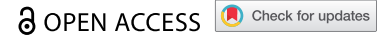


BRIEF REPORT



## DGK $\zeta$ exerts greater control than DGK $\alpha$ over CD8<sup>+</sup> T cell activity and tumor inhibition

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### ABSTRACT

Two isoforms of diacylglycerol kinases (DGKs), DGK $\alpha$  and DGK $\zeta$ , are primarily responsible for terminating DAG-mediated activation of Ras and PKC $\theta$  pathways in T cells. A direct comparison of tumor growth between mice lacking each isoform has not been undertaken. We evaluated the growth of three syngeneic tumor cell lines in mice lacking either DGK $\alpha$  or DGK $\zeta$  in the presence or absence of treatment with anti-PD1 and determined that (i) mice deficient in DGK $\zeta$  conferred enhanced control of tumor relative to mice deficient in DGK $\alpha$  and (ii) deficiency of DGK $\zeta$  acted additively with anti-PD1 in tumor control. Consistent with this finding, functional and RNA-sequencing analyses revealed greater changes in stimulated DGK $\zeta$ -deficient T cells compared with DGK $\alpha$ -deficient T cells, which were enhanced relative to wildtype T cells. DGK $\zeta$  also imparted greater regulation than DGK $\alpha$  in human T cells. Together, these data support targeting the  $\zeta$  isoform of DGKs to therapeutically enhance T cell anti-tumor activity.

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Diacylglycerol kinase alpha; diacylglycerol kinase zeta; T lymphocyte; tumor immunology

## Introduction

Significant clinical advances have resulted from targeting inhibitory cell surface receptors on T cells to generate responses against tumor. To date, the most effective strategies have targeted PD-1 or its ligand PD-L1 resulting in responses ranging from 15–50% in many cancers, many of which are durable.<sup>1</sup> Major efforts in the field of cancer immunotherapy are currently directed toward increasing the subtypes of cancers in which immunotherapies are effective, and in improving the efficacy of PD-1 blockade in responsive tumor subtypes. Most of this work has been directed at extracellular inhibitory receptors on T cells, such as CTLA-4, LAG3, or VISTA,<sup>2</sup> or at extracellular ligands present within the tumor milieu, such as adenosine<sup>3</sup> or prostaglandins.<sup>4</sup> However, intracellular inhibitory regulators in T cells may also represent potential targets to enhance T cell activity against cancer. These include diacylglycerol kinases (DGKs), proteins that metabolize the second messenger diacylglycerol (DAG), terminating critical pathways downstream of T cell receptor (TCR) signal transduction. DAG binds to at least two proteins, RasGRP1 and PKC $\theta$ , which are required for efficient TCR signal transduction.<sup>5</sup> Two isoforms of DGKs, DGK $\alpha$  and DGK $\zeta$ , have been identified as critical regulators of TCR signaling.<sup>6</sup> Deletion of either isoform of DGK results in enhanced ERK1/2 activation and increased functional responses of CD8<sup>+</sup> T cells including cellular proliferation and secretion of the cytokines IFN $\gamma$ , TNF $\alpha$ , and IL-2.<sup>7,8</sup> We have previously demonstrated that deletion of DGK $\zeta$  results in enhanced T cell activity against tumor<sup>8,9</sup>, and that

deletion of DGK $\alpha$  or DGK $\zeta$  increases the potency of CAR-T cells, both *in vitro* and *in vivo*.<sup>10</sup> Moreover, mice deficient in DGK $\zeta$  have been shown to efficiently reject tumors derived from syngeneic EL4-ova thymoma<sup>8</sup> or C1498 lymphoma cell lines.<sup>9</sup> Thus, both DGK $\alpha$  and DGK $\zeta$  are thought to represent proteins amenable to therapeutic modulation to enhance T cell activity in cancer treatment.

The purpose of this *short report* is to directly compare the roles that DGK $\alpha$  and DGK $\zeta$  play in regulating CD8<sup>+</sup> T cell function and maintaining a permissive environment for tumor growth. While direct comparisons between mice lacking either DGK $\alpha$  or DGK $\zeta$  have evaluated certain aspects of biochemistry and physiology,<sup>7,11,12</sup> a direct comparison of tumor responsiveness has not been performed. Moreover, biochemical and transcriptional studies of *human* T cells genetically lacking each isoform have not been extensively evaluated. The studies presented here confirm that loss of DGK $\zeta$  generates a greater enhancement of transcriptional changes, T cell function, and anti-tumor responsiveness in mice than loss of DGK $\alpha$ .

## Methods

### Mice

DGK $\alpha$ <sup>-/-</sup><sup>13</sup> and DGK $\zeta$ <sup>-/-</sup><sup>14</sup> mice bred onto C57BL/6 background have been described. Mice were bred and housed in the Medical College of Wisconsin (MCW) Biomedical Resource Center. C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All work involving animals

was approved by the MCW Institutional Animal Care and Use Committee.

### Antibodies and flow cytometry

The following monoclonal anti-mouse antibodies and flow cytometry reagents were obtained from eBioscience (San Diego, CA): anti-CD4 (GK1.5), anti-CD8 (53–6.7), anti-CD44 (1M7) and anti-granzyme B (GB11). Murine anti-CD3 (2C11) and anti-CD28 (37.51) were from BD Pharmingen. Flow cytometric analysis was done on a BD Biosciences LSRII flow cytometer, and resulting data analyzed using FlowJo software (Tree Star, Inc).

### Assessment of proliferation and cytokine production

Purified T cells from the spleen were labeled with CellTrace CFSE (Invitrogen) using protocols and reagents supplied by the manufacturer.  $1 \times 10^5$  cells were placed in individual wells of a 96-well plate, pre-coated with 100  $\mu$ l of indicated concentrations of plate-bound anti-CD3 and 5  $\mu$ g/ml anti-CD28 antibodies in PBS. Three days later, cells were assessed for CFSE dilution and production of IFN as described.<sup>15</sup>

### RNA-seq of *in vitro*-stimulated naïve CD8<sup>+</sup> T cells

Naïve CD8<sup>+</sup> T cells were isolated from spleens of WT, DGK $\alpha^{-/-}$ , or DGK $\zeta^{-/-}$  mice using negative selection enrichment (StemCell Technologies) and cells were stimulated with plate bound anti-CD3 (1  $\mu$ g/mL) and anti-CD28 (5  $\mu$ g/mL) at a concentration of  $5 \times 10^5$  cells/well of a 24 well plate. Total RNA was extracted from Mouse T-cells using an RNeasy Mini Kit (Qiagen) and RNA quality was assessed on Eukaryote Total RNA Nano Series II Chips using an Agilent Bioanalyzer. RNA-seq library was prepared using TruSeq Stranded Total RNA Kit (Illumina) and processed in a 75bp paired end run. 40 million reads were then obtained using an Illumina NextSeq after normalization for RNA content between genotypes.

### Generation and stimulation of human CRISPR-modified T cells

CD8<sup>+</sup> T cells were isolated from human blood using a RosetteSep CD8 cell kit (StemCell), then stimulated with CD3/CD28 dynabeads (Invitrogen) at a 1:1 ratio. After 24 hours of stimulation, cells were washed and beads removed. The activated T cells were then mixed with gRNA and Cas9 protein (NEB) complex and electroporated using the 4D-Nucleofector system (Lonza). T cells were cultured in T cell media supplemented with 10 ng/ml of rHIL2 (Peptide). Protein levels were then evaluated with the antibodies targeting pERK (Cell Signaling), ERK (Cell Signaling), DGK $\alpha$  (Santa Cruz), and DGK $\zeta$  (Sigma). The following gRNA sequences were generated by Synthego (5'→3'): DGK $\alpha$  gRNA1: TGATGTCCTAAAGCTCTTCG, DGK $\alpha$  gRNA2: TTATAGGCCATTGGGTACGA, DGK $\zeta$  gRNA1: ACTCGCTGCACGGGGCCCCA, DGK $\zeta$  gRNA5: CTAGGAGTCAGCGACATATG.

## Results

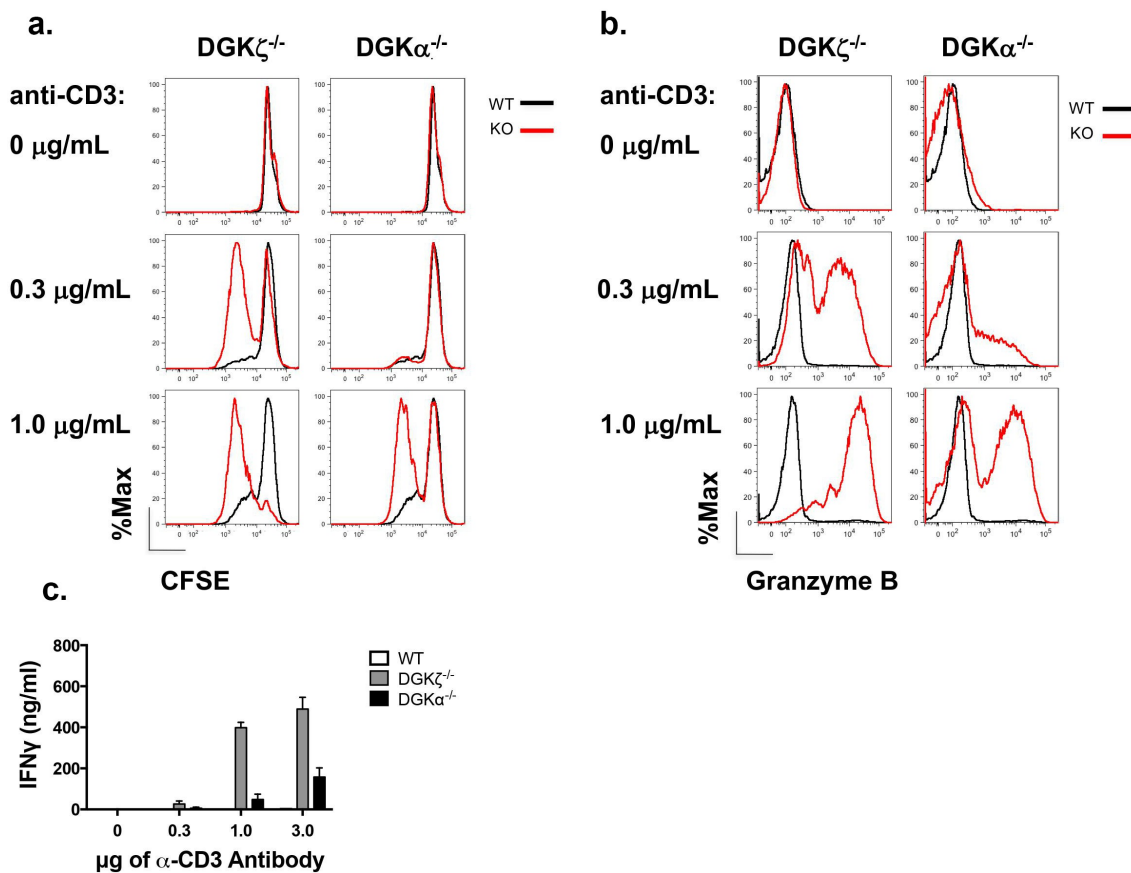
### Enhanced effector responses in stimulated DGK $\zeta^{-/-}$ versus DGK $\alpha^{-/-}$ or wt CD8<sup>+</sup> T cells

To begin to define the comparative impact of loss of DGK $\zeta$  or DGK $\alpha$  on naïve CD8<sup>+</sup> T cells, we evaluated functional changes including proliferation, expression of Granzyme B, and secretion of IFN $\gamma$  after limiting dilutions of *in vitro* TCR stimulation. While 72-hour stimulation with 1  $\mu$ g/mL of anti-CD3 drives minimal proliferation in T cells isolated from wildtype mice, comparable increase in proliferation in T cells lacking either DGK $\alpha$  or DGK $\zeta$  (Figure 1a) was observed. After stimulation with 0.3  $\mu$ g/mL of anti-CD3, however, only DGK $\zeta^{-/-}$  CD8<sup>+</sup> T cells proliferated (Figure 1a). Similarly, production of granzyme B was enhanced in CD8<sup>+</sup> T cells lacking either DGK $\alpha$  or DGK $\zeta$  at 1  $\mu$ g/mL of anti-CD3 stimulation, but was differentially enhanced in DGK $\zeta$  at lower concentrations (Figure 1b). Additionally, production of IFN $\gamma$  by T cells, as assessed by ELISA of culture media, was greater at all tested concentrations of anti-CD3 stimulation in DGK $\zeta^{-/-}$  relative to DGK $\alpha^{-/-}$  T cells (Figure 1c). These data indicate that DGK $\zeta$  more dominantly regulates naïve CD8<sup>+</sup> T cell functions than DGK $\alpha$  in response to TCR stimulation *in vitro*.

In order to more extensively define changes between naïve wildtype CD8<sup>+</sup> T cells and those deficient in DGK $\alpha$  or DGK $\zeta$ , we performed next generation RNA-seq analysis on naïve CD8<sup>+</sup> T cells stimulated with anti-CD3 and anti-CD28 for 48 hours *in vitro*. As a positive control, to confirm that the stimulatory conditions resulted in activation of naïve CD8<sup>+</sup> T cells, we concurrently evaluated IFN $\gamma$  in culture supernatants and found that, similar to Figure 1, IFN $\gamma$  levels were increased in media of DGK $\zeta^{-/-}$  T cells (526,076 pg/mL  $\pm$  143,823) relative to DGK $\alpha^{-/-}$  T cells (134,971 pg/mL  $\pm$  24,393) or wildtype cells (50,960 pg/mL  $\pm$  13,645) (data not shown). Using principal component analyses (PCA), RNA-seq results indicated that stimulated naïve DGK $\zeta^{-/-}$  CD8<sup>+</sup> T cells had more extensive changes than DGK $\alpha^{-/-}$  cells, relative to wildtype T cells (Figure 2a), a finding supported by assessment of highly significant changes in gene expression (fold change greater than 2 and *p*-value cutoff of  $1 \times 10^{-5}$ , Figure 2b) and in volcano plots assessing log-2 fold changes as a function of log-10-adjusted *p*-values (Figure 2c, 2d). As confirmation of the validity of the data set, individual genes known to be upregulated after stimulation of naïve CD8<sup>+</sup> T cells, e.g., IFN $\gamma$ , granzyme B (Gzmb), and Tbet (Tbx21) were evaluated and found to be increased in DGK $\zeta^{-/-}$  T cells when compared with DGK $\alpha^{-/-}$  T cells relative to wildtype counterparts (Figure 2e).

### Enhanced control of tumor growth in DGK $\zeta$ relative to DGK $\alpha$ or wildtype mice

To extend our analysis to *in vivo* functions reliant on CD8<sup>+</sup> T cell activity, we evaluated the capacity of DGK $\alpha^{-/-}$  and DGK $\zeta^{-/-}$  mice to control growth of subcutaneously or intravenously administered tumor. While mice deficient in either DGK $\alpha$  or DGK $\zeta$  have previously been shown to demonstrate improved control of tumor when compared to wildtype mice, a side by side comparison between the two genotypes has not been reported. We compared tumor growth of three models in



**Figure 1. Stimulated DGK $\zeta^{-/-}$  T cells demonstrate enhanced *in vitro* activation relative to DGK $\alpha^{-/-}$  or WT T cells.** Naïve CD8<sup>+</sup> T cells were isolated from spleens of mice of each genotype, labeled with CFSE, and  $1 \times 10^5$  cells/well of 96-well plate were stimulated with 5  $\mu\text{g/mL}$  of plate-bound anti-CD28 and the indicated amounts of anti-CD3. 72 hours later, cells were subjected to flow cytometry to evaluate for dilution of CFSE (a) and expression of granzyme B (b). Additionally, IFN $\gamma$  levels were assessed from culture media using ELISA averaged from duplicate wells (c). Data are from one of three representative experiments.

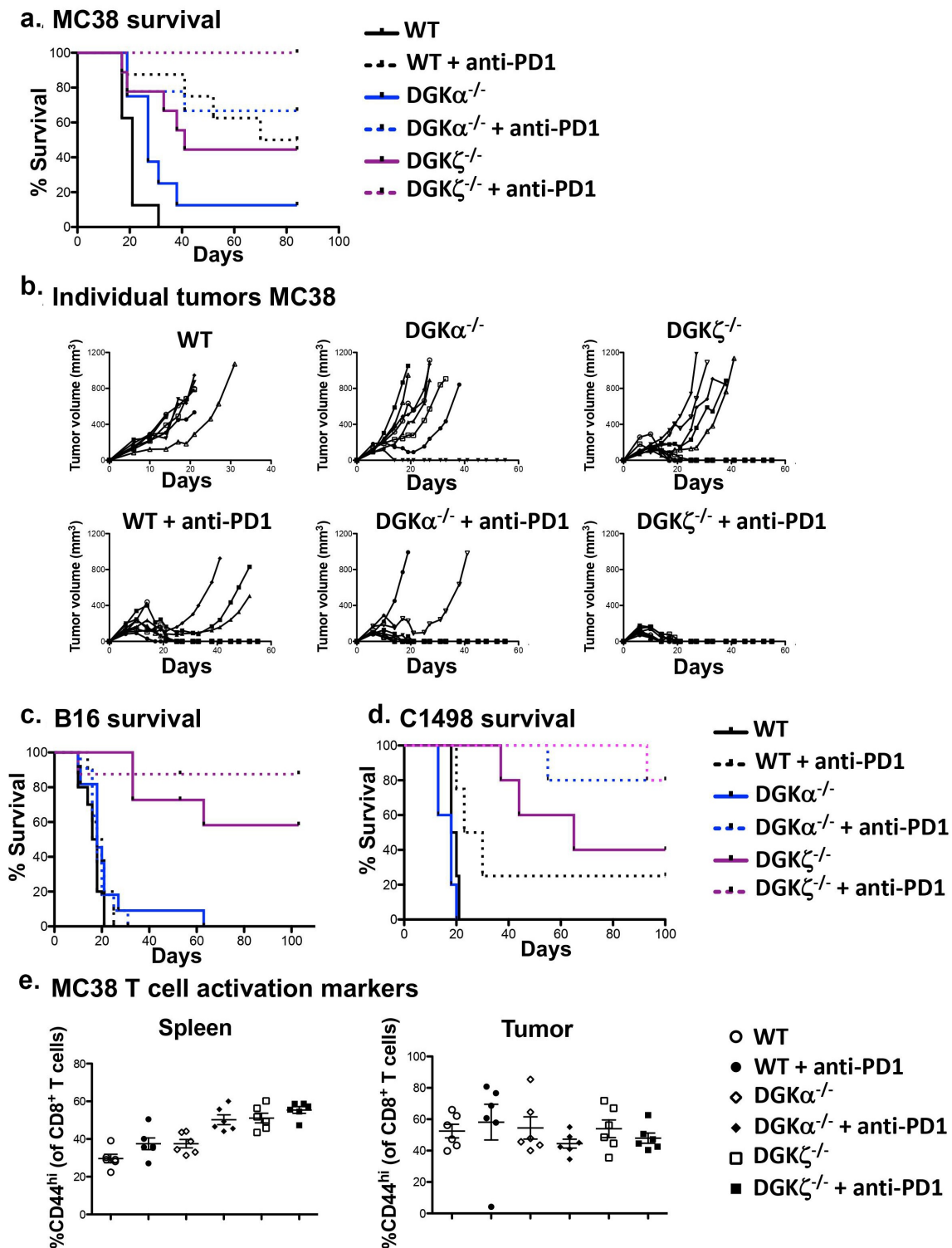
DGK-deficient or replete mice, either alone or in combination with anti-PD1, since anti-PD1 has been demonstrated to have broad clinical activity as an anti-cancer immunotherapy. The three tumor models were selected based on their well-characterized tumor response to anti-PD1-based therapy in immunocompetent C57Bl/6 mice. Specifically MC38 colon carcinoma cells have previously been reported to be sensitive to monotherapy anti-PD1 treatment,<sup>15</sup> B16F1 melanoma cells are minimally sensitive to treatment with anti-PD1,<sup>16</sup> and C1498 leukemia cells are intermediate in sensitivity to treatment with anti-PD1.<sup>9</sup> As has been described, wildtype mice implanted with MC38 carcinoma cells showed enhanced survival after treatment with anti-PD1 (Figure 3a, black lines). Comparable to untreated wildtype mice, DGK $\alpha^{-/-}$  mice demonstrated minimal ability to control growth of MC38 cells independent of anti-PD1 therapy (Figure 3a, blue lines). In contrast, DGK $\zeta^{-/-}$  mice demonstrated enhanced survival independent of anti-PD1 treatment, and addition of anti-PD1 treatment resulted in complete abrogation of tumor growth (Figure 3a, purple lines). The lack of tumor growth in DGK $\zeta^{-/-}$  mice could not be attributed to failed engraftment of tumor cells, since all mice demonstrated the presence of tumor at early (day 10) time points (Figure 3b). This indicates that DGK $\zeta$  plays a more significant role in limiting growth of MC38 tumors, when compared with DGK $\alpha$ , in a manner that appears to be additive in combination with anti-PD1

treatment. All surviving mice in these experiments demonstrated a capacity to reject a re-challenge of 10-fold higher amounts of MC38 cells ( $1 \times 10^7$  cells, data not shown) suggesting the presence of persistent memory T cells.

Evaluation of a second tumor cell line, B16 melanoma, revealed minimal control of tumor growth in wildtype or DGK $\alpha^{-/-}$  mice, with or without anti-PD1 therapy. In contrast, DGK $\zeta^{-/-}$  mice showed improved survival after administration of B16 melanoma cells independent of anti-PD1 therapy, although addition of anti-PD1 therapy may have improved survival (Figure 3c, purple lines). In a third model, we evaluated survival of mice after intravenous injection of C1498 cells. In this model, mice typically succumb from extensive implantation of tumor in the liver.<sup>9</sup> After iv injection of C1498 cells, we identified partial improvement in survival in anti-PD1 treated, versus untreated wildtype mice (Figure 3d, black lines). Similarly, DGK $\alpha^{-/-}$  mice demonstrated minimal control of C1498 cells (Figure 3d, solid blue line), but had greatly improved survival after treatment with anti-PD1 (Figure 3d, dotted blue line). As with other models, untreated DGK $\zeta^{-/-}$  mice demonstrated improved survival compared with DGK $\alpha^{-/-}$  or wildtype mice, in a manner enhanced with administration of anti-PD1 (Figure 3d, purple lines).

We further evaluated the activation status of CD8<sup>+</sup> T cells within spleen and tumor 10 days after tumor inoculation. Similar to previous evaluations with orthotopically implanted



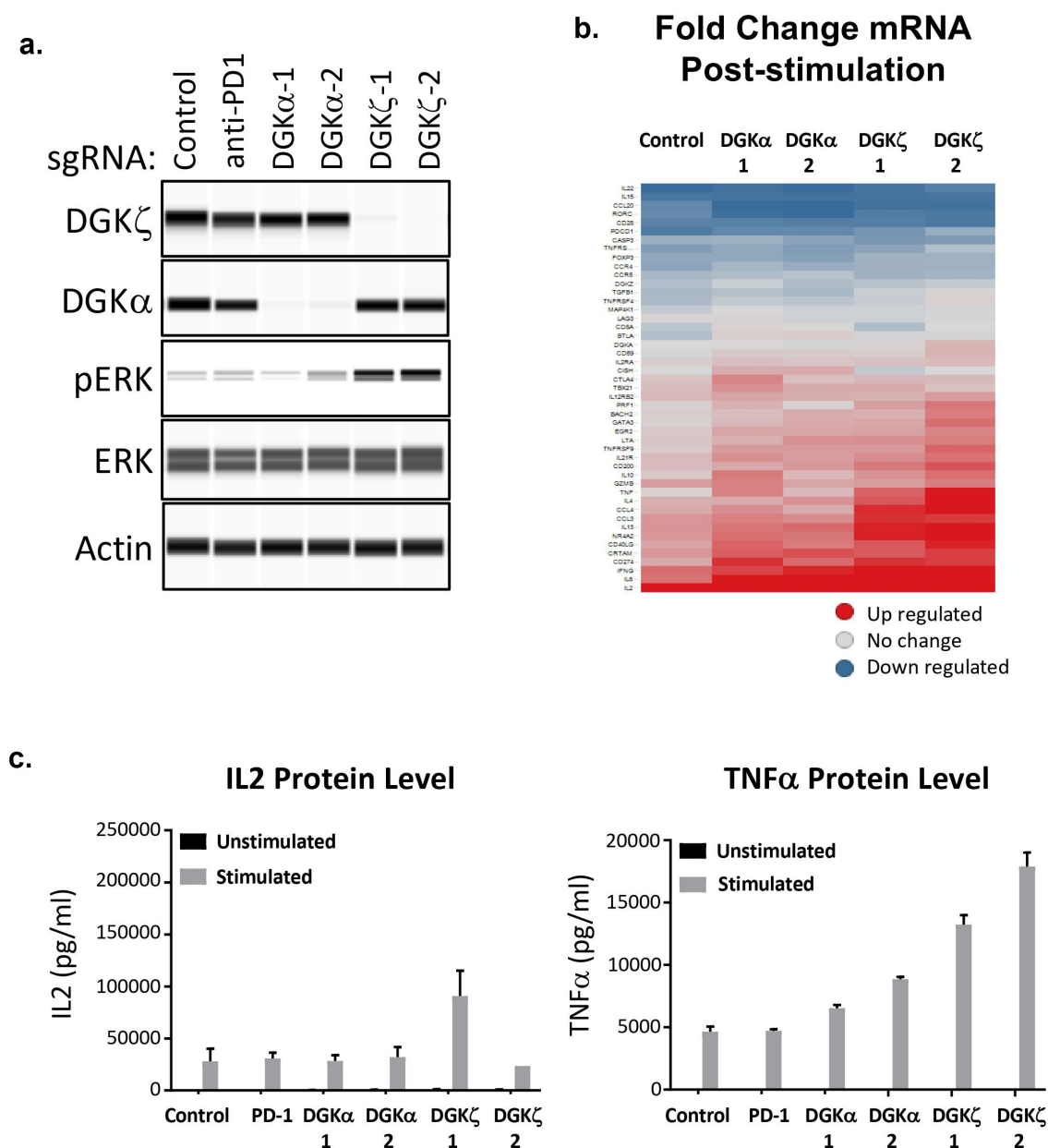


**Figure 3. Mice deficient in  $DGK\zeta^{-/-}$  demonstrate enhanced control of tumor relative to  $DGK\alpha^{-/-}$  or WT mice in a manner enhanced by anti-PD-L1 treatment.** (a, b) Mice of each genotype were inoculated with  $1 \times 10^6$  MC38 colon carcinoma cells subcutaneously, followed by injection with 10 mg/kg IgG or anti-PD1 6, 10 and 14 days after tumor inoculation. Mice were assessed for survival using Kaplan-Meier analyses (a) and tumor growth using bi-weekly caliper measurement and calculation of volume ( $1/2 \text{ length} \times \text{width}^2$ ) (b). Each group consisted of 8–9 mice and data are from one of three experiments. (c, d) Mice from each genotype were inoculated with  $5 \times 10^5$  B16F1 melanoma cells subcutaneously (c) or  $8 \times 10^6$  C1498 lymphoma cells intravenously (d), followed by IgG or anti-PD1 as described in (A), and evaluated for survival using Kaplan-Meier analysis. Data are representative from one of two experiments, using 10 (C) or 5 (D) mice per genotype and treatment group. (e) Mice from each genotype were inoculated with  $1 \times 10^6$  MC38 cells, treated with 10 mg/kg IgG or anti-PD1 on 4 and 7 days after tumor inoculation, and euthanized 10 days after tumor inoculation. Spleen and tumor were processed into single cell suspension and analyzed for expression of high levels of the activation marker CD44 on  $CD8^+$  T cells using flow cytometry. Data are representative from one of two experiments, with six mice in each treatment group.

previously used to evaluate changes in the functions of human CAR T cells lacking DGKs.<sup>21</sup> We utilized one control gRNA and two independent gRNAs for each *DGKA* or *DGKZ* gene. Nine days after electroporation, insertion or deletion (Indel) events at predicted sites of CRISPR-mediated changes were observed to be present in 99% of control gRNA, 98% of *DGKZ* gRNA1, 93% of *DGKZ* gRNA2, 76% of *DGKA* gRNA1, and 75% of *DGKA* gRNA2 (data not shown). After nine days in culture, cells were evaluated for protein expression of DGK $\alpha$  and DGK $\zeta$ . In both cases DGK $\alpha$  and DGK $\zeta$ -targeted cells demonstrated near-total loss of protein expression (Figure 4a).

To determine the impact of loss of DGK $\alpha$  or DGK $\zeta$  on activation of signaling mediators downstream of TCR stimulation, we evaluated ERK phosphorylation in T cells after

stimulation with anti-CD3 and anti-CD28 and found enhanced ERK phosphorylation in DGK $\zeta$ -targeted CD8<sup>+</sup> T cells relative to DGK $\alpha$ -targeted cells and control cells (Figure 4a). Similarly, TCR-dependent transcriptional changes were magnified in DGK $\zeta$ -targeted relative to DGK $\alpha$ -targeted CD8<sup>+</sup> T cells (Figure 4b). To assess whether increased TCR signaling resulted in enhanced effector cytokine levels, supernatant from cells stimulated with anti-CD3 and anti-CD28 were collected after 24 hours (Figure 4c). IL2 and TNF $\alpha$  protein levels increased greater in the DGK $\zeta$ -knockout T cells compared to wildtype or DGK $\alpha$  knockout cells. Collectively, these data indicate that, similar to murine CD8<sup>+</sup> T cells, DGK $\zeta$  plays a dominant role relative to DGK $\alpha$  in the regulation of TCR signaling in primary human CD8<sup>+</sup> T cells.



**Figure 4. CRISPR-Cas9 knockout of  $\zeta$  resulted in enhanced TCR signaling and T-cell activation compared to to DGK $\alpha$**  (a) Primary human CD8<sup>+</sup> T cells were electroporated with Cas9 and gRNA constructs targeting *DGKA* or *DGKZ* (two distinct gRNAs for each isoform) and protein levels of target gene, total ERK, pERK, and actin were assessed. (b) Cells were then stimulated with anti-CD3 and anti-CD28 and transcript changes of selected T cells target genes were measured at 24 hours. (c) Supernatants from indicated primary human CD8 + T cells stimulated with anti-CD3 and anti-CD28 for 24 hours were then monitored for IL2 and TNF $\alpha$  protein levels.

## Discussion

These studies described herein demonstrate enhanced signal transduction downstream of TCR stimulation in DGK $\zeta$ <sup>-/-</sup> relative to DGK $\alpha$ <sup>-/-</sup> murine T cells and are consistent with previous analyses. For instance, Guo et al. found increased amounts of active (GTP-bound) Ras in lysates of DGK $\zeta$ <sup>-/-</sup> T cells relative to DGK $\alpha$ <sup>-/-</sup> or wildtype counterparts after 15 minutes of TCR stimulation.<sup>7</sup> Similarly, Joshi and others identified enhanced Erk phosphorylation after stimulation of DGK $\zeta$ <sup>-/-</sup> T cells relative to DGK $\alpha$ <sup>-/-</sup> T cells,<sup>11</sup> with a greater magnitude of difference present in CD8<sup>+</sup> T cells compared with CD4<sup>+</sup> T cells. In our studies, function of DGK $\zeta$ <sup>-/-</sup> CD8 T cells were enhanced relative to DGK $\alpha$ <sup>-/-</sup> CD8 T cells and wildtype T cells after *in vitro* stimulation across three major functions of CD8<sup>+</sup> T cells, e.g., proliferation (as assessed by dilution of CFSE), cytokine production (as assessed by IFN $\gamma$  production), and cytotoxicity (as assessed by intracellular Granzyme B levels) (Figure 1). This pattern of qualitative changes among the three genotypes were also observed in evaluation of global transcription as assessed by RNA-seq (Figure 2). Intriguingly, the differences in functional changes between DGK $\alpha$ <sup>-/-</sup> and DGK $\zeta$ <sup>-/-</sup> T cells may be limited to certain differentiation states of CD8<sup>+</sup> T cells, since IFN $\gamma$  secretion has been demonstrated to be similar between DGK $\alpha$ <sup>-/-</sup> and DGK $\zeta$ <sup>-/-</sup> in certain models of infection,<sup>22</sup> and in CAR-T cells.<sup>21</sup> This may reflect differential expression of the two different isoforms depending on T cell activation status and effector or memory maturation.

Our studies also corroborate past reports demonstrating that elimination of DGKs, especially DGK $\zeta$ , can result in enhanced anti-tumor activity.<sup>8–10,15,23</sup> The results identified here augment these past findings by demonstrating that germline deletion of DGK $\zeta$  results in greater tumor rejection when compared with deletion of DGK $\alpha$ , and is additive with anti-PD1 therapy (Figure 3). These findings likely have important therapeutic implications in determining which isoform of DGK may represent the best target to augment T cell anti-tumor immunity. While crude inhibitors of DGKs, such as R59022, are available, these inhibitors lack the specificity, bio-availability, and potency to function as clinical therapeutic approaches. Further work is necessary to develop potent and specific inhibitors for DGK $\zeta$  or DGK $\alpha$ .

Our work also identifies that deletion of DGKs impacts human T cells in a similar manner as murine T cells. While work by others have demonstrated that CRISPR-mediated deletion of DGKs results in increased TCR-mediated ERK phosphorylation in CAR-T cells,<sup>21</sup> our studies add to these findings by identifying the breadth of functional changes in stimulated DGK $\zeta$ -targeted relative to DGK $\alpha$ -targeted human T cells in gene expression (Figure 4c). This suggests that the extensive body of work developed in the study of DGK-deficient mice is likely to be of relevance to human T cell immunity.

In summary, the studies outlined in this *short report* identify DGK $\zeta$  as a potent regulator of CD8<sup>+</sup> T cell function and anti-tumor responses with a favorable side effect profile and beneficial interaction with anti-PD1. Additional studies will further characterize the mechanism(s) underlying these features including insensitivity to inhibitory stimuli, and changes in effector T cell localization and survival.

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This paper is dedicated to the memory, scientific achievements, and collaborative spirit of Dr. Matthew Riese, the consummate physician scientist.

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## Declarations

### *Ethics approval and consent to participate*

All work involving animals was approved by the MCW Institutional Animal Care and Use Committee (AUC 2911).

## Availability of data and materials

The datasets during and/or analysed during the current study are available from the corresponding author on reasonable request.

The RNA-seq datasets generated during and/or analysed during the current study will be submitted to GEO (NIH) after acceptance prior to manuscript on-line availability.

## Competing interests

M.J.R. reports receiving consulting fees and research funding from Bristol-Myers Squibb. M.W., J.B., J.H., and H.S. are employees of Bristol Myers Squibb. S.W., J.G., C.C., and C.W., were employees of Bristol Myers Squibb at the time studies in this manuscript were conducted.

## Authors' contributions

Contribution: S.W. designed research, analyzed, and interpreted data, and drafted the manuscript; J.G. analyzed and interpreted data; C.C. designed and performed, analyzed and interpreted data, J.H. performed and analyzed data, C.W. designed and performed, analyzed and interpreted data, S.H. performed research, H.D. analyzed and interpreted data, E. W. performed research, collected, analyzed and interpreted data, S. T. analyzed and interpreted data, D.E. analyzed and interpreted data, M. R. designed experiments, analyzed and interpreted data, and drafted and edited the manuscript.

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