

Supplemental Materials and Methods

Human PBMCs and cell lines

PBMCs were prepared from healthy donor blood (Gulf Coast Blood Center, Houston, TX) and GBM patients undergoing resection at The University of Texas M.D. Anderson Cancer Center (M.D. Anderson), Houston, TX, by centrifugation on a Ficoll-Hypaque density gradient (Sigma-Aldrich, St. Louis, MO). Total PBMCs were treated for 3 days with conditioned medium collected after 72 hours from untransfected control gCSCs and from gCSCs upregulated with miRs. Cells were harvested and tested for inhibition of T-cell proliferation, T-cell apoptosis, and Treg induction. CD4⁺ T lymphocytes were purified by immunomagnetic depletion with the human naive CD4⁺ T-cell Isolation Kit II (Miltenyi Biotec, Auburn, CA). Naive CD4⁺ T-cells (CD3⁺CD4⁺CD45RA⁺CD45RO⁻) had a purity of over 96%. HeLa, GL261 and astrocyte cell lines (C8-D1A, C8-S and C8-D30) were acquired from ATCC (Manassas, VA).

Isolation of human GBM-infiltrating microglia/macrophages

After resected GBM surgical specimens were placed into single cell suspension, the GBM-infiltrating microglia/macrophages were isolated by performing Percoll gradient centrifugation followed by CD11b⁺ MACS positive selection (Miltenyi Biotec, Cambridge, MA) as we have previously described (1). The purity of the microglia/macrophages was >95%.

Human gCSCs

gCSCs were cultured in Dulbecco's modified Eagle's medium F-12 containing 20 ng/ml of epidermal growth factor, basic fibroblast growth factor (Sigma), and B27 (1:50; Invitrogen, Carlsbad, CA) as a neural stem cell-permissive medium (neurosphere medium) and passaged every 5-7 days. The characteristics of these cells, including the cytogenetics, limiting dilution

assays, tumorigenicity, CD133 expression, and immune-suppressive properties have been previously published (2, 3).

gCSC neuronal differentiation

Accutase-dissociated gCSC sphere cells were cultured in differentiation medium consisting of 10% fetal bovine serum and 100 ng/mL retinoic acid (4). Confluent monolayer cells were detached every 4 to 6 days by trypsinization and retinoic acid were replenished during the culture.

Glioma tissue microarray and in situ hybridization

This study was conducted according to LAB09-0463, which was approved by the institutional review board at MD Anderson and includes 235 patients with different glioma grades. The TMAs consisted of resected glioma tissues from GBM (n = 150), gliosarcoma (n = 6), anaplastic astrocytoma (n = 24), anaplastic mixed oligoastrocytoma (n = 9), anaplastic oligodendroglioma (n = 16), mixed oligoastrocytoma (n = 5), oligodendroglioma (n = 24), low-grade astrocytoma (n = 1), subependymoma (n=2), and normal brain (cortex; n = 19), and they have been previously described (5). For TMA construction, two 1-mm cores were obtained per tumor sample. The rationale for using a TMA was to facilitate an analysis of the largest number of tumor samples possible. The study neuropathologist (G.N.F.) gathered the tissue sections from the archived paraffin blocks and confirmed the tumor pathologic type. The time from resection to fixation was less than 20 minutes in all cases, in accordance with the Clinical Laboratory Improvement Amendments standard.

In situ hybridization was performed using the protocol developed by Nuovo et al (6), with some minor adjustments. Digoxigenin-labeled, locked nucleic acid-modified probes for miR-124 (hsa-miR-124) and the positive control (U6, hsa/mmu/rno) were purchased from Exiqon (Vedbek, Denmark). In brief, we placed 4- μ m sections of the TMA blocks in a heater at 59°C

overnight to attach cores to the silane-coated slide. Sections were deparaffinized with xylene (2 × 5 minutes), rehydrated with ethanol (100%, 50%, & 25% for 5 minutes each), and treated with diethylpyrocarbonate-treated water for 1 minute. Protease treatment was performed with pepsin solution (1.3 mg/ml) (Dako, Glostrup, Denmark) at 37°C for 50 minutes. After a post-fixation step in 4% paraformaldehyde, hybridization of the locked nucleic acid probe was carried out in a Hybrite (Abbott Laboratories, Abbott Park, IL) at 60°C for 5 minutes followed by 37°C overnight (12-18 hours). A low-stringency post-hybridization wash was performed at 4°C in standard sodium citrate containing 2% bovine serum albumin for 5 minutes, followed by incubation with anti-digoxigenin/alkaline phosphate conjugate antibodies (Enzo Diagnostics, Farmingdale, NY) in a heater at 37°C for 30 minutes. The blue color was developed by incubating the slide with nitroblue tetrazolium and bromchloroindolyl phosphate (Enzo Diagnostics) at 37°C. The colorimetric reaction was monitored visually and stopped by placing the slides in water when background coloring started to appear. The TMA was analyzed by the study neuropathologist (G.N.F.). In the assessment of miR-124 expression in gliomas, intervening neurons in the infiltrating component were not considered positive.

Luciferase assay

To determine whether miR-124 can bind to the STAT3 3'-UTR, we co-transfected HeLa reporter cells with the STAT3 3' UTR-luciferase reporter plasmid (pMirTarget, Origene, Rockville, MD) and miR-124 expression plasmid or scramble control plasmid (GeneCopoeia, Rockville, MD) with Lipofectamine 2000 transfection reagent (Invitrogen, Grand Island, NY). Renilla luciferase reporter plasmid was included as an internal control for transfection efficiency. The interaction between miR-124 and its target were measured by comparing the results of the cotransfection of STAT3 3' UTR-luciferase reporter and miR-124 plasmids with those of the 3' UTR-luciferase reporter plasmid and the scramble control plasmid. The luciferase assay was performed using

the Dual-Luciferase® reporter assay system (E1910, Promega, Madison, WI). Firefly luciferase activity was normalized by renilla luciferase activity.

Western blot

miR-124- or scramble RNA-transfected gCSCs were harvested, pelleted by centrifugation, and rinsed with ice cold PBS. The cells were lysed for 30 min in ice cold lysis buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1 mM EDTA) containing 1% Triton-X-100 and phosphatase and protease inhibitors (Sigma-Aldrich). The lysates were centrifuged at 14,000 rpm for 10 min at 4° C. The supernatants were collected and quantified for protein content. Equal amounts of proteins (20 µg) were electrophoretically fractionated in 8% sodium dodecyl sulfate (SDS)-polyacrylamide gels, transferred to nitrocellulose membranes, and subjected to immunoblot analysis with specific antibodies against STAT3, p-STAT3 (Tyr705), p-MAPK1/3 and actin (Cell Signaling Technology, Inc. Danvers, MA), MAPK1/3 (Millipore, Billerica, MA), Shc1 (BD Transduction Laboratories, San Jose, CA), IL-6R α and Tyk2 (Santa Cruz, Dallas, TX). Autoradiography of the membranes was performed using Amersham ECL Western-blotting detection reagents (Amersham Biosciences).

Immune-phenotypic characterization of gCSCs

The cell surface was stained with phycoerythrin (PE)-, FITC-, or APC-conjugated antibodies against the following proteins: MHC I, MHC II, CD40, CD80, CD86, and B7-H1 (BD Pharmingen, San Diego, CA) and CD133 (Miltenyi Biotech). To detect intracellular cytokines, we used phycoerythrin-conjugated antibodies against IL-2 and IFN- γ (R&D Systems, Minneapolis, MN). Intracellular STAT3 was assessed using PE-conjugated p-STAT3 (pY⁷⁰⁵) (BD Pharmingen). Total STAT3 expression was tested using PE-conjugated STAT3 (R&D Systems). Appropriate isotype controls were used for each antibody. Additionally, after treating gCSCs with precursor miR-124 or the negative control for 72 hours, we stored them in RNAlater

solution overnight at 4°C. Total RNA was extracted, and 100 ng were transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA). cDNA was used as a template for RT-PCR with SYBR Green PCR Master Mix. Forward and reverse primers for STAT3, MHC I, MHC II, CD40, CD80, B7-H1 and CD86 were used at a concentration of 50 µM (Sigma Aldrich). GAPDH (Sigma Aldrich) was used as the internal control.

ELISAs, cytokine and chemokine arrays

Supernatant medium conditioned by gCSCs transfected with miR-124 or scramble control was measured for cytokine concentrations using ELISAs (ELISA DuoSet ELISA development kits, R&D Systems). The supernatants were collected after 3 days in culture and stored at -20°C. For ELISA, the supernatants were added in triplicate to appropriate capture antibody-coated plates. After the plates were washed, horseradish peroxidase-conjugated detection antibody was added. The substrate used for color development was tetramethylbenzidine. The optical density was measured at 450 nm with a microplate reader (Spectra Max 190; Molecular Devices, Sunnyvale, CA), and cytokine concentrations were quantified with SoftMax Pro software (Molecular Devices). The detection limits for galectin-3 were 31 pg/mL; MIC-1, 8 pg/ml; IL-8, 16 pg/mL; and vascular endothelial growth factor, 8 pg/mL. Other cytokines and chemokines in gCSC supernatants were measured using Human Cytokine/Chemokine Array kits (Proteome Profiler, R&D Systems) following the manufacturer's instructions.

T cell proliferation, apoptosis, Treg induction and intracellular cytokine analysis

In general, the analysis of T-cell populations was based on the gated surface expression of FITC-conjugated anti-CD4 (RM4-4) and APC-conjugated anti-CD8 (53-6.7) antibodies. For T cell proliferation assays, 3×10^5 naïve CD4+ T cells/ml labeled with 2 µM CFSE were cultured in the presence of 1 µg/ml pre-bound anti-CD3/anti-CD28 antibodies and in some experiments with the conditioned medium from the gCSCs. After 72 hours, T cells proliferation was

measured by CFSE dilution via flow cytometer. An APC-conjugated anti-CD4 (RPA-T4) antibody was used for cell surface staining. The T-cell apoptosis assay was performed with the Annexin V/7-AAD staining kit (BD Pharmingen) according to manufacture instruction.

To detect forkhead box P3 FoxP3 protein expression, the surface-stained cells were further subjected to intracellular staining with PE-conjugated monoclonal antibodies to human FoxP3 (clone PCH101, eBioscience, San Diego, CA) using staining buffers and conditions specified by the manufacturer. To confirm that these Tregs were functional, Tregs induced by gCSC conditioned media were mixed with SP-DiIC18 (7) fluorescently-labeled autologous CD4⁺ T cells (1:1 ratio) and then plated into 96-well plates in the presence of allogeneic 3000R irradiated PBMCs (1×10^6 /mL, as antigen presenting cells) and 0.5 ug/ml anti-CD3 antibody in RPMI 1640 medium with 10% fetal bovine serum (8, 9). Functional suppression of autologous fluorescently-labeled CD4⁺ T cell proliferative responses was based on the analysis of responder cell division by flow cytometry.

For intracellular cytokine staining of T-cells, cells were stimulated for 6 hours in the presence of 50 ng/ml phorbol myristate acetate (PMA), 500 ng/ml ionomycin (Sigma-Aldrich), and 2 μ M monensin (GolgiStop, BD Sciences). The cells were then incubated with FITC-conjugated anti-CD4 and APC-conjugated anti-CD8 antibodies for surface staining followed by intracellular staining using PE-conjugated anti-human IFN- γ antibody (BD Pharmingen), PE-conjugated anti-mouse IFN- γ (4S.B3; BD Pharmingen), PE-conjugated anti-human IL-2, PE-conjugated anti-human TNF α antibodies (R&D), PE-conjugated anti-mouse TNF- α (MQ1-17H12; BD Pharmingen) and FIX/PERM buffers (BD Pharmingen) after membrane permeabilization according to the manufacturer's instructions.

For intracellular p-STAT3 detection, cells were first fixed with 2% paraformaldehyde at room temperature for 10 minutes. Thereafter, the cells were washed and permeabilized with 90% methanol on ice for 30 minutes, and then stained with PE-conjugated anti-p-STAT3 (Y705) antibody (BD Pharmingen) for 30 minutes at room temperature. Flow cytometry acquisition was

performed with a FACSCalibur (Becton Dickinson, San Diego, CA), and data were analyzed with FlowJo software (TreeStar, Ashland, OR).

T helper cell differentiation assay

Naive healthy donor CD4⁺ T-cells were transfected with miR-124 precursor and scramble control and then cultured in 24-well plates at a density of 1×10^6 cells per well in the presence of pre-bound anti-CD3/CD28 antibodies (BD Pharmingen) in T-cell medium (RPMI1640 supplemented with 10% FBS, 2mM L-glutamine, 1mM sodium pyruvate, 0.1mM nonessential amino acids, 10mM HEPES, 1X penicillin/streptomycin, 50 μ M 2-mercaptoethanol, at pH 7.04). Medium was supplemented with: IL-1 β (10 ng/ml), IL-6 (20 ng/ml), TNF- α (10 ng/ml), TGF- β 1 (3 ng/ml) and IL-23 (100 ng/ml) for Th17 differentiation; IL-12 (10 ng/ml) for Th1 differentiation; and TGF- β 1 (3 ng/ml) for inducible Tregs. Anti-IFN- γ and/or anti-IL-4 (BD Biosciences) were added to the cultures at a concentration of 10 μ g/ml for Th0, anti-IL-4 for Th1, and anti-IFN- γ for Th2. CD3/CD28 T-Cell Expander dynabeads (one bead per cell; Invitrogen) were used for Th cell restimulation for 2-3 rounds proceeding with Th cytokine staining.

Syngeneic subcutaneous model

Mice were maintained in the M.D. Anderson Isolation Facility in accordance with Laboratory Animal Resources Commission standards and conducted according to the approved protocol 08-06-11831. The murine glioma GL261 cell line was obtained from the National Cancer Institute-Frederick Cancer Research Tumor Repository. On the basis of PCR expression, miR-124 expression in GL261 cells was 350-fold less than in normal murine brain. These cells were cultured in an atmosphere of 5% CO₂ and 95% humidified air at 37°C in Dulbecco's modified Eagle's medium (Life Technologies; Grand Island, NY), supplemented with 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA), 1% penicillin/streptomycin (Life Technologies, Grand Island, NY), and 1% L-glutamine (Life Technologies). The GL261 glioma cell cultures were

divided every 3 days to ensure logarithmic growth. To induce subcutaneous tumors, logarithmically growing GL261 cells were injected into the right hind flanks of 6-week-old C57BL/6J female mice or nude mice at a dose of 4×10^5 cells suspended in 100 μ l of Matrigel basement membrane matrix (BD Biosciences). When palpable tumors formed (approximately 0.5 cm in diameter), the mice (n=10/group) were treated by local tumor injection or intravenous injection. Tumors were measured every other day. Mice that showed signs of morbidity, high tumor burden, or skin necrosis were immediately euthanized according to M.D. Anderson guidelines. Tumor volume was calculated with slide calipers using the following formula: $V = (L \times W \times H)/2$, where V is volume (mm^3), L is the long diameter, W is the short diameter, and H is the height. Likewise, miR-124 or scramble miR was delivered systemically via the tail vein every other day.

Ex vivo immune cytotoxicity assay and CD3+ T-cell isolation

Spleens from mice bearing tumors from subcutaneous injection of GL261 cells and treated by either miR-124 or scramble microRNA duplex for 2 weeks, were harvested and dissociated into single cell suspensions. After the erythrocytes in the spleens were lysed with 1x RBC lysis buffer (Sigma), splenocytes were washed once with RPMI 1640 medium and used as effector cells to target GL261 cells in modified cytotoxicity assays (8, 9). The ratios of splenocytes to 4 μ M CFSE-labeled GL261 target cells were 10:1, 40:1, and 100:1. Viability of CFSE-labeled GL261 cells was assessed using propidium iodide (BD Biosciences) staining and a FACSCalibur flow cytometer (BD Biosciences); data analysis was performed using FlowJo software. PBMCs from the blood and spleen were also used for T-cell purification using the CD3 negative selection kit (BD Biosciences). For glioma-infiltrating T-cell isolation, the gliomas were cut into small pieces and digested with Liberase TM (0.2 Wunsch unit/ml; Roche, Indianapolis, IN) for 2 hours at 37°C to make a single-cell suspension for CD3+ T-cell selection. The purity of CD3+ T-cells was > 94%.

Syngeneic intracranial glioma model

To induce intracerebral tumors in C57BL/6J mice, GL261 cells were collected in logarithmic growth phase, washed twice with PBS, mixed with an equal volume of 10% methyl cellulose in Improved modified Eagle's Zinc Option medium, and loaded into a 250- μ l syringe (Hamilton, Reno, NV) with an attached 25-gauge needle. The needle was positioned 2 mm to the right of bregma and 4 mm below the surface of the skull at the coronal suture using a stereotactic frame (Kopf Instruments, Tujunga, CA), as previously described (10). The intracerebral tumorigenic dose for GL261 cells was 5×10^4 in a total volume of 5 μ l. Mice were then randomly assigned to control and treatment groups (n=10/group). Animals were observed three times per week, and when they showed signs of neurological deficit (lethargy, failure to ambulate, lack of feeding, or loss of >20% body weight), they were compassionately killed. These symptoms typically occurred within 48 hours of prior to death. The brains were removed and placed in 4% paraformaldehyde and embedded in paraffin.

In vivo depletions

For studies of in vivo depletion of CD4+ and CD8+ T-cells in C57BL/6J mice via neutralization with monoclonal antibodies (mAbs), each mouse was injected i.p. with 0.4 mg rat anti-mouse CD8 (53-6.7), anti-CD4 (GK1.5), or normal IgG isotype as control antibody (all from Bio X Cell, West Lebanon, NH) in 200 μ l PBS on the same day that miR-124 treatment began, once daily for the following 3 consecutive days, and then twice a week for 2 additional weeks. Maintenance of the in vivo depletion throughout the experimental period was confirmed by flow cytometry of PBMCs with APC-anti mouse CD4 (RM4-5) and FITC-anti mouse CD8 α (53-6.7) (BD Biosciences).

T-cell adoptive transfer

CD3⁺ T-cells were isolated from splenocytes of GL261 tumor-bearing mice and transfected with scramble or therapeutic miRNAs by nucleofector technology (Lonza, CA). These miRNA modified T-cells were expanded by anti-CD3/anti-CD38 polyclonal stimulation for 48 hours, and miRNA expression levels were confirmed by RT-PCR. After the miRNA overexpression was confirmed, 6×10^6 T-cells/mouse were administered intravenously to mice subcutaneously implanted with GL261 cells.

Genetically engineered murine models

Vector constructs. RCAS-PDGFB generation has been previously described (11). RCAS-STAT3 was created by amplifying the sequence encoding the cDNA by PCR using specially designed primers to enable directional cloning into a Gateway entry vector. The proprietary Gateway LR recombination reaction between the entry vector containing STAT3 and a Gateway-compatible RCAS destination vector resulted in the RCAS-STAT3 vector, which was sequence verified.

DF-1 cell transfection. DF-1 immortalized chicken fibroblasts were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum in a humidified atmosphere of 95% air/5% CO₂ at 37°C. Live virus was produced by transfecting plasmid versions of RCAS vectors into DF-1 cells using FuGene6. These cells were replicated in culture.

Verification of STAT3 expression in vector. Untransfected DF-1 cells were grown in culture, transfected with RCAS-STAT3, and allowed to replicate for two to three passages. Cells were fixed with 4% paraformaldehyde, and immunocytochemical labeling was performed using standard methods. A rabbit polyclonal antibody against STAT3 (1:100; Cell Signaling Technology, Beverly, MA) and goat anti-rabbit Alexa Fluor 594 fluorescent conjugate (1:500; Molecular Probes, Carlsbad, CA) were used for detection. Prolong Gold anti-fade reagent with 4'-6-diamidino-2-phenylindole (DAPI) was used for labeling cell nuclei. Staining was visualized

with a Zeiss Axioskop 40 microscope. Expression was secondarily validated by Western blot analysis.

In vivo somatic cell transfer in transgenic mice. The transgenic Ntv-a mice are mixtures of different strains, including C57BL/6, BALB/c, FVB/N, and CD1. To transfer genes via RCAS vectors, DF-1 producer cells transfected with a particular RCAS vector (5×10^4 DF-1 cells in 1–2 μ l of PBS) were injected into the frontal lobes of Ntv-a mice at the coronal suture of the skull using a Hamilton Gastight syringe. The mice were injected on postnatal days 1 or 2, when the number of Nestin+ cells producing TVA is the highest. The mice were killed 90 days after injection or sooner if they demonstrated morbidity related to tumor burden. Their brains were removed and analyzed for tumor formation. Histologic verification of tumor formation and determination of tumor grade were performed by a neuropathologist (G.N.F.).

Animal randomization. Twenty-one days after introducing the glioma-inducing transgenes RCAS-PDGFB and RCAS-STAT3, we randomly assigned littermates to the treatment or control group (n=9/group). Mice were treated intravenously on Monday, Wednesday, and Friday for 3 weeks. After 90 days, the animals were compassionately killed, the CNS was fixed, and the tumors were analyzed immunohistochemically.

Quantification of p-STAT3 Expression in Tumors

Formalin-fixed, paraffin-embedded 4- μ m sections of the mouse brain tumors were stained to detect p-STAT3 expression using a rabbit polyclonal anti-p-STAT3 (Tyr705) antibody (1:50; Cell Signaling Technology) as previously described (12). Quantification was carried out by two independent observers analyzing the tumors with high-power fields (max: 400 \times objective and 100 \times eyepiece) of each specimen in the tumor regions. Each observer recorded the absolute number of cells with positive staining. The duplicate numbers were then averaged for the final number of cells with positive expression per specimen.

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