



Published in final edited form as:

Cancer Res. 2013 November 1; 73(21): . doi:10.1158/0008-5472.CAN-12-4124.

GM-CSF promotes the immunosuppressive activity of glioma-infiltrating myeloid cells through interleukin-4 receptor- α

Gary Kohanbash^{1,2,3}, Kayla McKaveney², Masashi Sakaki², Ryo Ueda², Arlan H. Mintz^{2,3}, Nduka Amankolor^{2,3}, Mitsugu Fujita^{2,3}, John R. Ohlfest⁶, and Hideho Okada^{2,3,4,5,§}

¹Department of Infectious Diseases and Microbiology, University of Pittsburgh Graduate School of Public Health, A419 Crabtree Hall, Pittsburgh, PA 15213

²Brain Tumor Program, University of Pittsburgh Cancer Institute, 1.19E Hillman Cancer Center, 5117 Centre Ave, Pittsburgh, PA, 15213

³Department of Neurological Surgery, University of Pittsburgh School of Medicine, 200 Lothrop Street, Pittsburgh, PA, 15213

⁴Department of Surgery, University of Pittsburgh School of Medicine, 200 Lothrop Street, Pittsburgh, PA, 15213

⁵Department of Immunology, University of Pittsburgh School of Medicine, 200 Lothrop Street, Pittsburgh, PA, 15213

⁶Department of Pediatrics, University of Minnesota, Minneapolis, MN 55455

Abstract

Malignant gliomas are lethal cancers in the brain and heavily infiltrated by myeloid cells. Interleukin-4 receptor- (IL-4R) mediates the immunosuppressive functions of myeloid cells, and polymorphisms in the IL-4R gene are associated with altered glioma risk and prognosis. In this study, we sought to evaluate an hypothesized causal role for IL-4R and myeloid suppressor cells in glioma development. In both mouse de novo gliomas and human glioblastoma cases, IL-4R was upregulated on glioma-infiltrating myeloid cells but not in the periphery or in normal brain. Mice genetically deficient for IL-4R 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-4R mediates IL-13 -induced production of arginase. Furthermore, glioma-derived myeloid cells suppressed T cell proliferation in an IL-4R -dependent manner, consistent with their identification as myeloid-derived suppressor cells. Granulocyte-macrophage colony-stimulating factor (GM-CSF) plays a central role for the induction of IL-4R 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-4R on myeloid-derived suppressor cells.

Keywords

MDSC; Glioma; IL-4R ; Arginase; Immunosuppression

[§]Corresponding author. Request for reprints: Hideho Okada, 1.19E Research Pavilion at Hillman Cancer Center, 5117 Centre Avenue, Pittsburgh, PA 15213. Phone: 412.623.1111; Fax: 412.623.1415; okadah@upmc.edu.

The authors have no conflict of interest to report.

INTRODUCTION

Malignant gliomas represent approximately 80% of all malignant brain tumors accounting for as many as 26,000 U.S. and European deaths annually, making them a significant unmet medical need (1). Prognosis for malignant glioma patients remain dismal with a median survival of approximately 15 months for glioblastoma following surgery and chemo/radiation therapy (2). Despite extensive research, treatment options for malignant gliomas remain limited. While immunotherapeutic approaches have demonstrated safety and promising preliminary activities (3), their effectiveness can be improved by overcoming the immuno-suppressive mechanisms induced by these tumors (2).

Myeloid cells are the most abundant hematopoietic cells in the human body and have diverse functions. Mounting evidence indicates that the tumor microenvironment alters myeloid cells, and the concept of myeloid-derived suppressor cells (MDSCs) has emerged (4, 5). MDSCs represent a heterogenic population of immature myeloid cells (IMCs) with an impaired ability to fully develop into macrophages, granulocytes, or dendritic cells and have highly pleiotropic abilities to suppress a variety of T-cell functions and promote tumor growth through effector molecules including arginase (4, 5).

In mice, MDSCs are identified as cells that simultaneously express the two markers CD11b and Gr1 (6-8), and are subdivided into two different subsets based on their expression of the two molecules Ly6C and Ly6G (4). CD11b⁺Ly6G⁻Ly6C^{high} cells have monocytic-like morphology and are termed monocytic-MDSCs (M-MDSCs), while CD11b⁺Ly6G⁺Ly6C^{low} cells have granulocyte-like morphology and are termed granulocytic-MDSCs (G-MDSCs). In cancer patients, MDSCs are defined as cells that express the common myeloid marker CD33 but lack markers of mature myeloid cells, such as the human leukocyte antigen (HLA)-DR (9-13). Human MDSCs can be divided into at least two subsets that likely parallel those in the mouse model: the CD15⁺ G-MDSCs and the CD14⁺ M-MDSCs. IL-4R expression on 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 (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 (SNPs) in IL-4R are associated with altered glioma risk and prognosis (20, 21), suggesting a possibility that IL-4R expression on myeloid cells may impact the glioma development. We therefore sought to determine whether IL-4R expression on myeloid cells plays a role in glioma development. Here we demonstrate, using a *de novo* glioma model and human malignant glioma tissues, GM-CSF, which is expressed at high levels in the glioma microenvironment, leads to up-regulation of IL-4R on CD11b⁺Gr1⁺ IMCs, thereby promoting the induction of arginase via IL-13. Our data demonstrate a novel immuno-suppressive 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 per an Institutional Animal Care and Use Committee-approved protocol.

Bone Marrow (BM)-MDSC Generation

A similar procedure has been previously described (22, 23). Briefly, red blood cell (RBC) depleted BM cells were isolated from WT or *I4ra*^{-/-} 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 (80 ng/ml) on days 4 and 9. All cytokines were purchased from Peprotech. CD11b⁺ cells were positively selected on day 10 and used in further experiments.

Arginase Activity Assay

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

MDSC-mediated T-cell Inhibition

CD8⁺ T-cells were isolated from WT BALB/c splenocytes (SPCs) using magnetic bead negative separation (Miltenyi Biotec), labeled with 100nM CFDA SE (Invitrogen) and incubated with varying amounts of day 10 cultured BM- or glioma-derived MDSCs for 5 days in the presence of anti-CD3/anti-CD28 Dynabeads (Invitrogen) and 30U/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 (mAbs) were obtained from Taconic; control IgG was obtained from Sigma-Aldrich. Mice with developing gliomas received i.p. injections of anti-Gr1 (0.25 mg/dose) 3x/week or anti-CD4 and anti-CD8 (0.5mg/dose) 2x/week starting on day 21 after induction of *de novo* glioma.

Real-time (RT)-PCR

The procedure has been described previously (7, 8). Primers and probes were obtained from Applied Biosystems. Human or mouse *GAPDH* was used as an internal control. All reactions were done in triplicate and relative expressions of RNAs compared to control samples were calculated using the C_T method.

Induction of *de novo* Gliomas by Intraventricular Transfection of Sleeping Beauty-Transposon-flanked Proto-Oncogenes

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-EGFRvIII (0.125 µg for each). For immunological evaluation of WT and *I4ra*^{-/-} tumors, we conducted bioluminescence imaging (BLI) using an IVIS200 (Caliper Life Sciences) and evaluated tumors of comparable size (BLI of 2×10⁸ luciferase units).

BM Chimera

BM chimera experiments were conducted as previously described (25). Briefly, RBC depleted BM cells were isolated from donor WT or *I4ra*^{-/-} mice. Host BALB/c-background WT mice received 10 Gy of total body irradiation followed by tail vein injection of 1 × 10⁶ viable BM cells. The efficiency of our BM chimera protocol was confirmed to be >96% using donor BM cells derived from enhanced green fluorescent protein (EGFP)-transgenic mice (Supplementary Figure 1).

Isolation of Murine Brain Infiltrating Leukocytes (BILs)

BILs were isolated using methods described previously (7, 26) using the Percoll (Sigma-Aldrich) isolation method. Due to 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 (PBMC)

De-identified fresh glioma tissues were obtained from the operating room per IRB-approved protocol, mechanically minced, resuspended in 70% Percoll (Sigma-Aldrich), overlaid with 37% and 30% Percoll, and centrifuged for 20 min at $500 \times g$. Enriched leukocyte populations were recovered at the 70%-37%. 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's 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-4R on glioma growth, we induced *de novo* gliomas by *Sleeping Beauty* (*SB*) transposon-mediated intraventricular transfection of the oncogenes *EGFRvIII*, *NRas*, and short hairpin (*sh*)*P53* in neonatal BALB/c-background WT and *Il4r*^{-/-} mice (hereby *SB* glioma). While the median symptom-free survival (SFS) for WT mice was 55.5 days, *Il4r*^{-/-} mice exhibited prolonged survival with a median SFS of 90 days ($p < 0.001$) (Figure 1A). As IL-4R is expressed on some MDSCs (15-17), we next evaluated whether the genetic deletion of *Il4r* impacts the glioma infiltration of myeloid cells, such as CD11b⁺Gr1⁺ cells, which are likely MDSC (Figure 1B and 1C). In WT mice, *SB* glioma-bearing brains demonstrated higher numbers of CD11b⁺Gr1⁺ cells compared with non-tumor bearing brains. Further, in the presence of the *SB* gliomas, WT CD11b⁺Gr1⁺ contained a higher percentage of IL-4R expressing cells than those in non-tumor bearing animals. Compared with WT animals, non-tumor bearing brains of *Il4ra*^{-/-} hosts had significantly fewer numbers of CD11b⁺Gr1⁺ cells which did not increase significantly in the presence of the *SB* tumor (Figure 1B and 1C).

To determine whether the different expression levels of IL-4R and numbers of CD11b⁺Gr1⁺ cells in tumor-bearing hosts are also seen in periphery, we analyzed SPCs derived from *SB* glioma-bearing mice (Supplementary Figure 2). Similar to our observation in the brain, WT but not *Il4r*^{-/-} hosts demonstrated an increase of CD11b⁺Gr1⁺ cells in the spleen following induction of *SB* glioma. However, unlike the brain, IL-4R expression levels on peripheral CD11b⁺Gr1⁺ cells remained low even in tumor-bearing animals. Thus IL-4R expression on CD11b⁺Gr1⁺ cells appears to be relatively limited to the cells infiltrating in the gliomas.

T-cells deficient of IL-4R or its major signaling molecule signal transducer and activator of transcription (STAT)-6 are typically skewed towards type-1 immune response, which is known to promote anti-tumor immunity (2, 27-29). To exclude a possibility that the prolonged survival of *Il4r*^{-/-} mice is due solely to enhanced anti-tumor T-cell response, we induced *SB* gliomas in WT and *Il4r*^{-/-} hosts in which CD4⁺ and CD8⁺ T-cells were

depleted (Figure 1D) and Supplementary Figure 3). Although depletion of T-cells significantly accelerated the growth of gliomas in both WT and *Il4ra*^{-/-} mice, *Il4ra*^{-/-} mice still demonstrated improved SFS over WT mice when both were depleted of T-cells. These data demonstrate that the improved survival of *Il4ra*^{-/-} mice is at least partially independent of T-cells.

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

To examine the impact of IL-4R on the glioma microenvironment, total RNA was extracted from WT or *Il4ra*^{-/-} *de novo* gliomas of similar size, and inflammation-associated genes were evaluated by RT-PCR (Figure 2A). The gliomas in WT mice demonstrated significantly higher levels of immunosuppressive *Tgfb* and *Arg1* than those in *Il4ra*^{-/-} mice. Notably, while similar levels of IL-13 were detected in WT and *Il4ra*^{-/-} tumors, IL-4 expression was undetectable.

To better understand the significance of IL-4R expression in CD11b⁺Gr1⁺ cells in the glioma, we isolated two subsets of CD11b⁺Gr1⁺ cells by fluorescence-activated cell sorting (FACS), CD11b⁺Ly6C^{high} monocytic cells and CD11b⁺Ly6G^{high} granulocytic cells, and analyzed MDSC-associated genes by RT-PCR (Figure 2B). While CD11b⁺Ly6C^{high} cells expressed higher levels of both *Tgfb* and *Arg1* than CD11b⁺Ly6G^{high} cells, *Arg1* expression was significantly lower in *Il4ra*^{-/-} CD11b⁺Ly6C^{high} cells than WT counterparts. These data suggest a significant role of IL-4R 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. While there are multiple methods to deplete CD11b⁺Gr1⁺ cells, such as with chemotherapeutics Sunitinib or Gemcitabine (30, 31), these also could have direct anti-tumor activities. Thus, we used anti-Gr1 (RB6-8C5) monoclonal mAb which efficiently depleted CD11b⁺Gr1⁺ cells in *de novo* glioma models in our previous studies (7, 8). To maintain complete depletion, we administered 50 mg/dose anti-Gr1 mAb 3x/week starting at day 23 (Figure 3A) (7, 8). Mice depleted of CD11b⁺Gr1⁺ cells experienced significantly prolonged SFS (Figure 3B) with 3 of 7 animals surviving past day 120 (median survival of 74 days), while all control mice treated with control isotype IgG died by day 68 (median survival of 55.5 days). BLI revealed that some (n=3) mice treated with anti-Gr1 mAb even experienced tumor regression below the level of detection (Figure 3C). These data demonstrate the importance of CD11b⁺Gr1⁺ cells in the development of *de novo* glioma.

BM chimeric mice reveal that *Il4ra* on BM cells is critical for accumulation of CD11b⁺Gr1⁺ cells in the brain

As we demonstrated in Figure 1, gliomas in *Il4ra*^{-/-} mice had fewer infiltrating CD11b⁺Gr1⁺ cells than in WT controls. To assess whether the difference was specifically due to intrinsic factors in BM cells, we evaluated the impact of *Il4ra* status on glioma-infiltration of CD11b⁺Gr1⁺ cells using a BM chimera system. Because induction of *de novo* gliomas is possible in neonatal mice only but not adult mice (24), as an alternative glioma model, BM chimera mice received stereotactic injections of cultured glioma cells established from syngeneic *de novo* glioma. In both SPCs and BILs, mice with WT BM displayed greater numbers of CD11b⁺Gr1⁺ cells but lower numbers of CD4⁺ and CD8⁺ T-cells compared with ones with *Il4ra*^{-/-} BM (Figure 4). These data suggest that IL-4R expression on BM-derived cells promotes the systemic distribution of CD11b⁺Gr1⁺ cells but may inhibit that of T-cells.

IL-4R α signaling promotes the T-cell-suppressing function of BM-derived and glioma-infiltrating CD11b⁺Gr1⁺ cells

Using mouse CD11b⁺Gr1⁺ cells derived from BM of WT or *Il4ra*^{-/-} mice, we first confirmed previously reported observations (6, 14) that the IL-13-IL-4R signaling mediates T-cell suppressing activities of IMCs via induction of arginase (Supplementary Figure 4). Notably, WT CD11b⁺Gr1⁺ cells suppressed T-cell proliferation and IFN- γ levels at lower BM-CD11b⁺Gr1⁺:T-cell ratios than *Il4ra*^{-/-} CD11b⁺Gr1⁺ cells. Furthermore, supplementation with an arginase inhibitor N^W-hydroxy-nor-arginine or L-arginine inhibited the T-cell suppressing activity of WT CD11b⁺Gr1⁺ cells. When we isolated CD11b⁺ cells from gliomas growing in the brain of WT or *Il4ra*^{-/-} mice, WT mouse-derived myeloid cells demonstrated more profound levels of inhibition on CD8⁺ T-cell proliferation compared with ones derived from *Il4ra*^{-/-} mice (Supplementary Figure 5). These data indicate that CD11b⁺Gr1⁺ BILs are indeed capable of suppressing T-cell proliferation in an IL-4R dependent manner. We hereby term CD11b⁺Gr1⁺ BILs MDSCs in the glioma environment.

GM-CSF up-regulates IL-4R α on BM cells and is overexpressed in gliomas

As IL-4R expression on CD11b⁺Gr1⁺ cells is increased in *de novo* gliomas, we next examined the factors in the glioma microenvironment that lead to the up-regulation of IL-4R. BM-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 *de novo* glioma-derived cell line for 4 days, and IL-4R expression was then measured by flow cytometry (Figure 5A). While G-CSF, GM-CSF and TCM treatment all up-regulated IL-4R 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* (Figure 5B) and human (Figure 5C) glioma tissues displayed higher GM-CSF expression levels compared with normal brains, contralateral brains (tested in mice only) and PBMC. When we evaluated protein levels of GM-CSF in patient-derived glioblastoma tissues by ELISA, we found that the levels (mean of 2.85 ng/gram tissue, n=8) (Figure 5D) were very similar to those we found effective to promote IL-4R up-regulation in mouse BM-derived MDSCs *in vitro* [experiments in Figure 5A using 250 U/ml (\approx 2.5 ng/ml)], suggesting that glioma-derived GM-CSF may be sufficient to induce IL-4R on myeloid cells in the glioma microenvironment.

Human Glioma-infiltrating CD14⁺HLA-DR⁻ monocytes express IL-4R α associated with suppressor functions

As murine M-MDSCs (CD11b⁺Ly6C^{high}), but not G-MDSCs (CD11b⁺Ly6G^{high}) express enhanced levels of *Arg1* (Figures 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-4R expression on human glioblastoma-infiltrating (n=7) and glioblastoma patient PBMC-derived (n=5) CD14⁺HLA-DR⁻ cells by flow cytometry (Figure 6A). Although glioblastoma tumor-infiltrating leukocytes (TILs) 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-4R was detected on 20-30% of CD14⁺HLA-DR⁻ TIL while IL-4R was barely detectable on corresponding populations in the PBMC. We further examined IL-4R expression on frozen glioblastoma TILs (n=13) and glioblastoma-patient derived PBMC (n=10) (Figure 6B left). Consistently, all glioblastoma-infiltrating CD33⁺CD14⁺HLA-DR⁻ cells, but not peripheral CD14⁺HLA-DR⁻ cells had detectable IL-4R⁺ cell populations, including those in 4 matched patient samples (Figure 6B right).

We next addressed whether IL-4R⁺ expression on glioblastoma-infiltrating CD33⁺CD14⁺HLA-DR⁻ cells was associated with immune suppressor functions. Using FACS, we isolated IL-4R⁺-positive and -negative sub populations of TILs, extracted total RNA and analyzed *TGFB* (Figure 6C). IL-4R⁺ cells had higher *TGFB* expression levels than their IL-4R⁻ counterparts. Although we also analyzed 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-4R⁺ positive and negative CD14⁺HLA-DR⁻ monocytes. We therefore examined IL-4R⁺-positive and negative populations in a leukapheresis-derived PBMC obtained from a glioblastoma patient (Figure 6D). Although there was a much smaller percentage (about 5% of CD14⁺HLA-DR⁻ cells) of IL-4R⁺ cells compared with those in TILs, CD14⁺HLA-DR⁻ IL-4R⁺ cells had higher levels of *ARG1* and *COX2* expression than their IL-4R⁻ counterpart. Importantly, glioblastoma-derived CD14⁺HLA-DR⁻ cells suppressed proliferation of autologous PBMC-derived CD8⁺ T-cells (Figure 6E) and IFN- γ production (Figure 6F), indicating that these cells are indeed MDSCs in glioblastoma. These data strongly suggest that IL-4R⁺ on 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 anti-tumor effector immune cell-functions and inhibiting the immune suppressor cells. Our data demonstrate for the first time, in glioblastoma patients and the *de novo* murine glioma model, that IL-4R⁺ is up-regulated on myeloid cells specifically in the tumor environment but not in the periphery. While we addressed our main focus on CD11b⁺Gr1^{+/high} cells as the most abundant BIL population in the brain (Figure 1B), we have also noted that all CD11b⁺Gr1⁺ cells, including CD11b⁺Gr1^{low} cells, express up-regulated IL-4R⁺ in the glioma microenvironment compared with those cells in the non-tumor bearing brain (Supplementary Figure 2).

Our studies using *in vitro* cultured cells and cells isolated from glioma-bearing hosts collectively suggest that GM-CSF, which is uniquely up-regulated in the glioma microenvironment, induces IL-4R⁺ 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). While we did not examine mechanism of GM-CSF up-regulation in human and mouse gliomas, it is possible that GM-CSF may be up-regulated 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 BM, systemic expansion and/or survival of these cells. Based on a study evaluating anti-IL-4R⁺ aptamer treatment (38), blockade of IL-4R⁺ resulted in increased apoptosis of MDSCs, suggesting that *Il4ra*^{-/-} MDSCs may be more prone to apoptosis. Our BM 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*^{-/-} BM had fewer CD11b⁺Gr1⁺ cells than mice receiving WT BM in both spleens and brains. More work is warranted to determine the precise mechanisms as to how *Il4ra* status impact the generation of CD11b⁺Gr1⁺ cells systemically.

Our findings are consistent with previous reports on IL-4R signaling for arginase expression (14, 16, 22). Interestingly, *Arg1* expression levels were significantly higher in Ly6C⁺ monocytic MDSCs than those in Ly6G⁺ granulocytic 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 appears 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. While 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 to *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). While the expression of *Tgfb* in murine MDSCs was not influenced by *Il4ra* expression, human IL-4R positive cells expressed elevated levels of *TGFB* compared with IL-4R negative cells. Our group and others have previously demonstrated the immune-suppressive role of *TGFB* and its impact on gliomas (41) and other cancers (16). Further studies are warranted to understand the differential regulation of *TGF*- 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-4R⁺ myeloid cells may exert suppressive functions on other immune cells, such as NK cells, or possibly promotion of tumor cell growth via non-immunological 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. While IL-4R plays a critical role in MDSCs, based on the partial abrogation of the MDSC-mediated T-cell suppression in *Il4ra*^{-/-} mice (Supplementary Figure 5), it is likely MDSCs have other non-IL-4R-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 glioblastoma patients (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 6 MDSC subpopulations in renal cell cancer patients identified 2 subtypes, CD14⁺HLA-DR^{-/lo} and CD11b⁺CD14⁻CD15⁺ cells, negatively associated with overall patients survival (45). In the current study, as the vast majority (approximately 75%) of the MDSCs in mouse SB gliomas are Ly6C⁺ monocytic 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 demonstrate a novel mechanism of immuno-suppression in the glioma microenvironment. GM-CSF, which is expressed at high levels in both human and mouse gliomas, promotes IL-4R expression on glioma-infiltrating myeloid cells with MDSC-properties, thereby leading to IL-13-mediated production of arginase. Arginase can then suppress anti-tumor immune cells, including T-cells, thereby promoting the development of glioma growth.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors would like to thank the following for their assistance: Dr. Masaki Terabe and Maria Sierra.

Grant Supports from: The NIH [2R01 NS055140, 2P01 NS40923, 1P01 CA132714] and Musella Foundation for Brain Tumor Research and Information. This project used UPCI shared resources (Animal Facility, Small Animal Imaging facility and Cytometry Facility) that are supported in part by NIH P30CA047904.

Reference List

1. Vredenburgh JJ, Desjardins A, Reardon DA, Friedman HS. Experience with irinotecan for the treatment of malignant glioma. *Neuro Oncol.* 2009; 11:80–91. [PubMed: 18784279]
2. Okada H, Kohanbash G, Zhu X, Kastenhuber ER, Hoji A, Ueda R, et al. Immunotherapeutic approaches for glioma. *Crit Rev Immunol.* 2009; 29:1–42. [PubMed: 19348609]
3. Okada H, Kalinski P, Ueda R, Hoji A, Kohanbash G, Donegan TE, et al. Induction of CD8+ T-Cell Responses Against Novel Glioma-Associated Antigen Peptides and Clinical Activity by Vaccinations With {alpha}-Type 1 Polarized Dendritic Cells and Polyinosinic-Polycytidylic Acid Stabilized by Lysine and Carboxymethylcellulose in Patients With Recurrent Malignant Glioma. *J Clin Oncol.* 2011; 29:330–6. [PubMed: 21149657]
4. Youn JI, Nagaraj S, Collazo M, Gabrilovich DI. Subsets of myeloid-derived suppressor cells in tumor-bearing mice. *Journal of Immunology.* 2008; 181:5791–802.
5. Youn J-I, Gabrilovich DI. The biology of myeloid-derived suppressor cells: The blessing and the curse of morphological and functional heterogeneity. *European Journal of Immunology.* 2010; 40:2969–75. [PubMed: 21061430]
6. Gabrilovich DI, Nagaraj S. Myeloid-derived suppressor cells as regulators of the immune system. *Nat Rev Immunol.* 2009; 9:162–74. [PubMed: 19197294]
7. Fujita M, Kohanbash G, Fellows-Mayle W, Hamilton RL, Komohara Y, Decker SA, et al. COX-2 blockade suppresses gliomagenesis by inhibiting myeloid-derived suppressor cells. *Cancer Res.* 2011; 71:2664–74. [PubMed: 21324923]
8. Fujita M, Scheurer ME, Decker SA, McDonald HA, Kohanbash G, Kastenhuber ER, et al. Role of Type 1 IFNs in Antiglioma Immunosurveillance—Using Mouse Studies to Guide Examination of Novel Prognostic Markers in Humans. *Clinical Cancer Research.* 2010; 16:3409–19. [PubMed: 20472682]
9. Almand B, Clark JI, Nikitina E, van BJ, English NR, Knight SC, et al. Increased production of immature myeloid cells in cancer patients: a mechanism of immunosuppression in cancer. *The Journal of Immunology.* 2001; 166:678–89. [PubMed: 11123353]
10. Schmielau J, Finn OJ. Activated granulocytes and granulocyte-derived hydrogen peroxide are the underlying mechanism of suppression of t-cell function in advanced cancer patients. *Cancer Res.* 2001; 61:4756–60. [PubMed: 11406548]
11. Zea AH, Rodriguez PC, Atkins MB, Hernandez C, Signoretti S, Zabaleta J, et al. Arginase-producing myeloid suppressor cells in renal cell carcinoma patients: a mechanism of tumor evasion. *Cancer Res.* 2005; 65:3044–8. [PubMed: 15833831]
12. Filipazzi P, Valenti R, Huber V, Pilla L, Canese P, Iero M, et al. Identification of a new subset of myeloid suppressor cells in peripheral blood of melanoma patients with modulation by a

- granulocyte-macrophage colony-stimulation factor-based antitumor vaccine. *J Clin Oncol.* 2007; 25:2546–53.
13. Hoechst B, Ormandy LA, Ballmaier M, Lehner F, Kruger C, Manns MP, et al. A new population of myeloid-derived suppressor cells in hepatocellular carcinoma patients induces CD4(+)CD25(+)Foxp3(+) T cells. *Gastroenterology.* 2008; 135:234–43. [PubMed: 18485901]
 14. Gallina G, Dolcetti L, Serafini P, De Santo C, Marigo I, Colombo MP, et al. Tumors induce a subset of inflammatory monocytes with immunosuppressive activity on CD8+ T cells. *The Journal of clinical investigation.* 2006; 116:2777–90. [PubMed: 17016559]
 15. Mandruzzato S, Solito S, Falisi E, Francescato S, Chiarion-Sileni V, Mocellin S, et al. IL4Ralpha+ myeloid-derived suppressor cell expansion in cancer patients. *J Immunol.* 2009; 182:6562–8. [PubMed: 19414811]
 16. Terabe M, Matsui S, Park JM, Mamura M, Noben-Trauth N, Donaldson DD, et al. Transforming growth factor-beta production and myeloid cells are an effector mechanism through which CD1d-restricted T cells block cytotoxic T lymphocyte-mediated tumor immunosurveillance: abrogation prevents tumor recurrence. *J Exp Med.* 2003; 198:1741–52. [PubMed: 14657224]
 17. Ostrand-Rosenberg S, Sinha P. Myeloid-derived suppressor cells: linking inflammation and cancer. *J Immunol.* 2009; 182:4499–506. [PubMed: 19342621]
 18. Kohanbash G, Okada H. Myeloid-derived suppressor cells in gliomas and glioma-development. *Immunol Invest.* 2012; 41:658–79. [PubMed: 23017140]
 19. Yang I, Han SJ, Kaur G, Crane C, Parsa AT. The role of microglia in central nervous system immunity and glioma immunology. *Journal of Clinical Neuroscience.* 2010; 17:6–10. [PubMed: 19926287]
 20. Schwartzbaum JA, Ahlbom A, Lonn S, Malmer B, Wigertz A, Auvinen A, et al. An international case-control study of interleukin-4Ralpha, interleukin-13, and cyclooxygenase-2 polymorphisms and glioblastoma risk. *Cancer EpidemiolBiomarkers Prev.* 2007; 16:2448–54.
 21. Scheurer ME, Amirian E, Cao Y, Gilbert MR, Aldape KD, Kornguth DG, et al. Polymorphisms in the interleukin-4 receptor gene are associated with better survival in patients with glioblastoma. *ClinCancer Res.* 2008; 14:6640–6.
 22. Highfill SL, Rodriguez PC, Zhou Q, Goetz CA, Koehn BH, Veenstra R, et al. Bone marrow myeloid-derived suppressor cells (MDSCs) inhibit graft-versus-host disease (GVHD) via an arginase-1-dependent mechanism that is up-regulated by interleukin-13. *Blood.* 2010; 116:5738–47. [PubMed: 20807889]
 23. Marigo I, Bosio E, Solito S, Mesa C, Fernandez A, Dolcetti L, et al. Tumor-induced tolerance and immune suppression depend on the C/EBPbeta transcription factor. *Immunity.* 2010; 32:790–802. [PubMed: 20605485]
 24. Wiesner SM, Decker SA, Larson JD, Ericson K, Forster C, Gallardo JL, et al. De novo induction of genetically engineered brain tumors in mice using plasmid DNA. *Cancer Res.* 2009; 69:431–9. [PubMed: 19147555]
 25. Epperly MW, Shields D, Niu Y, Carlos T, Greenberger JS. Bone marrow from CD18^{-/-} (MAC-1^{-/-}) homozygous deletion recombinant negative mice demonstrates increased longevity in long-term bone marrow culture and decreased contribution to irradiation pulmonary damage. *In Vivo.* 2006; 20:431–8. [PubMed: 16900771]
 26. Zhu X, Fallert-Junecko BA, Fujita M, Ueda R, Kohanbash G, Kasthuber ER, et al. Poly-ICLC promotes the infiltration of effector T cells into intracranial gliomas via induction of CXCL10 in IFN-alpha and IFN-gamma dependent manners. *Cancer Immunol Immunother.* 2010; 59:1401–9. [PubMed: 20549206]
 27. Fujita M, Zhu X, Ueda R, Sasaki K, Kohanbash G, Kasthuber ER, et al. Effective Immunotherapy against Murine Gliomas Using Type 1 Polarizing Dendritic Cells--Significant Roles of CXCL10. *Cancer Res.* 2009; 69:1587–95. [PubMed: 19190335]
 28. Okada H. Brain tumor immunotherapy with type-1 polarizing strategies. *Ann N Y Acad Sci.* 2009; 1174:18–23. [PubMed: 19769732]
 29. Sasaki K, Pardee AD, Qu Y, Zhao X, Ueda R, Kohanbash G, et al. IL-4 suppresses very late antigen-4 expression which is required for therapeutic Th1 T-cell trafficking into tumors. *J Immunother.* 2009; 32:793–802. [PubMed: 19752754]

30. Ko JS, Zea AH, Rini BI, Ireland JL, Elson P, Cohen P, et al. Sunitinib mediates reversal of myeloid-derived suppressor cell accumulation in renal cell carcinoma patients. *Clin Cancer Res.* 2009; 15:2148–57. [PubMed: 19276286]
31. Le HK, Graham L, Cha E, Morales JK, Manjili MH, Bear HD. Gemcitabine directly inhibits myeloid derived suppressor cells in BALB/c mice bearing 4T1 mammary carcinoma and augments expansion of T cells from tumor-bearing mice. *Int Immunopharmacol.* 2009; 9:900–9. [PubMed: 19336265]
32. Lin Y, Gustafson MP, Bulur PA, Gastineau DA, Witzig TE, Dietz AB. Immunosuppressive CD14+HLA-DR(low)- monocytes in B-cell non-Hodgkin lymphoma. *Blood.* 2011; 117:872–81. [PubMed: 21063024]
33. Vuk-Pavlovic S, Bulur PA, Lin Y, Qin R, Szumlanski CL, Zhao X, et al. Immunosuppressive CD14+HLA-DRlow/- monocytes in prostate cancer. *Prostate.* 2010; 70:443–55. [PubMed: 19902470]
34. Poschke I, Mougiakakos D, Hansson J, Masucci GV, Kiessling R. Immature immunosuppressive CD14+HLA-DR-/low cells in melanoma patients are Stat3hi and overexpress CD80, CD83, and DC-sig. *Cancer Res.* 2010; 70:4335–45. [PubMed: 20484028]
35. Mueller MM, Herold-Mende CC, Riede D, Lange M, Steiner HH, Fusenig NE. Autocrine growth regulation by granulocyte colony-stimulating factor and granulocyte macrophage colony-stimulating factor in human gliomas with tumor progression. *Am J Pathol.* 1999; 155:1557–67. [PubMed: 10550313]
36. Pylayeva-Gupta Y, Lee KE, Hajdu CH, Miller G, Bar-Sagi D. Oncogenic Kras-induced GM-CSF production promotes the development of pancreatic neoplasia. *Cancer Cell.* 2012; 21:836–47. [PubMed: 22698407]
37. Wang J, Liu Y, Li Z, Du J, Ryu MJ, Taylor PR, et al. Endogenous oncogenic Nras mutation promotes aberrant GM-CSF signaling in granulocytic/monocytic precursors in a murine model of chronic myelomonocytic leukemia. *Blood.* 2010; 116:5991–6002. [PubMed: 20921338]
38. Roth F, De La Fuente AC, Vella JL, Zoso A, Inverardi L, Serafini P. Aptamer-Mediated Blockade of IL4Ralpha Triggers Apoptosis of MDSCs and Limits Tumor Progression. *Cancer Res.* 2012; 72:1373–83. [PubMed: 22282665]
39. Nakamura K, Kitani A, Strober W. Cell contact-dependent immunosuppression by CD4(+)/CD25(+) regulatory T cells is mediated by cell surface-bound transforming growth factor beta. *J Exp Med.* 2001; 194:629–44. [PubMed: 11535631]
40. Ostrand-Rosenberg S. Myeloid-derived suppressor cells: more mechanisms for inhibiting antitumor immunity. *Cancer Immunol Immunother.* 2010; 59:1593–600. [PubMed: 20414655]
41. Ueda R, Fujita M, Zhu X, Sasaki K, Kasthuber ER, Kohanbash G, et al. Systemic inhibition of transforming growth factor-beta in glioma-bearing mice improves the therapeutic efficacy of glioma-associated antigen peptide vaccines. *Clin Cancer Res.* 2009; 15:6551–9. [PubMed: 19861464]
42. Rodrigues JC, Gonzalez GC, Zhang L, Ibrahim G, Kelly JJ, Gustafson MP, et al. Normal human monocytes exposed to glioma cells acquire myeloid-derived suppressor cell-like properties. *Neuro Oncol.* 2010; 12:351–65. [PubMed: 20308313]
43. Raychaudhuri B, Rayman P, Ireland J, Ko J, Rini B, Borden EC, et al. Myeloid-derived suppressor cell accumulation and function in patients with newly diagnosed glioblastoma. *Neuro-Oncology.* 2011; 13:591–9. [PubMed: 21636707]
44. Sippel TR, White J, Nag K, Tsvankin V, Klaassen M, Kleinschmidt-DeMasters BK, et al. Neutrophil Degranulation and Immunosuppression in Patients with GBM: Restoration of Cellular Immune Function by Targeting Arginase I. *Clinical Cancer Research.* 2011
45. Walter S, Weinschenk T, Stenzl A, Zdrojowy R, Pluzanska A, Szczylik C, et al. Multi-peptide immune response to cancer vaccine IMA901 after single-dose cyclophosphamide associates with longer patient survival. *Nature medicine.* 2012
46. Watters JJ, Schartner JM, Badie B. Microglia function in brain tumors. *JNeurosciRes.* 2005; 81:447–55.

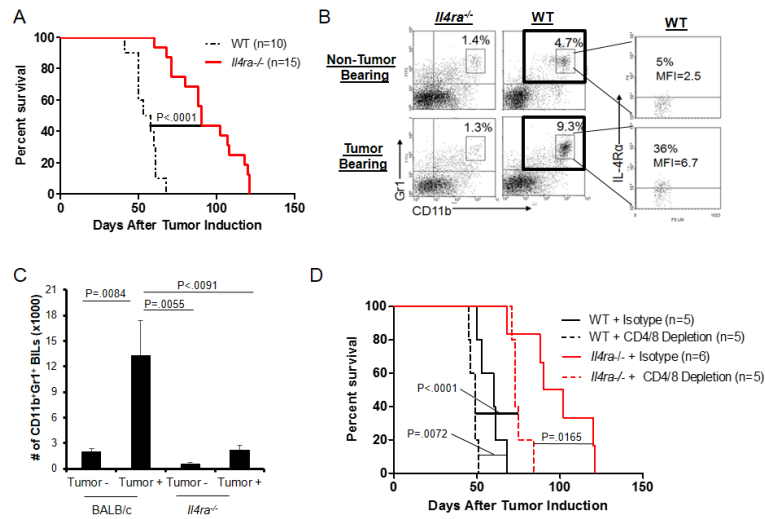


Figure 1. Effects of IL-4R on glioma development

De novo SB 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-4R expression on CD11b⁺Gr1⁺ cells. (C) Absolute numbers of CD11b⁺Gr1⁺ BILs in tumor-bearing or non-tumor bearing mice. Bars represent the mean and the standard deviation (SD) of results from 3-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.

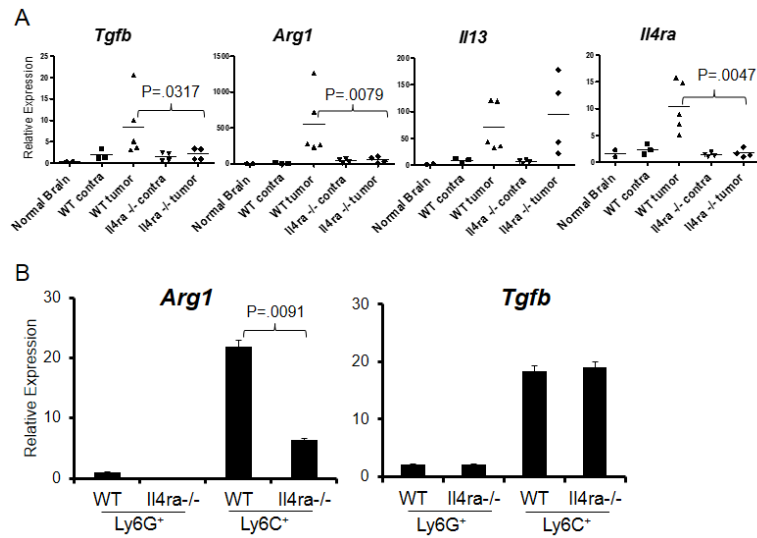


Figure 2. Effects of IL-4R on the glioma microenvironment and glioma-infiltrating myeloid cells

(A) Total RNA was isolated from brains of non-tumor-bearing mice (normal brain), the contralateral (contra) or tumor-bearing (tumor) hemispheres of brains derived from WT and *Il4ra*^{-/-} mice. mRNA expression levels of *Tgfb*, *Arg1*, *Il13* and *Il4ra* were analyzed by RT-PCR, relative to normal brain. (B) BILs from WT or *Il4ra*^{-/-} mice were sorted for cell populations double-positive for CD11b and Ly6C or CD11b and Ly6G. *Arg1* and *Tgfb* 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.

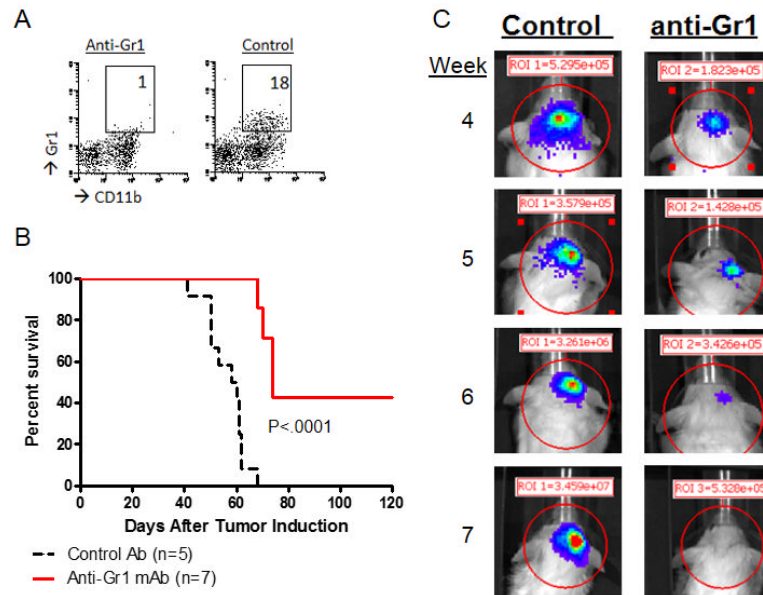


Figure 3. Depletion of CD11b⁺Gr1⁺ cells in mice bearing *de novo* gliomas
 Mice bearing *de novo* gliomas received anti-Gr1 mAb (A) CD11b⁺Gr1⁺ BILs from mice receiving control rat IgG or anti-Gr1 mAb. The number in each panel indicates the percentage of CD11b⁺Gr1⁺ cells in leukocyte-gated populations. (B) SFS was monitored in glioma-bearing mice treated with anti-Gr1 mAb or control IgG until day 120. (C) Representative of BLI showing complete regression of glioma following anti-Gr1 mAb treatment and rapid progression with control IgG treatment at Weeks 4-7 following the tumor induction.

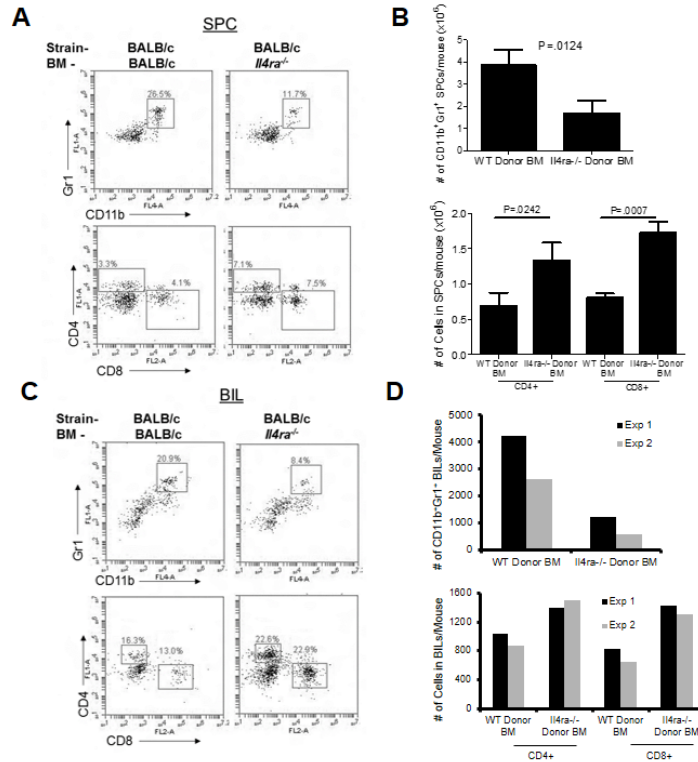


Figure 4. Critical role of IL-4R on BM cells in the immunological environment of glioma WT mice chimeric with either WT or *Il4ra*^{-/-} mouse-derived BM received intracranial injections of syngeneic glioma cells. At 3 weeks after glioma cell inoculation, SPCs and BILs were harvested and analyzed by 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 % 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 2 independent experiments.

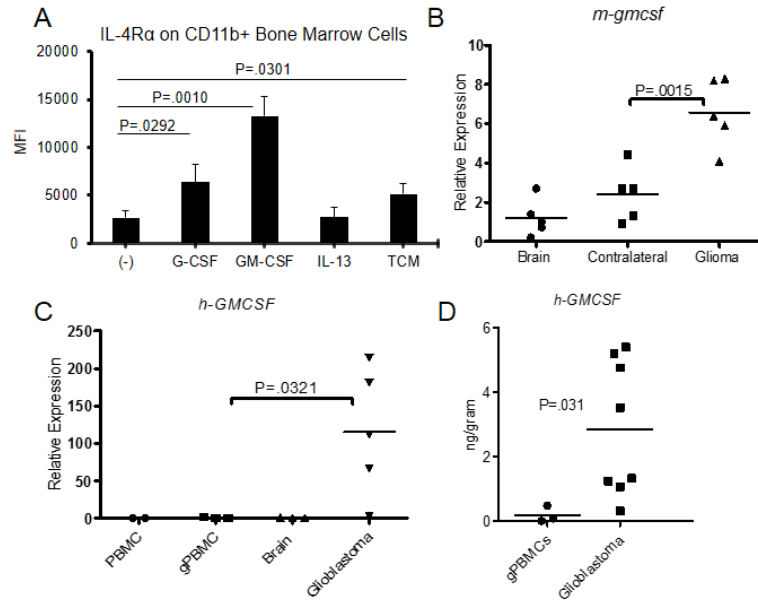


Figure 5. GM-CSF promotes IL-4R expression on myeloid cells and is up-regulated in human and mouse glioma tissues

BM cells were cultured in the presence of G-CSF (100 ng/ml), GM-CSF (250 U/ml), IL-13 (80 ng/ml), or TCM for 4 days. (A) IL-4R expression was determined by flow cytometry. Bars represent the mean and SD of mean fluorescence intensity (MFI) from 3 independent experiments. (B) Mouse *gmcsf* (*m-gmcsf*) was evaluated from brains of non-tumor-bearing mice (Brain), or the contralateral (Contralateral) or tumor-bearing (Glioma) hemispheres of WT mice bearing *de novo* glioma, relative to Brain. (C), Human *GMCSF* (*h-GMCSF*) expression was evaluated in total RNA isolated from healthy donor-derived PBMCs (PBMCs; n=2), glioma patient-derived PBMCs (gPBMCs; n=3), normal human brain tissue (Brain; n=3) or glioblastoma tissue (Glioblastoma; n=5), relative to PBMCs. (D) GM-CSF levels were evaluated by ELISA in protein extracts of gPBMCs and glioblastoma tissues. Values are adjusted to one gram of blood (for gPBMCs) or tumor tissue (for glioblastoma).

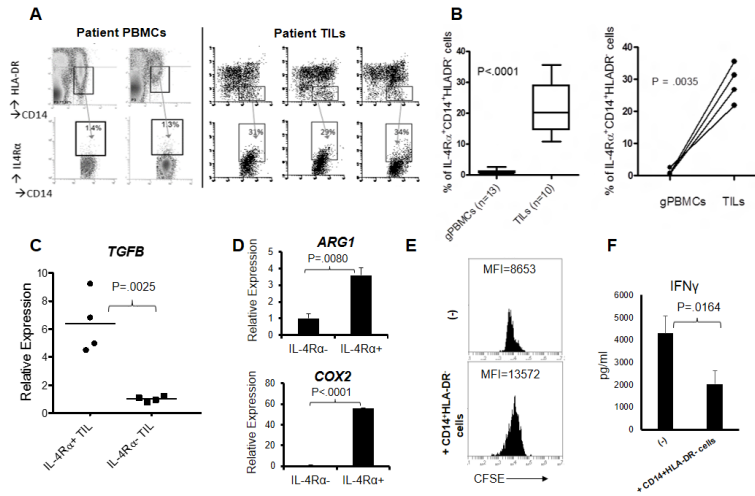


Figure 6. IL-4R expression on human tumor-infiltrating monocytes

TILs were isolated from fresh glioblastoma tissues by Percoll density separation; and glioblastoma patient-derived PBMCs were collected by Ficoll method. (A) IL-4R was analyzed on CD14⁺HLA-DR⁻ cells. (B) Percentages of CD14⁺HLA-DR⁻ cells expressing IL-4R in the PBMCs (n=13) and GBM tissues (n=10) (Left). Percentages of IL-4R⁺ cells among CD14⁺HLA-DR⁻ cells in the paired patient PBMCs and GBM tissues (n=4) (Right). (C) CD14⁺HLA-DR⁻ populations of TILs were sorted for two separate populations based on IL-4R⁺ or IL-4R⁻, and evaluated for expression levels of *TGFβ* in relative to those in PBMCs which are negative for IL-4R⁻. (D) CD14⁺HLA-DR⁻ populations of a GBM patient-derived leukapheresis sample were sorted for two separate populations based on IL-4R⁺ or IL-4R⁻, and evaluated by RT-PCR for expression levels of *ARG1* and *COX2*, relative to the IL-4R⁻ sample. (E) CD14⁺HLA-DR⁻ cells were sorted from glioblastoma-derived TILs and cultured with PBMC-derived, CFSE-labeled CD8⁺ T-cells for 5 days at a ratio of 1:4 (CD14⁺HLA-DR⁻ cells:T-cells). Proliferation was analyzed by flow cytometry gating on live lymphocytes; and (F) IFN- γ levels were assessed by ELISA.