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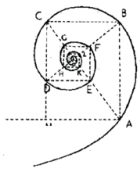

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IMMUNOTHERAPY AGAINST THE RADIAL GLIA MARKER GLAST EFFECTIVELY TRIGGERS SPECIFIC ANTITUMOR EFFECTORS WITHOUT AUTOIMMUNITY

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I

ABSTRACT

Glioblastoma (GB) is the most aggressive and fatal tumor of the central nervous system (CNS). Despite promising results of different therapeutic approaches, conventional treatments such as surgery, radiation and chemotherapy with Temozolomide (TMZ) are largely palliative. The identification of antigens preferentially expressed in GB and involved in its malignant phenotype is critical for developing therapeutic strategies.

Murine and human GB contain a fraction of cancer cells with stem-like features (CSC) and it has been proposed that only this population may be responsible for glioma recurrence. We previously found that GLAST, a membrane protein with relevant action in glutamate trafficking, is highly expressed in murine GL261 glioma stem-like cells (GSC).

In this study, we first investigated the functional consequences of GLAST enrichment using an immune-magnetic sorting approach to isolate GLAST+ cells from murine GSC. GLAST+ cells were significantly more tumorigenic than GLAST- or unsorted cells ($p < 0.0006$) when injected intracranially, showing the highly invasive nature of this subpopulation. GLAST expression and its impact on aggressiveness *in vivo* constituted a background of translational relevance.

To determine whether GLAST peptide-based immunotherapy protects against GL261-gliomas, we treated glioma-bearing mice with three subcutaneous injections of four GLAST-derived peptides emulsified with Montanide™ ISA 51 VG in association with granulocyte macrophage colony-stimulating factor (GM-CSF) injections. We found that immunization with GLAST peptides efficiently induces specific anti-tumor response in the murine GL261-glioma preventing the tumor progression in 40% of immunized mice. The immunization induced relevant effects on tumor microenvironment by up-regulating IFNγ and TNFα as well as down-regulating TGFβ1 and β2. GLAST expression significantly decreased in

gliomas from immunized mice, as evaluated by histological analysis and real-time PCR. Beyond these changes, trafficking of NK cells, CD8+ T cells and CD4+ T cells in the spleens and lymph nodes and their homing ability into the brain significantly increased in immunized mice when compared with controls.

A local chemotactic gradient characterized by expression of CXCL10 (which may be responsible for the recruitment of CTL), CCL3, CCL4 and CCL5 (which are involved in NK cell migration), and NKG2D ligand in immunized mice may play a role in the accumulation of immune cells (particularly of NK cells) at the sites of tumor formation.

Autoimmune reactions were not observed in immunized mice supporting evidence that GLAST may constitute a glioma antigen against which immune responses can be efficiently induced without toxicity.

In order to emphasize the translational relevance of GLAST as marker and glioma-associated antigen we will extend our studies to human GB.

GLAST expression was investigated by immunohistochemistry in 39 human primary GB. High percentage of GLAST+ cells, and a moderate or strong reactivity, was associated with a decreased overall survival ($p= 0.02$).

The main result of this study is the definition of GLAST as an attractive glioma-associated antigen for targeting in peptide-based immunotherapy in GB patients and a potential clinical marker in association with the patient prognosis.

SOMMARIO

Il Glioblastoma (GB) è il tumore più aggressivo e fatale del sistema nervoso centrale (SNC). Nonostante i promettenti risultati di diversi e nuovi approcci terapeutici, i trattamenti convenzionali quali la chirurgia, la radioterapia e la chemioterapia con Temozolomide (TMZ), sono ancora oggi palliativi. L'identificazione di antigeni preferenzialmente espressi nel GB, e coinvolti nel suo fenotipo maligno, è fondamentale per lo sviluppo di strategie terapeutiche.

GB murini e umani contengono una frazione di cellule tumorali con caratteristiche simil-staminali (CSC) ed è stato proposto che questa sottopopolazione cellulare sia la sola responsabile dell'insorgenza di recidive. Nel nostro laboratorio abbiamo scoperto recentemente che GLAST, un trasportatore di membrana con un ruolo fondamentale nel traffico di glutammato, è altamente espresso in cellule murine GL261 simil-staminali (GSC).

In questo studio abbiamo per prima cosa analizzato le conseguenze funzionali dell'arricchimento di GLAST in GSC murine, utilizzando una tecnica di separazione immunomagnetica che ci ha permesso di isolare cellule GLAST+. Quando iniettate intracranialmente le cellule GLAST+ hanno mostrato avere una natura altamente tumorigenica rispetto le cellule GLAST- o rispetto le cellule non sortate ($p < 0.0006$), evidenziandone la natura altamente invasiva. L'espressione di GLAST e il suo impatto sull'aggressività del tumore *in vivo* hanno costituito la base e un razionale di rilevanza traslazionale per questo studio.

Per determinare se un approccio di immunoterapia a base di peptidi fosse in grado di proteggere da tumori derivanti da GL261, abbiamo trattato topi con glioma con tre iniezioni sottocutanee di quattro peptidi, derivanti dalla sequenza aminoacidica di GLAST; i peptidi sono stati precedentemente emulsionati in Montanide™ ISA 51 VG e somministrati in associazione con

iniezioni locali di granulocite macophage-colony stimulating factor (GM-CSF).

Abbiamo evidenziato che l'immunizzazione con i peptidi di GLAST si è rivelata efficace nell'indurre una risposta immunitaria antitumorale sistemica e locale specifica, prevenendo la progressione del tumore nel 40% dei topi trattati.

L'immunizzazione ha indotto effetti rilevanti sul microambiente tumorale attraverso l'up-regolazione di IFNγ e TNFα e, contemporaneamente, la down-regolazione di TGFβ1 and β2.

L'espressione di GLAST, valutata mediante analisi isologica e Real-Time PCR, è risultata significativamente diminuita nei gliomi dei topi immunizzati.

Oltre a queste osservazioni, abbiamo evidenziato che i gliomi dei topi immunizzati, rispetto ai topi non trattati, mostrano un aumento di infiltrato di cellule natural killer (NK) e di cellule T citotossiche (CD8+) antigene-specifiche che esprimono la molecola di homing nota come very late antigen-4 (VLA-4).

Un gradiente chemiotattico locale osservato nei topi immunizzati, caratterizzato dall'espressione di CXCL10 (che può essere responsabile del reclutamento di CTL), CCL3, CCL4 e CCL5 (coinvolti nella migrazione delle cellule NK), e del ligando NKG2D, può aver giocato un ruolo fondamentale nell'accumulo di cellule immunitarie (in particolare di cellule NK) nei siti di formazione del tumore contribuendo ad un aumento della sopravvivenza. Molto importante, non sono state osservate reazioni autoimmuni nei topi immunizzati, fornendo una prova che GLAST può essere sfruttato come antigene contro il quale indurre un'efficace risposta immunitaria antitumorale senza tossicità.

Per sottolineare la rilevanza traslazionale di GLAST come marcatore e antigene glioma-associato amplieremo i nostri studi anche su GB umani. L'espressione di GLAST è stata studiata in 39 GB primari mediante

immunoistochimica. Un'alta percentuale di cellule GLAST+, e una sua forte o moderata reattività, può essere associata ad una minore sopravvivenza in pazienti affetti da GB ($p= 0.02$).

Il risultato principale di questo studio è stato la definizione di GLAST come un attraente antigene glioma-associato, target in un approccio di immunoterapia a base di peptidi e potenzialmente sfruttabile anche nel trattamento di GB umani. Inoltre abbiamo dimostrato che è possibile considerare GLAST come un potenziale marcatore clinico associabile alla prognosi del paziente.

ABBREVIATIONS

AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

APC: antigen presenting cell

bFGF: basic fibroblast growth factor

BSA: bovine serum albumin

CSC: cancer stem-like cell

CTL: cytotoxic T lymphocyte

CTLA-4: cytotoxic T-lymphocyte antigen-4

EAAT: excitatory amino acid transporter

EGF: epidermal growth factor

EGFR: epidermal growth factor receptor

EGFRvIII: epidermal growth factor receptor variant III

FABP7: fatty acid binding protein 7

FAK: focal adhesion kinase

FBS: fetal bovine serum

FDA: food and drug administration

GB: glioblastoma multiforme

GSC: glioma stem-like cell

GFAP: glial fibrillary acidic protein

GLAST: glutamate aspartate transporter

GM-CSF: granulocyte macrophage-colony stimulating factor

HIF-1α: hypoxia inducible factor-1α

HLA: human leukocyte antigen

ICAM-1: intercellular adhesion molecule-1

IDH1 and IDH2: isocitrate dehydrogenase 1 and 2

IDO: indoleamine 2,3-dioxygenase

IFNγ: interferon-γ

IL-: interleukin-

LAK: lymphocyte-activated killer cells

MAGE: melanoma antigen-encoding genes

MGMT: O-6-methylguanine-DNA methyltransferase

MHC: major histocompatibility complex

NK: natural killer

NMDA: N-methyl-D-aspartic acid

NOS: nitric oxide synthases

PCR: polymerase chain reaction

PDGFR: platelet-derived growth factor receptor

PGE2: prostaglandin E_2

PTEN: phosphatase and tensin homolog

SART: squamous cell carcinoma antigen recognized by T cells

STAT-3: signal transducer and activator of transcription-3

TAP: transporter associated with antigen processing

TGFβ: trasforming growth factor-β

TILs: tumor-infiltrating lymphocyte

TLR: toll-like receptor

TNFα: tumor necrosis factor-α

TRP: tyrosinase-related protein

VCAM-1: vascular cell adhesion molecule-1

VEGF: vascular endotelial growth factor

VEGFR: vascular endotelial growth factor receptor

VLA-4: very late antigen-4

WHO: world health organization

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FUTURE PERSPECTIVES

1.Introduction

1.1 Glioblastoma Multiforme

1.1.1 Introduction

Glioblastoma (GB) is the most aggressive form of gliomas, central nervous system (CNS) malignant tumors. Despite ceaseless efforts by researchers and physicians to find new therapeutic strategies, there have been no significant advances in the treatment of GB for several decades and most patients with GB die within one and half years of diagnosis. However, recent information regarding the genetic and epigenetic alterations in GB has helped elucidate the formation of this tumor in more detail [1].

1.1.2 Classification

Gliomas are histologically classified as astrocytomas, oligodendrogliomas and oligoastrocytomas. They are graded on the World Health Organization (WHO) grading system according to their degree of malignancy. The most malignant Grade IV gliomas (GB) exhibit advanced features of malignancy, which include necrosis, vascular proliferation and pleomorphism [2] [Fig.1]. Patients with GB generally die within one and half years from the time of diagnosis because of their strong resistance to conventional therapies, which include surgery, chemotherapy and irradiation.

Although until recently chemotherapy for GB did not substantially improve disease outcome when combined with other treatment methods. The median survival of the patients who received this combination treatment was 14.6 months. This is 2.5 months longer than the median survival time of patients who received radiation therapy alone. Therefore, combination therapy using temozolomide (TMZ) and radiation is now widely used worldwide.

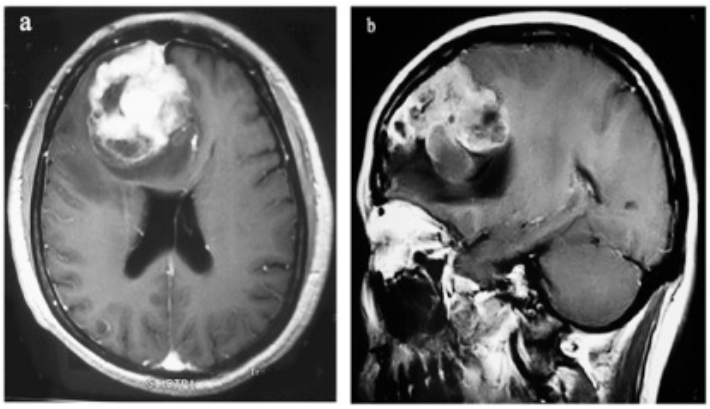

Fig. 1 Imaging of a 58-year-old female with GB. Contrast-enhanced MR images: (**a**) axial and (**b**) saggital showing a large enhancing mass in the deep site of the right frontal lobe and involved to midline. Surrounding edema and the cerebral compression with midline shift are also evident. The lesion has a mixed signal with hyperintense or hypointense signaling on T1-weighted gadolinium-enhanced images. Histopathological examination confirmed the diagnosis of GB (Xian Z et al, 2012).

Although numerous genetic alterations have been described in GB [3,4], such markers have proven to be of marginal utility in predicting outcome or guiding decisions regarding disease management.

GB have been subdivided into primary or secondary GB subtypes on the basis of their clinical presentation. Primary GB are generally found in older patients, while secondary GB are found in younger patients. Primary GB are not associated with prior symptoms or with evidence of detectable antecedent lower grade tumors. In contrast, secondary GB arise from lower grade gliomas within 5-10 years of diagnosis [5]. Although they have distinctive clinical histories, primary and secondary GB exhibit features that are histologically similar and are generally associated with an equally poor prognosis [Fig.2].

Several genes, such as *Tumor protein 53 (TP53), p16Ink4a, Phosphatase* and *Tensin Homolog Deleted from Chromosome 10 (PTEN)* and *Epidermal Growth Factor Receptor* (*EGFR)*, are altered in both primary and secondary GB [6,7].

These alterations are thought to occur in a sequential order during the malignant progression of gliomas. Loss or mutation of the *PTEN* tumor suppressor gene and amplification or overexpression of the *EGFR* oncogene are thought to be a characteristic of primary GB, whereas mutation of the *TP53* tumor suppressor gene appears to be an early event during the development of secondary GB [8-10]. Most importantly, abnormal activation of growth factor signaling and dysregulation of cell cycle checkpoints are found in both types of GB.

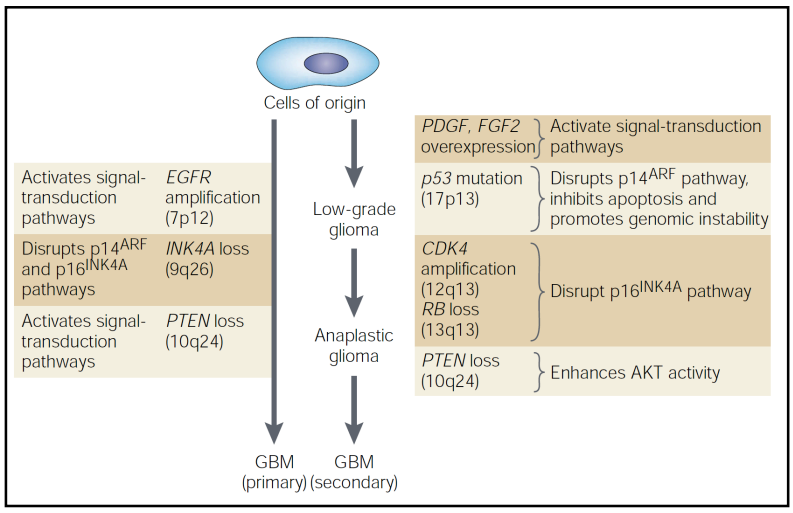

Fig. 2: Distinct molecular alterations between primary and secondary GB. GB formation is either *de novo* (primary GB) or due to the progression of a lower grade glioma to a higher grade one through the acquisition of additional mutations (secondary GB). The mutations listed are a subset of those found in these tumours that have some correlation with glioma grade and GB type. Also listed are some biological effects of these mutations and changes in gene expression that might contribute to their roles in gliomagenesis (Holland EC et al, 2001).

In a work of 2010, Verhaak proposed a classification of GB based on genomics and genetics identifying four distinct classes that are potentially responsive to different therapeutic approaches [11]. These subtypes are classified as: proneural, classical, mesenchymal and neural. According to this study, the proneural subtype is associated with GB that occur at a

young age and with secondary GB characterized by p53 and IDH1 mutations. This group of GB express oligodendroglial markers. Instead, the classic subtype presents chromosome 7 amplification, chromosome 10 deletion and amplification of epidermal growth factor receptor (EGFR). Mesenchymal GB show high expression of mesenchymal markers and a enhanced invasive capacity. Finally, the neural subtype is associated with neural markers.

The classification of GB into several subtypes, however, is at the canter of several debates and remains to be clarified. Phillips et al, for example, due to genetic characterizations carried out on different human GB lines analysis, divides GB in three classes: the proneural, the proliferative and mesenchymal subtype [12]. The proliferative subtype, or classic, had been proposed previously [13] and it was confirmed in this study by classifying those lines that exhibit a high proliferation mainly due to amplification of EGFR and PDGFR. In this study, the mesenchymal and the proneural subtypes coincide, in part, with the characteristics identified by Verhaak in the same molecular subtypes.

1.1.2.1 Activated growth factor signaling pathways

Various growth factors, such as platelet-derived growth factor (PDGF)-A, PDGF-B, epidermal growth factor (EGF), transforming growth factor (TGF)-β, insulin growth factor (IGF)-1 and fibroblast growth factor (FGF) are often produced and secreted in GB. In addition, the various growth factor receptors corresponding to each specific growth factor are often overexpressed in GB. Consequently, the autocrine/paracrine loops between ligand and receptor enhance their impact in gliomagenesis [14,16]. Several important signaling pathways, such as Ras-mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K)-AKT and Phospholipase-C gamma (PLCγ)-Protein Kinase C (PKC) are activated

downstream of growth factor receptors. Recent reports using genetically engineered mice suggest that these growth factor signaling cause the formation of gliomas directly [17].

1.1.2.2 Dysregulation of cell cycle checkpoints

The eukaryotic cell cycle is composed of four distinct phases: G1 phase, S phase (DNA synthesis), G2 phase and M phase (mitosis). Through the cell cycle, the genetic materials are correctly replicated and transmitted to the two daughter cells. Accurate regulation of the cell cycle is crucial for the survival of cells. Each phase has to progress in a sequential fashion and, to activate each phase, proper progression and completion of the previous phase is required. The engine of the cell cycle is the cyclin-cyclin-dependent kinase (CDK) complex. Cyclins constitute the regulatory subunit and CDK is the catalytic subunit of an activated heterodimeric kinase complex.

When cells receive promitotic extracellular signals, G1 cyclin-CDK complexes are activated and promote the expression of transcription factors, which leads to the increase of S-phase cyclin expression. The disruption of cell cycle checkpoints caused by the dysregulation of major tumor suppressors appears to be crucial for gliomagenesis. Firstly, the *TP53* gene is either mutated or deleted in certain gliomas, particularly in secondary GB. Secondly, the retinoblastoma (*RB*) gene is mutated in 10-25% of high-grade astrocytomas.

In addition, *RB* is functionally silenced in another 15% of astrocytomas via the amplification of its antagonist *CDK4.* Lastly, the *CDKN2A* locus encoding *p16INK4A* and *p14Arf* (which positively regulate the *RB* and *TP53* pathways, respectively) is deleted in approximately half of high-grade astrocytomas [18-19].

1.1.2.3 Isocitrate dehydrogenase 1 (IDH1) and IDH2 mutation in GB

Recently, a somatic mutation at amino acid 132 of the isocitrate dehydrogenase 1 (IDH1) protein was identified in more than 70% of WHO Grade II and III astrocytomas, oligodendrogliomas and secondary GB [20]. Surprisingly, tumors expressing an intact *IDH1* gene often have mutations at the analogous amino acid 172 of the IDH2 protein.

A recent report showed that forced expression of a mutated *IDH1* gene in cultured cells results in the reduction of the enzyme product α-ketoglutarate (α-KG), which leads to the increase of the levels of the hypoxia-induced factor-1α (HIF-1α), the stability of which is regulated by α-KG. Because HIF-1α is thought to promote tumor growth at low oxygen levels, mutation of the *IDH1* gene may facilitate tumor progression in GB. It should be noted that mutations in the *IDH1* or *IDH2* genes are identified in the vast majority of WHO Grade II or III gliomas and secondary GB [21].

1.1.2.4 Aberrant function of MicroRNA in GB

It has been speculated that at least one-third of all protein-coding genes in the human genome are partially regulated by microRNAs [22] MicroRNAs, which are single-stranded RNA molecules that comprise approximately 20-22 nucleotides, have been shown to play a central role in biological processes and distinct patterns of microRNA expression are seen in different normal cells and tissues [23]. MicroRNAs are noncoding RNAs from various chromosomal origins, which include intergenic, intronic, or exonic regions. The primary transcripts of microRNAs (Pri-miR) are transcribed from the genome by RNA polymerase II and are processed through a conserved mechanism to yield mature microRNAs. Mature microRNAs recognize partially complementary sequences in the 3'-UTR of their target genes and decrease the mRNA levels of the target by cleavage or by inhibiting translation initiation directly [24].

Many reports on the functions of microRNAs suggest that microRNAs have hundreds of potential targets and regulate multiple signaling pathways. Various levels of microRNA expression have been observed in cancers, including GB [25]. For example it has been reported that microRNA-21 (miR-21) is highly expressed in GB [26]. Inhibition of miR-21 in glioma cell lines results in the increase of caspase-dependent apoptosis, which suggests that miR-21 acts as an oncogene by inhibiting apoptosis. MiR-7 is down-regulated in GB and targets EGFR [27]. MiR-7 has also been reported to target insulin receptor substrate-2 (IRS-2), which is an adaptor protein that mediates PI3K signaling downstream of receptor tyrosine kinases and suppresses AKT activation.

In a recent publication of my group [28], Speranza M et al focused on NEED9, a novel target of mir-145. mir-145 is an important repressor of pluripotency in embryonic stem cells and a tumor suppressor in different cancer. We found that mir-145 is strongly down-regulated in GB specimens and corresponding glioblastoma-neurospheres (GB-NS, containing GB stem-like cells) compared to normal brain and low-grade gliomas (LGG). We focused the attention on HEF1/CasL/NEDD9, a scaffold protein involved in invasion in several types of cancer. We confirmed a significant down-regulation of NEDD9 in miRover-NS and a higher expression in GB compared to normal brain. We observed that intracranial injection of GB-NS over-expressing mir145 delays significantly tumor development: deriving tumors showed a significant down-regulation of NEDD9. Our results demonstrate the critical role of mir-145 and NEDD9 in regulating glioblastoma invasion and suggest a potential role of NEDD9 as a biomarker for glioma progression [28].

1.2 Medical treatment & management of glioblastoma

1.2.1 Introduction

Until relatively recently the treatment of GB with chemotherapeutic agents remained controversial due to lack of proven efficacy. Standard therapy for newly diagnosed GB consisted of maximum safe resection followed by fractionated external-beam radiotherapy [29]. Chemotherapy was often given concurrently with radiation or as an adjuvant, at time of initial diagnosis but after the completion of surgery and radiation, but its use was inconsistent and geographically variable. This began to change in the late 1980s with the investigation of temozolomide (TMZ), an orally administered alkylating agent. In 2005, a large Phase III trial of TMZ given concurrently with radiation therapy and as an adjuvant after radiation for newly diagnosed GB showed a significant survival benefit over radiation alone [1]. This regimen has since become the standard of care for patients with newly diagnosed GB. Further trials are ongoing to investigate the relative contributions of concurrent and adjuvant TMZ in treating GB and a variety of alternative TMZ administration.

Another recent approach to GB therapy is the use of molecularly targeted agents-low molecular weight kinase inhibitors and monoclonal antibodies that act as specific inhibitors of the signaling pathways implicated in tumor growth and survival. Angiogenesis has long been recognized as a key player in tumor growth and vascular proliferation is a defining feature of GB, so it is little surprise that therapies targeting the vascular endothelial growth factor (VEGF) system have been met with great enthusiasm in the neuro-oncology community. A wide variety of other signaling pathway agents are being evaluated both as monotherapies and in combination with traditional alkylating chemotherapy or alongside other targeted agents.

1.2.2 Cytotoxic chemotherapy for GB

1.2.2.1 Temozolomide

Temozolomide (TMZ) is an orally administered methylating agent with excellent bioavailability and blood-brain barrier penetration [1]. TMZ is a prodrug which is metabolized into 3-methyl-(triazen-1-yl)imidazole-4-carboxamide (MTIC). MTIC methylates DNA at a number of sites, most frequently N7-guanine and N3-adenine. While the conversion of O6-guanine to 06-methylguanine is less frequent, accounting for only about 5% of all methylation events, it induces a futile cycle of DNA mismatch repair which leads to double-stranded breaks and cellular apoptosis, thus serving as the primary mediator of TMZ-mediated cytotoxicity [30].

Temozolomide was first evaluated in glioma as a treatment of recurrent malignant glioma following radiation therapy, where it was shown to have good antitumor activity as a monotherapy and an acceptable side effect profile. Following the success of this approach, TMZ was evaluated in combination with radiation therapy as a treatment for newly diagnosed GB [31].

There are several mechanisms of tumor resistance to TMZ; the two with greatest implications for GB therapy are the O6-methylguanine DNA methyltransferase (MGMT) and poly(ADP-ribose) polymerase (PARP) systems. Methylation of O6-guanine, which as previously mentioned is the primary mediator of TMZ cytotoxicity, is repaired by the enzyme MGMT. MGMT is irreversibly inactivated in the process and de novo synthesis is required to maintain enzyme activity [32]. While MGMT is widely expressed in both normal human tissue and neoplasms, MGMT protein expression is heterogeneous within tumors due to frequent epigenetic silencing via gene promoter hypermethylation [33] As would be predicted from its mechanism, the level of MGMT protein within tumor tissue correlates with response to TMZ and MGMT promoter methylation predicts increased survival in patients with malignant glioma.

The irreversible inactivation of MGMT during DNA repair provides an a priori basis for the potential utility of dose-dense TMZ regimens by

suggesting that it may be possible to effectively overcome this chemotherapy resistance mechanism by designing a regimen in which increased or prolonged TMZ exposure leads to depletion of MGMT. MGMT can also be inhibited directly by O6-benzylguanine (O6-BG) and related agents and a number of recent studies have examined the combination of TMZ and MGMT inhibitors [34, 35].

1.2.2.2 Non-temozolomide systemic chemotherapy

Prior to the introduction of TMZ, the use of adjuvant chemotherapy along with surgery and radiation was inconsistent and geographically variable. When adjuvant therapy was employed, nitrosourea class compounds such as carmustine (BCNU) or lomustine (CCNU) were commonly chosen for their ability to cross the blood-brain barrier. CCNU was often combined with procarbazine, a DNA alkylating agent and vincristine, which disrupts microtubule formation, to create the regimen known as PCV. Despite decades of evaluation, no nitrosourea-based adjuvant treatment was unambiguously shown to confer a survival benefit beyond that achieved by radiation therapy alone in a Phase 3 randomized controlled trial. Several meta-analyses of have examined the available RCT data, the most rigorous of which showed a small but significant survival benefit equivalent to a 6% absolute increase in 1-year survival [36].

While TMZ has now supplanted the nitrosoureas as the adjuvant agent of choice as a monotherapy, combination therapies such as TMZ/ CCNU are being investigated which may offer survival benefits superior to TMZ alone [37]. Carboplatin is a platinum-based compound that interferes with DNA replication by producing cross-links within DNA strands. Intravenous carboplatin has modest activity against recurrent malignant glioma both in chemotherapy naive patients 31 Low-dose carboplatin has been shown to act as a radiosensitizer in some non-CNS solid tumors, but the addition of

carboplatin to radiation therapy for newly diagnosed malignant glioma did not increase survival time [38].

1.2.2.3 Surgically implanted carmustine (BCNU) coated wafers (Gliadel)

The relatively minimal antitumor activity of systemically administered nitrosourea-based chemotherapy has led to a variety of alternative approaches to chemotherapy delivery. The most studied method is the implantation of BCNU impregnated wafers (Gliadel) into the tumor cavity at the time of resection. These wafers then provide a controlled release of BCNU over a period of 2 to 3 weeks. This approach has several theoretical advantages over systemic chemotherapy, including the ability to deliver a greater dose to residual tumor and a more benign systemic side effect profile. A recent meta-analysis of the use of Gliadel for high grade glioma concluded that the addition of Gliadel to radiotherapy led to increased survival relative to radiotherapy alone for newly diagnosed tumors, but that there was no significant increase in survival with Gliadel therapy for recurrent disease [39].

1.2.3 Molecular-targeted therapies for GB

1.2.3.1 Angiogenesis pathways and agents: Vascular Endothelial Growth Factor (VEGF)

The role of angiogenesis in tumor survival and growth has been recognized since the early 1970s [40]. Tumors utilize many different mechanisms to produce their blood supply. The prototypical example is the vascular endothelial growth factor (VEGF) system. Gliomas, along with most other human solid tumors, express VEGF at elevated levels. VEGF expression is triggered by a number of factors, notably hypoxia and acidosis, which are commonly seen in high-grade tumors. VEGF then acts on endothelial cells to trigger increased vessel formation [41].

The first targeted anti-angiogenic agent shown to have efficacy against malignant glioma was bevacizumab (Avastin®), a monoclonal antibody against VEGF. Prior to its use in glioma, bevacizumab was shown to improve outcome in non-CNS malignancies such as colon cancer when given with cytotoxic chemotherapy [42], where it is thought to lead to improved chemotherapy response by allowing normalization of blood vessels. Given this background, initial trials of bevacizumab for GB patients combined the therapy with irinotecan, a conventional chemotherapeutic agent. Many studies of bevacizumab including combination therapy trials with other targeted agents are ongoing.

While bevacizumab is the best-studied of the VEGF pathway modifiers, several other agents are currently being evaluated. Aflibercept (VEGF Trap) is a soluble receptor that binds circulating VEGF and placental growth factor (PlGF), a related ligand of the VEGFR.

Small molecule kinase inhibitors aimed at VEGFR include vatalanib, pazopanib, cediranib and CT-322. While none of these therapies has shown unequivocal survival benefit in patients with glioma, cediranib therapy does lead to a rapid and dramatic radiographic response due to reversible vascular normalization [43]. In addition to the VEGF-specific therapies above, several agents currently under investigation act on VEGF in addition to other targets of glioma growth. Examples include sunutinib, vandetanib, sorafinib and axitinib. Anti-VEGF therapy is generally well tolerated.

1.2.4 Drug resistance in GB

Drug resistance in GB is one of the major factors that render this type of tumor incurable. EGFR is frequently amplified, overexpressed, or mutated in GB [9]. An activated form of EGFR mutant caused by the deletion of exons 2-7 (known as EGFRvIII) occurs in 20-30% of GB; EGFRvIII reduces

apoptosis and increases the proliferation of GB cells [44]. In addition, EGFRvIII has the capacity to malignantly transform murine *Ink4a/Arf* null neural stem cells (NSC) or astrocytes [45].

Therefore, EGFR is a strongly validated molecular target for GB treatment and the use of EGFR inhibitors is a reasonable therapeutic strategy for this disease. However, only 10-20% of GB patients respond to EGFR inhibitors. Although the mechanism of the resistance of GB to EGFR inhibitors is not known, the loss of PTEN is suggested to be associated with this mechanism. PTEN is commonly lost in GB and is an inhibitor of the PI3K signaling pathway, which is activated downstream of EGFR. It is suggested that loss of PTEN may dissociate EGFR inhibition from the PI3K pathway inhibition, which may result in the resistance of GB to EGFR inhibitors [46].

1.3 Cancer stem cells hypothesis

1.3.1 Introduction

The cancer stem cells (CSC) hypothesis has provided an alternative framework for understanding cancer heterogeneity, tumorigenesis and cancer progression. Recent identification of cancer-initiating stem cells in brain tumor [47, 48] and in other types of cancer suggested that CSC may play a central role in the propagation of several cancer types. CSC have also been shown to be responsible for prevalent radio-resistance and chemo-resistance in glioma [49].

Compared to conventionally cultured human cancer cell lines, CSC have been shown to recapitulate human brain tumors in phenotype and in cancer genetics and thus may more faithfully model mechanisms of tumorigenesis and tumor propagation.

The identification of glioma stem cells may have important applications in cancer therapy for glioma patients. Therapies targeting glioma stem cells may help overcome the persistent cancer resistance to chemotherapies

and radiation therapies. In addition, strategies targeting glioma stem cells may have positive impact on the development of immunotherapy for malignant brain cancer patients. Different hypothesis on the origin of CSC were postulated. They may be described as follow:

- Cancer cells arise from stem cells. Stem cells are distinguished from other cells by two characteristics: (1) they can divide to produce copies of themselves, or self-renew, under appropriate conditions and (2) they are pluripotent, or able to differentiate into most, if not all, mature cell types. If CSC arise from normal stem cells present in the adult tissue, de-differentiation would not be necessary for tumor formation. In this scenario, cancer cells could simply utilize the existing stem-cell regulatory pathways to promote their self-renewal. The ability to self-renew gives stem cells long lifespans relative to those of mature differentiated cells. It has therefore been hypothesized that the limited lifespan of a mature cell makes it less likely to live long enough to undergo the multiple mutations necessary for tumor formation and metastasis.
- The differentiation pathway from a stem cell to a differentiated cell usually involves one or more intermediate cell types. These intermediate cells, which are more abundant in adult tissue than are stem cells, are called progenitor or precursor cells. They are partly differentiated cells present in fetal and adult tissues that usually divide to produce mature cells. However, they retain a partial capacity for self-renewal. This property, when considered with their abundance relative to stem cells in adult tissue, has led some researchers to postulate that progenitor cells could be a source of CSC.
- Some researchers have suggested that cancer cells could arise from mature, differentiated cells that somehow de-differentiate to become more stem cell like. In this scenario, the requisite oncogenic (cancer causing) genetic mutations would need to drive the de-differentiation process as well as the subsequent self-renewal of the proliferating cells. This model leaves

open the possibility that a relatively large population of cells in the tissue could have tumorigenic potential; a small subset of these would actually initiate the tumor. Specific mechanisms to select which cells would be differentiated have not been proposed. However, if a tissue contains a sufficient population of differentiated cells, the laws of probability indicate that a small portion of them could, in principle, undergo the sequence of events necessary for de-differentiation. Moreover, this sequence may contain surprisingly few steps; researchers have recently demonstrated that human adult somatic cells can be genetically "re-programmed" into pluripotent human stem cells by applying only four stem-cell factors. These three hypotheses are summarized in [Fig.3].

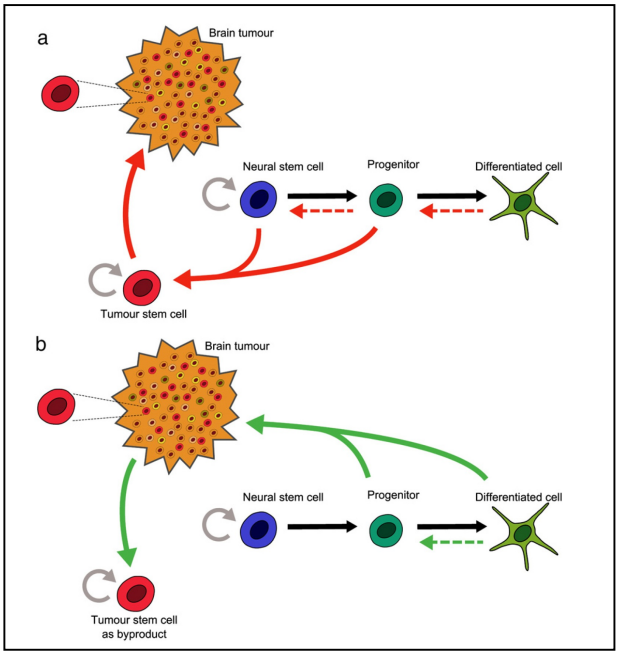

Fig. 3 Schematic representation of the possible relationships between neural stem cells (NSC), neural progenitors, CSC and brain tumours. (**a**) CSC originate from NSC or progenitor cells upon acquisition of genetic mutations and give rise to brain tumours, the maintenance of which is mediated by CSC. (**b**) Progenitor cells or even differentiated cells are the cell of origin of a brain tumour after occurrence of genetic mutations. Tumour stem cells found in brain tumours are a by product of tumorigenesis, required for tumour maintenance but not for tumour initiation. (**a, b**) Dotted arrows indicate possible de-differentiation of cells as an alternative cell of origin of tumours. Grey semi-circles signify self-renewal capacity of NSC and CSC (Sutter R et al, 2007).

1.3.2 Glioma stem cells as cancer initiating cells

GB can be initiated from brain tumor stem-like cells (BTSC) [48]. However, unlike normal tissue stem cells, CSC may be heterogeneous, meaning that all CSC from tumors of the same tissue origin and grade, or even from the same tumor, are not the same [50]. For example, both CD44+/CD24- and CD133+ cell populations had been identified from the same cancer in a BRCA breast cancer model, suggesting that one initial mechanism may lead to diverse CSC with different phenotypes [51].

The capability of BTSC to sustain brain tumor growth apparently lies in their active self-renewal and/or suppressed cell differentiation. Several major

signaling pathways that are critical in brain development have also been implicated in tumorigenesis, including: bone morphogenetic protein (BMP) [52] Notch [12] and Sonic Hedgehog (SHH) [53] EGFR [54] PTEN/PI3K/Mtor [55] PDGFR [56] and OLIG2 [57].

Recently, a gene expression profiling of gliomas has shown that SHH signaling is active in a subset of gliomas. This study further showed that SHH signaling is essential for glioma CSC self-renewal and CSC-initiated brain tumor growth. It is postulated that the relative homogenous population of CSC, rather than the heterogeneous tumor cells, may reveal key mechanisms of tumor initiation and propagation of primary tumors and hence predict clinical prognosis, therapy and drug response of tumors.

The identification of both SHH signaling-dependent and independent brain tumors in this study suggested that there are molecularly distinct subclasses of GB that have an effect on progression and prognosis. These findings were reminiscent of a recent study in which high-grade gliomas were classified into a proneural subclass and a mesenchymal subclass, resembling two stages in neurogenesis [12]. In that study, Phillips et al discovered a prognostic model utilizing PTEN-AKT and Notch signatures to predict poor versus better glioma prognosis, respectively.

In an other study, the SHH signaling-dependent brain tumors showed high activities in Notch and PDGFR pathways, while the SHH signaling-independent brain tumors showed PTEN-deficiency and high activity in PI3K-AKT pathway. In this study authors also described that brain tumor stem cells, with distinct signaling patterns, determined the different GB phenotypes and progression [58].

1.4 Basic concepts in glioma immunology

1.4.1 Introduction

Despite aggressive treatment with surgery, radiation and chemotherapy, average survival for glioblastoma patients remains only 14 months. More effective therapies are urgently needed. Immunotherapy (stimulating the immune system to attack the tumor) is one such promising therapy. Effective immunotherapy requires an understanding of the basic immunobiology of these tumors.

1.4.2 Basic concepts in immunology

The immune system made up of the various cellular and visceral components involved in recognizing and eliminating foreign and/or dangerous antigens. Anatomically, it is made up of primary and secondary lymphoid organs. Functionally, it consists of both innate and adaptive immune responses. Innate immunity deals with antigen-nonspecific responses to foreign or dangerous molecules. Some of this is accomplished at a purely molecular level with the complement system and systemic responses to inflammatory cytokines. At a cellular level, it is made up of macrophages, neutrophils and natural killer cells.

Activation of innate immunity is not antigen-specific but is induced in response to molecular factors common to multiple infectious or dangerous agents. In contrast to innate immunity, adaptive immune responses are antigen-specific. Foreign and/or dangerous antigens are scavenged from the environment, processed and presented by professional antigen presenting cells (APC) of the reticulo endothelial system, including dendritic cells and macrophages. APC form an important connection between adaptive and innate immunity. Adaptive immunity is comprised of humoral (antibody) mediated by B cells and cellular immune responses mediated by cytotoxic T cells. Both forms of adaptive immunity require signals from

helper T cells. Antigens are presented to T cells in the context of major histocompatibility complexes (MHC). Class I MHC are key for presentation to CD8+ cytotoxic T cells while class II MHC are important for presentation to CD4+ helper T cells. In addition to antigen presentation in the context of a MHC molecule, T-cell activation also requires a second costimulatory signal most commonly through the T-cell costimulatory molecules B7-1 or B7-2 on APC binding their ligand CD28 on T cells [59, 60] [Fig. 4].

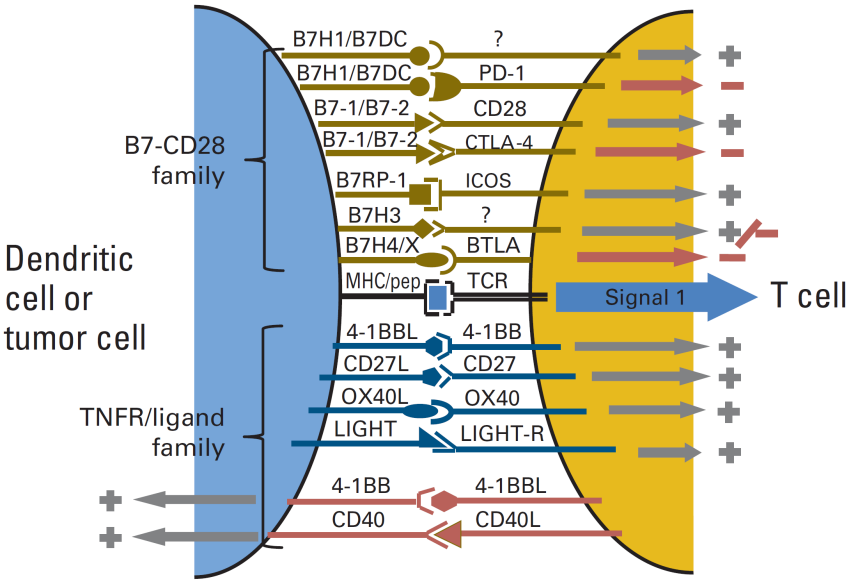

Fig. 4 Immunological synapse. Target recognition by T cells is two-step process. Specific interaction of T-cell receptor (TCR) with major histocompatibility complex (MHC) –peptide complexes displayed by tumor cells or antigen-presenting cells (APCs; eg, dendritic cells) provides first signal for T-cell recognition. Second event is coregulatory signal that determines whether T cell will become activated or anergic (nonreactive). T-cell coreceptors transmitting stimulatory (+) or inhibitory (+) signals on engagement of specific ligands expressed by tumor cells or APCs are depicted. Molecules in B7-CD28 and tumor necrosis factor receptor (TNFR) families are now being targeted for cancer immunotherapy. 4-1BBL, 4-1BB ligand; BTLA, B- and T-lymphocyte attenuator; CTLA-4, cytotoxic T-lymphocyte antigen 4; ICOS, inducible T-cell costimulator; LIGHT, homologous to lymphotoxin, shows inducible expression, competes with herpes simplex virus glycoprotein D, expressed by T cells; PD-1, programmed cell death 1 (Topalin SL, 2011).

1.4.3 Immunology in the CNS

The brain has long been considered an immunologically privileged site owing to its apparent inability to reject intracranial xenografts in older report, separation via the blood brain barrier and lack of obvious connections with the lymphatic system. However, unlike other immunologically privileged site like testes, no specific CNS-associated antigens have been described that are immunogenic systemically but evade immune detection within the brain. Furthermore, it is now clear that the blood brain barrier is only a relative barrier to lymphocyte tracking, particularly in pathological states. Connections between cerebrospinal fluid and cervical lymphatics have been documented [61, 62]. Abundant evidence demonstrates that intracranial xenografts are actually rejected very efficiently in all but the most immunocompromised hosts. Microglia, resident antigen presenting cells of the central nervous system, play an active part in very dynamic immune responses in the brain [63]. Multiple pathological states, ranging from multiple sclerosis to Alzheimer's disease to Parkinson's disease, bear witness to the ability of immune responses to occur within the brain. In short, it is clear that, while the central nervous system may be an immunologically distinct environment, it is not truly immunologically privileged.

1.4.4 Glioblastoma-associated antigens

A key assumption underlying most attempts at tumor immunotherapy (particularly strategies aimed at stimulating adaptive immune responses) is that tumors express specific abnormal antigens that can be recognized and eliminated by the immune system. Several lines of evidence support this assumption in cancer in general and glioblastomas in particular that can be demonstrated by discussion of a representative antigen.

In glioblastomas, mutant epidermal growth factor receptor-vIII is an excellent example of a tumor-specific antigen [64]. EGFRvIII is a truncated version of the epidermal growth factor receptor (EGFR) present in approximately 30% of glioblastomas. It has lost its extracellular domain and is constitutively active. It contains specific peptide sequences that have not been described in any normal human tissues and have been used to target vaccines [65]. Almost any tumor peptide has potential to function as a tumor antigen, leading to a bewildering number of potential targets. This begs the question: which antigens are the most attractive targets for immunotherapy? The National Cancer Institute of the United States has recently attempted to address this issue. They performed an in-depth review of 75 tumor-associated antigens that could be targeted by immunotherapy [66]. Antigens were ranked based on nine factors: therapeutic function, immunogenicity, role in oncogenicity, specificity, express level in tumors, cancer stem cell expression, percentage of patients with positive tumors and cellular location of expression. The highest ranked antigens included WT1, MUC1, LMP2, HPV E6/E7, HER2/Neu, EGFRvIII, MAGE A3 and NY-ESO-1. While the expression of some of these antigens (e.g., EGFRvIII) by glioblastomas is well established, the presence or absence of others (e.g., MUC1) have not been reported at all. Only a handful of systematic studies have been performed examining expression of potentially immunogenic antigens in glioblastomas have been performed. Many have included antigens that did not make the NCI's "top ten" (e.g., gp100, MART) or that were not considered in the NCI analysis at all (e.g., IL13Rα) [67]. For the development of new therapies against gliomas, it is required to identify tumor antigens as targets for specific immunotherapy.

Category of Antigens	Antigen
Cancer-testis (CT) antigens	MAGE1, SOX6
Tissue-specific antigens	Gp100, TRP-2
Mutated antigens	EGFRvIII
Others	IL13Ra2, EphA2, EphB6, AIM-2, HER-2, WT1, ARF4L, SART-3, SOX11, KIF1C, KIF3

***Tab. 1**. Human glioma antigens recognized by CTL*

1.5 Mechanisms of glioma-mediated immunosuppression

1.5.1 Introduction

A major contributing factor to glioma development and progression is its ability to evade the immune system. Here I would like to explore the mechanisms utilized by glioma to mediate immunosuppression and immune evasion. These include intrinsic mechanisms linked to its location within the brain and interactions between glioma cells and immune cells. Lack of recruitment of naïve effector immune cells perhaps accounts for most of the immune suppression mediated by these tumor cells. This is enhanced by increased recruitment of microglia, which resemble immature antigen presenting cells that are unable to support T-cell mediated immunity. Furthermore, secreted factors like TGFβ, COX-2 and IL-10, altered costimulatory molecules and inhibition of STAT-3 all contribute to the recruitment and expansion of regulatory T cells, which further modulate the immunosuppressive environment of glioma.

1.5.2 Intrinsic mechanism of immunosuppression

1.5.2.1 Altered human leukocyte antigen expression

Human leukocyte antigens (HLA) are the MHC molecules in humans. The class I antigens are comprised of three major (classical) genes- HLA-A, -B and -C; and three minor (non-classical) genes- HLA-E, -F and -G. The classical HLA class I molecules present intracellular antigenic peptides on the surface of altered cells, thus targeting the cells for lysis by cytotoxic CD8+ T cells (CTL). They are typically expressed by most nucleated cells in the body. Analysis of HLA class I antigens expression in 47 GB lesions revealed that expression was lost in approximately 50% of the samples [68]. The selective loss of HLA class I antigens would suggest a defect in antigen presentation by glioma cells and a concomitant impairment of CTL lysis. Recently, HLA-G, a non-classical HLA class I molecule, has been identified in malignant tumors and glioma cell lines and may be involved in tumor immune escape [69]. Another inhibitory molecule is the non-classical MHC class I molecule HLA-E. It is the only known ligand for CD94/NKG2A, an inhibitory receptor, expressed on NK cells and CD8+ T cells. The over-expression of HLA-E by glioma cells would render them resistant to NK cell and CTL cytotoxicity. Analysis of human long-term glioma cell lines, primary ex vivo polyclonal glioblastoma cell cultures and surgical glioblastoma specimens, revealed high expression of HLA-E [70].

1.5.2.2 The role of microglia

Microglia are the brain's resident macrophages. They differentiate from monocytes which migrate into the brain during embryogenesis. Microglia are distinguished from macrophages based on low expression of CD45 and high expression of CD11b (CD45dim CD11b+). Under normal physiological conditions, microglia roam the CNS, phagocytosing debris and maintaining homeostasis. It is likely that microglia may play dual roles during glioma

development through the expression of cytokines. Microglia have been postulated to support glioma cell proliferation through the production of IL-10 and IL-6 [71, 72]. IL-10 is also an immunosuppressive cytokine, which inhibits T-cell proliferation. Furthermore, microglia express a clearly Th2 skewed cytokine profile, which suppresses cytotoxic responses. Taken together, glioma infiltrating microglia support glioma tumorigenesis while promoting immune evasion.

1.5.3 Impairment of glioma and immune cell interactions

Cell-to-cell contact is required for lymphocytes to lyse tumor cells. Specifically, the release of the cytotoxic molecules, perforin and granzyme, by natural killer (NK) cells and CTL is cell contact dependent. Glioma cells have been shown to alter the extracellular matrix and this may have an impact on cell-to-cell contact. Recently, tenascin-C expression has been described in glioma cells. Tenascin-C belongs to a specialized class of ECM proteins, matricellular proteins, which function as adaptors and modulators of cell-matrix interactions [73]. The exact role of tenascin-C is unresolved, as it has been implicated in both cell adhesion and migration. Tenascin-C has also been shown to inhibit T-cell proliferation and IFNγ-production. The infiltration of lymphocytes into tumors was inhibited by tenascin-C [74]. Through the expression of tenascin-C, glioma cells could potentially enhance cell migration, while suppressing T-cell responses by limiting cell-to-cell contact.

1.5.4 Glioblastoma-derived immunosuppressive factors

1.5.4.1 TGF-β

It has long been recognized that GB secrete multiple immunosuppressive factors. Expression of transforming growth factor-β2 (TGFβ2; originally

described as glioblastoma-derived immunoinhibitory factor) by glioblastoma cells were originally described more than two decades ago [75]. More recently, TGFβ1 and -β2 have been shown to induce FoxP3 expression in anti-CD3 activated CD4+T cells, resulting in inducible regulatory T cells (Treg). Thus, the secretion of TGFβ by glioma cells mediates immunosuppression not only by acting directly on T cells and NK cells, but also indirectly through the induction of Treg.

1.5.4.2 Cyclooxygenase-2 and Prostaglandin E2

Cyclooxygenase-2 (COX-2) is an isoform of the cyclooxygenase enzyme or prostaglandin synthase responsible for the formation of important biological mediators called prostanoids (including prostaglandins, prostacyclin and thromboxane). COX-2 is an inducible enzyme, becoming abundant in activated macrophages and other cells at sites of inflammation. Inhibitors of COX-2 are used as nonsteroidal anti-inflammatory drugs (NSAID) for treating inflammation and pain. The over-expression of cyclooxygenase-2 (COX-2), the key enzyme for the synthesis of prostaglandin E2 (PGE2) from arachidonic acid, has also been well characterized in many types of cancers including colorectal, lung, urinary bladder and malignant gliomas [76]. PGE2, contributes to cellular immune suppression in cancer patients through an unknown mechanism. PGE2 is also a potent inducer of IL-10, which is produced by a variety of cells including monocytes, and exerts suppressive effects on dendritic cell maturation and Th1 responses [77]. PGE2 levels were significantly higher in patient glioma samples compared to control brain samples [78]. Furthermore, surgical removal of malignant brain tumors resulted in a decrease of PGE2 levels. PGE2 has been linked to tumor metastasis and immune evasion, such that the inhibition of PGE2 synthesis using COX inhibitors suppressed tumor growth [79].

1.5.4.3 IL-6 and VEGF

In addition to these frankly immunosuppressive factors, glioblastomas secrete several factors that are profoundly immunomodulatory. Some, such as interleukin-6 (IL-6) and colony stimulating factor-1 (CSF-1) have obvious immune functions such as shifting adaptive immunity to humoral (TH2) responses that may be less effective against solid tumors and/or attracting and stimulating monocytic cells [80]. Others, such as vascular endothelial growth factor (VEGF), impact immune responses in a more tangential fashion. Tumor-derived VEGF acts primarily as a pro-angiogenic factor and can be targeted for anti-angiogenic therapy (e.g., bevacizumab).

1.5.5 Induction of T cell anergy

1.5.5.1 Altered B7 Expression

While T cells infiltrate gliomas, in both animal models and primary patient samples, they often lack cytotoxic activity and fail to produce IL-2. This observed T-cell anergy has been linked to defective antigen presentation and poor co-stimulation by tumor cells. Antigen presentation to CD8+T cells is decreased during glioma tumorigenesis due to the loss of HLA class I expression. Additionally, glioma cells express very low levels of B7 costimulatory molecules [81]. Functional assays using heterogeneous ex vivo tumor preparations or pure populations of ex vivo tumor cells and microglia, demonstrated CD4+ T-cell activation only in the presence of exogenous B7 costimulation (provided by addition of soluble agonist anti-CD28 monoclonal antibody). Therefore, to overcome immunosuppression in glioma, strategies need to overcome the low levels of B7 costimulation.

1.5.5.2 STAT3

Signal transducer and activator of transcription-3 (STAT3) has been identified as a major molecular hub of several signaling pathways in several

types of cancer including glioblastoma, breast, lung, ovarian, pancreatic, skin and prostate cancer [82, 83].

The binding of STAT3 to its gene targets affects proliferation, survival, differentiation and development. It is a member of the STAT family of cytoplasmic latent transcription factors. Receptor engagement by members of IL-6 cytokine family like IL-6, oncostatin M and Leukemia inhibitory factor, or growth factors like platelet-derived growth factor (PDGF), fibroblast growth factor (FGF) and epithelial growth factor (EGF) activate STAT3 by tyrosine phosphorylation. Kinases that induce STAT3 activation are receptor kinases like Janus kinase (JAK) family members associated with, FGFR, EGFR, PDGFR or nonreceptor-associated kinases like Ret, Src or Bcl-Abl. Postactivation STAT3 works to regulate expression of *bcl2*, *bclxL*, *mcl1* and *cyclinD1* among others.

Other than promoting oncogenesis, active STAT3 also enables tumor growth by suppressing tumor recognition by the immune system [84]. STAT3 promotes tumor immune evasion by inhibiting pro-inflammatory cytokine signaling and amplifying Tregs. Inhibiting STAT3 activity by dominant-negative or antisense STAT3 expression increased expression of proinflammatory IFNγ, TNFα, IL-6, RANTES. STAT3 is also involved in maintaining immature dendritic cells and promoting tumor immune tolerance. Mice lacking STAT3 had elevated levels of MHC class II, CD80 and CD86. Antitumor activity of T cells, NK cells and neutrophils was increased and dendritic cell maturation was enhanced by inhibition of STAT3 [85]. STAT3 activity was shown to be elevated in tumor-associated Treg cells that maintain tumor immune evasion. In these Treg, elevated activation of STAT3 increased proliferation and promoted expression of FoxP3, TGFβ and IL-10, all of which inhibit CD8+ T-cell differentiation, dendritic cell maturation and promote proliferation of Treg.

In gliomas, activation of STAT3 has been associated with the inhibition of T-cell response. Treg have been shown in the blood and within the tumor microenvironment of glioblastoma patients and are thought to contribute to the lack of effective immune responsiveness against glioblastomas. In a tissue microarray using tumor samples from 129 glioma patients, Abou-Ghazal et al. showed correlation between prognosis of activated STAT3 residues and tumor-infiltrating Treg [86]. Low incidence of activated STAT3 was observed in normal brain or low-grade astrocytomas.

1.5.5.3 PDL-1

Programmed Death Ligand-1 (PDL-1) or B7-H1 is a member of the B7 family and is a ligand for PD-1 (Programmed Death-1), a member of CD28 family of T-cell regulators. B7-H1 is primarily inhibitory when expressed on tumors. It is significantly expressed on the surface of many human cancers, while undetectable in normal tissues [87]. B7-H1 is found to be highly expressed in carcinomas of colon, breast, ovarian, lung and melanoma cancer, oral squamous cell carcinoma, head and neck cancers and glioma among others [88-90]. The late downstream effects of signaling initiated through the B7-H1:PD-1 unidentified receptor network result in negative regulation of immune function [91]. The use of blocking monoclonal antibodies for B7-H1 or PD-1 increase cytokine production and antitumor CTL activity in experimental tumor models in mice [92].

Expression and immune regulatory activity of B7-H1 has been recently identified in human glioma cells in vitro and *in vivo*. In 12 different glioma cell lines, Wintrelle et al observed constitutive expression of B7-H1 mRNA [90]. High B7-H1 expression was observed in all 10 malignant glioma specimens upon immunohistochemical analysis, whereas no B7-H1 expression could be detected on normal brain tissues. In conclusion, B7-H1 may significantly influence the outcome of T-cell tumor interactions and

correspond to a novel mechanism by which glioma cells evade immune recognition and destruction.

1.5.6 The induction of apoptosis

1.5.6.1 Fas

The induction of apoptosis is a key immune evasion strategy for multiple malignancies, including glioma. Fas/APO-1 is a cell surface receptor that mediates apoptosis when it reacts with Fas ligand (FasL) or anti-Fas antibody. The expression of Fas has been demonstrated on perinecrotic glioma cells, a histological hallmark of glioblastomas, suggesting a correlation between Fas expression and cell death [93]. Since glioma cells are not always able to turn off Fas expression they must use other strategies to evade Fas-induced apoptosis. A potential endogenous antagonist of Fas is soluble Fas which lacks the transmembrane domain. The soluble form of the Fas mRNA was detected in one anaplastic astrocytoma and in two glioblastomas [94]. Interestingly, some glioma cells co-expressed Fas and FasL suggesting that soluble Fas expression by the glioma cells prevented tumor cell-induced apoptosis. The expression of FasL by glioma cells may act as a glioma immune evasion mechanism.

Apoptosis was induced in T cells by FasL+ tumor explants and tumor cell lines. According to Didenko et al, T cells that had undergone apoptosis expressed Fas and were colocalized with FasL-expressing tumor cells [95].

Since glioma cells have the ability to express both Fas and FasL, they have evolved mechanisms to prevent tumor cell apoptosis, while simultaneously inducing apoptosis in T cells.

1.5.6.2 CD70

The expression of CD70 is a novel mechanism utilized by glioma cells to induce apoptosis in T cells and consequently evade the immune response. CD70, TNF-related cell surface ligand, is normally expressed on mature dendritic cells, activated T and B cells. The binding of CD70 to its cognate receptor CD27, is thought to play an important role in T-cell, B-cell and NK cell activation. CD27 is constitutively expressed by T cells, B cells and NK cells [96]. Signaling via CD27 by activated T cells is critical for survival and memory cell formation. In contrast, the ligation of CD27 has also been shown to induce apoptosis in activated T and B cells. CD27 costimulation has also been shown to enhance Treg function [97].

CD70+ glioma cells were able to induce apoptosis in PBMC. *In vitro* experiments using glioma cells, in which Fas and TNFα were blocked, revealed that CD70 was sufficient to induce apoptosis in T cells [98].

1.5.6.3 Gangliosides

Tumor-derived gangliosides are important mediators of T-cell apoptosis and hence, they represent one mechanism by which tumors evade immune destruction [99]. Gangliosides are most highly expressed in cells of the CNS, where they comprise approximately 5-10% of the lipids.

1.5.7 Recruitment of immunosuppressive lymphocytes

1.5.7.1 Expression of chemokines

Chemokines are a family of cytokines helping in the migration of responder cells by inducing directed chemotaxis. These proteins are very small in size (8-10 kDa) and have a characteristic two cysteine residues joined by disulfide bonds (cystine). The different members of chemokine family share gene sequence and amino acid homology. They are classified into several categories according to spacing of the cystine moieties that are key to

forming their 3-dimensional shape and accordingly, different families of receptors for these molecules have been described [100]. Their role has been implicated in various processes including angiogenesis and CNS development. Most importantly, chemokines play a central role within the immune system, as the secretion of these molecules leads to migration of leucocytes [101]. Chemokines appear to play a significant role in various diseases, including cancer.

Chemokines is suspected to contribute to the process of the chronic inflammation that can predispose to cancer formation and progression [102]. Chemokines might also elicit an intrinsic effect on tumor cells. For instance, multiple human cancers including leukemias, lymphomas, gliomas and various epithelial carcinomas express CXC receptor 4 (CXCR4) and respond to its ligand CXC ligand 12 (CXCL12). This ligand-receptor interaction promotes the migration and metastatic establishment of tumor cells [103].

One of the initial reports of chemokines secretion by gliomas was made in 2001 [104]. The authors demonstrated that human glioma cell lines secrete MCP-1 (CCL2) and IL-8 on stimulation with anti-Fas antibody or soluble Fas ligand in a time and dose-dependent manner.

With regards to the tumoral migration of Treg, cancers express a series of chemokines that promote the infiltration by these regulatory lymphocytes. For instance, chemokine CCL22 promotes the migration of Treg into prostate and ovarian carcinomas [105].

In a work of 2008, Jordan JT et al [106] reported that human glioma cell lines express CCL22 in addition to CCL2, although only CCL2 was secreted by samples from GB patients. This has been investigated in the human glioma cell lines D-54, U-87, U-251 and LN-229 as well as in tumor cells from eight patients with GB. The authors further reported that Treg from these brain tumor patients had significantly higher expression of the CCL2 receptor CCR4 than the Treg from healthy controls. Migration

experiments have suggested that Treg migration is mediated by CCL2 and CCL22, which was blocked by antibodies to the chemokine receptors CCR2 and CCR4.

1.5.7.2 Myeloid-derived suppressor cells

Myeloid-derived suppressor cells (MDSC) were originally named natural suppressors. These cells act independently of MHC interactions and inhibit the antigen-induced proliferation of T cells and antibody production by B cells. MDSC were isolated from the bone marrow of tumor patients and named for their ability to suppress T-cell proliferation [107].

These cells lack expression of surface molecules characteristic of T, B, macrophage, or NK cells. Myeloid-derived suppressor cells are immature myeloid cells that have the potential to generate mature granulocytes, macrophages and dendritic cells. In mice, they are identified based on co-expression of CD11b and Gr-1 [108]. Human MDSC equivalents are CD34+, CD33+, CD15- and CD13+, with variable expression of CD11c and HLA-DR and were originally identified in patients with head and neck cancer [109].

Tumor-derived factors promoted MDSC recruitment and maturation into immunosuppressive cells and inhibited the differentiation of dendritic cells. Interestingly, glioma cells are known to produce both IL-6 and colony stimulating factor-1 (CSF-1), which may aid in the recruitment and/or expansion of MDSC in patients [110].

1.5.7.3 Regulatory T cells

Regulatory T cells (Treg; CD4+ CD25+ FOXP3+) are a fraction of the T-cell population that suppress immune activation and thereby maintain immune system homeostasis and tolerance to self-antigens. Functional depletion of Treg cells induces autoimmunity, facilitate transplantation tolerance and also increases immunity to tumors. Treg cells consist of 1-10% of total

CD4+T cells in thymus, peripheral blood and lymphoid tissues and could conceivably recognize a wide spectrum of self and nonself antigens [111].

Treg development takes place directly in the thymus and leave thymus as mature with defined phenotype. This is unlike the development of other T-cell subsets, which are induced upon antigen exposure. They are dependent on IL-2 stimulation for their development, peripheral expansion and suppression function. Blocking the IL-2R on Treg cells leads to a loss of their regulatory activity, suggesting a possible role for IL-2 for suppressor function. IL-2 may be required in the production of Treg and alterations in this pathway may block Treg cell development. Functionally, IL-2 promotes proliferation and survival in T cells either by activating the signal transducer and activator of transcription 5 (STAT5), a transcription factor, or by up-regulation of anti-apoptotic molecules, Bcl-2 [112]. Treg express forkhead box P3 (FoxP3), a transcription factor that plays a central role in defining their function [113]. FoxP3 is essentially expressed in CD4+ CD25+ CD8- thymocytes but not in any other thymic cells. FoxP3 deficient mice suffer from autoimmune symptoms and die from inflammatory diseases In humans, Type I diabetes, thyroiditis and inflammatory bowel diseases is associated with mutations on the FoxP3 gene. Existence of other populations of T regulatory lymphocytes has been reported in recent publications.

The precise means by which Treg suppress effector T-cell-mediated immune responses have not been definitively characterized. Some studies suggest roles of cytokines in their regulation and others support the contribution of cell-to-cell contact with effector T cells on APC, where membrane bound TGFβ and cytotoxic T-lymphocyte protein (CTLA-4) plays an important role [114].

An increased fraction of Tregs has been reported to infiltrate the tumor contributing to the immunodeficient status associated with glioma [110]. A lack of immune rejection of neoplastic cells is believed to be maintained by

Treg in many malignancies including colorectal, esophageal, pancreatic, breast, lung, ovarian and brain tumors [115-118]. It is therefore very important to understand the biology and function of Treg for its potential therapeutic benefits.

In 2006, a study was reported from the Heimberger laboratory [119]. GB patient tumors were analyzed for glioma-infiltrating microglia/macrophage (GIM) and their effect on antitumor immune responses. The authors revealed that GIMs failed to induce T-cell proliferation. They demonstrated a corresponding lack of effector/activated T cells and there was a prominent population of Treg (CD4+CD25+FoxP3+) infiltrating the tumor.

1.6 Active Immunotherapy, an attractive approach against cancer. Dendritic cells- and peptide-based vaccination

1.6.1 Introduction

There are increasing numbers of reports demonstrating that systemic immunotherapy using dendritic cells (DC) is capable of inducing an antitumor response within the immunologically privileged brain, confirming that the CNS may not be an absolute barrier to DC-based immunotherapy [120-122]. Therefore, the possibility that the immune system can mediate interactions with lesions in brain tumors suggests excellent potential for investigating novel immunotherapies. For example, DC-based immunotherapy might be a new treatment modality for patients with brain tumors. To induce an antitumor immune response against glioma cells, antigen presenting cells (APCs) such as DC may be required to efficiently internalize, process and present glioma-specific antigens to the immune system [123].

1.6.2 DC in immunobiology

The immune system has evolved as a highly complex and adaptive mechanism to distinguish non-self and self. Foreign cells, including host cells bearing intracellular pathogens, are recognized and destroyed by cellular immune responses, which depend on the T-cell receptor for specific recognition of cell-surface antigens. T cells that directly kill foreign cells are commonly $CD8^+$, generally recognizing a cell-surface complex of major histocompatibility complex (MHC) Class I molecules and foreign peptides derived from intracellular proteins processed in the foreign cells through a cytoplasmic pathway.

DC are rare, hematopoietically derived leukocytes that form a cellular network involved in immune surveillance, antigen capture and antigen presentation [123]. DC can differentiate from both myeloid precursors and

peripheral blood monocytes, maturing and migrating via the afferent lymphatics to T-cell-rich areas of draining lymph nodes and the spleen where they develop antigen-presenting function. DC that have encountered antigens have been shown to undergo maturation that renders them capable of migrating to regional lymph nodes and activating antigen-specific T cells [124]. Activated DC express high levels of Class I and II MHC antigens, as well as adhesion molecules (CD11a and CD54) and accessory molecules (CD40, CD80 and CD86).

During the maturation process, they lose their ability to process and present soluble antigen and become extremely potent stimulators of T-lymphocytes [125]. It has now become clear that DC may play an important supportive role in promoting and maintaining antigen-specific T cells *in vivo*. Although DC play central roles in immunity, they also have a critical role in maintaining self-tolerance. DC are unique, highly potent APCs capable of sensitizing naive $CD4^+$/$CD8^+$ cells [126]. While CD8-T cells are critical effectors in antitumor immune responses, $CD4^+$ T cells also have an important role. Possible mechanisms by which $CD4^+$ T cells may promote antitumor cytotoxic T cells (CTL) development and function include production of cytokines such as interferon (IFN)-γ, modification of DC and maintenance of $CD8^+$T-cell numbers. Originating from $CD34^+$ bone marrow stem cells, human DC precursors are found in the bone marrow as well as peripheral blood and, in a more mature form, in lymphoid and non-lymphoid tissues. Currently, three different subtypes of DC have been defined: Langerhans cells, interstitial DC and plasmacytoid DC.

Candidate APCs in the CNS include parenchymal cells, such as astrocytes, oligodendrocytes and endothelium and nonparenchymal hematogenous-derived immune cells including microglia, perivascular macrophages, other macrophage populations and DC [127].

Macrophages in the leptomeninges and choroid plexus perform a variety of functions in common with other tissue macrophages, such as removing

tissue debris from the CSF in the subarachnoid space, which would otherwise cause obstruction to the CSF drainage pathways.

1.6.2 DC in tumor immunology

The ability of DC to initiate or regulate immunity has led to the study of their use as cellular vaccine adjuvants for the immunotherapy of cancer [Fig. 5]. With techniques available for the isolation and bulk propagation of DC in vitro, great efforts have been made to use DC in various immunization strategies. [128].

DC are efficient at recruiting, selecting and expanding naive T cells with antigen specificity within lymphoid organs. These T cells may be exported into tumor sites as immune effectors capable of directly killing targets and releasing several cytokines that facilitate additional immune responses. DC can sensitize both $CD4^+$ and $CD8^+$ T cells to specific antigens, while $CD4^+$ T cells are critical for priming CTL. Therefore, both $CD4^+$ and $CD8^+$ T cells are equally important in tumor immunology.

Many strategies for delivering antigens into DC have been established in murine models and are now undergoing evaluation in clinical trials. These include the use of synthetic peptides where the tumor antigen is known [129, 130], stripped peptides derived from Class I molecules from tumors [131], tumor RNA [132] and tumor lysates [133,120].

The advantages of vaccinating with total tumor-derived material, such as tumor cell lysates or tumor-derived mRNA, are that the identity of the tumor antigens need not be known and the use of multiple tumor antigens reduces the risk of developing antigen-negative escape mutants. Another promising and efficient approach is by using cells produced by the fusion of DC and tumor cells as a vaccine [134].

DC are equipped with a sophisticated molecular array of cell components representing the antigen-processing machinery (APM). The APM is

essential for the uptake and processing by DC of tumor-derived antigens, allowing tumor-derived epitopes to be cross-presented to T cells. The ability of DC to traffic and to localize in appropriate regions of lymphatic tissues is critical for the success of DC-based vaccines. Most clinical trials manipulate DC through ex vivo culture to assure accurate antigen delivery and DC activation. DC are typically administered intradermally, intravenously, or, in special circumstances, intraperitoneally.

Human tumors express a variety of protein antigens recognizable by the immune system and these antigens are potential targets for cancer vaccination therapy. Unfortunately, the tumor antigens are self-derived and are generally considered weak antigens. Selection of tumor antigen and appropriate loading of in vitro-generated DC with the antigen is an initial and crucial step in the development of an efficient DC-based cancer vaccine. DC-based therapy in patients with cancer has now progressed to Phase II trials. With the safety of DC transfers established, the challenge of the ongoing clinical studies will be to determine effective therapeutic doses and to obtain evidence for clinical efficacy for this form of immunotherapy.

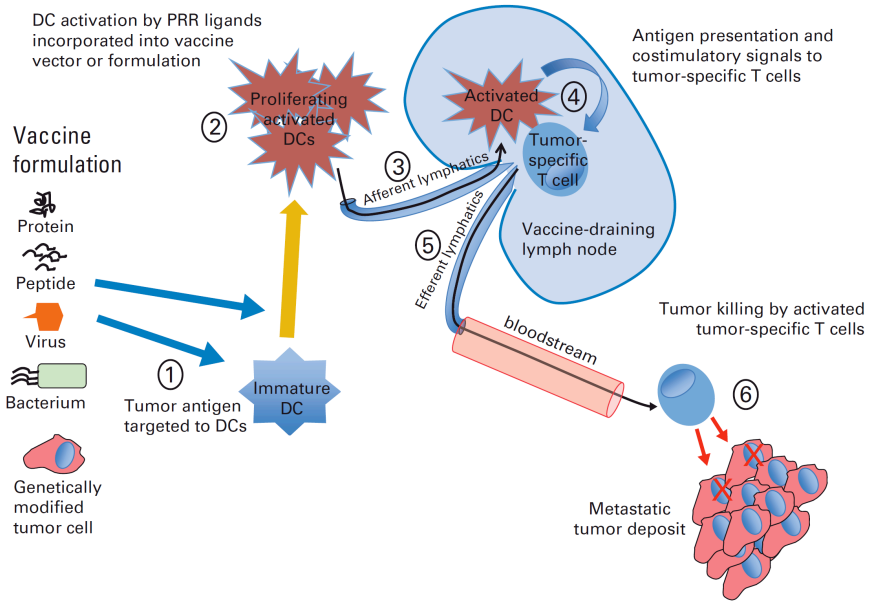

Fig. 5 Critical role of dendritic cells (DCs) in generating vaccine-induced antitumor immune responses. Cancer vaccines consist of many diverse formulations, in which antigen is in form of protein or peptide, recombinant virus or bacterium, or engineered tumor cell. Ultimately, antigen must be targeted to DCs (**1**). For cancer vaccine to be effective, DCs must be activated, either through incorporation of pattern-recognition receptor (PRR) agonist into vaccine or via activation properties of vector (ie, virus or bacterium). Ideal viral or bacterial vaccine vectors can infect DCs and, in so doing, activate them (**2**). Steps 1 and 2 can be accomplished ex vivo, as with DC vaccines (ie, sipuleucel-T). Activated DCs loaded with tumor antigen traffic to draining lymph node via afferent lymphatics (**3**). In lymph node, they present processed antigen to T cells along with costimulatory signals in form of cytokines and membrane ligands, thereby activating tumor-specific T cells (**4**) that are otherwise in tolerant state. Activated T cells leave draining lymph nodes via efferent lymphatics (**5**) and ultimately enter bloodstream via thoracic duct. They exit bloodstream in peripheral tissues, where they seek out and recognize tumor deposits expressing cognate tumor antigen and exert antitumor effects (**6**) (Topalin SL, 2011).

1.6.4 Clinical trials of DC-based vaccines

There are several reports concerning clinical trials of DC based vaccines for patients with glioma. The antigen sources in the trial varied from peptides eluted from autologous glioma cells, fusions of DC and tumor cells, to tumor lysates.

Yu et al [135] reported a Phase I clinical trial of DC pulsed with peptides eluted from autologous glioma cells. Two patients had recurrent anaplastic

astrocytoma (AA) and 7 glioblastoma (GB). Peptide-pulsed DC were injected intradermally in the deltoid region 3 times biweekly. DC vaccination elicited systemic cytotoxicity in seven patients and intratumoral cytotoxic and memory T-cell infiltration was detected in two patients. This Phase I study demonstrated the feasibility, safety and bioactivity of DC vaccines.

Liau et al [136] also reported a Phase I clinical trial of DC pulsed with peptides eluted from autologous glioma cells. Twelve GB patients were enrolled and three biweekly intradermal vaccinations were given. There were no serious adverse events observed and six patients developed systemic antitumor CTL responses. Unfortunately, the induction of systemic effector cells did not translate into objective clinical responses, particularly for patients with actively progressing tumors and/or those with tumors expressing high levels of TGFβ.

Yu et al [137] reported another Phase I trial of DC pulsed with tumor lysates of autologous glioma cells. Four patients had recurrent AA and 10 GB. Six of 10 patients demonstrated robust systemic cytotoxicity as demonstrated by IFNγ expression by peripheral blood mononuclear cells (PBMC) in response to tumor lysates after vaccination. The median survival for patients with recurrent GB in this study was 133 weeks.

Rutkowski et al [138] reported a Phase I trial of DC pulsed with tumor lysates of autologous glioma cells. One patient had recurrent AA and 11 GB. There were no serious adverse effects, with no clinical or radiological evidence of autoimmune reactions in any of the patients in these studies, with the exception of 1 patient who repetitively developed peritumoral edema. Two of the 6 patients with complete resection are still showing a continuous complete response (CR) after 3 years.

Liau et al [139] have reported a tumor lysate-pulsed DC vaccine in combination with the TLR-7 agonist, Imiquimod, following radio-chemotherapy for newly diagnosed GB. Thirteen patients received 3

immunizations at 2-week intervals, following completion of a 6-week course of radio-chemotherapy. Patients without tumor progression received booster vaccinations combined with topical administration of the Imiquimod. All immunizations were well tolerated, with only mild side effects. Increased levels of $CD8^+$ T cells reactive against tumor antigens were detected in 5 patients. The median progression free survival (PFS) is 18.1 months and median overall survival (OS) is 33.8 months. Newly diagnosed GB patients are currently enrolling for a multi-centre Phase II trial to test the efficacy of their autologous DC vaccine.

Kikuchi et al [140] reported on immunotherapy involving fusions of DC and glioma cells. Three patients had recurrent AA and 5 GB. Clinical results showed that there were no serious adverse effects and 2 partial responses (PRs).

Kikuchi et al [141] also reported another immunotherapy with fusions of DC and glioma cells combined with recombinant IL-12. Nine patients had recurrent AA and 6 GB. Clinical results showed that there were no serious adverse effects, 4 PRs and 1 minor response (MR) in patients with AA.

Yamanaka et al [142, 143, 143a] reported on therapy using DC pulsed by tumor lysates. Twenty-four patients with recurrent malignant glioma (6 Grade III and 18 Grade IV patients) were evaluated in a Phase I/II clinical study. DC were injected intradermally, or both intratumorally and intradermally every 3 weeks. The protocols were well tolerated with only local redness and swelling at the injection site in several cases, while clinical responses showed 1 patient with PR and 3 MRs. Increased ELISPOT and DTH responses after vaccination provided good laboratory markers to predict the

clinical outcome of patients receiving DC vaccination. The OS of patients with GB was 480 days, which was significantly better than that in the control group.

Wheeler et al [144] reported a Phase II trial of DC pulsed with tumor lysates

of autologous glioma cells. Thirty-four GB patients were enrolled. Seventeen patients exhibited a positive vaccine response quantified by IFNγ responsiveness after three vaccinations. Vaccine responders exhibited significantly longer post treatment times to tumor progression (TTP) and survival (TTS) relative to non responders. Vaccine-induced responses elicited therapeutic benefits primarily by sensitizing tumors to chemotherapy.

Vleeschouwer et al [145] reported a Phase II trial of DC pulsed with tumor lysates of autologous glioma cells. Fifty-six patients with relapsed GB were treated with at least three vaccinations. The median PFS and OS were 3 and 9.6 months, respectively, with a 2-year OS of 14.8%. Vaccine-related edema in one patient with gross residual disease before vaccination was the only serious adverse event.

In a recent work of my group, Pellegatta et al [146] reported the possible benefits of an immunotherapeutic strategy based on mature dendritic cells (DC) loaded with autologous tumor-cell lysates in 15 patients affected by recurrent GB. The median progression-free survival (PFS) of this patient cohort was 4.4 mo, and the median overall survival (OS) was 8.0 mo. Patients with small tumors at the time of the first vaccination (< 20 cm3; n = 8) had significantly longer PFS and OS than the other patients (6.0 vs. 3.0 mo, p = 0.01; and 16.5 vs. 7.0 mo, p = 0.003, respectively). CD8+ T cells, CD56+ natural killer (NK) cells and other immune parameters, such as the levels of transforming growth factor β, vascular endothelial growth factor, interleukin-12 and IFNγ, were measured in the peripheral blood and serum of patients before and after immunization, which enabled us to obtain a vaccination/baseline ratio (V/B ratio). An increased V/B ratio for NK cells, but not CD8+ T cells, was significantly associated with prolonged PFS and OS. Patients exhibiting NK-cell responses were characterized by high levels of circulating IFNγ and E4BP4, an NK-cell transcription factor.

Furthermore, the NK cell V/B ratio was inversely correlated with the TGFβ2 and VEGF V/B ratios. These results suggest that tumor-loaded DC may increase the survival rate of patients with recurrent GB after effective tumor debulking, and emphasize the role of the NK-cell response in this therapeutic setting.

DC based strategies appear promising as an approach to successfully induce antitumor immune responses and prolong survival in patients with glioma. DC therapy of glioma seems to be safe and without major side effects, thus its efficacy should be determined in randomized and controlled clinical trials. Every patient with glioma will be evaluated for the molecular genetic abnormalities in their individual tumors and novel immunotherapeutic strategies based on pharmacogenomics will be offered according to the genetic findings.

1.6.5 Peptide-based immunotherapy in glioma

Recent advances in molecular biology and tumor immunology have resulted in the identification of a large number of tumor-associated antigens (TAA) that could be used for cancer vaccination, since their epitopes associated with HLA class I molecules were recognized by cytotoxic T-lymphocytes (CTL).

Peptide-based vaccines represent a major focus of cancer vaccine research that offers exciting clinical possibilities. Peptides used as cancer vaccines usually consist of introducing small peptides (typically 7-14 amino acids in length) that are immunologic and expressed by targeted cells [147, 148]. Peptides are capable of binding to a particular HLA class I antigen. Such peptides are processed by host antigen presenting cells (APC), which travel to the lymph nodes and sensitize circulating T-lymphocytes, known as cytotoxic T cells (CTL) [Fig. 6].

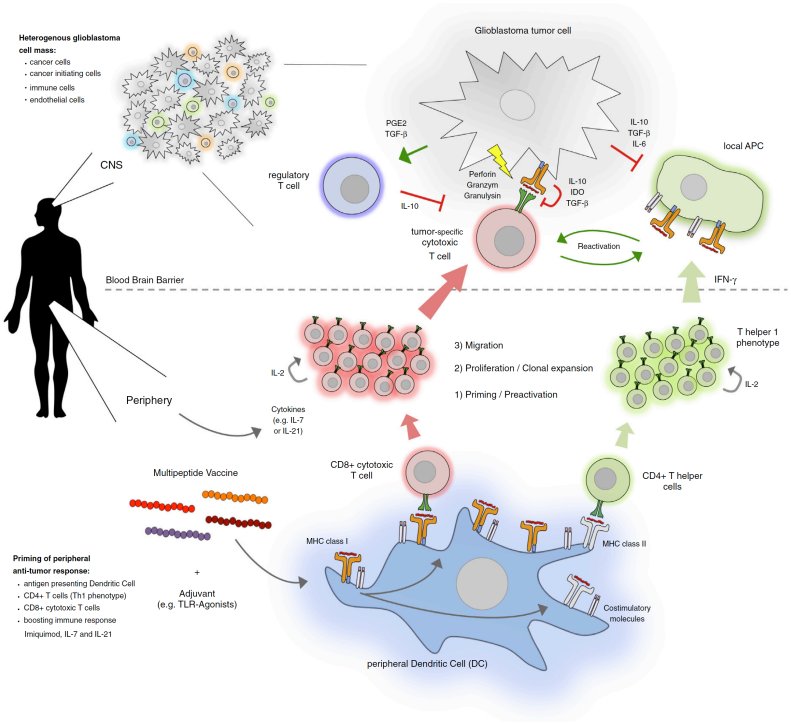

Fig. 6 Peptide-based immunotherapy. *The schematic overview depicts the idealized vaccination-induced peripheral priming of a tumor-specific immune response. After peptide uptake and processing peripheral antigen presenting cells, especially dendritic cells, present the antigen via HLA-class I and -II molecules to tumor-specific T cells. After TCR-peptide: HLA and costimulatory molecule-induced activation, T cells proliferate and execute their effector functions. CD4 + T helper cells promote the perpetuation of the mounted immune response by activating local antigen presenting cells (APC), while CD8 + cytotoxic T cells directly lyse tumor cells through induction of apoptosis by, i.e. perforin, granzyme and granulysin. Glioblastoma cells try to evade immune-mediated lysis by attracting regulatory T cells, thereby promoting an anti-inflammatory milieu.*

A peptide suitable for the individual patient is generally mixed with an adjuvant followed by subcutaneous administration every 7-14 days to form a vaccine. CTL recognizing a peptide on APC become activated in association with clonal expansion in the nodes. These activated CTL come out through lymph nodes or blood circulation, migrate and infiltrate into the brain, recognize the corresponding peptide-HLA complex on glioma cells and eliminate glioma cells, which in turn results in tumor regression.

Several clinical trials of peptide-based immunotherapy for cancer have been conducted in the past decade, but major clinical responses were rarely obtained [149]. Recently, objective responses for peptide vaccinations including WT1 peptide [150, 151], personalized peptide and EGFRvIII peptide [64] are obtained and reported. These clinical trials for malignant gliomas were reviewed in this chapter.

1.6.5.1 EGFRvIII-peptide vaccination

The majority of glioblastoma cases harbor mutations in the Epidermal Growth Factor Receptor (EGFR). In particular, the epidermal growth factor receptor variant III (EGFRvIII) is a consistent tumor-specific mutation that is widely expressed in malignant gliomas and other neoplasms. This mutation encodes a constitutively active tyrosine kinase that enhances tumorgenicity and migration and confers radiation and chemotherapeutic resistance. The EGFRvIII mutation arises from an in-frame deletion/ fusion that results in constitutively active EGF signaling. This in-frame deletion mutation splits a codon resulting in the creation of a novel glycine at the fusion junction between normally distant parts of the molecule and producing a sequence re-arrangement which creates a tumor-specific epitope for cellular or humoral immunotherapy in patients with malignant gliomas.

An EGFRvIII-specific peptide that spans this deletion/fusion has shown to be an immunogenic antigen [64] and was found to be an attractive target antigen for immunotherapy against malignant gliomas. In a Phase I clinical trial conducted at Duke University, 20 patients of malignant glioma were enrolled and vaccinated with the EGFRvIII peptide. Following surgical resection, each patient underwent leukapheresis to obtain peripheral blood mononuclear cells (PBMC) for autologous mature DC generation and baseline immunologic monitoring. Three patients with anaplastic astrocytomas and 13 patients with glioblastomas were vaccinated.

DC were pulsed for 2 h, with 500 μg of EGFRvIII-specific peptide, PEPvIII (LEEKKGNYVVTDHC) (Multiple Peptide Systems, San Diego CA) which was conjugated 1:1 with keyhole limpet hemocyanin (KLH) (Biosyn, Carlsbad, CA). The first 3 patients received 3 x 10^7 mature DC per vaccination intradermally beginning 2 weeks following completion of radiation therapy, the remaining patients received one-third of their total generated DC per injection (up to 1.1 x 10^8 cells).

The unique EGFRvIII junctional peptide has been utilized as a peptide vaccine to promote anti-tumor host immune responses against EGFRvIII-positive tumors [152]. Rodents challenged with EGFRvIII-positive cells subcutaneously or intracerebrally displayed significant tumor reduction and increased survival rates following administration of DC loaded with an EGFRvIII synthetic peptide conjugated to keyhole limpet hemocyanin (KLH) [153] or with EGFRvIII–KLH only [154].

Only a few trials have reported on EGFRvIII-specific therapy in glioma patients. Two clinical trials using vaccines against EGFRvIII have enrolled newly diagnosed malignant glioma and GB patients, and encouraging results have been reported [65, 155].

The first study evaluated the safety and immunogenicity of a DC-based vaccine targeting the EGFRvIII antigen. Patients with newly diagnosed GB after surgery and radiotherapy received intradermal vaccinations with autologous mature DC pulsed with an EGFRvIII-specific peptide conjugated to KLH. No allergic reactions or serious adverse events were encountered. EGFRvIII-specific immune responses were evident in most patients. Median time to progression from vaccination was 6.8 months (95% CI: 2.5-8.8), and median survival time was 18.7 months from vaccination and 22.8 months from time of histological diagnosis.

The second study was a Phase II trial that investigated the immunogenicity of an EGFRvIII peptide vaccine and the survival in patients with newly diagnosed, EGFRvIII-expressing GB and minimal tumor burden.

Intradermal vaccinations were administered until tumor progression. The 6-month progression-free survival (PFS-6) rate after vaccination was 67 and 94% after diagnosis. The median OS was 26.0 months greater than that observed in a control group matched for eligibility criteria, prognostic factors, and temozolomide treatment (p= 0.0013). The development of specific antibody (p= 0.025) or delayed-type hypersensitivity (p= 0.03) responses to EGFRvIII had a significant effect on OS.

Strikingly, at recurrence, 82% of patients had lost EGFRvIII expression (p= 0.001). Thus, the results of this trial provide the first evidence of the relevance of tumor immunoediting in shaping immune responses to glioma [156]. The disappearance of EGFRvIII-positive cells after immunotherapy indicates that the immune system primed against cells expressing the EGFRvIII peptide may kill them effectively. In spite of this the tumor may survive: this may be due to continued EGR signaling due to EGFR amplification in the absence of EGFRvIII. Data on maintained amplification of EGFR were not available in the manuscript by Sampson et al. Thus, investigation on the EGFR pathway of relapsing tumors after EGFRvIII peptide vaccination will provide important hints on the relevance, redundancy and flexibility of this critical pathway of tumorigenesis. Recent data have shown a deletion of the NFKBIA gene in approximately 22% of GB [157]. This deletion is usually absent in GB with EGFR amplification, suggesting that NF-kB deletion can substitute for EGFR amplification in the pathogenesis of GB. The observation prompts the study of the NF-kB status in GB before and EGFRvIII peptide immunotherapy treatment and consequent immunoediting.

1.7 Stem cells, radial glial cells, and a unified origin of brain tumors

1.7.1 Neural stem cells and the origin of brain tumors

As described in chapter 1.3.2, neural stem cells (NSC) have been implicated not only in brain development and neurogenesis but also in tumourigenesis; GB can be initiated from alterated neural stem-like cells.

The origin of NSC is still unclear, however a recent hypothesis suggests that they might originate from radial glial cells, primary embryonic stem cells derived from neuroepithelial cells [158]. Adult stem cells are defined as cells giving rise to daughter cells with equal developmental potential (self-renewal) and capable to generate all differentiated cell types in a given tissue (multipotency in most cases; unipotency e.g. in a spermatogenic stem cell). Cells with stem cell properties have been identified in four distinct anatomical areas of the postnatal mammalian brain: (i) the subventricular zone (SVZ) beneath the lateral wall of the lateral ventricles, (ii) the subgranular zone (SGZ) in the dentate gyrus of the hippocampus, and more recently also (iii) in the subcallosal zone (SCZ) between the hippocampus and the corpus callosum [159] and (iv) in the cerebellum at the boundary between internal granular layer (IGL) and white matter.

There is now evidence that all brain tumors originate from a special type of stem cell called the “radial glial cell” (RGC). Currently, the consensus of opinion is that most brain tumors also originate from the so-called “brain tumor stem cells” (BTSC) (of which RGCs are a subtype) [160]. When brain tumor stem cells mutate (4–7 mutations are needed before degeneration occurs), they can generate phenotypically diverse tumors. Within a specific tumor, brain tumor stem cells constitute only a small fraction of the total malignant cells [161].

It is interesting to note that in the human brain, most stem cells are located in the SVZ. Both supra- and infratentorially and when stimulated with carcinogens, cells in the SVZ become tumorigenic faster than those located

elsewhere. In the SVZ, stem cells exist in the form of RGC. RGC remain quiescent until they receive transformational signals. It is not clear if RGC, after receiving transformation signals, return to their initial stem cell configuration and then become tumorigenic or if they transform to tumor progenitor cells directly [162].

1.7.2 Inside the radial glia

Radial glial cells are a widespread cell type throughout the developing CNS of vertebrates [Fig. 7]. They are best known for their role in guiding migrating neurons [163] and a precursors during neurogenesis [164]. They arise early in development from neuroepithelial cells. Radial phenotype is typically transient, but some cells, such as Bergmann glia in the cerebellum an Müller glia in the retina, retain radial glia-like morphology postnatally. According to recent research, during the late stages of cortical development, radial glial cells divide asymmetrically in the ventricular to generate radial glial cells, postmitotic neurons and intermediate progenitor cells. Intermediate progenitor cells then divide symmetrically in the subventricular zone to generate neurons. During gliogenesis, radial glial cells differentiate into astrocytes [165].

The term radial glial cell refers to their two major characteristics, their long radial processes extending from the ventricular zone (VZ) to the pial surface and their glial properties, such as the content of glycogen granules or the expression of the astrocyte-specific glutamate transporter (GLAST) or the glial fibrillary acidic protein (GFAP) [163, 166]. Another radial glia-specific protein is the brain lipid binding protein (FABP7), expression of which could be induced by Notch-1 activation, in particular, when acted upon by reelin. Interestingly, Notch-1, then activated before birth, induces radial glia differentiation, but postnatally induces the differentiation into astrocytes [167]. Very interesting Pellegatta and colleagues performing

gene expression profile analysis on CSC, identified several up-regulated genes related to the Radial Glia. In particular GLAST and FABP7 are highly expressed in murine (GL261) and human glioma stem-like cells (GSC) [168]. In another study of De Rosa et al [169], FABP7 was identified as one of the most highly expressed genes in neurospheres cells from GB compared to their adherent counterpart. The authors found that down-regulation of FABP7 expression in NS by small interfering RNAs significantly reduced cell proliferation and migration. *In vivo* reduced tumorigenicity of GB cells with down-regulated expression of FABP7 was associated to decreased expression of the migration marker doublecortin. These data emphasize the role of FABP7 expression in GB migration.

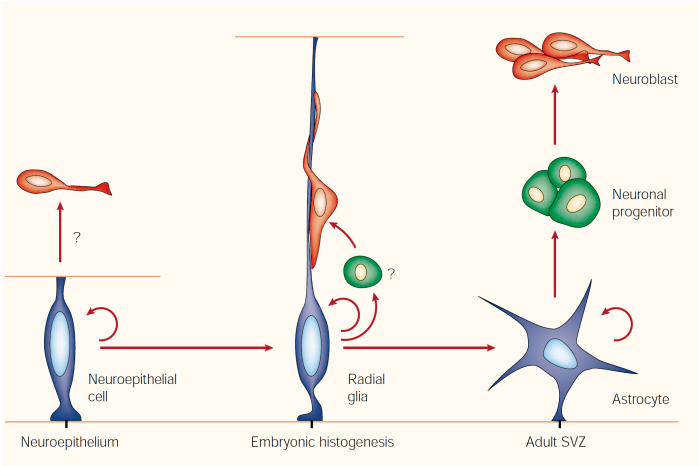

Fig. 7 Unified hypothesis for neural stem-cell development. **Left panel**: Neural stem cells (purple) in the early neuroepithelium extend from the ventricle to the pia. **Middle panel**: Like neuroepithelial stem cells, many radial glia (purple) also contact the ventricular and pial surfaces. Radial glia might be neural stem cells, perhaps an elongated form of the neuroepithelial cell. Radial glia are known to divide symmetrically (not shown) or asymmetrically (arrows) to produce neurons (red) that migrate into the cortex along the fibre of their progenitor. Radial glia might produce neurons directly or indirectly through transit amplifying cell types (green). **Right panel**: Radial glia transform into cortical astrocytes later in development. Cells derived from radial glia might come to reside in the adult subventricular zone (SVZ) (blue). Like radial glia and neuroepithelial cells, some SVZ astrocytes contact the ventricle and extend a short 9+0 cilium into the cerebrospinal fluid. These astrocyte-like cells behave as stem cells in that they self-renew and produce neurons (arrows), possibly through intermediate cell types (green) (Alvarez-Buylla A et al, 2001).

1.8 Glutamate signalling in physiological and pathological brain

1.8.1 Introduction

Glutamate, a nonessential amino acid, is the major excitatory neurotransmitter in the central nervous system. As such, glutamate has been shown to play a role in not only neural processes, such as learning and memory, but also in bioenergetics, biosynthetic and metabolic oncogenic pathways.

Glutamate has been the target of intense investigation for its involvement not only in the pathogenesis of benign neurodegenerative diseases (NDD) such as Parkinson's disease, Alzheimer's disease, schizophrenia, multiple sclerosis, and amyotropic lateral sclerosis (ALS), but also in carcinogenesis and progression of malignant diseases. In addition to its intracellular activities, glutamate in secreted form is a phylogenetically conserved cell signaling molecule. Glutamate binding activates multiple major receptor families including the metabotropic glutamate receptors (mGluRs) and ionotropic glutamate receptors (iGluRs), both of which have been implicated in various signaling pathways in cancer. Inhibition of extracellular glutamate release or glutamate receptor activation via competitive or non-competitive antagonists decreases growth, migration and invasion and induces apoptosis in breast cancer, melanoma, glioma and prostate cancer cells. In this chapter, I discuss the current state of glutamate signaling in brain and notions regarding glutamate involvement in brain cancer. These findings provide the notions that glutamate and/or glutamate transporter would be a interesting target of anti cancer therapies especially in gliomas.

1.8.2 Overview on glutamatergic neurotransmission

The amino acid glutamate is recognized as the primary excitatory neurotransmitter in the mammalian central nervous system (CNS). However, recent developments have shown that glutamate receptors are

broadly expressed in tissues other than the brain by cells that have not traditionally been associated with glutamate-mediated signalling. For example, the proliferation of several tumour cells is highly sensitive to glutamate receptor antagonists [169].

Glutamate was originally speculated to serve a metabolic function in the CNS [170], as it was found within numerous intracellular compartments including the cytosol and mitochondria of all CNS cell types. However, it is now known that despite its ubiquitous nature, levels of extracellular glutamate are indeed tightly regulated, thus allowing glutamate to function as the major excitatory neurotransmitter in the mammalian CNS. The tight control of glutamatergic neurotransmission is an energy-costly process, requiring multiple regulatory processes and high levels of glucose and oxygen consumption.

It is important to note that in addition to its role as a neurotransmitter, glutamate also serves as a metabolic precursor to GABA and as a component of various amino acid-based derivatives, e.g. the antioxidant glutathione. Consistent with glutamate's key role in multiple aspects of brain physiology, metabolic studies have determined that virtually all of the glucose that enters the CNS is eventually converted to glutamate

Cytosolic glutamate crosses the vesicular membrane via the activity of vesicular glutamate transporters (VGLUTs) [171]. VGLUTs are multimeric proton/glutamate antiporters. The loss of VGLUT expression via targeted knockout strategies results in the loss of glutamate packaging into synaptic vesicles and deleterious neuropsychiatric sequelae [172]. In a Ca^{2+} and soluble N-ethylmaleimide sensitive factor attachment protein receptor (SNARE)-dependent manner [173, 174], glutamate is released into the synaptic cleft to bind to and elicit its effects on postsynaptic receptors. Dysregulated excitatory neurotransmission, resulting in high concentrations of extracellular glutamate, and especially increased levels of extrasynaptic glutamate, leads to cellular damage (hence, the term “excitotoxicity”) [175,

176]. Fast synaptic transmission is mediated by a-amino-3-hydroxy-5-methyl-4-isoaxazolepropionate acid receptor (AMPA-R), whereas N-methyl-D-aspartate receptor (NMDA-R) activation is typically implicated in coincidence detection and associative learning. In addition, both neurons and glial cells express metabotropic glutamate receptors that couple to various second messenger pathways.

Following binding to synaptic glutamate receptors (Glu-R), Glu is either transported back into presynaptic neuronal terminals or taken up into nearby glial cells. Glial Glu uptake appears to be the predominant route for the degradation of Glu from the synaptic space making it among the most recognized functions of non-malignant astrocytes. Glu clearance occurs primarily via one of two Na^+-dependent transporters [Fig. 8]. They are localized in close proximity of glutamatergic synapses. In fact, throughout the CNS, most glutamatergic synapses are tightly ensheeted by astrocytic processes placing their Glu transporters near synaptic release sites. It is generally believed that this arrangement ensures the rapid and effective containment of Glu spillage out of the synaptic cleft.

Five such high-affinity transporters have been identified to date [177]. EAAT1 (GLAST) is abundantly detected in the neocortex and cerebellum but appears to be restricted to astrocytes. EAAT2 (GLT-1), the chief glutamate transporter in the forebrain, is expressed mostly in astrocytes but also, to a limited extent, in neurons. EAAT3 (EAAC1) is neuron-specific and enriched in GABAergic presynaptic nerve endings. EAAT4 has only been detected in the dendrites of cerebellar Purkinje neurons. Finally, EAAT5 is retina-specific. The location of the EAATs relative to the geometry of synapse places them in a critical position to prevent glutamate spillover and activation of extrasynaptic glutamate transporters [178].

Considering individual astrocytes serve large numbers of synapses with minimal overlap in the synapses served by neighboring astrocytes, the failure of a single astrocyte could impair glutamate removal at thousands of

synapses in some brain regions. Interestingly, dysfunction of EAATs has specifically been implicated in the pathology of several neurodegenerative disorders [179].

Once in the cytosol, glutamine synthetase, an astrocyte and oligodendrocyte-specific enzyme, converts glutamate into glutamine in an ATP-requiring reaction with ammonia. Both astrocytes and neurons contain glutamine transporters that, under appropriate electrophysiological conditions, lead to the net exchange of glutamine from astrocytes-to neurons. In neurons, the mitochondrial phosphate-specific enzyme, glutaminase, reconverts inert glutamine-to-glutamate for subsequent repackaging into synaptic vesicles. The cycling of glutamate/glutamine in astrocytes and neurons has been termed “the glutamine cycle”

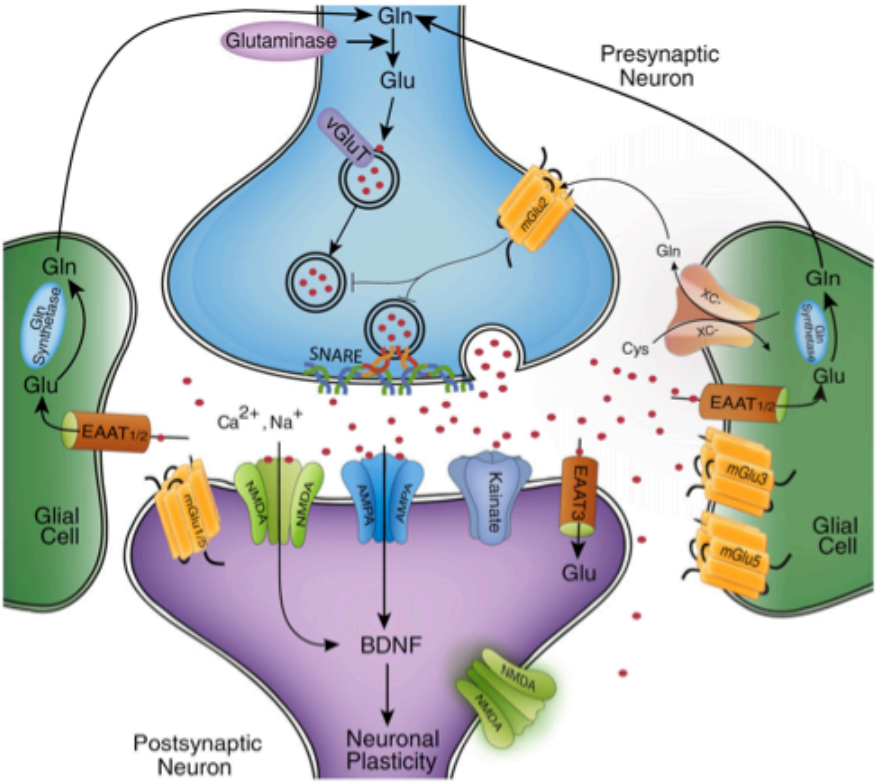

Fig. 8 Glutamatergic neurotransmission. Due to the risk of excitotoxic damage in the wake of excessive glutamatergic stimulation, precise physiological control of glutamate must be maintained in the mammalian CNS. Glutamine (Gln) is converted to glutamate (Glu) by glutaminase [though glutamate may also be derived from the TCA cycle (not shown)]. Glu is packaged into presynaptic vesicles by vesicular Glu transporter (VGLUT) proteins and synaptically released in a voltage-dependent manner through vesicular interactions with SNARE proteins. Synaptically-released Glu is recycled from the extracellular space by excitatory amino acid transporters (EAATs) expressed predominantly on astroglia. In astrocytes, Glu is converted to Gln by Gln synthetase and exported extracellularly to be taken up again by neurons. Additionally, system x-C is a cystine/glutamate antiporter expressed on glia that also contributes to Glu recycling. Glu receptors are present on presynaptic and postsynaptic neurons as well as on glial cells. These include both ionotropic receptors (NMDA, AMPA/KA) and metabotropic receptors (mGluRs). The effect of Glu is determined by the receptor subtype, localization (synaptic, perisynaptic and extrasynaptic), and interactions with various scaffolding and signaling proteins (not shown) in the postynaptic density. Glu receptor stimulation results not only in rapid ionotropic effects but also synaptic plasticity, e.g. long-term potentiation (LTP) and long-term depression (LTD), via cognate signal transduction cascades (Mark J. Niciu et al, 2012).

1.8.2.1 GLAST

The glutamate transporters EAAT1 know also as GLAST, belong to the SLC1 family. Sodium-dependent glutamate transport is an electrogenic process [180], consisting of two distinct half-cycles: first, glutamate is co-transported with three sodium ions and a proton and subsequently the binding sites reorient upon counter transport of potassium. Under physiological conditions, the inwardly directed sodium gradient and outwardly directed potassium gradient as well as the interior negative membrane potential promote the accumulation of the transmitter into the cell against its concentration gradient [181, 182], but at elevated external potassium concentrations the transporters mediate its efflux out of the cells. According to the transport cycle, elevated external potassium concentrations will render an increased proportion of transporters in the in ward facing conformation. In addition to the ion-coupled glutamate translocation, glutamate transporters mediate a thermodynamically uncoupled chloride flux activated by two of the molecules they transport, sodium and glutamate. Glutamate transporters have a non-conventional topology containing two re-entrant loops and two transmembrane domains (7 and 8) in their carboxyl terminal half [183]. Moreover, the two re-entrant loops are in close proximity.

1.8.3 Glutamate signaling in glioma

Mounting evidence suggesting that the glutamatergic system contributes to the pathophysiology of brain tumors is opening opportunities for the development of new models of pathogenesis, improved diagnostic tools and novel treatment strategies. A more complete understanding of glutamates' roles in the pathogenesis and pathophysiology of brain tumors may allow for an increasingly rational approach to drug development for these common, disabling illnesses.

Recent studies have implicated glutamate (Glu) signaling in the development and progression of malignant diseases. Glu, a non-essential amino acid, is the major excitatory neurotransmitter in the central and peripheral nervous systems. In addition, it functions as a source of energy for rapidly dividing cells such as lymphocytes and cancer cells [184].

Evidence is emerging for a role of Glu and its receptors in regulation of tumor growth. Several groups reported that glutamate receptor antagonists limit tumor growth Recent data show that blocking expression of selected GluR subunits inhibits proliferation of cancer cells in vitro [185, 186].

Glutamate neurotoxicity has been implicated in stroke, head trauma, multiple sclerosis, schizophrenia, ischemia and neurodegenerative diseases [187-190]. Three mechanisms seem to be responsible for Glu altered homeostasis control: a reduced reuptake of the neurotransmitter from the synaptic space, an inverse activity of Glu receptor and a down-regulation of these transporters expression.

A dysfunction in Glu transport has also been reported in malignant gliomas [191]. The comparison of Glu transport into astrocytes versus their malignant counterparts showed an almost complete absence of Na^+-dependent Glu uptake into gliomas.

The authors investigated glutamate uptake in glioma cells surgically isolated from glioma patients (GB) and in seven established human glioma cell lines, including STTG-1, D-54 MG, D-65 MG, U-373 MG, U-138 MG, U-251 MG, and CH-235 MG. All glioma cells studied showed impaired glutamate uptake that of normal astrocytes. Furthermore, Western blots showed a loss of protein expression for EAAT2 and a mislocalization of EAAT1 to the nuclear Membrane [192]. Within 12 h, a monolayer of glioma cells maintained in a culture flask achieved concentrations exceeding 500 μM [191]. This suggests that gliomas behaved exactly opposite to astrocytes, releasing Glu rather than sequestering it. Exposure of cultured hippocampal neurons to glioma-conditioned medium elicited sustained

[Ca^{2+}] elevations that were followed by widespread neuronal death. Similarly, coculturing of hippocampal neurons and glioma cells, either with or without direct contact, resulted in neuronal death. These findings suggest that a deliberate release of Glu may promote tumor expansion through the release of excitotoxic concentrations of Glu [193] from the tumor as discussed further below. The initial studies that described the release of Glu and a loss of Glu homeostasis showed the assiduous release of glutamate from cultured human glioma cells but also demonstrated a complete loss of the Glt-1 and GLAST glutamate transporters in patient biopsy tissues. Follow-up studies demonstrate Glu release in tumor-bearing animals and glioma patients. To accomplish the former, Behrens et al. [194] implanted RG2 glioma cells into the cortex of Fisher rats and used microdialysis probes to sample and measure peritumoral Glu. They found that the peritumoral region showed fourfold elevated Glu compared with either the tumor or the remaining brain. A similar conclusion was drawn by Nedergaard and coworkers [195] who used a bioluminescence detection method to visualize Glu in living brain slices acutely isolated from tumor-bearing animals. This study went further and demonstrated that C6 glioma cells that lacked the ability to release Glu failed to grow solid tumors when implanted into rats. Together, these studies provided compelling evidence that gliomas release Glu *in vitro*, *in situ*, and *in vivo*.

In neurons, the release of Glu occurs exclusively through synaptic vesicles and requires a Ca^{2+}-dependent fusion event. Biophysical and pharmacological evidence suggests that Glu release from gliomas does not use these mechanisms but instead occurs predominantly via the system x_c^2 cystine Glu exchanger [191]. This abundant amino acid carrier is Na^+ independent and transports cystine into the cell in exchange for Glu being released. It requires both extracellular cystine and intracellular Glu to function. Elimination of Glu via multiple pathways is important for cell

survival, as discussed below, and can serve to provide alternate sources of energy for glycolysis-impaired cells and protection from free radicals.

Elimination of Glu via multiple pathways is important for cell survival and can serve to provide alternate sources of energy for glycolysis-impaired cells and protection from free radicals. Another class of transporters, the excitatory amino acid transporters EAAT1-5 has been implicated in Glu release from neurons and glial cells at least under pathophysiological condition. Under conditions of energy failure, such as stroke, EAAT transporters can run backward exporting rather than importing Glu [196, 197]. The released Glu has a multitude of targets all of which benefit the tumor yet compromise the patient [198]; malignancy degree of glioma appears to be related with the quantity of glutamate released and present in the tumoral microenvironment. An high and prolonged exposure at Glu leads to activation on numerous pathways which have an effect in enhancing tumor growth. A greater concentration of Glu at synaptic level increases the activation of NMDA and AMPA receptors on the neural membrane causing hyperexcitability which explains the frequent seizures, noticed in the 80% of patients with low-grade gliomas [199]. Both NMDA and AMPA receptor signaling are thought to be critical for activating a series of complex pathways responsible for maintaining synaptic strength and the encoding and ‘‘survival’’ of long-term memories. Conceptually, it is perhaps not surprising that tumors of glial origin manipulate Glu receptors to promote limitless proliferation and survival via the activation jof Glu receptors.

AMPA receptors are highly expressed in glioblastoma and perform critical functions in glioma biology and enhance its malignant phenotype. They are heteromultimers composed of the GluR1–GluR4 subunits. glioblastoma appear to express low levels of the GluR2 subunit, which contributes to AMPA-R calcium permeability. Rzeski et al. demonstrated that antagonism of AMPA Glu receptors or the removal of calcium from the culture medium

in different tumor types including glioma decreased cell proliferation via both decreased cell division and increased cell death, suggesting that a common tumor proliferation mechanism involves Glu and Glu receptors [200]. Similarly, overexpression of AMPA-R promoted glioma proliferation in vitro, and reintroduction of GluR2 via adenoviral vector blocked this proliferation and induced apoptosis. Thus, glioma regulation of AMPA receptor subunit expression and calcium permeability may represent a novel mechanism for signal transduction pathway activation. Several mechanisms by which AMAP-Rs are able to modulate cell proliferation and survival have recently been described as AKT activation [201]. Additionally, it was recently demonstrated that AMPA-R stimulation promotes the highly oncogenic epidermal growth factor receptor EGFR or platelet-derived growth factor (PDGFR) expression [202].

Elevate Glu concentration may be also responsible to glioma invasion capability. Interestingly, in gradient chambers, glioma cells migrated toward elevated Glu concentrations, suggesting that Glu could be considered a chemoattractant. [203]. Because glioma cells frequently show chain migration, particularly along blood vessels, the leading cell, through release of Glu, may stimulate trailing cells to follow. Recent studies have elucidated several mechanistic ideas as to how AMPA-R on glioma cells may regulate cell motility and tumor invasiveness. Overexpression of GluR1, the most abundant subunit of the AMPA-R in glioma cells, results in an increase in glioma adhesion to extracellular matrix (ECM) components such as collagen. AMPA-Rs appear to accumulate at focal adhesion sites where they may indirectly mediate interactions between the ECM and integrins. The enhanced adhesion to the ECM is mediated via increased surface expression of b1-integrin and subsequent activation of focal adhesion kinase (FAK). Overexpression of the GluR1 subunit of the AMPA-R in glioma cells activates FAK independent of AMPA-R activation. Although the mechanism through which GluR1 expression can promote FAK activation is

unknown, it may be related to FERM protein or integrin engagement. Stimulation of the AMPA-R with Glu or AMPA promotes cellular detachment mediated through Rac1 and causes an increase in transwell migration in vitro and an increase in tumor invasion in vivo [204].

2.Aim of the study

Cancer immunotherapy strategies, aimed to re-educate the immune system to eradicate the tumor, met some recent success in prostate carcinoma and melanoma. However, the capacity of escaping the immune response, now considered one hallmark of cancer, limits the efficacy of immunotherapy.

A number of studies have shown that in glioblastoma (GB) and other cancers a sub-population of cells, defined as cancer stem-like cells, express stem-programs and is responsible for tumor perpetuation. The reacquisition of stem cell features may be critical for tumor survival under environmental challenges. Thus, targeting GB stem-like cells (GSC) is one strategy to increase the potential efficacy of GB immunotherapy.

The first aim of this study was to understand whether GLAST could be defined as a marker of GSC by investigating its involvement in tumorigenesis. GLAST positive cells were first isolated from murine GSC using an immunomagnetic sorting and compared with GLAST negative or unsorted GSC. *In vivo* experiments showed that GLAST-positive cells are more aggressive and lethal compared to GLAST-negative or unsorted cells. The observations obtained have allowed us to define GLAST as a potential marker of a more aggressive sub-population of GSC and moreover a specific therapeutic target.

Accordingly with our preliminary observations, the second aim was to develop a strategy of immunotherapy based on the use of GLAST-derived peptides in a murine model of malignant glioma. In order to predict their immunogenicity, we identified four high scoring peptides using different computational algorithms. Synthesized peptides were emulsified in an adjuvant, Montanide™ ISA 51 VG, and administered subcutaneously in GL261 glioma-bearing mice. The effectiveness of peptides was evaluated in combination with granulocyte-macrophage colony-stimulating factor (GM-CSF) used as an adjuvant. Activation of a specific anti-tumor immune response was investigated at different time points. Very important findings confirmed that immune activation, as a result of our vaccination protocol, is

related to a decrease of tumor invasiveness and lethality, with consequent significant increase of survival in treated mice.

The last aim was to emphasize the translational relevance of GLAST as marker and glioma-associated antigen in human GB. By immunohistochemistry 32 GB specimens were analysed for GLAST expression. An high reactivity is significantly related to a decreased overall survival. In light of this observation and in terms of clinical research, GLAST expression in glioblastoma could constitute another important weapon by which GSC may proliferate and migrate.

3.Materials and Methods

3.1 Cells culture

GL261 murine glioma stem cells (GSC) were maintained in DMEM-F12 Glutamax^{TM} (Life Technologies), B-27 supplement 1X (Life Technologies), penicillin/streptomycin 1X, human recombinant epidermal growth factor (EGF; 20 ng/ml; Peprotech), and human recombinant fibroblast growth factor-2 (FGF-2; 20 ng/ml; Peprotech).

The spleens and draining lymph nodes of immunized and control mice were surgically removed and mechanically processed in RPMI 1640 basal medium (LONZA) containing 5% fetal bovine serum, 20 mM HEPES, 2% L-glutamine and penicillin/streptomycin.

Erythrocytes were lysed with ice-cold ACK buffer (0.15 M NH_4Cl, 1 mM $KHCO_3$, 0.1 mM Na_2 EDTA pH 7.4), and lymphocytes were resuspended in RPMI 1640 supplemented with 10% fetal bovine serum, 2% penicillin/streptomycin, 2% L-glutamine, 50 μM β-mercaptoethanol, 100 mM sodium pyruvate and 100x nonessential amino acids.

We also added 10 U/ml of human recombinant IL-2 (Roche) to the medium for all in vitro experiments.

3.2 Immunomagnetic sorting of GLAST positive cells

To isolated GLAST positive cells, first 5-8 x 10^7 GL261-GSC were stained with the polyclonal anti-rabbit EAAT1 antibody (ab416 Abcam) in ice cold PBS + FCS 10% at the dilution of 1:200 for 30' at room temperature. A secondary anti rabbit IGg-PE conjugated Antibody was used at the concentration of 1:200 in ice cold PBS + BSA 3% at the same condition. Subsequently, the cells were magnetically labelled with Anti-PE MicroBeads (Miltenyi Biotec) according to the manufacturer's instruction. Then the cell suspension was isolated on a MACS Column system, which was placed in the magnetic field of a MACS Separator (Miltenyi Biotec).

Magnetically-labelled cells (GLAST positive) are retained in the column while the unlabeled cells (GLAST negative) run through. After removal of the column from the magnetic field, the magnetically retained cells can be eluted as the positively selected cell fraction.

3.3 Glioma model

In vivo experiments were performed using 6 old female C57BL/6N mice (Charles River Laboratory). 1 × 105 GL261-GSC are directly injected into the brain using a stereotactic frame. For tumor cells implantation the following stereotactic coordinates respect to the bregma were used: 0.7 mm posterior, 3 mm left lateral, 3.5 mm deep into the nucleus caudatum.

3.4 MRI studies

Magnetic resonance imaging (MRI) was performed on a 7 Tesla Bruker BioSpec 70/30 USR Preclinical System at different days after tumor implantation.

T2-weighted RARE sequence and T1-weighted spin echo sequences had been performed before and after intraperitoneal injection of contrast medium (Gadolinium DTPA).

3.5 Peptide Prediction

To determine whether GLAST peptide-based immunotherapy is protective against GL261 gliomas, we used the SYFPEITHI (http://www.syfpeithi.de/) and BIMAS (www.bimas.cit.nih.gov/molbio/hla_bind/) binding-motif algorithms to identify high-scoring target peptides containing both the murine major histocompatibility complex (MHC) H-2Db and the human HLA-A*0201.

We selected four peptides: Pep1 (44–52) YLFRNAFVL, score 23; Pep2 (103–11) SLVTGMAAL, score 18; Pep3 (368–376) TLPITFKCL, score 16; and Pep4 (403–411) ALYEALAAI, score 15 (**Tab. 1**). The complete sequence of GLAST is available in the UniProt database (www.uniprot.org/). Lyophilized peptides were purchased from Primm (Primm S.r.l) and resuspended at a final concentration of 5 mg/ml/peptide using SEPICLEARTM 01 PPI (SEPPIC), a lipoamino acid compound designed to dissolve poorly soluble peptides.

Peptide Sequences	Rammensee Score
$Pep1_{(44-52)}$ **YLFRNAFVL**	23
$Pep2_{(103-111)}$ **SLVTGMAAL**	18
$Pep3_{(368-376)}$ **TLPITFKCL**	16
$Pep4_{(403-411)}$ **ALYEALAAI**	15

Tab. 1 Table of selected peptides. Peptides were predicted basing on computational algorithms. Peptides are restricted for both murine MHC H-2Db and human HLA-A*0201 and were synthesized from Primm S.r.l.

3.6 In vivo experiment

A total of 38 C57BL/6N mice (Charles River Laboratory), 19 immunized and 19 controls (10 mice per group for survival studies and 9 mice per group for immunological and histological studies), were injected with 1×10^5 GL261 cells/mouse into the nucleus caudatum using a stereotactic frame. The stereotactic coordinates with respect to the bregma were as follows: 0.7 mm posterior, 3 mm left lateral and 3.5 mm deep into the nucleus caudatum. The immunized mice received subcutaneous (sc) injections of

all four peptides (15 μg/peptide) into different areas of the flank on days 4, 11 and 18 after tumor implantation (day 0). The peptides were emulsified with Montanide™ ISA 51 VG (1:1) (SEPPIC) using two 2-ml sterile Luer Lock glass syringes (Artiglass S.r.l) and a three-way stopcock connector (MOVI S.p.A.) as recommended by the manufacturer. The immunized mice received a total of 3 μg of recombinant murine granulocyte macrophage colony-stimulating factor (GM-CSF) (Miltenyi Biotec) spread out over three injections into the same area of the peptide injections, beginning one day before the first vaccination. The control mice were treated with vehicle only (Montanide) on the same days as the immunized mice were vaccinated and injected with GM-CSF according to the experimental schema [Fig. 3A results].

3.7 Cytotoxicity assay

Cytotoxicity assay. Lymphocytes from immunized and control mice were isolated on day 15 after tumor implantation. We tested their ability to recognize and lyse GL261 cells *in vitro*. A total of 2 × 106 splenocytes were pre-stimulated for 5 days in the presence of 5 × 10^5 3-Gy-irradiated naïve splenocytes as antigen presenting cells and 5 μg/mL of each peptide. The cells were cultured in 24-well tissue culture plates in a final volume of 2 mL/well of complete RPMI 1640 supplemented with 10 U/mL IL-2. Splenocytes from healthy mice were irradiated (43855F Cabinet X-ray System, Faxitron® X-ray Corporation) and used as antigen presenting cells. Pre-stimulated lymphocytes were tested for GL261-specific cytotoxicity using different effector:target (E:T) ratios (10:1, 20:1 and 40:1). NIH 3T3 cells were used as negative controls. To quantify cell lysis, a non-radioactive cytotoxic assay (Cytotoxicity detection kit plus LDH, Roche) was performed according to the manufacturer's instructions. Absorbances for

the various cell groups were used to calculate the percentage of specific cytotoxicity according to the following equation: (effector:target cell mix − effector cell control) − low control/(high control − low control) × 100, where the high and low controls correspond to the background target absorbance and the maximum target absorbance, respectively.

3.8 Proliferation assay

Immunized and control mice were sacrificed 15 days after tumor implantation, and a total of 2 × 106 splenocytes were primed for 5 days in the presence of 5 × 10^5 3 Gy-irradiated naïve splenocytes as antigen presenting cells, 5 µg/mL of all peptides and 10 U/ml of IL-2. After pre-stimulation, 5 × 10^5 splenocytes were incubated for 24 h or 48 h in the presence of single peptides (5 µg/ml) and IL-2 and tested for their ability to proliferate. The number of viable cells was assessed using MTT Reagent (Millipore). The data are expressed as the percentage of proliferation, which is calculated according to the following equation: (OD stimulated splenocytes – OD splenocytes without peptide)/OD stimulated splenocytes × 100.

3.9 Isolation of CNS-infiltrating lymphocytes

CNS-infiltrating lymphocytes were isolated using a tumor dissociation kit (mouse, Miltenyi Biotec) on day 15 after tumor implantation. Briefly, glioma-bearing hemispheres (where GL261 cells were injected) from immunized and control mice (n = 4/group) were explanted, cut into small pieces of 2–4 mm, and dissociated using GentleMACS (Miltenyi Biotec) according to manufacturer's instruction. The cells were then suspended in PEB buffer (PBS/0.5% bovine serum albumin/ 2 mM EDTA) for labelling and flow cytometry evaluation.

3.10 Flow cytometry

Lymphocytes from spleens, cervical draining lymph nodes and explanted gliomas of immunized and control mice were used for immune monitoring. Briefly, 1.5 × 10^6 cells were stained in PBS for 30 min at 4°C with the following antibodies: anti-CD4 PE-Cy5 (BD Bioscience), anti-CD3 FITC (Biolegend), anti-CD8-FITC (BD Bioscience), anti-CD8-PE (Biolegend), and anti-CD49d-APC (Miltenyi Biotec). For NK cell detection, an anti-NKp46-PE antibody (Miltenyi Biotec) was used according to the manufacturer's instructions. For GLAST positive cells evaluation we used an Anti-GLAST (ACSA-1) PE (Miltenyi Biotec) Flow cytometry acquisition was performed on a MACSQuant instrument, and the data were analyzed with the MACSQuantify Software (Miltenyi Biotec).

3.11 RNA extraction and Real-time PCR (RT-PCR)

Total RNA was isolated from freshly harvested GL261 gliomas, lymphocytes and paraffin-embedded samples from immunized and control mice and used for gene expression analysis. Similar studies were also performed on cells from in vitro experiments. RNA was extracted with TRIzol reagent (Life Technologies) using the RNeasy MINI KIT (Qiagen) and the RNase-Free DNase Set (Qiagen). For paraffin-embedded samples, the Absolutely RNA FFPE kit (Stratagene) was used according to the manufacturer's instructions. cDNA was synthesized from total RNA using oligo (dT) and M-MLV Reverse Transcriptase (Life Technologies). Specific primers for target genes were designed for Fast SYBR Green chemistry (Applied Biosystems) and purchased from Primm S.r.l. The relative mRNA levels were measured using a 7500 Real-Time PCR System (Applied Biosystems) and calculated using the ΔΔCt method. The expression levels

of the target genes were normalized to the expression level of β2-microglobulin. The sequences of the primers are reported in the [Tab. 2].

Primers	Sequence 5'- 3'
E4BP4	fw 5'-AAAACAACGAAGCTGCCAAA-3 rev 5'-CCAAAACCAGGTCATTGAGG-3'
CD49d	fw 5'-CAAACCAGACCTGCGAACA-3' rev 5'- TGTCTTCCCACAAGGCTCTC-3'
CXCL10	fw 5'-GCTGCCGTCATTTTCTGC-3' rev 5'-TCTCACTGGCCCGTCATC-3'
CCL5	fw 5'-CCCTCACCATCATCCTCACT-3' rev 5'-GAGAGGTAGGCAAAGCAGCA-3'
CCL2	fw 5'-CATCCACGTGTTGGCTCA-3' rev 5'-GATCATCTTGCTGGTGAATGAGT-3'
CCL4	w 5'-GCCCTCTCTCTCCTCTTGCT-3' rev 5'-GAGGGTCAGAGCCCATTG-3'
CCL3	fw 5'-CAAGTCTTCTCAGCGCCATA-3' rev 5'-GGAATCTTCCGGCTGTAGG-3'
NKG2D	fw 5'-TGAAGTCACCTGTGTTTATGCAG-3' rev 5'-CACTGTCAAAGAGTCATCCAACA-3'
GLAST (mouse)	fw 5'-GATGCTGCAGATGCTGGTCTT-3' rev 5'-TATCTAGGGCCGCCATTACGT-3'
GLSAT (human)	fw 5'-CGGACAAATTATTACAATCAGCA-3' rev 5'-ATTCCAGCTGCCCCAATACT-3'

Tab 2. Sequence of primers designed for RT-PCR SybrGreen chemistry.

3.12 Histology and immunohistochemistry

Immunohistochemical analysis of GLAST (Santa Cruz), Ki67 (BD Bioscience), CD8 (R&D Systems), CD4 (R&D Systems), and CD11b (BD

Bioscience) was performed on paraffin-embedded sections. For double immunofluorescence, the tumor sections were incubated with anti-GLAST and anti-nestin antibodies overnight at 4°C and then incubated with Alexa Fluor 488-conjugated anti-rabbit secondary antibody. Quantitative analyses were performed on three to five independent fields per tumor by counting the number of cells in the photographed fields using the 40× objective of a Leica DM-LB microscope.

3.13 Statistical analysis

The differences between groups were analyzed using two-tailed Student's t-tests and were considered statistically significant when $p < 0.05$.

4.Results

4.1 Results I: Characterization of the radial glia marker GLAST in a murine model of malignant glioma

4.1.1 Enrichment of GLAST expressing cells in GL261 GSC increases tumour progression

Our first evidence that glioma stem-like cells (GSC) can express GLAST came from a gene expression profile analysis performed on murine GL261 cells that compared neurospheres (NS) and adherent cells (AC) [168]. To investigate the functional consequences of GLAST enrichment we isolated GLAST expressing GL261 GSC using the magnetic cell sorting technology. The positive fraction was 10- to 14-fold enriched for GLAST expressing cells [**Fig. 1A**]. When we injected unsorted, GLAST+ and GLAST- cells into mouse brain, we found that GLAST+ cells were significantly more aggressive than GLAST- cells or unsorted cells as confirmed by Kaplan Meier survival analysis [**Fig. 1B**].

Brain magnetic resonance imaging (MRI) performed on a 7 Tesla Bruker BioSpec 70/30 USR Preclinical System at 22 days after tumor injection confirmed a different pattern of progression of GL261-derived gliomas from GLAST+ or GLAST- cells. Gliomas generated from GLAST- and unsorted cells were still confined to the homolateral hemisphere while tumors from GLAST+ cells appeared more aggressive and infiltrate the contralateral hemisphere [**Fig. 1C**].

We then explanted gliomas developed from unsorted, GLAST+, and GLAST- cells, and examined GLAST expression patterns using flow cytometry and morphological analysis [**Fig. 2 A-B**]. Gliomas from GLAST+ cells showed high GLAST expression whereas gliomas from GLAST- cells partially reacquired GLAST expression. The presence of many clusters from tumor mass in gliomas derived from GLAST+ cells is an evidence of their invasive capability.

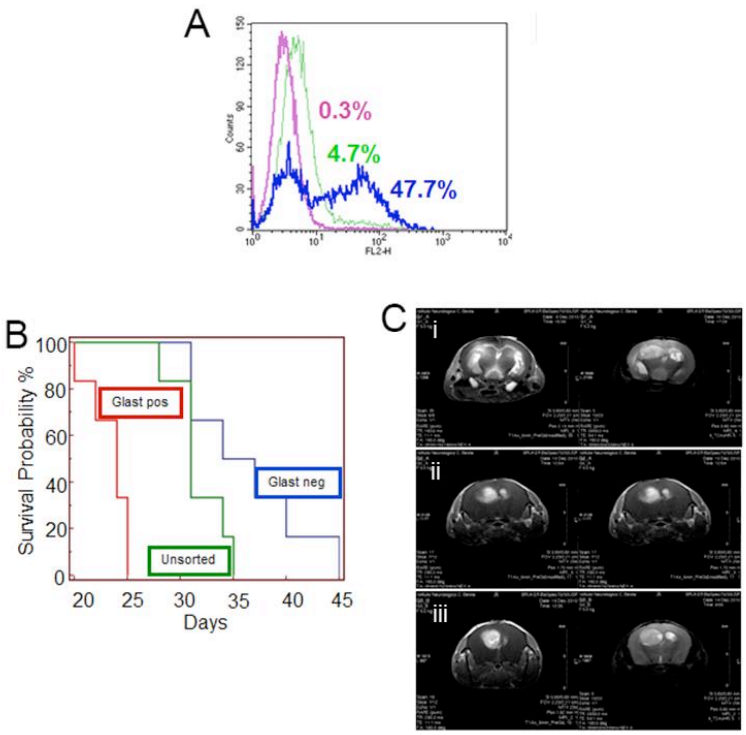

Fig. 1 Enrichment of GLAST expressing cells in GL261 GSC increases tumour progression. **A** Flow cytometry histograms showed the percentage of enrichment and depletion after magnetic separation: unsorted GL261 population contains 4.7% GLAST positive cells, as determined by flow cytometry analysis. After immune separation the positive fraction was enriched about of 10 fold, expressing 47.7%; on the contrary the negative fraction was depleted completely containing only 0.3% of GLAST positive cells. **B** Kaplan Meier survival analysis confirmed that mice injected with GLAST+ GL261 cells had significantly lower survival rate than mice injected with unsorted or GLAST– cells (p=0.003 GLAST+ vs GLAST-; p=0.0006 GLAST+ vs unsorted; p=0.2 GLAST- vs unsorted) **C** T1 (right panel) with contrast medium and T2 (left panel, 20 slices; thickness 0.8 mm, no gap; TR: 2,658 ms/TE: 54 ms/RARE Factor 8/FOV: 2.20 9 2.20 cm2; averages 6; image matrix 256 9 256; in plane resolution 0.086 mm; acquisition time 8 min 30 s) representative MRI images confirmed a different pattern of glioma formation. GLAST+ derived gliomas (**Ci**) are more infiltrative the surrounding parenchyma and the contralateral hemisphere than GLAST- (**Cii**) or unsorted (**Ciii**) derived tumors.

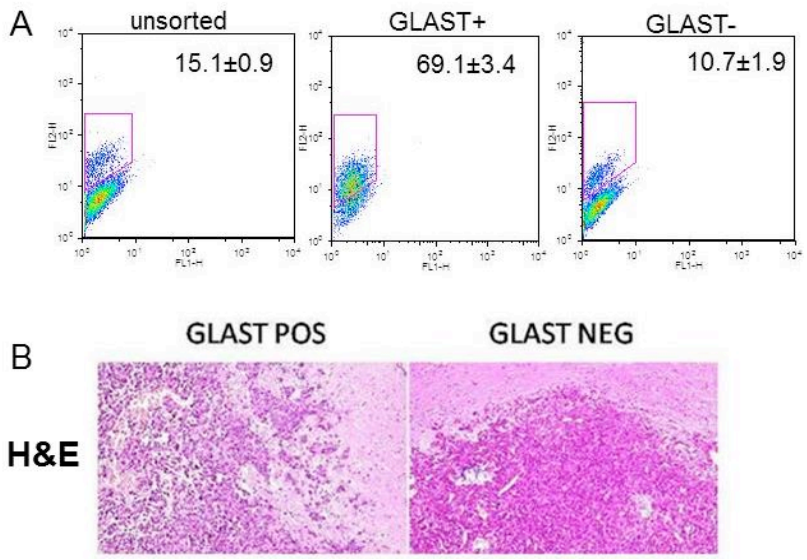

Fig. 2 GLAST expression is re-acquired in vivo. **A** Flow cytometry analysis on explanted gliomas showed a high GLAST expression in gliomas from GLAST+ cells. GLAST- glioma showed a partially reacquisition of GLAST expression. **B** H&E morphological analysis performed on paraffin embedded gliomas showed a relevant presence of cell clusters from the central tumor mass as an unique characteristic of gliomas derived from GLAST+ cells (left panel). Tumor from GLAST- cells (right panel) appeared very regular with a net border showing a slower progression.

4.2 Results II: Immunotherapeutic approach with GLAST-derived peptides; activation of a specific anti-glioma immune response

4.2.1 Vaccination with GLAST-peptides results in long-term survival and cytotoxicity

The expression of GLAST in GL261-malignant gliomas and our preliminary results, in particular the evidence that GLAST enrichment impacts on progression and invasion of gliomas, support the idea that GLAST could be considered a good marker and target for GB immunotherapy.

To determine whether GLAST peptide-based immunotherapy is protective against GL261 gliomas, C57BL/6 syngeneic mice were vaccinated three times with GLAST-derived peptides beginning four days after intracranial

injection of GL261 GSC [**Fig. 3A**]. Survival analysis demonstrated that GLAST peptides provide significant protection against GL261 GSC. All control animals (glioma-bearing mice treated with vehicle only) died by day 25, whereas treatment with GLAST peptides emulsified with Montanide ISA-51 in combination with granulocyte macrophage colony-stimulating factor (GM-CSF) injections extended the survival of mice to 40% ($p < 0.002$ vs. control) [**Fig. 3B**].

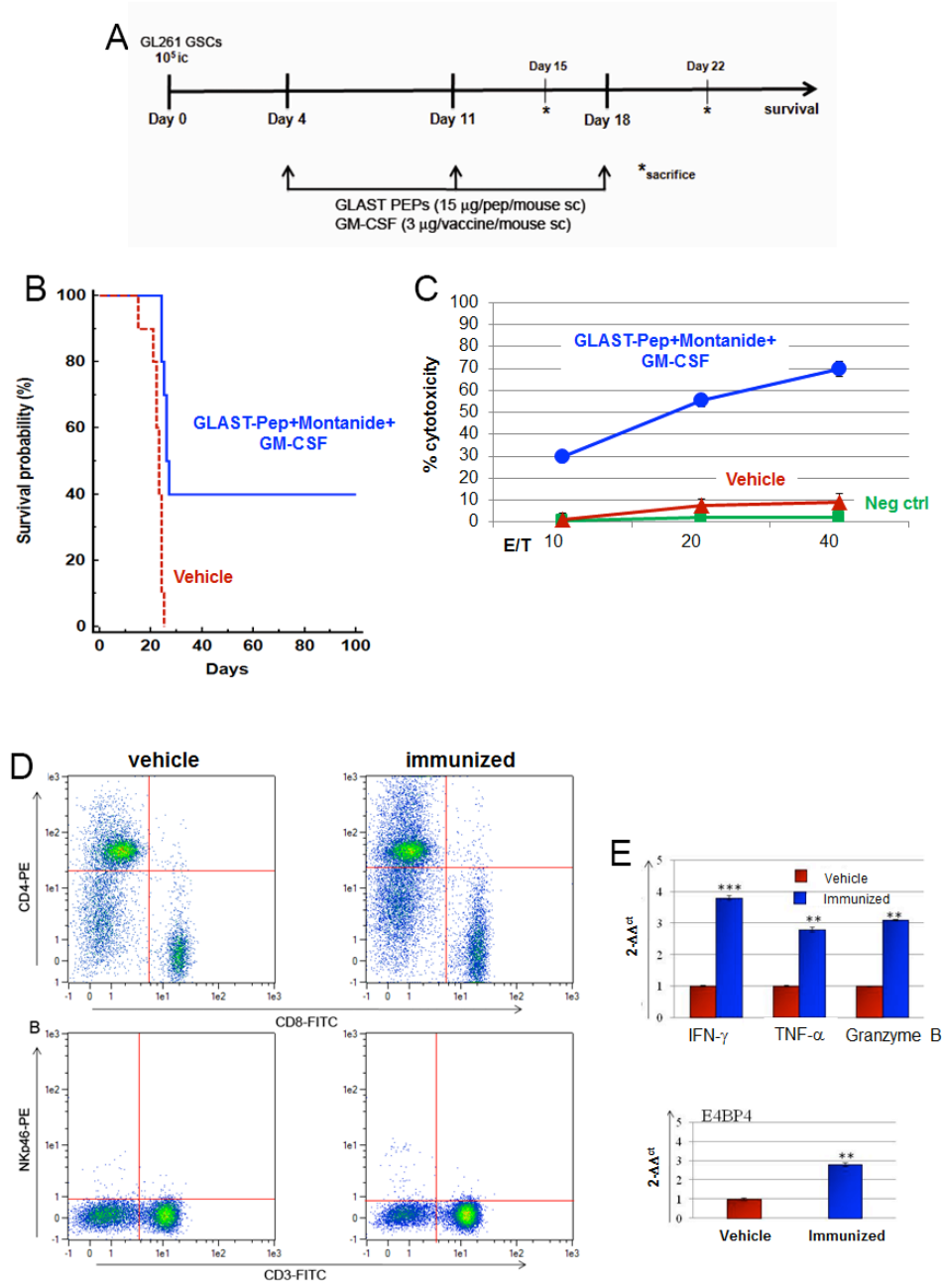

Fig. 3 Vaccination with GLAST peptide results in long-term survival and induces specific antitumor cytotoxicity. **A** C57BL/6N mice were injected i.c. with GL261 GSC. Immunized mice received three s.c. vaccinations on days 4, 11 and 18 with all peptides (15 µg/single peptide) emulsified in Montanide™ ISA 51 VG. A total of 3 µg of GM-CSF/mouse were administered during each treatment. Control mice were treated

with vehicle only. On days 15 and 22, three mice/group were sacrificed for immune monitoring and histological analysis. **B** Kaplan-Meier survival analysis showed that mice immunized with GLAST peptide (n = 10) survived significantly longer than control mice (vehicle, n = 10) ($p < 0.002$). **C** In vitro LDH cytotoxicity assay revealed that splenocytes from immunized mice recognize and lyse GL261 target cells but not NIH 3T3 cells. **D** Flow cytometry performed on splenocytes from immunized mice showed that the percentages of CD4+ and CD8+ T cells and NK cells were significantly increased when compared with controls (upper parts, CD4+ cells: 16.20 ± 0.60% vs. 8.20 ± 1.30%, $p = 0.01$; CD8+ cells: 13.50 ± 2.10% vs. 4.10 ± 0.07%, $p = 0.02$; lower part, NKp46+ CD3- cells: 3.00 ± 0.30 vs. 0.90 ± 0.07, $p = 0.009$). Data were obtained from three different evaluations and are reported as the mean % ± SD of immunized vs. control mice. **E** RT-PCR analysis showed that the splenocytes of immunized mice express higher levels of IFNγ, TNFα and granzyme B. Infiltration of NK cells was investigated by analyzing the relative expression of the transcription factor E4BP4. $^{**}p < 0.001$; $^{***}p < 0.0001$.

To determine whether GL261-specific effector cells were generated in response to peptide vaccination, pre-stimulated splenocytes were assayed for in vitro cytotoxicity 15 d after tumor implantation. The splenocytes were re-stimulated with peptides and tested 5 days later for cytotoxic activity against GL261 GSC or NIH 3T3 cells as a negative control, using an LDH release assay. The splenocytes from mice treated with GLAST peptides displayed strong cytotoxic activity against GL261 GSC when compared with those from control mice [**Fig. 3C**]. The specificity of the cytotoxicity was confirmed by the total absence of reaction against NIH 3T3 cells.

To characterize the direct effects of peptide vaccination in association with GM-CSF treatment on T-cell function, spleens and cervical draining lymph nodes were harvested 15 days after tumor implantation and multiple cell populations were quantified by flow cytometry. We observed significant increases in NK cells, CD8+ T cells and CD4+ T cells in the spleens [**Fig. 3D**] and lymph nodes [**Fig 4**] of immunized mice when compared with controls. RT-PCR analysis of splenocytes revealed that GLAST peptide administration also enhanced the expression of interferonγ (IFNγ), tumor necrosis factor α (TNFα) granzyme B and the NK cell-specific transcription factor E4BP4 [**Fig. 3E**]. These data support the specificity of the vaccine-induced effector response and the involvement of NK cells in the response to GLAST peptide immunization.

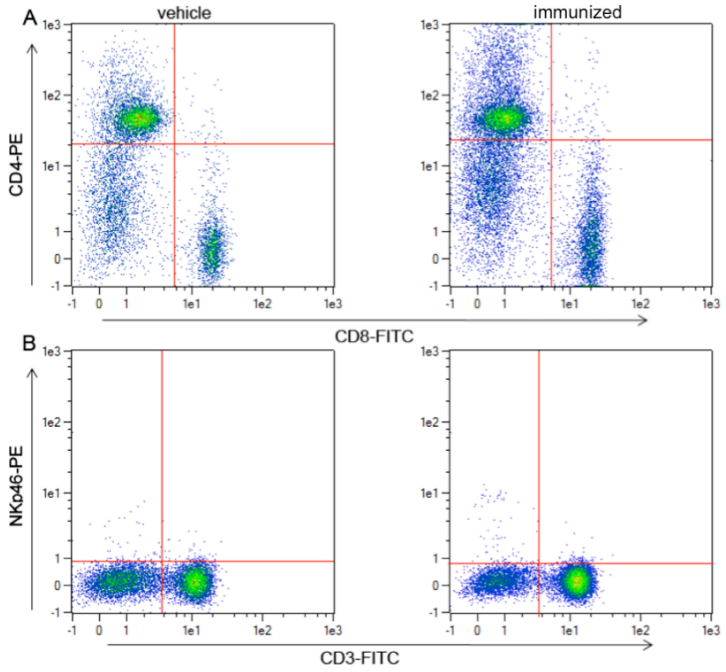

Fig. 4 GLAST-peptide administration activates systemic immune response. In cervical lymph nodes from immunized mice the percentage of CD4, CD8-T and NK cells increased significantly when compared to controls: **A** CD4+ 23.50 ± 2.10 vs. 15.20 ± 0.10, p =0.04; CD8+ cells 13.60 ± 1.40 vs. 5.10 ± 0.70, p = 0.01; **B** CD3- NKp46+ cells 2.00 ± 0.50 vs. 0.20 ± 0.07, p = 0.01 immunized vs. vehicle mice respectively. The panels show representative dot plots obtained from three different evaluations 15 days after tumor implantation.

4.2.2 Immunization with peptides specifically targets GLAST positive glioma cells and modulates tumor microenvironment

Fifteen days after tumor implantation, GLAST expression was significantly decreased in gliomas from immunized mice. Histological analysis showed that GLAST expression disappeared during the treatment course [**Fig. 5A**]. GLAST expression was 5.2 ± 2.1-fold lower in the tumors of immunized mice than in those of mice treated with vehicle only [**Fig. 5B**]. We also observed a significant reduction in Ki67-positive cells ($p < 0.0001$) in immunized vs. control mice [**Fig. 5C**]. Immunofluorescence analysis confirmed that GL261 gliomas from control mice strongly expressed

GLAST and co-expressed nestin on day 22, whereas GLAST and nestin expression were not detected in gliomas from immunized mice [**Fig. 6**].

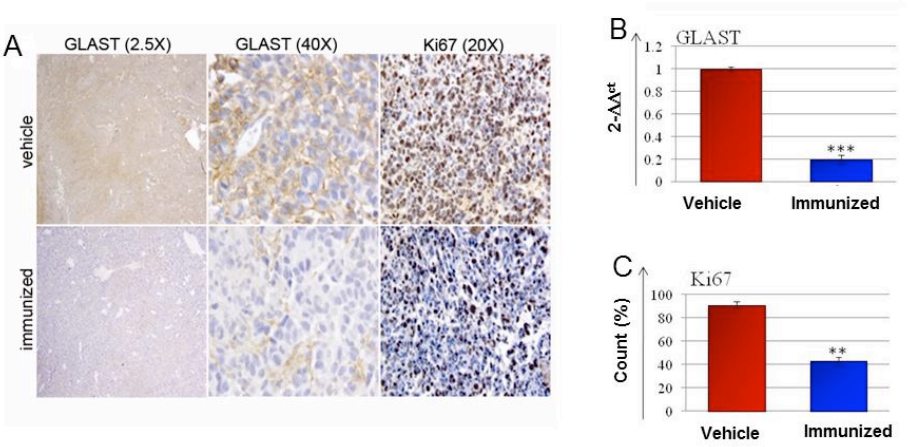

Fig. 5 GLAST expression disappears in gliomas from immunized mice. **A** Histological analysis showed strong and diffuse positivity for GLAST in gliomas from vehicle mice. GLAST expression was decreased in gliomas from immunized mice (magnification 2.5× and 40×). **B** RT-PC R confirmed the significant decrease in GLAST expression in gliomas from immunized mice when compared with control mice (n = 3/group). ***p < 0.0001. **C** Quantitative analysis of Ki67-positive cells summarized as the mean ± SD of the number of cells counted in 5 different 40× tumor sections. **p < 0.001.

To characterize the effect of peptide vaccination on the tumor microenvironment, we investigated the expression of key molecules involved in immune activation (IFNγ and TNFα) or suppression (transforming growth factor, TGFβ1 and TGFβ2) by RT-PCR analysis of freshly harvested tumors from immunized and control mice (n = 3/group). IFNγ and TNFα expression levels were 9.60 ± 0.05- and 16.80 ± 0.03-fold higher (p < 0.0001), respectively, in immunized mice than in control mice. Moreover, the levels of granzyme B, the serine protease released by CTL, and E4BP4 were 25.60 ± 0.12- and 15.00 ± 0.05-fold higher (p < 0.0001 and p < 0.01), respectively [**Fig. 7A**]. In contrast, TGFβ1 and TGFβ2 expression levels were 3.4- and 2.6 ± 0.2-fold lower, respectively, in

immunized mice than in control animals ($p < 0.001$) [**Fig. 7B**]. Similar expression patterns were observed by RT-PCR analysis of paraffin-embedded gliomas (data not shown).

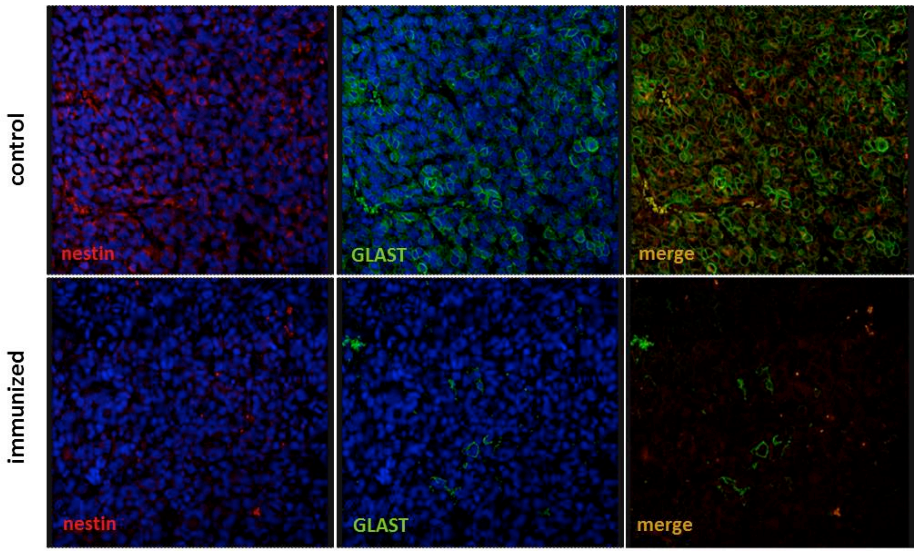

Fig. 6 Nestin/GLAST double positive cells disappear in gliomas from immunized mice. Gliomas from control mice treated with vehicle investigated 22 days after tumor implantation showed high levels of nestin (red) and GLAST (green) double positive cells (upper panel). In gliomas from mice immunized with GLAST peptides and sacrificed on day 22 double positive cells disappeared (lower panel). Three representative mice for each group have been investigated and representative images are displayed.

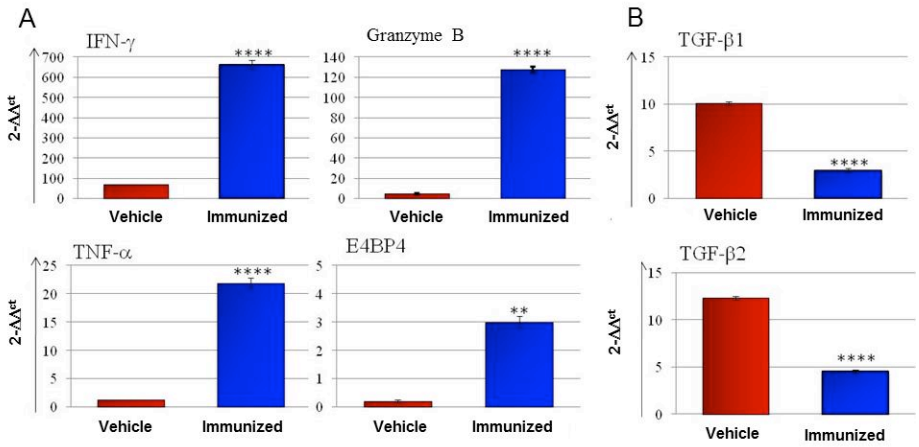

Fig. 7 Peptide immunization exerts immune modulation on tumor monicroenvironment. **A** Relative expression of IFNγ, TNFα, granzyme B, the transcription factor E4BP4 and **B** TGBβ1 and TGFβ2 were investigated using RT-PC R on RNA from immunized and control mice. **p < 0.001, ***p <0.0001, ****p <0.00001.

4.2.3 Peptides immunogenicity and efficacy are related to granuloma formation

To verify the immunogenicity of the GLAST-derived peptides, we first considered the appearance of potential local reactions during treatment. Only in immunized mice we observed the formation of a nodule at each injection site immediately after vaccination, enabling the slow, localized release of antigen. In immunized mice that displayed prolonged survival or specific immune reactions, we found four separate granulomas appearing as localized nodules with different sizes at the locations where each of the four peptides was administered. Non-necrotizing granulomas showing a high proliferation index and lymphocyte accumulation associated with macrophages were found at the injection sites of peptide 1 [**Fig. 8A**]. Granulomas that formed at the injection sites of peptides 2, 3 and 4 were characterized by the prevalence of macrophages, the total absence of

proliferating cells, CD8+ and CD4+ T cells, and noticeable central necrosis possibly caused by the activity of Th1 cells [**Fig. 8B**]. In particular, IFNγ is considered a major cause of necrosis in established granulomas as a consequence of exacerbated immune responses.

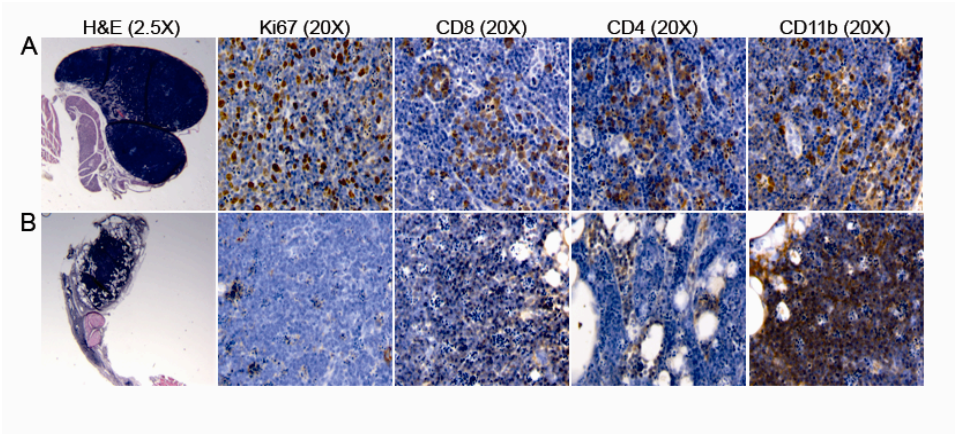

Fig. 8 Local granulomas formation reflects peptide immunogenicity. **A** Granulomas derived from the injection site of peptide 1 appeared well developed and exhibited non-necrotic tissues. They were highly proliferative, as indicated by Ki67 staining and were infiltrated by CD4+, CD8+ and CD11b+ cells. **B** Granulomas from peptide 4 injection sites exhibited central necrosis, a total absence of Ki67-positive cells, CD8+ and CD4+ T lymphocytes and a massive CD11b+-cell infiltration.

To test the in vitro immunogenicity of the four peptides used for vaccinations, splenocytes from control and immunized mice were primed in vitro using a mixture of irradiated antigen-presenting cells (APCs) and peptides. Five days later, the splenocytes were tested for their ability to proliferate in the presence of GLAST peptides. Lymphocytes from immunized mice proliferated significantly more than lymphocytes from vehicle receiving mice. The ability of peptides 2, 3 and 4 to induce robust proliferation correlated well with their in vivo immunogenicity [**Fig. 9**].

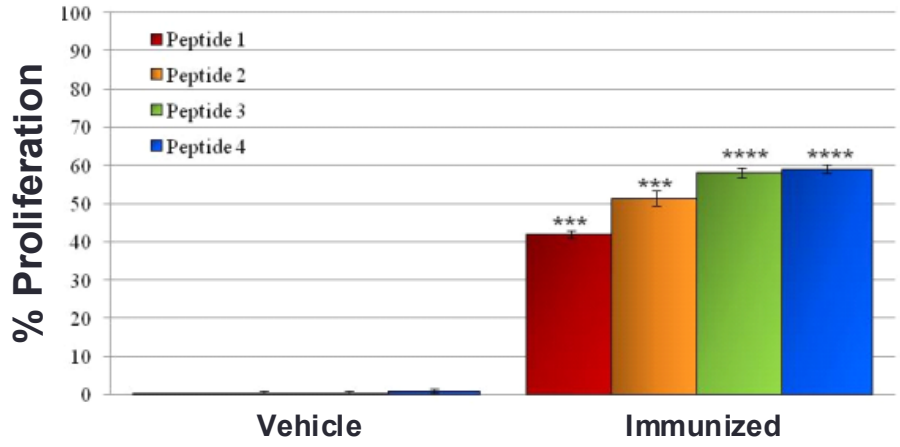

Fig. 9 Proliferation test reveals the immunogenicity of GLAST-derived peptides in vitro. Splenocytes derived from immunized mice proliferated significantly more than splenocytes derived from vehicle mice. This was particularly evident in the presence of peptides 3 and 4 that appeared more immunogenic in vitro ***p < 0.0001, ****p < 0.00001.

4.2.4 The lack of autoimmune reactions and demyelination supports the safety of GLAST-peptide administration

GLAST is not a glioma-specific antigen, and the risk of vaccinating with this antigen must be weighed carefully owing to the possibility of inducing autoimmune reactions targeting the CNS. To test whether the administration of GLAST-derived peptides might damage healthy areas of the CNS, we evaluated brain sections from immunized mice by hematoxylin and eosin (H&E) and Luxol Fast Blue (LFB) staining. We examined brain samples collected at different time points after immunization and observed that highly myelinated areas, such as the *corpus callosum* and the internal capsule, were densely stained with LFB, demonstrating that demyelination had not occurred in these mice. We also observed the integrity of the SVZ, where GLAST is expressed by radial glia-derived neural stem cells [**Fig. 10 A-B**] and of the cerebellum (Bergmann glia). There was no evidence of

immune cell infiltration, and GLAST positivity was comparable in immunized and control mice [**Fig. 10 C-D**].

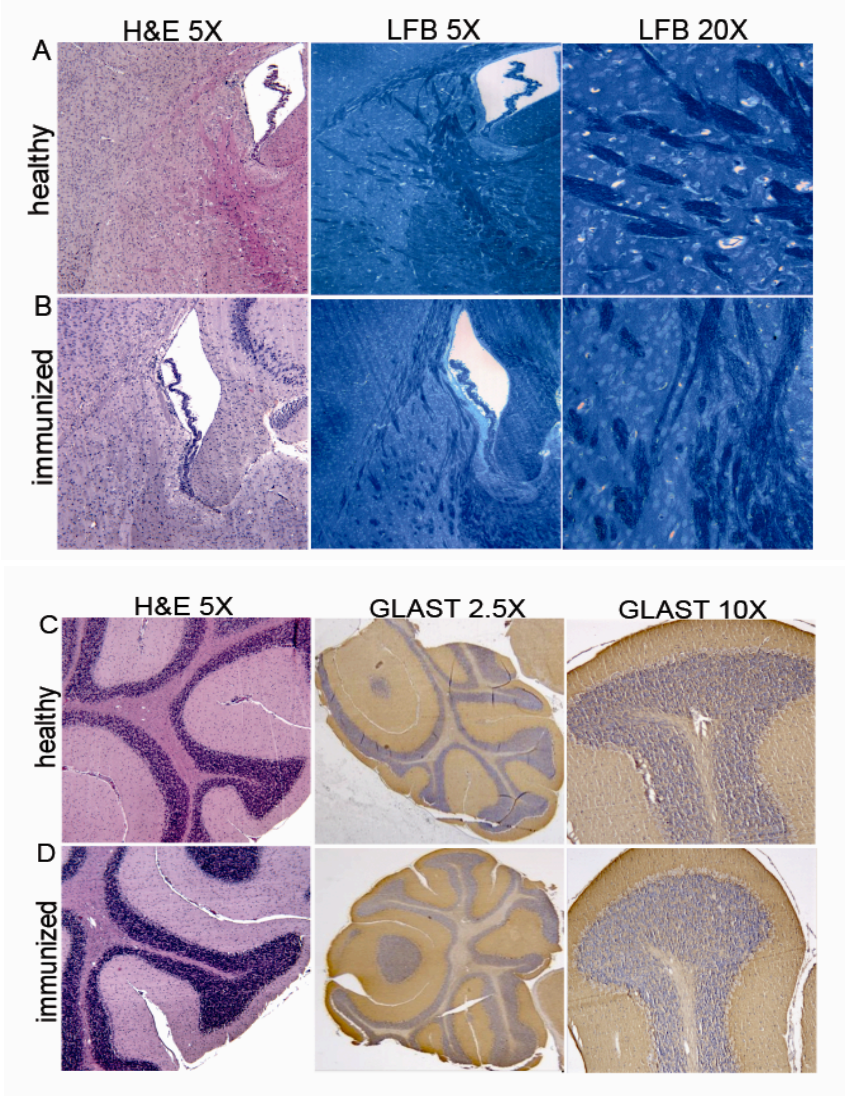

Fig. 10 Lack of autoimmune reactions and demyelination support the safety of GLAST-peptide immunization. **A-B** H&E and LFB staining of the subventricular zone revealed the total absence of demyelination in the brains of immunized (lower panel) when compared with healthy brains (upper panel). Highly myelinated areas appear densely stained with LFB (magnification 5× and 20×). **C-D** H&E staining of cerebellar tissue showed a structural integrity (magnification 5X) that was also confirmed by strongly positive GLAST staining at the level of Bergmann glial processes in immunized mice when compared with healthy mice (magnification 2.5X and 20X). Representative images from three mice are shown.

4.2.5 Increased local recruitment and tumor tropism of antigen-specific T cells in immunized mice appears to be related to enhanced expression of chemoattractants

The absence of autoimmune signs supports the idea that activated immune cells selectively travel to the tumor site. We isolated infiltrating lymphocytes from freshly harvested tissues (n = 4 for each group) and evaluated the percentage of CD4+ and CD8+ T cells within the CD3+ T cell population by flow cytometry. Representative plots show that CD4+ T and CD8+ T cell infiltration of tumor tissues was significantly increased in immunized mice ($p < 0.001$ and $p = 0.01$, respectively) [**Fig. 11**]. In addition, the percentage of CD8+ T cells expressing VLA-4 was higher in immunized than in control mice ($p = 0.004$), supporting the efficient CNS homing of CTL activated upon peptide vaccination. The frequency of NKp46+CD3- NK cells was very low in tumors from vehicle-treated mice and appeared to be slightly, but significantly, increased in tumors from immunized mice ($p = 0.002$).

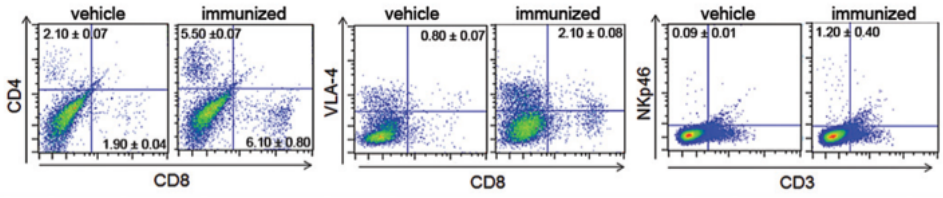

Fig. 11 Local immune infiltration increases in immunized mice. CNS-infiltrating CD4+ and CD8+ T cells and NK cells isolated from the glioma-bearing hemisphere on day 15 were more abundant in immunized mice than in control mice. The numbers reported in the dot plots represent the frequency of the investigated subpopulations.

We next hypothesized that the attraction and recruitment of immune cells may be directed by the presence of chemoattractant factors. We thus examined the expression of CCL2 (which is involved in attracting

immunosuppressive regulatory T cells, Treg), CXCL10 (which is responsible for specific CTL recruitment), CCL3, CCL4 and CCL5 in immunized and control mice 15 d after tumor implantation. We performed RT-PCR and compared the results from the left hemisphere, where tumor cells were implanted, to those from the contralateral hemisphere and from tumor-free brain tissue. We found that CCL2 expression was 5.80 ± 0.10-fold lower and CXCL10 expression was 11.70 ± 0.01-fold higher in immunized mice, compared with their control counterparts ($p < 0.0001$) [**Fig. 12A**]. Moreover, CCL3 and CCL4 expression was 2.20 ± 0.10- and 2.00 ± 0.30-fold higher, respectively ($p < 0.001$). CCL5 was expressed at lower levels than the other chemokines in control mice and was increased significantly in immunized mice (15.00 ± 0.70-fold higher, $p < 0.0001$) [**Fig. 12B**].

These data suggest that the CCL3/CCL4/CCL5 axis may play a role in the accumulation of immune cells (and particularly of NK cells) at the sites of tumor formation. To test this hypothesis, we investigated the expression of the NKG2D ligand (NKG2DL), and found that its was expressed at very low levels or was absent in control mice, while it was upregulated in immunized mice, suggesting that the GLAST peptide-induced anti-tumor immune response involves NKG2D-mediated NK cell recognition of tumor cells [**Fig. 12B**]. In further support of the hypothesis that infiltrating and local cells cooperate to create a chemotactic gradient, we found that the expression levels of these chemokines were increased in immunized mice after a single injection of GLAST peptides (data not shown).

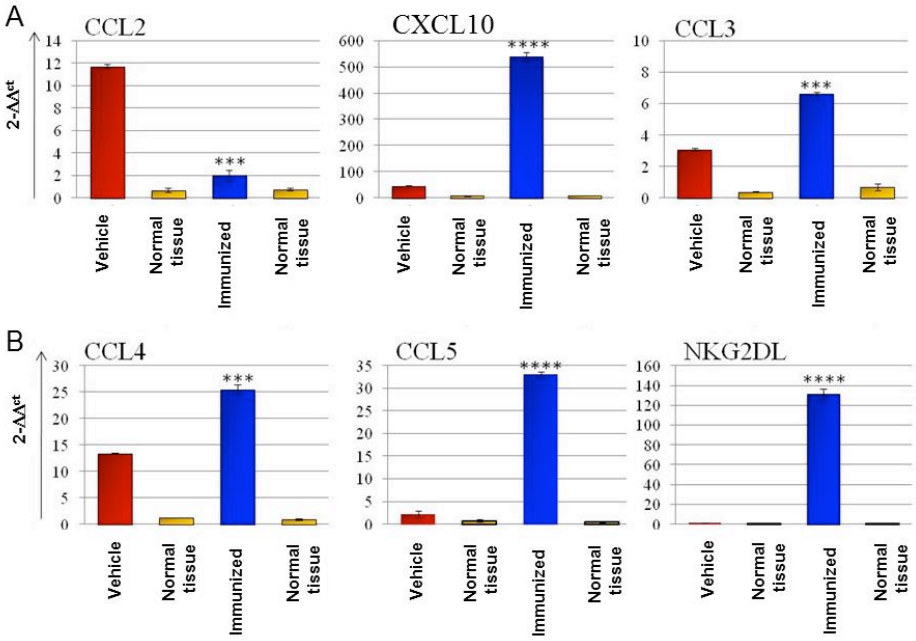

Fig. 12 Tumor microenvironment shows increased levels of chemoattractant factors. **A** CCL2 expression was measurable in vehicle control mice and decreased significantly in immunized mice. On the contrary CXCL10 and CXCL3 expression level was higher in immunized mice when compared with control mice. **B** CCL4, CCL5 and NKG2DL were expressed at higher levels in immunized than in control mice. mRNA levels from the hemisphere where the tumor cells were implanted and from the contralateral hemisphere were normalized to the relative quantity of β2-microglobulin. The relative expression of chemokines was compared with that detected in normal brain tissue.

4.3 Results III: Definition of GLAST as a clinical marker

In order to emphasize the translational relevance of GLAST as marker and glioma-associated antigen we will extend our studies to human GB. Through immunohistochemistry we examined a series of 50 human GB specimens; we found strong GLAST expression in the large majority of samples, specifically located on the plasma membrane of the tumour cells [**Fig. 13**]. To validate the relevance of GLAST expression in GB, we selected a group of patients (n=36) who were treated with surgery,

radiotherapy, and temozolomide [1], and we correlated their GLAST expression with overall survival (OS). We found that a high percentage of GLAST+ cells and a strong or moderate reactivity correlated with a lower overall survival of patients (p= 0.02), supporting a potential role of GLAST as a clinical marker in the prognosis of GB patients [**Fig. 14**].

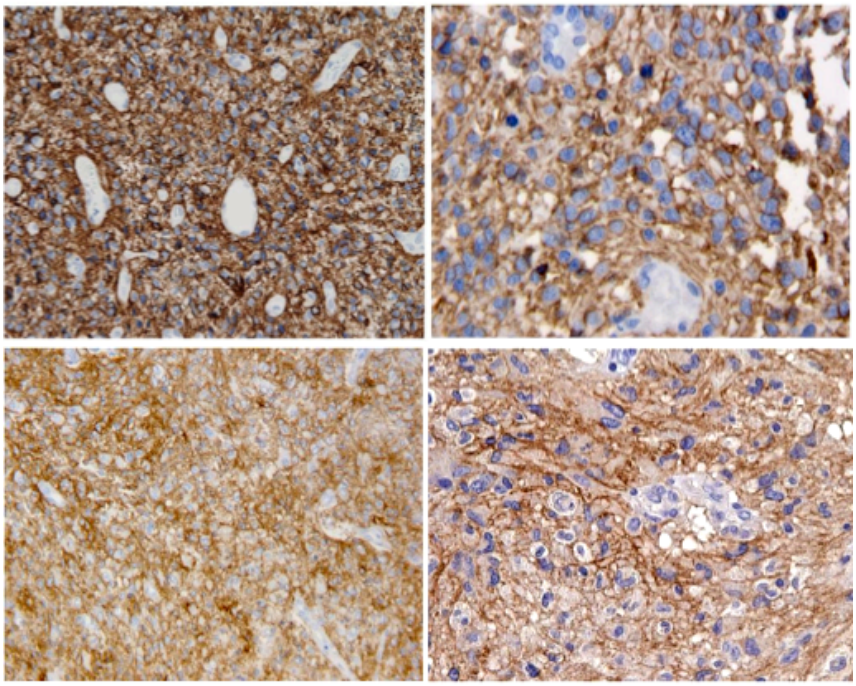

Fig. 13 Glioblastoma specimens show strong GLAST reactivity. GLAST expression was investigated in 50 paraffin-embedded human GB specimens. GLAST is preferentially localized in the plasma membrane of tumour cells; a high percentage of GLAST+ cells was detected in the majority of samples analysed.

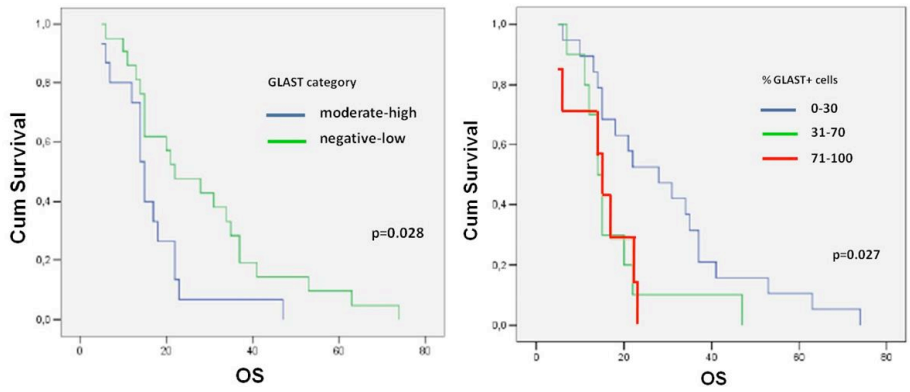

Fig. 14 GLAST expression correlates with OS of GB patients. GLAST expression was investigated by immunohistochemistry and compared with the OS in a cohort of 36 GB patients that received standard cure with radio- chemotherapy. A-B Patients with negative-low expression of GLAST survived significantly longer than patients with a moderate-high percentage of GLAST+ cells (p=0.028).

5.Discussion

Many clinical trials involving the immune system are now ongoing in several types of cancer including glioblastoma (GB). They are based on the rationale that tumor associated antigens (TAA), preferentially expressed by tumor cells, are recognized by effectors immune cells.

There are four important issues to consider in designing an effective cancer vaccine: identify potent tumor rejection antigens; stimulate an effective anti-tumor immune response; avoid autoimmune pathologies; and prevent the immune escape. The identification of GB cell subpopulations, expressing stem programs, provides the background for immunological studies aimed at their targeting. Murine and human GB contain a fraction of cells with stem cell-like features (GSC) and it has been proposed that only this population may be responsible for glioma recurrence. It was found by several groups, including our, that GB populations enriched with GSC can give rise to gliomas resembling closely the original tumor and rather different from the experimental gliomas generated by brain injection of established cell lines [48, 205]. These glioma stem-like cells have been found to preserve genetic alterations of their originating tumor and are tumorigenic in nude mice [206, 207]. We have found that GSC can be also obtained by established cultures from murine gliomas, like GL261. GL261-GSC have similar features to human GSC, as we extensively demonstrated in 2006. Dendritic cells (DC) loaded with GL261-GSC were significantly more effective than DC loaded with serum-cultured GL261 at inducing an immune rejection of highly malignant gliomas that were otherwise lethal in about one month. With these experiments, we provided a proof of principle that the use of a cellular population enriched in GSC for DC loading may increase the efficacy of anti-glioma (and possibly anti-tumor) immunotherapy [168].

Some studies have investigated the relationship between the GSC and the immune system. The results underscore for example the relevance of the GSC in the immuno-escape mechanism [208-211]. The relationship

between the immune system and cancer is dynamic and complex. Individual human tumors harbor a multitude of somatic gene mutations and epigenetically dysregulated genes, the products of which are potentially recognized as foreign antigen [64, 210]. However, the overriding relationship between the immune system and growing cancer is one of tolerance, in which, paradoxically, foreign molecules expressed by tumor cells are viewed as self. Growing cancers contain tumor-infiltrating lymphocytes (TILs), which are ineffective at tumor elimination in vivo but can exert specific functions (eg, proliferation, cytokine secretion, cytolysis) outside the immunosuppressive and tolerogenic tumor microenvironment. This is because the tumor milieu contains suppressive elements including regulatory T cells [110] and myeloid-derived suppressor cells (MDSC) [213]; soluble factors such as interleukin 6 (IL-6), IL-10, vascular endothelial growth factor (VEGF), and transforming growth factor beta (TGFβ) [75].

Important observations regarding the ability of NK cells and CD8 + T cells to recognize and destroy GSC are published [214-216] and cancer immunotherapy has become an interesting therapeutic option in several type of cancers.

The identification of a specific marker associated to cancer stem-like cells is mandatory, however this urgency conflicts with the evidence that strategies directed against one antigen only may have limited efficacy. Two aspects are important for appropriate therapeutic design: to overcome tolerance in order to elicit specific anti-tumor response and to avoid undesired autoimmune side effects.

In characterizing expression profiles of GL261-GSC we have found that expression of a group of five genes, including GLAST, is significantly up-regulated [168]. All these genes are expressed by radial glia. Interestingly, Buylla et al showed that radial glia is a potential source of adult neural stem cells [217]. To better define and characterize GSC we needed a surface

marker that would enable isolation; we have addressed the possibility to isolate GSC using GLAST as a marker.

GLAST, an astrocyte specific glutamate/aspartate transporter, is involved in the glutamate uptake in the CNS, and it is required for normal neurotransmission. Glutamate release by several membrane transporters promotes growth of malignant glioma and it appeared involved in invasion and angiogenesis [169, 203]. Glutamate-secreting gliomas promote an inflammatory response within the surrounding tissue, possibly induced by the neural death or by glutamate itself. The high expression of GLAST on the GSC plasma membrane supports the idea that it may be a good target for an immunotherapeutic approach. Furthermore, results obtained in the GL261-GSC model suggest an involvement of GLAST in conferring a more aggressive phenotype.

In the first part of this project we wanted to understand whether GLAST could be defined as a marker of GSC by investigating his involvement in tumorigenesis. GLAST positive cells were first isolated from murine GL261 using an immunomagnetic sorting and compared with GLAST negative sub-population or unsorted cells. In vivo experiments showed that GLAST positive cells are more aggressive and lethal compared to the GLAST negative sub-population. In vivo monitoring performed by MRI at different time points after GL261 implantation have confirmed the hypothesis that GL261 GLAST positive-derived tumors are more aggressive compared with GLAST negative-derived tumors, causing a more extended lesion due to the migration of cells through the ventricles to the contralateral hemisphere. These preliminary observations have allowed us to define GLAST as a potential marker of a more aggressive sub-population of GSC and moreover a specific therapeutic target.

In several published studies expression of aberrant protein in cancer cells was used to develop vaccines: HER-2 for example has been used as a target in breast and ovarian cancer [218], MUC-1 and WT-1 in several

types of cancer, including lung, stomach and in some haematological cancers [219], EGFRvIII has been reported as a suitable target in GB [152] but until now GLAST has not previously been reported to be expressed in GSC.

In term of preclinical studies, we provided an immunotherapeutic approach with four GLAST-derived peptides. In our study, GLAST-derived peptides were emulsified with the adjuvant Montanide™ ISA 51 VG and administered in conjunction with GM-CSF were effective in prolonging survival, enhancing systemic and local immunity, and modifying the tumor microenvironment.

Loss of GLAST expression in gliomas from immunized mice provided evidence of the induction of a specific antitumor immune response against GLAST-expressing tumor cells that resulted in subsequent immunoediting and tumor escape [156, 220]. A recent example of immunoediting was provided by studies involving immunotherapy targeting the tumor-specific EGFRvIII mutation, which is highly expressed in GB [155].

In spite of immunoediting, however, we found that targeting the radial glia marker GLAST significantly increased the survival of mice bearing GL261 gliomas. We also observed a remarkable modulation of the glioma microenvironment and an increase in peripheral NK cells in immunized mice. In addition, the NK cell-specific transcription factor E4BP4 [221] was expressed at higher levels in gliomas from immunized mice than in those from control mice, confirming the contribution of NK cells to the antitumor response.

NK-cell infiltration led to increased IFNγ expression, and its local accumulation exacerbated CXCL10 production in the glioma microenvironment. CXCL10, in turn, was responsible for the recruitment of antigen-specific CTL, defined as Tc1, to GL261 gliomas. The production of IFNγ and the expression of VLA-4 by antigen-specific Tc1 cells were found

to be critical for efficient infiltration into the glioma mass [222].

NK cells are able to recognize the loss of MHC I molecule, an important event in tumor immunoediting. Recent studies also show the potential of an NK-cell response in fighting GSC. In agreement with these findings, we detected upregulation of NKG2DL expression in gliomas from immunized mice. NKG2DL is weakly expressed or absent in GSC [223], and TGFβ seems to be responsible for both this decreased expression of NKG2DL [224, 225] and the downregulation of NKG2D receptor on NK cells [226]. Thus, the down-regulation of TGFβ observed in mice immunized with GLAST peptides could influence NKG2DL expression and reverse the suppression of NK cells. The efficacy of our therapeutic strategy could be hampered by immune tolerance, as GLAST is expressed by the normal CNS tissue; conversely, the absence of tolerance could result in autoimmune reactions. Our data suggest that the absence of toxicity following GLAST-derived peptide immunization may be attributed to the recruitment of activated immune cells to the tumor site by a chemotactic gradient. In particular, gliomas in GLAST peptide-treated mice displayed upregulation of CXCL10 concomitant with downregulation of CCL2, an important chemokine involved in Treg migration [106]. Furthermore, we found that CCL5 was highly expressed in gliomas of immunized mice.

This chemokine may play a dual role depending on its source. CCL5/RANTES (regulated upon activation, expressed and secreted by normal T cells) inuces chemotaxis in T cells, monocytes, dendritic cells, NK cells, eosinophils and basophils [227-229]. When produced by CD8+ T cells, CCL5 is associated with effector functions, together with CCL3 and CCL4 [230-232], and antigen-specific T cells are activated in response to CCL5 [233]. In contrast, tumor secreted CCL5 is deleterious and acts as an immunosuppressor, and its inhibition improves the efficacy of immunochemotherapy [234]. GL261 gliomas treated with vehicle express

low levels of CCL5, whereas gliomas from mice immunized with GLAST peptides express much higher levels of CCL5. These findings support the idea that, in our experimental system, CCL5 expression is related to the presence of infiltrating immune cells.

In conclusion, both NK cells and CD8+ T cells can play an important role in tumor rejection: NK cells may be one important source of IFNγ a crucial cytokine that mediates CD8+ T-cell activation [235]; our protocol of immunotherapy with GLAST-derived peptide delayed and, at least in some cases, abolished tumor growth, resulting in the survival of 40% of the immunized mice. Recent studies suggest that some chemotherapeutic agents possess immune stimulatory effects [236, 237]. We thus plan to design immunochemotherapy combinations in an attempt to potentiate the specific antitumor immunity achieved following GLAST peptide immunization. In addition, a more complete appraisal of the safety of GLAST immunotherapy is essential to assess the translational potential of these findings.

The main result of this study is the definition of GLAST as an attractive glioma-associated antigen for targeting in peptide-based immunotherapy in GB patients. To understand if GLAST could also represent a good predictive marker in association with the overall survival of patients with GB we examined a series of GB specimens by immunohistochemistry. We found that a high percentage of GLAST+ cells and a strong or moderate reactivity correlated with a lower overall survival of patients. Several published work described the pathophysiology of GLAST signalling and help us to understand our observation. In fact, as previously mentioned, GLAST has a physiological role in the glutamate uptake after neurotransmission; extracellular concentration of glutamate is maintained very low which preventing neurotoxicity and CNS damage [195]. However, in several pathological conditions such as ischemia, hypoxia, and neurodegenerative disorders, astrocytes can reverse glutamate uptake

acquiring a skills release of the neurotransmitter [188]. Cells from glioma have been shown to have the same altered activity: the uptake of glutamate appears to be reduced and its elevated extracellular concentrations suggest a strong release [211]. In a study of Ye and colleagues, glutamate transporters were characterized in several human glioblastoma cells. They found an over- expression of the cystine-glutamate transporter, a reduction or a complete absence of the Glt-1 transporter, and a translocation of GLAST in the nuclear membrane [192]. These differences and the abnormal activity of glutamate transporters on the tumor cells are the major causes of the extracellular glutamate accumulation. *In vivo,* the release of glutamate in the tumor microenvironment was confirmed both in glioma models [195] and in humans [238]. Malignancy rate appears to be related to the amount of glutamate released and its presence in the tumor microenvironment [195]. The high extracellular glutamate levels may have two important consequences: to act as a growth factor for tumor cells themselves, to induce apoptosis of parenchyma cell [203] Infact, the higher concentration of glutamate at the synaptic level increases the activation of NMDA and AMPA receptors causing neuronal hyperexcitability resulting in a Ca^{2+}-dependent neurotoxicity. The increase in intracellular Ca^{2+} induces a mitochondrial dysfunction and the activation of different lipase, protease, phosphatase and nuclease that induce apoptotic pathway.

The evaluation of important roles of GLAST in aberrant glutamate release could support its relevance in the biology of glioblastoma. In our murine model, the association of immunotherapy and chemotherapy may represent an attractive approach to modulate chemokine expression in tumor microenvironment, reinforcing the recruitment of additional effector cells and enhancing the therapeutic efficacy of vaccinations.

6. Future Perspectives

Development of therapeutic vaccines has been the subject of several phase I or phase II studies especially based on the use of DC loaded with glioma-derived tumor antigens. These studies provided data on safety and efficacy in clinical trials using autologous tumor lysate-loaded DC [137, 138, 240].

At the Fondazione I.R.C.C.S. Istituto Neurologico “Carlo Besta” our experience with DC immunotherapy started in 2006 in close collaboration with the Cell Factory located within our Institute. Six patients suffering from GB were treated in 2006-08 on a compassionate basis, whereas in 2010 and 2011 respectively, two phase I studies called DENDR2 (EudraCT 2008-005038-62) and DENDR1 (EudraCT 2008-005035-15), coordinated by Dr. Finocchiaro, were approved by the Ministry of Health and started. In both studies we are testing the effects of immunotherapy with DC loaded with tumor lysate in conjunction with chemotherapy (CT) with TMZ in patients with first diagnosis (DENDR1) and recurrence (DENDR2) of GB.

The therapeutic program of DENDR1 includes radical surgical resection of the tumor, followed by radiotherapy (RT) and concomitant adjuvant CT with TMZ according to the Stupp protocol [1]. Immunotherapy follows concomitant RT/CT, and it includes seven injections of tumor lysate-loaded DC overall.

The therapeutic program of DENDR2 for recurrent GB patients includes surgical resection of the tumor, followed by immunotherapy with 5 injections of tumor lysate-loaded DC and simultaneous treatment with TMZ. RT and TMZ chemotherapy are not performed on these patients because they have already experienced this treatment before and they have presented recurrence despite that.

Nevertheless, it is already clear that the definition of novel targets and the modification of immune suppression at the tumor site are important points to be addressed if DC immunotherapy has to added to the list of standard

treatments available for GB. At the same time the existence of cancer stem cells requires deeper studies to be accepted and understood.

As a preclinical data, we have first demonstrated in the GL261 model that DC targeting GB cancer stem cells (GSC) are particularly effective in eliciting anti-tumor responses [168]. A patent has been derived from this study (US2010071081 -A1-; www.epo.org).

In preliminary experiments in the GL261 model of malignant glioma we have found that mice vaccinated with DC loaded with GSC lysate survive longer than others vaccinated with tumor lysate (unpublished data). In our laboratories we isolate and culture GSC from fresh GB specimens growing in the absence of serum and in the presence of EGF and bFGF as neurospheres after mechanical and enzymatic dissociation. We appreciate that NS may mirror much more closely than previous, serum-based glioma cell lines, the actual biology of GB [241, 242]. These GSC have been used as an *in vitro* model in many of our published studies [28, 169, 243, 244].

Recently, in our Institution the Cavitation Ultrasonics Surgical Aspirator (CUSA) is preferably used for GB surgery. CUSA facilitates the removal of large tumors from inside out and delivers an irrigating solution that converts the fragmented tissue into an emulsion and then aspirates the particles directly into a sterile bag.

The bag contains some larger pieces of tissue, debris and high amounts of erythrocytes, and occasionally necrotic or reactive tissue. Tissue fragments in CUSA bags after several rounds of spinning ad washing are dissociated using the GentleMacs Dissociator (Miltenyi Biotec) that provides a closed system and reproducible results. We have optimized a gentle and effective protocol starting from appropriate gentleMACS programs, which allows obtaining a high yield of viable tumor cells. After processing cell suspensions are cultured as neurospheres in DMEM/F12, B27 supplement, human recombinant b-FGF and EGF.

During the last year we obtained from the Department of Neurosurgery of Istituto Besta a total of 41 CUSA. GB were the most represented brain tumors (78%). Using tumor fragments obtained from surgery and combining a mechanical dissociation with enzymatic disaggregation on a series of primary GB we previously obtained GSC in 52% of the cases. This percentage is now increased to 70% with the use of surgical material in CUSA and the optimized processing. This improvement and the use of a closed system providing a wider margin of safety encouraged us to incorporate this process in a clinical trial protocol which plans to use GSC as a source of antigens for DC replacing the whole lysate from GB specimens used in current immunotherapy protocols presently active at Istituto Besta. Another contribution for our study would be related to the identification of GSC markers and their investigation as a clinical marker in association with the prognosis of brain tumor patients.

The identification of five genes related to the radial glia signature, including GLAST (Glutamate Aspartate transporter) stems from our studies on expression profiles of murine GL261 GSC and confirmed in human GB GSC [168]. GLAST has been selected as a GSC marker and its enrichment impacts on tumor aggressiveness and progression, constituting a background for translational relevance

In our study we showed that immunization with GLAST peptides efficiently promotes specific anti-tumor response in the murine glioma GL261, preventing the tumor progression only in 40% of immunized mice. In term of preclinical studies, we hypothesize that chemo-immunotherapy combination using GLAST peptides may convert the glioma microenvironment, especially through chemokine expression, into a site permissive for an efficient immune response and also act as an adjuvant able to instigate and sustain both systemic and local tumor response [**Fig.1**].

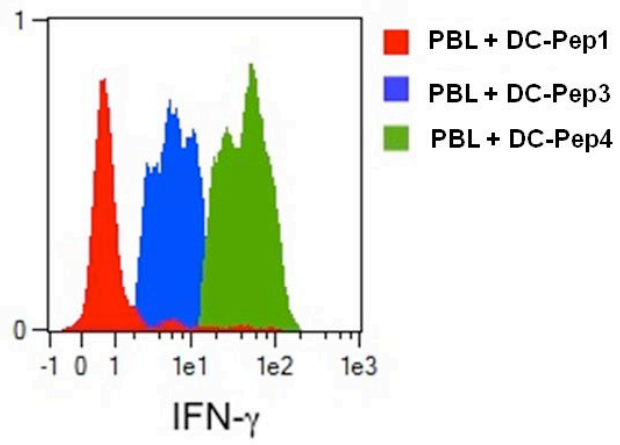

Fig. 1 Cytotoxic T lymphocytes produce IFNγ after peptide-loaded DC stimulation. CD3+CD8+ were gated and evaluated for the IFN-γ production by flow cytometry. Peptide 3 and 4 induced a significant response compared to peptide 1 and 2 (not shown) that failed to stimulate T cells.

7.References

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8. Scientific Production Relative To The Present Work

Published paper

Gabriele Cantini, Federica Pisati, Sara Pessina, Gaetano Finocchiaro and Serena Pellegatta. Immunotherapy against the radial glia marker GLAST effectively triggers specific antitumor effectors without autoimmunity. Oncoimmunology (2012) 1:6, 884-893
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Poster presentation

- Effective Immunotherapy against the neural stem cell marker GLAST in a murine model of malignant glioma.
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Abstract presented at conferences

- Immunotherapy Against the Neural Stem Cell Marker GLAST is Effective in a Murine Model of Malignant Glioma.
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