Phosphoproteomics reveals GITR agonism differentially regulates inflammatory vs. regulatory T cell subsets.

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INTRODUCTION

Triggering the co-stimulatory receptor glucocorticoid-induced TNFR-related protein (GITR) on T cells has been identified as a promising cancer immunotherapeutic strategy, yet the signaling events by which GITR agonism induces antitumor immunity are not well understood. GITR is expressed on activated CD4$^+$ and CD8$^+$ T cells, and is constitutively expressed on regulatory T cells (T_{regs}). While GITR appears to act as a conventional co-stimulatory receptor in effector CD4$^+$ and CD8$^+$ T cells, the role of GITR on T_{regs} remains controversial.

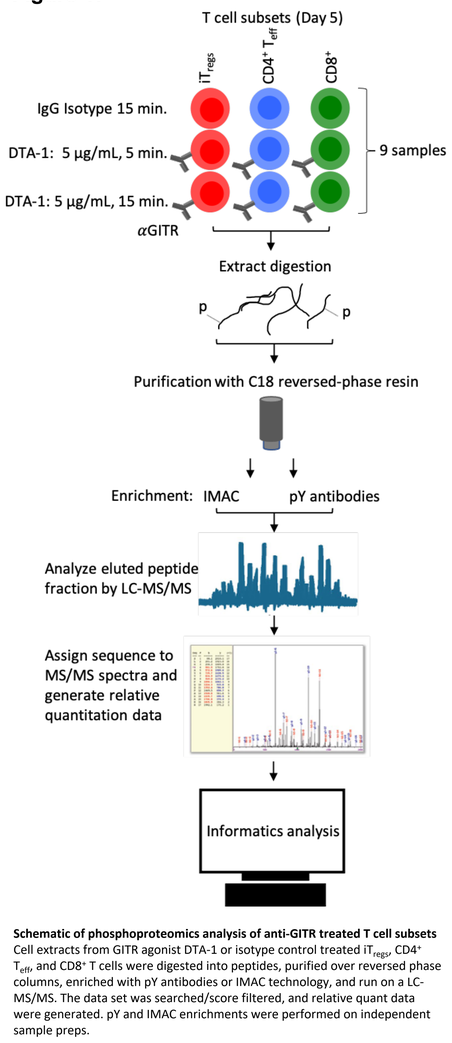
Here we utilized a phosphoproteomics approach to identify and compare GITR-mediated intracellular signaling events in induced-T_{regs} (iT_{regs}), CD4$^+$, and CD8$^+$ cytotoxic T cells. The GITR agonist monoclonal antibody DTA-1 was used to stimulate the T cell subsets and GITR agonism was validated via NFκB pathway activation and phospho-JNK induction. PTMScan® immobilized metal affinity chromatography (IMAC) beads were used to enrich Ser/Thr/Tyr-modified peptides, and PTMScan® Phospho-Tyrosine Rabbit mAb (P-Tyr-1000) was used to capture an additional pool of phospho-Tyr modified peptides (Cell Signaling Technology, Inc.).

CONCLUSIONS

- The phosphoproteomics data revealed distinct protein phosphorylation events between DTA-1-treated iT_{regs}, CD4$^+$ T_{eff}, and CD8$^+$ T cells.
- DTA-1 induced phospho-p38 MAPK pathway activation in iT_{regs}, but not in CD4$^+$ T cells or CD8$^+$ cytotoxic T cells.
- Induction of p38 MAPK signaling is functionally important as inhibition of p38 abrogated GITR-induced proliferation of iT_{regs}.
- These data demonstrate that GITR agonism induces unique phospho-proteome profiles between T cell subsets, and this differential signaling leads to unique responses that will need to be considered when targeting GITR with immunotherapeutic strategies.

Methods

Figure 1:



Schematic of phosphoproteomics analysis of anti-GITR treated T cell subsets Cell extracts from GITR agonist DTA-1 or isotype control treated iT_{regs}, CD4$^+$ T_{eff}, and CD8$^+$ T cells were digested into peptides, purified over reversed phase columns, enriched with pY antibodies or IMAC technology, and run on a LC-MS/MS. The data set was searched/score filtered, and relative quant data were generated. pY and IMAC enrichments were performed on independent sample preps.

Results

Table 1:

	IMAC	pY
Total Number of Detected Peptides	18,830	771
Total Number of Significant FC	5,645	244

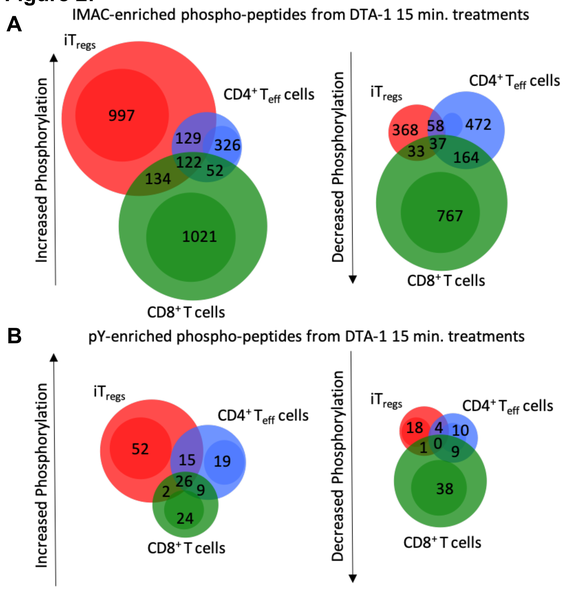
iT_{regs}		
Time Point	15 min.	
Enrichment	IMAC	pY
Increase	1382	95
Decrease	496	23

CD4$^+$ T_{eff} cells		
Time Point	15 min.	
Enrichment	IMAC	pY
Increase	629	69
Decrease	731	23

CD8$^+$ T cells		
Time Point	15 min.	
Enrichment	IMAC	pY
Increase	1,329	61
Decrease	1,001	48

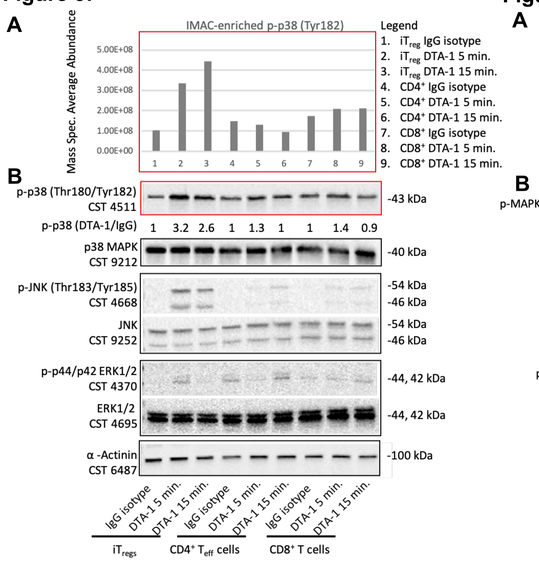
Summary of anti-GITR treatment IMAC and pY Enrichment Results. IMAC and pY-enriched phospho-peptide data from T cell subsets treated with 5 µg/mL IgG isotype control for 15 min. or 5 µg/mL DTA-1 for 5 or 15 min. Data are from two replicate LC-MS/MS runs per sample and were filtered to reflect nonredundant phosphorylation sites. The number of significant increases and decreases are shown for each T cell subset at 15 min. DTA-1 timepoints relative to IgG isotype control. Significant fold change increases are defined as the number of upregulated phospho-peptides ≥ 2.5 fold change and the number of decreases are defined as the number of downregulated phospho-peptides ≤ -2.5 fold change.

Figure 2:



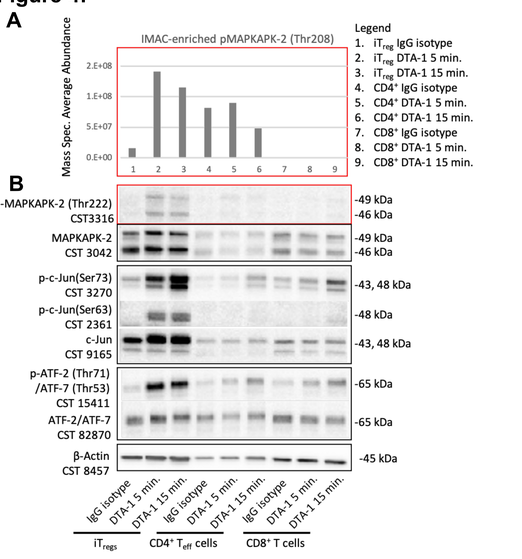
Venn diagrams of DTA-1-regulated phosphorylation events comparing iT_{regs}, CD4$^+$ T_{eff}, and CD8$^+$ T cells . (A) The number of unique IMAC-purified phospho-peptides that had elevated phosphorylation ≥ 2.5 fold (left), and reduced phosphorylation ≤ -2.5 fold change (right). (B) Venn diagrams showing the number of pY antibody-purified phospho-peptides that had elevated phosphorylation ≥ 2.5 fold change (left), and reduced phosphorylation ≤ -2.5 fold change (right). The relative size of the T cell subsets in the diagrams corresponds with the number of unique phospho-peptides detected. (A) is scaled for IMAC-purified peptides and can be compared across increases and decreases, and (B) is scaled for pY-purified peptides and can be compared across increases and decreases.

Figure 3:



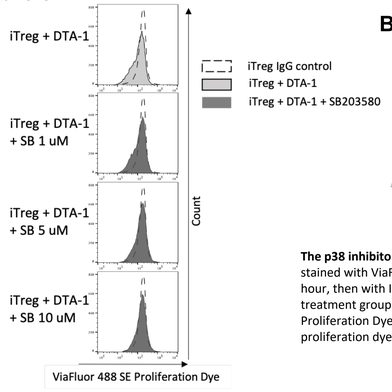
GITR triggering induces p38 phosphorylation in iT_{regs} but not in CD4$^+$ T_{eff} cells and CD8+ T cells. (A) Mass spectrometry measured average abundance (intensity) of p-p38(Tyr182) detected in the T cell subsets. (B) Western blot analysis of the three main phospho and total MAPK signaling pathway members. P-p38 is highlighted in red boxes to easily compare phospho-proteomics and western blotting detection. Column layout of samples in (A) corresponds with western blot lanes in (B). Relative fold change of p-p38 is shown normalized to IgG control for each subset.

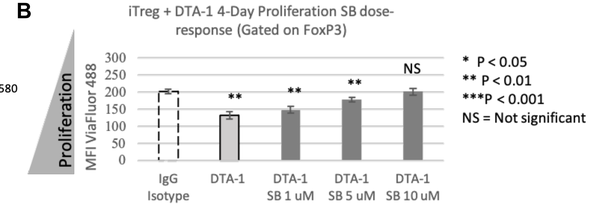
Figure 4:



GITR triggering induces signaling downstream of p38 phosphorylation in iT_{regs} differentially compared to CD4$^+$ T_{eff} cells and CD8$^+$ T cells. (A) Mass spectrometry measured average abundance (intensity) of p-MAPKAPK-2(Thr208) detected in the T cell subsets. P-MAPKAPK-2 (Thr208) was not detected in the CD8$^+$ T cell population. P-MAPKAP-2 (Thr222) is the human phospho site that the antibody product is named for, and (Thr208) is the mouse site. (B) Western blot analysis of phospho and total proteins downstream of p38.

Figure 5:





The p38 inhibitor, SB203580, abrogates DTA-1 induced proliferation of iT_{regs} in a dose-dependent manner. iT_{regs} were stained with ViaFluor 488 SE Proliferation Dye and were pretreated with SB203580 (1, 5, or 10 µM) or vehicle control for 1 hour, then with IgG isotype or DTA-1 (5 µg/ml) for 4 days. SB203580 was administered every 12 hours to respective treatment groups at the indicated concentration. (A) Histograms showing flow cytometry analysis of ViaFluor 488 SE Proliferation Dye gated on FoxP3$^+$ cells. (B) Column graph showing statistical comparison of average MFI of the proliferation dye of the indicated treatment groups (n=4). Statistical significance is shown relative to IgG Isotype control.

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