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Advanced Age Increases Immunosuppression in the Brain and Decreases Immunotherapeutic Efficacy in Subjects with Glioblastoma

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Abstract

Purpose: Wild-type isocitrate dehydrogenase-expressing glioblastoma (GBM) is the most common and aggressive primary brain tumor with a median age at diagnosis of ≥65 years. It accounts for ~90% of all GBM and has a median overall survival (OS) of <15 months. Although immune checkpoint therapy has achieved remarkable survival benefits in a variety of aggressive malignancies, similar success has yet to be achieved for GBM among phase III clinical trials to-date. Our study aimed to understand the relationship between subject age and immunotherapeutic efficacy as it relates to survival from glioma.

Experimental Design: 1) Clinical data: Glioblastoma patient datasets from the cancer genome atlas, Northwestern Medicine Enterprise Data Warehouse, and clinical studies evaluating immune checkpoint blockade (ICB) were stratified by age and compared for OS. 2) Animal models: Young, middle-aged and older adult wild-type and IDO knock-out syngeneic mice were intracranially-engrafted CT-2A or GL261 glioma cell lines and treated with or without CTLA-4/PD-L1 mAbs, or radiation, anti-PD-1 mAb and/or a pharmacologic IDO enzyme inhibitor.

Results: Advanced age was associated with decreased GBM patient survival regardless of treatment with ICB. The advanced age-associated increase of brain IDO expression was linked to the suppression of immunotherapeutic efficacy and was not reversed by IDO enzyme inhibitor treatment.

Conclusions: Immunosuppression increases in the brain during advanced age and inhibits anti-glioma immunity in older adults. Going-forward, it will be important to fully understand the factors and mechanisms in the elderly brain that contribute to the decreased survival of older GBM patients during treatment with ICB.

Keywords

Senescence; neuroimmunology; immunotherapy; brain tumor; IDO

INTRODUCTION

As complex organisms age, there are changes in the architecture and function of the immune system. These changes are collectively termed ‘immunosenescence’, which reflects the dysregulation of an organism’s capacity for responding to novel immunological challenges, as well as a diminished capacity to initiate long-term immunological memory. Progressive immune aging is associated with the long-term sequelae of thymic involution[1] and T cell lymphopenia[2–5], loss of T cell receptor diversity[6], an increased inequality of clonal leukocyte pools[7], as well as a higher regulatory T cell ($CD4^+CD25^+FoxP3^+$):$CD8^+$ cytolytic T cell ratio in the peripheral blood[8]. In addition to altering the lymphoid compartment, progressive aging also alters myeloid cell development in the bone marrow[9,10]. In the central

nervous system (CNS), bone marrow-derived dendritic cells take up residency in the parenchyma[11–13], microglia express higher basal levels of MHCII and CD11b[14], and the potential for microglia to express immunosuppressive TGFβ and IL-10 is increased[15,16]. Senescent astrocytes increase in the aged brain and release higher levels of pro-inflammatory factors as part of the senescence-associated secretory phenotype (SASP)[17,18]. Notably, the eradication of senescent cells improves the healthspan in mouse models of premature aging[19].

Cancer is the second most lethal disease associated with advanced aging in the United States. Although the general mortality rate for individuals with cancer has declined over the past 40 years, it has steadily risen for those diagnosed with primary brain and other CNS cancers[8]. Glioblastoma (GBM) is the most common and aggressive primary brain tumor, accounting for ≥50% of malignant brain tumors with a median age at diagnosis of 65 years old[20]. The median overall survival (mOS) for patients with GBM is 15–20 months[21–23], but is significantly lower among elderly individuals[8,24]. It has been suggested that $CD8^+$ recent thymic emigrants (RTEs) account for the prognostic power of age on clinical outcome in GBM patients[25] because they are significantly decreased in older individuals, perhaps due to the synergy between glioma-induced- and advanced age-induced thymic atrophy[26]. Also notable is that long-term survivorship (LTS) is negligible among wild-type isocitrate dehydrogenase (IDH) elderly GBM patients[27], which characterize the majority of diagnoses. Immune checkpoint blockade (ICB) has provided a survival benefit, and in some cases, durable LTS in patients with end-stage melanoma[28], non-small cell lung carcinoma[29], renal cell cancer[30] and a growing list of other malignancies. In contrast, phase III studies treating with anti-PD-1 mAb, nivolumab, in recurrent GBM patients [NCT02017717], and nivolumab in combination with standard radiotherapy in patients with newly-diagnosed *MGMT*-unmethylated GBM [NCT02617589], failed to demonstrate an improved mOS. We recently hypothesized that one reason for the dismal outcomes of GBM patients is the under-appreciation for- and the unique interactions between-the CNS, immune system, and advanced age in subjects with glioma.

We previously found a higher mortality rate among older C57BL/6 mice with intracranial syngeneic GL261 glioma as compared to younger counterparts[31]. We then discovered a suppression of treatment efficacy in older adult mice with brain tumors that were simultaneously treated with radiation, anti-PD-1 mAb, and an indoleamine 2,3 dioxygenase 1 (IDO) enzyme inhibitor; a strategy now undergoing phase I clinical evaluation [NCT04047706][32]. Strikingly, there were no differences in the intratumoral leukocyte populations when comparing young and older subjects treated with trimodal immunotherapy. However, there was an increase in the expression of immunosuppressive IDO in the older adult brain regardless of tumor burden[31,32]. Importantly, IDO expression also increases in the human brain during advanced age and independent of whether there is a pre-existing brain tumor[8]. IDO is a rate-limiting enzyme that metabolizes the essential amino acid tryptophan into kynurenines. The associated enzyme activity is thought to be the primary mechanism of how IDO mediates immunosuppression, although it can also suppress the immune response independent of enzyme activity[33]. Arising from this multi-mechanistic immunosuppressive potential, we questioned the nature of increased IDO expression in the

brain and its relationship to immunotherapeutic efficacy in older adults with malignant glioma.

Here, we confirm a strong association between advanced age and decreased mOS in patients with GBM, demonstrating that the decreased mOS is also observed among older recurrent GBM patients treated with adjuvant ICB therapy. A lifespan analysis of syngeneic mice engrafted with glioma showed a selective dependence on $CD4^+$ and/or $CD8^+$ T cells for modulating the magnitude of LTS among the different age groups. This T cell dependence was particularly pronounced among older adult mice that lacked any LTS and died faster as compared to younger counterparts. We next evaluated the immunosuppressive contribution and targetability of IDO among young and older adult mice with intracranial glioma treated with immunotherapy. Strikingly, IDO exerted an overarching suppressive action on immunotherapeutic efficacy in older adults as compared to younger counterparts with glioma. Unexpectedly, the advanced age-mediated suppressive effect of IDO was not reversed by treatment with a potent IDO metabolic inhibitor. To the best of our knowledge, this is the first report to describe an increased level of IDO-mediated immune suppression during advanced age and reflects a new targetable mechanism for enhancing immunotherapeutic efficacy in elderly patients with incurable GBM.

MATERIALS AND METHODS

The Cancer Genome Atlas (TCGA)

Survival data for GBM patients was accessed from the TCGA using the University of California Santa Cruz Xena portal on 02-01-2019. All GBM patient analyses associated with the TCGA analysis were performed in a de-identified fashion and this work therefore neither requires informed patient consent nor approval by the Northwestern University Institutional Review Board (NU IRB).

Northwestern Medicine Enterprise Data Warehouse (NM EDW)

Patient medical records from the Northwestern Medicine Enterprise Data Warehouse (EDW) were accessed for individuals diagnosed with GBM, or those with lung, breast, kidney or colon cancer CNS metastases. Inclusion criteria for patients with GBM are included in Supp. Fig. 1 and as described[34]. For lung, breast, kidney or colon cancer CNS metastases, only subjects with an initial diagnosis at Northwestern and a reported date of death were analyzed. This study was granted exemption status from the NU IRB based on the de-identified GBM patient records provided for analysis and did not require informed patient consent.

Recurrent GBM (rGBM) patients treated with immune checkpoint blockade (ICB)

Data on rGBM patient age, sex, MGMT promoter methylation status, IDH status and median overall survival (mOS) were extracted and analyzed from three independent clinical data sets including: (i) the Ivy Consortium's multi-institution, randomized, open-label pilot study of the anti-PD-1 antibody pembrolizumab in patients with recurrent, surgically resectable GBM[35]; (ii) the Northwestern University-sponsored study entitled, 'tremelimumab and durvalumab in combination or alone in treating patients with recurrent malignant glioma'

[NCT02794883]; (iii) a patient cohort treated with pembrolizumab or nivolumab at Northwestern University and Columbia University, made available by Zhao *et al.*, 2019[36].

Mice and cell lines

Wild-type C57BL/6 (Cat#000664) and IDOKO (Cat#005867; C57BL/6 background) mice were obtained from Jackson Laboratories and maintained in the Northwestern University Center for Comparative Medicine (CCM). Wild-type mice were purchased at 4-, 6-, 8-, 23-, 47-, 60-, or 75-weeks of age, while IDOKO mice were maintained and aged in the CCM facility. All procedures involving animals were conducted in accordance with the Institutional Animal Care and Use Committee (IACUC) of Northwestern University. Unmodified GL261 cells were obtained from the NIH/NCI at Frederick (Frederick, MD), luciferase-expressing GL261 (GL261.fl) were provided by Dr. Orin Bloch's laboratory, and CT-2A cells were provided by Dr. Thomas Seyfried (Boston College, Boston, MA). Cell lines were authenticated prior to use. All cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 μg/mL) and streptomycin (100 mg/mL) at 37°C. All products used for cell culture were purchased from Gibco Invitrogen.

Orthotopic intracranial injection model

Mice were intracranially injected at 6-, 8-, 23-, 47- or 72–86-weeks of age with 5×10^2 - 5×10^4 syngeneic glioma cells and is further elucidated for each experiment in the figure legends. NIH guidelines on the care and use of laboratory animals were followed for all surgical procedures. Mice were anesthetized with 0.1 mL/g solution containing ketamine HCl (90 mg/kg) and xylazine (10 mg/kg) by intraperitoneal injection, and subcutaneously injected with meloxicam (2 mg/kg) prior to surgery and 24 hours following surgery for pain management. The surgical site was shaved and prepared with iodine followed by 70% ethanol. A 1 cm 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. Injections were conducted with a stereotactic frame and a 2.5 μL volume of glioma cells reconstituted in PBS and intracranially-injected (i.c.) to a depth of 3 mm using a 22-gauge Hamilton syringe. The skin was stapled after removal of the needle.

In vivo treatments

Mice were treated with whole brain radiation (RT; 2 Gy/day × 5 days), intraperitoneal anti-PD-1 mAb or PD-L1 mAb (clone J43 and 10F.9G2, respectively; 500 mg loading dose followed by a 100 mg maintenance dose every 3 days for 3 treatments total; BioXCell), and oral gavage suspended in ORAplus (Perrigo) with daily BGB-5777 or BGB-7204 (100 mg/kg; BeiGene). Mice were anesthetized with 0.15 mL solution containing ketamine HCl (90 mg/mkg) and xylazine (10 mg/kg) with an intraperitoneal injection prior to subject placement in a lead box with head-only exposure and irradiation with 2 Gy via cesium-137. For leukocyte depletion, mice were injected with 200 mg anti-CD4 mAb (clone GK1.5; InVivoMab), anti-CD8 mAb (clone YTS 169.4; InVivoMab), or anti-NK1.1 mAb (clone PK136; InVivoMab) every 3 days, beginning at 3 days prior to intracranial (i.c.) engraftment with glioma cells and continuing until study removal due to endpoint criteria and/or death.

Depletion of gut microbiota

Mice were treated daily with an antibiotic cocktail through oral gavage containing 500 mg/L Vancomycin (Sigma), 1 g/L Metronidazole (Sigma) and 1 g/L Neomycin (Sigma) as described[37]. Additionally, the mice were placed in cages with water bottles containing 1 g/L Ampicillin (Sigma). The water was changed weekly and the cages were changed every 3 days. Antibiotic treatment began 4 weeks prior to intracranial injection and continued until the experimental endpoint. Gut depletion was confirmed by quantifying mouse fecal samples for 16S RNA with RT-PCR.

Bone marrow chimeras

The bone marrow of recipient mice was ablated with 4.75 Gy irradiation, twice during the same day, with a 4-hour rest period between doses. During the rest period, cells were collected from the femurs and tibias of age- and sex-matched donor mice by flushing the bones with cold DMEM lacking FBS. A single cell suspension was generated by filtering the bone marrow through a 100 μm filter. Cells were counted and re-suspended in cold PBS at a final concentration of 5×10^6 cells/100 μL. After the final irradiation, recipient mice received 200 μL (1×10^7 cells) of donor bone marrow cells with a 30-gauge needle through the tail vein. Mice then received antibiotic-containing 2 g/L of Neomycin Sulfate for 8 weeks. Approximately 3 months after BM engraftment, the recipient mice were intracranially-injected with 5×10^4 GL261 cells.

Statistical analysis

Overall survival (OS) for cancer patients was defined as the time from diagnosis to death or last known alive time. For mouse studies, overall survival was defined as the day of tumor cell engraftment until reaching endpoint criteria and/or death. OS was estimated using the method of Kaplan-Meier. Median survival and its confidence intervals were estimated based on the Kaplan-Meier estimates. OS was compared between groups using logrank test or Cox proportional hazards regression models. Cox models were used to estimate hazard ratios (HRs). Proportional hazards assumption was checked. For predictors with multiple group levels (e.g. age groups) pairwise comparisons between groups were made based on the fitted Cox model, and p-values were adjusted for multiple pairwise comparisons using Scheffe's method because group sizes were unequal. Overall statistical significance of multi-level predictors was assessed using Wald test based on the fitted Cox model. Multivariable Cox models were fitted to obtain adjusted effects of age on overall survival. IDO mRNA expression relative to GAPDH (Fig. 5B) was summarized using mean and standard error of the mean (SEM) and compared between age, genotype, and treatment groups using a linear regression model which included a 3-way interaction term. Pairwise comparisons of the 6 resulting groups were made based on the fitted model and the *p*-values were adjusted using Scheffe's method. Statistical analyses were conducted using RStudio and R v. 3.6.1 statistical software; Cox models were fitted using the survival package in R; emmeans package was used to calculated adjusted *p*-values.

RESULTS

Overall survival is decreased among older adults with GBM but not in those with brain metastases arising from other cancers

We analyzed overall survival and estimated median OS (mOS) across age groups for (n=535) GBM patients from the TCGA (Fig. 1A,B), as well as n=499 with indications of *de novo* GBM from the NM EDW (Fig. 1C,D). Older subjects had decreased OS and shorter mOS. TCGA-associated GBM patients of the age groups <20–34 (n=39), 35–44 (n=47), 45–54 (n=121), 55–64 (n=151), 65–74 (n=115) and 75–84+ (n=62) years old have a mOS of 748, 618, 476, 442, 327 and 211 days, respectively (Fig. 1A). NM EDW-associated GBM patients of the age groups <20–34 (n=29), 35–44 (n=36), 45–54 (n=107), 55–64 (n=148), 65–74 (n=119) and 75–84+ (n=59) have a mOS of 1281, 831, 654, 510, 456 and 306 days, respectively. The TCGA- and NM EDW-associated median survival of GBM patients <65 years of age is 485 days (TCGA; Fig 1B and Supp. Fig. 2A) and 636 days (Fig. 1D and Supp. Fig. 2B), respectively. This survival is significantly better compared to TCGA and NM EDW-associated GBM patients ≥65 years of age, which is 290 days (p<0.0001, logrank test) and 383 days (p<0.0001, logrank test), respectively. Similar decreases in mOS were observed across both sexes with progressive aging although this was more pronounced among females in the oldest age groups (Supp. Fig. 3A,B). To determine if this same trend was present in other highly aggressive malignancies occurring within the CNS, we analyzed data from various CNS brain metastases. While these metastases each represent distinct, highly aggressive malignancies, the trend for lower survival among older individuals is not observed. The mOS for patients with lung cancer CNS metastases is 305 days for patients <65 years of age and 257 days for patients ≥65 years of age (p=0.081; Fig. 1E). For breast cancer CNS metastases, mOS is 341 days for patients <65 years of age and 268 days for patients ≥65 years of age (p=0.215; Fig. 1F). These figures are 243 days for patients <65 years of age and 336 days for patients ≥65 years of age for patients with kidney cancer CNS metastases (p=0.670; Fig. 1G). Finally, the mOS for patients with colon cancer CNS metastases is 168 days for patients <65 years of age and 150 days for patients ≥65 years of age (p=0.755; Fig. 1H). Collectively, these data suggest that advanced age is a significant negative factor in patients with GBM but is not generalizable to all types of cancer in the CNS.

To determine whether the negative effects of advanced age in human GBM patients can be experimentally recapitulated, we analyzed the mOS and LTS of C57BL/6 mice intracranially-engrafted with CT-2A glioma cells at 6, 23, 47 and 75 weeks of age. The mOS and LTS were 39 days and 13%, 43 days and 40%, 41.5 days and 27% and 40.5 days and 0%, respectively, among 6, 23, 47 and 75 week-old animals treated with control IgG (Fig. 2A,C,D,E,F; n=15/group). To determine whether different subsets of host T cells affect the survival of mice challenged with CT-2A glioma, similar age groups were intracranially-engrafted with 500 CT-2A glioma cells and co-depleted of $CD4^+$ and $CD8^+$ T cells based on independent confirmatory experiments that our depletion strategy was highly effective (Supp. Fig. 4). The mOS and LTS was 38 days and 13%, 40 days and 13%, 41 days and 14%, and 25.5 days and 0%, respectively, in 6, 23, 47, and 75 week-old mice co-treated with $CD4^+$ and $CD8^+$ T cell-depleting antibodies (Fig. 2B,C,D,E,F; n=15/group). These data

collectively suggest three primary conclusions: (i) T cells provide different levels of protection that depend on host age; (ii) older adult T cells are critical for preventing a catastrophic decrease of mOS from glioma; (iii) older adults with glioma have poorer LTS outcomes as compared to younger counterparts.

Advanced age is associated with decreased survival of human patients and mice with malignant glioma treated with ICB

Because we had identified an age-associated lower mOS in two independent GBM patient databases, we next questioned whether this effect extended to subjects with GBM treated with immunotherapy. Relevant information was extracted from patient medical records including age, sex, MGMT promoter methylation status, and IDH status for recurrent GBM (rGBM) in patients treated with various forms of ICB in the adjuvant setting (Fig. 3A). Ninety-two patients were dichotomized into groups defined as <65 years of age (n=65) and ≥65 years of age (n=27; Fig. 3B). rGBM patients <65 years of age and treated with ICB had a higher mOS of 278 days compared to patients ≥65 years of age treated similarly with a mOS of 154 days (Fig. 3A; p=0.024). This negative association between older age and shorter OS among subjects treated with ICB, combined with the negative effect of age in preclinical models (Fig. 2A,B,F), prompted us to generate a table of analogous age groups between C57BL/6 mice and humans, including weaning, puberty, sexual maturity/growth plate closure, reproductive senescence, and life expectancy (Fig 3C). Based on our previous work evaluating anti-CTLA-4 mAb/anti-PD-L1 mAb treatment in syngeneic mice, we asked whether age negatively affects the survival benefit of this treatment. Similar to the clinical setting, 78–82 week old mice (n=14) with syngeneic glioma have a mOS of 25.5 days and 27% LTS compared to 8–12 week old mice (n=15) treated similarly with a mOS not reached and 67% LTS (Fig 3D; p=0.0097). The outcomes collectively suggest that: (i) advanced age negatively affects mOS in human GBM patients treated with ICB; (ii) older adult mice with glioma treated with ICB recapitulate the negative survival outcomes of human subjects with GBM.

Advanced age suppresses immunotherapeutic efficacy through a non-tumor, non-hematopoietic mechanism

To explore the basis for how advanced age negatively affects immunotherapeutic efficacy in subjects with glioma, we considered developing a bone marrow chimeric mouse model with different ages of host non-tumor stroma and hematopoietic cells. However, previous work in melanoma suggested that the gut microbiota play an important role in immunotherapeutic efficacy[38,39]. Combined with the required use of antibiotics during generation of bone marrow chimeric mice, we first evaluated the effects of antibiotic treatment on immunotherapeutic efficacy. Given our previous work validating the combination of radiation (RT), anti-PD-1 mAb and an IDO enzyme inhibitor to improve the survival of mice with well-established glioma[36], which serves as the basis of our ongoing clinical trial in newly-diagnosed GBM patients [NCT04047706], we investigated the effects of antibiotics in animals using this strategy. Eight week-old WT mice with intracranial GL261 treated with RT, anti-PD-1 mAb and IDO enzyme inhibitor had a mOS of 36 days and a 45% LTS compared to the control group with a mOS of 24 days and no LTS (Fig. 4A; p=0.01). Antibiotic-mediated gut microbiota depletion (Supp. Fig. 5) had no effect on OS of

immunotherapy-treated subjects with a mOS=31.5 days and 44% LTS (p=0.907) compared to similarly treated mice not administered antibiotics (mOS=36.0 days). Armed with confirmatory evidence that antibiotics do not disable this immunotherapeutic approach, we next generated young and older adult chimeric WT mice reconstituted with either young or older adult bone marrow.

There was no difference in the OS of young WT mice with young or older adult bone marrow and treated with trimodal immunotherapy (Fig. 4B; p=0.972). Similarly, there was no difference in the OS between older adult WT mice with young or older adult bone marrow and treated with trimodal immunotherapy (p=0.190). In contrast, there was an increase in mOS and LTS in young mice given young or older adult bone marrow, compared to older adult mice with young (not significant) or older (p=0.019 and 0.004; respectively) adult bone marrow (p<0.05). These data collectively suggest that advanced age suppresses immunotherapeutic efficacy through a non-hematopoietic-, non-tumor stroma-dependent mechanism.

IDO lowers mOS in older mice through a mechanism that is not reversed by pharmacologic IDO enzyme inhibitor treatment

We previously validated the therapeutic effects of combined RT, anti-PD-1 mAb and IDO inhibitor treatment in multiple glioma models utilizing the IDO enzyme inhibitor BGB-5777[32]. To determine how therapeutic efficacy of BGB-5777 compares to that of a novel clinical-grade IDO enzyme inhibitor, BGB-7204, OS was evaluated in 8 week-old C57BL/6 mice with GL261 treated with RT and anti-PD-1 mAb combined with either BGB-5777 or BGB-7204. Both IDO inhibitors synergized with RT and anti-PD-1 mAb to improve OS and LTS compared to the control group with a mOS of 30 days and no LTS (Fig. 5A; p=0.05). There was no difference in OS between mice with intracranial glioma treated with either BGB-5777 or BGB-7204 in combination with RT and anti-PD-1 mAb (p=0.999). Because there was no difference in therapeutic efficacy and given the superior PK/PD properties of BGB-7204 (Supp. Fig. 6), all subsequent experiments utilizing the IDO enzyme inhibitor employed the clinical-grade compound.

We next confirmed our previous observations of higher IDO expression levels in the older adult brain compared to younger counterparts (Fig. 5B). Interestingly, the age-associated higher IDO expression was more marked in the non-tumor sample taken from the contralateral hemisphere, than in intratumoral areas. To determine the significance of this observation, we evaluated the effect of IDO inhibitor pre-treatment on OS in young and older WT mice. IDO inhibitor pre-treatment began at 7 days prior to intracranial engraftment with GL261 in 8–12 week-old WT mice. This resulted in a trend for increased survival with mOS = 19 days, longer than in the young control group with mOS = 15 days (Fig. 5C; n=15 mice/group). In contrast, older adult WT mice pre-treated with IDO enzyme inhibitor had mOS = 15 days, identical to the older control group that also had mOS = 15 days (Fig. 5D; n=11–13 mice/group). Given the unexpected age-dependent differences in survival, we asked whether IDO differentially affected the OS of young and older adult animals by evaluating IDO-KO mice injected with GL261. As expected from the results of the IDO inhibition experiments, 8–12 week-old IDOKO mice had significantly longer OS (mOS = 21

days) than the young WT group (mOS = 15 days) (Fig. 5C; p=0.014; n=14–15 mice/group). Unexpectedly, 72–78 week old IDOKO mice also had a significantly longer OS (mOS = 19 days) than the older WT group with a mOS = 15 days (Fig. 5D; p=0.027; n=9–11 mice/group). These data suggest that the pre-treatment efficacy of pharmacologic IDO enzyme inhibition is absent in older adults with glioma.

To ensure that the older animals succumbed to brain tumor burden, and not other aging-related factors, bioluminescence imaging evaluated brain tumor growth (Fig. 5B–D). The steady increase in luminescence intensity and visualization of tumor luminescence in young and older adult animals suggested that mice succumbed to the brain tumor and not to other unexpected co-morbidities (Fig. 5E, Supp. Fig. 7).

IDO suppresses immunotherapeutic efficacy during advanced age and is not reversed by pharmacologic enzyme inhibitor treatment

With the discovery that IDO levels are higher in older adult brains, but that the negative survival-associated effects of IDO are not reversed by enzyme inhibition, we evaluated whether IDO behavior differ in a more therapeutically-relevant model. Accordingly, 8–12 and 78–86 week-old WT and IDOKO mice injected with GL261 were simultaneously treated with RT, ant-PD-1 mAb and the IDO inhibitor (Fig. 6A). In-line with our previous findings[36], there was a greater improvement in OS in young than older WT mice with brain tumors (p<0.0001; n=19–20 mice/group). However, there was no significant difference in OS between GL261-bearing young WT and IDOKO mice treated with trimodal immunotherapy. Older adult IDOKO mice with brain tumors treated with RT, anti-PD-1 mAb and the IDO inhibitor showed a marked improvement in OS with mOS = 19.5 days and 29% LTS, relative to older treated WT mice with a mOS = 16 days and 0% LTS (P<0.0001; n=19–21 mice/group). Notably, however, the older IDOKO mice with glioma treated with immunotherapy still did not exhibit OS rescued to the level of younger counterparts, suggesting there are additional age-dependent immunosuppressive factors contributing to suppression of the anti-glioma immune response and/or responsiveness to glioma immunotherapy. There were no sex differences among the different groups treated with trimodal immunotherapy (Fig. 6B). Thus, the advanced age-mediated increase of brain IDO levels suppresses immunotherapeutic efficacy in both sexes and independent of its ability to suppress the immune response through enzyme activity. A working hypothesis for current and independent observations is presented in Fig. 6C.

DISCUSSION

The most prominent feature of aging is a gradual loss of functional reserve that results in degeneration at the molecular, cellular, tissue and organismal levels[40]. In mammals, age-related degeneration gives rise to well-recognized pathologies, including sarcopenia, atherosclerosis and heart failure, osteoporosis, macular degeneration, pulmonary insufficiency, renal failure, and neurodegenerative conditions including Alzheimer’s and Parkinson’s disease, stroke, dementia and many others. Although species vary in their susceptibility to specific age-related pathologies, collectively, these pathologies arise with almost exponential kinetics beginning at approximately 50–60 years of age in humans[41,42].

Deleterious alterations in the immune system that accompany human aging are commonly referred to as 'immunosenescence'[43]. It is clear that aging does not always lead to an inevitable decline in immunological functions but rather causes modifications. During development, thymic involution at puberty drastically reduces the output of naïve T cells and during aging, after long-term residence in the host, the remaining naïve T cells exhibit impaired functionality, such as differentiation into effector cells after antigen stimulation, reduced production of cytokines, and a more restricted T-cell receptor repertoire[44,45]. Epigenetic age-related changes include the methylation of cytokine gene promoters that impair optimal immune functions[46] and decrease the potency of T lymphocytes in aged individuals as compared to cells of the same phenotype from younger counterparts.

Despite an aggressive standard of care treatment that includes maximal surgical resection, stereotactic radiation, and chemotherapy with the DNA alkylating agent temozolomide, mOS is ~15 months and ~31 months for individuals with *de novo* and secondary GBM, respectively47. Among elderly patients, mOS is markedly reduced to only 4–5 months, according to population-based studies[48]. Attempts to explain the shorter survival of elderly GBM patients include (i) less favorable tumor biology; (ii) less aggressive care due to older patient frailty; (iii) greater treatment toxicity due to less physiological reserve; (iv) competing comorbidities that may shorten life[48]. Based on the overwhelming majority of *de novo* GBM diagnoses, its associated onset during advanced aging, the promising outcomes that immunotherapy has yielded for many cancer patients but not for individuals with GBM[49,50], and the well-established relationship between advanced age, immunosenescence and neuroimmunological changes in the brain, we explored the relationship between age-dependent increases in CNS immunosuppression with outcome for subjects with glioma during immunotherapeutic treatment.

We evaluated two independent databases (TCGA and the NM EDW) for the relationship between *de novo* GBM patient mOS and subject age. Our analysis showed both databases indicated a progressive decrease in mOS with increasing subject age. This finding was most obvious upon gross stratification of elderly human patients with GBM as compared to younger counterparts. Given the rapid course of disease often observed among individuals with brain metastases, we expected a similarly negative association of elderly individuals with CNS metastatic infiltration. In contrast, individuals ≥65 years of age with lung, breast, kidney or colon CNS metastases did not have decreased OS as compared to younger counterparts. These conflicting outcomes may reflect a different malignant sensitivity to aging in the brain that is attributable to the parenchymal source of immunosuppression in the CNS and its negative relationship to a wholly parenchymal-resident tumor (ie. GBM), as compared to metastatic disease that is less protected by the blood brain barrier due to its partial residence in the perivascular space[51].

While there is a paucity of comprehensive datasets arising from the study of immunological protection during tumor challenge, there exists a large amount of information related to vaccine efficacy across the lifespan, albeit, segmented across large categories[52]. The data suggest that, while early- and mid-life vaccination-mediated immunological protection is strongest, the robustness of immune defenses lessen during advanced aging. We therefore questioned whether mice at different ages would show differences in mOS and/or long-term

survival (LTS) after intracranial engraftment of a highly aggressive immune checkpoint therapy-resistant murine glioma cell line. Strikingly, the highest level of survival was observed among middle-aged 23- and 47-week old mice as compared to 6-week old mice, which are age analogous to ~28–30-, ~38–40- and ~14–15-year old humans respectively. We hypothesize that the lack of immune system maturity of the 6-week old mice plays a role in the decreased survival as compared to 23- and 47- week old mice. In the 75-week old setting, we hypothesize that the aging immune system as well as the enhanced immunosuppression in the brain are synergistic factors contributing to the decreased survival as compared to younger counterparts. Notably, the simultaneous depletion of $CD4^+$ and $CD8^+$ T cells ablated the survival advantage among middle-aged subjects. Interestingly, at least 13% of subjects from the 6-, 23-, and 47-week old groups survived long-term regardless of T cell lymphopenia, suggesting the existence of a T cell-independent protective mechanism to glioma challenge among these age groups. Most dramatically, the 75-week old mice with glioma which are age-analogous to ~58–59 year old humans, had 0% LTS and a mOS that was further decreased when co-depleted of $CD4^+$ and $CD8^+$ T cells. These findings show for the first time that there are age-dependent changes in OS from malignant glioma that depend in part but not exclusively on the normal presence of $CD4^+$ and/or $CD8^+$ T cells, and highlight subject age as an important factor for survival outcomes in the lymphopenic setting during advanced age.

With subject age confirmed to play a role in immune-mediated survival among adult subjects with glioma, and our new findings that older individuals exhibited poorer survival from GBM as compared to younger counterparts, we next questioned whether recurrent glioblastoma (rGBM) patients treated with immune checkpoint blockade have a survival difference that can be stratified by age. Similar to our TCGA and NM EDW analysis, elderly rGBM patients treated with immune checkpoint blockade had a significant decrease in mOS as compared to younger counterparts. An age-associated decrease in OS was also observed in older adult syngeneic mice with intracranial glioma treated simultaneously with anti-CTLA-4 mAb/anti-PD-L1 mAb.

Nearly 40 years ago, Flood *et al.* were among the first to describe an aging-dependent decreased capability of mounting idiotypic and anti-idiotypic responses required for controlling the growth of ultraviolet light-induced fibrosarcomas in syngeneic mice[53]. More recently, it was discovered that relapse-free destruction of large long-established tumors expressing a very high-affinity tumor-specific antigen can be achieved through adoptive transfer of splenocytes from young 6-month old, but not 16-month old mice[54]. In each of these studies, the tumor was highly immunogenic; in contrast, GBM is well recognized for its low immunogenicity[55]. We therefore questioned whether the hematopoietic compartment or non-hematopoietic non-tumor stroma was primarily responsible for suppressing anti-glioma immune responses during advanced age. Unexpectedly, it was the aged non-hematopoietic non-tumor stroma that primarily prevented immunotherapeutic efficacy. These data collectively suggest that a non-hematopoietic factor, not of tumor cell origin, suppresses the anti-glioma immune response at advanced age, but the nature of this factor remains unknown thus far.

Multiple findings of increased IDO expression in the normal human and mouse brain during advanced age make this factor a good candidate for the source of non-hematopoietic non-tumor stroma-mediated immune suppression[31,32]. This hypothesis prompted us to question the role of IDO in suppressing the anti-glioma immune response during aging. When expressed, IDO suppresses immune responses both by acting as a rate-limiting enzyme that converts tryptophan into kynurenines[56], as well as through non-enzyme-mediated intracellular signaling[33]. Given the dual immunosuppressive mechanisms attributed to IDO, we addressed the potential role of tryptophan metabolism in suppressing the elderly glioma immune response by studying young and older adult subjects treated with or without a pharmacologic IDO enzyme inhibitor. Rather unexpectedly, the data collectively suggest that IDO suppresses the anti-glioma immune response independent of its enzymic function. This observation raises multiple intriguing questions including (i) how does IDO suppress the anti-GBM immune response; (ii) what is the cellular origin of immunosuppressive IDO during advanced age; (iii) and is this generalizable to other age-related diseases of the central nervous system? These questions remain unanswered at this time.

Among the resident cell types in the brain, astrocytes are the most abundant. They possess proliferative capacity, are crucial for neuron survival[57], mediate ion homeostasis, growth factor responses, and neurotransmitter functions[58]. Previous work demonstrated that astrocyte dysfunction is associated with multiple advanced aging-associated neurodegenerative diseases including Alzheimer's disease (AD) and Parkinson's disease (PD)[59,60]. Importantly, senescent astrocytes have been associated with aged- and AD-brain tissues[61], while other studies have identified factors capable of inducing astrocyte senescence[59,61,62]. These studies provide a potential link between aging and senescent astrocytes that release inflammatory molecules. Given the exquisite sensitivity of IDO to inflammatory signals, it is tempting to hypothesize that senescent astrocytes increase brain IDO expression, thereby indirectly contributing to the suppression of anti-glioma immunity in elderly subjects.

Together, our novel observations highlight a negative effect of advanced age in subjects with malignant glioma that is partly dependent on IDO. They also suggest that the current generation of IDO enzyme inhibitors will be ineffective at reversing the full extent of immunosuppressive IDO effects during advanced age and provides the rationale for designing and creating next generation modalities that fully reverse IDO-mediated immune suppression. Targeting the non-enzymic function(s) including the IDO immunoreceptor tyrosine signaling motifs that control protein stability and promote non-canonical NF-κB activation[33] could prove to be more effective than targeting the catalytic domain. Finally, our work highlights an important implication in the field of neuro-oncology seeking to improve care and outcomes in elderly GBM patients – current preclinical modeling that takes place in young subjects and/or subjects ablated for the immune system do not fully mimic the differential effects of age across the lifespan, nor do they allow for understanding the advanced age-associated consequences on GBM initiation, growth, and/or response to therapy. Ultimately, our work supports the future evaluation of age-appropriate glioma models to fully understand the biological underpinnings that contribute to the negative effects of immune aging in elderly individuals with GBM.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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TRANSLATIONAL RELEVANCE

Adult glioblastoma (GBM) is the most common primary malignant brain tumor, divided into two distinct subtypes: (i) *de novo* GBM, which comprises ~90% of diagnoses and possesses wild-type isocitrate dehydrogenase (IDH); (ii) secondary GBM, which accounts for the other ~10% of diagnoses and has mutated IDH. *De novo* GBM arises without any previous history of glioma, whereas secondary GBM transforms from a lower grade glioma into GBM. Individuals with *de novo* GBM have a median age of ~68–70 years, whereas individuals with secondary GBM have a median age of 48 years. Based on the majority of GBM diagnoses presenting as *de novo* cases, onset at advanced age and the promising outcomes that immunotherapy has yielded for many different cancers, we focused on the outcomes and effects of advanced age on treatment with immune checkpoint blockade in subjects with malignant glioma. We found that: (i) contrary to emerging reports for treatment of metastatic melanoma and certain other solid tumors by checkpoint blockade, GBM patient survival is inversely correlated with age such that elderly patients have worse survival than younger patients regardless of treatment by checkpoint blockade; (ii) older adults with glioma do not become long-term survivors and depend on T cells for maximal survival; (iii); IDO (indoleamine 2,3 dioxygenase) is relatively increased in the elderly brain and decreases immunotherapeutic efficacy against glioma. These data collectively suggest that future therapeutic efforts should focus on reversing the maladaptive effects of aging in GBM patients to enhance immunotherapeutic efficacy.

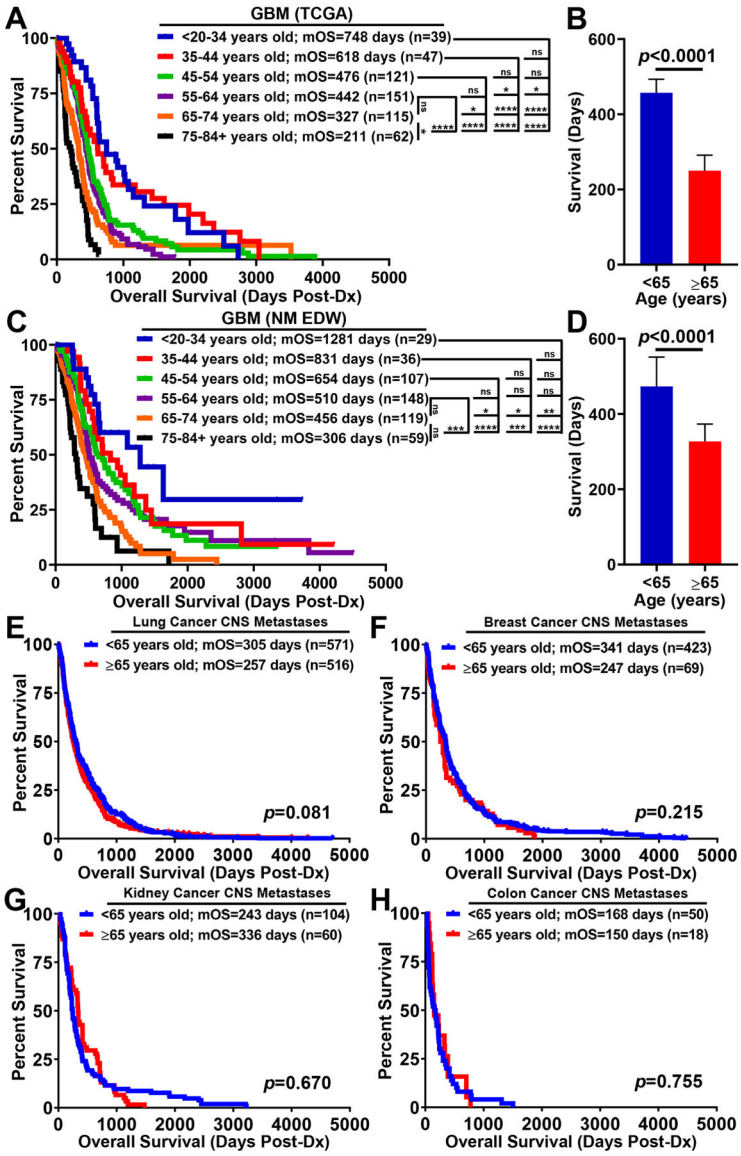

Figure 1. Advanced aging is associated with decreased overall survival (OS) among glioblastoma (GBM) patients, but not in human subjects with lung-, breast-, kidney-, or colon- metastases in the brain.

Human subject OS analyzed from the medical records **(A,B)** for the wild-type isocitrate dehydrogenase (wtIDH) cohort of GBM patients in the cancer genome atlas (TCGA) and **(C,D)** GBM patients in the Northwestern Enterprise Data Warehouse (NW EDW) and analyzed as described in Supp. Fig. 1. Subjects were stratified by **(A,C)** <20–34 (blue)-, 35–44 (red)-, 45–54 (green)-, 55–64 (purple)-, 65–74 (orange)-, and 75–84+ (black)-years of age, or by **(B,D)** <65 (blue)- or ≥65 (red)-years of age. The NW EDW database was also evaluated for the OS of human subjects with brain metastases originating from the **(E)** lung, **(F)** breast, **(G)** kidney, and **(H)** colon and stratified by <65 (blue)- or ≥65 (red)-years of age. Overall survival estimated using the method of Kaplan-Meier (A, C, E-H). Age groups compared using Cox proportional hazards models; pairwise groups comparisons adjusted

using Scheffe's method (A, C). Median OS with 95%CI (B, D). Age groups compared using logrank test (E-H). *p<0.05; **p<0.01 ***p<0.001; ****p<0.0001

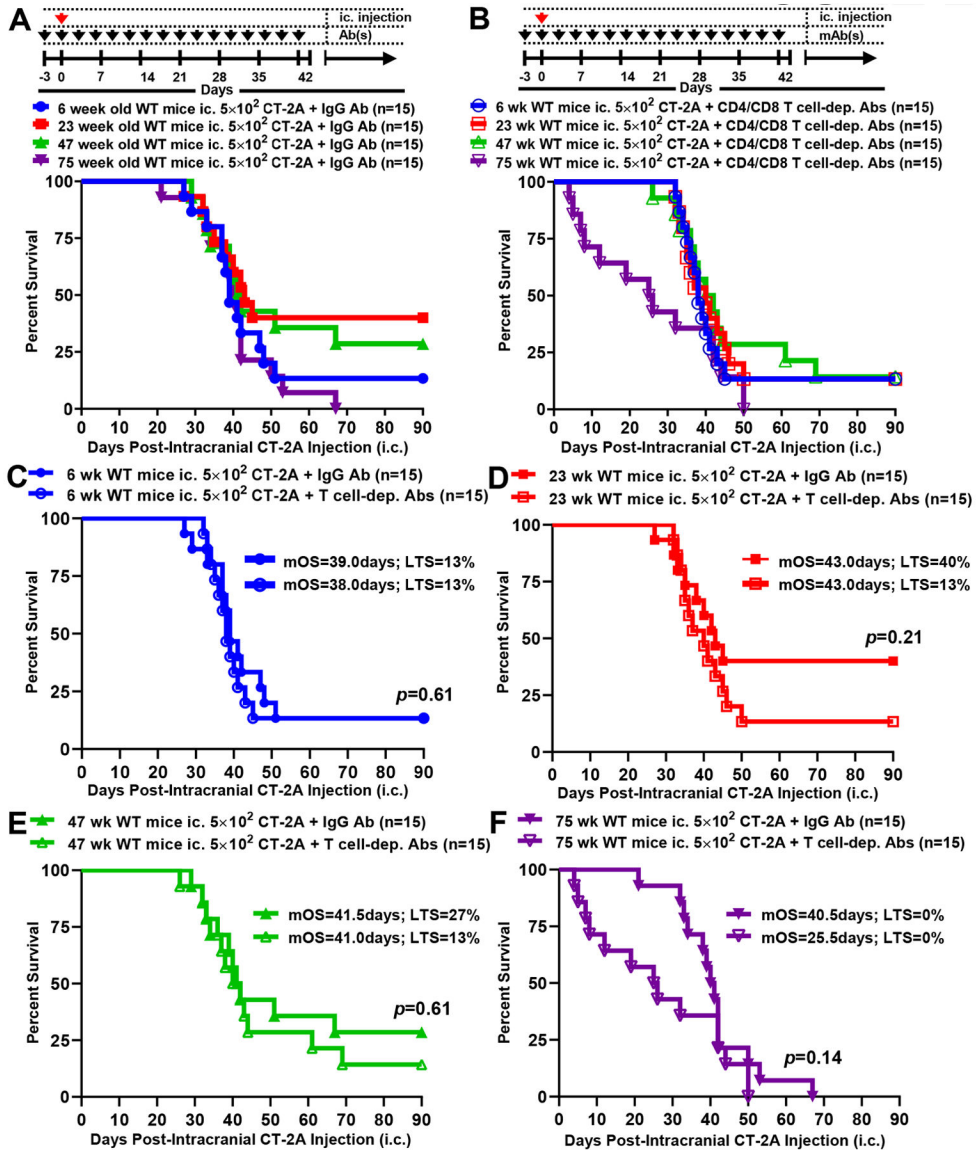

Figure 2. Advanced aging limits sporadic long-term survivor (LTS) generation in syngeneic mice with intracranial CT-2A glioma.

Timeline and overall survival analysis of 6-, 23-, 47-, and 75-week old C57BL/6 wild-type (WT) mice intracranially-engrafted with 500 CT-2A cells and administered **(A)** non-specific IgG Ab or **(B)** $CD4^+$ and $CD8^+$ T cell-depleting Abs. Individual age groups treated with IgG Ab or T cell-depleting Abs were further stratified by **(C)** 6-, **(D)** 23-, **(E)** 47-, and **(F)** 75-week old subjects, respectively. Overall survival estimated using the method of Kaplan-Meier; age groups compared using Cox proportional hazards models (A-F).

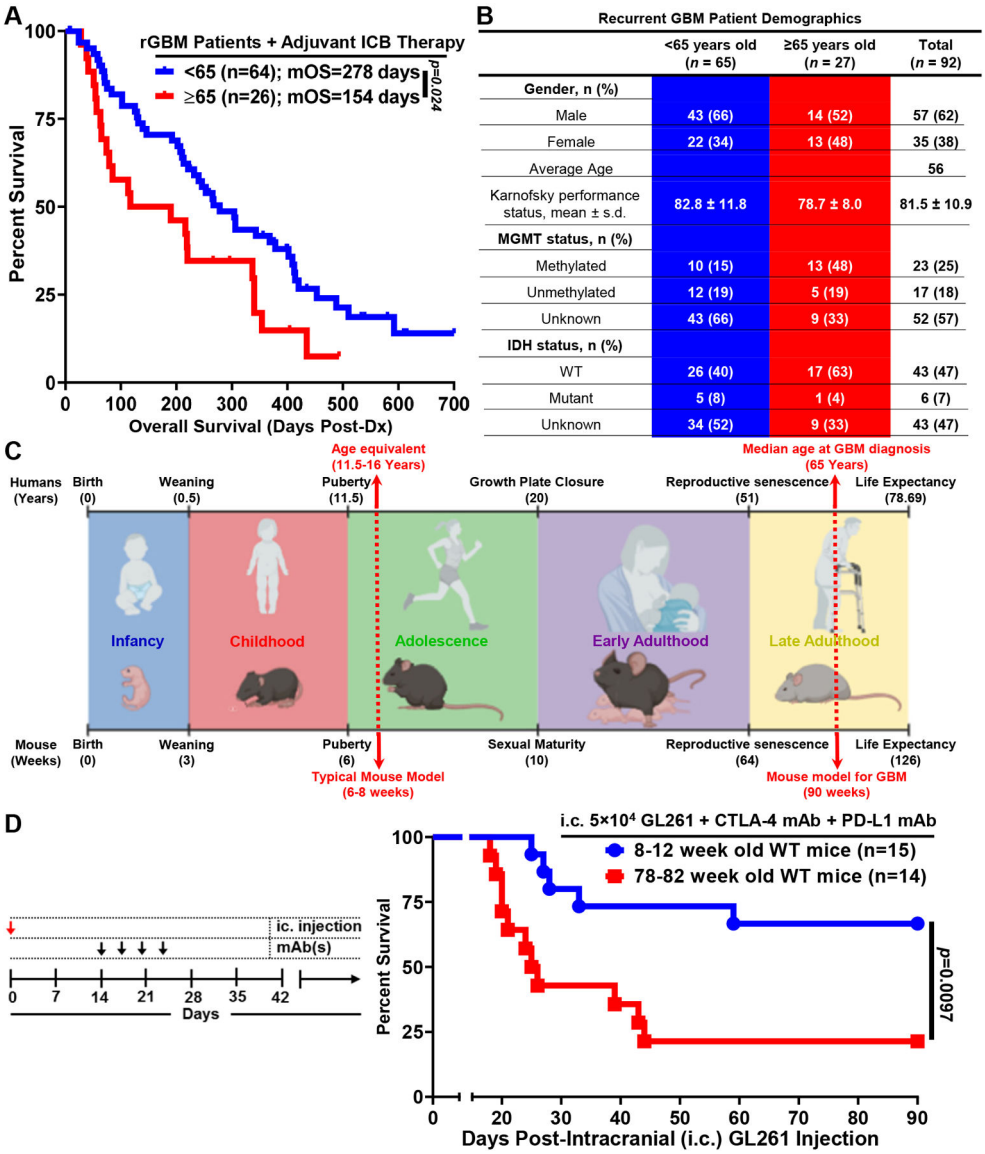

Figure 3. Advanced aging is associated with decreased overall survival (OS) of recurrent glioblastoma (rGBM) patients and syngeneic mice treated with immune checkpoint blockade therapy.

(A) OS and **(B)** patient demographics of rGBM patients treated with adjuvant nivolumab (anti-PD-1 mAb), pembrolizumab (anti-PD-1 mAb), libtayo (anti-PD-1 mAb), durvalumab (anti-PD-L1 mAb), tremelimumab (anti-CTLA-4 mAb), or a combination of durvalumab and tremelimumab, and ultimately stratified by <65 (blue)- or ≥65 (red)-years of age**. (C)** Schematic illustration (created with biorender.com) of lifespan milestones and comparative ages between humans and C57BL/6 mice at the time of birth, weaning, puberty, sexual maturity, reproductive senescence, and life expectancy. The first dotted red line denotes the analogous mouse and human age range for common syngeneic mouse glioma modeling at 6–12 weeks old, which corresponds to the age of 11.5–16 year old human. The second dotted red line denotes the analogous mouse and human age ranges for the median age of a human GBM patient diagnosis at 64 years old in humans, which corresponds to 87 weeks

old in mice. **(D)** Treatment timeline and OS of C57BL/6 WT mice intracranially-engrafted with 5×10^4 GL261 cells and simultaneously treated with anti-CTLA-4 and anti-PD-L1 mAbs beginning at day 14 after tumor cell injection. Overall survival estimated using the method of Kaplan-Meier; age groups compared using Cox proportional hazards models (A, D); *$p<0.05$; **$p<0.01$

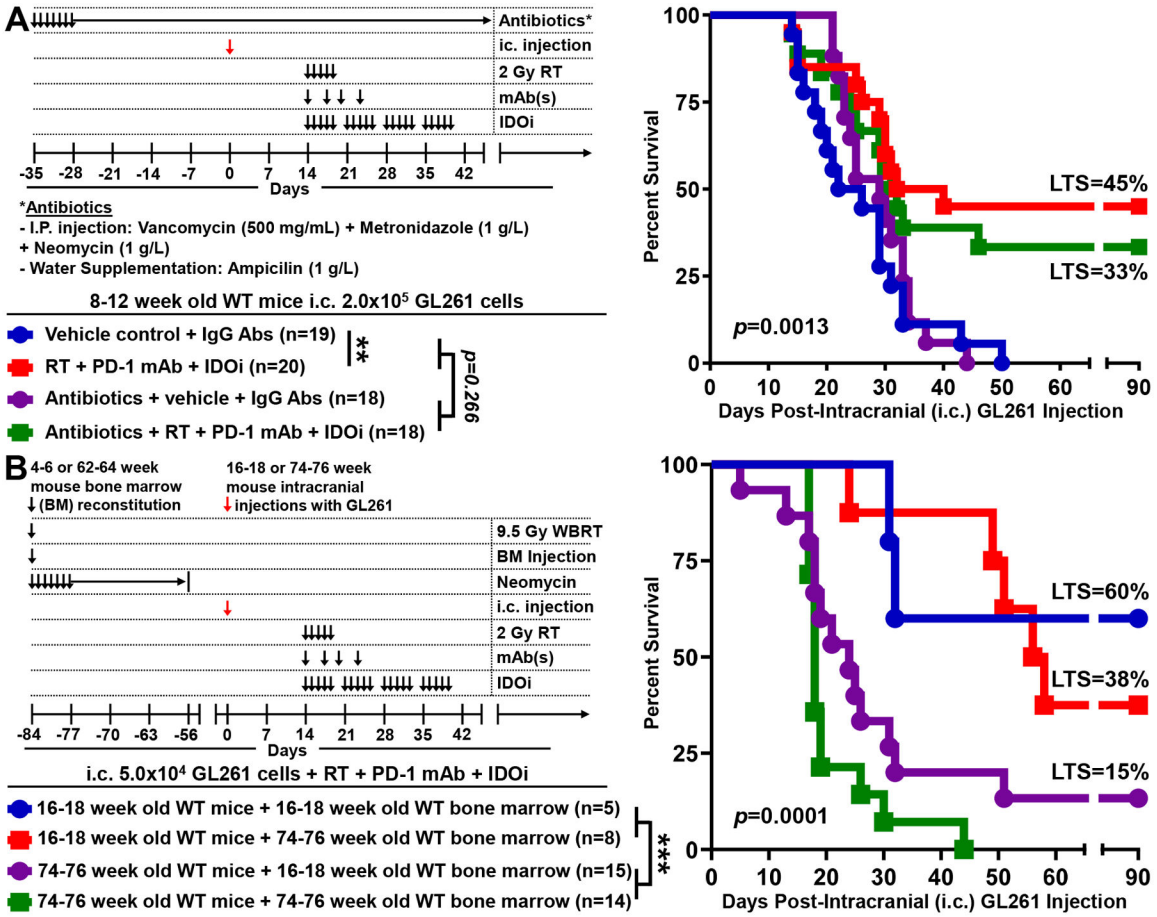

Figure 4. Advanced aging licenses non-tumor-, non-hematopoietic-stroma to suppress immunotherapeutic efficacy in subjects with malignant glioma.

(A) Timeline and overall survival (OS) analysis of 8–12 week old C57BL/6 wild-type (WT) mice treated with or without intraperitoneally (i.p.)-administered vancomycin, metronidazole, and neomycin, as well as ampicillin via the water supply, beginning at day -35, followed by intracranial-engraftment with 2.0×10^5 GL261 cells at day 0, and treatment with 2 Gy whole brain radiation (RT) × 5 days total, one 500 μg loading dose followed by three 100 μg maintenance doses of anti-PD-1 mAb (clone J43), and the IDO enzyme inhibitor (IDOi; BGB-7204), beginning at day 14 after tumor cell injection and as previously described[32]. **(B)** Timeline for the mixed age bone marrow chimeric mouse experiment. Briefly, 4–6 week old and 62–64 week old C57BL/6 WT mice were sublethally irradiated with 9 Gy whole body radiation (WBRT) and reconstituted with the bone marrow from either 4–6 week old or 62–64 week old C57BL/6 WT mice during the same day. Mice received treatment with neomycin between days -84 to -56. Approximately three months later, all groups were intracranially-engrafted with 5.0×10^4 GL261 cells, and treated with RT, anti-PD-1 mAb, and IDO1 enzyme inhibitor, beginning at day 14 after tumor cell injection and as described in (A). The resulting OS analysis is presented as Kaplan Meier survival curves. Overall survival estimated using the method of Kaplan-Meier (A, B). Age groups compared using Cox proportional hazards models; pairwise groups comparisons adjusted using Scheffe's method (A, B). $^{**}p\leq0.01$; $^{***}p\leq0.001$

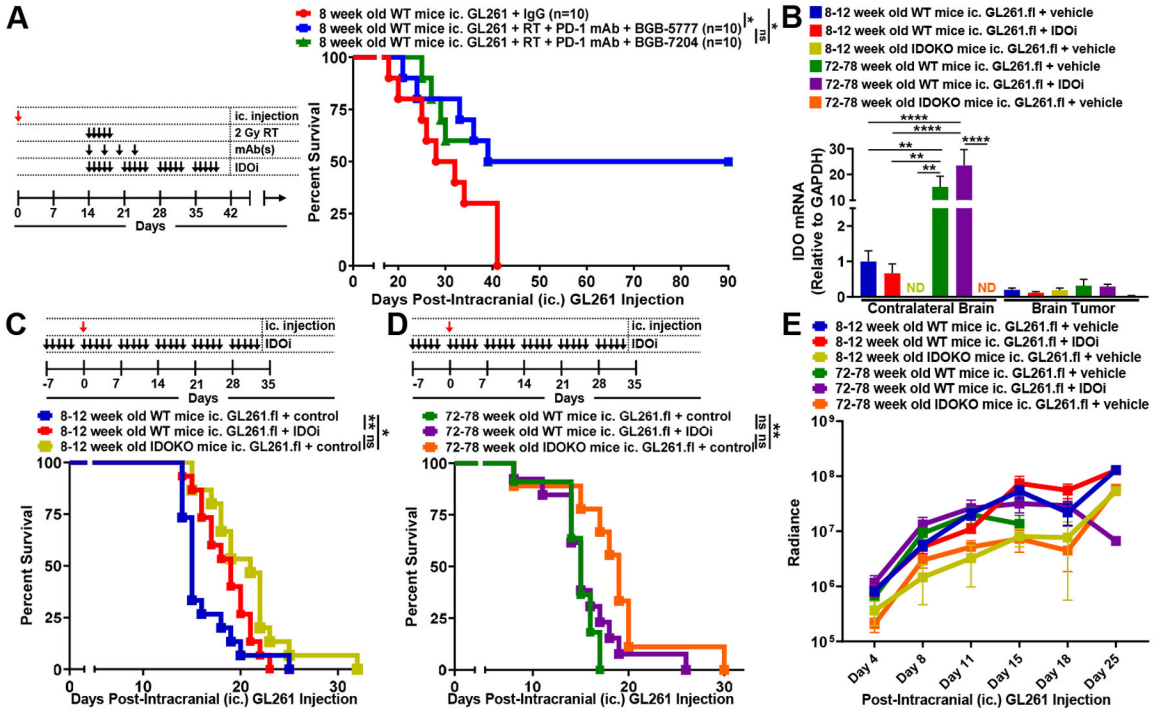

Figure 5. Advanced aging licenses non-tumor cell IDO to decrease overall survival (OS) despite the treatment with a pharmacologic IDO enzyme inhibitor in subjects with malignant glioma.

(A) Timeline and OS analysis of 8 week old wild-type (WT) C57BL/6 mice intracranially-engrafted with 2.0×10^5 GL261 cells and treated with IgG Abs, or with 2 Gy whole brain radiation (RT) × 5 days total, one 500 μg loading dose followed by three 100 μg maintenance doses of anti-PD-1 mAb (clone J43) and combined with either BGB-5777 (tool compound IDO enzyme inhibitor which we previously published with[36]) or BGB-7204 (clinical grade IDO enzyme inhibitor) beginning at day 14 after tumor cell injection. **(B-F)** IDO expression- and OS-analysis of 8–12 week old and 72–78 week old, WT and IDOKO (C57BL/6 background) mice, pre-treated with vehicle alone or with BGB-7204 (IDOi), followed by the intracranial-engraftment with 5.0×10^4 GL261.fl cells modified to express luciferase. **(B)** IDO mRNA expression reflects the levels in the contralateral brain without tumor, as well as the syngeneic brain tumor. **(E)** Bioluminescence of GL261.fl brain tumors expressed as a percentage of the final radiance for each mouse. Overall survival estimated using the method of Kaplan-Meier; age groups compared using Cox proportional hazards models; pairwise groups comparisons unadjusted (A) or adjusted using Scheffe's (C, D). Mean ± SEM; groups compared using linear regression model; pairwise groups comparisons adjusted using Scheffe's method. *p<0.05; **p<0.01; ****p<0.0001

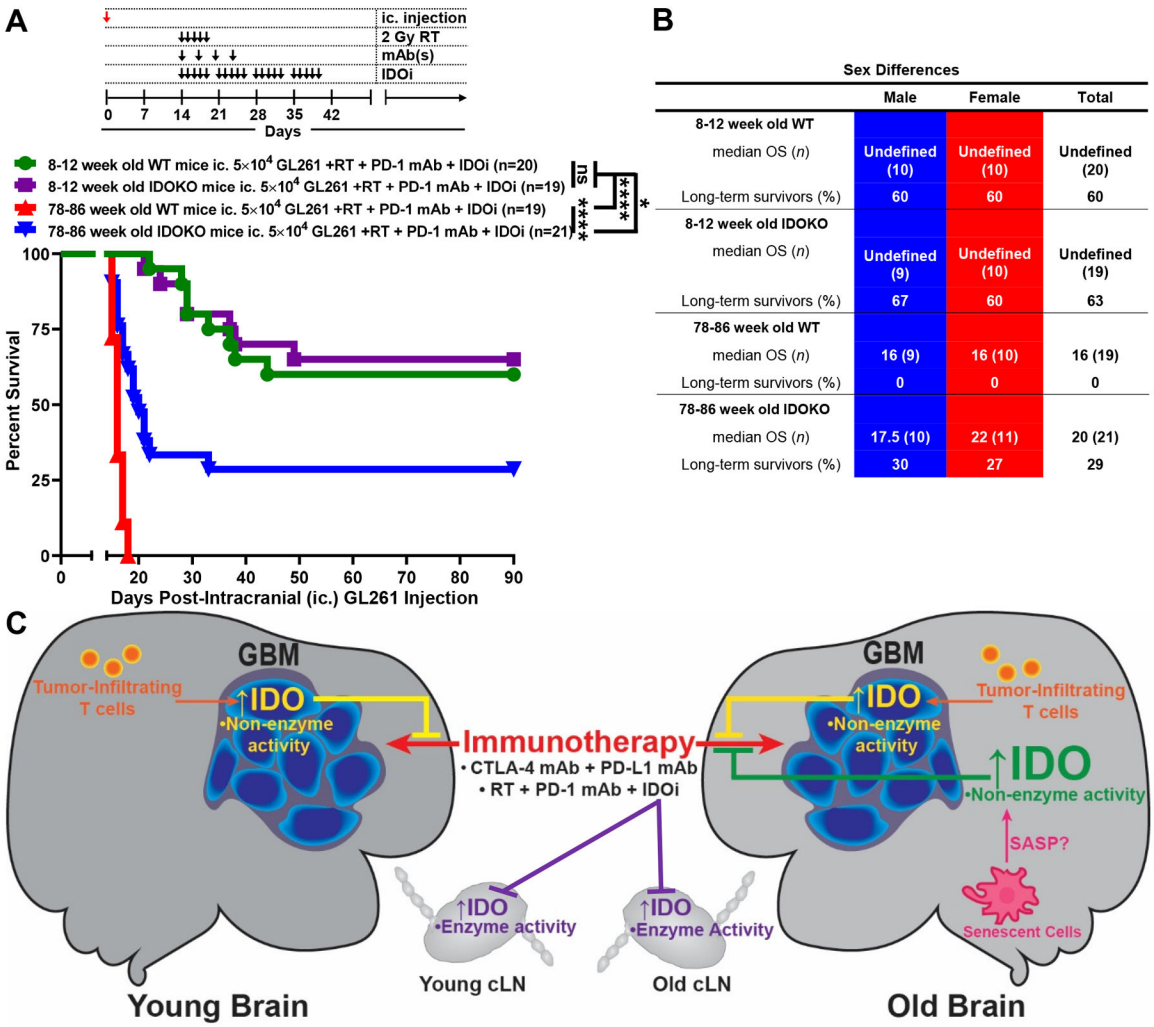

Figure 6. Advanced aging enhances the immunosuppressive effects of IDO, which is not reversed during the treatment with a pharmacologic enzyme inhibitor in subjects with malignant glioma.

(A) Timeline and overall survival (OS) analysis of 8–12 week old- and 78–86 week old-C57BL/6 wild-type (WT) and IDOKO (C57BL/6 background) mice intracranially-engrafted with 5.0×10^4 GL261 cells and treated with 2 Gy whole brain radiation (RT) × 5 days total, one 500 μg loading dose followed by three 100 μg maintenance doses of anti-PD-1 mAb (clone J43), and IDO enzyme inhibitor (IDOi; BGB-7204) beginning at day 14 after tumor cell injection. **(B)** Composition and numbers of young and older adult male and female WT mice that are represented in (A) and their association with median and long-term OS. **(C)** A working hypothesis for how IDO suppresses: (i) the anti-glioma immune response; (ii) responsiveness to glioma immunotherapy, in subjects with malignant glioma. Greater than 90% of human patient-resected glioblastoma (GBM) is IDO positive and for which, intratumoral IDO expression levels increase with higher tumor-infiltrating T cell levels[63] but are not changed with aging[8]. Glioma cell IDO increases intratumoral immunosuppressive regulatory T cells (Tregs; $CD4^+FoxP3^+$)[64] regardless of the treatment with an IDO enzyme inhibitor[65] and the absence of IDO metabolism by glioma cells[66,67]. In contrast, IDO metabolism is detectable in the brain tumor-draining cervical lymph nodes (cLN)[66].

Advanced age increases IDO expression in the brain (Fig. 5B)[8,31,36] and decreases immunotherapeutic efficacy against glioma despite IDO enzyme inhibitor treatment. Given their association with mediating inflammation, senescent cells with the senescence-associated secretory phenotype (SASP) indirectly upregulate immunosuppressive IDO expression in the brain during advanced aging[59,61], due to the high sensitivity for IDO expression by pro-inflammatory molecule expression[68]. Overall survival estimated using the method of Kaplan-Meier (A). Age groups compared using Cox proportional hazards models; pairwise groups comparisons adjusted using Scheffe's method (A). *$p<0.05$; ****$p<0.0001$