Supplementary materials and methods

Cell culture
The cell lines, U-373 MG and U87, were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Euroclone, Milan - Italy), supplemented with 10% fetal bovine serum (FBS) (Life Technologies, Carlsbad, CA - USA), 2 mM GlutaMax (Life Technologies) and 100 mg/ml penicillin/streptomycin (Euroclone). pHGG cells were processed and cultured in GBM media: DMEM with 10% FBS, 2 mM GlutaMax, 100 mg/ml penicillin/streptomycin, 7.5% Sodium bicarbonate solution (Merck KGaA, Darmstadt, Germany), 100mM Sodium Pyruvate (Merck) and 1% Non-Essential Amino Acids (Euroclone). All cell lines were maintained in a humidified atmosphere containing 5% CO₂ at 37°C, routinely tested for mycoplasma infection and authenticated by short-tandem repeat analysis (Eurofins Genomic, Ebersberg, Germany). For the pHGG, DNA derived from autologous PBMCs was used as reference.

Healthy donor PBMCs and pHGG
Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats obtained from healthy donors after informed consent was signed, in accordance with the rules set by our Institutional Review Board (OPBG Ethical committee, Rome, N°969/2015) using Lymphoprep™ density gradient medium (Eurobio, Courtaboeuf, France). T lymphocytes were then isolated using the Pan-T Cell Isolation Kit (Miltenyi, Bergisch Gladbach, Germany), according to the manufacturer’s instructions, and used for the in vitro and in vivo experiments. pHGG samples, including paraffin-embedded specimens and fresh biopsies, were obtained from patients undergoing surgery at OPBG; the diagnosis was confirmed by the OPBG neuropathologist (F.D.C.) and the study was approved by the Institutional Review Board.

Immunohistochemistry (IHC)
Post-resection pHGG tissues were paraffin-embedded, sectioned, stained with hematoxylin and eosin (H&E) and with an EphA2 antibody (A2/EPHA2, Rabbit anti-human monoclonal antibody (mAb) - Immunological Sciences, Rome, Italy) and examined by a pathologist (F.D.C.) in a blinded fashion. A quantification score of the
antigen was established, evaluating both the intensity and percentage of positive cells within the tumor.

For the tissues derived from the animal studies, samples were collected, fixed, processed and stained according to standard procedures. We performed H&E and IHC for Ki-67 (MIB-1, Mouse anti-human mAb - Agilent technologies, Santa Clara, CA, USA, 1:200).

**Facs analysis**

Expression of cell surface molecules was determined by FACS using standard methodology. Anti-human mAb were purchased from BD Biosciences (Franklin Lakes, NJ, USA), Miltenyi, eBiosciences (Santa Clara, CA, USA). The characterization of T cells has been performed gating on CD45 positive cells, in order to exclude tumor cells. The following mAbs were used: CD4-PerCP, CD8-BUV395, CD25-PE and -BB515, CD45-APC-H7 and -BUV805, CD45RA-APC, CD45RO-BV510, CD56-FITC, CD62L-BV605, CD95-BV421, CD127-PeCy7, CD197-BV421, FoxP3-PE, Ki67-Alexa647, Lag3-FITC and PD1-BV786. Samples were acquired with a BD LSR-Fortessa X-20 and analyzed using FACS-Diva (BD Biosciences) and Flowlogic softwares (Miltenyi). For each sample, a minimum of 20000 events were acquired.

**Immunofluorescence data**

Primary and immortalized cell lines were grown in bidimensional, black, 12-well tissue culture plates with glass bottom (Euroclone). Cells were fixed with 4% paraformaldehyde at room temperature (RT) for 10 minutes, washed three times with Phosphate-buffered saline (PBS), permeabilized with 0.5% Triton X-100 solution (Merck) for 10 minutes at RT and then blocked with appropriate serum according to the species of the secondary antibody for 1h at RT. Primary antibodies directed against Nestin (Merck, 1:400), SOX2 (Cell Signaling, Danvers, MA, USA, 1:400), GFAP (Agilent, 1:50), CNPase (Merck, 1:200), TUJ-1 (Covance, Princeton, NJ, USA, 1:2000), Olig-2 (Merck, 1:200), and Musashi-1 (Merck, 1:200) were added and incubated overnight at 4°C. Cells were then washed in PBS three times and incubated with Alexa Fluor488/555-conjugated secondary antibodies for 1h at RT. Nuclei were counterstained with DAPI, samples mounted with Vectashield (Vector Laboratories) and examined using a Leica DM2500 fluorescence microscope or a Zeiss LSM700 confocal microscope.
Milliplex assay and TGFβ ELISA
Supernatants from co-cultures were collected after 24h to measure the following cytokines by MILLIPLEX® Multiplex assay using Luminex® according to the manufacturer's instructions: GM-CSF, IFN-γ, IL10, IL12p70, IL13, IL15, IL17A, IL17E, IL17F, IL1β, IL2, IL21, IL22, IL23, IL27, IL28A, IL31, IL33, IL4, IL5, IL6, IL9, MIP3α, TNFα and TNFβ. For the detection of TGFβ, simple-plex ELISA has been performed using the ELLA biosystem (ProteinSimple, San Jose, CA, USA).

Xenograft mouse model for in vivo studies
Six/seven-week-old female NOD.Cg-PrkdcscidIl2rgtm1Wjl/SzJ mice were purchased from Charles River and maintained in the animal facility Plaisant Castel Romano, Rome, Italy. Mice were used to establish two different murine models, each one consisting of 4 groups and 6 animals/group: a subcutaneous (s.c.) and an orthotopic model. In the orthotopic model, U373, gene-modified to express the firefly luciferase and the green fluorescence protein (FF.Luc/GFP), were stereotaxically implanted (0,2x10⁶/2μl) in the brain using the following coordinates: 2 mm posterior to the bregma, 1 mm lateral to the midline and 3 mm ventral from the surface of the skull at an infusion rate of 1μl/min. Seven/10 days after OA (6x10⁶vp/2μl) or EAd (6x10⁷vp/2μl) or OA+EAd (6x10⁶vp+6x10⁷vp/4μl) were inoculated intratumorally (i.t.). After 2 days, T cells (5x10⁴/2μl) were inoculated i.t. and 4 days later mice received a second dose of viruses i.t. with anti-CD28 (αCD28) mAb i.p. (7,5μg) and i.t. (1μg) (BD Bioscience). For the s.c. model, mice were implanted with 3x10⁶ U373 FF.Luc/GFP cells in the right flank in presence of matrigel (BD Bioscience). Ten/13 days later, viruses were infused i.t. with the same concentration used in the previous model. At day 13-16, mice received T cells (10x10⁶) i.v. followed by a second dose of viruses at day 17-20 i.t. in presence of αCD28. Tumor growth was measured weekly with the Xenogen-IVIS Imaging System (PerkinElmer, Waltham, MA, USA) and reported as total photon/sec/cm²/sr, as previously described(53).

Data and Code Availability
The RNA sequencing data will be deposited and accessible on the NCBI-GEO database once the manuscript is accepted for publication.
### Supplementary tables

#### Primers Sequences

**Epha2-BiTE**
- Forward 5'-TAACAGCGTGAAGGGCCGGTTCACCA
- Reverse 5'-TAAATGGTCACTCTGTCGCCCACGCTGG

**E1A**
- Forward 5'-TCCGGTTTCTATGCCAAACCT
- Reverse 5'-TCCTCCGGTGATAATGACAAGA

**GAPDH**
- Forward 5'-CATGCCTTCTTGCCTCTTGTCTCTTAGAT
- Reverse 5'-CCATGGGTGGAATCATATTGGAACATGTAA

---

#### Supplementary table 1. Sequences of forward and reverse primers: EpHα2-BiTE, E1A and GAPDH

<table>
<thead>
<tr>
<th></th>
<th>NT</th>
<th>OA</th>
<th>EAd</th>
<th>OA+EAd</th>
</tr>
</thead>
<tbody>
<tr>
<td>INFγ</td>
<td>6,05±2,20</td>
<td>6,26±2,04</td>
<td>1257,31±806,05</td>
<td>1573,93±813,11</td>
</tr>
<tr>
<td>IL2</td>
<td>2,39±2,39</td>
<td>2,69±2,68</td>
<td>269,49±86,33</td>
<td>278,79±106,16</td>
</tr>
<tr>
<td>IL6</td>
<td>163,66±14,64</td>
<td>107,37±19,52</td>
<td>1421,18±1096,72</td>
<td>2367,76±433,69</td>
</tr>
<tr>
<td>TNFα</td>
<td>1,08±0,49</td>
<td>1,14±0,45</td>
<td>478,18±259,44</td>
<td>523,60±251,37</td>
</tr>
<tr>
<td>IL10</td>
<td>1,47±0,63</td>
<td>1,26±0,38</td>
<td>101,46±25,18</td>
<td>132,29±13,45</td>
</tr>
</tbody>
</table>

#### Supplementary table 2. Concentration (pg/ml) of the most relevant Th1/Th2 cytokines released in the supernatant 24h after co-culture, expressed as mean±SD.

<table>
<thead>
<tr>
<th></th>
<th>NT</th>
<th>OA</th>
<th>EAd</th>
<th>OA+EAd</th>
</tr>
</thead>
<tbody>
<tr>
<td>INFγ</td>
<td>40,58±28,03</td>
<td>37,25±14,01</td>
<td>1157,25±610,41</td>
<td>1369,72±566,06</td>
</tr>
<tr>
<td>IL2</td>
<td>38,34±27,04</td>
<td>27,64±9,26</td>
<td>513,54±427,54</td>
<td>487,68±400,58</td>
</tr>
<tr>
<td>IL6</td>
<td>86,00±33,98</td>
<td>60,17±5,41</td>
<td>425,82±159,55</td>
<td>487,27±81,01</td>
</tr>
<tr>
<td>TNFα</td>
<td>15,11±7,43</td>
<td>13,05±2,83</td>
<td>238,25±45,05</td>
<td>420,64±81,13</td>
</tr>
<tr>
<td>IL10</td>
<td>6,63±0,89</td>
<td>7,50±0,66</td>
<td>311,82±239,52</td>
<td>389,56±242,48</td>
</tr>
</tbody>
</table>
Supplementary figure legends

Suppl. Figure 1. Replication of OA and EAd in pHGG cells. Quantification by qPCR of the expression of E1A and EphA2-BiTE, 5 days after infection of pHGGs (n=5). Data are expressed as median ± SEM.

Suppl. Figure 2 Characterization of the T cells in co-culture with tumor infected cells. A) Percentage of CD4/CD8 subpopulation ratio in co-culture with either U373 or (B) pHGGs tumor cells and (C,D) analysis of naïve/memory compartments of CD4+ and CD8+ T cells by FACS, in co-culture with either U373 (C) or pHGGs (D). Data from 9 healthy donors of T cells are expressed as median ± SEM.

Suppl. Figure 3. After co-culture with infected tumor cells, T cells show an enhanced activation profile. Analysis of activation and exhaustion profile of the CD4+ and CD8+ subpopulations of T cells by FACS, after co-culture with infected U373 (A) or pHGGs (B) tumor cells. (C) Characterization of the activation markers CD95 (left graph) on T cells and CD25 (right graph) on cytotoxic, CD8+ T cells after co-culture with U87 tumor cells infected with a lower MOI of EAd (250 vp/cell). Data from an overall of 12 healthy donors of T cells are expressed as median ± SEM.

Suppl. Figure 4. Gene modulation of TGFβ family molecules and their receptors on tumor cells following the different treatments. Gene expression variation of TGFβ-1, 2, 3 and the induced isoform (TGFβI) as well as of the TGFβ receptor-1, 2 and 3 on tumor cells after treatment with either OA, EAd, OA+EAd or NT, in absence (-) or in presence of T cells and with or without αCD28. Data from 3 independent experiments are expressed as mean ± SEM.

Suppl. Figure 5 Pathway enrichment analysis on T lymphocytes following the different treatments. (A) Pathway enrichment based on gene ontology in signal transduction and inflammation genes in T lymphocytes following OA, EAd or OA+EAd treatment. In red are represented the up regulated genes while in green the down-regulated ones. (B) Volcano plots (log2 fold-change on the x-axis vs -log2 adjusted p-
value on the y-axis) of the up/down regulated genes of the transduction and inflammation pathways, after treatment (OA or EAd vs NT).

Suppl. Figure 6 Pathway enrichment analysis on the benefit of αCD28 on T lymphocytes following the different treatments. Contribution of αCD28 on the pathway enrichment based on gene ontology on signal transduction and inflammation genes in T lymphocytes following NT, OA, EAd and OA+EAd treatment. In red are represented the up-regulated genes while in green the down-regulated ones.

Suppl. Figure 7 In vivo antitumor activity of the combined approach and impact of the αCD28 costimulation molecule on the tumor control in the OA+EAd group of treatment. (A) Bioluminescence signal of the engrafted subcutaneous tumors evaluated by IVIS imaging system over time; all the treated mice per group are shown; (B) Bioluminescence signal of the engrafted orthotopic tumors evaluated by IVIS imaging system over time; all the treated mice per group are shown; (C) Evaluation of the tumor growth expressed as bioluminescence signal change in OA+EAd groups of treatment, without or with administration of αCD28. Data are summarized as median ± SEM.

Suppl. Figure 8. The combined treatment shows no toxicity on normal cells. (A) Percentage of apoptotic cells analyzed by FACS (7AAD/AnnexinV apoptotic signature) in MSCs (n=3), HSCs (n=3) and NBTCs (n=3) normal cells. Data of T cells from 3 healthy donors for each cell type are expressed as median ± SEM.