# #4982. Anti-mCTLA-4 Treatment Results in Early and Late Immune Response Effects in a Murine Model of Colorectal Carcinoma

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## Introduction and Background

- During pre-clinical research, analysis of the tumor immune response at a single post-treatment timepoint is often standard when therapeutic mechanism of action is investigated.
- The use of a single sampling point for analysis can limit research on new immune modulation therapies as it provides only a partial view into the dynamic nature of the developing immune response in the tumor.
- In this study we examined the kinetics of tumor-directed immune infiltration and activation on day 11 and day 17 post-implantation using the CT26 model for colorectal carcinoma.
- The CT26 model is moderately responsive to checkpoint inhibition. To examine early and late effects that checkpoint inhibition has on the immune response, tumor-bearing mice were treated with anti-mCTLA-4. Tumor analysis corresponded to day 4 and day 10 post-treatment.
- Endpoints analyzed consisted of:
  - 11 distinct tumor-infiltrating subsets
  - CD8+ T cell activation marker expression
  - T cell cytokine analysis
  - Effector/Memory T cell phenotype
- We hypothesized that multiple sampling points would uncover unique immune subset specific profiles of infiltration and activation in the CT26 tumor.

## Materials and Methods

- CT26 cells were implanted subcutaneously into the right axilla of BALB/c mice. Mice were randomized into treatment groups following establishment of tumors. Anti-mCTLA-4 antibody (clone 9D9) was acquired from Bio X Cell and dosed IP. Tumor progression was monitored by caliper measurements.
- For immune profiling, tumors were dissociated into single cell suspensions (gentleMACS™, Miltenyi), labeled with fluorescent antibodies, and analyzed by an Attune™ NxT flow cytometer (Thermo Fisher Scientific). The immune subsets were then delineated using FlowJo (FlowJo, LLC). The number of cells/gram of tumor was quantified using Precision Count Beads™ (BioLegend). For effector/memory T cell analysis, a spleen from a naïve BALB/c mouse was analyzed for comparison.
- Tumor-infiltrating immune subsets were immunophenotyped using the Covance MI-CompLeukocyte™ package and MI-T Effector/Memory™ panel.
- For IFNγ and granzyme B analysis, tumor-derived cells were stimulated ex vivo with PMA and ionomycin for 5 hours in the presence of brefeldin A. Following incubation, non-adherent cells were collected and immuno-stained with fluorescent antibodies targeting cell surface receptors. The cells were then fixed, permeabilized and stained with anti-IFNγ and granzyme B antibodies prior to sample acquisition.

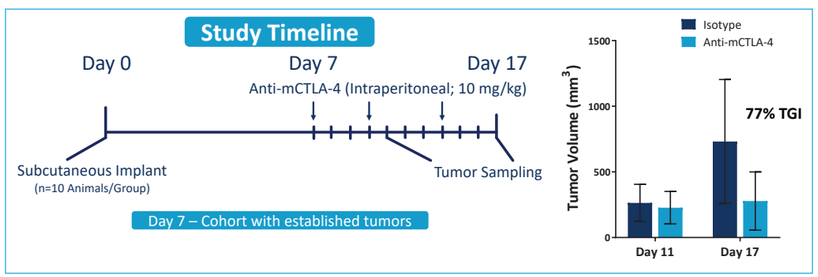


**Figure 1. Anti-mCTLA-4 Inhibits CT26 Tumor Growth.**
**(Left) In vivo study design and timeline.**
**(Right ) Mean tumor volumes on days 11 and 17 post implant (n=10/group).**

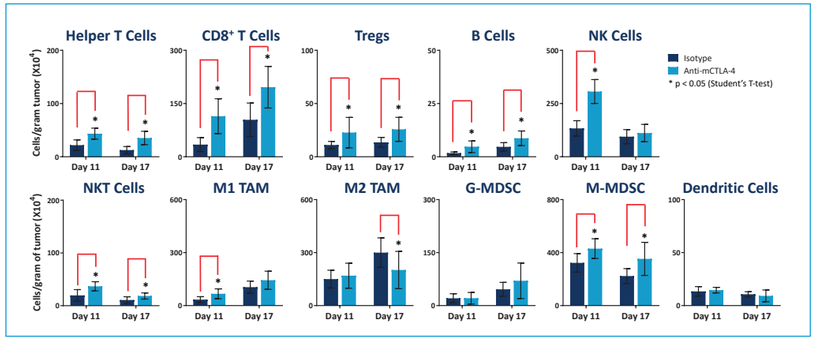


**Figure 2. TIL Kinetics are Regulated by Anti-mCTLA-4.**
**A increase in the infiltration of CD8+ T cells, B cells, macrophages and G-MDSCs was observed from day 11 to day 17.**
**Helper T cell, NK, NKT and M-MDSC infiltration peaked at the day 11 timepoint.**
**Anti-mCTLA-4 treatment enhanced infiltration of all T cell subsets, NKT cells and M-MDSCs at day 11 and day 17.**
**Anti-mCTLA-4 treatment triggered marked increases in NK and M1 TAM infiltration at day 11 only.**
**Anti-mCTLA-4 inhibited the infiltration of M2 TAMs at day 17.**

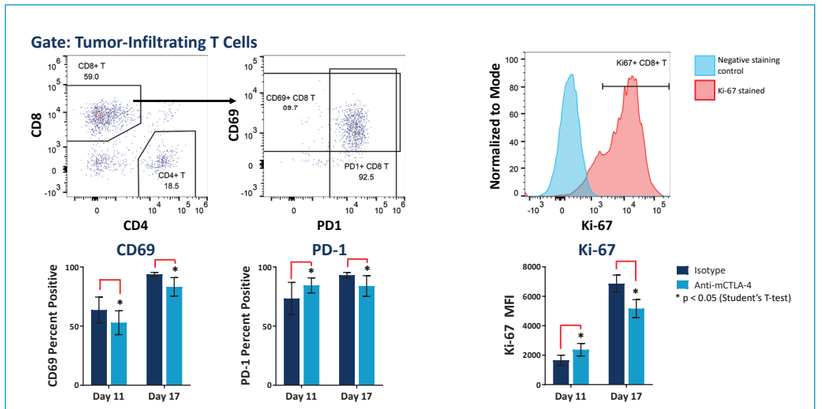


**Figure 3. Anti-mCTLA-4 Alters the Kinetics of T Cell Activation.**
**(Left) CD69, PD-1 and Ki-67 expression increased on CD8+ T cells from day 11 to day 17.**
**(Right) Anti-mCTLA-4 delayed CD69 upregulation but upregulated PD-1 and Ki-67 expression at the day 11 timepoint.**

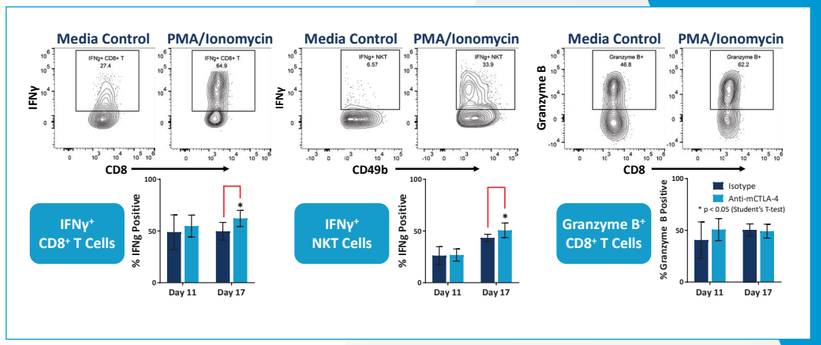


**Figure 4. Anti-mCTLA-4 Increases IFNγ in CD8+ T Cells and NKT Cells.**
**(Top) Representative plots to illustrate IFNγ and granzyme B responses following treatment with PMA and ionomycin (Gate: Tumor CD3+ cells).**
**(Bottom) Anti-mCTLA-4 treatment enhanced IFNγ (but not granzyme B) production in CD8+ T cells and NKT cells.**

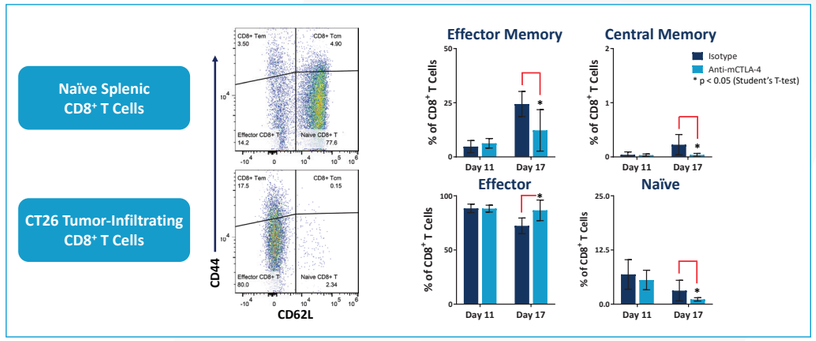


**Figure 5. Anti-mCTLA-4 Prolongs Effector CD8+ T Cell Infiltration.**
**Anti-mCTLA-4 triggered an increase in effector CD8+ T cells in the tumor and a corresponding decrease in naïve and memory T cells at day 17.**

## Results and Conclusions

- Using the CT26 model, we profiled the kinetics of immune subset infiltration in tumors with or without checkpoint blockade. The results demonstrate that kinetic analysis can reveal trends that would go unnoticed if analysis was instead performed at a single time point.
- Notably, while the tumor infiltration of most subsets increased from day 11 to day 17, Helper T cell, NK, NKT and M-MDSC subsets peaked at day 11. Furthermore, the effects of anti-mCTLA-4 had temporal components, which included NK and TAM infiltration, Ki-67 expression in CD8+ T cells, IFNγ responses in CD8+ T cells and NKT cells and memory T cell formation.
- Taken together, these data emphasize that a model-specific understanding of how the immune response develops during tumor progression is important. These characteristics can drive model selection and sampling time decisions when different target endpoints are considered for analysis.

*Presented at the AACR Annual Meeting 2019, March 31- April 3, 2019 – Atlanta, GA*

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