#A34. Combined Focal Radiation and Anti-mCTLA-4 Antibody Therapy Modulates Myeloid Subset Phospho-STAT3 Levels in the 4T1 Tumor Microenvironment, and Results in Tumor Growth Inhibition

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Introduction and Background

Signaling through signal transducer and activator of transcription factor 3 (STAT3) in myeloid subsets triggers gene expression that has direct and indirect suppressive effects on several immune subsets.

Selective targeting of STAT3 in myeloid cells has become an attractive therapeutic approach as it can prevent adverse effects that may otherwise be triggered by broad inhibition of the pathway.

To facilitate development of drugs with selectivity for phospho-protein targets like STAT3, we have developed a phospho-flow panel that can measure phosphoprotein states in distinct myeloid subsets simultaneously.

The 4T1 syngeneic breast cancer model was used due to its high prevalence of granulocytic (G) MDSCs, as well as other immunosuppressive myeloid subsets.

Our results demonstrate that phospho-STAT3 (pSTAT3) levels were differentially regulated in MDSC and tumor-associated macrophage (TAM) subsets following in vivo treatment with focal radiation, anti-mCTLA-4, or the combination.

Materials and Methods

- 4T1-Luc cells were implanted in the lower mammary fat pad of female Balb/c mice. Mice were randomized into treatment groups following establishment of tumors. Checkpoint inhibitor antibody was acquired from Bio X Cell and dosed IP. Image-guided irradiation was performed under 1-2% isoflurane on the Small Animal Radiation Research Platform (SARRP; Xstrahl, Inc.). Treatment was delivered to a 10 Gy total dose in 2 equally weighted beams. Tumor progression was monitored by caliper measurements.
- For immune profiling, tumors were collected when the control group reached a mean volume of 350-500 mm^3. After dissociation into single cell suspensions (gentleMACS™, Miltenyi), samples were labeled with a comprehensive leukocyte panel and analyzed by flow cytometry (Attune™ NxT, Invitrogen). The immune subsets were then delineated using FlowJo (Tree Star, Inc.). The number of cells/gram of tumor was quantified using Precision Count Beads™ (Biolegend).
- For phospho-flow, cell samples were fixed and permeabilized after antibody labeling for surface markers, and then stained with a fluorescently labeled anti-p-STAT3 antibody (S727).
- Statistical analysis was performed using a student's t-test.

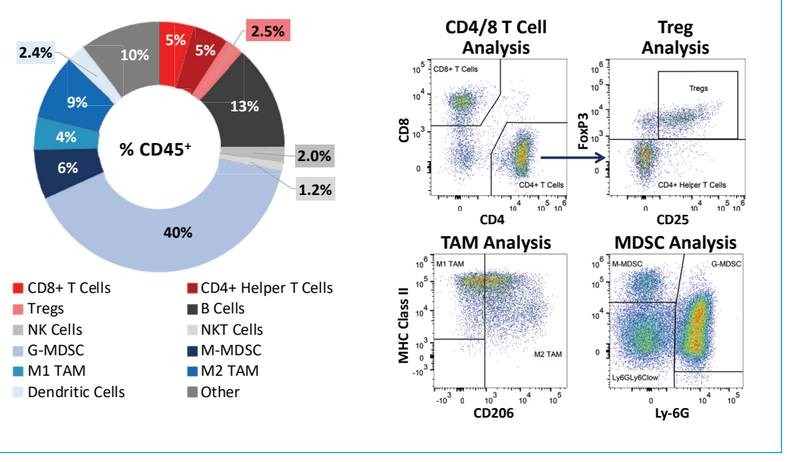

Figure 1. 4T1 Tumor Immunophenotype is Myeloid Dominated.
Baseline immunophenotype in 4T1 tumors. Data represents percentages of subsets among total CD45+ cells (mean of n=6 untreated mice). Pseudocolor plots show data from a representative mouse, which illustrates selected immune subset gating.

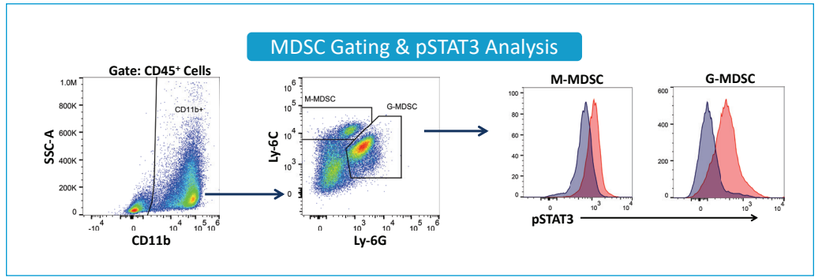

Figure 2. pSTAT3 is Detected in 4T1 Tumor MDSC Subsets.
pSTAT3 is detectable in M-MDSC and G-MDSC by phospho-flow analysis. pSTAT3 signal (red histogram peaks) is compared to the fluorescence minus one negative control signal (blue). Data is representative of n=5 mice.

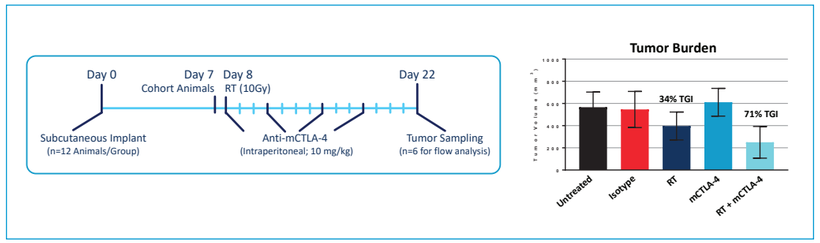

Figure 3. Radiation and Anti-mCTLA-4 Inhibits 4T1 Tumor Growth.
(Left) In vivo study design and timeline. (Right) Mean tumor volume (mm^3) on day 21 post-implant (n=12/group). Tumor growth inhibition (TGI) was quantified where indicated.

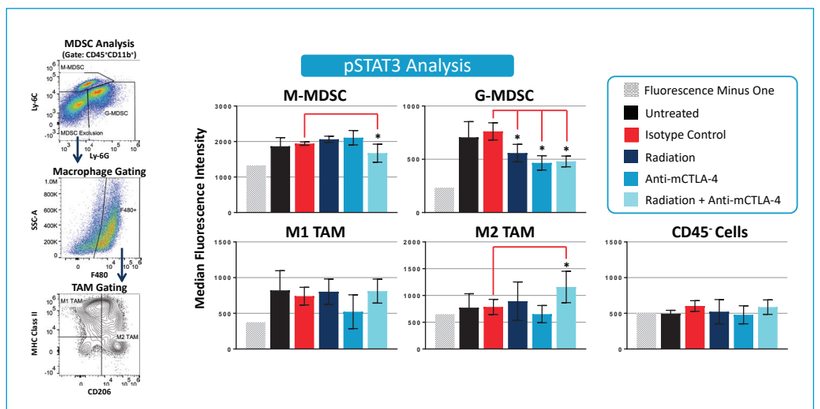

Figure 4. Myeloid pSTAT3 Levels are Altered by In Vivo Therapy.
(Left) In vivo study design and timeline. (Right) Mean tumor volume (mm3) on day 21 post-implant (n=12/group). Tumor growth inhibition (TGI) was quantified where indicated.

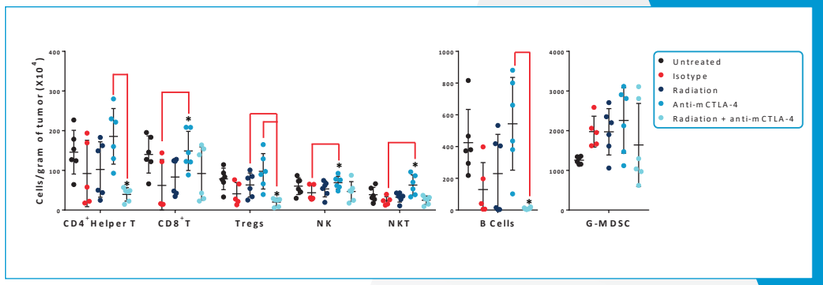

Figure 5. Lymphocyte Subset Infiltration in the 4T1 Tumor.
Immune subset infiltration into the tumor was measured using the MI-CompLeukocyte panel. Changes in response to combined treatment include significant reductions in Tregs, and B cells. M-MDSC, M1 TAM, M2 TAM and DCs did not change in response to treatment (not shown). * p value < 0.05

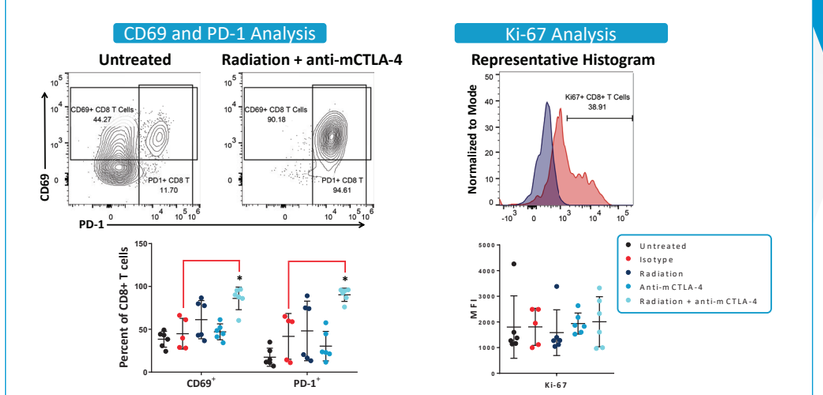

Figure 6. Upregulated Activation Markers on CD8+ T Cells.
(Left) Combined treatment with radiation and checkpoint blockade triggered CD69 and PD-1 to be expressed on >90% of T cells. (Right) Ki-67 was unchanged at the timepoint evaluated. * p value < 0.05.

Results and Conclusions

- Ex vivo phospho-flow analysis demonstrated that the differential effects of in vivo therapy on phospho-protein levels within distinct tumor-derived subsets can be detected. This enabled us to gain insight into how cell signaling may help shape anti-tumor immunity in response to therapy.
- Remarkably, pSTAT3 levels decreased and increased in MDSC and TAM subsets respectively, following radiation and checkpoint blockade. And maximum pharmacodynamic changes were, in some cases, observed following single agent treatment while in other cases, combined therapy was required to trigger the greatest dynamic change. Future work is needed to elucidate the mechanism by which radiation and anti-mCTLA-4 therapy alters myeloid STAT3 phosphorylation.
- An exciting finding was the near complete depletion of B cells and Tregs caused by combined therapy, but additional work would be needed to determine what, if any, role this plays in the biology of the model and its response to therapies.

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