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Role of type-1 interferons in anti-glioma immunosurveillance - Using mouse studies to guide examination of novel prognostic markers in humans

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Abstract

Purpose—We hypothesized that the type-1 interferons (IFNs) would play a pivotal role in anti-glioma immunosurveillance through promotion of type-1 adaptive immunity and suppression of immunoregulatory cells.

Experimental Design—We induced *de novo* gliomas in $Ifnar1^{-/-}$ (deficient for type-1 IFN receptors) or wild type (WT) mice by intracerebroventricuar transfection of *NRAS* and a short hairpin RNA against *P53* using the *Sleeping Beauty* (*SB*) transposon system. We analyzed survival of 587 glioma patients for single-nucleotide polymorphisms (SNPs) in type-1 IFN-related genes.

Results—$Ifnar1^{-/-}$ mice exhibited accelerated tumor growth and death. Analyses of brain tumor-infiltrating lymphocytes (BILs) in $Ifnar1^{-/-}$ mice revealed an increase of cells positive for $CD11b^+Ly6G^+$ and $CD4^+FoxP3^+$, which represent myeloid-derived suppressor cells (MDSCs) and regulatory T-cells (Tregs), respectively, but a decrease of $CD8^+$ CTLs compared with WT mice. $Ifnar1^{-/-}$ mouse-derived glioma tissues exhibited a decrease in mRNA for the CTL-attracting chemokine CXCL10, but an increase of CCL2 and CCL22, both of which are known to attract immunoregulatory cell populations. Dendritic cells (DCs) generated from the bone marrow of $Ifnar1^{-/-}$ mice failed to function as effective antigen-presenting cells (APCs). Moreover, depletion of $Ly6G^+$ cells prolonged the survival of mice with developing gliomas. Human epidemiological

studies revealed that SNPs in *IFNAR1* and *IFNA8* are associated with significantly altered overall survival of patients with WHO grade 2–3 gliomas.

Conclusions—The novel *SB*-induced murine glioma model led us to discover a pivotal role for the type-1 IFN pathway in anti-glioma immunosurveillance and relevant human SNPs that may represent novel prognostic markers.

Introduction

Gliomas are the most common malignant brain tumors. Despite their dismal prognosis, limited information is available regarding their etiology and the prognostic factors that influence patients' survival. It is therefore critical to gain a better understanding of the complex biological interactions that regulate glioma development and growth.

Animal models that mimic the complexity of human gliomas would be useful in understanding glioma biology and predicting therapeutic responses. In this regard, a novel *SB* transposon-mediated *de novo* murine glioma model has been recently developed in which tumor initiation and progression can be monitored by bioluminescent imaging (1). These murine tumors share many features with the human disease including glial marker expression, pseudopalisading necrosis, and brain invasion. In contrast to traditional transplanted models, these tumors evolve with the host immune system; herein we demonstrate that they are profoundly infiltrated by regulatory immune cells that suppress anti-tumor immunity, which is similar to human gliomas (2). Therefore, this *de novo* glioma model allows us to address immunological mechanisms of gliomagenesis.

With regard to tumorigenesis, the concept of cancer immunosurveillance has been proposed; that is, the immune system can protect the host against tumor development (3). Among the relevant molecules, type-1 IFNs have been suggested as central coordinators in the dynamic relationship between the host immune system and cancers (4). In particular, hematopoietic cells in the host (rather than tumor cells) have been shown to be the crucial targets of the antitumor activity of endogenous type-1 IFNs (4).

In recent years, a number of SNPs across the genome have been identified in association with cancers (5). With regard to SNPs in immune system modulators, previous epidemiological studies including ours have demonstrated that SNPs in *Interleukin* (*IL*)-*4R* are associated with altered glioma risks (6) and prognosis (7,8). Another recent study has demonstrated that a common variant (V249I) in the chemokine receptor *Cx3cr11* is associated with increased survival and reduced tumor infiltration by microglia in glioma patients (9). These studies suggest that cytokines and chemokines may be critically involved in the pathways that regulate glioma development and prognosis.

Based on these findings, we hypothesized that defects in the type-1 IFN pathway would play an important role in the pathogenesis and clinical course of gliomas. In the current study, we show that the *Ifnar1*-deficiency in mice accelerates gliomagenesis and is associated with an increased infiltration of MDSCs and Tregs along with a decreased infiltration of DCs and CTLs. In particular, DCs derived from *Ifnar1$^{-/-}$* mice exhibit altered antigen-presenting functions. Furthermore, monoclonal antibody (mAb)-mediated depletion of MDSCs prolongs the survival of tumor-developing mice, implicating that MDSCs promote glioma development. Moreover, type-1 IFN-inducing treatment delays gliomagenesis in mice in an *Ifnar1*-dependent manner. Finally, we show significant effects of SNPs in type-1 IFN-related genes (*IFNAR1* and *IFNA8*) on survival of patients with WHO grade 2–3 gliomas. Collectively, these findings indicate a pivotal role of the type-1 IFN pathway in gliomagenesis and support the development of type-1 IFN-based strategies for prevention of malignant transformation in glioma patients.

Materials and Methods

Animals

WT C57BL/6 mice (H-2^b) were obtained from Taconic Farms. C57BL/6-background *Ifnar1*$^{-/-}$ mice were kindly provided by Dr. Murali-Krishna Kaja (University of Washington, WA); they are deficient for α-subunit for type-1 IFN receptor (10). Pmel-1 mice were obtained from The Jackson Laboratory; they are C57BL/6-background and transgenic for an hgp100$_{25-33}$-specific T cell receptor that cross-reacts to an H-2D^b/mgp100$_{25-33}$ complex (11). Animals were handled in the Animal Facility at the University of Pittsburgh per an Institutional Animal Care and Use Committee-approved protocol.

Antibodies, gp100-specific tetramer, and peptides

The following mAbs were obtained from BD Biosciences: anti-CD11c (HL3), anti-CD4 (VH129.19), anti-CD8 (53-6.7), CD86 (GL1), anti-I-A^b (AF6-120.1), anti-IFN-γ (XMG1.2), anti-IL-4 (11B11), anti-H2-K^b (AF6-88.5), and isotype-matched controls. Anti-CCR7 (4B12) mAb was obtained from BioLegend. The following mAbs were obtained from eBioScience: anti-CD11b (M1/70), anti-FoxP3 (NRRF-30), and anti-Ly6G (RB6-8C5). Anti-CD25 mAb (PC61) was kindly provided by Dr. Masaki Terabe (National Cancer Institute, Bethesda, MD). Control IgG was obtained from Sigma-Aldrich. H-2D^b/mgp100$_{25-33}$ tetramer was obtained from National Institute of Allergy and Infectious Disease Tetramer Facility at Emory University Vaccine Center (Altanta, GA). The following peptides were synthesized in University of Pittsburgh Peptide Synthesis Facility: H-2D^b-binding human/mouse gp100 (h/mgp100) $_{25-33}$ (KVPRNQDWL), H-2D^b-binding Garc1$_{77-85}$ (AALLNKLYA), and H-2D^b-binding EphA2$_{671-679}$ (FSHHNIIRL).

Intracerebroventricular DNA injection

The procedure has been described previously (1). *In vivo*-compatible DNA transfection reagent (*In vivo*-JetPEI™) was obtained from Polyplus Transfection. The following DNA plasmids were used: *pT2/C-Luc//PGK-SB13*, *pT/CAGGS-NRASV12*, and *pT2/shP53*.

In vivo bioluminescent intensity (BLI) measurement

The procedure has been described previously (12). Luciferin was obtained from Caliper Life Sciences.

Tumor cell culture

YAC1 murine lymphoma cell line was obtained from ATCC. All cells were maintained in a mouse complete medium consisting of RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 mg/ml streptomycin, and 10 μM L-glutamine in a humidified incubator in 5% CO_2 at 37°C. All of the reagents described here were obtained from Invitrogen Life Technologies.

Intracranial cell injection and BIL isolation

The procedure has been described previously (13).

Preparation of CTLs and DCs

The procedure has been described previously (13). The following recombinant murine cytokines were obtained from R&D Systems: granulocyte/macrophage colony-stimulating factor, rmIFN-α, and rmIL-12. Recombinant human IL-2 (rhIL-2) was obtained from PeproTech. Lipopolysaccharide (LPS) was obtained from Sigma-Aldrich. We used 3×10^6 CTLs or 1×10^6 DCs.

Quantitative real-time polymerase chain reaction (RT-PCR)

The procedure has been described previously (14). The primers and probes for the following genes were obtained from Applied Biosystems: *Ccl2* (Mm00441242_m1), *Ccl22* (Mm00436439_m1), *Cxcl10* (Mm99999072_m1), *Gp100* (Mm00498996_m1), *Epha2* (Mm00438726_m1), and *Trp2* (Mm00441984_m1).

Cytokine and chemokine release assay

The ELISA kits were obtained as follows: mIFN-γ from BD Biosciences; mIL-12p70 from eBioscience; mCXCL10 and mCCL22 from R&D Systems. All assays were conducted according to the manufacturers' instructions.

In vivo CTL proliferation assay and cytolytic assay

The procedure has been described previously (13). Carboxyfluorescein diacetate succinimidyl ester (CFSE) was obtained from Invitrogen.

mAb-mediated cell depletion assay

The protocol was established on the basis of previous studies (15,16). Mice with developing gliomas received intraperitoneal (i.p.) injections of anti-Ly6G mAb (clone RB6-8C5, 0.25 mg/ dose) on days 21, 23, 25, and 27 after tumor induction. Some mice received i.p. injections of anti-CD25 mAb (clone PC61, 0.25 mg/dose) on days 21 and 24 after tumor induction.

Administraion of polyinosinic-polycytidylic acid stabilized by lysine and carboxymethylcellulose (poly-ICLC)

Poly-ICLC was kindly provided by Oncovir (Washington, D.C.). Mice with developing gliomas received intramuscular (i.m.) injections of either poly-ICLC (2.5 mg/kg/dose) or mock PBS starting on days 21 and 24 after tumor induction, and weekly thereafter.

Human subjects

The population used in this study has been described previously (17). Adults (aged 18 and older) newly diagnosed with a glioma (ICD-O-3 codes 9380-9481) in Harris County, TX were recruited between 2001 and 2006. The original study population (n=761) was restricted to non-Hispanic whites with WHO grade 2–4 gliomas (n=587) for the genetic analyses. Blood samples for DNA extraction were collected from the cases before initiation of chemotherapy or radiation therapy. The male to female ratio was 1.4:1. Treatment and survival data were collected from medical record review for all cases. The study was approved by the institutional review boards of all participating institutions, and written informed consent was obtained from each participant.

SNP selection and genotyping

Given our interest in type-1 IFNs, SNPs present on the Illumina Human 610 Quad SNP Chip in the following genes were selectively addressed: *IFNAR1*, *IFNAR2*, *IFNA1*, *IFNA2*, *IFNA4*, *IFNA5*, *IFNA6*, *IFNA7*, *IFNA8*, *IFNA13*, *IFNA14*, *IFNA16*, and *IFNA21*. DNA samples were excluded if less than 95% of loci were successfully genotyped. For all SNPs utilized in this analysis, ≥95% of the samples was genotyped successfully and each SNP had a GenCall score of ≥0.25. In addition, duplicate samples were used for quality control; >99% of all genotyping results were concordant between original and duplicate samples.

Statistical analyses

In mouse studies, the statistical significance of differences between two groups was determined by Student's t-test; one-way analysis of variance with Holm's post hoc test was conducted for

a multiple group comparison. Log-rank test was used to determine significant differences in survival curves among groups. All mouse data were analyzed by R Environment version 2.10.1. In human studies, survival time was calculated beginning at the date of hospital registration. Log-rank test was used to determine significant differences in survival curves stratified by genotype. The proportional hazards assumption for each model was tested using log-log plots; there was no evidence that the proportional hazards assumption was violated for any of the models. All human data were analyzed by SAS version 9.1. P<0.05 was considered to be statistically significant.

Results

Ifnar1$^{-/-}$ mice exhibit accelerated tumor growth and death following SB-based induction of gliomas

We first evaluated the role of type-1 IFN pathway on development of gliomas induced by *SB*-mediated transfection of *NRAS* and *shp53* (silencing RNA against *P53*) in *Ifnar1*$^{-/-}$ or WT mice. The induced tumors exhibited characteristics of human gliomas such as pseudopalisading necrosis and invasion (Supplementary Fig. 1). *Ifnar1*$^{-/-}$ mice exhibited accelerated tumor growth (Fig. 1A, left panel) and death (right panel) compared with WT mice.

To characterize the induced gliomas further, we isolated tumor cells from the tumor tissues of *Ifnar1*$^{-/-}$ or WT mice, and established a total of 41 cell lines *in vitro* (WT: 19; *Ifnar1*$^{-/-}$: 22). All cell lines exhibited spindle-shape morphology and grew at a comparable rate (data not shown). *Ifnar1*$^{-/-}$ and WT tumors expressed similar levels of the GAAs gp100 (Fig. 2D) and EphA2 *in vivo* (Supplementary Fig. 2A) as well as MHC class I and II *in vitro* (Supplementary Fig. 2B). To determine their tumorigenicity *in vivo*, we injected three cell lines established from each of *Ifnar1*$^{-/-}$ and WT mice into the brains of *Ifnar1*$^{-/-}$ and WT mice. Data in Fig. 1B represent experiments with one cell line from each genotype; we observed similar results in all cell lines (data not shown). *Ifnar1*$^{-/-}$ glioma cells failed to grow in WT hosts (Fig. 1B, black solid lines), while these cells grew aggressively in *Ifnar1*$^{-/-}$ hosts (red solid lines). In contrast, WT mouse-derived glioma cells grew aggressively both in *Ifnar1*$^{-/-}$ and WT hosts (dashed lines). These data suggest that the environment of *Ifnar1*$^{-/-}$ hosts allowed the growth of gliomas that would not have grown in the immunologically competent microenvironment of the central nervous system (CNS).

We next sought to determine whether the observed rejection of *Ifnar1*$^{-/-}$ glioma cells in WT hosts involved CTL-mediated recognition. To this end, we evaluated CTL responses mounted in cervical lymph nodes (LNs) of mice that had received inoculation of *Ifnar1*$^{-/-}$ or WT glioma cells in the brain. CTLs isolated from *Ifnar1*$^{-/-}$ hosts exhibited reduced tumoricidal function against the inoculated cell lines compared with WT hosts (Fig. 1C). Moreover, consistent with the Fig. 1B, the WT hosts that had received *Ifnar1*$^{-/-}$ tumor cells demonstrated the highest levels of CTL activity against the same *Ifnar1*$^{-/-}$ glioma cells (Fig. 1C). Additional experiments showed minimum lysis of YAC1 target cells, supporting that these responses were not merely antigen-independent natural killer cell responses (Supplementary Fig. 3). In accordance with the CTL data, LN cells derived from WT mice bearing *Ifnar1*$^{-/-}$ tumors produced the highest level of IFN-γ among the groups (Fig. 1D). Taken together, these data suggest that the *Ifnar1*$^{-/-}$ tumor cells are more immunogenic than the WT tumor cells in their ability to induce CTL responses.

Ifnar1$^{-/-}$ mice exhibit increased tumor-infiltration of CD11b$^+$Ly6G$^+$ and CD4$^+$FoxP3$^+$ cells but decreased Tc1 effector cells and CD11c$^+$ DCs

We next evaluated whether the defect of the type-1 IFN pathway would impact the immunological microenvironment of *de novo* gliomas by flow cytometric analyses of BILs.

Gliomas induced in *Ifnar1*⁻/⁻ mice were infiltrated with remarkably increased numbers of CD11b$^+$Ly6G$^+$ cells (35% vs 18% of leukocyte-gated cells) and CD4$^+$FoxP3$^+$ cells (11% vs 4%), which are considered to be MDSCs and Tregs, respectively (Fig. 2A). Although the small numbers of these cells obtained from small brain tumors in mice did not allow functional analyses of these cells *ex vivo*, these data suggest that the type-1 IFN pathway plays a critical role in limiting the accumulation of MDSCs and Tregs in the glioma microenvironment. To determine the effects of host type-1 IFN pathway on the recruitment of type-1 effector CTLs (Tc1) into the *SB*-induced tumors (which express gp100; Fig. 2D), *ex vivo* activated gp100-reactive Tc1 derived from Pmel-1 mice (13,18) were intravenously (i.v.) transferred into *Ifnar1*⁻/⁻ or WT mice bearing established *SB*-induced gliomas (Fig. 2B). On the fifth day following the i.v. transfer, *Ifnar1*⁻/⁻ mice demonstrated a lesser degree of tumor-infiltration by CD8$^+$gp100-tetramer$^+$ Tc1s (2%) compared with WT mice (8%) with *SB*-induced tumors.

We hypothesized that the observed alterations of BIL populations in *Ifnar1*⁻/⁻ mice would be at least partially associated with changes in chemokine profiles. This hypothesis was supported by a recent study demonstrating that IFN-α down-regulates CCL22, a Treg-attracting chemokine (14). In addition, CCL2 has been suggested to be the principal chemokine for MDSC migration in gliomas (19) and Tregs (20). On the other hand, CXCL10 represents type-1 effector CTL-attracting chemokines (13,18,21). We therefore performed quantitative RT-PCR to evaluate the mRNA expression levels of *Ccl2*, *Ccl22*, and *Cxcl10* in the brain hemisphere bearing *SB*-induced gliomas (Fig. 2C). There was a significant up-regulation of *Ccl2* (295%) and *Ccl22* (267%), while *Cxcl10* was reduced (54%), in *Ifnar1*⁻/⁻ mice compared with WT mice.

Ifnar1⁻/⁻ DCs fail to induce glioma-associated antigen (GAA)-reactive CTLs in vivo

Among a variety of host immune cells that respond to type-1 IFNs, we directed our primary focus on APCs, such as DCs. We generated DCs from bone marrow cells derived from either *Ifnar1*⁻/⁻ or WT mice. When the DCs were matured with LPS, *Ifnar1*⁻/⁻ DCs exhibited lower levels of the co-stimulatory molecule CD86 compared with WT DCs, while other maturation markers were expressed at similar levels on both DC types (Fig. 3A, upper panels). *Ifnar1*⁻/⁻ DCs failed to up-regulate IL-12p70 and CXCL10 production levels in response to IFN- α stimulation compared with WT DCs (Fig. 3A, lower panels). IFN-α stimulation down-regulated CCL22 production levels by WT DC, but not by *Ifnar1*⁻/⁻ DCs. These data suggest that the absence of the type-1 IFN signaling induces a substantial shift of the chemokine production profile in APCs.

To evaluate the antigen-presenting function of *Ifnar1*⁻/⁻ vs WT DCs *in vivo*, these DCs were loaded with synthetic hgp100 $_{25–33}$ peptide and injected into *Ifnar1*⁻/⁻ mice subcutaneously (s.c.) as vaccines. The vaccination with WT DCs efficiently induced the proliferation (Fig. 3B, upper panels) and cytolytic ability (Fig. 3B, lower panels) of antigen-specific CTLs *in vivo*, while *Ifnar1*⁻/⁻ DCs failed to promote these responses. These data suggest the critical roles of type-1 IFN pathway in the function of APCs.

Our data in Fig. 2B demonstrated diminished tumor-infiltration of gp100-reactive CTLs in *Ifnar1*⁻/⁻ mice. We then evaluated whether supplementation of WT DCs in the microenvironment of *Ifnar1*⁻/⁻ glioma would recover the trafficking of i.v. infused effector Tc1 cells (Fig. 3C). Intratumoral injections of WT DCs, but not *Ifnar1*⁻/⁻ DCs, recovered the efficient trafficking of Tc1 cells to the gliomas in *Ifnar1*⁻/⁻ mice (6% vs 2%), indicating that the type-1 IFN pathway in APCs is particularly important for attraction of CTLs in the glioma microenvironment regardless of IFN signaling in cells that are not APCs. Taken together, these data implicate the type-1 IFN pathway as important for attracting effector Tc1 cells and reducing immunoregulatory cells in the glioma microenvironment.

MDSCs play a significant role in the described phenomena

Based on the data shown in Fig. 2 and previous reports on Tregs [reviewed in (22)], we hypothesized that MDSC and Tregs would play a substantial role in promotion of gliomagenesis. To address this, we depleted these cell populations by systemic administration of mAbs. Depletion of Ly6G$^+$ cells reduced the infiltration of CD11b$^+$Ly6G$^+$ cells in tumor sites (Fig. 4A) and prolonged the survival of both *Ifnar1$^{-/-}$* and WT mice (Fig. 4B). In contrast, when anti-CD25 mAb was used for depletion of Tregs (23), the recipient mice did not show any prolongation of survival in response to the treatment (Fig. 4C), although the treatment reduced the number of CD4$^+$FoxP3$^+$ cells in the tumor site (Fig. 4D). Taken together, the data suggest that MDSCs, but not Tregs, played a significant role in promotion of gliomagenesis in this murine model.

Treatment with poly-ICLC prolongs the survival of mice bearing SB-induced gliomas in an Ifnar1-dependent manner

The data described above suggest pivotal roles of the host type-1 IFN pathway in delaying and/ or reducing development of *SB*-induced gliomas. We next sought to determine whether augmentation of the type-1 IFN pathway in WT mice compared with *Ifnar1$^{-/-}$* mice would also delay or prevent the growth of *SB*-induced gliomas by i.m. administration of poly-ICLC as a potent type-1 IFN-inducer (24). Treatment of *SB*-induced mice significantly prolonged the survival of mice in an *Ifnar1*-dependent manner, with 2 of 6 treated WT mice still alive on day 100 after birth (Fig. 5), while none of control WT mice treated with PBS lived longer than 75 days.

SNPs in type-1 IFN-related genes correlate with overall survival of glioma patients

We performed a survival analysis in a population of 587 patients with WHO grade 2–4 gliomas (Supplementary Table 1) for whom genotype data were available to begin to understand the impact of type-1 IFN-related genes on prognosis. Cox regression analyses demonstrated no significant association of IFN-related SNPs with the survival of patients with grade 4 glioblastoma multiforme (GBM; data not shown). However, when the grade 2–3 gliomas were analyzed, we found that patients with the AA genotype of *IFNAR1* rs1041868 have significantly poorer overall survival (HR=3.6; 95% CI=1.5–8.3) than those with the AG or GG genotypes. Similarly, patients with the AC genotype of *IFNA8* rs12553612 were more likely to die than those with the AA genotype (HR=2.5; 95% CI=1.1–5.9). Furthermore, Kaplan-Meier survival curves indicated that median survival for patients with the *IFNAR1* rs1041868 AA genotype was 24 months compared with 90 months for patients with the AG/GG genotypes; the same effect was seen for the *IFNA8* rs12553612 AC genotype compared with the AA genotype (Fig. 6). Adjustment of the models by age at diagnosis, extent of surgery, and chemotherapy use did not affect the point estimates (data not shown). It was unlikely that these factors would confound the association between genotype and survival time. We also examined the effect of having one or both of these significant SNPs. However, only one patient carried both variant SNPs; we were therefore unable to examine the joint effect. None of the other SNPs examined were significantly associated with overall survival (data not shown). We further confirmed that each of the genotype groups included patients from both histologic groups, indicating that the observed effects are because of the genotypes but not because the groups with worse survival (AA in rs1041868 and AC in rs12553612) were composed of those with worse histology (grade 3 glioma). Taken together, these data indicate that type-1 IFNs play an important role in gliomagenesis in mice and may present valuable prognostic factors in humans.

Discussion

This is one of the first reports documenting the status of immune cell infiltration and chemokine expression in the microenvironment of *de novo* murine gliomas. This model allowed us to determine the role of endogenous type-1 IFNs in glioma development in mice and identify type-1 IFN SNPs that are associated with altered prognosis of glioma patients. Such interconnected studies that bridge *de novo* murine glioma models and human epidemiological data will help us further elucidate novel factors contributing to risk and prognosis for this highly fatal disease, and to develop possible prevention strategies.

A critical role of type-1 IFNs in cancer development has been shown previously in methylcholanthrene-induced skin tumor model (25), where the authors primarily focused on the immunoediting process of cancers. In particular, they demonstrated that tumors arising in the absence of type-1 IFN responsiveness are more immunogenic than tumors arising in immunocompetent mice. We observed similar findings (Fig. 1). As *Ifnar1*$^{-/-}$ and WT cell lines showed no significant differences in expression of GAAs and MHC, further studies are warranted to delineate the factors that underlie the unique immunogenicity of tumors arising in *Ifnar1*$^{-/-}$ hosts. Nevertheless, in our current study, we demonstrated specific changes in immune cell populations and chemokine productions in the glioma microenvironment (Fig. 2). In particular, our data suggest that responsiveness of DCs to type-1 IFNs affects their antigen-presenting function (e.g. changes in CD86 expression) as well as the chemokine production profiles in the tumor microenvironment (Fig. 3), which may be responsible for the changes in the percentages and absolute numbers of infiltrating MDSCs and Tregs. We also evaluated the significance of MDSCs in glioma development by mAb-mediated depletion (Fig. 4A and 4B). Taken together, the novel *de novo* glioma model provided us with unique opportunities to identify the roles of type-1 IFN pathway in anti-glioma immunosurveillance.

A variety of strategies have been described to deplete MDSCs, such as gemcitabine (26) and sunitinib (27). We used anti-Ly6G mAb (RB6-8C5) to deplete Ly6G$^+$ CD11b$^+$ cells (Fig. 4A and 4B) because anti-Ly6G mAb reacts relatively specifically to MDSCs, sparing effector immune cell populations, such as CTLs. RB6-8C5 was originally developed as a mAb recognizing a surface antigen, granulocyte receptor 1 (Gr-1) (28), which is a member of the Ly6 gene family. Then, Ly6G, a granulocyte surface marker, was found to be the major antigen detected by RB6-8C5 (29). Although Ly6G is expressed on neutrophils, DCs, and subsets of monocytes, macrophages, and lymphocytes (15), we have found that systemic administration of the RB6-8C5 did not significantly affect the numbers of these other immune cell populations (data not shown).

We used the anti-CD25 mAb (PC61) to deplete Tregs (Fig. 4C and 4D). This strategy is widely accepted and clinically applied as Daclizumab (30), and our regimen is based on a previous study employing a similar dose and schedule (16). Although there is a theoretical concern is that this strategy might deplete activated T cells that express CD25 (31), it is also known that expression of CD25 is transient following activation (32). Indeed, we observed no significant effect of anti-CD25 mAb on CD8$^+$ T cell subpopulations in BILs (data not shown). Therefore, this does not appear to be a significant concern in most cancer studies.

We demonstrated that SNPs in *IFNAR1* and *IFNA8* have significant associations with overall survival in patients with the grade 2–3 gliomas (Fig. 6). It is ultimately important to determine whether these SNPs have a significant impact on the biological function of type-1 IFN pathways. The *IFNAR1* rs1041868 is located in intron 10, and *IFNA8* rs12553612 is located in the 5′ untranslated region of the *IFNA8* gene. While the location of these SNPs does not suggest putative functionality, further studies are warranted to determine whether these SNPs impact gene expressions. In addition, these SNPs could be in linkage disequilibrium with as

yet unidentified functional SNPs, which could be further elucidated in future studies. Previous studies have shown a significant immunological impact of several SNPs in other IFN-related genes, such as ones in *Toll-Like Receptor* (*TLR*) *3* (33,34) and *TLR4* (35). Therefore, if the SNPs in *IFNAR1* and *IFNA8* indeed affect the biological function of the gene products, it will substantiate that these SNPs alter the status of immunological surveillance and could influence the prognosis of glioma patients. Such observations will also prompt us to evaluate whether these SNPs dictate altered risks for occurrence of glioma in general populations.

Despite the significant effects of these SNPs in patient with the grade 2–3 gliomas (Fig. 6), these did not demonstrate a significant association with survival in GBM patients. This may imply that the profound immune suppression and aggressive growth of GBM may overshadow the potential role of type-1 IFN pathway. In humans, grade 2–3 gliomas are considered to represent pre-malignant tumors prior to their transformation into secondary GBMs (36). In our mouse model, *SB*-induced gliomas appear relatively dormant at days 20–30 after birth (Fig. 1) and therefore may simulate the grade 2–3 gliomas before they start growing more aggressively. We also observed that poly-ICLC treatment started on day 21 after birth leads to therapeutic effects in a host type-1 IFN-dependent manner (Fig. 5). Taken together, these findings suggest that IFN-based therapy may be most appreciated in the lower-grade gliomas rather than GBMs. In addition, since IFN-α treatment failed in a phase III trial for patients with high-grade gliomas due to dose-limiting toxicity (37), toxicity would be a major concern if type-1 IFNs were used in patients particularly with low-grade gliomas. On the other hand, poly-ICLC has been shown to be safe and well-tolerated in glioma patients (38–40) and thus seems to be more suitable for treatment of glioma patients.

Due to the low minor allele frequencies in the SNPs of interest, we analyzed the grade 2–3 gliomas as a group with more similar survival separately from the grade 4 GBMs to have adequate power for the analysis. Future analyses, on a larger number of patients, could determine if further differences exist between patients with grade 2 versus grade 3 gliomas. If the functional significance of the SNPs reported here can be identified, such information may be used to select patients who are likely to benefit from future immunotherapy trials. In summary, the current study indicates a pivotal role of type-1 IFN pathway in gliomagenesis and supports the development of type-1 IFN-based strategies for immunological prevention of gliomas.

Translational Relevance

Although gliomas are the most common type of primarymalignant brain tumors in adults, only limited information is availableregarding their etiology and prognostic factors that influence patient survival. In this study, using a novel murine glioma model (where *de novo* gliomas can be induced in mice using the *Sleeping Beauty* transposon system), we demonstrate that the absence of type-1 IFN signaling causes dynamic changes in the immunological microenvironment in murine gliomas, thereby accelerating the tumor growth. In addition, we show that SNPs in *IFNAR1* and *IFNA8* are associated with glioma patients' survival. Cumulatively, these findings imply that active collaboration using the novel murine glioma model and human genetics may foster the discovery of novel risk and/ or prognostic factors for gliomas, thereby leading to the development of effective immunotherapeutic strategies and ways to reduce occurrence of gliomas.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

References

1. Wiesner SM, Decker SA, Larson JD, et al. De novo induction of genetically engineered brain tumors in mice using plasmid DNA. Cancer Res 2009;69:431–9. [PubMed: 19147555]

2. Parney IF, Waldron JS, Parsa AT. Flow cytometry and in vitro analysis of human glioma-associated macrophages. Laboratory investigation. J Neurosurg 2009;110:572–82. [PubMed: 19199469]

3. Dunn GP, Old LJ, Schreiber RD. The three Es of cancer immunoediting. Annu Rev Immunol 2004;22:329–60. [PubMed: 15032581]

4. Dunn GP, Koebel CM, Schreiber RD. Interferons, immunity and cancer immunoediting. Nat Rev Immunol 2006;6:836–48. [PubMed: 17063185]

5. Pharoah PD, Dunning AM, Ponder BA, Easton DF. Association studies for finding cancer-susceptibility genetic variants. Nat Rev Cancer 2004;4:850–60. [PubMed: 15516958]

6. Schwartzbaum J, Ahlbom A, Malmer B, et al. Polymorphisms associated with asthma are inversely related to glioblastoma multiforme. Cancer Res 2005;65:6459–65. [PubMed: 16024651]

7. Scheurer ME, Amirian E, Cao Y, et al. Polymorphisms in the interleukin-4 receptor gene are associated with better survival in patients with glioblastoma. Clin Cancer Res 2008;14:6640–6. [PubMed: 18927306]

8. Wrensch M, Wiencke JK, Wiemels J, et al. Serum IgE, tumor epidermal growth factor receptor expression, and inherited polymorphisms associated with glioma survival. Cancer Res 2006;66:4531–41. [PubMed: 16618782]

9. Rodero M, Marie Y, Coudert M, et al. Polymorphism in the microglial cell-mobilizing CX3CR1 gene is associated with survival in patients with glioblastoma. J Clin Oncol 2008;26:5957–64. [PubMed: 19001328]

10. de Weerd NA, Samarajiwa SA, Hertzog PJ. Type I interferon receptors: biochemistry and biological functions. J Biol Chem 2007;282:20053–7. [PubMed: 17502368]

11. Overwijk WW, Tsung A, Irvine KR, et al. gp100/pmel 17 is a murine tumor rejection antigen: induction of "self"-reactive, tumoricidal T cells using high-affinity, altered peptide ligand. J Exp Med 1998;188:277–86. [PubMed: 9670040]

12. Ohlfest JR, Demorest ZL, Motooka Y, et al. Combinatorial antiangiogenic gene therapy by nonviral gene transfer using the sleeping beauty transposon causes tumor regression and improves survival in mice bearing intracranial human glioblastoma. Mol Ther 2005;12:778–88. [PubMed: 16150649]

13. Fujita M, Zhu X, Ueda R, 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]

14. Muthuswamy R, Urban J, Lee JJ, Reinhart TA, Bartlett D, Kalinski P. Ability of mature dendritic cells to interact with regulatory T cells is imprinted during maturation. Cancer Res 2008;68:5972–8. [PubMed: 18632653]

15. Daley JM, Thomay AA, Connolly MD, Reichner JS, Albina JE. Use of Ly6G-specific monoclonal antibody to deplete neutrophils in mice. J Leukoc Biol 2008;83:64–70. [PubMed: 17884993]

16. Terabe M, Ambrosino E, Takaku S, et al. Synergistic enhancement of CD8+ T cell-mediated tumor vaccine efficacy by an anti-transforming growth factor-beta monoclonal antibody. Clin Cancer Res 2009;15:6560–9. [PubMed: 19861451]

17. Okcu MF, Selvan M, Wang LE, et al. Glutathione S-transferase polymorphisms and survival in primary malignant glioma. Clin Cancer Res 2004;10:2618–25. [PubMed: 15102663]

18. Fujita M, Zhu X, Sasaki K, et al. Inhibition of STAT3 promotes the efficacy of adoptive transfer therapy using type-1 CTLs by modulation of the immunological microenvironment in a murine intracranial glioma. J Immunol 2008;180:2089–98. [PubMed: 18250414]

19. Huang B, Lei Z, Zhao J, et al. CCL2/CCR2 pathway mediates recruitment of myeloid suppressor cells to cancers. Cancer Lett 2007;252:86–92. [PubMed: 17257744]

20. Jordan JT, Sun W, Hussain SF, DeAngulo G, Prabhu SS, Heimberger AB. Preferential migration of regulatory T cells mediated by glioma-secreted chemokines can be blocked with chemotherapy. Cancer Immunol Immunother 2008;57:123–31. [PubMed: 17522861]

21. Nishimura F, Dusak JE, Eguchi J, et al. Adoptive transfer of type 1 CTL mediates effective anti-central nervous system tumor response: critical roles of IFN-inducible protein-10. Cancer Res 2006;66:4478–87. [PubMed: 16618775]

22. Okada H, Kohanbash G, Zhu X, et al. Immunotherapeutic approaches for glioma. Crit Rev Immunol 2009;29:1–42. [PubMed: 19348609]

23. Zou W. Regulatory T cells, tumour immunity and immunotherapy. Nat Rev Immunol 2006;6:295–307. [PubMed: 16557261]

24. Zhu X, Nishimura F, Sasaki K, et al. Toll like receptor-3 ligand poly-ICLC promotes the efficacy of peripheral vaccinations with tumor antigen-derived peptide epitopes in murine CNS tumor models. J Transl Med 2007;5:10. [PubMed: 17295916]

25. Dunn GP, Bruce AT, Sheehan KC, et al. A critical function for type I interferons in cancer immunoediting. Nat Immunol 2005;6:722–9. [PubMed: 15951814]

26. Suzuki E, Kapoor V, Jassar AS, Kaiser LR, Albelda SM. Gemcitabine selectively eliminates splenic Gr-1+/CD11b+ myeloid suppressor cells in tumor-bearing animals and enhances antitumor immune activity. Clin Cancer Res 2005;11:6713–21. [PubMed: 16166452]

27. Ko JS, Zea AH, Rini BI, 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]

28. Tepper RI, Coffman RL, Leder P. An eosinophil-dependent mechanism for the antitumor effect of interleukin-4. Science 1992;257:548–51. [PubMed: 1636093]

29. Fleming TJ, Fleming ML, Malek TR. Selective expression of Ly-6G on myeloid lineage cells in mouse bone marrow. RB6–8C5 mAb to granulocyte-differentiation antigen (Gr-1) detects members of the Ly-6 family. J Immunol 1993;151:2399–408. [PubMed: 8360469]

30. Rech AJ, Vonderheide RH. Clinical use of anti-CD25 antibody daclizumab to enhance immune responses to tumor antigen vaccination by targeting regulatory T cells. Ann N Y Acad Sci 2009;1174:99–106. [PubMed: 19769742]

31. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. J Immunol 1995;155:1151–64. [PubMed: 7636184]

32. Malek TR. The biology of interleukin-2. Annu Rev Immunol 2008;26:453–79. [PubMed: 18062768]

33. Dhiman N, Ovsyannikova IG, Vierkant RA, et al. Associations between SNPs in toll-like receptors and related intracellular signaling molecules and immune responses to measles vaccine: preliminary results. Vaccine 2008;26:1731–6. [PubMed: 18325643]

34. Yang Z, Stratton C, Francis PJ, et al. Toll-like receptor 3 and geographic atrophy in age-related macular degeneration. N Engl J Med 2008;359:1456–63. [PubMed: 18753640]

35. Apetoh L, Ghiringhelli F, Tesniere A, et al. Toll-like receptor 4-dependent contribution of the immune system to anticancer chemotherapy and radiotherapy. Nat Med 2007;13:1050–9. [PubMed: 17704786]

36. Purow B, Schiff D. Advances in the genetics of glioblastoma: are we reaching critical mass? Nat Rev Neurol 2009;5:419–26. [PubMed: 19597514]

37. Buckner JC, Schomberg PJ, McGinnis WL, et al. A phase III study of radiation therapy plus carmustine with or without recombinant interferon-alpha in the treatment of patients with newly diagnosed high-grade glioma. Cancer 2001;92:420–33. [PubMed: 11466698]

38. Salazar AM, Levy HB, Ondra S, et al. Long-term treatment of malignant gliomas with intramuscularly administered polyinosinic-polycytidylic acid stabilized with polylysine and carboxymethylcellulose: an open pilot study. Neurosurgery 1996;38:1096–103. [PubMed: 8727138]

39. Butowski N, Chang SM, Junck L, et al. A phase II clinical trial of poly-ICLC with radiation for adult patients with newly diagnosed supratentorial glioblastoma: a North American Brain Tumor Consortium (NABTC01–05). J Neurooncol 2009;91:175–82. [PubMed: 18797818]

40. Butowski N, Lamborn KR, Lee BL, et al. A North American brain tumor consortium phase II study of poly-ICLC for adult patients with recurrent anaplastic gliomas. J Neurooncol 2009;91:183–9. [PubMed: 18850068]
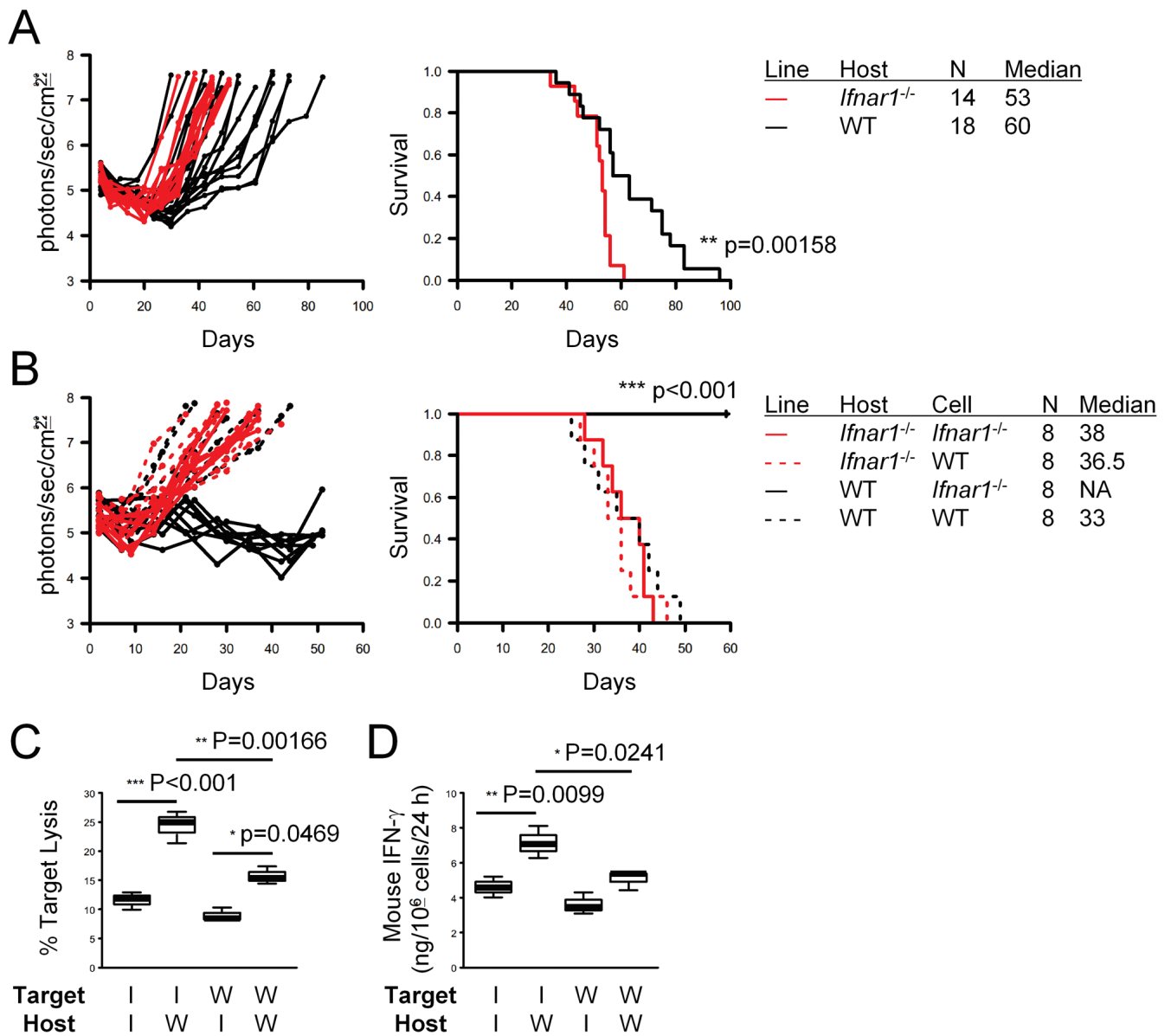
Figure 1. *Ifnar1*$^{-/-}$ **mice exhibit accelerated tumor growth and death following** *SB***-based induction of gliomas**

(**A**) CNS gliomas were induced in C57BL/6-background *Ifnar1*$^{-/-}$ (red lines) or WT (black lines) neonatal mice by intraventricular transfection of the following plasmids: *pT2/C-Luc// PGK-SB1.a3* (0.2 μg), *pT/CAG-NRas* (0.4 μg), and *pT/shp53* (0.4 μg). Tumor growth (left) and symptom-free survival (right) were monitored. P-values are based on log-rank test. (**B**) Glioma cells derived from *Ifnar1*$^{-/-}$ (solid lines) or WT (dashed lines) mice were injected into the basal ganglia of *Ifnar1*$^{-/-}$ (red lines) or WT (black lines) host mice (1×10^5/mouse). Tumor growth (left) and symptom-free survival (right) were monitored. (**C**) ^{51}Cr release assay was performed to evaluate cytolytic ability of cervical LN cells obtained from the mice bearing *Ifnar1*$^{-/-}$ cells (solid lines in **B**) against the *Ifnar1*$^{-/-}$ and WT cell lines. (**D**) ELISA was performed to evaluate the amount of mIFN-γ secretion by the CTLs used in (**C**) per the last 24 hrs. (**C** and **D**) I: *Ifnar1*$^{-/-}$; W: WT. Lines within boxes denote means; box upper and lower

bounds indicate SD; whiskers indicate minimum and maximum values. P-values are based on Holm's post hoc test.
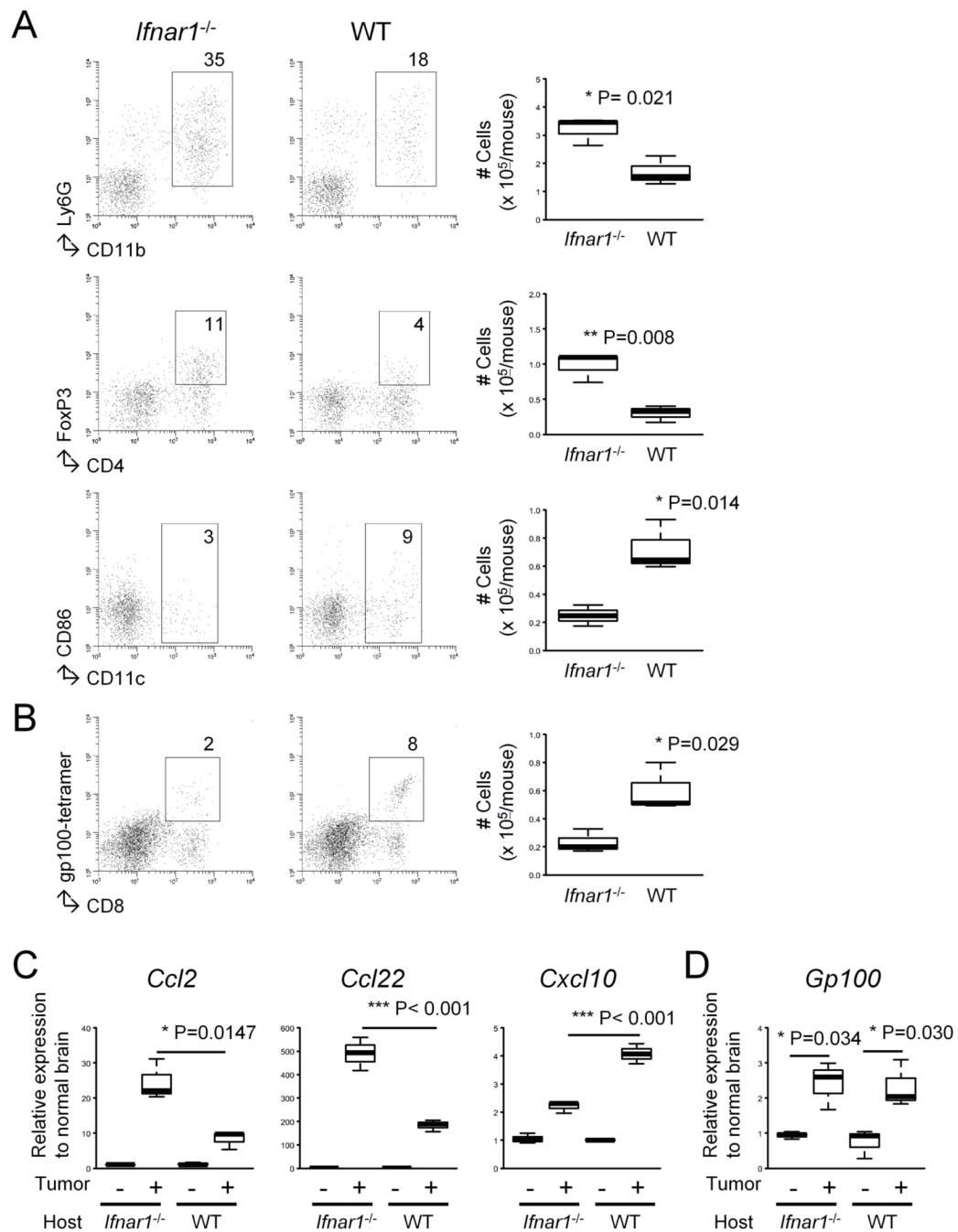
Figure 2. *Ifnar1*−/− mice demonstrate increased tumor-infiltration of CD11b+Ly6G+ and CD4+FoxP3+ cells but decreased Tc1 effector cells and CD11c+ DCs
(**A** and **B**) The mice bearing *SB*-induced tumors were sacrificed at around days 50–60, and BILs were isolated based on similar tumor size observed by BLI. BILs obtained from 3 mice in a given group were pooled and then evaluated by flow cytometry for the following subpopulations: CD11b+Ly6G+, CD4+FoxP3+, CD11c+CD86+ (**A**) and CD8+gp100 tetramer+ (**B**). Numbers in dot plots indicate percentage of gated subpopulations in leukocyte-gated BILs. Absolute numbers are depicted in the rightmost panels. P-values are based on Student's t-test. (**C** and **D**) Total RNA was extracted from each mouse brain (3 mice/group).

Quantitative RT-PCR was performed to evaluate the mRNA expression levels of the following molecules: *Ccl2*, *Ccl22*, *Cxcl10* (**C**) and *Gp100* (**D**).
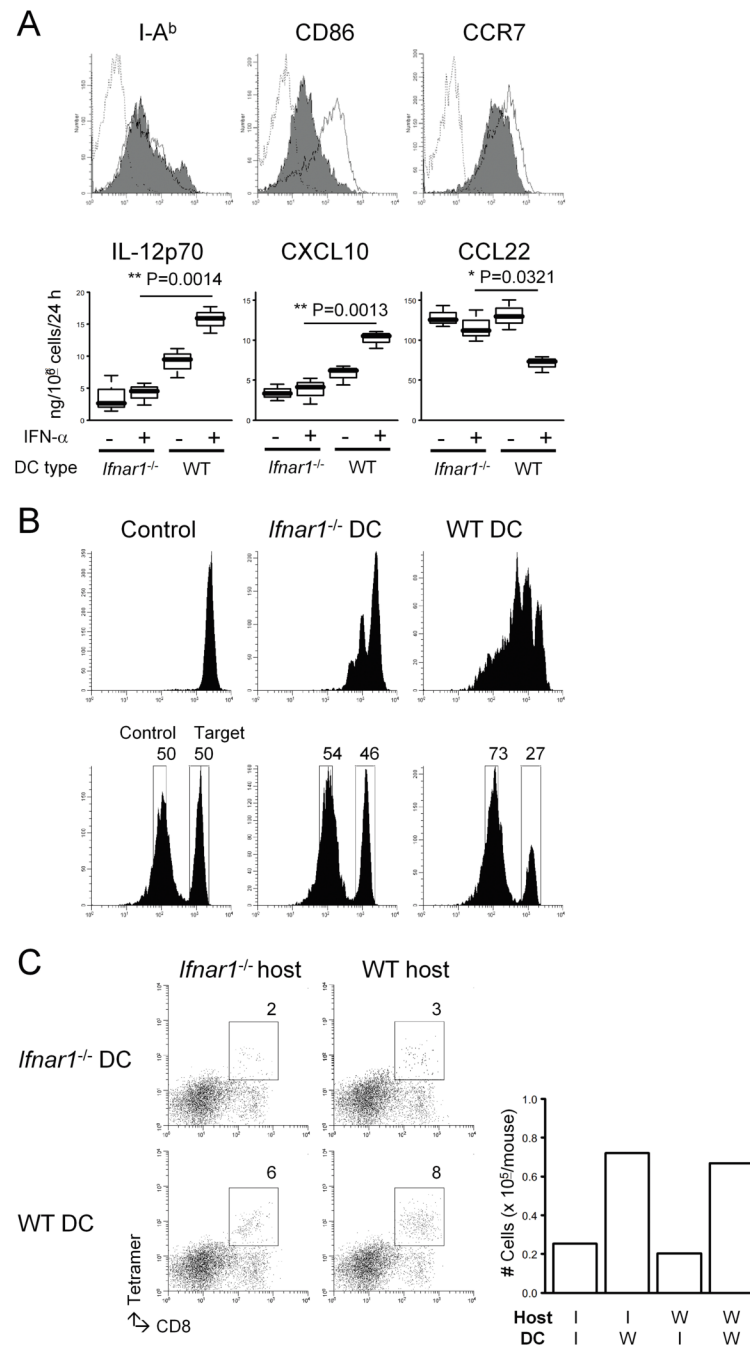
Figure 3. *Ifnar1*$^{-/-}$ **DCs demonstrate defects in their APC function and the ability to attract CTLs in the glioma sites**

(**A**) Bone marrow-derived mature DCs were prepared as described in Materials and Methods. (Upper panels) Flow cytometry was performed to evaluate the following surface markers: I-A^b (an MHC class II), CD86, and CCR7. Histograms represent the following DC types: *Ifnar1*$^{-/-}$ (shaded), WT (open), and WT DCs stained with isotype control IgG (dashed line). (Lower panels) Immature DCs were matured with 250 ng/ml LPS in the presence or absence of 10 ng/ml rmIFN-α. ELISA was performed to evaluate the production levels of the following molecules per the last 24 hours: mIL-12p70, mCXCL10, and mCCL22. (**B**) WT mice received s.c. immunization with either *Ifnar1*$^{-/-}$ or WT DCs loaded with 5 μg/ml hgp100$_{25-33}$ (upper)

or Garc1$_{77-85}$ (lower) peptide on Day 0. Control mice received PBS. (Upper panels) On day -1, mice received an i.v. injection of 5×10^6 naive Pmel-I CD8$^+$ T cells labeled with CFSE. On day 6, inguinal LN cells were collected to evaluate the proliferation of CSFE-labeled gp100-reactive cells by flow cytometry. (Lower panels) On day 6, the Garc1$_{77-85}$-specific cytolytic ability of effector T cells was evaluated by *in vivo* cytolytic assay as described in Materials and Methods. (**C**) Mice with developing gliomas received intratumoral injections of DCs derived from *Ifnar1*$^{-/-}$ or WT mice (1×10^5/mouse) on day 45 after birth, and i.v. Tc1 infusions on the same day (3×10^6/mouse). Mice were then sacrificed on day 5 following the DC injection for flow-cytometric evaluation of Tc1 infiltration into the tumor sites. Numbers in dot plots indicate percentage of CD8$^+$gp100-teramer$^+$ cells in lymphocyte-gated BILs. Absolute numbers are depicted in the rightmost panel.
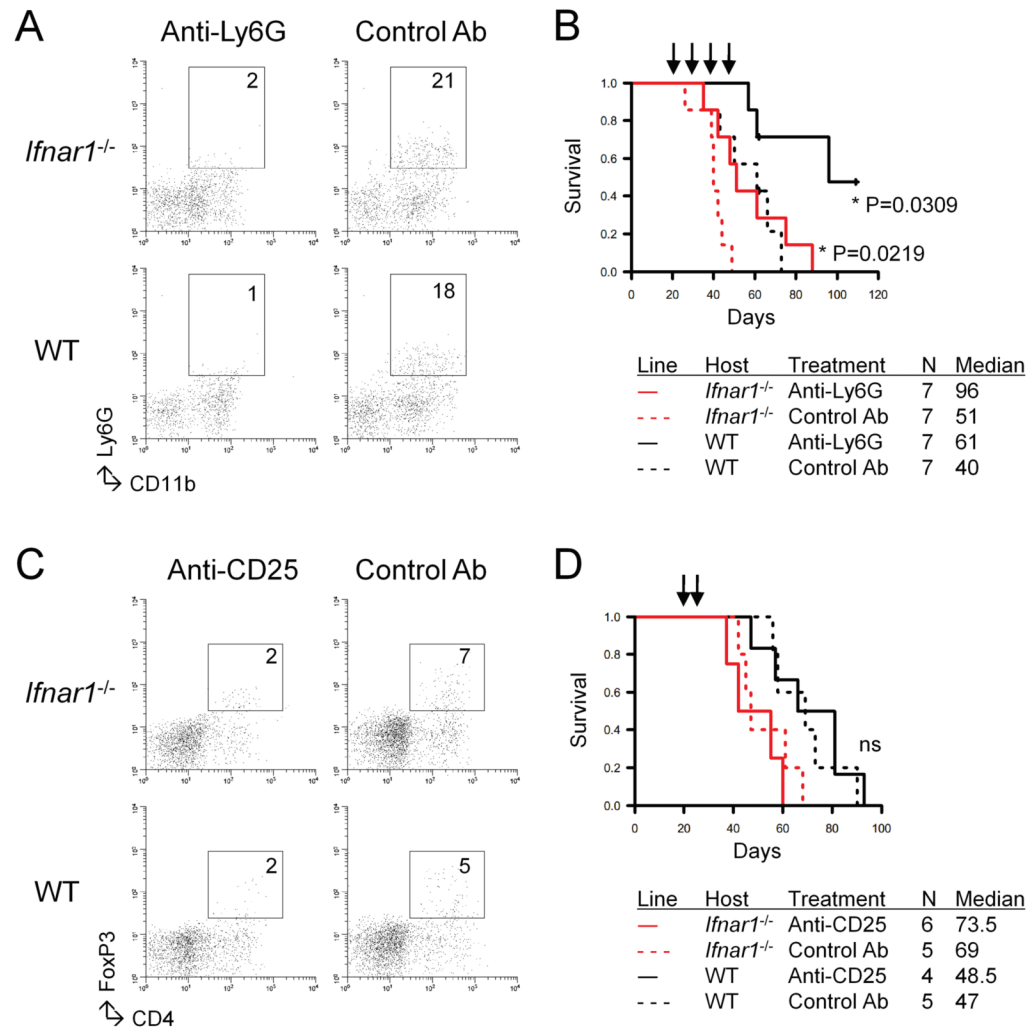
Figure 4. mAb-mediated depletion of Ly6G$^+$ cells, but not CD25$^+$ cells, inhibit the growth of *SB*-induced gliomas

(**A** and **B**) *Ifnar1$^{-/-}$* or WT mice with developing gliomas received i.p. injections of anti-Ly6G mAb (RB6-8C5; 0.25 mg/dose) or control IgG on days 21, 23, 25, and 27 after tumor induction. (**A**) The mice were sacrificed, and BILs were isolated for CD11b$^+$Ly6G$^+$ subpopulations. (**B**) Symptom-free survival was monitored. (**C** and **D**) *Ifnar1$^{-/-}$* or WT mice with developing gliomas received i.p. injections of anti-CD25 mAb (PC61; 0.25 mg/dose) or control IgG on days 21 and 24 after tumor induction. (**C**) The mice were sacrificed, and BILs were isolated for CD4$^+$FoxP3$^+$ subpopulations. (**D**) Symptom-free survival was monitored.
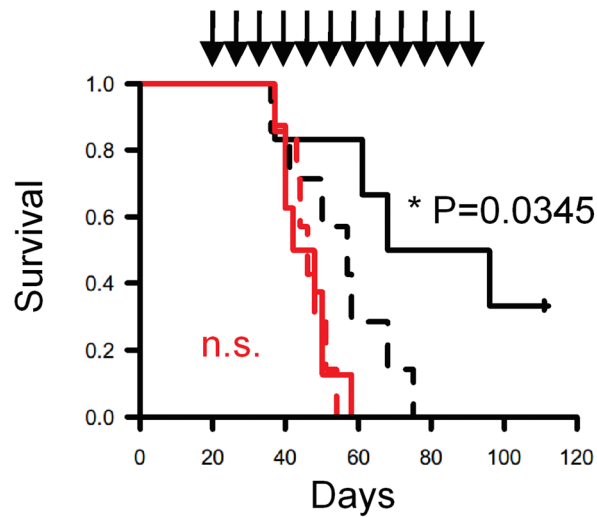
Figure 5. Treatment with poly-ICLC prolongs the survival of mice bearing *SB*-induced gliomas in an *Ifnar1*-dependent manner

Mice with developing gliomas received i.m. injections of either poly-ICLC (2.5 mg/kg/dose) or mock PBS starting on days 21 and 24 after birth, and weekly thereafter. Symptom-free survival was monitored.
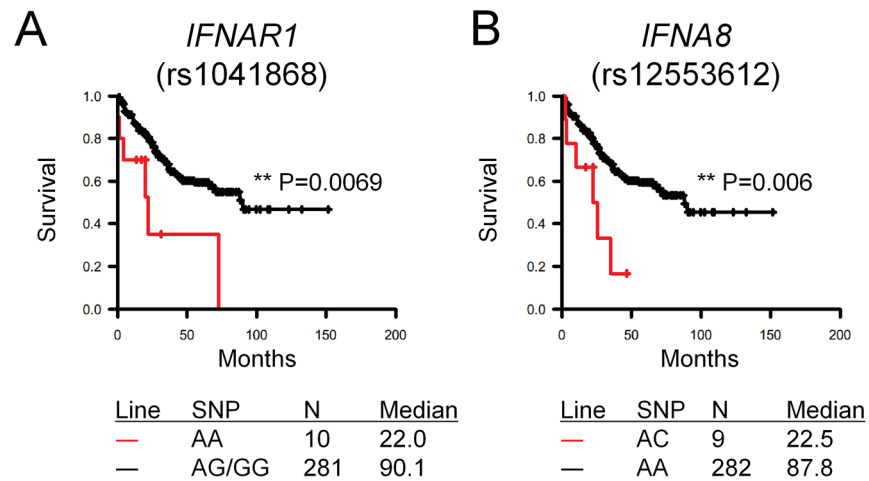
Figure 6. Association of SNPs in IFN-related genes and the survival of patients with WHO grade 2–3 gliomas

Overall survival was evaluated among 304 glioma patients with grade 2–3 gliomas by genotype for SNPs in IFN-related genes. (**A**) Patients with AA genotype (red line) for *IFNAR1* rs1041868 exhibited a significantly shorter survival than those with the AG/GG genotypes (black line). (**B**) Patients with AC genotype (red line) for *IFNA8* rs12553612 exhibited a significantly shorter survival than those with the AA genotype (black line).