Exploring Proximal Biomarkers of CBL-B Inhibition in Human Peripheral Blood Mononuclear Cells

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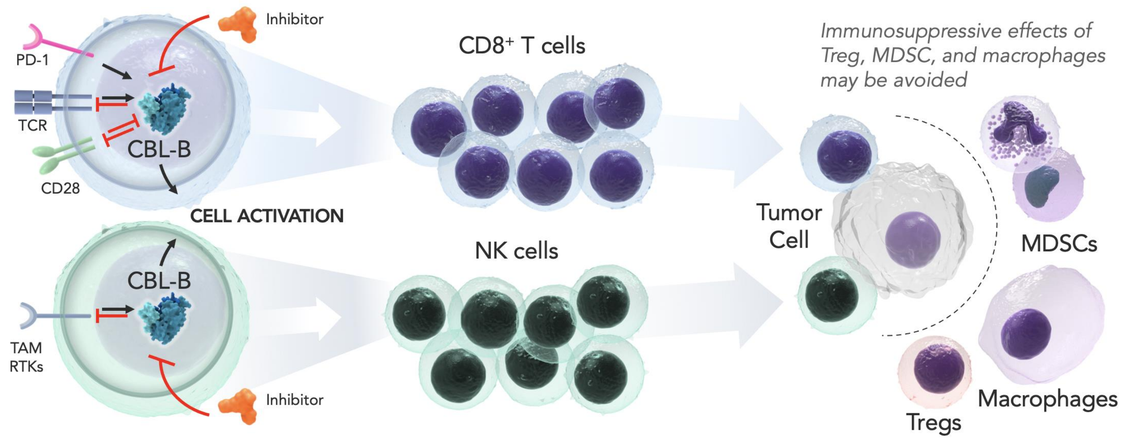
Introduction

- Casitas B-lineage lymphoma proto-oncogene b (CBL-B) is an E3 ubiquitin-protein ligase that serves as a critical regulator of immunity. Substantial preclinical evidence supports CBL-B inhibition as a potent driver of anti-tumor immunotherapy[1-4].
- HotSpot has previously disclosed our discovery of an orally bioavailable, selective, small molecule, allosteric CBL-B inhibitor (CBL-Bi), HST-1011, that has advanced into Phase 1/2 clinical trial (NCT05662397).
- As part of the translation of mechanism of action to clinical exploration, we sought to identify and characterize proximal biomarkers capable of monitoring CBL-Bi clinically.

CBL-B Inhibition Enhances Anti-tumor Immunity Through Several Key Biological Mechanisms



1 Lowers threshold for effector cell activation

2 Drives effector cell proliferation and inflammation

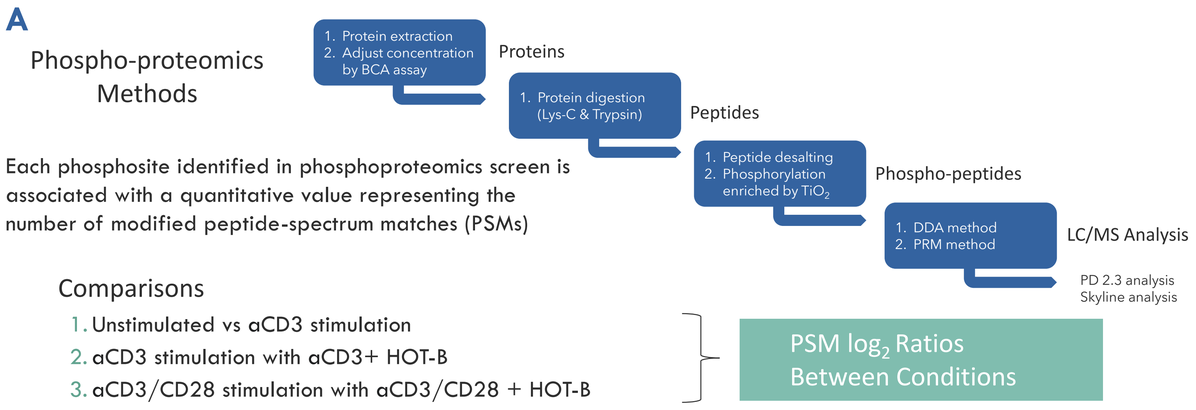
3 Reduces susceptibility to suppression

Methods

- A phospho-proteomics study was conducted to investigate alterations in phosphorylation sites on proteins in Jurkat cells activated by anti-CD3 antibody cross-linking (aCD3) and anti-CD28 costimulation (aCD28), in the presence and absence of CBL-Bi.
- Selected phosphorylation changes were measured *in vitro* using a Meso Scale Discovery (MSD) assay and flow cytometry in human peripheral blood mononuclear cells (PBMCs).
- A flow-based assay was employed to measure the biomarkers in mouse PBMCs from animals treated with CBL-Bi.
- CBL-Bi's impact on potential substrates of CBL-B's E3 ligase activity was monitored for changes in treated human PBMCs using flow cytometry.
- Two HotSpot allosteric CBL-B inhibitors, HOT-A and HOT-B, were used interchangeably throughout the studies.

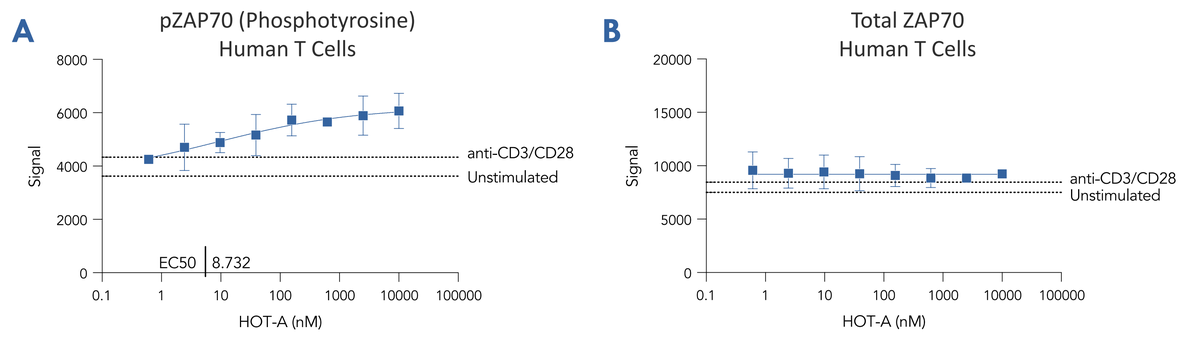
Results

Figure 1. Phospho-proteomics Identified Phosphosite Modifications Caused by CBL-Bi in Jurkat Cells



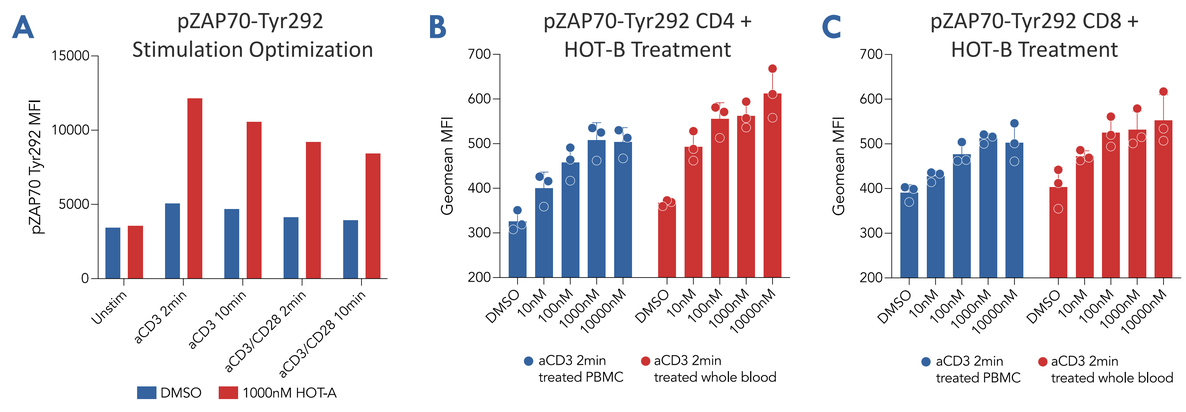
(**A**) Workflow of the phospho-proteomics study to identify alterations in phosphorylation sites on proteins in Jurkat cells activated by anti-CD3 antibody cross-linking and anti-CD28 costimulation (aCD3/CD28) for 5min, in the presence and absence of 1000nM CBL-Bi HOT-B. (**B**) Correlation plots comparing PSMs (Log2) identified in cells treated with aCD3 to those treated with aCD3 and HOT-B showed significant differences in serine (S), threonine (T), and tyrosine (Y) phosphorylation. (**C**) Representative bar plots of PSM values of phosphorylation sites on ZAP70, CD3E, LAT, and PLCG1.

Figure 2. Dose-dependent Increase in Total ZAP70 Tyrosine Phosphorylation in Activated Human T Cells Upon CBL-Bi



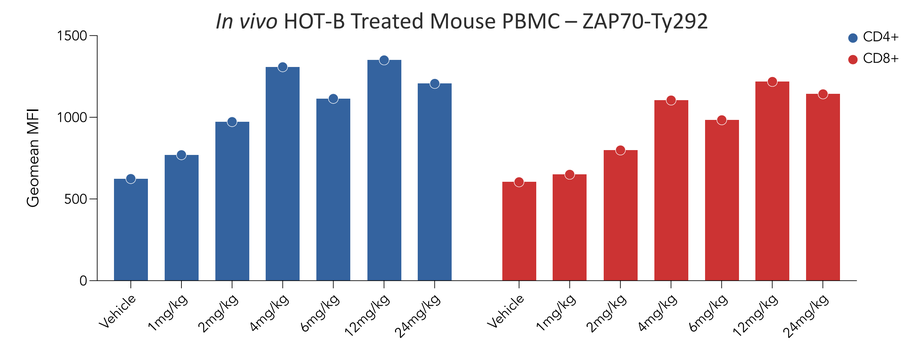
MSD detection of pZAP70(Tyr) (4G10) (**A**) and total ZAP70 (**B**) in human T cells stimulated with aCD3/CD28 for 10 min and increasing concentrations of HOT-A CBL-Bi.

Figure 3. Phosphorylation of ZAP70 at Tyrosine 292 (pZAP70-Tyr292) by CBL-Bi with Anti-CD3 Stimulation in Human PBMCs and Whole Blood



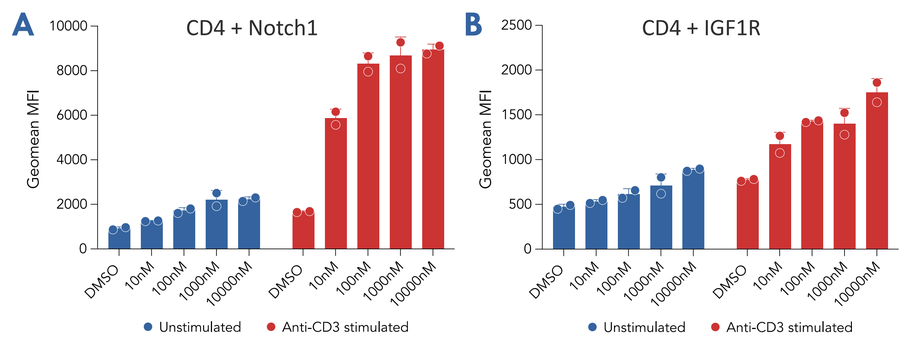
(**A**) Human PBMCs were stimulated with or without aCD3 or aCD3/aCD28 cross-linking, in the presence or absence of 1μM HOT-A. pZAP70(Tyr292) in human CD4+ PBMCs was detected by flow cytometry. (**B-C**) Human PBMCs or whole blood was stimulated *in vitro* with anti-CD3 cross-linking for 2 minutes in the presence or absence of HOT-B (10-10000 nM). The MFI of pZAP70-Tyr292 in CD4+ (**B**) and CD8+(**C**) was measured by flow cytometry.

Figure 4. CBL-Bi Increased pZAP70(Tyr292) in Mouse PBMCs *in vivo*



Female BALB/c mice were treated with vehicle or HOT-B (1mg/kg-24mg/kg) for 1 hour. PBMCs were isolated and restimulated with anti-CD3 cross-linking. Phosphorylation of ZAP70 at tyrosine 292 in mouse CD4+ and CD8+ T cells was detected by flow cytometry.

Figure 5. Validation of Notch1 and IGF1R, Potential Substrates of CBL-B, as Proximal Biomarkers Modulated by CBL-Bi in Human PBMCs



Human PBMCs were treated *in vitro* with or without anti-CD3 cross-linking in the presence or absence of HOT-B (10-10000nM) for 24 hours. Surface expression of Notch1 and IGF1R were characterized by flow cytometry. The MFI of Notch1 (**A**) and IGF1R (**B**) in CD4+ T cells are shown.

Conclusions

- Evaluating the effect of CBL-Bi on TCR signaling enabled identification of a repertoire of proximal biomarkers with potential to monitor and optimize CBL-Bi clinically.
- Phosphorylation of ZAP70 in T cells was demonstrated to be a reliable marker for CBL-Bi activity in human and mouse T cells. However, implementing a phospho-flow assay clinically presents additional challenges.
- Notch1 and IGF1R are potential substrates of CBL-B which have the potential to be feasibly assayed in a clinical setting, and both showed robust dose-dependent CBL-Bi effects preclinically[5].
- We believe clinical validation and exploration are warranted to assess the utility of these proximal biomarkers and their association with downstream pharmacodynamics following treatment with HST-1011 in the clinic.

References

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