Concurrent Dexamethasone Limits the Clinical Benefit of Immune Checkpoint Blockade in Glioblastoma

SUPPLEMENTAL FIGURES

Supplemental Figure S1. Flow cytometry gating strategies.

Flow cytometry gating strategy displayed using one of the representative IgG-treated control GL261-luc2 mouse spleens: (A) Singlets from FSC-H by FSC-A; then cells by size from FSC-A by SSC; then alive cells by negative Live/Dead APC-Cy7; then CD45-BV605+ leukocytes; then T cells by CD3-BV785+ and other cells by CD3-BV785- (then NK cells by NK1.1-FITC+, then Ki67-PE+ NK cells). T cells were further divided into CD4-BV650+/CD8-BV711- and CD4-BV650-/CD8-BV711+ T cells, each of which were further gated for Ki67-PE+ proliferative cells and CD69-BV510+ cells. CD4-BV650+/CD8-BV711- T cells were also divided into FOXP3-BV421+ regulatory T cells. (B) Singlets from FSC-H by FSC-A; then cells by size from FSC-A by SSC; then alive cells by negative Live/Dead APC-Cy7; then CD45-BV711+ leukocytes; divided into CD11c-BV510+ dendritic cells (further divided into CD86-PECy7+/CD80-BV650+ cells) and CD11c-BV510- cells, which were further divided into CD11b^{lo} CD45^{hi} cells, CD11b^{hi} CD45^{hi} myeloid cells, and CD11b^{hi} CD45^{lo} cells (e.g. microglia in brain) by CD45-BV711 and CD11b-BV785. CD11b^{hi} CD45^{hi} myeloid cells were subsequently divided into Ly6C^{hi} Ly6G- monocytes and Ly6C$^{low\text{-}int}$ Ly6G- macrophages by Ly6C-BV605 and Ly6G-PerCPCy5.5, as well as into CD86-PECy7+/CD80-BV650+ cells and PDL1-BV421+ cells. Each contour represents 5% of events.

Supplemental Figure S2. Neither dexamethasone administered prior to initiation of anti-PD-1 therapy nor dexamethasone alone affects survival in the GL261-luc2 glioblastoma mouse model. (A) Representative longitudinal bioluminescence imaging obtained serially from the same mice over time, demonstrating increased tumor growth when low (1 or 2.5 mg/kg) or high (10 mg/kg) doses of dexamethasone were co-administered during PD-1 therapy, compared to anti-PD-1 without dexamethasone. Kaplan-Meier OS estimates are depicted, with comparison by Cox regression. (B) For re-challenge experiments to assess immunologic memory responses to tumor, $1x10^5$ GL261 non-luciferase-transduced cells were injected intracranially into the contralateral hemisphere in a cohort of mice that were previously treated and survived over 100 days (from Figure 1A). A similar tumor cell inoculum was administered to a cohort of treatment naïve mice as a control. Re-challenged mice were followed for a minimum of 128 additional days and received no additional therapy. (C) To evaluate dexamethasone's effect when administered prior to anti-PD-1 therapy in GL-261-luc2 mice (n=18/group), dexamethasone (10 mg/kg) was administered daily on days 1-5 after intracranial implantation. Anti-PD-1 antibody was administered using the dose-intensive schedule, *i.e.* delivered IP beginning on day 6 (500 µg loading dose) followed by 7 additional doses (250 µg) at 3-day intervals.

ns, not significant; *$p<0.05$; **$p<0.01$; ***$p<0.001$; Dex, dexamethasone; αPD1, anti-PD1; 95CI, 95% confidence interval; NR, not reached.

Supplemental Figure S3. **The effect of concurrent dexamethasone on intratumoral and systemic regulatory T cells and the CD8+ to CD4+ FOXP3+ ratio in the GL261-luc2 glioblastoma mouse model.** As in Figure 3, tissue was collected at day 16 of a dose-intensive regimen of anti-PD-1, with dexamethasone (10 mg/kg) administered IP on days 6-16. Tissue (n=4-8/group) was harvested on day 16 and analyzed by flow cytometry. Immune cell counts were evaluated by multiple linear regression, normalized to the corresponding IgG control group's mean count (dashed gray line), and displayed as mean ± SE. Differences between treatment groups for (A) regulatory CD45+ CD3+ CD4+ FOXP3+ T cells and (B) CD8+ to CD4+ FOXP3+ (Treg) ratios are depicted.

cLN, cervical lymph node; Dex, dexamethasone; ns, not significant, $p \geq 0.05$; *$p<0.05$; **$p<0.01$; ***$p<0.001$

Supplemental Figure S4. Visualization of dexamethasone's effects on CD4, CD8, and regulatory T lymphocyte subsets in the tumor-microenvironment by cyclic immunofluorescence. (A) Multiplex immunofluorescence micrographs of GL261-luc2 tumor tissue from mice treated with IgG (top image) or dexamethasone (bottom image). Images show CD4 (red), CD8α (yellow), FOXP3 (blue), and Hoechst nuclear dye (gray). Images show approximately 20% of tumor cross-sectional area, scale bar 200 μm. (B) High-magnification micrographs of individual lymphocytes in the GL261-luc2 tumor microenvironment showing conventional CD4+ FOXP3- T (top), cytotoxic CD8+ T (middle), and regulatory CD4+ FOXP3+ T cell subsets. Scale bar 5 μm. (C) Immune cell frequency normalized to the total number of tumor cells per treatment group (IgG vs. dexamethasone).

SUPPLEMENTAL METHODS

Murine Radiotherapy Delivery

Mice were anesthetized using 1-3% isoflurane/medical air inhalation and positioned using orthogonal laser pointers on the gantry. Fluoroscopic “scout” images were acquired to position the brain at the center of the collimated-field, and a low dose cone beam CT was subsequently acquired for precise placement of the isocenter. The reconstructed CT DICOM was then uploaded to the RT_Image software (shared by Dr. Edward E. Graves, Stanford University, CA), to generate the treatment plan. The treatment beam was set up as 225 keV, 13 mA passed through a 0.3 mm copper filter and was delivered over a collimated square field of 1x1 cm^2. This device has a calibrated beam output of 3.55 Gy/min at 0.5 cm depth, at isocenter, in water for this configuration.

Characterization of the Tumor-Immune Microenvironment with Cyclic Immunofluorescence

Cyclic immunofluorescence (CyCIF) was performed on formalin-fixed paraffin-embedded brain tissue sections from GL261-implanted mice as previously described (38,39). Tissue sections were stained with Hoechst dye to label cell nuclei and immunolabeled with immune and mesenchymal cell lineage markers: CD8α (clone: 4AM15), CD4 (clone: 4SM95), FOX3 (clone: FJK-16s), and Vimentin (clone: D21H3). Imaging was performed at 20x (2x2 binning) using a CyteFinder slide-scanning fluorescence microscope (RareCyte Inc). Raw images were flatfield corrected as described, then stitched and registered using ASHLAR (https://github.com/labsyspharm/ashlar) (40). Images were segmented by training a U-Net convolutional neural network architecture on Hoechst signal intensity to identify individual cells (41). Custom-written Python scripts were then used to computationally extract quantitative protein expression data on individual cells for downstream analysis. Immune cell frequencies were reported as the ratio of each immunophenotype relative to the number of tumor cells (*i.e.* Vimentin+ cells negative for immune lineage markers) in the corresponding tissue section.