PD-1 blockade activates conventional CD4+ T cells and the innate immune response during glioblastoma eradication

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ABSTRACT

Blockade of immune cell co-inhibitory receptor PD-1 using monoclonal antibodies enables anti-tumor immune responses in various solid tumors and lymphoid malignancies. Our laboratory previously demonstrated PD-1 blockade elicits an anti-tumor immune response resulting in tumor rejection and long-term survival in approximately 25-50% of mice with an orthotopic GL261 glioblastoma, despite lacking a corresponding accumulation of CD8+ cytotoxic T cells in the tumor or draining lymph nodes. In this investigation, we evaluated the role of conventional CD4+ T cells and the innate immune response in PD-1 mediated anti-glioma immunity using multiplex technologies for immunohistochemistry and flow cytometry. In response to anti-PD-1 monotherapy, intratumoral CD4+ T cells expressed significantly elevated levels of proteins required for T cell proliferation, activation, and effector function. CD4+ T cell activation was accompanied by the classical activation and M1 polarization of resident microglia and tumor-infiltrating macrophages, including down-regulation of PD-L1 and upregulation of MHC class II surface expression. We also demonstrated that depletion of either CD4+ or CD8+ T cells was sufficient to completely ablate anti-PD-1 mediated tumor eradication and long-term survival. Our data suggests CD4+ T cells and myeloid cells may play a prominent role in the eradication of glioblastoma by PD-1 blockade.

BACKGROUND

- Combination PD-1 and CTLA-4 antibody blockade induces tumor-eradication and long-term survival in approximately 75% of mice bearing advanced stage glioblastoma tumors in the syngeneic GL261 model.
- Intratumoral accumulation of CD8+ cytotoxic T cells was observed in mice treated with both anti-PD-1 and anti-CTLA-4.
- PD-1 monotherapy induced anti-tumor immunity in 25-50% of glioblastoma-bearing mice despite a decrease in CD8+ cytotoxic T cells.
- PD-1 mAb single agent therapy resulted in fewer CD4+ T regulatory cells and myeloid-derived suppressor cells, as well as enhanced NK cell activation.

METHODS

C57BL/6 mice (The Jackson Laboratory) were orthotopically implanted with a luciferized syngeneic GL261 cell line (a kind gift of David Zagzag, New York University). All animal experiments were approved by the Dana-Farber Animal Care and Use Committee. The mouse anti-mouse monoclonal antibody (mAb) PD-1 - 332.8H3 (mouse IgG1, k) was generated in PD-1-deficient mice in the laboratory of Dr. Gordon Freeman, as described previously.[2]

Formalin-fixed, paraffin embedded brains were sectioned and sequentially stained with antibodies from Cell Signaling Technology, Inc. followed by TSA-based amplification. Images were spectrally unmixed with a Mantra™ Quantitative Pathology Workstation (PerkinElmer). See panel optimization information in Table 1-2.

Table 1

Antibody	Product #	Dilution	Fluorophore	Order
CD4 (D7D2Z)	25229	1:50	AF350	1st
CD3e (D4V8L)	99940	1:300	Cy5	2nd
PD-1 (D7D5W)	84651	1:100	FITC	2nd
CD8 (D4W2Z)	98941	1:300	AF594	3rd
TIM-3 (D3M9R)	83882	1:1600	AF555	4th
F4/80 (D2S9R)	70076	1:500	Cy5.5	5th

Table 2

Antibody	Product #	Dilution	Fluorophore	Order
CD4 (D7D2Z)	25229	1:50	AF350	1st
Granzyme B (D6E9W)	46890	1:150	AF555	2nd
CD8 (D4W2Z)	98941	1:300	AF594	3rd

CONCLUSIONS

- **CD4+ and CD8+ T cells, but not NK cells, are required for the therapeutic benefit of PD-1 blockade in GL261 tumors.**
- **PD-1 blockade increased conventional CD4+ T cell accumulation in tumors.**
- **PD-1 therapy increased CD4+ T cell activation. Granzyme B+ CD4+ T cells can be detected in PD-1 treated tumors.**
- **PD-1–treated tumors had lower PD-1^{hi} and TIM-3+ immune cells, and elevated PD-1^{mid} cells, suggesting a shift toward activation versus exhaustion.**
- **PD-1 therapy enhanced the classical activation of resident and tumor-infilitrating myeloid populations.**

REFERENCES

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Gool, S.W. et al. Cancer Immunol Immunother. 2011; 60:153–60
Akbari, O. et al. Mucosal Immunol. 2010; 3:81–91.

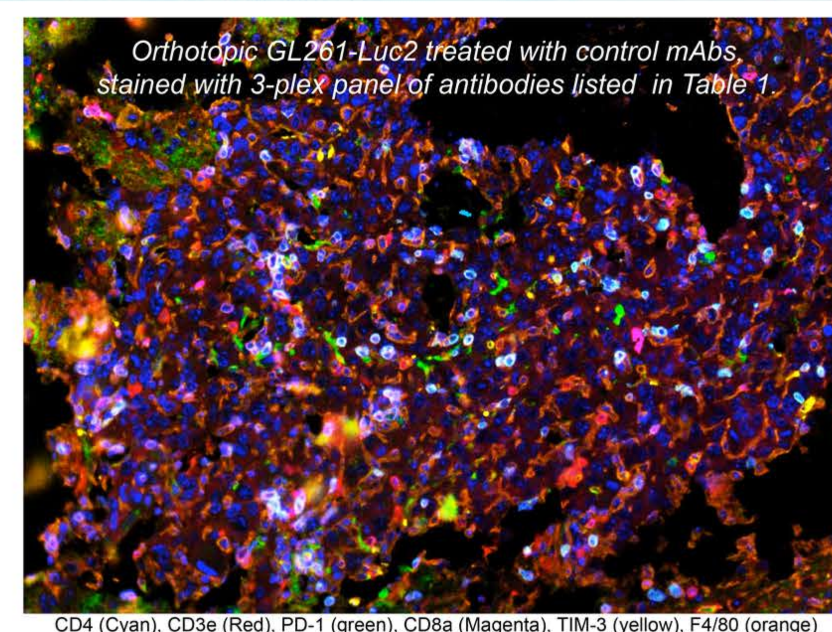
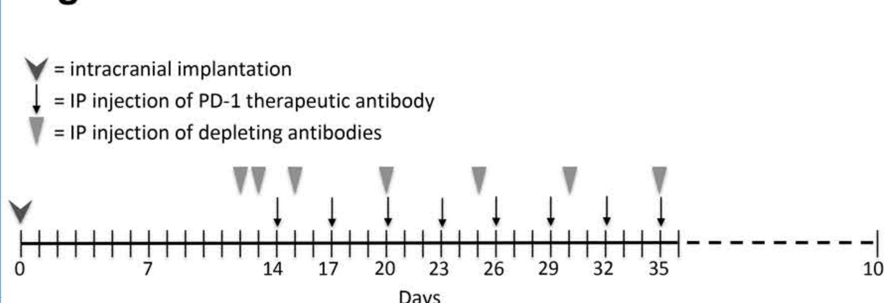


Figure 1A



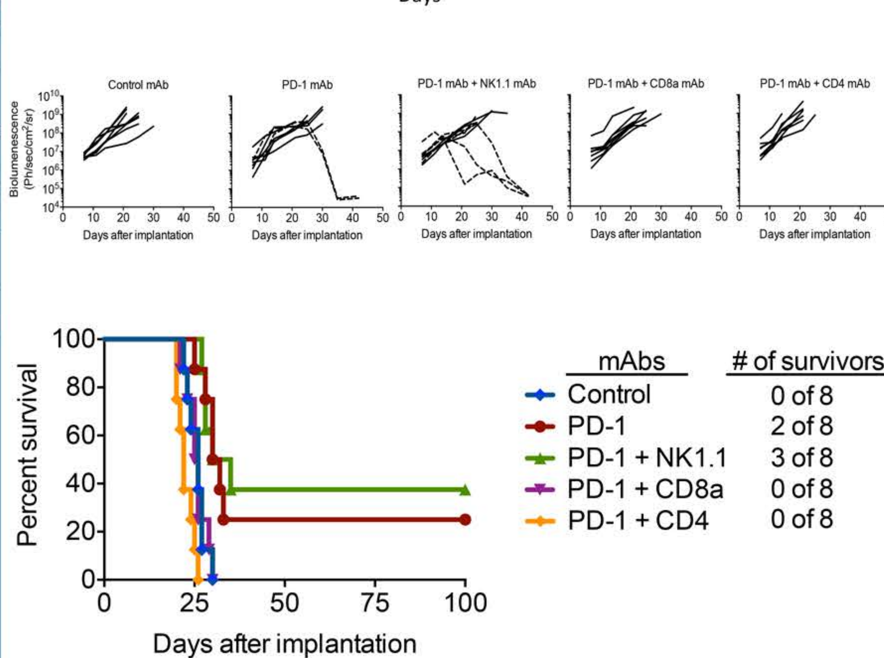


Figure 1B

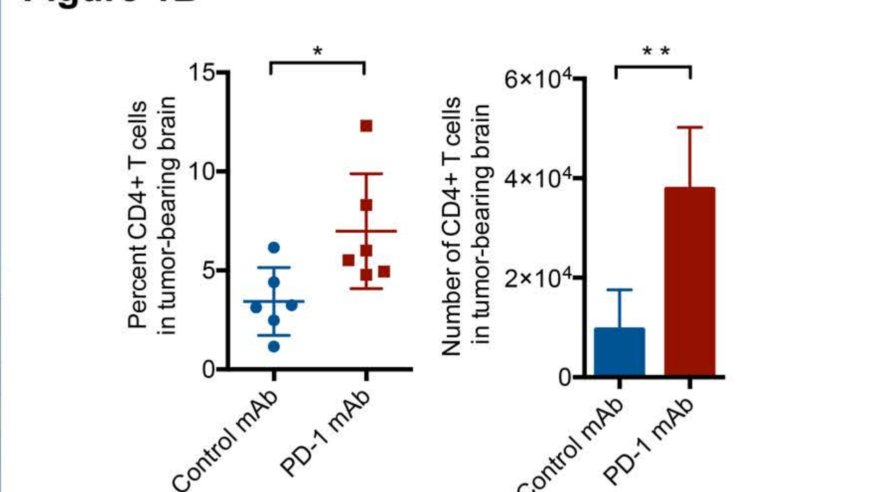


Figure 1C

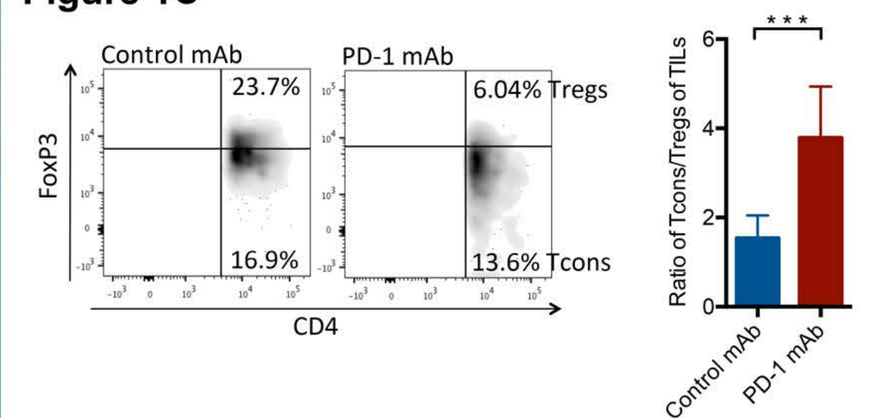


Figure 1. CD4 and CD8, but not NK cells, are required for successful response to PD-1 therapy in mouse models of glioblastoma. C57BL/6 mice were intracranially inoculated with 1 X 10^5 GL261-Luc2 cells on day 0. Following confirmation of tumor by bioluminescence, mice were treated with indicated depleting antibodies on days 12, 13, 15, 20, 25, 30, and 35. Mice were concurrently treated with PD-1 mAbs (332.8H3; mouse IgG1, k) or isotype controls on days 14, 17, 20, 23, 26, 29, 32 and 35. (A) Monitoring and treatment schema *(top)*. Graphs of bioluminescence measured on days 7, 11, 14, 21, 25, 30 and 35 *(middle)*. Kaplan-Meier survival curves of long-term survivors (100 days) initially treated with the PD-1 mAbs, isotype controls, and/or indicated depleting antibodies *(bottom)*. (B) T cell infiltrates within intracranial tumors following PD-1 blockade were analyzed by flow cytometry. CD3+CD4+ T cells as a percentage of live CD45+ immune cells *(left)*. Absolute number of live CD45+CD3+CD4+ T cells within the tumor-bearing brain *(right)*. (C) Representative flow cytometry plots of CD4+FoxP3+ regulatory T cells (Tregs) and CD4+FoxP3- conventional T cells. Values in top right corner represent the percent of Tregs or Tcons gated on CD45+CD3+ tumor-infiltrating lymphocytes (TILs). Bar graph depicting the ratio of Tcons/Tregs *(right)*. All graphs include values for individually analyzed mice, and the mean + SEM of 6 mice per treatment group. Two-tailed Student's T test was used to determine statistical significance (*P<0.05; **P P<0.01; ***P<0.001)

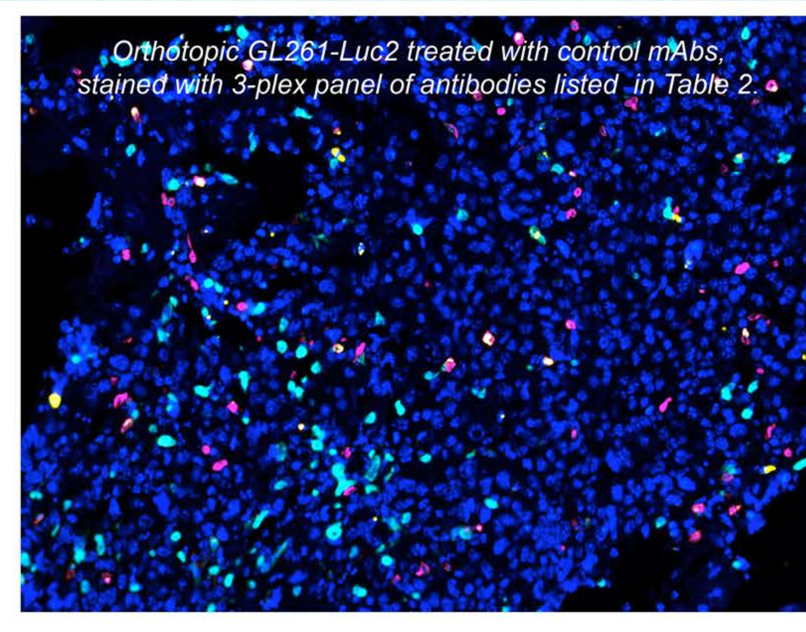


Figure 2A

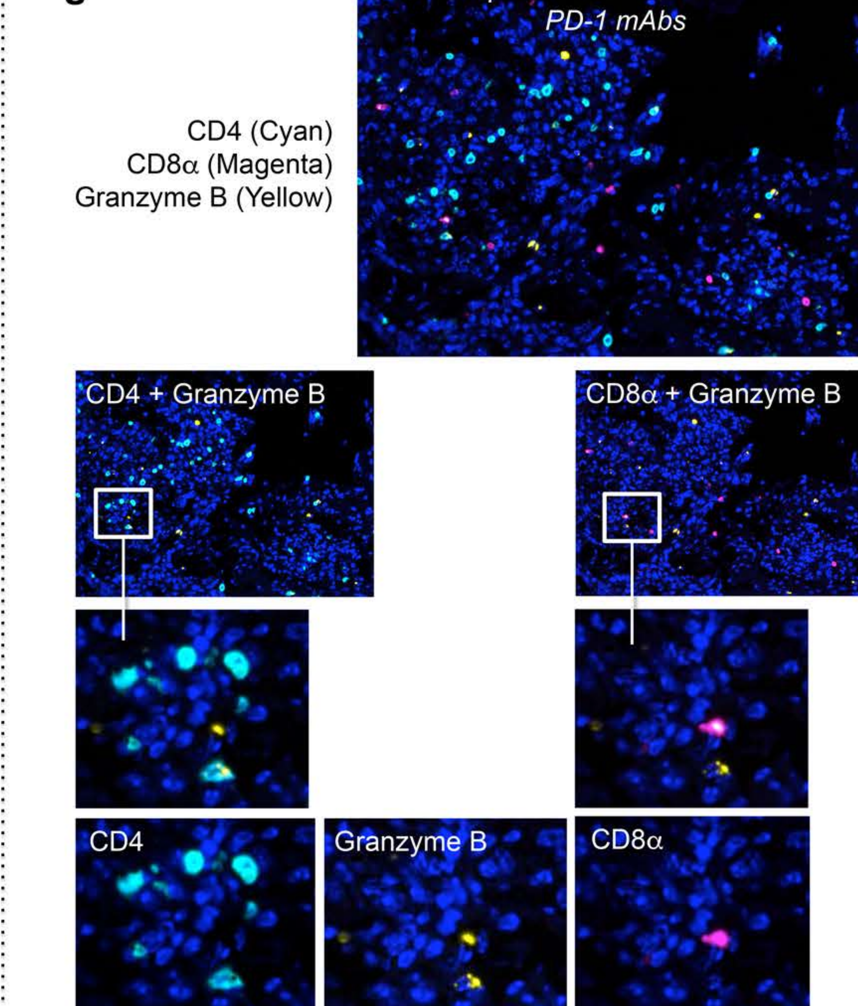
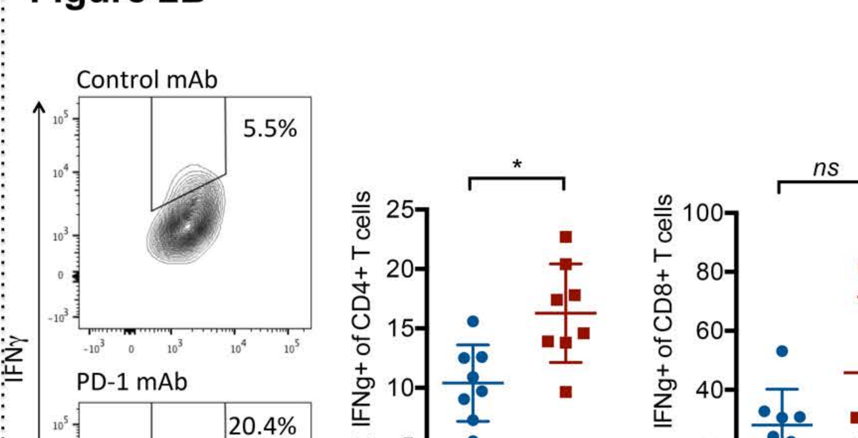


Figure 2B



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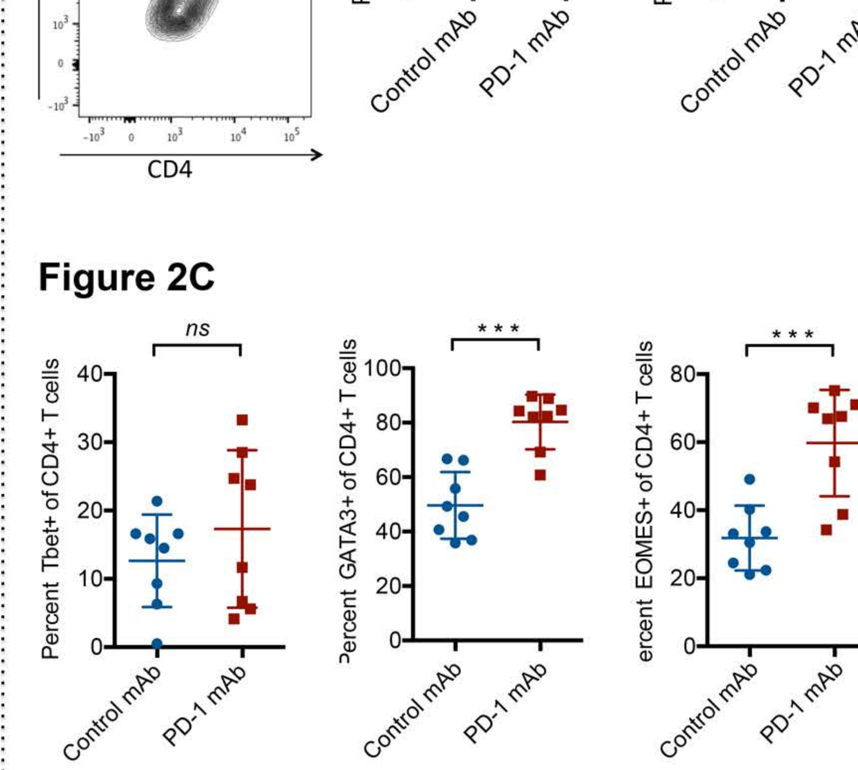
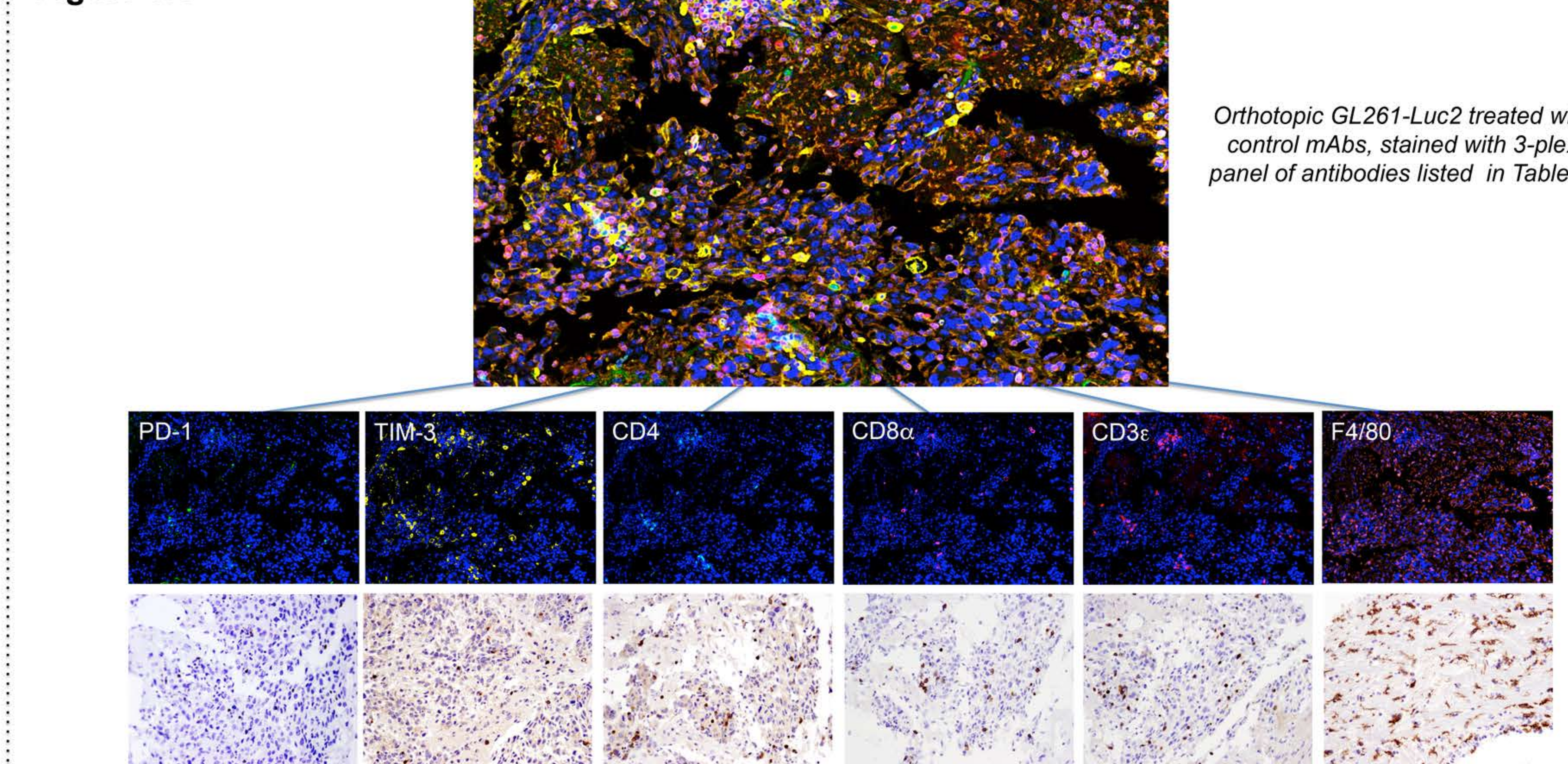


Figure 2. PD-1 monotherapy induces intratumoral CD4 T cell activation. GL261-Luc2 tumors were established with advanced disease and treated with isotype control IgG or PD-1 mAbs on days 14, 17, 20, and 23. Brains were harvested on day 24, fixed, and embedded in paraffin. (A) Representative multiplex immunohistochemistry images of CD4+ *(cyan)*, CD8+ *(magenta)*, and Granzyme B+ *(yellow)* cells within the tumor. (B) Representative flow cytometry plots of CD4+IFN-γ+ T cells. Values in top right corner represent the percent of IFN-γ+ gated on CD45+CD3+CD4+ T cells. (C) Percentages of live CD45+CD3+CD4+ cells that are T-bet+, GATA3+, and EOMES+ detected by flow cytometry. All graphs include values for individually analyzed mice, and the mean + SEM of 6 mice per treatment group. Two-tailed Student's T test was used to determine statistical significance (*P<0.05; **P P<0.01; ***P<0.001)

Figure 3A



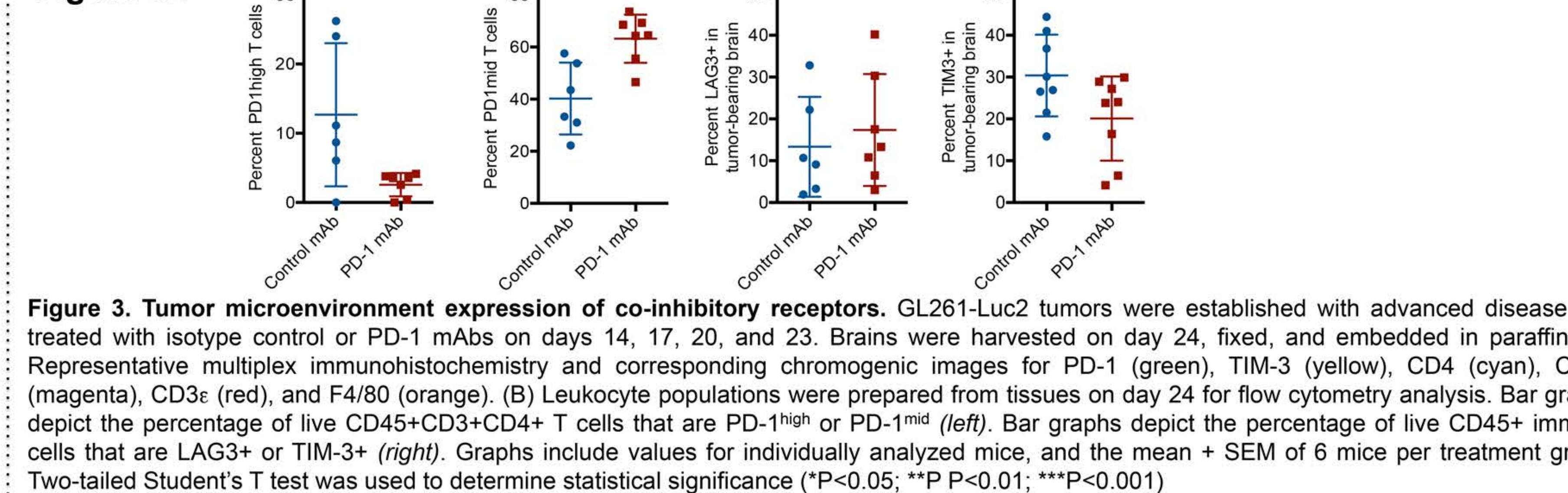


Figure 3B

Figure 3. Tumor microenvironment expression of co-inhibitory receptors. GL261-Luc2 tumors were established with advanced disease and treated with isotype control or PD-1 mAbs on days 14, 17, 20, and 23. Brains were harvested on day 24, fixed, and embedded in paraffin. (A) Representative multiplex immunohistochemistry and corresponding chromogenic images for PD-1 (green), TIM-3 (yellow), CD4 (cyan), CD8α (magenta), CD3ε (red), and F4/80 (orange). (B) Leukocyte populations were prepared from tissues on day 24 for flow cytometry analysis. Bar graphs depict the percentage of live CD45+CD3+CD4+ T cells that are PD-1^{high} or PD-1^{mid} *(left)*. Bar graphs depict the percentage of live CD45+ immune cells that are LAG3+ or TIM-3+ *(right)*. Graphs include values for individually analyzed mice, and the mean + SEM of 6 mice per treatment group. Two-tailed Student's T test was used to determine statistical significance (*P<0.05; **P P<0.01; ***P<0.001)

Figure 4A

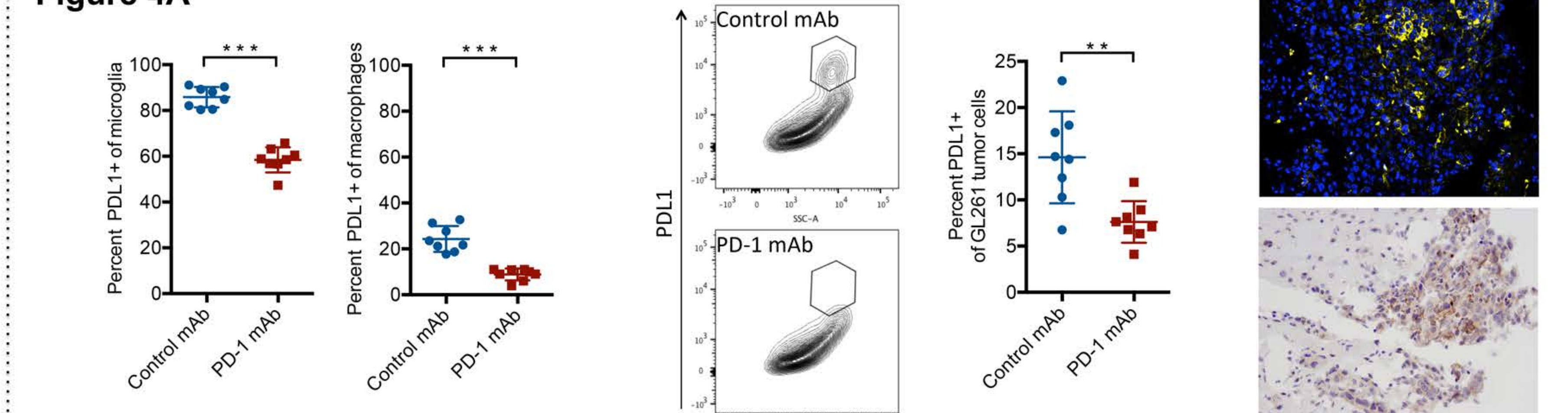


Figure 4B

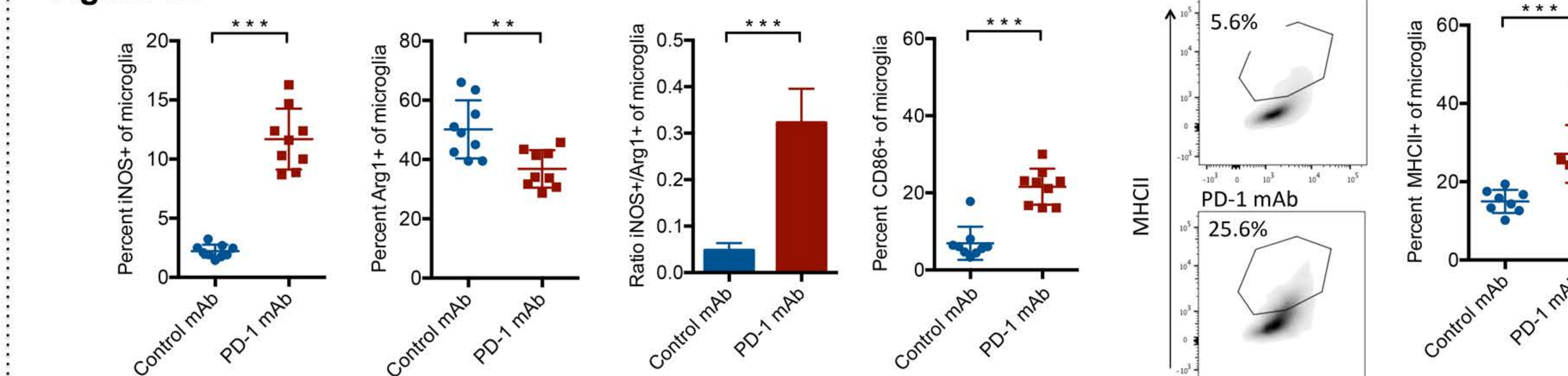


Figure 4C

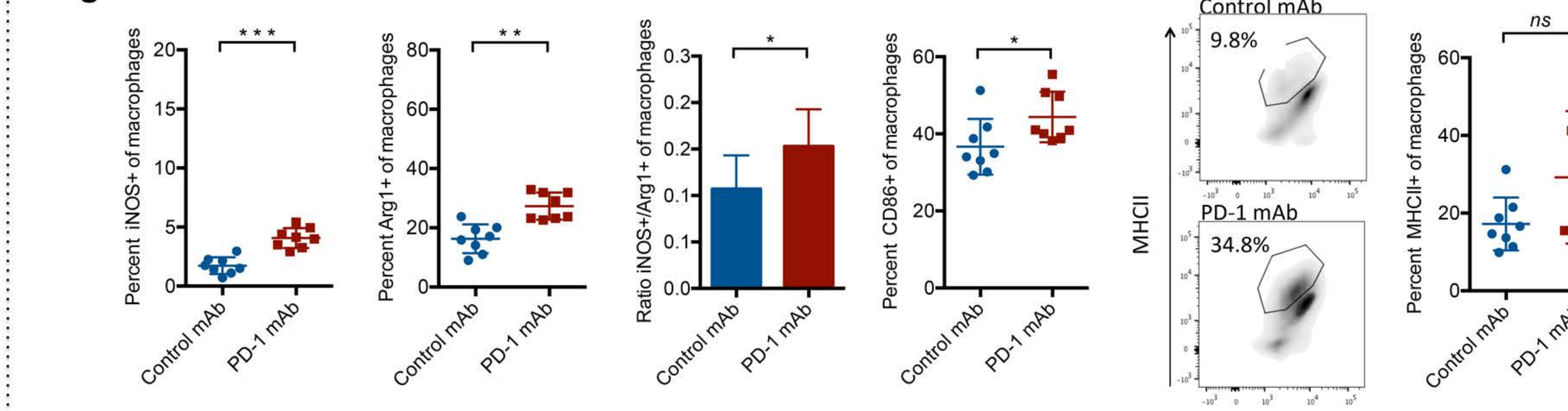


Figure 4. Phenotypic analysis of M1- and M2-polarized resident microglia and tumor-infiltrating macrophages. GL261-Luc2 tumors were established with advanced disease and treated with isotype control or PD-1 mAbs on days 14, 17, 20, and 23. (A) Leukocyte populations were prepared from tissues on day 24 for flow cytometry analysis. Percentages of live CD45^{lo}CD11b+ microglia, CD45^{hi}CD11b+ macrophages, or CD45-GFAP+ tumor cells that are PD-L1+. Representative multiplex immunohistochemistry and corresponding chromogenic images for PD-L1 *(yellow)*. (B) iNOS+, Arg1+, or CD86+ as a percentage of live CD45^{low}CD11b+ microglia. Bar graph depicts the ratio of iNOS+/Arg1+ gated on microglia. Representative flow cytometry plots of MHC class II (MHCII). Values in top left corner and graph *(right)* represent the percent of MHCII+ within the microglia population. (C) iNOS+, Arg1+, or CD86+ as a percentage of live CD45^{high}CD11b+ tumor-infiltrating macrophages. Bar graph depicts the ratio of iNOS+/Arg1+ gated on macrophages. Representative flow cytometry plots of MHC class II (MHCII). Values in top left corner and graph *(right)* represent the percent of MHCII+ cell within macrophage population. All graphs include values for individually analyzed mice, and the mean + SEM of 6 mice per treatment group. Two-tailed Student's T test was used to determine statistical significance (*P<0.05; **P P<0.01; ***P<0.001)

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