#1668. Comprehensive 10-Color Flow Cytometry Analysis of the Neuroblastoma Intratumoral Immune Response Using the Murine Syngeneic Neuro-2a Tumor Model

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Introduction

Background

Neuroblastoma is the most prevalent solid tumor in childhood and the most common tumor in infants less than 1 year of age. In spite of aggressive multi-modal therapeutic approaches, the five-year survival rate in patients with high-risk neuroblastoma remains poor at <50%.

Although anti-CTLA-4 and anti-PD-L1 can suppress neuroblastoma growth in certain mouse models, the role that the immune system has in controlling neuroblastoma growth is not well characterized.

This study provides a comprehensive profile of the neuroblastoma immune response using the murine syngeneic Neuro-2a model and investigates how anti-PD-1 therapy shapes that response in the tumor microenvironment (TME).

Methods

- Groups of mice implanted with Neuro-2a tumors were dosed with anti-mouse PD-1 antibody, isotype-matched control antibody, or left untreated.
- Tumor burden was measured at 17 days post-implantation.
- MI-CompT™ and MI-TAM™ panels were used to profile the following T cell and myeloid subsets by flow cytometry:
  – CompT: CD4+ and CD8+ T cells, regulatory T cells (Tregs), activated/exhausted CD8+ T cells
  – TAM: Monocytic and granulocytic myeloid-derived suppressor cells (M-MDSC, G-MDSC), macrophages, M1 and M2 tumor-associated macrophages (TAM).
- CD8+ T cell pro-inflammatory cytokine responses were measured ex vivo in tumor-derived cells from isotype and anti-PD-1 treated groups.

Results

- Neuro-2a tumors contained relatively few T cells and were dominated by MDSC subsets and M2 polarized macrophages.
- Both isotype and anti-PD-1 treatment increased intra-tumoral T cells and decreased the prevalence of suppressive myeloid subsets.
- Anti-PD-1 antibody treatment but not isotype control enhanced CD8+ T cell cytokine responses to in vitro re-stimulation.

Materials and Methods

- Female A/J mice were purchased from Jackson Laboratories and were implanted subcutaneously in the low axilla with Neuro-2a cells (n=4-7/group).
- Mice were treated via intra-peritoneal injection with In Vivo Plus anti-mouse PD-1 antibody from Bio X Cell (West Lebanon, NH) at a fixed 200µg/injection, every third day for a total of five doses.
- For immunophenotyping, tumors were processed into single cell suspensions using the gentleMACS™ Dissociators (Miltenyi Biotec). Samples were acquired on an Attune™ NxT Flow Cytometer (Thermo Fisher Scientific) and data was analyzed using FlowJo software (Tree Star).
- For cytokine analysis, tumor-derived cells were cultured at a concentration of 1X10^6/ml treated with PMA (40 ng/ml) and Ionomycin (500 ng/ml) for five hours in the presence of Brefeldin A. Intracellular interferon gamma (IFNγ) and tumor necrosis factor alpha (TNFα) expression were analyzed by flow cytometry.

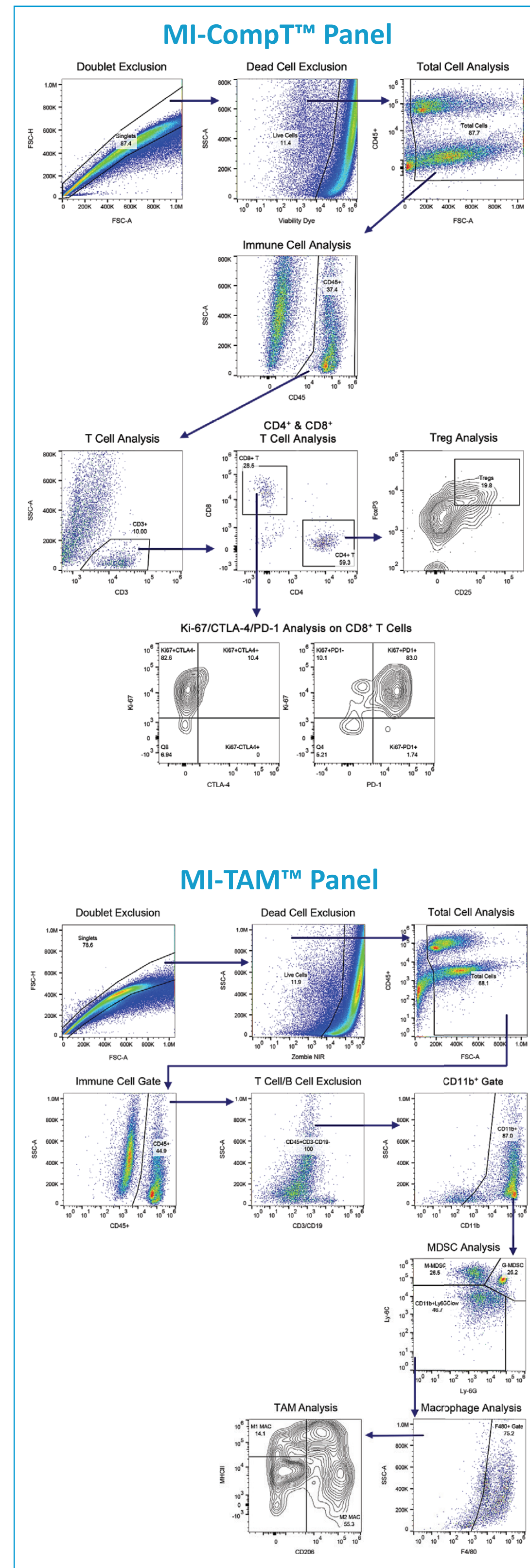

Figure 1. Gating Strategies.
Immunophenotyping of Neuro-2a tumors using the MI-CompT™ and MI-TAM™ panels.

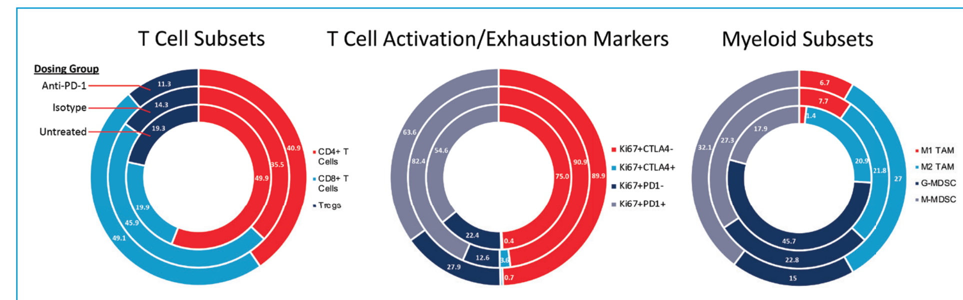

Figure 2. T Cell and Myeloid Subset Quantification in Neuro-2a Tumors Using the MI-CompT™ and MI-TAM™ Panels.
The MI-CompT™ and MI-TAM™ panels can quantify a wide range of T cell and myeloid subsets in tumors and other tissue. The effects that in vivo isotype control and anti-PD-1 antibody treatment have on the expansion of immune subset in the TME was assessed. Each partition in the charts above represents the percentage of the indicated endpoint from each treatment group measured as follows: CD3+ and CD11b+ cells as a percent of total intra-tumoral CD45+ cells. CD4+ and CD8+ T cells as a percent of CD3+ cells. Tregs as a percent of CD4+ T cells. Activation/Exhaustion marker expression as a percent of CD8+ T cells. M1/M2 TAM, and G/M-MDSC as a percent of CD11b+ cells.

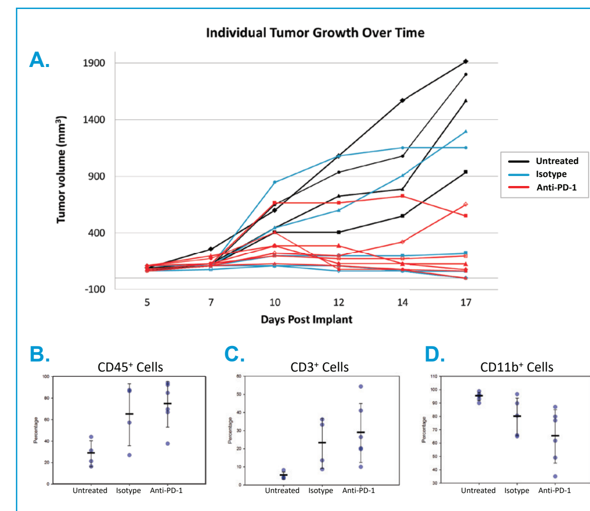

Figure 3. Tumor Burden Inversely Correlates with T Cell Percentages in the Tumors of Treated Mice
The individual tumor growth was measured three times per week (A). On day 17, tumors were harvested and analyzed. CD45+ cells were measured as a percent of total tumor-derived cells (B). T cells and myeloid cells identified as positive for CD3 and CD11b expression respectively were measured as a percent of total CD45+ cells (C). Taken together, these results indicate that tumor growth inhibition correlates with an increase in the recruitment and expansion of T cells in the tumors of antibody treated mice.

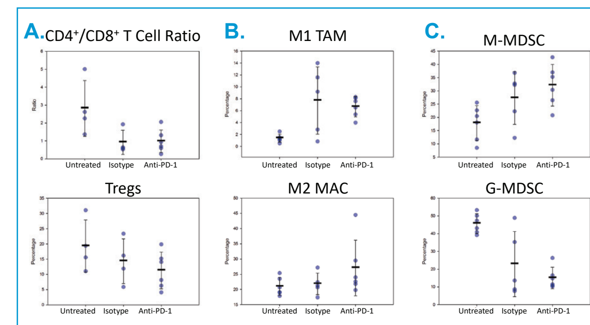

Figure 4. TME Immune Profile Shifts Toward an Anti-Tumor Phenotype in Isotype and Anti-PD-1 Antibody Treated Mice.
To gain insight into the effects that in vivo antibody treatment has on pro- and anti-tumor responses in the TME, the percentage of several key immune subsets were measured as described in Fig. 2. In the T cell compartment (A), a decrease in the CD4+/CD8+ T cell ratio was observed in both isotype and PD-1 antibody treatment groups. The percentage of Tregs also decreased in both treatment groups. In the macrophage compartment (B), an increase in the percentage of M1 TAMs was observed. Finally an increase in monocytic-MDSCs and a corresponding decrease in granulocytic–MDSCs was observed (C).

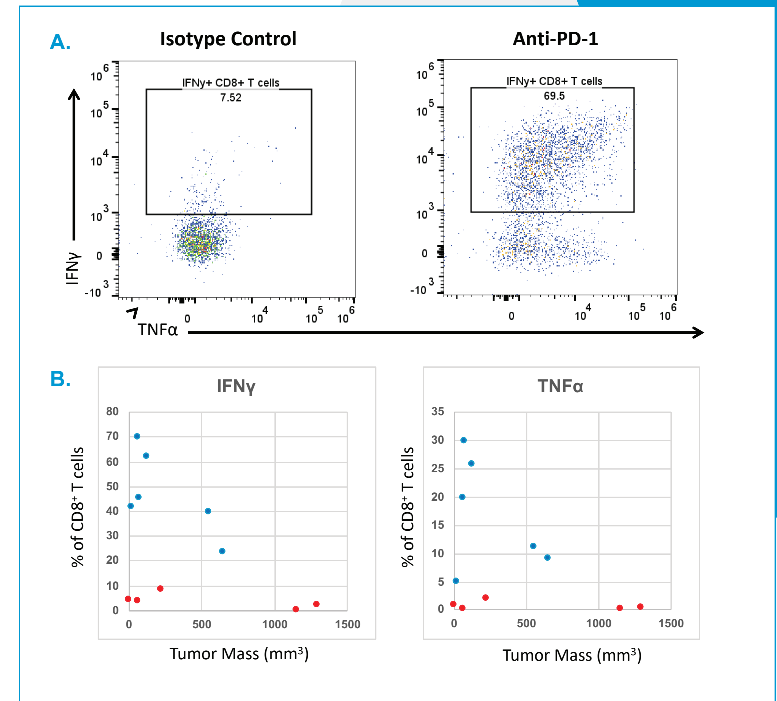

Figure 5. Anti-PD-1 Treatment Elevates IFNγ and TNFα Responses in CD8+ T cells, which Inversely Correlates with Tumor Burden.
To gain insight into the effects that anti-PD-1 antibody treatment has on CD8+ T cell function in the TME, tumor-derived cells were treated ex vivo with PMA and Ionomycin and de novo IFNα and TNFα expression was measured in gated CD8+ T cells by flow cytometry. Representative dot plots that demonstrate anti-PD-1 treatment enhances the potential of CD8+ T cells to produce IFNγ and TNFα are shown (A). Furthermore, an overall inverse correlation between cytokine production and tumor mass is observed (B). This trend is seen in the anti-PD-1 treated group (blue data points) as well as isotype treated group (red data points) albeit on a smaller magnitude. Together, these data indicate that pro-inflammatory cytokines produced from CD8+ T cells in the TME play an important role in the inhibition of Neuro-2a tumor growth.

Conclusions

- Treatment with anti-PD-1 antibody shifts the intra-tumoral immune profile towards an inflammatory and anti-tumor phenotype characterized by decreased myeloid subsets and increased T cell subsets.
- Anti-PD-1 antibody treatment triggered a reduced CD4+/CD8+ T cell ratio in the TME, reduced Tregs, and increased M1 polarized TAMs. Remarkably, a similar trend was observed in the isotype control antibody treated group.
- Compared to the isotype treated group, treatment with anti-PD-1 antibody dramatically increases the potential of cytotoxic CD8+ T cells to produce pro-inflammatory cytokines. These elevated cytokine levels in turn likely contribute to the corresponding tumor growth inhibition and may distinguish the mechanism by which the isotype antibody and the anti-PD-1 antibody exert the anti-tumor effects we observed.

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