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# Dual Inhibition of DGKα and DGKζ Increases T Cell and NK Cell Activity

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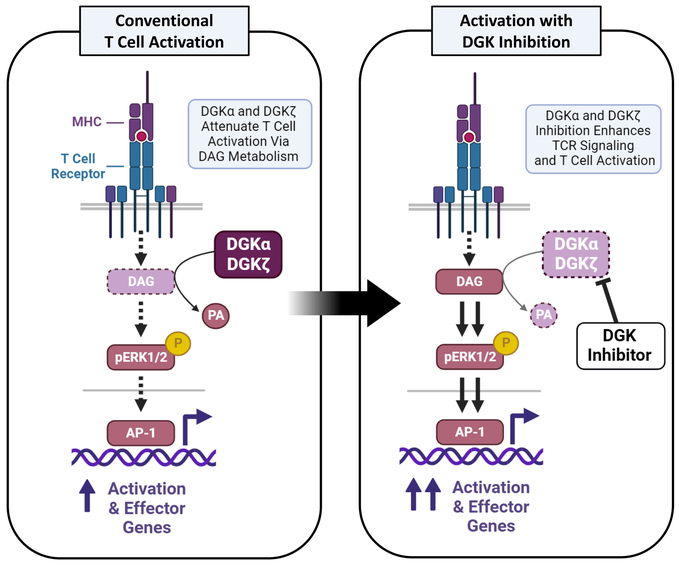
## Background & Summary

### Overview

- Diacylglycerol (DAG) is rapidly generated during T cell activation and serves as a critical second messenger to promote downstream signaling.
- Diacylglycerol kinases (DGKs) attenuate immune cell activation by phosphorylating DAG to form phosphatidic acid (PA), resulting in decreased downstream signaling[1].
- Blocking DGK enzymatic activity can delay the conversion of DAG to PA, enhancing intracellular signaling and increasing the strength of immune cell responses[1-3]. In T cells, blocking DGK kinase activity leads to increased activation and may delay T cell exhaustion and promote effector activity.
- **Here, we demonstrate that co-inhibition of DGKα and DGKζ, the primary DGK family members expressed by immune cells, increases T cell and NK cell activity *in vitro* beyond the level observed with solitary DGKα or DGKζ enzyme inhibition.**

References: 1. Mérida *et al.* (2015) *Sci. Signal.*; DOI: 0.1126/scisignal.aaa0974; 2. Jung *et al.* (2018) *Cancer Res.*; DOI: 10.1158/0008-5472.CAN-18-0030; 3. Riese *et al.* (2013) *Cancer Res.*; DOI: 10.1158/0008-5472.CAN-12-3874; Mechanism of Action Figure created with BioRender.com

### DGK Inhibition Mechanism of Action




## Results

### DGKα and DGKζ are Expressed by Immune Cells and Tumor Infiltrating Lymphocytes

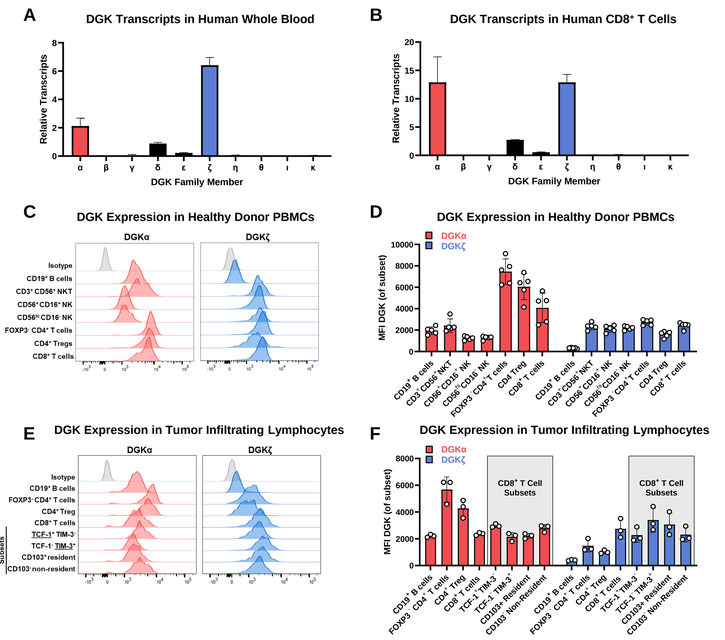



**Figure 1. Analysis of DGK expression in human blood from healthy donors and dissociated tumor samples from non-small cell lung cancer biopsies.** Transcript analysis of the ten DGK family members in **(A)** human whole blood and **(B)** purified CD8+ T cells. Assessment of DGKα and DGKζ expression by flow cytometry in **(C,D)** human healthy donor peripheral blood mononuclear cells and **(E,F)** dissociated tumor biopsies from non-small cell lung cancer patients. Representative histograms are shown from a single donor. MFI – median florescent intensity

### Dual DGKα and DGKζ Deletion using CRISPR/Cas9 Increases T Cell Activation and Cytokine Secretion Following Co-stimulation

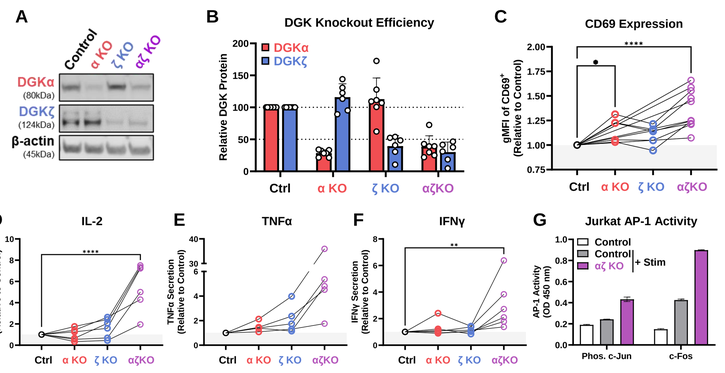



**Figure 2. Assessment of human T cell activation phenotypes following CRISPR/Cas9-targeting of DGKs. (A,B)** Immunoblot and relative protein quantification following knockout (KO) of DGKα (α KO), DGKζ (ζ KO), or both (αζ KO) in purified human CD8+ T cells using CIRPSR/Cas9. **(C-F)** Assessment of activation (CD69) and cytokine production (IL-2, TNFα, and IFNγ) following CD3/CD28 co-stimulation. **(G)** AP-1 activity in Jurkat cells with both DGKα and DGKζ deleted (αζ KO) using CRISPR/Cas9. AP-1 members were quantified by ELISA after capture using oligonucleotides containing an AP-1 binding site (TPA-response element). * p < 0.05, ** p < 0.01, **** p < 0.0001

## Results

### Small Molecule-Based Dual Inhibition of DGKα and DGKζ Increases T Cell and NK Cell Responses More Than Single Target DGK Inhibition

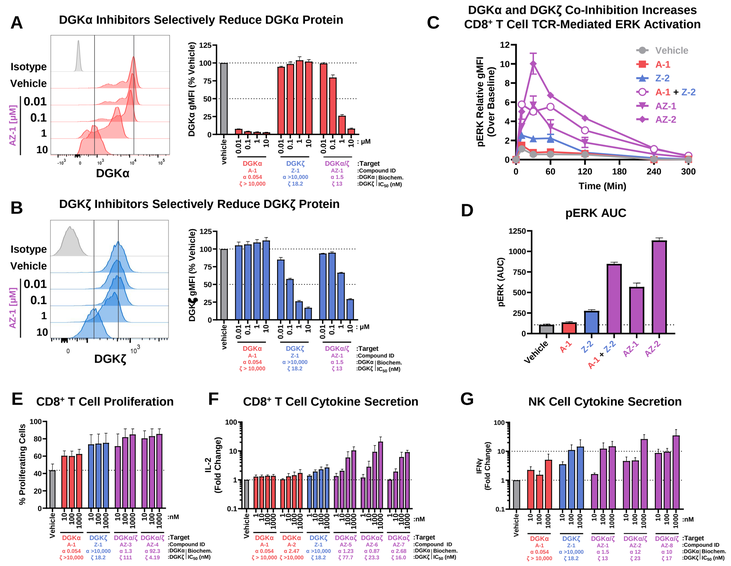



**Figure 3. Effects of DGKα and DGKζ selective and dual targeting inhibitors on T cells and NK cells.** Various small molecule inhibitors targeting DGKα (A-1), DGKζ (Z-1-2), or both enzymes together (AZ-1-8) were tested in a variety of cellular assays. For each compound, the biochemical potencies, based on ADP-Glo assays, are listed bellow the unique compound ID. Protein expression of DGKα **(A)** and DGKζ **(B)** decreased with small molecule inhibitor treatment in a dose-dependent and target-specific manner as measured by flow cytometry 48-hours after compound treatment. **(C)** ERK phosphorylation in primary human CD8+ T cells following CD3/CD28 co-stimulation in the presence of 1 μM of each inhibitor. The effect of co-targeting DGKα and DGKζ was demonstrated using dual-targeting inhibitors (AZ-1, AZ-2), potencies listed in (H)), and using the combination of a selective DGKα inhibitor (A-1, DGKα $IC_{50}$ 0.054 nM, DGKζ $IC_{50}$ = < 10 μM) and a selective DGKζ inhibitor (Z-2, DGKα $IC_{50}$ >10 μM, DGKζ $IC_{50}$ = 24.7 nM). **(D)** Area under the curve (AUC) based on results form (C). **(E)** Quantification of proliferating human CD8+ T cells over a 5-day period following CD3/CD28 co-stimulation with DGK inhibitor treatment. **(F)** Human CD8+ T cell IL-2 production over 72-hours following CD3/CD28 co-stimulation with DGK inhibitor treatment. **(G)** Human NK cell IFNγ secretion after overnight incubation with IL-2 in the presence of DGK inhibitors.

### Antigen-Specific Immune Cell Activation and Cytokine Production Showed the Greatest Increase using Dual Inhibition of DGKα and DGKζ in Murine OT-I Splenocyte Cultures

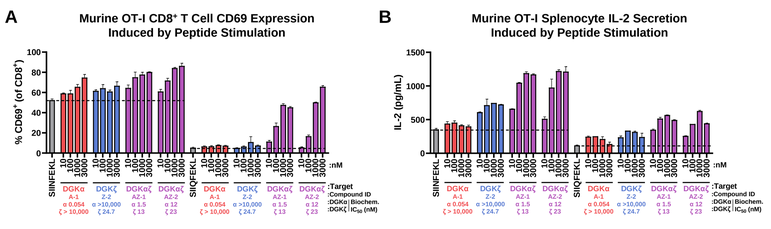



**Figure 4. Characterization of OT-I mouse splenocyte responses to cognate peptide exposure *in vitro* with DGK inhibitor treatment.** OT-I splenocytes were treated *in vitro* with high affinity (SIINFEKL) or moderate affinity (SIIQFEKL) ovalbumin peptides in the presence of DGK inhibitors. **(A)** CD69 expression was assessed 24-hours after stimulation with 0.001 ng/mL of peptide in the presence of compound. **(B)** IL-2 production was quantified 72-hours after stimulation with 1 ng/mL of peptide in the presence of compound.

## Results

### Dual Inhibition of DGKα and DGKζ Increases Human CD8+ T Cell Cytokine Production in Response to MHC Class I Restricted Viral Peptide Stimulation

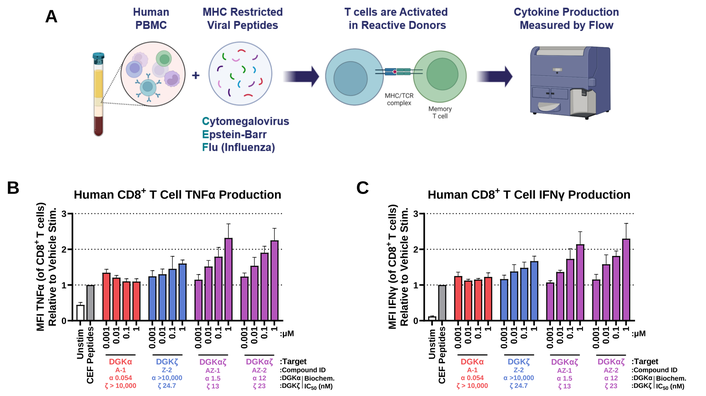



**Figure 5. Analysis of cytokine production by human CD8+ T cells in reactive donors after exposure to MHC-I restricted viral peptides.** **(A)** Depiction of the experimental principle. Human PBMCs isolated from donors known to be reactive to CEF (cytomegalovirus, Epstein-Barr, and influenza) viral peptides were activated by peptide stimulation *in vitro*, and re-stimulated with peptide in the presence of DGK inhibitors 6-days later. Analysis of intracellular cytokine staining was then performed to assess the production of **(B)** TNFα and **(C)** IFNγ. Figure (A) created with Biorender.com.

### High Throughput Inhibitor Evaluation Demonstrates Improved Cellular Potency when Selectivity for DGKα Over DGKζ Decreases in Favor of Co-Inhibition

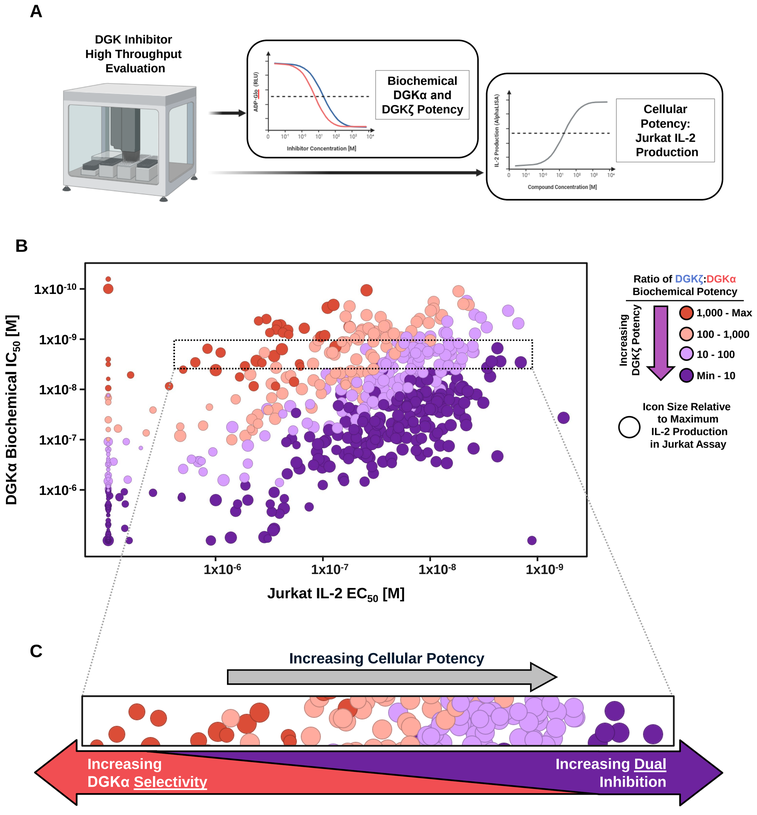



**Figure 6. High throughput DGK inhibitor potency analysis. (A)** Schematic outlining the assays used for compound characterization. For each compound, DGKα and DGKζ biochemical potency was assessed (ADP-Glo) along with Jurkat cellular potency (AlpaLISA), based on Jurkat IL-2 production following CD3/CD28 co-stimulation. The maximum level of Jurkat IL-2 production was also recorded relative to a reference compound. **(B)** Cumulative analysis from >500 compounds. DGKα biochemical potency (x-axis) and Jurkat cellular potency (y-axis) are shown with icon colors representing the degree of DGKα selectivity over DGKζ, binned based on the ratio of DGKζ / DGKα biochemical potency. **(C)** A selected cross-section from (B) highlighting the natural stratification and increased cellular potency of inhibitors targeting both DGKα and DGKζ compared to DGKα selective inhibitors, when DGKα potency is held constant. Figure (A) created with BioRender.com.

## Conclusions

- DGKα and DGKζ were expressed by human immune cells and tumor infiltrating CD8+ T cells.
- Pharmacological or genetic targeting of DGKα and/or DGKζ resulted in increased T cell and NK cell responses to activating stimuli, including antigen-specific T cell stimulation.
- High throughput characterization of > 500 compounds showed improved cellular potency when DGKα and DGKζ were inhibited together, relative to DGKα alone.
- Dual inhibition of DGKα and DGKζ produced superior increases in T cell activation, TCR downstream signaling, and cytokine production compared to inhibitors selective for only DGKα or DGKζ.