Definition of the immunological properties of cancer stem cells isolated from human glioblastoma

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The cancer stem cell hypothesis

Cancer stem cell (CSC)
Nontumorigenic cancer progenitor cell
Nontumorigenic cancer cell

Tumor response following conventional cancer therapies
Tumor recurrence with expansion of CSC pool
Treatment with CSC-targeted therapy
Tumor regression

Cao S et al. (2013) Cancer stem cell in and g Ingram
Nat Clin Pract Oncol 10 10.1038/ncponc0642
Neurospheres and the neurosphere assay

CSCs can be defined as:

1) self-renewing cells;

2) cells that give rise to the variety of differentiated cells found in the malignancies (multipotency);

3) cells able to generate a phenocopy of the original malignancy in immunocompromised mice (tumorigenic ability).
Self renewal, tumorigenicity and differentiation ability of GBM CSCs

By Mazzeni S. et al. Submitted
**EXPRESSION OF NEURAL STEM CELL-ASSOCIATED MOLECULES AND OF TRANSCRIPTION FACTORS BY GBM CSCs AND FBS TUMOR CELLS.**

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Molecule</th>
<th>c-Myc</th>
<th>Nestin</th>
<th>Nanog</th>
<th>S100A4</th>
<th>S100A6</th>
<th>Sall4</th>
<th>SOX2</th>
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<tbody>
<tr>
<td>080125 CSCs</td>
<td></td>
<td>2</td>
<td>n.d.</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>5</td>
<td>7</td>
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<tr>
<td>080125 FBS</td>
<td></td>
<td>2</td>
<td>n.d.</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>8</td>
<td>3</td>
</tr>
<tr>
<td>080418 CSCs</td>
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<td>2</td>
<td>2</td>
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<td>4</td>
<td>38</td>
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<tr>
<td>080418 FBS</td>
<td></td>
<td>4</td>
<td>15</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>20</td>
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<tr>
<td>080325 CSCs</td>
<td></td>
<td>1</td>
<td>n.d.</td>
<td>3</td>
<td>4</td>
<td>2</td>
<td>3</td>
<td>15</td>
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<tr>
<td>080325 FBS</td>
<td></td>
<td>1</td>
<td>n.d.</td>
<td>4</td>
<td>1</td>
<td>1</td>
<td>n.d.</td>
<td>8</td>
</tr>
</tbody>
</table>

Data are represented as MRFI that is the ratio between the mean of intensity of fluorescence of the cells stained with the selected mAb and that of the negative control; bold value means MRFI ≥ 2.
Expression of MHC and APM molecules and NKG2DLs in GBM-derived CSCs and FBS tumor cells

- The expression of:
  - MHC class I and II;
  - Antigen processing machinery (APM), using 21 different mAbs directed against HLA molecules, their heavy chains, β2-microglobulin immunoproteasome, constitutive proteasome subunits, chaperon molecules, TAPs etc.;
  - NKG2DLs;
- has been tested in 11 different GBM CSCs and, for 5 of them, in their paired tumor cells grown in the presence of FBS (FBS tumor cells).
Expression and modulation of MHC molecules and NKG2DLs in GBM CSCs vs FBS tumor cells

080125 CSC

080418 CSC

080125 FBS

080418 FBS
APM expression and modulation in CSCs vs FBS tumor cells

A

080125 FBS

GBM cell line

080125 GFD

B

080418 FBS

GBM cell line

080418 GFD
THE CELL LINES WERE TREATED in vitro WITH 10 nM OF 5-AZA-CdR FOR 4 DAYS. THE EXPRESSION OF MHC, APM MOLECULES AND OF NKG2DLs WAS EVALUATED BY CYTOFLUORIMETRIC ANALYSIS; DATA ARE REPRESENTED AS MRFI.
TAA expression in GBM CSCs

Survivin, COA-1 AND SOX2 WERE COMMONLY EXPRESSED IN BOTH CSCs AND FBS TUMOR CELLS; NO EXPRESSION OF MAGE, NY-ESO-1, GP100 AND IL-13Rα2 WAS FOUND.
CSCs can elicit autologous T cell-mediated immune responses

PT. # 070104
Reactivity against CSC or FBS tumor cell lines in autologous setting by T lymphocytes isolated from 4 GBM patients

<table>
<thead>
<tr>
<th>Patient #</th>
<th>T cell line #</th>
<th>Autologous CSC recognition</th>
<th>Autologous FBS tumor cell recognition</th>
<th>MHC-restriction</th>
<th>Cytokine release</th>
<th>TH type subset</th>
</tr>
</thead>
<tbody>
<tr>
<td>070104</td>
<td>2</td>
<td>+++ &lt;sup&gt;b&lt;/sup&gt;</td>
<td>N. A.</td>
<td>MHC I</td>
<td>IFN-γ</td>
<td>TH1</td>
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<tr>
<td>3</td>
<td>+</td>
<td></td>
<td>N. A.</td>
<td>MHC II</td>
<td>IFN-γ</td>
<td>TH1</td>
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<tr>
<td>10</td>
<td>+</td>
<td></td>
<td>N. A.</td>
<td>MHC I</td>
<td>IFN-γ</td>
<td>TH1</td>
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<tr>
<td>080325</td>
<td>1</td>
<td>+</td>
<td>+++</td>
<td>MHC II</td>
<td>IL-5</td>
<td>TH2</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td></td>
<td>++</td>
<td>MHC I</td>
<td>IFN-γ</td>
<td>TH1</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td></td>
<td>++</td>
<td>MHC II</td>
<td>IL-5</td>
<td>TH2</td>
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<tr>
<td>080125</td>
<td>4</td>
<td>+</td>
<td>++</td>
<td>MHC II</td>
<td>IL-5</td>
<td>TH2</td>
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<tr>
<td>080418</td>
<td>1</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>IL-5</td>
<td>TH2</td>
</tr>
</tbody>
</table>

<sup>a</sup>: FBS tumor cells are not available;
THE EXPRESSION OF IMMUNE-REGULATORY MOLECULES WAS EVALUATED BY IF AND CYTOFLUORIMETRIC ANALYSIS;

DATA ARE REPRESENTED AS MRFI;

B7-1 AND B7-2 WERE NOT DETECTED ON THESE CELL LINES.
PBMc were stimulated in vitro with mitogens with or without GBM CSCs or FBS tumor cells; after 72 and 120 hrs the proliferative ability of CD3+ gated cells was analyzed by CFSE staining and cytofluorimetric analysis. This experiment has been repeated twice. The proliferative index and the division index were also calculated.
Whole transcriptome analysis of CSCs vs. FBS tumor cells

GFD vs FBS comparison:
Fold > 1.5, p < 0.005, permutation p < 0.017
469 genes, 393 gene pass 80% present filter.

See gene data list analysis output file and attached.
Genes involved in canonical pathways

- Mitochondrial Dysfunction
- Oxidative Phosphorylation
- Antiproliferative Role of Somatostatin Receptor 2
- Glutathione Metabolism
- Butanoate Metabolism
- Ubiquinone Biosynthesis
- Eicosanoid Signaling
- Methane Metabolism
- Oncostatin M Signaling
- Actin Cytoskeleton Signaling
- PI3K/AKT Signaling
- N-Glycan Biosynthesis
- Melanocyte Development and Pigmentation Signaling
- IGF-1 Signaling

The graph shows the percentage of downregulated, no change, upregulated, and no overlap with dataset genes in different pathways. The y-axis represents the percentage, and the x-axis lists various pathways.
Immune related Gene profile signature

- Genes with immunological function were differentially expressed in CSCs vs FBS tumor cells.

In particular the proteosome maturation protein and the proteasome activator subunit 1 were down-modulated (-1.8 and -2.4 fold change, respectively) in CSCs compared to FBS tumor cells correlating with alterations we found at protein level of APM molecule (i.e. MB1) expression.

- Genes related to IFN signaling, such as IFN regulatory factor binding 2 protein (2.7 fold), Tax1 (3.2 fold), IFNGR1 (1.7 fold) and the TNF receptor-associated factor 2 (TRAF2) (1.8 fold) were also under expressed in CSCs in comparison with FBS tumor cells.

Notably, IL-6 and IL-8 were also found down-modulated in CSCs vs FBS tumor cells and these findings correlating with protein secretion levels we have detected in the supernatants of these cell lines.

- No differential gene expression of IFN-α, IFN-β, TNFR and IL-1 was detected between CSCs and FBS tumor cells.

Conversely, genes involved in JAK-STAT signal pathway were found up-regulated (2 fold) in CSCs compared to FBS tumor cells, in line with the previously reported evidence that members of this protein family were aberrantly activated in a variety of tumors including GBM (Bromberg, 2002; Brantley et al., 2008).
Secretion of suppressive or pro-inflammatory cytokines by CSCs vs. FBS tumor cells

Cytokine detection in the supernatants was detected by ELISA or Searchlight assays; no detection in the sups of IL-10, IL-13 and TNF-α was observed.
Immunobiological differences between CSC and FBS tumor cell lines.

<table>
<thead>
<tr>
<th>Immune-related molecules/activity</th>
<th>MHC I</th>
<th>MHC II</th>
<th>NKG2DLs</th>
<th>APM</th>
<th>IFN modulation (^a)</th>
<th>5-Aza-(CdR) (^b)</th>
<th>T cell-mediated recognition</th>
<th>Suppressive activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSCs</td>
<td>+</td>
<td>-/+</td>
<td>+/-</td>
<td>+</td>
<td>+</td>
<td>+/-</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>FBS</td>
<td>++</td>
<td>-/+</td>
<td>+/-</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+/-</td>
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</table>

<table>
<thead>
<tr>
<th>Stem cell-associated molecule and/or TAA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nestin</td>
</tr>
<tr>
<td>CSCs</td>
</tr>
<tr>
<td>FBS</td>
</tr>
</tbody>
</table>

\(^a\): modulation of the expression of MHC, NKG2D and APM molecules following \textit{in vitro} treatment of the cells with either IFN-\(\alpha\) or \(\gamma\),

\(^b\): modulation of the expression of MHC, NKG2D and APM molecules following \textit{in vitro} treatment of the cells with the demethylating agent 5-Aza-(CdR).
Conclusions

A Low immunogenic profile was found in both CSCs and FBS tumor cells isolated from GBM patients, with higher defective APC pattern in CSCs;

the immune profile can be rescued, though more efficiently in FBS tumor cells, by treatment with IFNs or with 5-Aza-CdR of GBM cell lines;

T cell-mediated immune responses can be isolated from GBM patients, though mostly TH2-mediated subset;

Differential gene signature, including immune related genes, was detected in CSCs vs FBS tumor cells; in some cases we could confirm these results at the protein levels (ELISA; SEarchLight).
Future directions

- To identify the TAA recognized by anti-GBM CSC T lymphocytes;
- To carry out functional experiments based on gene profile data to possibly identify CSC-associated markers and/or TAA;
- To investigate whether the ability of CSCs to elicit T cell-mediated immune responses can be increased by usage of antagonist mAbs directed against negative immune regulatory molecules (anti-CTLA-4 or -PD-L1);
- To translate all the obtained information to design CSC-specific immunotherapy protocols for GBM patients.
ACKNOWLEDGEMENT

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- Rossella Galli
- Soldano Ferrone
A HYPOTHETICAL MODEL OF THE RELATIONSHIP BETWEEN PRIMARY TUMOR-DERIVED TUMOR STEM CELLS AND GBM TUMOR CELL LINES

<table>
<thead>
<tr>
<th></th>
<th>NBE-cultured GBM cells</th>
<th>Serum-cultured GBM cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proliferation</td>
<td>Constant</td>
<td>Limited growth, reaches plateau, followed by exponential growth</td>
</tr>
<tr>
<td>Clonogenicity/Tumorigenicity</td>
<td>Clonogenic and tumorigenic regardless of passages</td>
<td>Not at early passages</td>
</tr>
<tr>
<td>Differentiation potential</td>
<td>Induce to become glial and neuronal lineages</td>
<td>Do not respond to differentiation stimuli</td>
</tr>
<tr>
<td>Telomerase activity</td>
<td>Positive</td>
<td>Negative initially, but became positive at late passages</td>
</tr>
<tr>
<td>Tumor histology</td>
<td>Extensive migration/infiltration Phenocopy primary human GBMs</td>
<td>Fail to show infiltration similar to common glioma lines</td>
</tr>
<tr>
<td>Global gene expression</td>
<td>Similar to primary human GBMs</td>
<td>Different from primary tumors similar to common glioma lines</td>
</tr>
<tr>
<td>NSC-related genes</td>
<td>Nestin, Sox2, CD133, Musashi, Bmi</td>
<td>-</td>
</tr>
<tr>
<td>Genotype</td>
<td>Same as parental tumors regardless of passages</td>
<td>Additional genomic alterations not found in parental tumors</td>
</tr>
</tbody>
</table>

LEE J. ET AL., CANCER CELL 2006
### in vivo Expression of MHC and APM molecules in GBM lesions

<table>
<thead>
<tr>
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<th>070104</th>
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<td><strong>SOX2</strong></td>
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<td><img src="image11.png" alt="Image" /></td>
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## Tumors Containing Cancer Stem Cells

<table>
<thead>
<tr>
<th>Tumor Type</th>
<th>Markers</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chronic myeloid leukemia (CML)</td>
<td>BCR-ABL CD34⁺ CD38⁻</td>
<td>(Barret et al, 2003)</td>
</tr>
<tr>
<td>Melanoma</td>
<td>CD20⁺</td>
<td>(Fang D et al, 2005)</td>
</tr>
<tr>
<td>Prostate</td>
<td>CD44⁺⁺ CD11⁺ CD133⁺</td>
<td>(Collins AT et al, 2005)</td>
</tr>
<tr>
<td>Liver cancer</td>
<td>CD90⁺ CD44⁺</td>
<td>(Yang ZF et al, 2008)</td>
</tr>
<tr>
<td>Glioma</td>
<td>AC133⁺ and/or EGFR⁺</td>
<td>(Sinh SK et al, 2004; Galli R et al, 2004; Liu G et al 2006)</td>
</tr>
</tbody>
</table>
Aims

To characterize the immune profile of CSCs isolated from GBM patients;

to analyze the immunobiological functions of GBM CSCs compared to their autologous differentiated tumor cells (grown in vitro in the presence of FBS and, referred as FBS tumor cells);

to define whether CSCs can represent suitable targets for immune based therapeutic interventions for GBM patients.
IFN-α modulation of the expression of antigen processing molecules by CSCs

![Bar chart showing MRFI values for different molecules under different conditions](image)

**MRFI**: ratio between the mean fluorescence intensity of cells stained with the selected mAb and that of cells stained with isotype-matched control mouse immunoglobulins.
Pro-angiogenic factor release by both GBM CSCs and FBS tumor cells

VEGF, FGF-b AND ANGIPOIETIN 2 WERE ALSO FOUND IN THE SUPS, WHILE NO SECRETION OF MPIF1 AND EOTAXIN WAS FOUND.