Inhibition of the E3 ligase CBL-B enhances the effector function and proliferation of natural killer (NK) cells

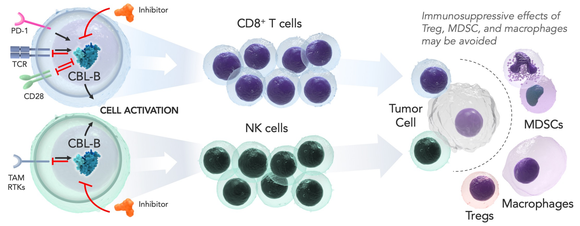
Yilin Qi*, Jun Kuai*, Yingzhi Bi*, Samira Jaeger, David Greco, Ken Carson, Wei Liu, Hadi Danaee, Timothy Reilly, Geraldine Harriman, Fang Wang†

HotSpot Therapeutics, Inc, One Design Center Place, Suite 19-600, Boston, MA 02210, USA; *equal contribution; †corresponding author

Introduction

- Casitas B lymphoma-b (CBL-B), the E3 ubiquitin ligase, is a critical intracellular checkpoint protein in cytotoxic T and NK cells[1-4]
- Ablation of CBL-B results in enhanced IFNγ and perforin release by primary human NK cells, promoting NK cell-mediated cancer cell killing[5-6]
- Mice with *cblb* knockout spontaneously reject tumor growth[7-8]. While *cblb*$^{-/-}$ CD8 T cells play a key role in tumor rejection, NK cells also play a role[5]
- HotSpot has identified a series of allosteric, selective CBL-B inhibitors which exhibited potent *in vitro* and *in vivo* properties. We observed strong activation of NK cells which led to profound NK-mediated tumor killing
- Therefore, we believe inhibition of CBL-B to activate cytotoxic T and NK cells represents a promising opportunity to enhance immune-mediated tumor suppression

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

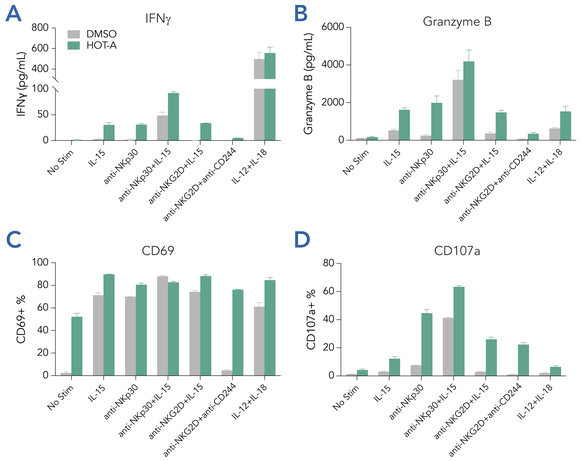
3 Reduces susceptibility to suppression

Method

- HotSpot's allosteric CBL-B inhibitor HOT-A was used through the study
- Human NK cells were enriched using the EasySep human NK cell isolation kit (STEMCELL) or the anti-CD56 microbeads (Miltenyi)
- Flow cytometry-based assays were applied to characterize the activation, proliferation, tumor killing, and tumor infiltration of NK cells
- IFNγ and granzyme B levels in the medium were measured by ELISA
- Human NK single-cell secretome was characterized by IsoPlexis
- Gene expression of tumors from CT26 mouse model was analyzed by nanostring

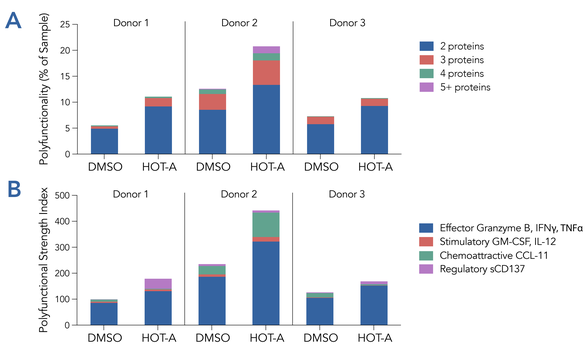
Results

Figure 1. HotSpot CBL-B Inhibitor, HOT-A, Enhanced the Activation of NK Cells *in vitro*



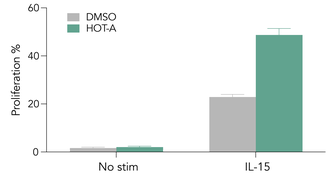
Human NK cells were enriched from human PBMC. They were treated with no stimulation control (No Stim), IL-15 (10ng/ml), plate-coated anti-NKp30 (10μg/ml) +/- IL-15, plated coated anti-NKG2D (10μg/ml) + IL-15 or anti-CD244 (10μg/ml), IL-12 (10ng/ml) + IL-18 (10ng/ml) in the presence or absence of HOT-A (1μM) for 24 hours. The culture medium was collected to measure IFNγ (A) and Granzyme B (B) levels. The cells were analyzed by flow cytometry on surface marker CD69 (C) and CD107a (D).

Figure 2. HOT-A Increased NK Single-cell Polyfunctionality



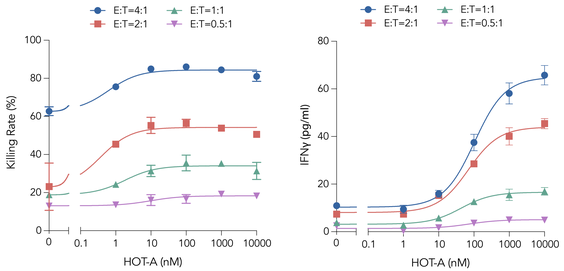
Human PBMCs were recovered with IL-2 (10ng/ml) + IL-15 (10ng/ml) in the presence or absence of HOT-A (100nM) for 24 hours. NK cells were then enriched and stimulated with IL-12 (10ng/ml) + IL-18 (10ng/ml) for 24 hours. After stimulation, cells were stained with Cell Stain 405 and loaded to IsoCode chip for IsoLight assay. Human NK single-cell secretome (32 proteins) were detected. Polyfunctionality (A) and polyfunctional strength index (B) were analyzed from three donors.

Figure 3. CBL-B Inhibition Enhanced NK Cell Proliferation



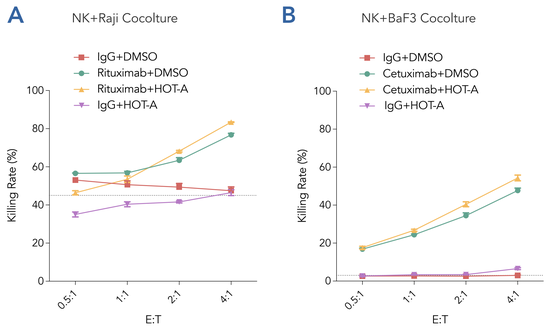
Human NK cells were enriched from human PBMCs and labeled with cell trace violet (CTV). They were treated with no stimulation control (No stim) or IL-15 (10ng/ml) in the presence or absence of HOT-A (100nM). On day 5, cells were analyzed by flow cytometry for proliferation. The percentage of CTV^{low} NK cells were shown.

Figure 4. CBL-B Inhibition Enhanced NK Cell Medicated Cytotoxic Activity Against K562 Cells



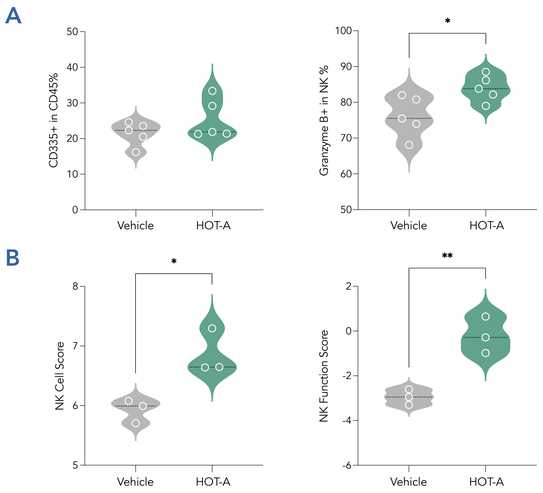
Human NK cells were pretreated with DMSO control or HOT-A (10μM, 1μM, 100nM, 10nM, 1nM) for 1 hour. Then, CTV labeled K562 cells were added to set up cocultured at different E:T ratio (0.5:1, 1:1, 2:1 and 4:1). After 4 hours' incubation, killing rate was measured as percentage of 7AAD+K562 cells; The culture medium was collected to measure IFNγ level.

Figure 5. No Significant Effect Observed on ADCC Mediated Cytotoxicity with CBL-B Inhibitor



Human NK cells were pretreated with or without HOT-A (100nM). CTV-labeled Raji cells were pretreated with IgG or Rituximab (1μg/ml). CTV-labeled BaF3-EGFR-ASV (BaF3) cells were pretreated with IgG or Cetuximab (1μg/ml). After 1 hour of pretreatment, set up coculture of human NK cells with Raji cells (A) or with BaF3 cells (B) at different E:T ratio (0.5:1, 1:1, 2:1 and 4:1). After 4 hours' incubation, killing rate was characterized by percentage of PI positive cells in the tumor cells.

Figure 6. CBL-B Inhibition Enhanced NK Cell Function in Tumor *in vivo*



Female BALB/c mice were inoculated with CT26 tumor cells, randomized, and treated with vehicle or HOT-A. (A) The immunoprofiling of the tumor immune microenvironment from both groups was done by flow cytometry on day 4 after dosing . The frequency of CD335+ in CD45+ cells and Granzyme B+ in NK cells were shown. (B) On day 13, tumor mRNA was extracted and analyzed using nanoString™ mouse PanCancer immune profiling panel. NK Cell Score and NK Function Score were calculated based on the signature gene expression. Statistics were calculated using t-test. *p<0.05; **p<0.01

Conclusions

- HOT-A, a novel allosteric small molecule that potently inhibits CBL-B E3 ubiquitin ligase activity, enhanced NK cell activation and effector functions *in vitro* and *in vivo*.
- Data suggests that inhibition of CBL-B mediated multifaceted anti-tumor immune reaction, including activation of NK cells. We believe more studies are warranted to fully explore the potential of CBL-B inhibitors in tumor immunology.

References

1. Lutz-Nicoladoni C, Wolf D, Sopper S. *Front Oncol.* 2015;5:58.
2. Jafari D, Mousavi MJ, Keshavarz Shahbaz S, et al. *Clin Exp Immunol.* 2021;204(1):14-31.
3. Bachmaier K, Krawczyk C, Kozieradzki I, et al. *Nature.* 2000;403(6766):211-216.
4. Chiang YJ, Kole HK, Brown K, et al. *Nature.* 2000;403(6766):216-220.
5. Paolino M, Choidas A, Wallner S, et al. *Nature.* 2014;507(7493):508-512.
6. Lu T, Chen L, Mansour AG, et al. *J Immunol.* 2021;206(4):677-685.
7. Loeser S, Loser K, Bijker MS, et al. *J Exp Med.* 2007;204(4):879-891.
8. Chiang JY, Jang IK, Hodes R, Gu H. *J Clin Invest.* 2007;117(4):1029-1036.