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Characteristics of The Immune and Molecular Microenvironment of The Intra-and Extra-Parenchymal Tumors of The Central Nervous System

PhD thesis

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INTRODUCTION

Tumors of the central nervous system (CNS)

Primary brain tumors are a various group of neoplasms arising from different cells of the CNS. They are either benign or malignant. Benign brain tumors grow relatively slowly and do not tend to invade other parts of the brain. Malignant brain tumors are fast growing, more aggressive tumors that spread to other areas of the brain.

Brain tumor types

Due to the biological and histological heterogeneity, brain tumors are divided into two main groups; glioma and non-glioma.

Glioma

Glioma is a malignant primary brain tumor, originating from transformed progenitor glial or neural stem cells. The World Health Organization (WHO) has characterized glioma into two sub-groups; low-grade glioma (LGG) grade I and II and high-grade glioma (HGG) grade III and IV, depending on the histological and the genetic features. According to the phenotype, glioma can be classified into astrocytic, ependymal, and oligodendrocyte subtypes.

Astrocytoma

Astrocytoma is a category of glioma originating from a particular type of glial cells called astrocytes. It accounts for the vast majority of gliomas (75%). The WHO classified astrocytic tumors into different degrees based on their level of aggressiveness including pilocytic astrocytoma (grade I), diffuse astrocytoma (grade II), anaplastic astrocytoma (grade III) and glioblastoma (grade IV).

Glioblastoma

Glioblastoma (GBM) is considered as the most aggressive malignant form of astrocytic lineage. It displays extensive molecular and cellular heterogeneity. GBM represents 16% of all primary brain tumors and more than 54% of all glioma types.

GBM remains an irremediable disease with a median overall survival rate of 15 months, even though the use of a combination of therapies. The poor survival outcome in GBM patients were correlated to the molecular aberrations due to mutations in distinct genes. One of the most critical gene alterations in secondary GBM and also in LLG is a mutation in isocitrate dehydrogenase (IDH). O^6-methylguanine

deoxyribonucleic acid (DNA) methyltransferase (MGMT) is another marker which is ordinarily tested as part of the routine clinical examination in GBM patients. MGMT is a DNA repair enzyme that effectively protects cells against methylating agents such as temozolomide (TMZ). MGMT promoter methylation is mainly abundant in secondary GBM with 75% versus 36% in primary GBM. Consequently, methylation of MGMT promoter in patients allow alkylating agents to be more effective.

Non-glioma

Non-glioma is a group of tumors that originate from other type of brain cells except glial cells. These tumors include medulloblastomas, pituitary adenomas, meningiomas and central nervous system lymphomas.

Meningioma

Meningioma (MNG) arises from the meninges consequently is not a brain tumor, but it constitutes 20-30% of all primary intracranial tumors. Meningioma arises from the arachnoidal cap cells of the leptomeninges. According to the WHO, 70-80% meningioma is generally benign (grade I). However, 5-20% have the potential to become atypical (grade II) and 1-3% malignant tumors (grade III).

Incidence of brain tumors

Although brain tumors are rare, they are among the highest causes of mortality. Worldwide, around 240,000 cases of primary CNS and malignant brain tumors are reported per year. In 2019, approximately 86,970 patients were diagnosed in the United States with primary brain tumors, among which 26,170 patients were diagnosed with primary malignancy and 60,800 with non-malignant tumor. CNS tumors in young adults and children account for 20% and 30% of cancer deaths, respectively. According to estimates from the WHO in Hungary, 765 brain and CNS cancer cases and almost 661 cancer deaths occurred in 2020.

Survival of CNS tumors

The prognosis for brain tumor is extremely dependent on their grade. Generally, brain tumors are organized according to the grading systems that order them from least grade (grade I) to the most aggressive grade (grade IV), as previously described. Only, 40% of people live longer than one year after being diagnosed with a malignant brain tumor, and even less (around 20%) live for 5 years.

Current therapeutic approaches for tumors in the CNS

Environmental and genetic factors are both play a role in the pathogenesis of brain tumors. The treatment is highly dependent on the stage and location of the tumor.

Surgery

The first and the most common treatment modality for patients diagnosed with brain tumors is surgery. It helps to reduce the pressure on the CNS and enhances the efficacy of chemo- and radiation therapy. Patients with low grade primary tumors can be effectively cured with surgery if the tumor is at an operable location. However, high grade tumors are often treated with radio- and/or chemotherapy before and also after surgery. Combination therapy is routinely applied in higher grade gliomas (grade II and VI).

Radiotherapy

Radiotherapy can cause DNA damages to both normal and tumor tissues. In inoperable and non-completely resected LGG cases, radiation is the treatment of choice. The majority of HGG cases are treated with combination therapy, including postoperative adjuvants; radio and/or chemotherapy. Unfortunately, not all tumor cells are sensitive to the radiation. Hypoxic tumors are considered more radioresistant, because their DNA is less likely to be damaged. Additionally, meningiomas (grade II and III) are also treated with radiotherapy after surgical resection. However, frequent recurrence of meningiomas require re-treatment (re-resection and re-irradiation).

Chemotherapy

Through different mechanisms chemotherapy is used to manage tumors, including blocking a distinct signaling pathway, and depleting nutrients critical for cell growth. It is a major challenge to treat brain tumors with chemotherapy due to the presence of the BBB. The chemotherapeutic drug most frequently used to treat tumors of the brain is temozolomide (TMZ) (commercially called Temodar). TMZ is an orally applied alkylating agent and it induces cell cycle arrest. TMZ is most effective in GBM patients who lack the expression of MGMT. The anti-tumor effect of TMZ is due to its ability to methylate DNA at O^6-guanine (5%), N^7-guanine (>70%) and N^3 adenine (>9%).

Immunotherapy

Life quality of a brain tumor patient is significantly reduced, due to long-term toxicities and the adverse effects of the standard treatments. There are a limited number of immunotherapy clinical trials for brain tumors. Additionally, immune checkpoint inhibitors (ICI) are still under investigation.

Ipilimumab, an anti–cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) antibody was initially developed for treatment of melanoma. It is an immunoglobulin G1 (IgG1) human monoclonal antibody directed against CTLA-4 that leads to increased T-cell activation. The first anti-programmed death-1 (PD1) antibody was nivolumab for treating patients with advanced stages of non-small cell lung cancer (NSCLC). It is a fully human monoclonal antibody able to inhibit immunoregulatory cell surface receptor protein PD-1. It is expressed highly on the surface of $CD4^+$ and $CD8^+$ T-cells that are chronically exposed to antigens. Programmed death ligand-1 (PD-L1) is the ligand of PD-1, its' interaction maintains peripheral tolerance. PD-L1 is expressed by some tumor cells to evade immune elimination.

Chemotherapy resistance

Chemoresistance is the ability of cancer cells to avoid response to chemotherapy and the tumor cells carry on proliferating. Statistical data revealed that over 90% mortality of tumor patients is attributed to drug resistance.

Drug transporters

One of the largest drug transporter families is the adenosine triphosphate (ATP)-binding cassette (ABC) transporter family. ABC transporters are transmembrane proteins that transport a range of substrates across extra/intra-cellular membranes. In humans, 49 different ABC transporters were identified. They are divided into 7 subfamilies, from A to G, based on their domain organization and sequence similarities. Many studies focus on the influx and efflux mechanisms to evaluate the potency of drugs in tumor therapy. Current studies focus on ABCB1 and ABCG2 that are highly involved in drug resistance. ABCB1, also called multi-drug resistant (MDR) protein 1 (MDRP1) and is abundantly expressed in cell membranes of the kidneys, liver and blood-barriers. The efflux transporter ABCG2 is widely expressed in stem cell populations.

Expression of drug transporters in brain tumors

One of the complications associated with CNS tumors is their weak response to anti-neoplastic drugs. The effectiveness of chemo and immunotherapy is impaired by the BBB/BBTB. Interestingly, the expression of ABC transporters in BBB/BBTB has directly been related to chemoresistance versus several of their anticancer drug substrates. Although actively investigated there is still limited knowledge about the expression and the activity of ABC transporters in CNS tumors. Certainly, ABCB1, ABCG2, ABCC1, ABCC4, and ABCC5 up-regulation has been reported in glioma cells in recent research. Thus, brain tumors can be regarded as being located behind a multibarrier system, which defends the tumor cells from chemotherapeutics agents.

MATERIALS AND METHODS

Ethical statement

Brain tumor samples (**Table 1**) were collected at the Departments of Neurosurgery and Pathology, Clinical Centre, University of Pecs, Hungary. In accordance with the Declaration of Helsinki patients had given written informed consent and the project was approved by the Medical Research Council, Hungary (0194/16 (10833-/2016/EKU).

Patient samples

Twenty-two brain tumor samples were collected for this study.

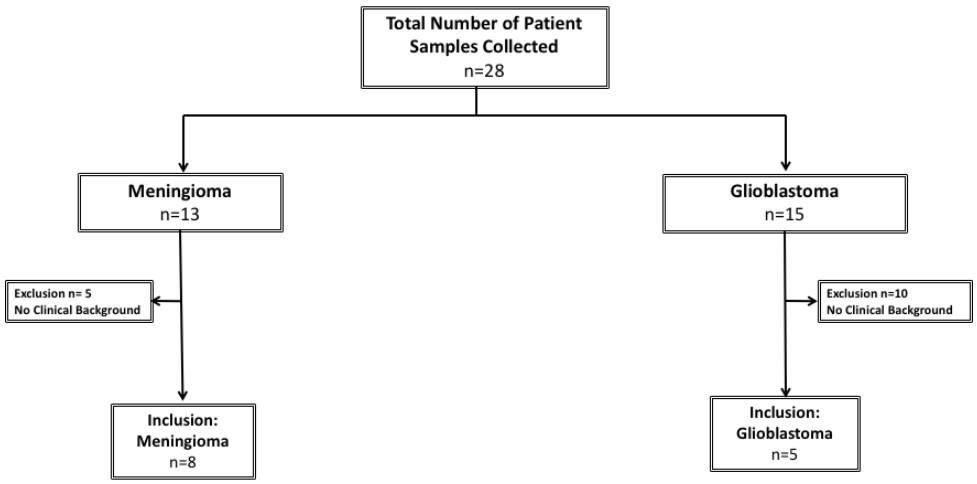

Figure 1. Brain tumor samples inclusion criteria flow chart.

Table 1. Summary of patient data-tumor type, age range, diagnosis, and therapy preceding surgery. Not applicable (N/A), negative (-).

Tumor Type	N°	Code	Age Range	IDH	Diagnosis	Radio- or Other Therapy before Surgery
MNG	1	M1	50–60	N/A	Grade I meningioma	-
	2	M2	70–80	N/A	Grade I meningioma	-
	3	M3	40–50	N/A	Grade I meningioma	-
	4	M4	70–80	N/A	Grade I transitional meningioma	-
	5	M5	60–70	N/A	Grade I meningioma brain invasion	-
	6	M6	40–50	N/A	Grade I meningioma	-
	7	M7	40–50	N/A	Grade I meningioma	+
	8	M8	70–80	N/A	Grade I meningioma	-
GBM	1	G1	60–70	(-)	Grade IV Glioma	-
	2	G2	70–80	(-)	Grade IV Glioma	-
	3	G3	60–70	(-)	Grade IV Glioma	+
	4	G4	40–50	(-)	Grade IV Glioma	-
	5	G5	60–70	(-)	Grade IV Glioma	-

RNA isolation and Reverse transcription

Total RNA was isolated from tumor samples using NucleoSpin RNA isolation kit (Macherey-Nagel, Düren, Germany). RNA concentration was measured by Nanodrop 2000 (ThermoFisher Scientific, Waltham, MA, USA). Reverse transcription was performed using random primers and a high-capacity RNA to cDNA kit (ThermoFisher Scientific, Waltham, MA, USA). All generated cDNA samples were stored at −20 °C until used. Total RNA of five pooled normal human brain samples were purchased from a commercial source (BioChain Institute, San Francisco, CA, USA). All generated cDNA samples were stored at −20 °C until used.

Real-time quantitative polymerase chain reaction (qRT-PCR)

qRT-PCR reactions were carried out using Luminaris Color HiGreen qPCR master mix (ThermoFisher Scientific). Amplification was made by PikoREAL 96 PCR system (ThermoFisher Scientific). The reference genes were glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and/or β-actin (by taking the average of their Ct values, in both cases). The relative quantification (RQ) was calculated compared to gene expression levels of the normal tissues. Sequence specific primers were used to detect the following genes: β-actin, GAPDH, FOXP3, IDO1, IL-10, INF-gamma, CD27, CD163, CTLA4, CD28, TGF-beta, CD4, PD1, PDL1, CD19, CD56, CD8, CD3, PAX5, CD70, B7-1, B7-2, ABCB1, and ABCG2.

Hematoxylin-Eosin staining

Five μm thick tissue sections were stained in Mayer's hematoxylin solution (Sig-ma-Aldrich, St. Louis, MO, USA) for 10 min, washed, then exposed to 0.25% acetic acid and eosin solution. Sections were mounted using Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA). Images were taken using an Eclipse Ti-U inverted microscope (Nikon Inc., Tokyo, Japan).

Immunohistochemistry

Five μm thick slides were cut from formalin-fixed, paraffin embedded tissue blocks corresponding to the surgical samples (GBM and MNG) used for qRT-PCR. First, the slides were rinsed in heated xylene and were washed with a descending dilution series of ethanol (97%–80%–70%–50%) to remove paraffin. After deparaffinization the slides were rehydrated in distilled water and DAKO antigen Target Retrieval Solution (DAKO, Agilent, Santa Clara, CA, USA) at 97 °C for 20–30 min. Endogenous

peroxidase activity was blocked for 15 min with Tris Buffer Saline (TBS, pH 7.4) containing 3% H_2O_2. Slides were washed three times with TBS containing Tween 20 (TBST) (0.05%, pH 7.4). Pre-blocking was carried out with 3% bovine serum albumin (BSA) in TBS for 20 min before overnight incubation with the appropriate primary antibody at 4 °C. Slides were then washed with TBS three times. The reactions were visualized using Envysion System (DAKO). For nuclear counterstaining, hematoxylin staining was performed. Finally, slides were mounted with Faramount Aqueous Mounting Medium (DAKO). Histological evaluation was performed with the help of Panoramic MIDI digital slide scanner (3Dhistech, Budapest, Hungary). The number of positive cells was assessed per mm^2 except for the CD68 positive cell count that was assessed per 0.08 mm^2. Image analysis was performed using the ImageJ software with the IHC toolbox plug-in. The list of antibodies and dilutions are summarized in **Table 3**.

Immunofluorescent staining

Five µm thick slides were cut from formalin-fixed, paraffin embedded tissue blocks corresponding to the surgical samples used for qRT-PCR. After deparaffinization and antigen retrieval the sections were pre-blocked with 5% BSA in TBST for one hour before applying the primary antibodies anti-CD19 and anti-CD45 for overnight incubation at 4 °C. CD19 and CD45 were detected using an anti-mouse IgG Alexa Fluor 488 (1:200) and anti-rabbit IgG Alexa Fluor 555 (1:200) secondary antibodies (ThermoFisher Scientific), respectively. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (ab142859) (1:1000) (Abcam Plc., Cambridge, UK). Images were obtained using an Olympus IX-81 (OLYMPUS Corporation, Tokyo, Japan) fluorescence microscope. The list of antibodies and dilutions are summarized in **Table 3**.

Table 3. List of antibodies

Antibody	Clone	Source	Isotype	Source	Dilution
Anti-CD4	4B12	Mouse	IgG1, kappa	Thermo	1:20
Anti-CD8	C8/114B	Mouse	IgG1, kappa	Thermo	1:50
Anti-CD3	Polyclonal	Rabbit	/	Dako	1:400
Anti-CD45	2B11+ PD7/26	Mouse	IgG1, kappa	Dako	1:400
Anti-CD19	EPR5906	Rabbit	IgG	Abcam	1:500 –
Anti-CD79	JCB117	Mouse	IgG1, kappa	Dako	1:200
Anti-PAX5	Polyclonal	Rabbit	/	Thermo	1:50
Anti-CD68	PGM1	Mouse	IgG3, kappa	Dako	1:200
Anti-PD1	NAT105	Mouse	IgG1, kappa	Abcam	1:50
Anti-PDL1	22C3	Mouse	IgG1	Dako	1:50

Statistical analysis

Statistical analysis was performed using the SPSS software version 20 (IBM, USA). Grouped data were presented as logRQ ± technical error. Non-grouped data are presented as 1/dCt individually and average ± standard error of the mean (SEM) using one-way and two-way analysis of the variance (ANOVA). $P < 0.05$ was considered as significant. Figures were generated using the GraphPad Prism 8 software (2018, GraphPad Software, Inc., USA).

RESULTS

Variable infiltration of T, B, NK and macrophage cells into meningioma and glioblastoma

Based on qRT-PCR analysis of cell surface markers, both MNG and GBM brain tumor samples have shown a slightly increased $CD45^+$ white blood cell (WBC) infiltration compared to normal brain. To identify the main cell types within the WBC population, screening of the expressions of $CD3^+$ T-cell, $CD56^+$ NK and $CD19^+$ B-cell markers were performed. The transcript levels of the T-cell marker CD3 in MNG were found to be significantly higher than in the normal brain, while in the GBM samples CD3 expression was not different. The NK cell marker CD56 was significantly reduced in all MNG samples compared to both normal brain and GBM. In both MNG and GBM samples the mRNA levels of CD19 B-cell marker were not different from the normal brain. IHC supported the initial findings, as the tested individual MNG samples had generally higher protein expression of the CD3 T-cell marker than what was detected in GBM. Neither tumors, nor infiltrating $CD45^+$ lymphocytes have stained positive for CD19. In certain areas of GBM sections some congregation of $CD19^+CD45^+$ double positive cells were detected, whereas such areas were not found in MNG section.

The immune microenvironment is actively suppressive in both meningioma and glioblastoma

Initial screening showed that the immune microenvironment appeared different at T and NK cells levels in MNG and GBM. These findings, at first glance might explain some of the characteristically different behavior of the tumors, indicating a more active tumor suppressive microenvironment in MNG. The cytotoxic T-cell marker (CD8) was markedly increased in MNG compared to normal brain and significantly higher than in GBM samples. The co-stimulatory molecule, CD28 which is essential for T-cell activation was also present in both tumor types but only detected at a significantly higher level in MNG compared to normal brain. The pro-inflammatory and anti-tumor INF-γ mRNA levels were also slightly increased in both MNG and GBM samples compared to normal controls. Although the cytotoxic ($CD8^+$) T-cells were only found in certain areas of the studied tumor samples, their higher presence in MNG samples suggested a potentially successful immune checkpoint intervention for MNG as the CD8 ration to CD4 was higher in MNG than in GBM. Further analysis of T-cell

markers revealed that the $CD4^+$ helper T-cell (Th) marker message levels were slightly elevated in both tumor types along with the regulatory T cell (Treg) marker forkhead box P3 (FOXP3). The presence of the $CD4^+$ T-cells was also supported by IHC. In contrast to the $CD8^+$ T-cells marker, which was localized to specific tumor areas, evenly distributed CD4 staining was detected in both types of brain tumors. This indicates that the presence of $FOXP3^+CD4^+$ Th cells throughout both tumor tissues were mostly immuno-suppressive Treg cells. IHC of the tumor-associated macrophage (TAMs) marker CD68 further supported an immunosuppressive microenvironment. Both tumor types were strongly and evenly positive for CD68. qRT-PCR analysis of another TAM marker CD163 strongly supported the initial observation, as both MNG and GBM expressed CD163 message levels way above the detected levels in the normal brain. Amongst the known functions of TAMs are the expression of IL-10 and transforming growth factor β (TGFβ) that suppress the T-cell mediated anti-tumor immune response. The anti-inflammatory TGFβ and IL-10 were expressed at greater levels in both MNG and GBM compared to the normal brain, which indicates the presence of active TAMs. As both cytokines are involved in creating the immune-suppressive environment by the inhibition of the polarization of naïve T-cells into Th1 and NK cells, the low level of NK cell marker CD56 in MNG was supported by the increased message levels of the above-mentioned cytokines. Additionally, IL-10 is known to be over-expressed by both $CD163^+$ TAMs and immunosuppressive Treg ($CD4^+$ $FOXP3^+$Treg) cells. As the Treg marker FOXP3 message levels were higher in both tumor types than in the normal brain controls, the results indicate an actively immunosuppressive microenvironment in both brain tumor types. Although cytotoxic T-cell levels in MNG were higher than in GBMs, and the expression of the co-stimulatory CD28 was also present in both tumor types, the mRNA levels of CD27, a member of the TNF receptor superfamily and co-stimulatory immune checkpoint molecule for activated T-cell survival was increased compared to CD28. As CD27/CD70 interaction promotes lymphocyte apoptosis, it is likely that activated immunosuppressive lymphocytes persist in both MNG and GBM. CD27 also aids differentiation of plasma cells from B cells if CD27 can interact with its ligand CD70.

Immune checkpoint targets in CNS tumors

The immune checkpoint targets (PDL1-PD1, B7-CTLA4) in MNG and GBM were different. qRT-PCR analysis in some individual cases showed nearly 100-fold increase

of PD1 in MNG compared to normal brain control. Some individual MNG samples also showed at least 3-fold increase in PD1 expression, compared to GBM and normal controls. In contrast, PDL1 message levels were slightly increased by mRNA detection and PDL1 protein was not detectable in either tumor types. As PD1 is found on T-cells, but its ligand PDL1 was not detected at increased levels in either tumor types compared to normal controls. The above results indicate that simple inhibition of the PDL1-PD1 immune checkpoint is highly unlikely to be effective in tumor elimination. Levels of CTLA-4, a strong inhibitor of T lymphocyte co-stimulation, were higher than in normal controls. The ligands of CTLA-4, B7-1 (CD80) and B7-2 (CD86), were expressed at higher levels in both MNG and GBM than in normal brain control. Based on this data it can be assumed that T lymphocytes are inactive in both brain tumor microenvironments, due to CTLA-4 induced inhibition that competitively binds B7-1 and B7-2. Additionally, indoleamine 2,3-dioxygenase 1 (IDO1) was vastly increased in both types of tumor microenvironments. It is well studied that the metabolic product kynurenine generates and enhances the activities of $CD4^+$ $FOXP3^+$ Treg cells and myeloid-derived suppressor cells, as well as promote angiogenesis indicating a suppressed immune microenvironment in both GBM and MNG.

ABCB1 and ABCG2 are differently expressed in glioblastoma and meningioma

Drug transporters expression was measured in both brain tumors; MNG and GBM compared to normal brain, by qRT-PCR. Non-diseased brain mRNA was used as normal control for the brain tumors. Recent studies have shown, that both ABCB1 and ABCG2 play a key role in the regulation of drug penetration of the BBB. In the CNS tumors, however, neither MNG nor GBM expressed significantly higher levels of ABCB1 than the normal brain tissue. In MNG ABCG2 expression was slightly higher than in GBM or normal brain, but the increase was non-significant and only affected some individual patient samples. Both ABCB1 and ABCG2 mRNA expression levels were barely detectable in GBM. Interestingly, in MNGs highly expressed MDR1 expression was detected.

DISCUSSION

Theoretically, the immune system should be able to eliminate brain tumors. Not surprisingly, oncologists were hopeful that immune checkpoint antibodies would revolutionize therapy. Unfortunately, the results were controversial. To understand the reasons why immune checkpoint therapies often fail, several studies have investigated the immune microenvironment of GBMs, but not MNGs. Although the two tumor types couldn't be more different histologically, the clinical problem remains the same: how to debulk or remove a tumor completely without damaging the brain tissue. Hence, we compared the immune markers of the highly aggressive GBMs and the slow growing neoplasm MNGs with each other and the normal brain. Initially, we focused on two immune checkpoint targets, PD1 and CTLA-4, as monoclonal antibodies developed to target these two checkpoints, and they are mostly successful against several tumor types.

CTLA-4 is a protein receptor expressed by activated T-cells to provide control for the immune system over T-cell activities. CTLA-4 has about 30% homology with CD28 (T-cells co-stimulatory protein), and both molecules competitively bind to B7-1 (CD80) and B7-2 (CD86) ligands on the surface of APCs. Therefore, the expression of CTLA-4 receptor ligands were also tested. In the periphery, tissues control T cells via expression of PD-L1 as PD-L1 is the ligand for PD-1 an additional inhibitory co-receptor that is expressed on the surface of T-cells and their interaction maintains peripheral tolerance. Certain tumors exploit this system to evade the immune system by expressing high levels of PDL1. Based on our data, it is hardly surprising as neither GBM, nor MNG express the ligands for PD-1 or CTLA-4. In the absence of immune suppression activating ligands, immune checkpoint monoclonal antibodies cannot have the expected therapeutic effect. The low level of NK cell marker, $CD56^{+}$ explains the lack of efficacy of NK cell targeting therapy. CD56 is the archetypal phenotypic marker of NK cells but can actually be expressed in other cell types, therefor other NK markers should be applied (CD16, CD161), however those are also not exclusive NK-specific markers. The lower level of CD56/NCAM in MNG samples were initially surprising, however, an early article draw my attention to the differences between low grade and high grade meningiomas. Based on that information low grade meningiomas express low levels of NCAM.

For instance, TGF-β is documented as a key inhibitory cytokine of NK cells which limits the number and anti-metastatic function of NK cells and is highly expressed in the studied tumors. While T-cells are present in both tumor types, $CD8^+$ cytotoxic T-cells are only in abundance in MNG providing the false impression that MNG could be targeted with immune checkpoint therapy. The initial observation, however, is misleading.

The microenvironment in GBM and MNG are highly immunosuppressive as $CD68^+$ and $CD163^+$ anti-inflammatory M2 type TAMs infiltrate both GBM and MNG. TAMs, which secrete anti-inflammatory and immune suppressive cytokines (e.g., TGF-β and IL-10), enhance the expansion of immune suppressive $CD4^+$ T-reg cells inhibiting the functions of $CD8^+$ cytotoxic T and NK cells. Additionally, both tumor types express IDO. IDO is a heme-containing enzyme that catalyzes the first and rate-limiting step in the kynurenine pathway, which is the O_2-dependent oxidation of L-tryptophan to N-formylkynurenine. INF-γ that is highly expressed in both GBMs and MNGs, stimulates tissue macrophages to produce a higher level of IDO-1, which via alteration of cytokine levels, inhibits the proliferation of effector T-cells. The immune-suppressive role of IDO-1 was supported previously by studies using Trp metabolites that induce the differentiation of Treg cells and increase apoptosis of effector T-cells through inhibiting the mechanistic target of rapamycin complex 1 (m-TORC1).

Currently, several IDO-1 inhibitors are under clinical evaluation and the results are promising using IDO inhibitors in combination with anti-PD1 in preclinical models of GBM.

Overall, activation or inhibition of the immune system depends on the balance between co-stimulatory and co-inhibitory pathways. In aggressive tumors, the immune system is often suppressed which secures the survival of the abnormal cells. It appears that combination therapy is necessary to overcome the strongly immune-suppressive brain tumor milieu. Using the appropriate immune checkpoint inhibitors in combination with IDO-1 inhibitors might be an alternative treatment for the inoperable brain tumors, refractory MNGs, and chemoresistant GBMs.

It was described in lung cancers that the canonical WNT pathway can induce ABCB1 and ABCG2 drug transporter expression. In GBM ABC transporter expression is stage dependent. It is also known that TMZ is a weak substrate of ABCB1which also explains the developing drug resistance to TMZ over time.

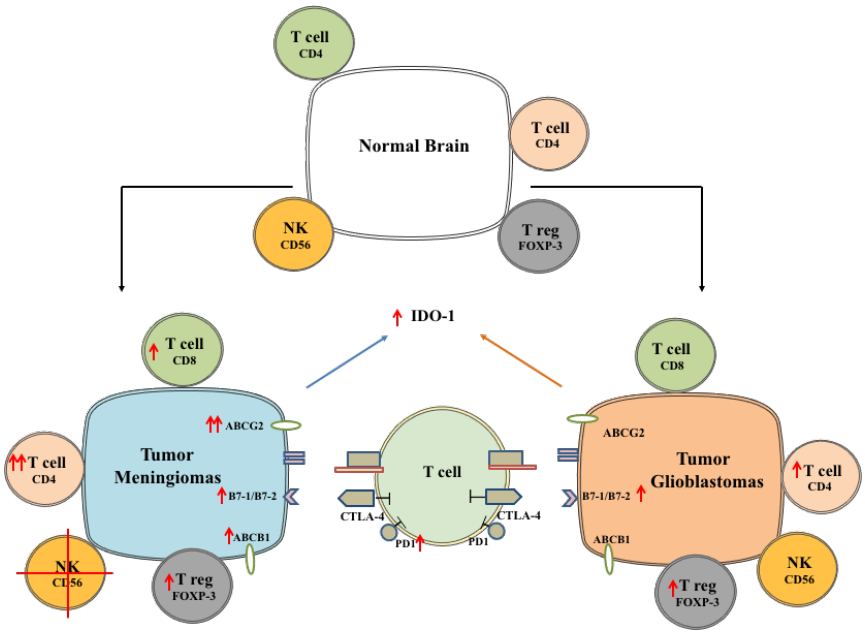

Figure 2. Schematic summary of the characteristic immune and molecular microenvironments of intra- and extra-parenchymal CNS tumors.

CONCLUSION

Overall, the altered immune- and molecular microenvironments not only result in increased proliferation but also in tumor resistance against radio-immuno- and chemotherapy.

The balance between co-stimulatory and co-inhibitory pathways determines activation or inhibition of the immune system. In aggressive tumors, such as GBM, the immune system is often suppressed which ensures the survival of the tumor cells. It appears that combination therapies are essential to overcome the strongly immune-suppressive brain tumor environment. Using IDO1 inhibitors in combination with the appropriate immune checkpoint inhibitors might be an alternative treatment for both parenchymal and extra-parenchymal therapy-resistant brain tumors.

Although further studies are essential, the difference between MNG and GBM are clear. MNG patient samples have no NK cells, so even targeted therapy using specific antibodies would not be able to activate NK cells that carry FcγR as there aren't any NK cells to recognize the antibody and eliminate the tumor cell. Even macrophages are likely to be of TAMs. The $CD8^+$ cytotoxic T-cells are present in MNG and they also express PD1, however, the tumor is negative for PDL1. Therefore, immune checkpoint inhibitors would not likely to have any effects. The presence of a large number of $CD4^+$ Th cells, $CD68^+$ TAMs, and IDO1 point to the immunosuppressive tumor milieu. Our data reveal the increased levels of ABCB1 and ABCG2 in MNG which might indicate reduced ability to respond to chemotherapeutic agents. Despite MNG samples are defined as slow growing neoplasms, they show a clear tendency to recurrence after resection.

Although GBMs have a normal level of NK marker, have shown a restricted level of cytotoxic T-cell level than normal and no PD1 staining. Meanwhile, there is an abundance of $CD4^+$ Th cells, $CD68^+$ TAMs, and significantly elevated levels of IDO1. Moreover, both brain tumor types have significantly increased B7-2 (CD86) expression, which is the ligand of CTLA-4 on T-cells. CTLA-4 has shown a role in turning down T-cell activity. Although CTLA-4 is there on all T-cells, its level is not higher than in the control in either MNG or GBM, it can also lead to a complex immunosuppressive signal. The immunosuppressive $CD4^+$ Treg cells that are in abundance in both MNG and GBM do not express CTLA-4, which can lead to continuous activation of the immunosuppressive $CD4^+$ Treg cells.

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Publications

The thesis is based on the following publications

Soltani A, Kajtar B, Abdelwahab EHMM, Steib A, Horvath Z, Mangel L, Jaromi L, Pongracz JE. Is an Immunosuppressive Microenvironment a Characteristic of Both Intra- and Extraparenchymal Central Nervous Tumors? *Pathophysiology*. 2021; **28**(1):34-49. doi:10.3390/pathophysiology28010004. Q1.

Jaromi L, Csongei V, Vesel M, Abdelwahab EMM, **Soltani A**, Torok Z, Smuk G, Sarosi V, Pongracz JE. KRAS and EGFR Mutations Differentially Alter ABC Drug Transporter Expression in Cisplatin-Resistant Non-Small Cell Lung Cancer. *International Journal of Molecular Sciences*. 2021; **22**(10):5384.doi:10.3390/ijms22105384. Q1.

(impact factor: 4.5)

Poster presentations related to this thesis

Soltani A, Jaromi L, Pongracz JE. PD-L1 expression in human non-small cell lung cancer, 3D conference, 2017, Pecs, Hungary.

Soltani A, Kajtar B, Abdelwahab EHMM, Steib A, Horvath Z, Mangel L, Jaromi L, Pongracz JE. Immunosuppressive microenvironment in both glioma and meningioma. XVIth Congressus Pharmaceuticus Hungaricus, 2020, Debrecen, Hungary.