HPK1 Inhibition Relieves Suppression Downstream of TCR Activation to Drive Enhanced Cytokine Production, an Effect that is Further Enhanced by Immune Checkpoint Blockade

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Abstract 854

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INTRODUCTION

- T cell activation is critical in the initiation and potentiation of anti-tumor immune responses.
- Hematopoietic Progenitor Kinase 1 (HPK1/MAP4K1) is a member of the MAP4K family whose activity restrains T cell activation through phosphorylation of SLP-76 (pSLP-76) at Serine 376, leading to disassembly of the TCR complex.
- Mouse genetic deletion and kinase dead mutants of HPK1 have been shown to enhance T cell activity and combine with immune checkpoint inhibition.
- Therefore, we sought to demonstrate that inhibitors of HPK1 activity can increase T cell activation and combine with immune checkpoint blockade to amplify anti-tumor T cell responses.

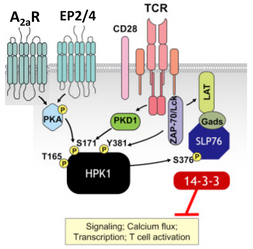

Figure 1. HPK1 functions as a negative regulator of TCR signaling. Schematic from Wu, P. *et al.* Structure 2019 Jan 2;27(1):125-133.

METHODS

- **T cell experiments:** PBMC and CD8$^+$ T cells were isolated from human blood and cells were activated using anti-CD3/CD28 stimulation, Staphylococcal enterotoxin A (SEA) or CEF peptide pool. Supernatants were assayed for IL-2, IFN-γ and TNF-α using cytokine bead array (CBA). Activation markers and SLP-76 phosphorylation (S376) were assayed by flow cytometry. OT-1 splenocytes were isolated from OT-1 mice and activated using ovalbumin-specific residues (257-264) of varying affinity.
- **CRISPR knockout:** HPK1^{KO} CD8$^+$ T cells were generated using Amaxa nucleofection of cells with Cas9 and gene-specific sgRNA sequences. Cells were recovered for at least 7 days prior to use.
- **Compounds and antibodies:** Arcus HPK1 inhibitors and HPK1 reference compound (You et al. *J Immunother. Cancer*. 2021) were used. Anti-PD-1 antibody zimberelimab was used to block the effects of PD-1/PD-L1 signaling.

RESULTS

HPK1 is Broadly Expressed in Immune Cells and Correlates with Immune Infiltration in Normal Human Tissue

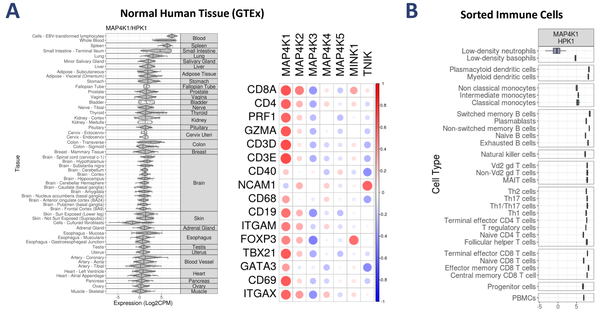

Figure 2. (A) HPK1 (MAP4K1) gene expression is highly enriched in tissues with high immune cell content (left panel) and strongly correlates with immune-related genes in normal tissues compared to other MAP4K family members (right panel). **(B)** Gene expression data from sorted immune cell populations shows that HPK1 expression is found at high levels in nearly all immune cells with moderate expression observed in circulating monocytes.

Genetic Deletion of HPK1 Amplifies CD8$^+$ T cell Activation

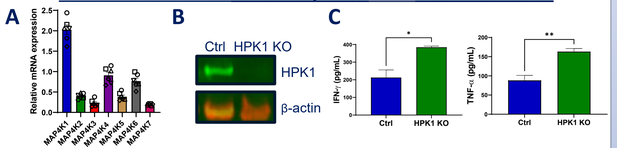

Figure 3. (A) mRNA expression of MAP4K1-7 relative to housekeeping gene HPRT1 in primary human CD8$^+$ T cells from 6 donors. **(B)** CRISPR knockout of HPK1 in primary human CD8$^+$ T cells was confirmed by Western blot. **(C)** CD3/28/2 activated HPK1^{KO} CD8$^+$ T cells display increased IFN-γ (left panel) and TNF-α (right panel) secretion. * p < 0.05, ** p < 0.01 paired T test.

Arcus HPK1 Inhibitor Displays Selectivity for HPK1 over a Majority of Kinases

A

	Arcus HPK1 inh_1	Arcus HPK1 inh_2	HPK1 REF inh
HPK1 IC_{50} (nM)	2.45* (n=2)	0.987 (n=2)	2.94 (n=5)
Jurkat pSLP-76 IC_{50} (nM)	47.4 (n=2)	169 (n=1)	103 (n=3)
Jurkat IL-2 EC_{50} (nM)	68.1 (n=2)	232 (n=2)	478 (n=3)

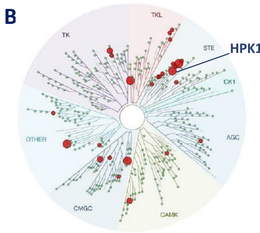

Figure 4. Arcus HPK1 inhibitor displays excellent potency and selectivity for HPK1 over most kinases. (A) HPK1 biochemical potency, and cellular potency measuring activation-induced pSLP-76 (S376) and IL-2 secretion were determined for Arcus inh_1, Arcus inh_2 and HPK1 REF inh. * High ATP biochemical assay. **(B)** Arcus HPK1 inh_1 selectivity was profiled using Eurofins KINOMEscan scanMAX assay and screened against 468 kinases at 100 nM concentration.

Pharmacological Inhibition of HPK1 Increases T cell Activation and Cytokine Secretion

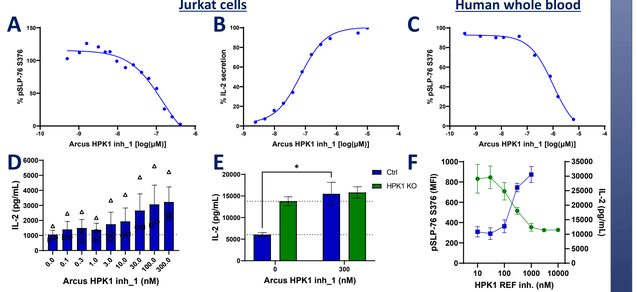

Figure 5. Inhibition of HPK1 decreases phosphorylation of SLP-76 (S376) and increases T cell activation. Arcus HPK1 inh_1 **(A)** decreases pSLP-76 (S376), **(B)** increases IL-2 secretion in Jurkat cells and **(C)** decreases CD3/28 activation-induced pSLP-76 (S376) in human whole blood. **(D)** Arcus HPK1 inh_1 increases IL-2 secretion from human CD8$^+$ T cells from 3 donors activated using CD3/28 stimulation. **(E)** The selectivity of Arcus HPK1 inh_1 was confirmed in human CD8$^+$ T cells. Ctrl and HPK1^{KO} T cells were pre-treated with Arcus HPK1 inh_1 and activated using CD3/28 stimulation, prior to IL-2 measurement after 72 h. * p < 0.05 Two-way ANOVA (Šidák's multiple comparison test). (F) Inhibition of pSLP-76 S376 (green line) correlates with an increase in IL-2 secretion (blue line) in a matched human CD8$^+$ T cell donor using HPK1 REF inhibitor.

Concentration-dependent Inhibition of HPK1 Kinase Activity Correlates with Increased Cytokine Secretion in Human Whole Blood

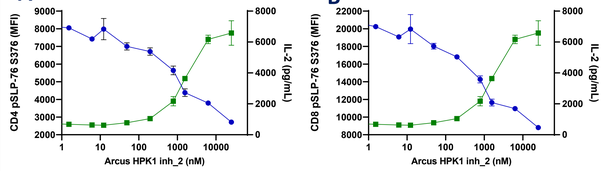

Figure 6. HPK1 inhibition decreases pSLP-76 (S376) and increases IL-2 secretion in a concentration-dependent manner in T cells in human whole blood. Measurement of CD3/28 activation-induced pSLP-76 (S376) (blue line) and IL-2 secretion (green line) in **(A)** CD4$^+$ and **(B)** CD8$^+$ T cells in human whole blood from the same donor pre-treated with given concentrations of Arcus HPK1 inh_2.

HPK1 Inhibition Relieves the Effect of Immunosuppressive Signals Found in the TME

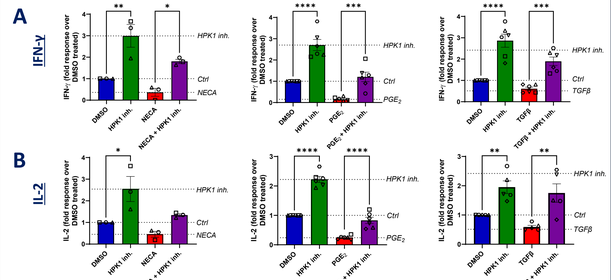

Figure 7. Inhibition of HPK1 enhances IFNγ and IL-2 secretion and restores cytokine secretion in the presence of suppressive signals. HPK1 inhibition using HPK1 REF inhibitor increases **(A)** IFNγ and **(B)** IL-2 secretion alone or in the presence of suppressive adenosine (NECA, 5 μM), prostaglandin (PGE_2, 10 nM) and TGFβ (TGFβ , 3 ng/mL) signaling in human CD8$^+$ T cells. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001 One-way ANOVA (Šidák's multiple comparison test).

HPK1 Inhibition Enhances Cytokine Secretion in OT-1 Splenocytes

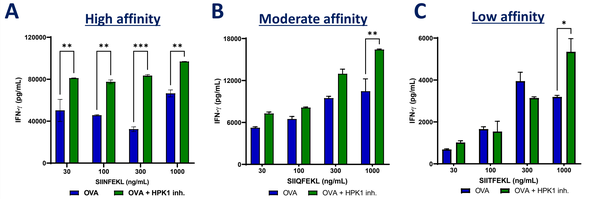

Figure 8. HPK1 inhibition enhances T cell activation in response to higher affinity TCR/peptide interactions. HPK1 inhibition using HPK1 REF inhibitor increases IFN-γ secretion in high **(A)**, moderate **(B)** and low **(C)** affinity ovalbumin peptide activated OT-1 splenocytes. * p<0.05, ** p<0.01, *** p<0.001 Two-way ANOVA followed by Tukey test.

HPK1 Inhibition Combines with Immune Checkpoint Blockade to Further Enhance T cell Activation in Response to SEA

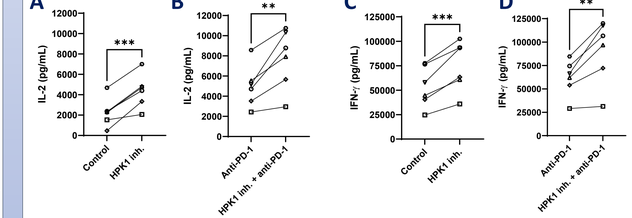

Figure 9. Combination of HPK1 inhibition and PD-1 blockade enhances PBMC cytokine secretion in response to SEA. PBMCs from healthy human donors were pre-treated with HPK1 REF inhibitor in the absence (A, C) or presence (B, D) of anti-PD-1 (zimberelimab) for 1 h prior to activation with Staphylococcal enterotoxin A (SEA). Measurement of **(A, B)** IL-2 and **(C, D)** IFNγ after 72 h.. **p ≤ 0.01, ***p ≤ 0.001 paired T test.

Combination of HPK1 Inhibition with Immune Checkpoint Blockade Further Amplifies Antigen-Specific T cell Activation

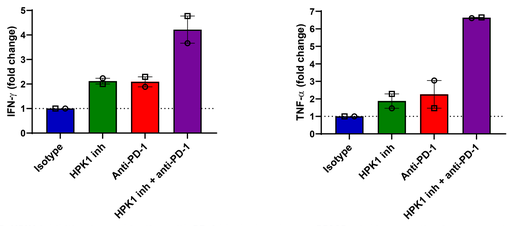

Figure 10. HPK1 inhibition in combination with PD-1 blockade enhances PBMC cytokine secretion in response to antigen recall. PBMCs from healthy human donors were pre-incubated with HPK1 REF inhibitor and anti-PD-1 (zimberelimab) for 1 h prior to incubation with a CEF peptide pool (2 μg/mL each peptide). After 6 days, measurement of IFNγ (left panel) and TNFα (right panel) secretion was performed.

CONCLUSIONS

- HPK1 (MAP4K1) is the most abundantly expressed member of the MAP4K family in CD8$^+$ T cells and genetic deletion of HPK1 in human CD8$^+$ T cells increases cytokine secretion.
- Arcus HPK1 inhibitor shows kinome selectivity and reproduces the phenotype of genetic deletion in CD8$^+$ T cells, decreasing pSLP-76 (S376) and increasing IL-2 secretion.
- Arcus HPK1 inhibitor decreases pSLP-76 (S376) in human whole blood in a concentration-dependent manner and concurrently increases IL-2 secretion.
- HPK1 inhibition can restore cytokine secretion suppressed by inhibitory signals - adenosine (NECA), PGE_2 and TGFβ in human CD8$^+$ T cells.
- HPK1 inhibition increases cytokine secretion in OT-1 splenocytes with the most enhanced effect observed in high-affinity antigen-activated splenocytes
- Inhibition of HPK1 combines with PD-1 blockade using zimberelimab to further enhance T cell activation in response to SEA or antigen recall.
- **These data highlight HPK1 as an attractive target for immunotherapy as inhibiting HPK1 augments T cell activation.**