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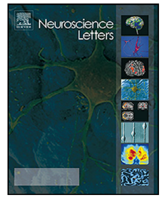

Research article

Advanced age negatively impacts survival in an experimental brain tumor model

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HIGHLIGHTS

- Advanced age decreases survival in an immunocompetent brain tumor model.
- IDO1 expression is significantly higher in aged brain.
- Age does not affect the phenotype or quantity of brain tumor infiltrating lymphocytes.

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ABSTRACT

Glioblastoma (GBM) is the most common primary malignant brain tumor in adults, with an average age of 64 years at the time of diagnosis. To study GBM, a number of mouse brain tumor models have been utilized. In these animal models, subjects tend to range from 6 to 12 weeks of age, which is analogous to that of a human teenager. Here, we examined the impact of age on host immunity and the gene expression associated with immune evasion in immunocompetent mice engrafted with syngeneic intracranial GL261. The data indicate that, in mice with brain tumors, youth conveys an advantage to survival. While age did not affect the tumor-infiltrating T cell phenotype or quantity, we discovered that old mice express higher levels of the immunoevasion enzyme, IDO1, which was decreased by the presence of brain tumor. Interestingly, other genes associated with promoting immunosuppression including CTLA-4, PD-L1 and FoxP3, were unaffected by age. These data highlight the possibility that IDO1 contributes to faster GBM outgrowth with advanced age, providing rationale for future investigation into immunotherapeutic targeting in the future.

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1. Introduction

Glioblastoma (GBM) is the most common primary malignant brain tumor within the adult central nervous system (CNS) [1,2]. The median age of an adult patient diagnosed with GBM is 64 years old [3]. GBM is a highly aggressive brain tumor thought to arise by malignant transformation of glial precursor cells and/or mature glia. Despite maximal surgical resection, radiotherapy, and chemotherapy, overall survival (OS) remains at 14.6 months post-diagnosis with 26% of patients surviving two years [4].

The dismal prognosis of GBM is, in part, due to the potently immunosuppressive brain tumor microenvironment. This is reflected by the accumulation of regulatory T cells (Tregs) [5,6], myeloid derived suppressor cells (MDSC) [7,8], IDO1 activity [9,10], increased expression for immune checkpoint molecules, CTLA-4 and PD-1/PD-L1 [11–13] and immunosuppressive cytokine expression [14–16]. This is commensurate with a progressive impairment of the adaptive immune system with age [17]. Notably, $CD8^+$ recent thymic emigrant levels are associated with the prognostic impact of age on clinical outcomes in GBM patients [18].

The median age of an adult human GBM patient diagnosis is equivalent to ∼74 weeks of age in mice. Given that the majority

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of brain tumor studies occur in relatively young mice, equivalent to 6–12 weeks old, we hypothesized that age may negatively impact the immune response to brain tumors. Here, we report that old immunocompetent mice engrafted syngeneic GL261 cell-based brain tumors have decreased survival when compared to young counterparts. Unexpectedly, there was no impact of age on the tumor-infiltrating T cell response. However, we found a substantial upregulation of mRNA for the immunosuppressive enzyme, IDO1, in old mouse brain when compared to young- or tumor bearing-brain. These data show for the first time that old mice with brain tumors have decreased survival and implicates IDO1 as a potential contributing factor that contributes to tumor growth.

2. Materials and methods

2.1. Mice and cell lines

Young (6-week old) and aged (72-week old) mice C57BL/6 (wild-type; Cat# 000664) were obtained from Jackson Laboratories and maintained in the Northwestern University Center for Comparative Medicine. Mice were intracranially injected at 8 or 74 weeks of age, respectively. All surgical procedures were completed in accordance with NIH guidelines on the care and use of laboratory animals. Mice were euthanized by injection of a lethal dose of Ketamine (200 mg/kg) and Xylazine (10 mg/kg) followed by cervical dislocation. GL261 cells were obtained from the NCI (Frederick, MD) and cultured in Dulbecco modified Eagle medium (DMEM) and supplemented with 10% fetal bovine serum (FBS), in addition to penicillin (100 μg/mL) and streptomycin (100 mg/mL) at 37 °C. All products used for cell culture were purchased from Gibco Invitrogen.

2.2. Orthotopic intracranial injection model

Mice were anesthetized with 0.15 mL solution containing ketamine HCl (90 mg/mkg) and xylazine (10 mg/kg) with an intraperitoneal injection and administered meloxicam (2 mg/kg) through subcutaneous injection for pain management. The surgical site was shaved and prepared with iodine and 70% ethyl alcohol. A midline incision was made, followed by drilling a parietal burr hole 2 mm posterior to the coronal suture and 2 mm lateral to the sagittal suture. Mice were placed in a stereotactic frame and 2×10^5 GL261 cells in 2.5 μL saline were intracranially-injected (ic) with a 22-gauge needle at a depth of 3 mm. After needle removal, the skin was stapled.

2.3. Flow cytometry and T-cell stimulation

Brain tumor (BT), cervical lymph node (cLN) and spleen were isolated at 3 weeks post-intracranial injection (ic.) and mashed through a sterile 70 μm nylon mesh cell strainer (Fisher Scientific) with the plunger end of a 3 mL syringe into ice-cold DMEM. Single cell suspensions of BT were mixed in a PBS/30% Percoll solution and slowly pipetted onto a 70% Percoll cushion. Samples were centrifuged at 1200 × *g* for 30 min with no brake. The top layer was aspirated and the buffy coat, between the 30% and 70% percoll layers, was isolated and washed in cold PBS. Single cell suspensions of BT, cLN and spleen were then divided into three groups for staining: 1) unstimulated T cells, 2) stimulated T cells and 3) antigen-presenting cells. For stimulation, T cells were co-incubated with Cell Stimulation Cocktail (PMA/Ionomycin/Brefeldin A/Monensin; eBioscience) for 5 h in DMEM at 37 °C. Cells were incubated with antibodies in PBS + 2% FBS for 30 min on ice and according to Table 1. Samples were then permeabilized overnight at 4 °C using Fix/Perm Buffer (eBioscience) and incubated accordingly. Cellular frequency was determined with an LSR Fortessa flow cytometer (BD) and Flowjo analysis software (TreeStar, Cupertino, CA).

Table 1
The experimental design for flow cytometric evaluation. All antibody staining, except (*), was performed prior to overnight (o/n) permeablization. (*) indicates staining was performed after o/n permeablization.

Group	Marker	Clone	Tag
1	anti-mouse CD3	145-2C11	eFluor450
	anti-mouse CD4	GK1.5	PE-Cy7
	anti-mouse CD8	53–6.7	FITC
	anti-mouse/human CD44	IM7	APC
	anti-mouse/rat FoxP3	FJK-16s	PE
2	anti-mouse CD3	145-2C11	PE-Cy7
	anti-mouse CD11b	M1/70	FITC
	anti-mouse CD11c	N418	PE
	anti-mouse CD45	30-F11	eFluor450
	anti-mouse Ly-6G (Gr1)	RB6-8C5	APC
3	anti-mouse CD3	145-2C11	eFluor450
	anti-mouse CD4	GK1.5	PE-Cy7
	anti-mouse CD8	53–6.7	APC
	anti-mouse IFN-γ	XMG1.2	FITC
	anti-mouse/rat IL-17A	eBio17B7	PE

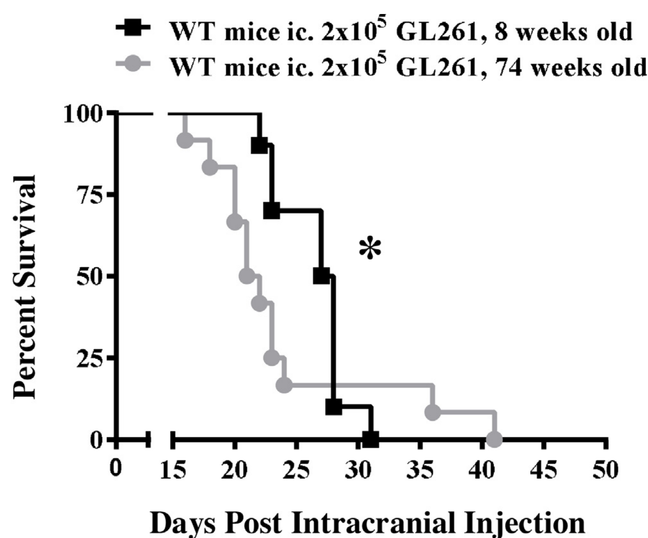

Fig. 1. The overall survival of young and old mice with brain tumors. 2×10^5 GL261 cells were intracranially-injected into 8 week old (black, n = 10) or 74 week old (grey, n = 12) C57BL/6 mice. The Kaplan-Meier curve represents mouse survival times over a time course of 50 days. *Gehan-Breslow-Wilcoxon test $P = 0.0292$.

2.4. HPLC analysis

HPLC analysis was carried out as previously described by Zhai, et al. [19].

2.5. Statistical analysis

Data are represented as the mean ±SEM. The statistical significance of the differences in mRNA expression and tumor-infiltrating T cell response was determined by two-tailed unpaired Student-*t* test for two groups and one-way ANOVA for groups of three or more followed by Turkey's post-hoc test. Overall survival was defined as the time from implantations until death. Survival curves were plotted using the Kaplan-Meier method and compared by Gehan-Breslow-Wilcoxon test. Data were analyzed using Prism 6.0 software (GraphPad Software). A *P* value less than 0.05 was considered significant.

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