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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 an 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. 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 myeloid-derived suppressor cells.

Keywords

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

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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). $CD11b^{+}Ly\text{-}6G^{-}Ly6C^{high}$ 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-4Rα 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-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 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-4Rα 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 *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 (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 $\Delta\Delta C_T$ 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 *Il4ra*$^{-/-}$ tumors, we conducted bioluminescence imaging (BLI) using an IVIS200 (Caliper Life Sciences) and evaluated tumors of comparable size (BLI of 2×10^{8} luciferase units).

BM Chimera

BM chimera experiments were conducted as previously described (25). Briefly, RBC depleted BM 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 1×10^{6} 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 × 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

Il4rα$^{-/-}$ 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 (SB)* transposon-mediated intraventricular transfection of the oncogenes *EGFRvIII, NRas*, and short hairpin (*sh)P53* in neonatal BALB/c-background WT and *Il4rα*$^{-/-}$ mice (hereby *SB* glioma). While the median symptom-free survival (SFS) for WT mice was 55.5 days, *Il4rα*$^{-/-}$ mice exhibited prolonged survival with a median SFS of 90 days (p <0.001) (Figure 1A). As IL-4Rα is expressed on some MDSCs (15-17), we next evaluated whether the genetic deletion of *Il4rα* 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^{+}Gr-1^{+} cells compared with non-tumor bearing brains. Further, in the presence of the *SB* gliomas, WT CD11b^{+}Gr-1^{+} contained a higher percentage of IL-4Rα 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^{+}Gr-1^{+} 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-4Rα and numbers of CD11b^{+}Gr-1^{+} 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 *Il4rα*$^{-/-}$ hosts demonstrated an increase of CD11b^{+}Gr1^{+} cells in the spleen following induction of *SB* 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 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 *Il4rα*$^{-/-}$ mice is due solely to enhanced anti-tumor T-cell response, we induced *SB* gliomas in WT and *Il4rα*$^{-/-}$ 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-4Rα 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-4Rα 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-4Rα expression on MDSCs in the glioma microenvironment, especially though *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-4Rα expression on BM-derived cells promotes the systemic distribution of CD11b^{+}Gr1^{+} cells but may inhibit that of T-cells.

IL-4Rα 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-4Rα 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-4Rα-dependent manner. We hereby term CD11b$^+$Gr1$^+$ BILs MDSCs in the glioma environment.

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

As IL-4Rα 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-4Rα. 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-4Rα expression was then measured by flow cytometry (Figure 5A). While G-CSF, GM-CSF and TCM treatment all up-regulated 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* (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-4Rα up-regulation in mouse BM-derived MDSCs *in vitro* [experiments in Figure 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 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-4Rα 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-4Rα was detected on 20-30% of CD14$^+$HLA-DR$^-$ TIL while 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) (Figure 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 4 matched patient samples (Figure 6B right).

We next addressed whether IL-4Rα expression on glioblastoma-infiltrating $CD33^+CD14^+HLA\text{-}DR^-$ cells was associated with immune suppressor functions. Using FACS, we isolated IL-4Rα-positive and -negative sub populations of TILs, extracted total RNA and analyzed *TGFB* (Figure 6C). IL-4Rα$^+$ cells had higher *TGFB* expression levels than their IL-4Rα$^-$ 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-4Rα positive and negative $CD14^+HLA\text{-}DR^-$ monocytes. We therefore examined IL-4Rα-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 $CD14^+HLA\text{-}DR^-$ cells) of IL-4Rα$^+$ cells compared with those in TILs, $CD14^+HLA\text{-}DR^-$ IL-4R$^+$ cells had higher levels of *ARG1* and *COX2* expression than their IL-4R$^-$ counterpart. Importantly, glioblastoma-derived $CD14^+HLA\text{-}DR^-$ 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-4Rα on $CD14^+HLA\text{-}DR^-$ 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-4Rα 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-4Rα 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-4Rα 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-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 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-4Rα 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-4Rα positive cells expressed elevated levels of *TGFB* compared with IL-4Rα 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-β 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 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-4Rα 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-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 $CD33^+HLA\text{-}DR^-$ 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, $CD14^+HLA\text{-}DR^{-/lo}$ 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 $CD14^+HLA\text{-}DR^-$ 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-4Rα 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.

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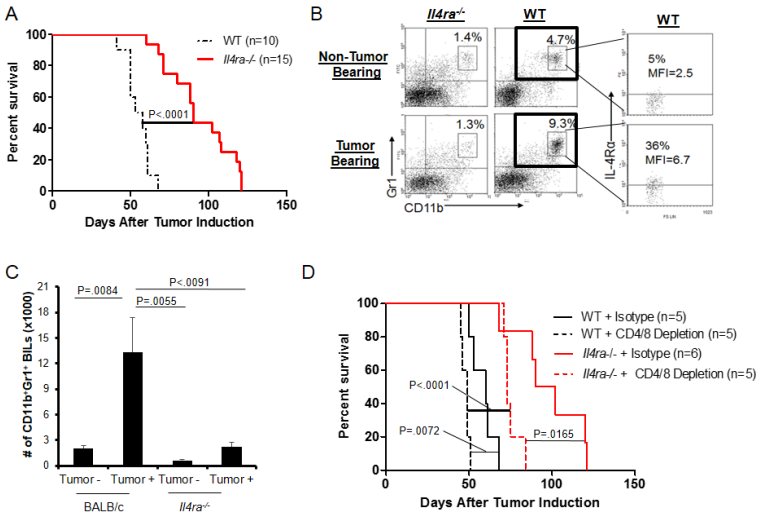

Figure 1. Effects of IL-4Rα on glioma development

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-4Rα 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.

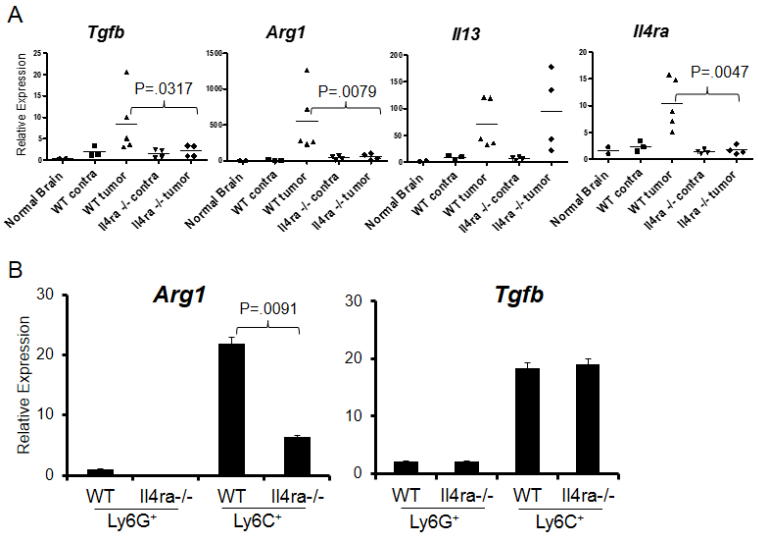

Figure 2. Effects of IL-4Rα on the glioma microenvironment and glioma-infiltrating myeloid cells

(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.

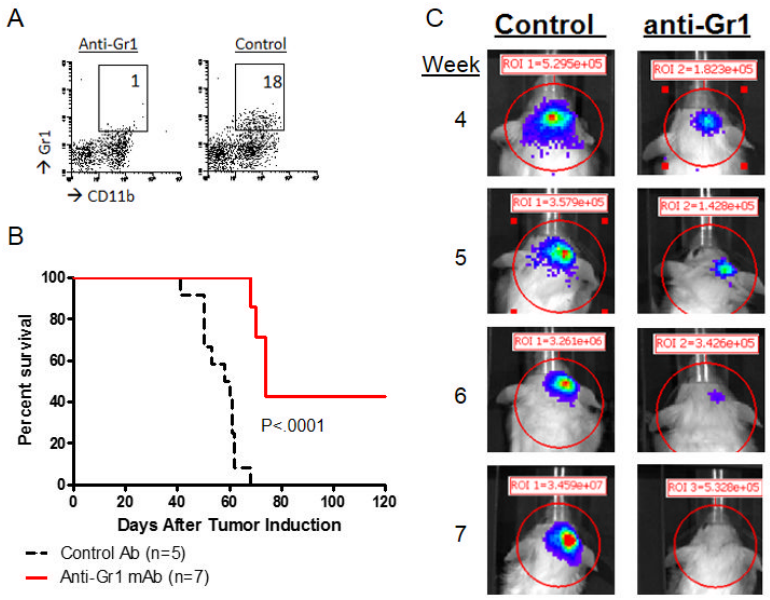

Figure 3. Depletion of CD11b$^+$Gr1$^+$ cells in mice bearing *de novo* gliomas

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.

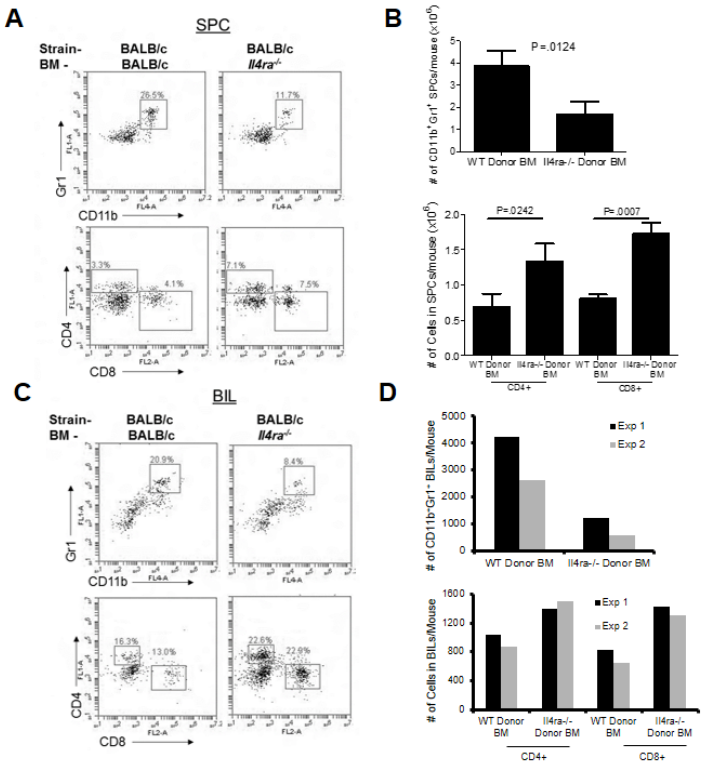

Figure 4. Critical role of IL-4Rα on BM cells in the immunological environment of glioma

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.

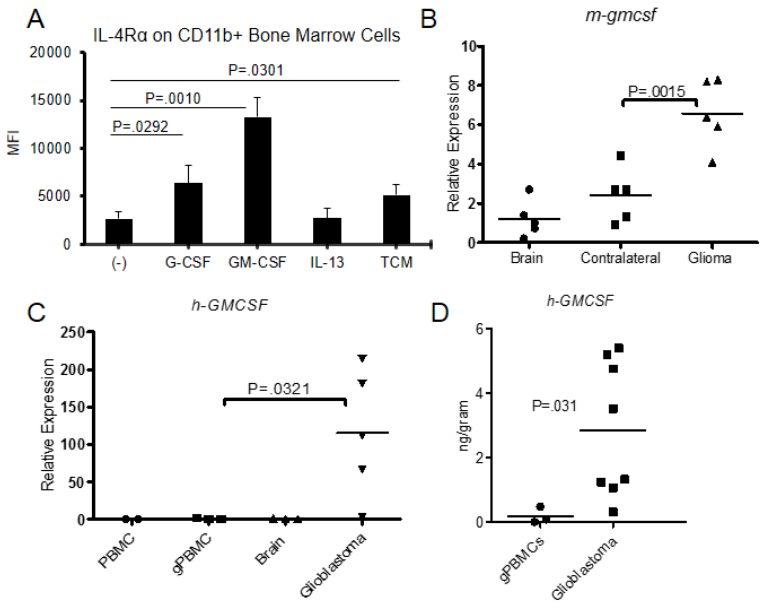

Figure 5. GM-CSF promotes IL-4Rα expression on myeloid cells and is up-regulated in human and mouse glioma tissues

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-4Rα 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).

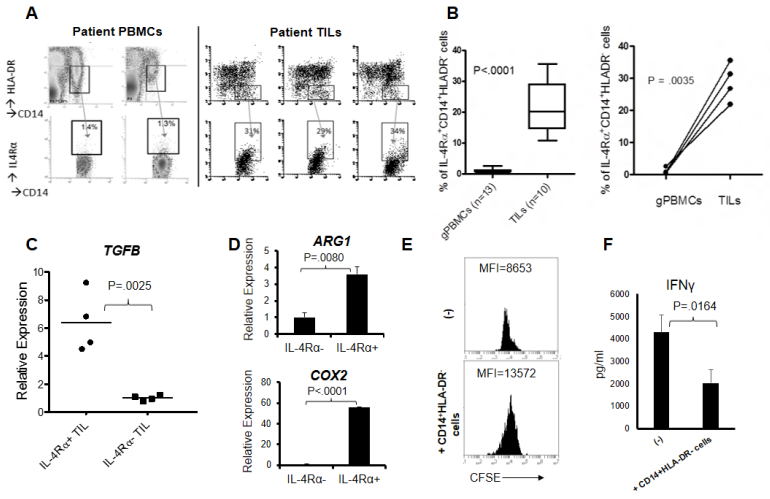

Figure 6. IL-4Rα expression on human tumor-infiltrating monocytes

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