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Cbl Inhibition Increases the Response of Cytotoxic T Cells to Sub-Optimal Stimulation and Drives Enhanced Anti-Tumor Responses Alone or in Combination with PD-1 Blockade

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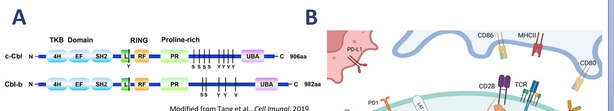
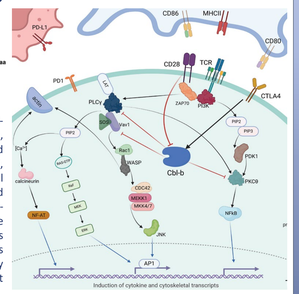
Introduction

- Casitas B-lineage lymphoma-b (Cbl-b) and c-Cbl proteins belong to a small class of RING-type E3 ubiquitin ligases that target multiple proteins for ubiquitination and degradation.
- The domain architecture for Cbl-b and c-Cbl consists of a highly conserved N-terminal half that includes a tyrosine kinase-binding (TKB) domain and a RING-finger domain. The more variable C-terminal region contains ubiquitin-association (UBA) domains and other protein interaction motifs (Figure 1A).
- Cbl-b is expressed in immune cells and is known to play a central role in inhibiting effector T cell activity through its E3 ubiquitin ligase activity. Cbl-b has been shown to negatively regulate multiple signaling proteins downstream of the T cell receptor (TCR), suggesting that inhibition may yield enhanced anti-tumor T cell activity (Figure 1B).
- Herein, we describe a series of experiments using a dual Cbl-b/c-Cbl small molecule inhibitor, to demonstrate that Cbl blockade enhances T cell activity under standard and sub-optimal stimulation conditions.



Modified from Tang et al., *Cell Immunol*. 2019



Modified from Augustin et al., *J Immunother Cancer*. 2023

Figure 1. (A) Diagram of the structure domains of c-Cbl and Cbl-b proteins. Cbl-b and c-Cbl are composed of a helix (4H) bundle, an EF hand and an SH2 domain. The TKB domain is connected through a conserved helical linker (L) to a RING finger (RF), which contributes to the E3 ligase activity. The C-terminal region includes proline-rich (PR) motifs, multiple serine and tyrosine phosphorylation sites, and a leucine zipper/ubiquitin-association (UBA) domain. **(B)** Intracellular Cbl-b signaling in the tumor microenvironment. CD28 co-stimulation inhibits downstream Cbl-b signaling, while CTLA4 stimulation promotes Cbl-b activity. On activation, Cbl-b ubiquitinates several key proteins that inhibit T cell effector function and prevent effective anti-tumor responses.

Methods

- **Isolated CD8⁺ T cell experiments:** CD8⁺ T cells were isolated from healthy human blood by negative selection and stimulated as indicated +/- Cbl inhibitor, then analyzed by flow cytometry and cytokine bead array.
- **OT-I splenocytes experiments**: Spleens from OT-I mice were harvested and processed to generate a single cell suspension. Splenocytes were stimulated with ovalbumin peptides of different affinities +/- Cbl inhibitor and cytokine production was assessed by cytokine bead array.
- ***In vivo* tumor models:** CT26 or MC38 cells were implanted subcutaneously and when tumors reached ~75mm³ mice were dosed with αPD-1 (10 mg/kg, IP, Q5D) and/or Cbl inhibitor A0322275 (30 mg/kg, PO, QD) as indicated. Tumor volumes were observed, and tumors collected for tumor infiltrating lymphocytes analysis by flow cytometry.
- **Cancer cells experiments**: Cancer cells were cultured according to suppliers' recommendations. Cells were plated +/- Cbl inhibitor for various time-points, as indicated, and cell titer glow reagent was added for cell viability assessment.
- **Cbl inhibitor compound information:** Cbl-b/c-Cbl inhibitor, A0322275, from patent application WO2020264398.

Results

Cbl Inhibitor Increases Cytokine Secretion And Proliferation of Human Primary CD8⁺ T Cells

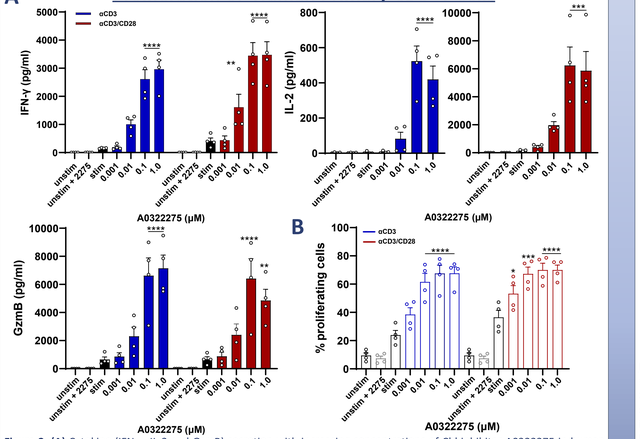


Figure 2. (A) Cytokine (IFN-γ, IL-2 and GzmB) secretion with increasing concentrations of Cbl inhibitor A0322275 in human primary CD8⁺ T cells stimulated with plate-bound αCD3 or αCD3 + αCD28 for 3 days. **(B)** Percentage of proliferating CD8⁺ T cells in the presence of increasing concentrations of A0322275. Proliferation of cells was assessed by cell trace violet dilution. **These results demonstrate a concentration-dependent increase of T cell activation with Cbl inhibition.** One-way ANOVA, Dunnett's multiple comparison test *=p<0.05, **=p<0.01, ***=p<0.001, ****=P<0.0001 compared to respective stimulated condition.

Cbl Inhibition Increases the Frequency of Highly Polyfunctional T Cells Across All CD8⁺ T Cell Subpopulations

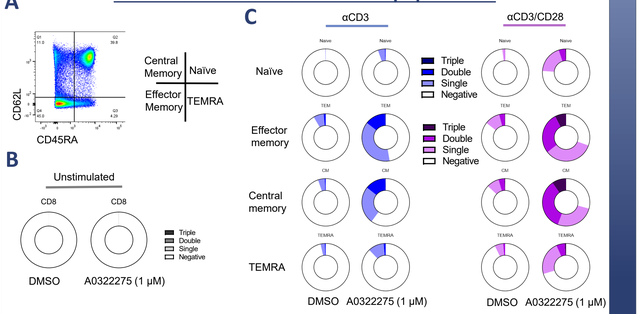


Figure 3. (A) Primary human CD8⁺ T cells were classified into different subpopulations according to the expression of CD62L and CD45RA. **(B)** Unstimulated cells in the absence or presence of Cbl inhibitor A0322275 and frequency of IFN-γ⁺, TNF⁺ and/or IL-2⁺ cells **(C)** CD8⁺ T cell subpopulations were stimulated with αCD3 or αCD3/CD28 in the presence of Cbl inhibitor for 24 hours and frequency of triple, double, single and negative cells for cytokines was quantified.

Serially-Stimulated CD8⁺ T Cells Have Restored Activity With Cbl Inhibition

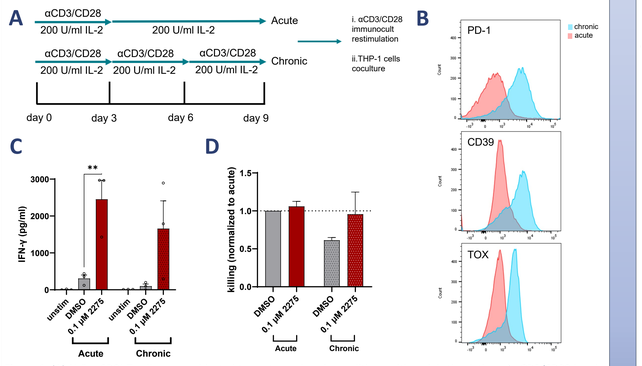


Figure 4. (A) Naïve CD8⁺ T cells were subjected to acute or chronic conditions with serial stimulation of αCD3/CD28 beads and IL-2 for 9 days. After this period, cells were restimulated with αCD3/CD28 immunocult for 24 hours or co-cultured with CTV-labelled αCD3-coated THP-1 cells at 1:2.5 ratio overnight. **(B)** PD-1, CD39 and TOX markers were assessed by flow cytometry by the end of day 9 to confirm distinct stimulations phenotypes. **(C)** IFN-γ levels secreted following restimulation in the presence of Cbl inhibitor A0322275. **(D)** Killing ability of CD8⁺ T cells normalized to acute condition**.** One-way ANOVA, Dunnett's multiple comparison test **=p<0.01 compared to DMSO.

Antigen-Specific Responses Are Increased With Cbl Inhibition and Different Affinity Peptides

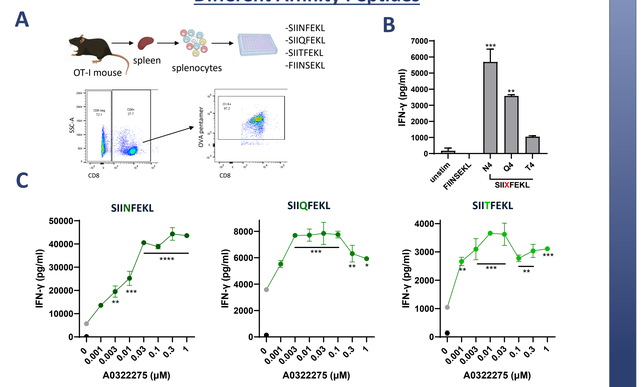


Figure 5. (A) Spleens from OT-I mice were harvested and processed for splenocytes. Cells were stimulated with ovalbumin peptides with different affinities for 3 days +/- Cbl inhibitor as indicated. Dot plots show the percentage of positive CD8⁺ T cells and OVA TCR pentamer. **(B)** IFN-γ levels secreted by different OVA peptides. **(C)** IFN- γ levels are increased in the presence of Cbl inhibitor with all peptides stimulation. One-way ANOVA, Dunnett's multiple comparison test *=p<0.05, **=p<0.01, ***=p<0.001, ****=P<0.0001 compared to respective untreated condition.

Cbl Inhibition Synergizes with PD-1 Blockade to Reduce Tumor Volume and Increases Tumor Antigen Specific T Cells

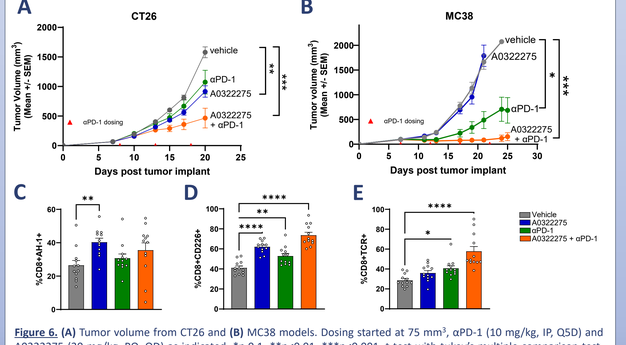


Figure 6. (A) Tumor volume from CT26 and **(B)** MC38 models. Dosing started at 75 mm³, αPD-1 (10 mg/kg, IP, Q5D) and A0322275 (30 mg/kg, PO, QD) as indicated. *p,0.1, **p<0.01, ***p<0.001, t test with tukey's multiple comparison test. **(C)** Percentage of CD8⁺AH-1⁺ **(D)** CD8⁺CD226⁺ and **(E)** CD8⁺TCR⁺ in tumors of CT26-tumor bearing mice at day 20. Similar results were observed in MC38 tumors. :*p<0.1; **p<0.01, ****p<0.0001, ordinary one-way ANOVA with tukey's multiple comparison test.

There is No Effect of Cbl Inhibition on Growth and Survival of Cancer Cells *in vitro*

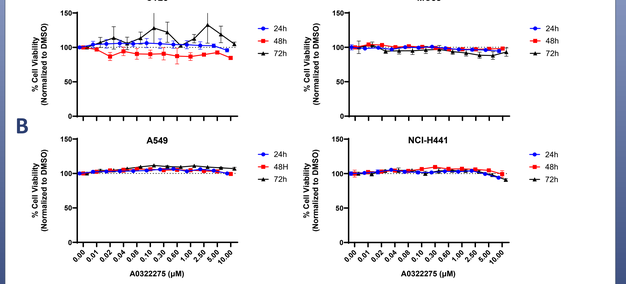


Figure 7. (A) Mouse syngeneic cancer cell lines and **(B)** Human cancer cell lines were treated with different concentrations of Cbl inhibitor A0322275 as indicated and CellTiter Glo assay performed at the end of 24, 48 or 72 hours. Percentage of cell viability normalized to DMSO is shown.

Conclusions

- Cbl-b is a negative regulator of T cell activation through its E3 ubiquitin ligase activity which leads to the degradation or altered cellular localization of key components downstream of the TCR.
- Altogether, our data demonstrate that pharmacologic Cbl inhibition robustly enhances T cell activity *in vitro* and *in vivo* under standard and sub-optimal stimulation conditions and provide a mechanistic rationale for targeting Cbl-b/c-Cbl to amplify anti-tumor immune responses.