Role of Type 1 IFNs in Antiglioma Immunosurveillance—Using
Mouse Studies to Guide Examination of Novel Prognostic
Markers in Humans

Mitsugu Fujita1,5, Michael E. Scheurer6, Stacy A. Decker7, Heather A. McDonald6, Gary Kohanbash4,5, Edward R. Kastenhuber6, Hisashi Kato5, Melissa L. Bondy8, John R. Ohlfest7, and Hideho Okada1,2,3,5

Abstract

Purpose: We hypothesized that the type 1 IFNs would play a pivotal role in antiglioma 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 mice by intracerebroventricular transfection of NRas and a short hairpin RNA against P53 using the Sleeping Beauty transposon system. We analyzed the survival of 587 glioma patients for single nucleotide polymorphisms (SNP) in type 1 IFN–related genes.

Results: Ifnar1−/− mice exhibited accelerated tumor growth and death. Analyses of brain tumor–infiltrating lymphocytes in Ifnar1−/− mice revealed an increase of cells positive for CD11b+Ly6G+ and CD4+FoxP3+, which represent myeloid-derived suppressor cells and regulatory T cells, respectively, but a decrease of CD8+ cytotoxic T lymphocytes (CTLs) compared with wild-type 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 generated from the bone marrow of Ifnar1−/− mice failed to function as effective antigen-presenting cells.

Moreover, depletion of Ly6G+ cells prolonged the survival of mice with developing gliomas. Human epidemiologic studies revealed that SNPs in IFNAR1 and IFNA8 are associated with significantly altered overall survival of patients with WHO grade 2 to 3 gliomas.

Conclusions: The novel Sleeping Beauty–induced murine glioma model led us to discover a pivotal role for the type 1 IFN pathway in antglioma immunosurveillance and relevant human SNPs that may represent novel prognostic markers. Clin Cancer Res; 16(13); 3409–19. ©2010 AACR.

Gliomas are the most common malignant brain tumors. Despite their dismal prognosis, limited information is available about their etiology and the prognostic factors that influence the 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 in predicting therapeutic responses. In this regard, a novel Sleeping Beauty (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 glioma marker expression, pseudopalisading necrosis, and brain invasion. In contrast to traditional transplanted models, these tumors evolve with the host immune system; herein, we show that they are profoundly infiltrated by regulatory immune cells that suppress antitumor immunity, which is similar to human gliomas (2). Therefore, this de novo glioma model allows us to address immunologic 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
decreased infiltration of dendritic cells (DC) and CTLs. (MDSC) and regulatory T cells (Treg) along with an increased infiltration of myeloid-derived suppressor cells accelerates gliomagenesis and is associated with an current study, we show that the pathogenesis and clinical course of gliomas. In the type 1 IFN pathway would play an important role in 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 myeloid-derived suppressor cells (MDSC) and regulatory T cells (Treg) along with a decreased infiltration of dendritic cells (DC) 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 the survival of patients with WHO grade 2 to 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 the prevention of malignant transformation in glioma patients.

Materials and Methods

Animals
Wild-type (WT) C57BL/6 mice (H-2b) were obtained from Taconic Farms. C57BL/6-background Ifnar1−/− mice were kindly provided by Dr. Murali-Krishna Kaja (University of Washington, Seattle, 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 hgp10025-33–specific T-cell receptor that cross-reacts to an H-2Dβ/mgp10025-33 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-Aβ (AF6-120.1), anti–IFN-γ (XMG1.2), anti–IL-4 (11B11), anti–H-2Kb (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-2Dβ/mgp10025-33 tetramer was obtained from the National Institute of Allergy and Infectious Disease Tetramer Facility at Emory University Vaccine Center (Atlanta, GA). The following peptides were synthesized in the University of Pittsburgh Peptide Synthesis Facility: H-2Dβ–binding human/mouse gp100 (h/mgp100) 25-33 (KVPRNQDWL), H-2Kβ–binding Garc117-85 (AALLNKLYA), and H-2Dβ–binding EphA2671-679 (FSHHNIIRL).

Intracerebroventricular DNA injection
The procedure has been previously described (1). In vivo compatible DNA transfection reagent (In vivo-JetPEI) was obtained from Polyplus Transfection. The following DNA plasmids used were as follows: pT2/C-Luc/[PGK-SB13, pT/CAGGS-NRasV12, and pT2/shP53.

In vivo bioluminescent intensity (BLI) measurement
The procedure has been previously described (12). Luciferin was obtained from Caliper Life Sciences.

Tumor cell culture
YAC1 murine lymphoma cell line was obtained from the American Type Culture Collection. All cells were maintained in a mouse complete medium consisting of RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin, 100 mg/mL.
streptomycin, and 10 μmol/L L-glutamine in a humidified incubator in 5% CO₂ at 37°C. All of the reagents described here were obtained from Invitrogen Life Technologies.

**Intracranial cell injection and brain tumor–infiltrating leukocyte (BIL) isolation**

The procedure has been previously described (13).

**Preparation of CTLs and DCs**

The procedure has been previously described (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 was obtained from PeproTech. Lipopolysaccharide was obtained from Sigma-Aldrich. We used 3 × 10⁸ CTLs or 1 × 10⁶ DCs.

**Quantitative real-time PCR**

The procedure has been previously described (14). The primers and probes for the following genes were obtained from Applied Biosystems: Cdl2 (Mm00412421_m1), Ccl2 (Mm00436439_m1), Cxcl10 (Mm99999072_m1), Gp100 (Mm04989961_m1), Epha2 (Mm00438726_m1), and Trp2 (Mm00449841_m1).

**Cytokine and chemokine release assay**

The ELISA kits were obtained as follows: mIFN-γ from BD Biosciences, mIL-12p70 from eBioscience, and 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 previously described (13). Carboxyfluorescein diacetate succinimidyl ester was obtained from Invitrogen.

**Ab-mediated cell depletion assay**

The protocol was established on the basis of previous studies (15, 16). Mice with developing gliomas received 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.

**Administration of polynosinic-polycytidylic acid stabilized by lysine and carboxymethylcellulose (poly-ICLC)**

Poly-ICLC was kindly provided by Oncovir. 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 tumor induction, and weekly thereafter.

**Human subjects**

The population used in this study has been previously described (17). Adults (ages 18 y 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 to 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 as follows: IFNAR1, IFNAR2, IFNA1, IFNA2, IFNA4, IFNA5, IFNA6, IFNA7, IFNA8, IFNA13, IFNA14, IFNA16, and IFNA21. DNA samples were excluded if <95% of loci were successfully genotyped. For all SNPs used 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 ANOVA 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 the 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. S1). Ifnar1−/− mice exhibited accelerated tumor growth (Fig. 1A, left) and death (right) 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 glioma-associated antigens Gp100 (Fig. 2D) and Epha2 in vivo (Supplementary Fig. S2A) as well as MHC class I and II in vitro (Supplementary Fig. S2B). 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, red solid lines), whereas 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.

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 (LN) 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 showed 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 only
Fig. 2. *Ifnar1−/−* mice show 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 to 60, and BILs were isolated based on similar tumor size observed by BLI. BILs obtained from three 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, 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 (three mice/group). Quantitative real-time PCR was performed to evaluate the mRNA expression levels of the following molecules: *Ccl2*, *Ccl22*, *Cxcl10* (C), and *Gp100* (D).
antigen-independent natural killer cell responses (Supplementary Fig. S3). 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 affect the immunologic microenvironment of de novo gliomas by flow cytometric analyses of brain tumor-infiltrating leukocytes (BIL). Gliomas induced in Ifnar1−/− mice were infiltrated with remarkably increased numbers of CD11b+Ly6G+ cells (35% versus 18% of leukocyte-gated cells) and CD4+FoxP3+ cells (11% versus 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 i.v. transferred into Ifnar1−/− or WT mice bearing established SB-induced gliomas (Fig. 2B). On the 5th day following the i.v. transfer, Ifnar1−/− mice showed 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 showing that IFN-α downregulates 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 a Tc1-attracting chemokines (13, 18, 21). We therefore performed quantitative real-time 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 upregulation of Ccl2 (295%) and Ccl22 (267%), whereas Cxcl10 was reduced (54%), in Ifnar1−/− mice compared with WT mice.

Ifnar1−/− DCs fail to induce glioma-associated antigen–reactive CTLs in vivo

Among a variety of host immune cells that respond to type 1 IFNs, we directed our primary focus on antigen-presenting cells (APC), such as DCs. We generated DCs from bone marrow cells derived from either Ifnar1−/− or WT mice. When the DCs were matured with lipopolysaccharide, Ifnar1−/− DCs exhibited lower levels of the costimulatory molecule CD86 compared with WT DCs, whereas other maturation markers were expressed at similar levels on both DC types (Fig. 3A, top). Ifnar1−/− DCs failed to upregulate IL-12p70 and CXCL10 production levels in response to IFN-α stimulation compared with WT DCs (Fig. 3A, bottom). IFN-α stimulation downregulated 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−/− versus WT DCs in vivo, these DCs were loaded with synthetic hgp10025-33 peptide and injected into Ifnar1−/− mice s.c. as vaccines. The vaccination with WT DCs efficiently induced the proliferation (Fig. 3B, top) and cytolytic activity (Fig. 3B, bottom) of antigen-specific CTLs in vivo, whereas 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 showed 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% versus 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 ref. 22), we hypothesized that MDSC and Tregs would play a substantial role in the 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 two of six treated WT mice still alive on day 100 after birth (Fig. 5), whereas 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 to 4 gliomas (Supplementary Table S1) for whom genotype data were available to begin to understand the effect of type 1 IFN–related genes on prognosis. Cox regression analyses showed 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 to 3 gliomas were
analyzed, we found that patients with the AA genotype of *IFNAR1* rs1041868 have significantly poorer overall survival (hazard ratio, 3.6; 95% confidence interval, 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 (hazard ratio, 2.5; 95% confidence interval, 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). 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 epidemiologic 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), in which the authors primarily fo-
cused on the immunoediting process of cancers. In partic-
sular, they showed that tumors arising in the absence of
type 1 IFN responsiveness are more immunogenic than tu-
mors arising in immunocompetent mice. We observed
similar findings (Fig. 1). As Ifnar1−/− and WT cell lines
showed no significant differences in expression of glioma-
associated antigens and MHC, further studies are war-
ranted to delineate the factors that underlie the unique
immunogenicity of tumors arising in Ifnar1−/− hosts.
Nevertheless, in our current study, we showed 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 func-
tion (e.g., changes in CD86 expression) as well as the
chemokine production profiles in the tumor microenvi-
ronment (Fig. 3), which may be responsible for the
changes in the percentages and absolute numbers of infil-
trating MDSCs and Tregs. We also evaluated the significance
of MDSCs in glioma development by mAb-mediated deple-
tion (Fig. 4A and B). Taken together, the novel de novo gli-
oma model provided us with unique opportunities to
identify the roles of type 1 IFN pathway in antiglioma im-
munosurveillance.

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 B) 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 anti-
gen, granulocyte receptor 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 D). This strategy is widely accepted and
clinically applied as Daclizumab (30), and our regimen

**Fig. 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.

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**Fig. 6.** Association of SNPs in IFN-related genes and the survival of patients with WHO grade 2 to 3 gliomas. Overall survival was evaluated among 304 glioma patients with grade 2 to 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).

**A**

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is based on a previous study using a similar dose and schedule (16). Although there is a theoretical concern 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 seem to be a significant concern in most cancer studies.

We showed that SNPs in IFNAR1 and IFNA8 have significant associations with overall survival in patients with the grade 2 to 3 gliomas (Fig. 6). It is ultimately important to determine whether these SNPs have a significant effect 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. Although the location of these SNPs does not suggest putative functionality, further studies are warranted to determine whether these SNPs affect 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 immunologic effect of several SNPs in other IFN-related genes, such as ones in TLR3 (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 immunologic 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 to 3 gliomas (Fig. 6), these did not show 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 to 3 gliomas are considered to represent premalignant tumors before their transformation into secondary GBMs (36). In our mouse model, SB-induced gliomas appear relatively dormant at days 20 to 30 after birth (Fig. 1) and therefore may simulate the grade 2 to 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, because 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 is 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 to 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 immunologic prevention of gliomas.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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