig. S5). These data indicate that CD11b<sup>+</sup>/Gr1<sup>+</sup> BILs are indeed capable of suppressing T-cell proliferation in an IL-4Ra–dependent manner. We hereby term CD11b<sup>+</sup>/Gr1<sup>+</sup> BILs MDSCs in the glioma environment.

**GM-CSF upregulates IL-4Ra on bone marrow cells and is overexpressed in gliomas**

As IL-4Ra expression on CD11b<sup>+</sup>/Gr1<sup>+</sup> cells is increased in <i>de novo</i> gliomas, we next examined the factors in the glioma microenvironment that lead to the upregulation of IL-4Ra. Bone marrow-derived cells were cultured with G-CSF (100 ng/mL), GM-CSF (250 U/mL), IL-13 (80 ng/mL), or tumor-conditioned media (TCM) from the culture of a <i>de novo</i> glioma-derived cell line for 4 days, and IL-4Ra expression was then measured by

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**Figure 3.** Depletion of CD11b<sup>+</sup>/Gr1<sup>+</sup> cells in mice bearing <i>de novo</i> gliomas. Mice bearing <i>de novo</i> gliomas received anti-Gr1 mAb. A, CD11b<sup>+</sup>/Gr1<sup>+</sup> BILs from mice receiving control rat IgG or anti-Gr1 mAb. The number in each panel indicates the percentage of CD11b<sup>+</sup>/Gr1<sup>+</sup> cells in leukocyte-gated populations. B, SFS was monitored in glioma-bearing mice treated with anti-Gr1 mAb or control IgG until day 120. C, representative of BIL showing complete regression of glioma following anti-Gr1 mAb treatment and rapid progression with control IgG treatment at weeks 4 to 7 following the tumor induction.
Figure 4. Critical role of IL-4Rx on bone marrow (BM) cells in the immunologic environment of glioma. WT mice chimeric with either WT or Il4ra−/− mouse-derived bone marrow received intracranial injections of syngeneic glioma cells. At 3 weeks after glioma cell inoculation, SPCs and BILs were harvested and analyzed by flow cytometry. A and C, CD11b+Gr1+ double-positive cells (top) and CD4+ and CD8+ cells (bottom) in SPCs (A) and BILs (C). The numbers in each histogram indicate the percentage of gated populations in leukocyte- (CD11b+Gr1+ cell analysis) or lymphocyte-gated (CD4+ and CD8+ cell analysis) cells. Data represent results from one of five spleens or a pooled BIL sample in one of two experiments with similar results. B and D, absolute numbers of CD11b+Gr1+ cells (top) and CD4+ or CD8+ cells (bottom). Bars represent the mean and SD in five spleens (B). In pooled BILs (D), bars represent the mean from each of the two independent experiments.
flow cytometry (Fig. 5A). Although G-CSF, GM-CSF, and TCM treatment, all upregulated IL-4Rα expression in three independent experiments, GM-CSF treatment had the most pronounced effect. We thus evaluated our hypothesis that the glioma microenvironment exhibits elevated levels of GM-CSF compared with normal brains or peripheral blood cells. Indeed, in both mouse de novo (Fig. 5B) and human (Fig. 5C), glioma tissues displayed higher GM-CSF expression levels compared with normal brains, contralateral brains (tested in mice only), and PBMC. When we evaluated the protein levels of GM-CSF in patient-derived glioblastoma tissues by ELISA, we found that the levels (mean of 2.85 ng/g tissue, n = 8; Fig. 5D) were very similar to those we found effective to promote IL-4Rα upregulation in mouse bone marrow-derived MDSCs in vitro (experiments in Fig. 5A using 250 U/mL; ≈2.5 ng/mL), suggesting that glioma-derived GM-CSF may be sufficient to induce IL-4Rα on myeloid cells in the glioma microenvironment.

**Human glioma-infiltrating CD14⁺ HLA-DR⁻ monocytes express IL-4Rα with suppressor functions**

As murine M-MDSCs (CD11b⁺Ly6C⁹⁹), but not G-MDSCs (CD11b⁺Ly6Chigh) express enhanced levels of Arg1 (Fig. 2B), and as it has been reported that human monocytic CD33⁺ CD14⁺ HLA-DR⁻ cells have T-cell suppressive (i.e., MDSC) functions (32–34), we next evaluated IL-4Rα expression on human glioblastoma-infiltrating (n = 7) and glioblastoma patient PBMC-derived (n = 5) CD14⁺ HLA-DR⁻ cells by flow cytometry (Fig. 6A). Although glioblastoma tumor-infiltrating leukocytes (TIL) and PBMC cannot be isolated by the same method (see Materials and Methods), using identical forward and side scatter gating on CD14⁺ HLA-DR⁻ monocytes in both types of samples, IL-4Rα was detected on 20% to 30% of CD14⁺ HLA-DR⁻ TIL, whereas IL-4Rα was barely detectable on corresponding populations in the PBMC. We further examined IL-4Rα expression on frozen glioblastoma TILs (n = 13) and glioblastoma patient-derived PBMC (n = 10; Fig. 6B, left). Consistently, all glioblastoma-infiltrating CD33⁺CD14⁺ HLA-DR⁻ cells, but not peripheral CD14⁺ HLA-DR⁻ cells had detectable IL-4Rα⁺ cell populations, including those in four matched patient samples (Fig. 6B, right).

We next addressed whether IL-4Rα expression on glioblastoma-infiltrating CD33⁺CD14⁺ HLA-DR⁻ cells was associated with immune suppressor functions. Using FACS, we isolated IL-4Rα⁺-positive and -negative subpopulations of TILs, extracted total RNA, and analyzed TGFB (Fig. 6C). IL-4Rα⁺ cells had higher TGFB expression levels than their IL-4Rα⁻ counterparts.
counterparts. Although we also analyzed the expression of ARG1 and COX2, possibly due to limited numbers of human glioblastoma-infiltrating cells, expression of these molecules was below our limit of detection in both IL-4Rx-positive and -negative CD14+ HLA-DR+ monocytes. We therefore examined IL-4Rx-positive and -negative populations in a leukapheresis-derived PBMC obtained from a patient with glioblastoma (Fig. 6D). Although there was a much smaller percentage (about 5% of CD14+ HLA-DR+ cells) of IL-4Rx+ cells compared with those in TILs, CD14+ HLA-DR+ IL-4Rx+ cells had higher levels of ARG1 and COX2 expression than their IL-4Rx− counterpart. Importantly, glioblastoma-derived CD14+ HLA-DR+ cells suppressed proliferation of autologous PBMC-derived CD8+ T cells (Fig. 6E) and IFN-γ production (Fig. 6F), indicating that these cells are indeed MDSCs in glioblastoma. These data strongly suggest that IL-4Rx+ CD14+ HLA-DR+ cells in the tumor microenvironment is important for the immunosuppressive activity of these cells.

**Discussion**

An ideal immunotherapy for gliomas would maximize the therapeutic index by both improving antitumor effector immune cell-functions and inhibiting the immune suppressor cells. Our data show for the first time, in patients with glioblastoma and the de novo murine glioma model, that IL-4Rx is upregulated on myeloid cells specifically in the tumor environment but not in the periphery. Although we addressed our main focus on CD11b+Gr1− high cells as the most abundant B1L population in the brain (Fig. 1B), we have also noted that all CD11b+Gr1− cells, including CD11b+Gr1low cells, express upregulated IL-4Rx in the glioma microenvironment compared with those cells in the nontumor-bearing brain (Supplementary Fig. S2).

Our studies using *in vitro* cultured cells and cells isolated from glioma-bearing hosts collectively suggest that GM-CSF, which is uniquely upregulated in the glioma microenvironment, induces IL-4Rx expression on myeloid cells, thereby
facilitating IL-13-induced arginase expression and resulting T-cell suppression. Our data are consistent with a previous report that GM-CSF and the GM-CSF receptor on gliomas correlates with advanced tumor stage (35). Although we did not examine mechanism of GM-CSF upregulation in human and mouse gliomas, it is possible that GM-CSF may be upregulated through an oncogenic Ras-dependent mechanism (36, 37), which was expressed in our de novo gliomas. Although Ras mutations are uncommon in human gliomas, activation of the Ras pathway is typical via signaling from receptor tyrosine kinases that are often overexpressed in human gliomas.

**IL4ra**−/− gliomas are infiltrated by significantly fewer CD11b+Gr1+ cells than gliomas in WT mice. It remains elusive whether this is due to differential mobilization from bone marrow, systemic expansion, and/or survival of these cells. On the basis of a study evaluating anti-IL-4Rα aptamer treatment (38), blockade of IL-4Rα resulted in increased apoptosis of MDSCs, suggesting that IL4ra−/− MDSCs may be more prone to apoptosis. Our bone marrow chimera experiments further revealed that the increased number of CD11b+Gr1+ cells in WT mice is intrinsic to hematopoietic cells as total body irradiated mice receiving IL4ra−/− bone marrow had fewer CD11b+Gr1+ cells than mice receiving WT bone marrow in both spleens and brains. More work is warranted to determine the precise mechanisms as to how IL4ra status impacts the generation of CD11b+Gr1+ cells systemically.

Our findings are consistent with previous reports on IL-4Rα signaling for arginase expression (14, 16, 22). Interestingly, Arg1 expression levels were significantly higher in Ly6C+ M-MDSCs than those in Ly6G+ G-MDSCs. Together with the fact that approximately 75% of CD11b+BILs were Ly6C+ cells in our mouse model (data not shown), T-cell inhibition by MDSC in our model seems to be largely mediated by arginase.

Higher levels of Tgfb were detected in de novo gliomas in WT mice compared with those in IL4ra−/− mice. Although CD11b+Ly6C+ cells expressed higher levels of Tgfb compared with CD11b+Ly6G+ populations in both WT and IL4ra−/− mice, there was not a significant difference in the Tgfb expression levels between WT and IL4ra−/− cells. This may mean that the higher Tgfb levels in the whole glioma tissue in WT mice may be attributed to the higher number of glioma-infiltrating myeloid cells in WT mice compared with IL4ra−/− mice. It is also possible that, as an indirect MDSC-mediated mechanism, other cells in the glioma environment, such as regulatory T cells, which can be induced by MDSCs, may also contribute to the higher Tgfb expression in WT gliomas (39, 40). Although the expression of Tgfb in murine MDSCs was not influenced by IL4ra expression, human IL-4Rα–positive cells expressed elevated levels of TGFβ compared with IL-4Rα–negative cells. Our group and others have previously shown the immune-suppressive role of TGFβ and its impact on gliomas (41) and other cancers (16). Further studies are warranted to understand the differential regulation of TGFβ expression between murine and human MDSCs.

Our finding that IL4ra−/− mice have prolonged SFS compared with WT mice in the absence of T cells suggests that cells other than T cells are also important for the prolonged survival of IL4ra−/− mice bearing de novo gliomas. Thus, it is important to note that in the absence of T cells, IL-4Rα−/− myeloid cells may exert suppressive functions on other immune cells, such as natural killer cells, or possibly promotion of tumor cell growth via nonimmunologic mechanisms, such as enhancement of angiogenesis. It is also noteworthy that, although the median survival of mice treated with anti-Gr1 mAb was shorter than that of IL4ra−/− mice, 3 of 7 animals receiving anti-Gr1 mAb for depletion of Gr1+ cells survived for longer than 120 days. Although IL-4Rα plays a critical role in MDSCs, based on the partial abrogation of the MDSC-mediated T-cell suppression in IL4ra−/− mice (Supplementary Fig. S5), it is likely MDSCs have other non-IL-4Rα-dependent mechanisms of immune suppression, and thus the depletion of Gr1+ cells may have more robust impacts than the disruption of IL4ra.

In humans, healthy donor-derived human CD14+ monocytes exposed to glioma cells acquire MDSC-like properties, including increased production of IL-10, TGF-β, and B7-H1, and a heightened ability to induce apoptosis in activated lymphocytes (42). Patients with glioblastoma have more circulating CD33+/HLA-DR+ MDSCs in their peripheral blood than do normal donors (42, 43). Furthermore, significant increases in arginase 1 activity levels have been observed in plasma of patients with glioblastoma (43, 44). Interestingly, T-cell suppression in glioblastoma was completely reversed through the pharmacologic inhibition of arginase 1 or with arginine supplementation (44). In regard to MDSC subpopulations, a recent study examining six MDSC subpopulations in patients with renal cell cancer identified two subtypes, CD14+HLA-DR− and CD11b+CD14+CD15+ cells, negatively associated with overall patient survival (45). In the current study, as the vast majority (~75%) of the MDSCs in mouse Sleeping Beauty gliomas are Ly6C+ M-MDSCs expressing Arg1, we focused on the monocytic CD14+HLA-DR+ population in human glioblastoma. Although glioblastoma is densely infiltrated by microglia/macrophages (19, 46), to our knowledge, our current study is one of the first to characterize the phenotype and function of MDSCs in human gliomas.

Our findings show a novel mechanism of immunosuppression in the glioma microenvironment. GM-CSF, which is expressed at high levels in both human and mouse gliomas, promotes IL-4Rα expression on glioma-infiltrating myeloid cells with MDSC properties, thereby leading to IL-13–mediated production of arginase. Arginase can then suppress antitumor immune cells, including T cells, thereby promoting the development of glioma growth.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

**Conception and design:** G. Kohanbash, A. Mintz, N. Amankulor, M. Fujita, H. Okada

**Development of methodology:** G. Kohanbash, K. McKavey, A. Mintz, N. Amankulor, M. Fujita, J.R. Ohlheisz, H. Okada

**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** G. Kohanbash

**BILs** were high in both human and mouse gliomas, promoting IL-4Rα expression on glioma-infiltrating myeloid cells with MDSC properties, thereby leading to IL-13–mediated production of arginase. Arginase can then suppress antitumor immune cells, including T cells, thereby promoting the development of glioma growth.

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