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Supplemental Information

Glioblastoma Bystander Cell Therapy: Improvements in Treatment and Insights into the Therapy Mechanisms

Marta Guerra-Rebollo, Carolina Nogueira de Moraes, Cristina Alcoholado, Carolina Soler-Botija, Lourdes Sanchez-Cid, Olaia F. Vila, Oscar Meca-Cortés, Sara Ramos-Romero, Nuria Rubio, José Becerra, Jeronimo Blanco, and Cristina Garrido

SUPPLEMENTAL FIGURES

Figure S1

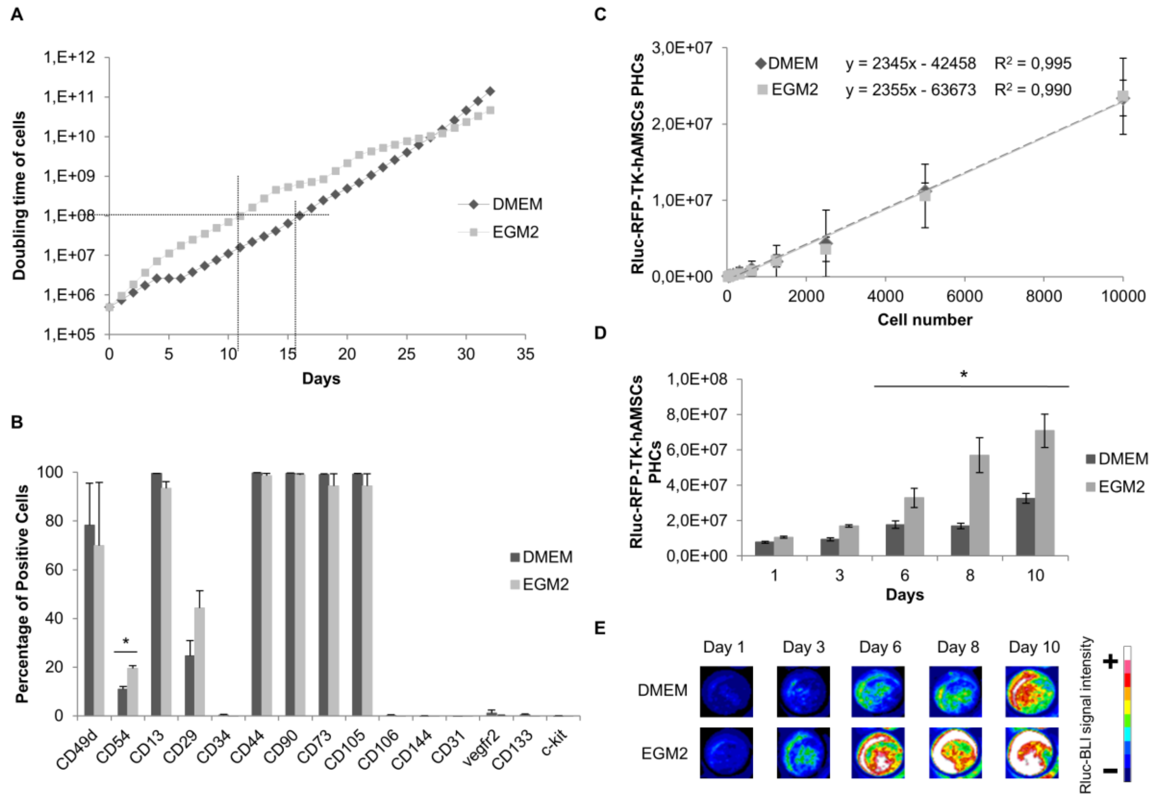

Figure S1. Characteristics of Rluc-RFP-TK-hAMSCs grown in DMEM and in EGM2. (A) Graph comparing the proliferation rate of Rluc-RFP-TK-hAMSCs in DMEM and EGM2. The dotted lines mark the times required for the production of 10^8 cells. (B) Histogram showing the frequency of surface marker expressed by Rluc-RFP-TK-hAMSCs grown in DMEM and EGM2. Data shown are the mean ± SD of two independent experiments. Statistically significant differences (*) were considered when $p < 0.05$ by t test comparison. (C) Standard curve showing linear correlation between light events and cell number for Rluc-RFP-TK-hAMSCs grown in DMEM (n = 5) and in EGM2 (n = 5). Data are represented as means ± SD. (D) Histogram comparing the growth rates of Rluc-RFP-TK-hAMSCs grown in DMEM (n = 6) and EGM2 (n = 6). Data shown are the mean ± SD from three independent experiments (* significant differences were considered when $p < 0.05$ by t test comparison). (E) BLI images of representative wells showing Rluc activity after 1, 3, 6, 8, and 10 days of culture in DMEM and EGM2 medium. Color bar shows the arbitrary standard rainbow color scale used to depict Rluc light intensities (white = highest; dark blue = lowest) in BLI images.

Figure S2

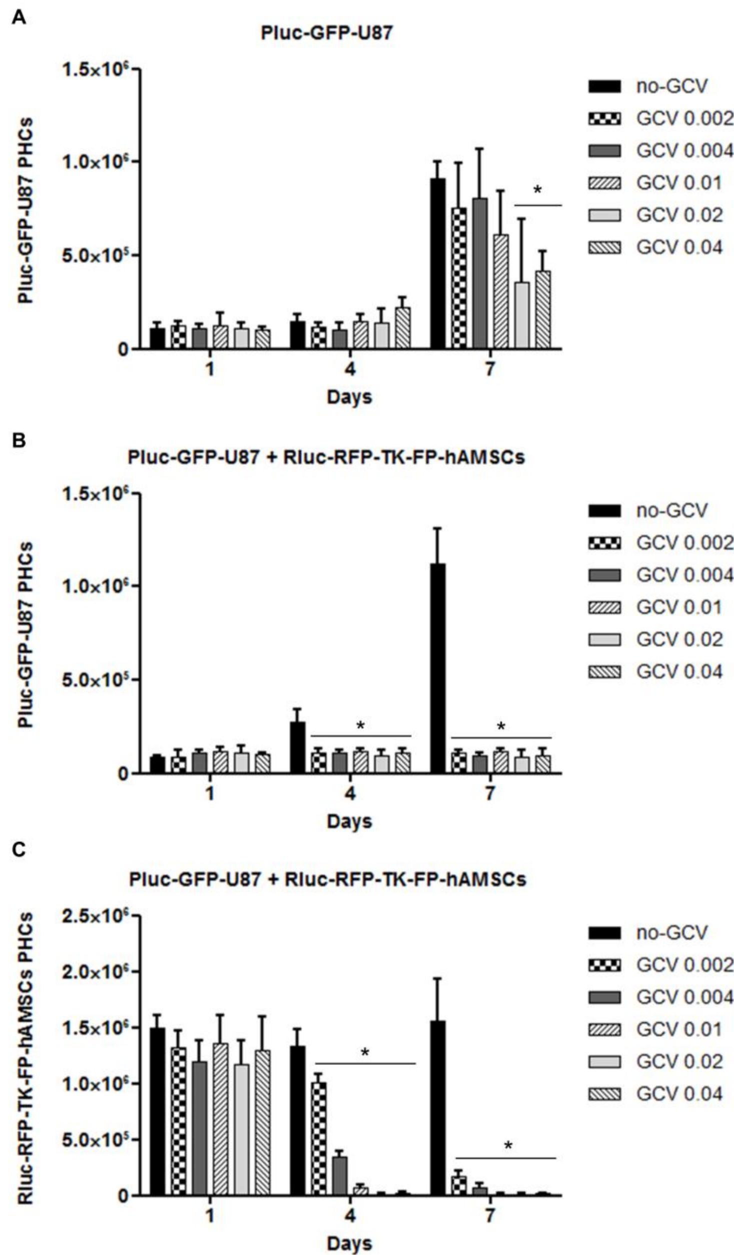

Figure S2. GCV effect on cell growth. (A) Histogram showing concentration dependent cytotoxic effect of GCV on Pluc-GFP-U87 cells. (B) Histogram showing concentration dependent bystander cytotoxic effect of GCV on Pluc-GFP-U87 tumor cells mediated by Rluc-RFP-TK-FP-hAMSCs. (C) Histogram showing concentration dependent cytotoxic effect of GCV on Rluc-RFP-TK-FP-hAMSCs co-cultured with Pluc-GFP-U87 tumor cells. Data bars represent the average of triplicate wells (means ± SD) from triplicate experiments (* significant differences were considered when $p < 0.05$ by anova test comparison adjusted by Bonferroni method).

Figure S3

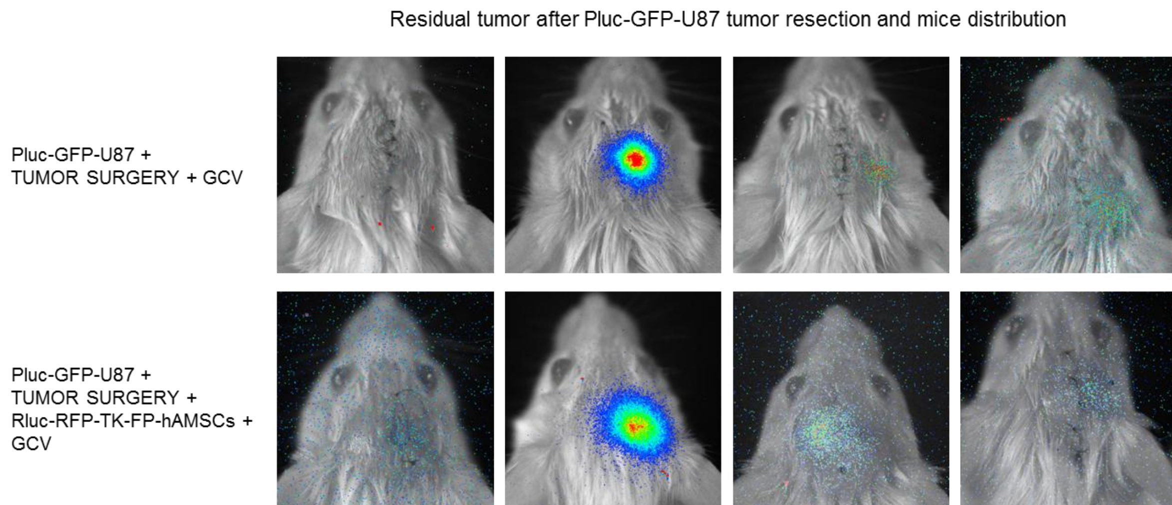

Figure S3. Mice distribution based on residual tumor after Pluc-GFP-U87 tumor resection. Tumor debulking was monitored by BLI signal of remaining Pluc-GFP-U87 cells after surgery. Animals were distributed in the two indicated homogeneous groups according to the level of residual tumor image right after Pluc-GFP-U87 tumor resection.

Figure S4

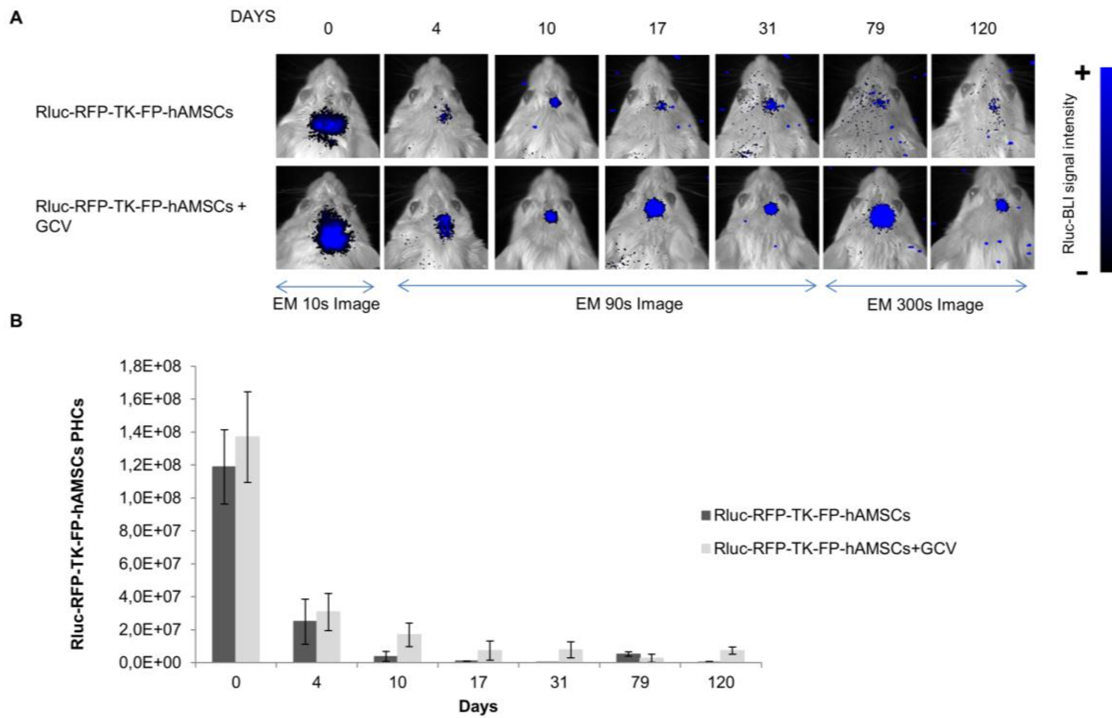

Figure S4. BLI monitoring of Rluc-RFP-TK-FP-hAMSCs implanted in the brain of tumor-free mice treated or not with GCV. (A) Representative bioluminescence high sensitive images (EM) showing the changes in light production by Rluc-RFP-TK-FP-hAMSCs implanted on day 0 in tumor-free mice that were either treated or not with daily intraperitoneal GCV (50 mg/kg) beginning day 4th after cell implantation. The bioluminescence images were superimposed on black and white images of the corresponding mouse heads. Color bar shows the arbitrary standard rainbow color scale used to depict relative light intensities (blue = highest; black = lowest). (B) Graph describing changes in BLI photon counts recorded from Rluc-RFP-TK-FP-hAMSCs implanted mice (n = 4 mice/group) (mean ± SD). No statistically significant differences were detected (t test).

Figure S5

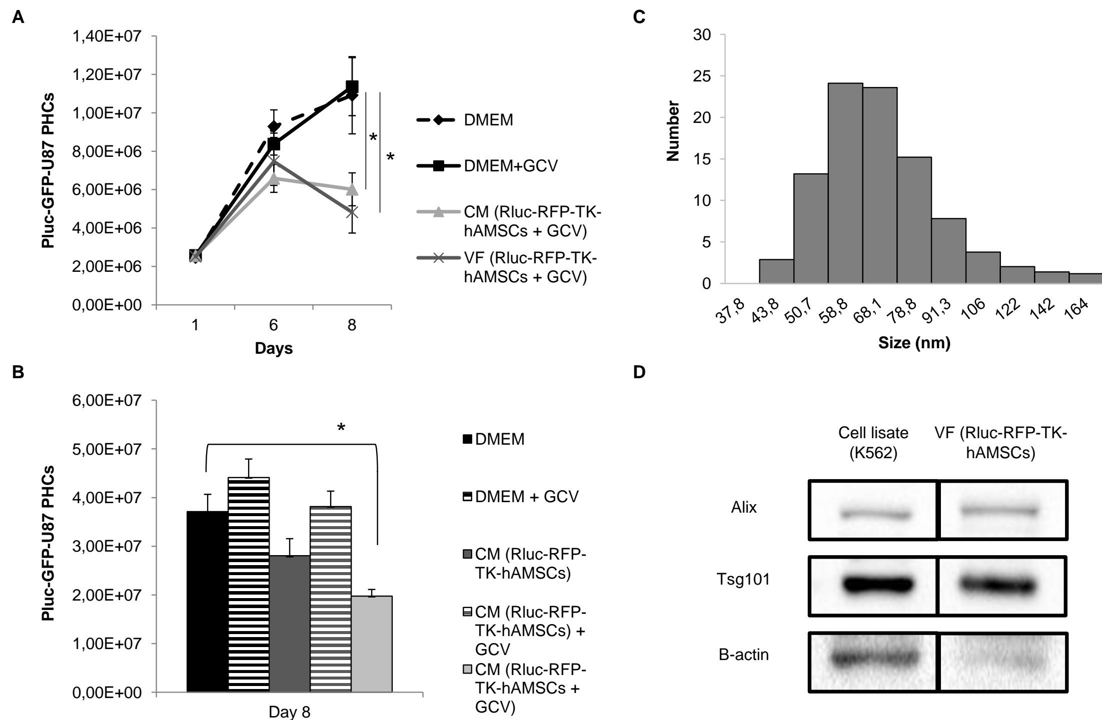

Figure S5. Conditioned medium and ultracentrifuge sub-cellular vesicle fraction from Rluc-RFP-TK-hAMSCs + GCV mediate a cytotoxic effect on Pluc-GFP-U87 tumor cells. (A) Growth of Pluc-GFP-U87 cells in GCV treated conditioned medium (0.004 µg/µl; 48h) from Rluc-RFP-TK-hAMSCs (n = 5) or in the ultracentrifuge extracellular vesicle fraction (VF) from the same GCV treated conditioned medium (n = 5). Cells grown in DMEM ± GCV (0.004 µg/µl) were used as controls. Data points represent the Pluc-BLI signal intensity (mean ± SD). Significant differences (*) were considered when $p < 0.05$ by one-way anova test adjusted by the Bonferroni method, relative to the DMEM group. (B) Pluc-GFP-U87 cells were incubated with conditioned medium from Rluc-RFP-TK-hAMSCs pre-treated with GCV (0.004 µg/µl, 48h); conditioned medium from Rluc-RFP-TK-hAMSCs supplemented with or without GCV at the time of cultivation, or in DMEM ± GCV (n = 6, each condition). Data points represent the Pluc-BLI signal intensity (mean ± SD). Significant differences (*, $p < 0.05$) were found between DMEM group and CM (Rluc-RFP-TK-hAMSCs + GCV). Statistical analysis was made using one-way anova test adjusted by the Bonferroni method, relative to the DMEM group. (C) Histogram showing size distribution and number (Zetasizer Nano ZS) of VF isolated from Rluc-RFP-TK-hAMSCs conditioned medium by ultracentrifugation. (D) Western blot analysis showing the presence of exosomal markers Tsg101 and Alix in the ultracentrifuge isolated VF fraction. The figure compares protein signal intensities in the VF and in a K562 cell lysate, relative to Beta-Actin as internal standard.

SUPPLEMENTAL MATERIALS AND METHODS

Proliferation analyses. The average doubling time of cells was calculated during the logarithmic growth phases according to the following formula: doubling time = time (days) / log2 (N2 / N1), where N1 is the first cell count and N2 is the cell count at the end of logarithmic growth phases. The total cell number after the initiation of culture was also measured by seeding the hAMSCs at a density of 5×10^3 cells/cm^2.

Flow cytometry. Rluc-RFP-TK-hAMSCs and Rluc-RFP-TK-FP-hAMSCs were trypsinized, washed, and suspended in 100 µl of PBS with 1% bovine serum albumin (Sigma). Cells were incubated in the dark at 4°C for 30 min with 10µl of the following monoclonal antibodies conjugated with phycoerythrin: CD49d, CD54, CD106, CD73, CD90, CD144, CD13, CD44, CD29 (BD Biosciences), CD31, C-Kit, CD34, CD105 (Abcam), VEGFR (R&D Systems), and CD133 (Miltenyi). Unspecific binding was assessed by isotype control mouse IgG2a-PE and mouse IgG1k-PE (BD Bioscience). Cells were then washed in PBS and analyzed using a Gallios Flow Cytometer (Beckman Coulter).

GCV doses. Pluc-GFP-U87 cells were seeded alone or in co-culture with Rluc-RFP-TK-FP-hAMSCs in 96-well plates at 4:1 therapeutic cell: tumor cell ratio. Samples received GCV doses from 0.002 to 0.04 µg/µl, control samples did not received GCV. Triplicate samples of each culture condition were analyzed by BLI on days 1 (before starting GCV treatment), 4, and 7. The experiment was repeated three times.

Extracellular vesicle fraction preparation and analysis. Conditioned media or vesicle fraction (VF) were prepared from confluent Rluc-RFP-TK-hAMSCs cultures with GCV (0.004 µg/µl). Supernatants were collected from 48 hour cultures (using exosome-free FBS), centrifuged (300 × g, 5 min) and filtered through 0.22 µm filters. VF was isolated by successive centrifugations 1200 × g (20 min) and 10 000 × g (40 min) to eliminate cells and debris, followed by centrifugation at 110 000 × g (90 min). The resulting pellet was washed once in a large volume of PBS, centrifuged at 110 000 × g (90 min), re-suspended in 300 µl of PBS and storage at −80°C. VF size distribution was analyzed by dynamic light scattering (DLS) in a Zetasizer Nano ZS (Malvern Instruments). Protein concentrations were determined using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). Western blot analysis was used to identify Alix (1:1,000, ab186429, Abcam) and Tsg101 (1:1000, ab125011, Abcam), specific protein markers for exosomes in the vesicular fraction (40 µg of protein). Mouse anti-actin (1: 2000; Santa Cruz Biotechnology) in K562 cell lysate was used to detect actin as internal standard. A secondary HRP labeled antibody (1:10000; Abcam) for 1h at room temperature was used for detection. The signal was detected using

ECL (GE Healthcare Life Science) and visualized in the ChemiDocTM XRS+system (BioRad).

In vitro cytotoxic activity of conditioned media and VF. Pluc-GFP-U87 cells were seeded in 96-well plates at a density of 500 cells/well with 100 µl of medium. After 24 hours, culture medium was replaced with conditioned media or sub-cellular vesicle fraction suspension. Pluc-GFP-U87 cells cultured with DMEM with or without GCV were used as controls (n = 5, each condition). Luciferase activity of U87 cells was monitored by Pluc-BLI on days 1 (before adding conditioned media), 6, and 8 post conditioned media addition. The experiment was repeated three times. Experiments were also carried out comparing conditioned media from non-GCV pre-treated Rluc-RFP-TK-hAMSCs by adding or not GCV “a posteriori” to the Pluc-GFP-U87 cells; and conditioned media from GCV pre-treated Rluc-RFP-TK-hAMSCs (n = 6, each condition).