

# Immunotherapy against the radial glia marker GLAST effectively triggers specific antitumor effectors without autoimmunity

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The glutamate-aspartate transporter GLAST is a radial glia marker that is highly expressed in GL261 stem-like cells (GSCs). To target GLAST, we treated glioma-bearing mice with three subcutaneous injections of four GLAST peptides emulsified with Montanide ISA-51 in association with granulocyte macrophage colony-stimulating factor (GM-CSF) injections. Vaccination with GLAST peptides significantly prolonged survival, effectively enhanced systemic T-cell and NK-cell responses and promoted robust antitumor cytotoxicity. GLAST expression significantly decreased in gliomas from immunized mice, as evaluated by histological analysis and real-time PCR (RT-PCR). Moreover, the immunization protocol led to the upregulation of interferon $\gamma$  (IFN $\gamma$ ) and tumor necrosis factor $\alpha$  (TNF $\alpha$ ) as well as to the downregulation of transforming growth factor (TGF)  $\beta$ 1 and  $\beta$ 2 in the tumor. Beyond these changes, gliomas from immunized mice exhibited an increased recruitment of NK cells and antigen-specific CD8 $^+$  T cells expressing the tumor homing molecule VLA-4, as well as a local chemotactic gradient featuring expression of CXCL10 (which may be responsible for the recruitment of CTLs), CCL3, CCL4 and CCL5 (which are involved in NK-cell migration), and NKG2D ligand on glioma cells. Importantly, although GLAST is expressed in the central nervous system, autoimmune reactions were not observed in immunized mice. Altogether, these results support the contention that GLAST may constitute a glioma antigen against which immune responses can be efficiently induced without major safety concerns.

## Introduction

The current standard treatment for glioblastoma (GB) involves surgery, radiotherapy and chemotherapy with temozolomide (TMZ) and is associated with an overall survival (OS) of 14.6 mo.<sup>1,2</sup> Phase I/II clinical trials of anti-GB immunotherapy have demonstrated the feasibility and the safety of this approach. Moreover, immune-mediated antitumor responses have been detected in these studies.<sup>3–10</sup> Dendritic cells (DCs) loaded with multiple antigens (i.e., tumor cell lysates, tumor-eluted peptides or fusion products of DCs and GB cells) were used to reduce the risk of tumor escape due to antigen-loss variants. Interestingly, strategies focused on promoting antitumor immune responses against tumor-associated antigens in the central nervous system (CNS) have also been proposed.<sup>11,12</sup> In addition, the identification of GB cell subpopulations displaying stem cell gene expression programs provided the foundation for immunological studies aimed at targeting these cells. Thus, murine and human GB cell populations contain a fraction of cells with stem cell-like features, and it has been proposed that only this population may be responsible for glioma recurrence. Several groups, including ours, found that GB populations enriched in glioma stem-like

cells (GSCs) can give rise to gliomas that closely resemble the original tumor but that are rather different from the experimental gliomas generated by brain injections of serum-driven established cell lines.<sup>13,14</sup> GSCs have been found to maintain the genetic alterations of their originating tumor and are tumorigenic in nude mice.<sup>15,16</sup> We have found that GSCs can also be obtained from established cultures of murine gliomas, such as GL261 cells. DCs loaded with antigens from GL261 GSCs were significantly more effective than DCs loaded with antigens from serum-cultured GL261 cells in inducing immune rejection of highly malignant gliomas that were otherwise lethal in approximately one month. These findings provide a proof of principle that targeting cell populations enriched in GSCs may increase the efficacy of anti-glioma (and possibly anti-tumor) immunotherapy.<sup>17</sup> Characterization of the gene expression profiles of these cells revealed five genes related to radial glia, a source of neural stem cells located in the subventricular zone (SVZ) of the adult brain, that were upregulated in GL261 GSCs.<sup>18</sup> We focused our attention on the surface marker GLAST (astrocyte-specific glutamate-aspartate transporter), which plays an important role in glutamate uptake and the regulation of excitatory neurotransmission and prevents glutamate-mediated excitotoxicity in the

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Il Fondo di Gio

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**Flow cytometry.** Lymphocytes from spleens, cervical draining lymph nodes and explanted gliomas of immunized and control mice were used for immune monitoring. Briefly,  $1.5 \times 10^6$  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. Flow cytometry acquisition was performed on a MACSQuant instrument, and the data were analyzed with the MACSQuantify Software (Miltenyi Biotec).

**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  $\Delta\Delta C_t$  method. The expression levels of the target genes were normalized to the expression level of  $\beta 2$ -microglobulin. The sequences of the primers are reported in the **Supplemental Materials**.

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**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 $\times$  objective of a Leica DM-LB microscope.

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

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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## Supplemental Materials

Supplemental materials may be found here: [www.landesbioscience.com/journals/oncoimmunology/article/20637](http://www.landesbioscience.com/journals/oncoimmunology/article/20637)

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