Differences in T-cells Phosphorylated STAT Expression in Melanoma Patient Response Assessed by Flow Cytometry

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Background

STAT proteins Signal Transducer and Activator of Transcription proteins (STATs) mediate a number of cell functions. STAT proteins are phosphorylated upon activation, which is a critical modification for some functions. pSTATs are involved in regulating immune/tumor interactions and have shown to be of interest in cancer outcomes. Further, multiple pSTATs have been shown to be differentially expressed in melanoma samples, and may have therapeutic implications.

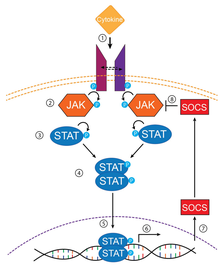


Figure 1: JAK – STAT pathway

1. Binding of cytokine to receptor
2. Recruitment of JAK/TYK to receptor; cross phosphorylation of JAKs; phosphorylation of receptor by JAK/TYK
3. Binding of STATs to phosphorylated receptors; phosphorylation of STATs by JAKs.
4. Phosphorylated STATs form hetero- or homodimers.
5. Phosphorylated STAT dimers migrate to the nucleus.
6. STAT bind to gene regions/motifs, initiate transcription.
7. SOCS proteins expressed in negative feedback loop.
8. SOCS proteins inhibit cytokine signaling

Rationale: We previously showed that pSTAT3(S727) expression in T-cells was positively associated with response in metastatic melanoma patients treated with αPD1 immunotherapy. This finding, in conjunction with other existing studies, indicate the potential importance of understanding T-cell pSTAT expression in differing melanoma patient responses. Consequently, we wished to assess potential associations of other pSTAT molecules in patient outcomes. Conventionally, pSTAT expression is evaluated by western blot assays, but this technology has several limitations. Flow cytometry is able to acquire multi-dimensional data at single cell resolution, relatively cheaply and rapidly. Therefore, we sought to generate and optimize a flow cytometry panel and protocol to simultaneously assess phosphorylation of all STAT proteins.

Methods

We developed a flow cytometry protocol to simultaneously assess eight phosphorylations of six STAT proteins, with two residues of both pSTAT1 and pSTAT3. Panels were optimized for fixation method, antibody clones and antibodies titrations. FlowJo software was used for data acquisition and analysis, with statistical analyses performed in R. The protocol was applied to two sample sets: demographic matched healthy donor vs melanoma PBMC, and PBMC from checkpoint immunotherapy, adjuvant treated metastatic melanoma patients.

Patient Demographics

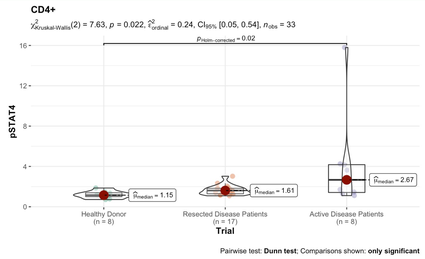
Healthy vs Melanoma PBMC (demographic matched):

Healthy Donor	Active Disease	Surgically Resected
N = 8	N = 8	N = 17

Adjuvant treated metastatic melanoma patients:
PBMC samples prior-to-treatment and week 13 of treatment from melanoma patients receiving combination αPD1 and αCTLA4 adjuvant immunotherapy:

Healthy Donor	Baseline	On Treatment
N = 10	N = 20	N = 18

Results: Healthy vs Melanoma



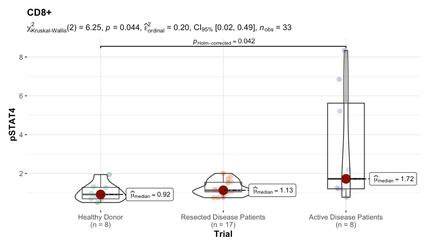


Figure 2:
pSTAT4 expressing CD4+ and CD8+ T-cells were increased in active disease patients compared to healthy donors (multiple comparison adjusted p=0.02 and 0.04, respectively). There was no significant difference between resected disease patients and healthy donors, or between resected disease patients and active disease patients.

Demographic matched PBMC samples were evaluated via flow cytometry to compare healthy donors, resected disease patients, and active disease patients for eight pSTAT residues. Patient samples were those collected prior to treatment.

pSTAT4 expression was found to be elevated in active disease patients in both CD4+ and CD8+ T-cells. Other pSTATs exhibited similar trends, suggesting further experimentation and exploration with increased sample sizes.

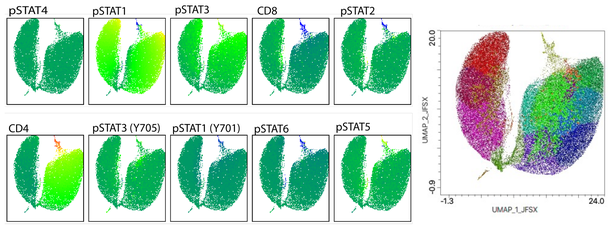
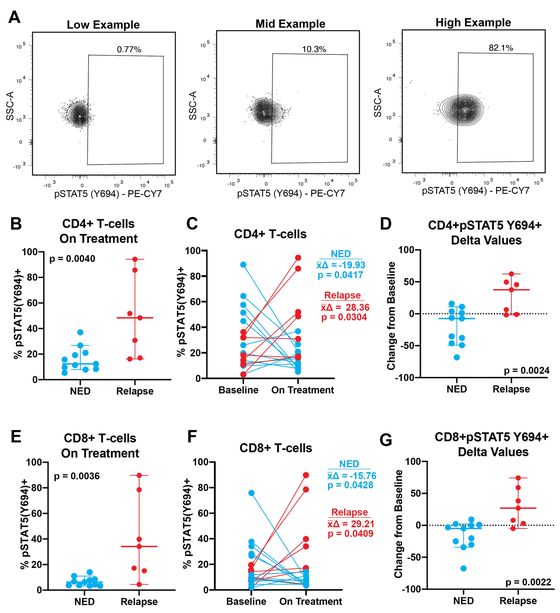


Figure 3:
A dimensionality reduction (umap) and Phenograph clustering were used to explore complex phenotypes. The clustering analysis showed clear separation between Umaps colored by each marker are shown in the left panels. The cluster analysis colored umap is on the right.

Results: Baseline vs On Treatment



PBMC from surgically resected stage III/IV melanoma patients treated with combination nivolumab and ipilimumab were evaluated by flow cytometry for the expression of STAT phosphorylation. Among others, we found significantly increased frequencies of pSTAT5(Y694) expression in both CD4+ and CD8+ T-cells in patients who relapsed. Paired, intra-patient analysis showed a significant increase in the frequency of pSTAT5+ T-cells from baseline to on-treatment (week 13) in relapsing patients and a reciprocal decrease in pSTAT5+ frequencies in no evidence of disease (NED) patients. This was observed in both CD4+ and CD8+ T-cells.

Figure 4:

- Panel A shows examples of differences in gated flow populations of pSTAT5 for samples with low, intermediate, and high expression.
- Panels B and E show the on-treatment frequencies of pSTAT5 CD4+ and CD8+ T-cells.
- Panels C and F show the change in pSTAT5 between baseline samples and on treatment samples for relapsing and NED patients.
- Panels D and G show the absolute change from baseline pSTAT5 values in relapsing and non-relapsing patients.

Panel Design

Vendor	Catalogue	Host	Target	Clone	Antigen	Fluorochrome
BioLegend	686412	Mouse	Human	A15158B	Stat1(p727)	AF647
BDBiosciences	562985	Mouse	Human	4a	Stat1 (pY701)	BV421
R&D	IC2890N	Rabbit	Human	1021D	Stat2 (Y689)	AF700
BDBiosciences	558557	Mouse	Human	49/p-Stat3	Stat3 (pS727)	PE
BDBiosciences	562673	Mouse	Human	4/P-STAT3	Stat3 (pY705)	PE-CF594
BDBiosciences	558136	Mouse	Human	38/p-Stat4	STAT4 (pY693)	AF488
BDBiosciences	560117	Mouse	Human	47/Stat5(pY694)	Stat5 (pY694)	PECY7
BDBiosciences	561195	Mouse	Human	18/P-Stat6	Stat6 (pY641)	PerCPCy5.5

Conclusions

We have developed and optimized a flow cytometry panel able to assess the expression of eight pSTATS. Our initial applications of this protocol has revealed differences in T-cell pSTAT signaling based on metastatic melanoma disease and patient outcomes on checkpoint immunotherapy. Given the simultaneous detection of pSTATs, this protocol lends itself to the use of dimension reduction techniques and cluster analyses to detect complex phenotypes.

The results of the two experiments suggest there are relationships between immune cell signaling, disease, and patient outcomes. Specifically, our preliminary data suggests elevated frequencies of pSTAT4 expressing T-cells in metastatic melanoma patients and increases in pSTAT5 expression during treatment as associated with relapse. Experiments are ongoing to validate these findings, explore potential associations of complex phenotypes with patient outcomes, and explore potential mechanistic relationships to patient outcomes.

References Support

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