A kinetic analysis of tumor gene expression and cellular changes following immune checkpoint inhibition in the MC38 colon carcinoma model

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1 Abstract

The clinical success of immune checkpoint therapies such as PD-1, PD-L1, and CTLA-4 inhibitory antibodies has stimulated interest in a wide range of approaches to cancer immunotherapy. Development of these new modalities requires robust, well characterized preclinical animal models to evaluate efficacy and potential toxicities. Preclinical efficacy assessments of novel immune-oncology therapies requires a functional immune system which limits the usefulness of traditional xenograft models and drive the use of humanized (human immune cell engrafted animals) and syngeneic model systems. Syngeneic mouse tumors are popular model systems in which to evaluate these novel therapies due the presence of fully intact mouse immune system and ease of accessibility. The MC38 mouse colon cancer model is popular for efficacy assessment studies due to its responsiveness to typical immune checkpoint inhibitors (ICI). We endeavored to characterize the kinetic immune response to checkpoint inhibitors (anti-PD-1 + anti-CTLA-4) in MC38 tumors using flow cytometry and gene expression analysis. Gene expression analysis provided a signature of gene changes that correlate to immune driven changes in tumor growth and may be used in preclinical pharmacodynamics studies as evidence of mode of action for other novel immune-oncology therapies. In addition, gene overrepresentation analysis highlighted the presence and involvement of B cell populations in the MC38 tumor environment which was supported by IHC data that show distinctive B cell staining in what resemble tumor associated tertiary lymphoid structures. These findings provide a greater understanding of the ICI response in the MC38 tumor model and may inform mode of action study designs for other novel immuno-oncology therapies.

2 Methods and Materials

In Vivo Studies:

C57BL/6 mice (Charles River) were implanted in the flank with 5×10^5 MC38 cells and dosing began when tumors reached a mean volume of 184 mm^3. Mice were dosed IP with either vehicle or anti-PD-1 + anti-CTLA-4 (ICI) on a Day 1, Day 4, and Day 8 schedule. For the kinetic response to ICI treatment, 10 animals were sacrificed and tumors collected for either flow cytometry (5 tumors) or gene expression (5 tumors) analysis 24 hours after each dose. Vehicle treated animals were sacrificed and sampled on Day 9.

Flow Cytometry Analysis:

Tumors were dissociated to single cell suspensions and immune cell populations were analyzed by flow cytometry. All data were collected on a LSRFortessa (Becton Dickinson) and analyzed with FlowJo software (Tree Star, Inc.). Initial sequential gating on singlets (FSC-H vs. FSC-A), leukocytes (SSC-A vs. FSC-A) and live cells was performed followed by identification of immune cells populations based upon the following phenotype markers:

CD4	(CD45+ CD3+ CD4+ CD8-)	gMDSC	(CD45+ CD3- CD11b+ Ly6G+ Ly6C^{low})
CD8	(CD45+ CD3+ CD8+ CD4-)	mMDSC	(CD45+ CD3- CD11b+ Ly6G- Ly6C^{high})
Treg	(CD45+ CD3+ CD4+ CD25+ FoxP3+)	Macrophage	(CD45+ CD3- CD11b+ F4/80+)
NK	(CD45+ CD3- CD49b+ CD11b^{low})	B cells	(CD45+ CD3- CD11b- CD45R+ [B220])

Expression: CD69, CD86, MHC II, CD25

Gene Expression Analysis:

Tumor was extracted following the QuantiGene tissue homogenization protocol. Gene expression was analyzed using a 30 gene QuantiGene Plex panel (ThermoFisher) run on a MAGPIX analyzer. Data were normalized to the Tbp housekeeping gene and expressed as fold change from the control group mean. Expression data for the Day 9 time point was additionally performed using a Clariome D transcriptome microarray. Resulting gene overrepresentation data was analyzed by BiNGO (Cytoscape).

3 Results

Figure 1. Gene Overrepresentation Analysis for PD-1 + CTLA-4 Inhibitor Differentially Up-regulated Genes at Day 9

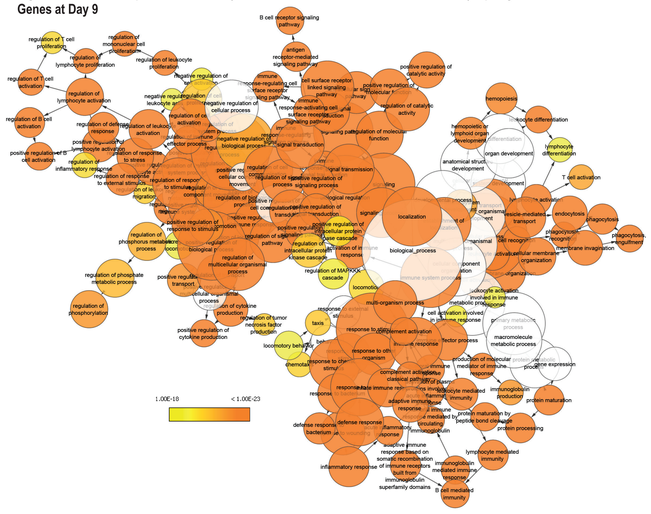


Figure 2. IHC Analysis of MC38 Tumor Infiltrating Leukocyte Populations. A. B Cell marker CD45R (B220) response to therapy in MC38 tumors; B. Sections showing tertiary lymphoid structure (B Cell).

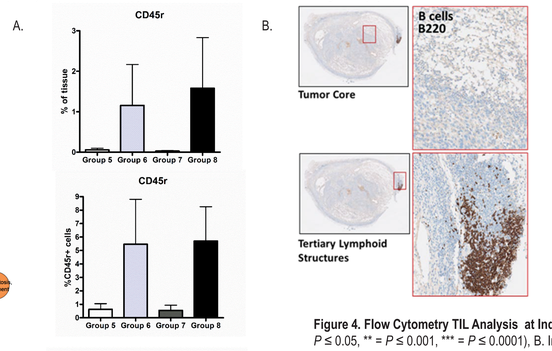


Figure 3. Tumor Growth Curves. A. Median growth curves for control and anti-PD-1 + anti-CTLA-4 treated groups; B. Spider plots of individual animal tumor growth.

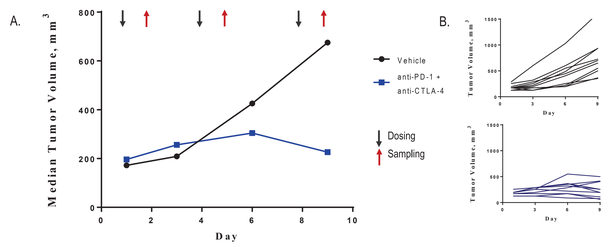


Figure 4. Flow Cytometry TIL Analysis at Indicated Time Points Following Either Vehicle or ICI Treatment. A. Mean and range data for each immune cell subpopulation (* = $P \leq 0.05$, ** = $P \leq 0.001$, *** = $P \leq 0.0001$), B. Immune cell populations presented as mean cells per mm^3 tumor volume,

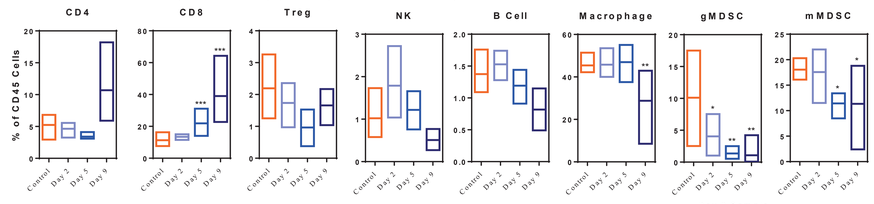
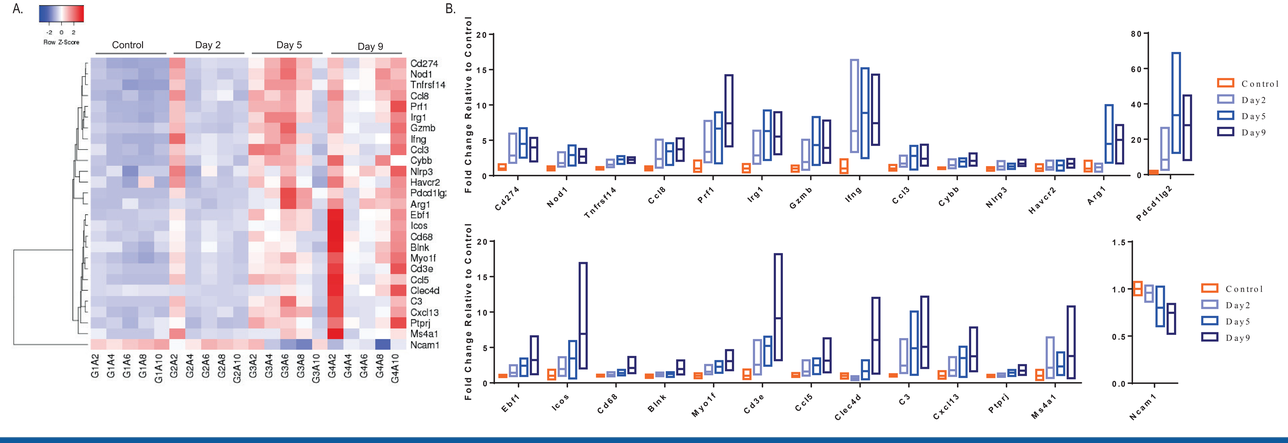


Figure 5. Gene Expression Time Course Data for ICI Treatment. A. Heatmap of individual tumor expression data at each time point (clustering method by average linkage and distance measurement by Pearson method); B. Box plots for mean and range for each gene in the QuantiGene Plex panel



4 Summary

- Kinetic changes in the TIL populations by flow cytometry were assessed in MC38 tumors following initiation of anti-PD-1 + anti-CTLA-4 antibody therapy.
- Microarray gene expression analysis identified a large number of differentially expressed genes involved in various aspects of immune function in response to ICI (anti-PD-1 + anti-CTLA-4) treatment in MC38. Expression of 27 genes were verified to change in response to the specific ICI treatment by the orthologous method of QuantiGene Plex.
- Kinetic changes in select gene expression were assessed in MC38 tumors following initiation of anti-PD-1 + anti-CTLA-4 antibody therapy.
- These data provide useful information on the kinetics of TIL population changes in response to ICI treatment.