Mutant Isocitrate Dehydrogenase I in Glioma Cells Decreases Adenosinergic Pathway Signaling in Glioma Tumor Microenvironment

A thesis submitted in partial fulfillment of the Degree of Bachelor of Science in Biology, Health, and Society with Honors

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Abstract

Gliomas are the most common malignant brain tumors in adults[1]. Low-grade gliomas (LGGs) are defined by the World Health Organization (WHO) grades I and II[2]. The WHO restructured the classification of tumors of the nervous system in 2021, focusing on mutations in the isocitrate dehydrogenase 1 (IDH1) gene[3]. The R132H mutation of IDH1 results in the reduction of α-ketoglutarate (αKG) to 2-hydroxyglutarate (2HG)[4]. 2HG has various effects on the tumor microenvironment and its signaling pathways[5]. The adenosinergic pathway is used to control tumor immune response[6]. Under normal physiological conditions, cellular damage causes the release of extracellular adenosine triphosphate (ATP), which binds to immune P2X7 receptors to trigger inflammatory antitumor responses[7]. However, tumors have adapted to turn extracellular ATP into anti-inflammatory adenosine (ADO) with the use of the adenosinergic pathway (AP), which allows them to avoid the antitumor responses[8]. Integral to the adenosinergic pathway are the receptors CD39 and CD73, the former of which is used to dephosphorylate ATP to adenosine diphosphate (ADP) and further to adenosine monophosphate (AMP), and the latter is used to hydrolyze the last phosphate on AMP to convert it to adenosine[8]. In an effort to analyze changes in the adenosinergic pathway due to mutant IDH1 (mIDH1), as well as the effect of mIDH1 on the expression of certain markers in T cells, we looked at CD73 and ATP expression in glioma cells and immune cells. It was observed that the secretion of ATP as well as the expression of CD73 was decreased in mIDH1 glioma cells. CD73 expression was increased in mIDH1 immune cells in the tumor microenvironment. These results lend themselves to the possibility of novel immunotherapies for the treatment of mIDH1 glioma patients using treatments that target adenosinergic pathway molecules.

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Introduction

Characterization of Low-Grade Gliomas (LGGs)

Gliomas are the most common primary intracranial tumor [9]. They represent 81% of malignant brain tumors and are among the most lethal cancers [9]. Current treatments for glioma include surgery, to resect a portion of the tumor [10]. Further treatment includes radiation therapy in combination with chemotherapy after tumor resection [10]. Those with low grade gliomas (LGGs) (World Health Organization (WHO) grade 2) have an approximate survival of 7 years post-diagnosis [11]. While this is better compared to patients with high grade glioma (WHO grade 3/4), all LGGs progress to high grade gliomas and are ultimately lethal [11]. The World Health Organization (WHO) published the fifth edition of the WHO Classification of Tumors of the Central Nervous System (CNS) in 2021, introducing changes to CNS tumor nomenclature and grading [12]. Among these changes in guidelines specifically regarding gliomas is the increased focus on the mutations in the genes coding for isocitrate dehydrogenase 1 (IDH1) [13]. The mutations were linked to a higher survival rate in patients, and the IDH1 mutations now characterize LGGs, which include astrocytomas and oligodendrogliomas [12,14]. The types of adult-type diffuse gliomas listed in the latest edition are IDH-mutant astrocytomas, IDH-mutant oligodendrogliomas, and IDH-wildtype glioblastomas (GBM) [12]. The WHO CNS5 has made the transition to within-tumor-type grading rather than entity specific grading, but has retained the ranges of grades used for tumor types in prior editions [12]. Grades are correlated to idealized clinical-biological behavior, where CNS WHO grade 1 tumors are those which are curable with surgery, and CNS WHO grade 4 tumors are highly malignant and will lead to death without therapy [12]. In the WHO CNS5, mutant-IDH astrocytomas range from CNS WHO grades 2 to 4, and mutant-IDH oligodendrogliomas range from CNS WHO grades 2 to 3 [12].
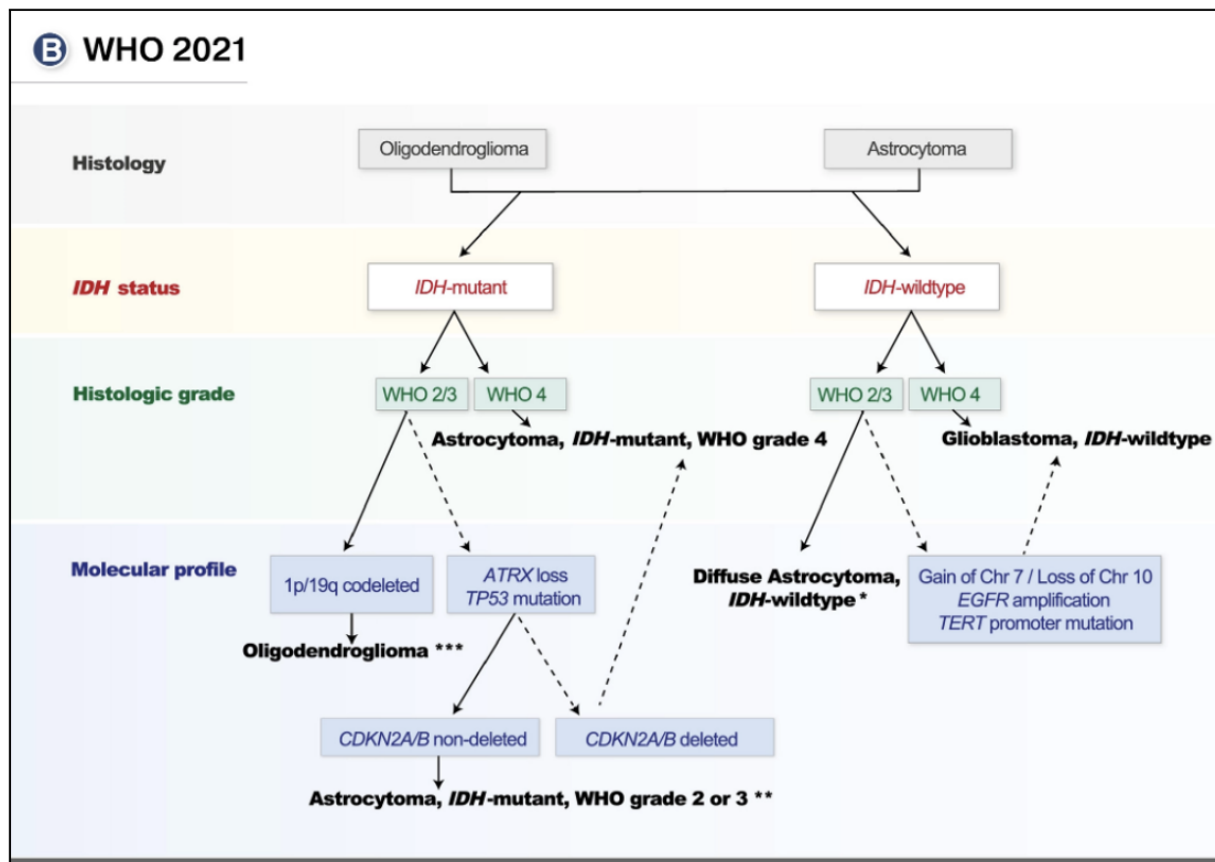
Figure 1: A map for the classification of diffuse gliomas based on histological and genetic features [15]

<u>Isocitrate Dehydrogenase 1 (IDH1) and the Adenosinergic Pathway</u>

Isocitrate dehydrogenase is a $NADP^+/NAD^+$ dependent homodimer. It is a catalyst for the oxidative decarboxylation of isocitrate to α-ketoglutarate (αKG) while also reducing $NADP^+/NAD^+$ to NADPH/NADH in the Krebs cycle. In gliomas, the mutation in IDH1 is a missense mutation at codon 132, where arginine is substituted primarily with histidine [16]. Within LGGs, mIDH1 exhibits an oncogenic gain-of-function mutation that allows for the reduction of α-ketoglutarate (αKG) to 2-hydroxyglutarate (2HG) [14]. 2HG has a multitude of effects on the tumor microenvironment, including changes in various signaling pathways [17].

An important pathway used to control immune response is the adenosinergic pathway, composed of the receptors CD39, CD73, and adenosine receptors (ARs) [18]. ATP is released

extracellularly due to cellular damage, such as hypoxia or nutrient deprivation [19]. Extracellular

ATP binds to P2X7 receptors on immune cells to trigger inflammatory antitumor responses [19].

Tumors are able to convert extracellular ATP into anti-inflammatory adenosine (ADO) using the

adenosinergic pathway (AP), which allows them to evade antitumor immune responses [19]. In the

AP, extracellular ATP is dephosphorylated to adenosine diphosphate (ADP) and adenosine

monophosphate (AMP) with the enzyme ectonucleoside triphosphate diphosphohydrolase-1,

known as CD39 [19]. The last phosphate on AMP is then hydrolyzed to produce adenosine by

5'-nucleotidase, known as CD73 [19]. The adenosine then binds to ARs, which are seven-pass

transmembrane G-protein coupled receptors (GPCRs) [19]. Under physiological conditions, levels

of extracellular adenosine are tightly controlled, but in conditions like cancer, adenosine is

produced extracellularly 100 times more than normal, leading to the increased signaling of

ARs[19]. ARs decrease the secretion of inflammatory mediators while increasing the secretion of

anti-inflammatory mediators, such as IL-10 and TGF-β [19].
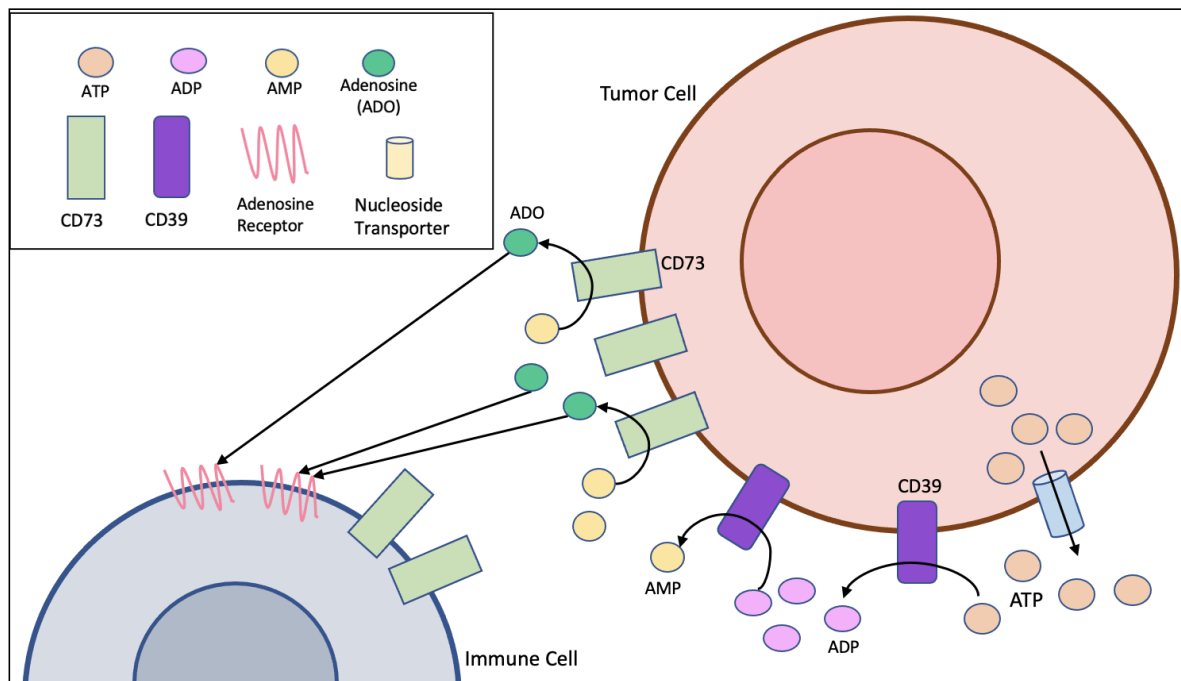
Figure 2: Adenosinergic pathway in physiological conditions.

The effects of mIDH1 on the adenosinergic pathway and its impact on the function of antitumor T cells have not yet been explored in full.

Tumor Immune Microenvironment

The central nervous system (CNS) was previously thought to be 'immune privileged' [20]. However, it was later found that this status depends on the existence of neuroinflammation during gliomagenesis [20]. When this happens, the blood-brain barrier (BBB) is compromised, leading to an infiltration of certain immune cells from the periphery [21]. These infiltrates consist of CNS resident microglia, myeloid cells, and T cells [22]. Tumor infiltrating lymphocytes (TILs) are composed of cytotoxic anti-glioma CD8+ T cells (CTLs) and CD4+ helper T cells as well as regulatory T cells (Tregs) [23].

Materials and Method

Mouse Glioma Models

The Sleeping Beauty (SB) Transposase system was used to create mIDH1 and wild-type IDH1 (wtIDH1) mouse models of glioma via genetic engineering. These mice had ATRX and p53 loss, which resemble human low grade astrocytomas. Using SB, genetic lesions were introduced into the genome of neonatal mice, from which the tumor was allowed to develop. While the wtIDH1 mice had genetic lesions in ATRX, NRAS-G12V, and p53, the mIDH1 mice had the same lesions with the addition of IDH1-R132H. These lesions were encoded in plasmids along with sleeping beauty transposase and luciferase, and either the wtIDH1 or mIDH1 plasmids were injected into the lateral ventricle of the neonatal mice.

Neurosphere Culture

The glioma neurospheres were generated from the brain tumors of the sleeping beauty mice. Using transcranial perfusion with Tyrode's solution, the mice are euthanized and the brains

are harvested to be examined under an epifluorescence microscope, under which the tumor expressing GFP and Katushka will be visible against healthy tissue. Using a non-enzymatic cell dissociation buffer, the tumor is dissociated and strained through a 70 micrometer strainer. The wild-type cell lines NPA and CPA and mutant NPAI and CPAI neurospheres were grown in DMEM-F12 media supplemented with 100 units/mL penicillin-streptomycin, 100ug/mL Normocin, B-27 supplement, N-2 supplement, and 20 ng/mL of fibroblast and epidermal growth factor (FGF and EGF) in a T-75 flask at 37 degrees Celsius, 5% CO2. The wild-type RPA cell line and the mutant RPAI cell line were grown in Neurocult media with the same supplements as DMEM-F12 in addition to platelet-derived growth factor alpha (PDGF-α). The glioma neurosphere population could be identified after three days in culture as clusters which were isolated into a new T-75/T-25 flask. Conditioned media and cells were collected along with the glioma neurospheres to measure the secreted proteins.

Implantation

Tumors were induced in mice via transfection of wtIDH1 or mIDH1 neurospheres into the right striatum. NPA (wtIDH1) neurospheres were implanted in one group of mice, and NPAI (mIDH1) neurospheres were implanted in another group. With 100 microliters total of ketamine and dexmedetomidine, the mice are anesthetized and the skull is shaved and prepared with iodine prior to implantation. The neurospheres are injected with a 22-gauge Hamilton syringe at a rate of 1 microliter per minute with the following coordinates: 0.5 mm anterior, 2.00 mm lateral, and 3.00 mm deep. After implantation, the mice were given buprenorphine (0.1 mg/kg) and carprofen (5 mg/kg) for analgesia.

In-vivo Imaging

An IVIS spectrum imaging system was used to measure bioluminescence after implantation. Automatic exposure, large binning, and aperture f=1 were the settings used with the IVIS spectrum. Using a combination of oxygen and isoflurane (1.5-2.5% isoflurane), the mice were anesthetized, and 100 microliters of luciferin solution was injected intraperitoneally in order to monitor tumor formation and progression. Luminescence was quantified using Living Image Software. Luminescent intensity was measured in units of sr x photons/cm^2 at the region of interest (ROI), which was indicated by the circle around the heads of mice. One image was collected 3 minutes after injection, and another was taken 5 minutes after injection, where maximal intensity was measured for both. Mice displaying symptoms such as lethargy, hunched posture, scruffy fur, or seizures displayed an intensity higher than 10^8.

<u>Single Cell Suspension and Purification for Flow Cytometry Analysis</u>

Brain, spleen, and blood were collected from the mice. The tumor within the brain was dissected after removal of the brain from the euthanized mice and strained through a 70 micrometer strainer connected to a 50 milliliter conical tube with a pistol and 30-40 milliliters of RPMI media with 5% FBS. The cells were centrifuged at 1500 rpm for 5 minutes at 4 degrees Celsius, and the supernatant was discarded. The pellet was resuspended in 7 milliliters of media and 3 milliliters of 90% isotonic Percoll was added in a 15 milliliter conical tube. Using a serological pipette, 1 milliliter of 70% isotonic Percoll was added to the bottom of the tube to create a density gradient. The solution with distinct layers was centrifuged at 2200 rpm for 30 minutes at room temperature. The tumor-infiltrating cells were collected carefully from the distinct white band that formed in between the two layers. Blood was collected directly after euthanizing the mice to prevent coagulation. For splenocyte isolation, extracted spleens are strained with a pistol through a 70 micrometer strainer attached to a 50 milliliter conical tube.

The strainer is washed with 5 milliliters of RPMI media prior to straining the spleens, and two more washes with 5 milliliters of RPMI media each were done during straining. The splenocytes and blood were centrifuged. The pellets were resuspended in 1 milliliter of RBC lysis buffer for the spleens and 8 milliliters for the blood in order to lyse the red blood cells. The cells were neutralized with DPBS after 1-2 minutes for a total volume of 10 milliliters. The cells were centrifuged at 1500 rpm for 5 minutes at 4 degrees Celsius and ready for flow cytometry.

Flow Cytometry

The cells were prepared for antibody staining first, prior to flow cytometry. Cell staining was performed with minimal exposure to light in order to minimize cellular metabolic activity and marker expression changes. The cells were resuspended in DPBS and had Amcyan added to them, which stains for dead cells as a viability dye. After incubation at room temperature for 20 minutes, the cells were centrifuged at 1500 rpm for 5 minutes at 4 degrees Celsius. After a wash with flow buffer and another centrifuge cycle, FC Block (CD16/CD32) was added to the cells to prevent any non-specific binding. The cells were then centrifuged again at 1500 rpm for 5 minutes at 4 degrees Celsius and washed again with flow buffer fluid. Cells were then stained with fluorescent conjugated antibody mixtures and then incubated for 30 minutes. The mixture included CD45, CD8, CD73, and CD4. The cells were then washed with flow buffer fluid and were ready for data collection on the FACSAria flow cytometer. The data was analyzed using FloJo Version 10.

ATP and BCA Assays

Mutant IDH1 glioma cells were counted and transferred from T-75 flasks into 15 milliliter conical tubes and then spun at 1500 rpm for five minutes. The supernatant was discarded and the remaining cell pellets were resuspended in DMEM-F12 or Neurocult media,

depending on the cell line. The NPA (wtIDH1), NPAI (mIDH1), CPA (wtIDH1), and CPAI (mIDH1) neurosphere pellets were resuspended in DMEM-F12 media, while the RPA (wtIDH1) and RPAI (mIDH1) neurosphere pellets were resuspended in Neurocult media. In each well of a 6-well plate, 500,000 cells were plated in 2 milliliters of media and cultured for 72 hours. Levels of ATP secreted from the glioma cells were measured by Molecular Probes™ ATP Determination Kit (A22066) according to manufacturer's instructions. According to pellet size, 100-200 microliters of RIPA buffer was added to the sample with 1X concentration of protease and phosphatase inhibitor cocktail. The cells were then centrifuged at 1500 rpm for 5 minutes and the supernatant was discarded. The neurosphere pellets were resuspended in appropriate amounts of RIPA buffer and transferred to new 1.5 milliliter tubes. The solution was then stored at -80 degrees Celsius overnight. After thawing, the samples were centrifuged at 12,000 RCF for 30 minutes at 4 degrees Celsius, and the supernatant was transferred into new 1.5 milliliter tubes on ice. Total protein was then quantified by Thermo Scientific™ Pierce™ BCA Protein Assay Kit according to manufacturer's instructions. The ATP secreted by the glioma cells was normalized to the total cell protein and recorded.

Results

Two groups of mice were used to generate cell lines for this experimental design. The neurospheres were prepared in accordance with the groups, with group 1 receiving NPA (wtIDH1) neurospheres, and group 2 receiving NPAI (mIDH1) neurospheres. Once implanted, the mice were monitored until they were late-stage symptomatic and then euthanized. Brain tissue was taken to isolate the tumor and prepared for analysis via flow cytometry, as depicted in Figure 3.
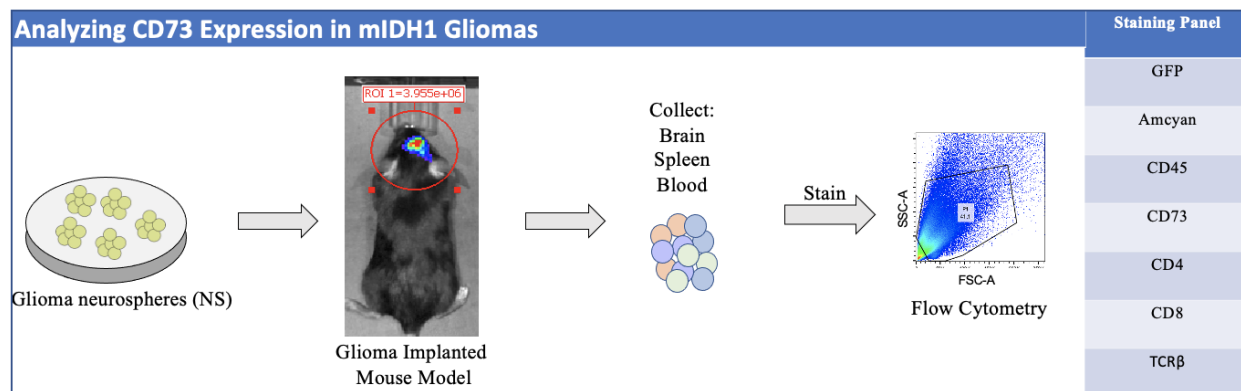
Figure 3: Experimental design in analyzing CD73 expression in mIDH1 gliomas. Mice were implanted with NPA or NPAI neurospheres and then harvested for tumor, spleen, and blood, then analyzed against the listed staining panel by flow cytometry.

CD73 Expression in Tumor Cells

Two human cell lines, SJGBMwt (wtIDH1) and SJGBMmu (mIDH1), as well as additional mouse cell lines RPA (wtIDH1) and RPAI (mIDH1) were included in *in vitro* analysis via flow cytometry. Survival analysis of the gliomas in the TCGA data according to NT5E (CD73) expression shows that gliomas with a low expression of CD73 have a lower survival probability than gliomas with a higher expression of CD73 (Figure 4A), showing that a difference in CD73 expression correlates with a difference in survival rate in mIDH1 glioma patients. We identified the GFP+ tumor cells (Figure 4B), and gated within this population to view CD73 expression across the wild-type and mutant cell lines (Figure 4C). Across NPA/NPAI, RPA/RPAI, and human SJGBMwt/SJGBMmu tumor cell lines *in vitro,* we observed reduced CD73 expression in the mutant IDH1 cell lines (Figure 4D). For example, in the NPA/NPAI cell lines, we observed 71.8% of tumor cells expressing CD73 in the NPA (wtIDH1) cell line, but only 48.2% of tumor cells expressing CD73 in the NPAI (mIDH1) cell line (Figure 4C).
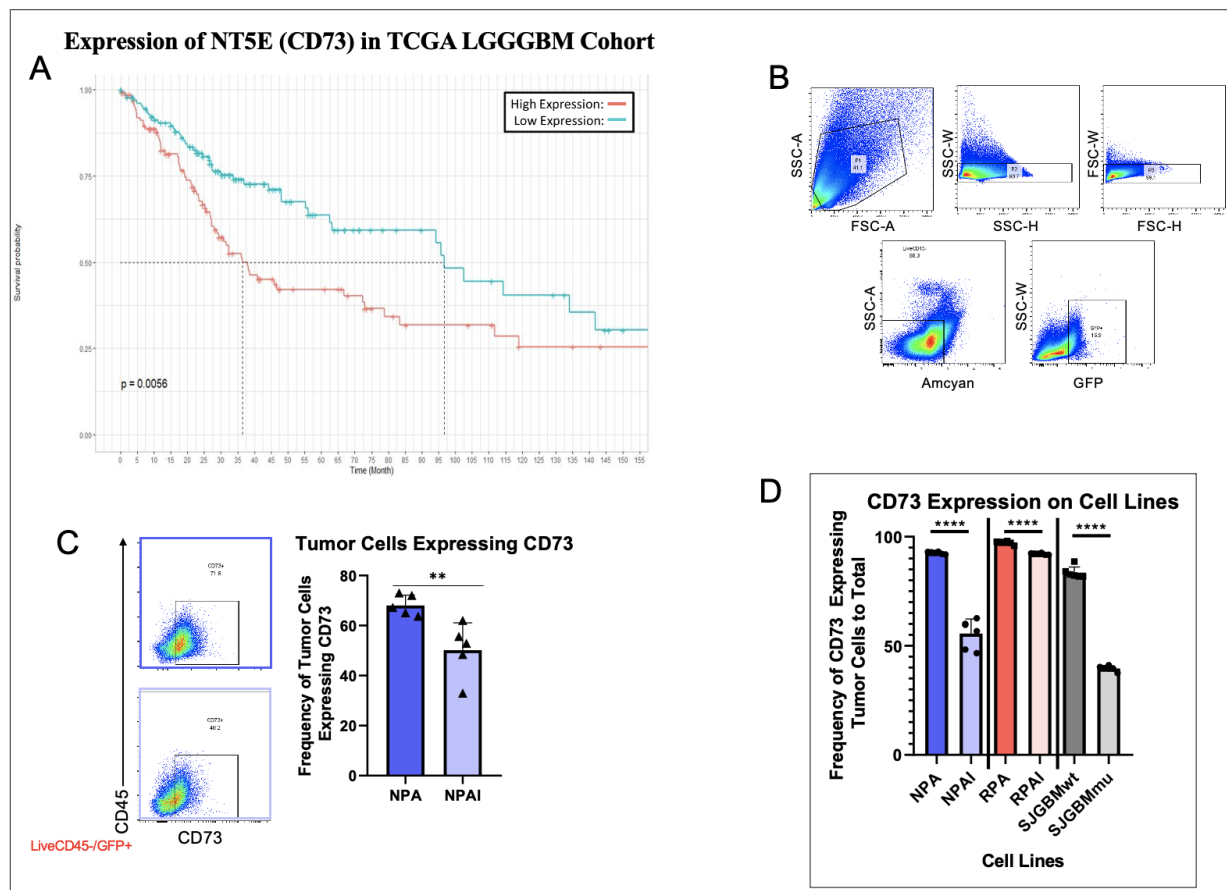
Figure 4: Mutant IDH1 glioma cells have reduced CD73 expression. **(A)** Survival analysis of gliomas in TCCG data according to NT5E (CD73) expression (high or low). **(B)** Gating strategy to identify GFP+ tumor cells from implanted mouse models. **(C)** Left panel shows gating with CD73+ cells, while the right panel shows the frequencies of tumor cells expressing CD73 in NPA/NPAI. **(D)** Frequency of tumor cells from multiple cell lines expressing CD73.

CD73 Expression in Immune Cells

CD8 T cells make up a large number of tumor infiltrating immune cells, as mentioned earlier. We identified the TCRβ+/CD8+ CD8 T cells from the glioma tumor microenvironment that we implanted into mouse models (Figure 5A). The expression of CD73 was analyzed in all CD45+ immune cells (Figure 5B). We then focused on the CD8 T cells to analyze their expression of CD73 (Figure 5C). Across the NPA/NPAI cell lines, both immune and CD8 T cells

had reduced CD73 expression in the NPA (wtIDH1) cell line, contrasting the results found in the tumor cells.
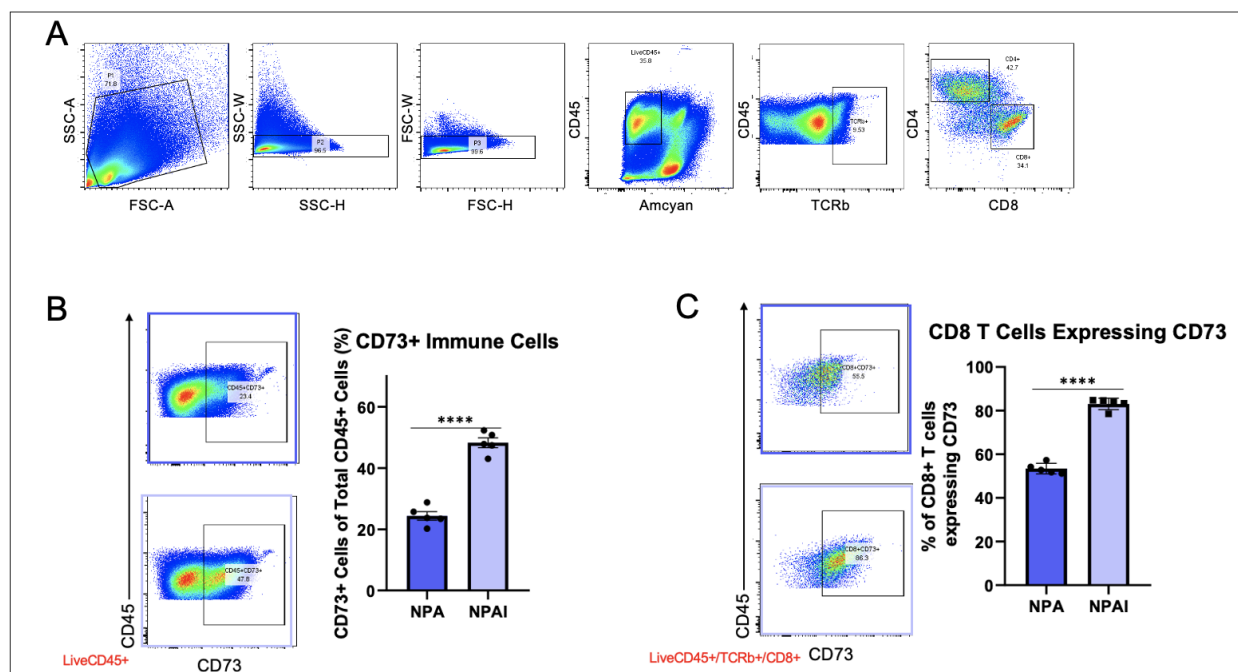

Figure 5: Mutant IDH1 immune cells have greater expression of CD73. **(A)** Gating strategy to identify CD8 T cells from the tumor microenvironment of the gliomas implanted into the mouse models. **(B)** The left panel has the gates representative of CD73+ immune cells, while the right panel shows the frequencies of immune cells that express CD73. **(C)** The left panel has gates representative of the CD73+ CD8 T cells, while the right panel shows the frequencies of CD8 T cells that express CD73.

ATP Secretions

Neurospheres from multiple cell lines were used to conduct an ATP assay and a total protein assay (Figure 6A). After normalizing the secreted concentration of ATP to the total protein concentration, we observed that the mutant IDH1 cell lines NPAI and RPAI secreted significantly reduced ATP concentrations than their wild-type counterparts. While the CPAI cell line secreted less ATP than CPA, this difference was not significant (Figure 6B).
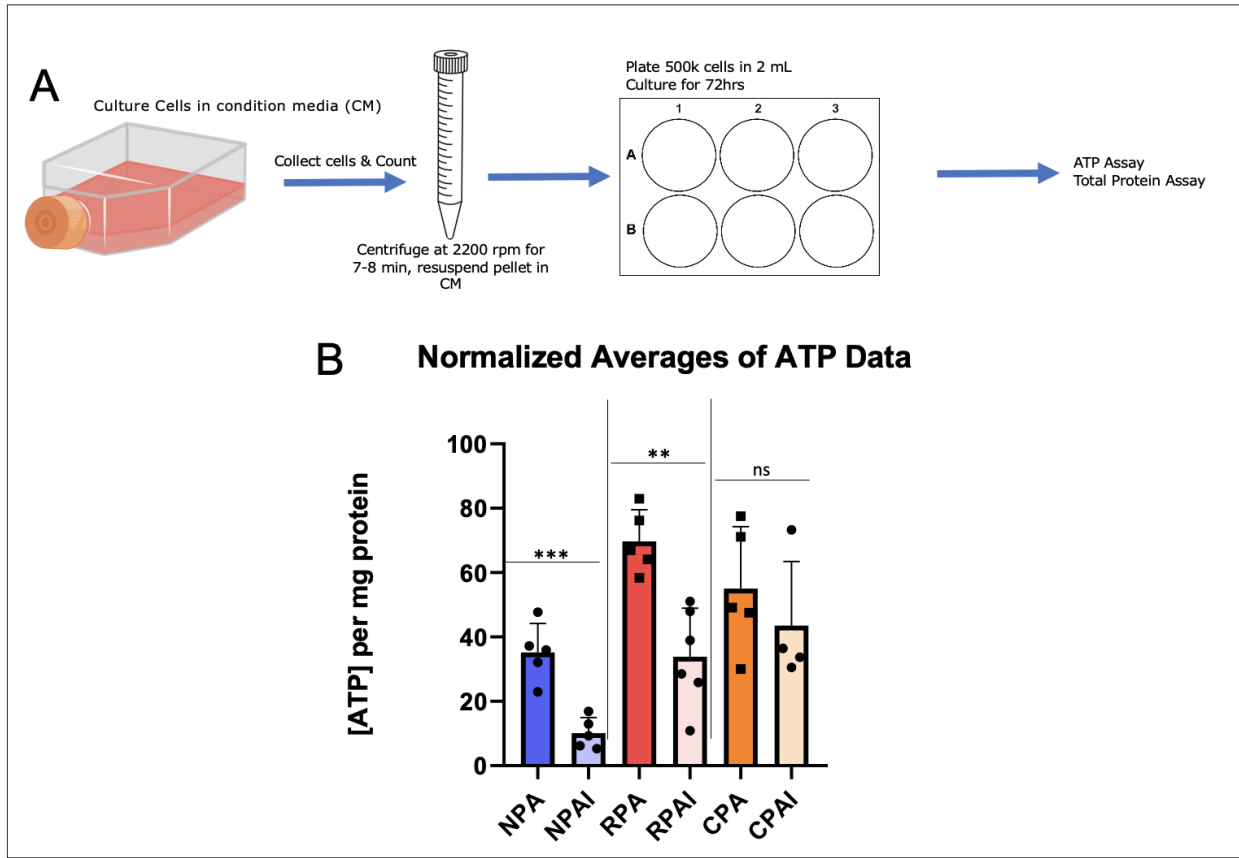
Figure 6: Mutant IDH1 gliomas have reduced secretion of ATP. **(A)** Experimental design to collect condition media and cells to use for ATP and BCA Assays. **(B)** Secreted ATP from each glioma cell line normalized to the total protein concentration.

Discussion and Conclusion

In the mutant IDH1 glioma cells, more CD8 T cells show increased expression of CD73. However, tumor cells showed less expression of CD73, which is required to convert AMP to adenosine. Despite the increased expression of CD73 in immune cells, the tumor microenvironment of mIDH1 gliomas harbors many more tumor cells than immune cells, which have a reduced expression of CD73. This leads to the conclusion that overall, the adenosinergic pathway is reduced in the mIDH1 glioma microenvironment, with CD73 expression being reduced in mIDH1 tumor cells. The ATP assays also revealed that mIDH1 tumor cells display a

lower level of ATP (as shown in the NPAI, RPAI, CPAI cell lines) compared to wtIDH1. Analyzing the changes in the tumor microenvironment, as a result of 2-HG, has led to multiple new possibilities in the realm of immune modulation in mIDH1 gliomas, which lend themselves to progress in novel immunotherapies. Moving forward from these results, more work will be done to analyze adenosinergic pathway molecules on other cell lines and in the periphery to possibly confirm what we are seeing in mIDH1 glioma cells. This includes looking at the adenosinergic pathway expression in myeloid cells as well as in the peripheral blood. Furthermore, measuring adenosine levels in the conditioned media of mIDH1 tumor cells *in vitro* would aid in determining the effect of the altered pathway on anti-inflammatory adenosine production. Lastly, in order to look at the effects of the altered pathway on survival, the use of CD73 and/or adenosine receptor inhibitors would be another promising step forward.

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