DGKζ exerts greater control than DGKa over CD8+ T cell activity and tumor inhibition

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ABSTRACT

Two isoforms of diacylglycerol kinases (DGKs), DGKa and DGKζ, are primarily responsible for terminating DAG-mediated activation of Ras and PKCθ 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 DGKa or DGKζ, in the presence or absence of treatment with anti-PD1 and determined that (i) mice deficient in DGKζ conferred enhanced control of tumor relative to mice deficient in DGKa and (ii) deficiency of DGKζ acted additively with anti-PD1 in tumor control. Consistent with this finding, functional and RNA-sequencing analyses revealed greater changes in stimulated DGKζ-deficient T cells compared with DGKα-deficient T cells, which were enhanced relative to wildtype T cells. DGKζ also imparted greater regulation than DGKa in human T cells. Together, these data support targeting the ζ isoform of DGKs to therapeutically enhance T cell anti-tumor activity.

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.1 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 VISTA2 or at extracellular ligands present within the tumor milieu, such as adenosine3 or prostaglandins.4 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 PKCθ, which are required for efficient TCR signal transduction.5 Two isoforms of DGKs, DGKa and DGKζ, have been identified as critical regulators of TCR signaling.6 Deletion of either isoform of DGK results in enhanced ERK1/2 activation and increased functional responses of CD8+ T cells including cellular proliferation and secretion of the cytokines IFNγ, TNFa, and IL-2.7,8 We have previously demonstrated that deletion of DGKζ results in enhanced T cell activity against tumor,9,10 and that deletion of DGKa or DGKζ increases the potency of CAR-T cells, both in vitro and in vivo.10 Moreover, mice deficient in DGKζ have been shown to efficiently reject tumors derived from syngeneic EL4-ova thymoma8 or C1498 lymphoma cell lines.9 Thus, both DGKa and DGKζ 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 DGKa and DGKζ play in regulating CD8+ T cell function and maintaining a permissive environment for tumor growth. While direct comparisons between mice lacking either DGKa or DGKζ have evaluated certain aspects of biochemistry and physiology,7,11,12 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 DGKζ generates a greater enhancement of transcriptional changes, T cell function, and anti-tumor responsiveness in mice than loss of DGKa.

METHODS

MICE

DGKa−/− and DGKζ−/− 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

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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 (GB1.5), anti-CD8 (53–67.6), anti-CD44 (1M7) and anti-granzyme B (GB11). Murine anti-CD3 (2G11) 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 × 10⁶ cells were placed in individual wells of a 96-well plate, pre-coated with 100 μl of indicated concentrations of plate-bound anti-CD3 and 5 μg/ml anti-CD28 antibodies in PBS. Three days later, cells were assessed for CFSE dilution and production of IFN as described.¹⁵

**RNA-seq of in vitro-stimulated naïve CD8⁺ T cells**

Naïve CD8⁺ T cells were isolated from spleens of WT, DGKα⁻/⁻, or DGKζ⁻/⁻ mice using negative selection enrichment (StemCell Technologies) and cells were stimulated with plate bound anti-CD3 (1 μg/mL) and anti-CD28 (5 μg/mL) at a concentration of 5 × 10⁵ 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⁺ 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 (Peprotech). Protein levels were then evaluated with the antibodies targeting pERK (Cell Signaling), ERK (Cell Signaling), DGKa (Santa Cruz), and DGKζ (Sigma). The following gRNA sequences were generated by Synthego (5’->3’): DGKA gRNA1: TGATGTCCCTAAAGCTCTTCG, DGKA gRNA2: TTATAGGCCATTGGTACGA, DGKZ gRNA1: ACTCGTGCACGGGCGCCCA, DGKZ gRNA5: CTAGGACGTCGGACATAG.

**Results**

**Enhanced effector responses in stimulated DGKζ⁻/⁻ versus DGKa⁻/⁻ or wt CD8⁺ T cells**

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

In order to more extensively define changes between naïve wildtype CD8⁺ T cells and those deficient in DGKa or DGKζ, we performed next generation RNA-seq analysis on naïve CD8⁺ 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⁺ T cells, we concurrently evaluated IFNγ in culture supernatants and found that, similar to Figure 1, IFNγ levels were increased in media of DGKζ⁻/⁻ T cells (526,076 pg/mL ± 143,823) relative to DGKa⁻/⁻ T cells (134,971 pg/mL ± 24,393) or wildtype cells (50,960 pg/mL ± 13,645) (data not shown). Using principal component analyses (PCA), RNA-seq results indicated that stimulated naïve DGKζ⁻/⁻ CD8⁺ T cells had more extensive changes than DGKa⁻/⁻ 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 1x10⁻⁶, 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⁺ T cells, e.g., IFNγ, granzyme B (Gzm b), and Tbet (Tbx21) were evaluated and found to be increased in DGKζ⁻/⁻ T cells when compared with DGKa⁻/⁻ T cells relative to wildtype counterparts (Figure 2e).

**Enhanced control of tumor growth in DGKζ relative to DGKa or wildtype mice**

To extend our analysis to in vivo functions reliant on CD8⁺ T cell activity, we evaluated the capacity of DGKζ⁻/⁻ and DGKa⁻/⁻ mice to control growth of subcutaneously or intravenously administered tumor. While mice deficient in either DGKa or DGKζ 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
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. B16F1 melanoma cells are minimally sensitive to treatment with anti-PD1, and C1498 leukemia cells are intermediate in sensitivity to treatment with anti-PD1. 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, DGKα−/− mice demonstrated minimal ability to control growth of MC38 cells independent of anti-PD1 therapy (Figure 3a, blue lines). In contrast, DGKζ−/− 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 DGKζ−/− 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 DGKζ plays a more significant role in limiting growth of MC38 tumors, when compared with DGKα, 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 (1x10³ 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 DGKα−/− mice, with or without anti-PD1 therapy. In contrast, DGKζ−/− 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. 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, DGKα−/− 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 DGKζ−/− mice demonstrated improved survival compared with DGKα−/− or wildtype mice, in a manner enhanced with administration of anti-PD1 (Figure 3d, purple lines).

We further evaluated the activation status of CD8+ T cells within spleen and tumor 10 days after tumor inoculation. Similar to previous evaluations with orthotopically implanted
KPC1242 cells, control of tumor growth in the MC38 model correlated with enhanced presence of activated (CD44^{hi}) CD8^{+} T cells in spleen of DGKζ^{−/−} mice (Figure 3e). Notably, however, there was no change in the levels of activated intratumoral (CD44^{hi}) CD8^{+} T cell (Figure 3e). This observation may reflect that the majority of intratumoral CD8^{+} T cells in the MC38 models are already in the activated state. Collectively, these data indicate that DGKζ^{−/−} mice demonstrate an enhanced ability to control growth of subcutaneously administered tumor, when compared with wildtype or DGKα^{−/−} mice, which may be further enhanced in combination with anti-PD1 treatment.

Primary human T cells deficient in DGK demonstrate enhanced functional and transcriptional changes

While biochemical and functional changes resulting from manipulation of DGKs in human T cell lines have been performed, these studies have relied on cell lines, such as Jurkat T cells, that have profound genetic defects in critical inhibitory signaling regulators such as PTEN. To evaluate the impact of loss of DGKα or DGKζ in human primary CD8^{+} T cells, we electroporated Cas9 and guide RNA (gRNA) complexes targeting DGKα or DGKζ into CD8^{+} T cells isolated from whole blood. A CRISPR-based approach has been
Figure 3. Mice deficient in DGKζ−/− demonstrate enhanced control of tumor relative to DGKα−/− or WT mice in a manner enhanced by anti-PD-L1 treatment. (a, b) Mice of each genotype were inoculated with 1 × 10⁶ 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 length x width²) (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 × 10⁵ B16F1 melanoma cells subcutaneously (c) or 8 × 10⁶ 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 × 10⁶ 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.\textsuperscript{21} We utilized one control gRNA and two independent gRNAs for each \textit{DGKA} or \textit{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 \textit{DGKZ} gRNA1, 93\% of \textit{DGKZ} gRNA2, 76\% of \textit{DGKA} gRNA1, and 75\% of \textit{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\(^+\) 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\(^+\) 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\(^+\) T cells, DGK plays a dominant role relative to DGK\(\alpha\) in the regulation of TCR signaling in primary human CD8\(^+\) T cells.

\begin{figure}[h]
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\caption{CRISPR-Cas9 knockout of \(\zeta\) resulted in enhanced TCR signaling and T-cell activation compared to \(\alpha\). (a) Primary human CD8\(^+\) T cells were electroporated with Cas9 and gRNA constructs targeting \textit{DGKA} or \textit{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.}
\end{figure}
Discussion

These studies described herein demonstrate enhanced signal transduction downstream of TCR stimulation in DGKζ−/− relative to DGKa−/− 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 DGKζ−/− T cells relative to DGKa−/− or wildtype counterparts after 15 minutes of TCR stimulation.7 Similarly, Joshi and others identified enhanced Erk phosphorylation after stimulation of DGKζ−/− T cells relative to DGKa−/− T cells,11 with a greater magnitude of difference present in CD8+ T cells compared with CD4+ T cells. In our studies, function of DGKζ−/− CD8+ T cells were enhanced relative to DGKa−/− CD8+ T cells and wildtype T cells after in vitro stimulation across three major functions of CD8+ T cells, e.g., proliferation (as assessed by dilution of CFSE), cytokine production (as assessed by IFNγ 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 DGKa−/− and DGKζ−/− T cells may be limited to certain differentiation states of CD8+ T cells, since IFNγ secretion has been demonstrated to be similar between DGKζ−/− and DGKa−/− in certain models of infection,22 and in CAR-T cells.21 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 DGKζ, can result in enhanced anti-tumor activity.3-10,15,23 The results identified here augment these past findings by demonstrating that germ-line deletion of DGKζ results in greater tumor rejection when compared with deletion of DGKa, 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, bioavailability, and potency to function as clinical therapeutic approaches. Further work is necessary to develop potent and specific inhibitors for DGKζ or DGKa.

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,21 our studies add to these findings by identifying the breadth of functional changes in stimulated DGKζ-targeted relative to DGKa-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 DGKζ as a potent regulator of CD8+ 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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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.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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