Decreased Diacylglycerol Metabolism Enhances ERK Activation and Augments CD8+ T Cell Functional Responses*

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Modulation of T cell receptor signal transduction in CD8+ T cells represents a novel strategy toward enhancing the immune response to tumor. Recently, levels of guanine exchange factors, RasGRP and SOS, within T cells have been shown to represent a key determinant in the regulation of the analog to the digital activation threshold of Ras. One important for regulating activation levels of RasGRP is diacylglycerol (DAG), and its levels are influenced by diacylglycerol kinase-ζ (DGKζ), which metabolizes DAG into phosphatidic acid, terminating DAG-mediated Ras signaling. We sought to determine whether DGKζ-deficient CD8+ T cells demonstrated enhanced in vitro responses in a manner predicted by the current model of Ras activation and to evaluate whether targeting this threshold confers enhanced CD8+ T cell responsiveness to tumor. We observed that DGKζ-deficient CD8+ T cells conform to most predictions of the current model of how RasGRP levels influence Ras activation. But our results differ in that the EC50 value of stimulation is not altered for any T cell receptor stimulus, a finding that suggests a further degree of complexity to how DGKζ deficiency affects signals important for Ras and ERK activation. Additionally, we found that DGKζ-deficient CD8+ T cells demonstrate enhanced responsiveness in a subcutaneous lymphoma model, implicating the analog to a digital conversion threshold as a novel target for potential therapeutic manipulation.

The activation of CD8+ T cells is an exquisitely regulated process that has evolved from the need to balance adequate responses to intracellular pathogens or nascent tumor cell development against the pathologic destruction of self-organs and tissues. Current clinical attempts to manipulate immune responses toward the control of malignancy have demonstrated difficulty achieving high levels of efficacy (1, 2). Recently, the notion of boosting T cell responses to tumor by modulating T cell receptor (TCR)2 signal transduction has been proposed, and model systems under development appear promising (3, 4); however, the ideal means to target TCR signaling in a way that optimally balances anti-tumor effects with autoimmunity have not yet been established. Recently, the activation of Ras has been identified as a novel threshold in TCR activation (5). We sought to determine whether modulation of the Ras activation threshold confers enhanced CD8+ T cell responsiveness to tumor.

Until recently, the activation of Ras within T cells was thought to result solely from the Ras guanine nucleotide exchange factor RasGRP1. RasGRP1 becomes activated after binding to diacylglycerol (DAG), a second messenger formed by the cleavage of phosphoinositol-bisphosphate by phospholipase Cγ1 after engagement of the TCR. Mice deficient in RasGRP1 demonstrate developmental arrest of T cells prior to thymic positive selection, an event known to be dependent on robust Ras/ERK signaling, and they fail to exhibit ERK phosphorylation after engagement of the TCR or treatment with phorbol myristate acetate, a DAG analog (6). This mechanism of Ras activation was thought to be relatively unique to T cells, because RasGRP1 expression is restricted to lymphocytes and components of the nervous system, whereas other Ras guanine nucleotide exchange factors, such as Son of Sevenless proteins (SOS1 and SOS2), are ubiquitously expressed in all tissue types. Recent data, however, derived from both mathematical modeling and biochemical analyses in the Jurkat T cell line, the DT40 B cell line, and primary T cells suggest a role for both RasGRP1 and SOS in Ras activation following engagement of the TCR (7). Under this proposed model, RasGRP1 appears to be primarily responsible for the activation of the first Ras molecules, which can then interact with an allosteric binding site for Ras-GTP on SOS (5, 8, 9). This then allows SOS to rapidly catalyze the activation of Ras molecules because of positive feedback regulation on the exchange activity for SOS. This refined understanding of Ras activation in T cells suggests that DAG-RasGRP1, an analog signaling module, primes efficient Ras activation by SOS.

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2 The abbreviations used are: TCR, T cell receptor; DAG, diacylglycerol; DGK, diacylglycerol kinase; OVAp, ovalbumin peptide (SIINFEKL); pERK, phosphorylated ERK.
thereby enabling a digital Ras response due to positive feedback regulation of SOS (10, 11).

As a means to assess CD8\(^+\) T cells with modulated Ras activation, we evaluated the CD8\(^+\) T cell responses in mice deficient in diacylglycerol kinase-\(\zeta\) (DGK\(\zeta\)). DGK\(\zeta\) and DGK\(\alpha\), the two predominant DGK isoforms in T cells, phosphorylate DAG after TCR stimulation, terminating DAG-mediated signal transduction (12–15). Eliminating DGK\(\zeta\) has been shown to decrease DAG metabolism in T cells (12), which would be expected to increase the number of DAG-RasGRP1 functional units produced after TCR activation, because DGK\(\zeta\) co-localizes with RasGRP shortly after nucleation of the proximal TCR activation complex and is important in dampening DAG-mediated signaling (16). We have previously demonstrated that CD4\(^+\) lymphocytes from mice deficient in DGK\(\zeta\) demonstrate qualitatively enhanced proliferation, interleukin-2 (IL2) production, and ERK phosphorylation after engagement of the TCR (12). In this study, we aimed to rigorously define how deletion of DGK\(\zeta\) affects ERK activation in CD8\(^+\) T cells after stimulation, to determine whether this pattern of activation is consistent with the two-component model of Ras activation within T cells, and to correlate modulation of this activation threshold to enhanced responses to subcutaneously implanted tumor.

**EXPERIMENTAL PROCEDURES**

Mice—DGK\(\zeta\)−deficient mice backcrossed to C57Bl/6 were described previously (12). C57Bl/6 mice containing a transgene for the OVAp T cell receptor (OT-I mice) were obtained from The Jackson Laboratories. All experiments were performed in mice 6–12 weeks old. Animal maintenance and experimentation were performed in accordance with the Institutional Animal Care and Use Committee at the University of Pennsylvania.

Immunoblotting—CD8\(^+\) T cells from mouse spleenocytes were purified with magnetic CD8\(^+\) T cell isolation kits as suggested by the manufacturer (Miltenyi Biotec). CD8\(^+\) T cell purity after MACS selection was routinely found to achieve >90% purity, as determined by flow cytometry. 1 × 10\(^6\) purified cells in 100 \(\mu\)l were subsequently incubated with an equal volume of 2.5 \(\mu\)g/ml \(\alpha\)-CD3 (500A2, Pharmingen), for the indicated times, or 1 \(\mu\)g/ml phorbol 12-myristate 13-acetate (Sigma) for 15 min. Reactions were terminated with the addition of 1 ml of ice-cold phosphate-buffered saline, and cells were pelleted with centrifugation. Cell pellets were resuspended in 45 \(\mu\)l of cell lysis buffer containing 1% Nonidet P-40 and protease inhibitors as described previously (17). Nuclei and cell debris were removed by centrifugation, and lysates were subjected to Western blotting, using primary antibodies from Cell Signaling at concentrations recommended by the manufacturer.

Flow Cytometric Detection of Intracellular ERK Phosphorylation—2 × 10\(^6\) spleenocytes from wild type and DGK\(\zeta\)-deficient mice were incubated with \(\alpha\)-CD3 (500A2) at the indicated concentration for 15 min in reaction volumes of 200 \(\mu\)l. Alternatively, 1 × 10\(^6\) CD45.2\(^+\) spleenocytes from OT-I or DGK\(\zeta\)-deficient OT-I were incubated for 15 min in the presence of 2 × 10\(^6\) CD45.1\(^+\) spleenocytes pre-loaded for 1–2 h with 10 \(\mu\)M OVAp or variant peptide. Reactions were stopped with 3 ml of 1× BD\(\text{TM}\) Phosflow (BD Biosciences) lys/fix buffer prewarmed to 37°C. Cells were washed and surface-stained in FACS buffer (PBS with 3% FCS and 0.01% sodium azide) and then equilibrated and probed for pERK in BD\(\text{TM}\) Perm/Wash buffer. Primary \(\alpha\)-pERK (Cell Signaling) was used at 1:200, and secondary \(\alpha\)-rabbit-Ax488 (Molecular Probes) was used at 1:100. Flow cytometry was performed with an LSR II cytometer (BD Biosciences) and analyzed using FlowJo software (Treestar).

**Mathematical Modeling of Ras Activation in DGK\(\zeta\)-deficient Mice**—To perform a stochastic simulation, we solved the Master equation (18) associated with the reactions shown in the coarse-grained signaling network in Fig. 1A. In this model, DGK deficiency is considered to enhance RasGRP activity and nothing else. Thus, the model explores the consequences of DGK deficiency with the assumption that its only effect is to enhance RasGRP activity.

The Master equation describes the intrinsically stochastic kinetics of a system undergoing changes following a series of coupled chemical reactions. We use the standard Gillespie algorithm (19) to solve the Master equation associated with the reactions shown in Table 1. The rate constants and the initial concentrations are shown in Tables 1 and 2, respectively.

### Table 1: Reactions and rates for the coarse grained model

| Reaction | Forward rate | Backward rate | Source |
|----------|--------------|---------------|--------|
| Signal + cytosolic SOS \(\leftrightarrow\) signal-SOS (membrane-bound) | 0.05 | 0.01 | Estimated |
| Signal + PIP\(_2\) \(\leftrightarrow\) signal + DAG | 0.05 | Estimated |
| DAG + DGK \(\leftrightarrow\) inactive complex + DGK | 0.05 | Estimated |

### Table 2: Concentrations for the coarse grained model

| Species | Concentration | Source |
|---------|---------------|--------|
| Signal | 0–12 molecules/(\(\mu\)m)\(^2\) | Estimated |
| Cytosolic SOS | 1875 molecules/(\(\mu\)m)\(^3\) | Estimated |
| RasGRP1 | 1250 molecules/(\(\mu\)m)\(^3\) | Estimated |
| DGK | 1250 molecules/(\(\mu\)m)\(^3\) | Estimated |
| PIP\(_2\) | 12 molecules/(\(\mu\)m)\(^3\) | Estimated |
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the rates related to the coarse grained “species” and DGK used for Fig. 1B are shown in Tables 1 and 2.

Measurement of in Vitro Cytokine Production—1 × 10^6 splenocytes from wild type, DGKζ−/−, OT-I, or DGKζ−/− OT-I were incubated with 500A2, OVAp (SIINFEKL), or altered mutant peptide for 5 h in the presence of 2 μg/ml brefeldin A and 1 × BD GolgiStop™. Cells were subsequently surface-stained, permeabilized with BD Cytofix/Cytoperm™ buffer, and stained for the presence of intracellular cytokines in BD™ Perm/Wash buffer. Alternatively, cells were assessed for intracellular cytokine production upon restimulation using 500A2 (5 μg/ml) after a 3-day incubation of 1 × 10^6 MACS-purified CD8+ T cells in individual wells of a 96-well plate coated with 2C11 (2.5 μg/ml) and α-CD28 (5 μg/ml). Fluorochrome-conjugated antibodies were from Pharmingen. Flow cytometry was performed and analyzed as described above. Dead cells were removed from analysis by excluding cells that stained positively with a fixable Live/Dead stain (Invitrogen).

Assessment of Proliferation and Cytotoxicity—Wild type and DGKζ-deficient splenocytes were labeled with CellTrace CFSE (carboxyfluorescein succinimidyl ester, Invitrogen) using protocols and reagents supplied by the manufacturer. CD8+ CD44high and CD8+ CD44low cells were isolated by flow sorting using a FACS Aria cytometer (BD Biosciences), with the assistance of the University of Pennsylvania Flow Cytometry Core, achieving cell purities in excess of 98%. After isolation, 1 × 10^5 cells were placed into individual wells of a 96-well plate, previously coated with α-CD3 (2C11, Pharmingen) overnight at 4 °C. Three days later, cells were surface-stained and subjected to flow cytometry for evaluation of CFSE dilution. IL2 production from supernatants of individual wells was determined using an IL2 ELISA kit (Pharmingen).

To assess proliferation using BrdU labeling, 8-week-old wild type or DGKζ-deficient mice were provided with drinking water containing 1 mg/ml BrdU for 12 days prior to euthanasia and determination of BrdU incorporation into CD8+ CD44high splenocytes using an α-BrdU staining kit (Pharmingen) and flow cytometry.

To evaluate cytotoxicity, 2 × 10^7 OT-I or DGK-deficient OT-I splenocytes were incubated for 3 days in 7 ml of media supplemented with IL2 (100 units/ml) and OVAp (10 μM). An aliquot of cells was subjected to flow cytometry to determine the percentage of effector cells present in the mixture (Vβ5+ CD8+), and the effector cells were incubated with 1 × 10^4 target cells in triplicate, after 1 × 10^7 target cells had been incubated with 50 μCi of ^51Cr and either media or OVAp for 1 h at 37 °C. After 4 h of incubation, cells were centrifuged, and the media were removed to assess for chromium release. Maximum possible release of chromium was determined from an aliquot of resuspended target cells not incubated with effector cells, and percent lysis was calculated as (×[Cr]sample/[^51Cr]max power).

EL4-ova Subcutaneous Tumor Model—EL4-ova cells were maintained in 25-cm² cell culture flasks at 37 °C in 5% CO₂ using media recommended by the distributor (ATCC). To test endogenous mouse responses, EL4-ova cells growing in log phase were washed twice in PBS, and 2.5 × 10^4 EL4-ova cells in 200 μl of PBS were implanted subcutaneously in the right flank of anesthetized mice. Three weeks later, mice were euthanized, and tumors were weighed. To evaluate for the presence of Ki-67 in tumor-infiltrating lymphocytes, tumors were physically disrupted into cell suspension, and cells were surface-stained for CD8, CD4, and CD44, permeabilized with FoxP3 staining buffer (eBioscience), and incubated with human α-Ki67-FITC (Pharmingen) prior to flow cytometry. Splenocytes from tumor-bearing mice were processed as described above. APC (allophycocyanin)-coupled Kb-OVAp (SIINFEKL) tetramer was a gift of John Wherry (University of Pennsylvania).

To assess OT-I-specific anti-tumor responses, CD8+ CD44low cells were flow-sorted from OT-I and DGKζ−/− OT-I (CD45.2+) splenocyte suspensions, and 5 × 10^5 of sorted cells were intravenously injected into anesthetized CD45.1+ mice. Forty eight hours later, mice were inoculated with 5 × 10^5 EL4-ova cells in the right flank. One week later, mice were euthanized, and tumors were excised and weighed. Splenocytes were stained for the expression of surface markers or stimulated to produce IL2 as described above.

RESULTS

ERK Phosphorylation Is Enhanced in DGKζ−/− CD8+ T Cells after TCR Stimulation—As an initial means to test the impact of DGKζ deficiency on CD8+ T cells, we evaluated whether TCR stimulation affected the phosphorylation of phospholipase Cγ1 (20), a protein upstream of the presumed site of DGKζ action, and ERK, a protein whose activation depends upon TCR-stimulated DAG production (21). As predicted, MACS-purified DGKζ-deficient CD8+ T cells demonstrated enhanced levels of ERK phosphorylation but similar levels of phospholipase Cγ1 phosphorylation compared with wild type cells after TCR stimulation as assessed by Western blotting of whole cell lysates (Fig. 2A).

We then used a computational model (Fig. 1A and Tables 1 and 2) to predict the effects of increased RasGRP activity. This model predicts a significant increase in the number of cells exhibiting high Ras activity (RasGTP) (Fig. 1B) and a modest increase in the amount of RasGTP per stimulated cell (data not shown). Because MAPK activation is triggered by Ras activation, we can expect phospho-ERK to show similar qualitative trends. The mathematical model also predicts an alteration of dose responses, such that a lower concentration of stimulus should induce more cells with robust Ras activation, and hence ERK phosphorylation, upon increasing RasGRP activity in CD8+ T cells with respect to wild type cells (Fig. 1B).

To test these model predictions obtained by increasing RasGRP activity, we studied the effects of DGKζ deficiency (because it influences RasGRP) by utilizing a flow cytometric assay that permits discrimination of molecular changes on a per cell basis. When comparing primary CD8+ wild type cells versus DGKζ-deficient cells, we observed a greatly increased percentage of pERK-positive cells after TCR stimulation in the mutant cohort (Fig. 2B, left panel, flow cytometry plots, and right panel, top graph), and a modest increase in the amount of phosphorylated ERK per positive cell, as deter-
cells became unresponsive at a similar concentration of stimulant as wild type CD8+ T cells (Fig. 2B, right panel, lower graph), suggesting an additional degree of complexity not previously anticipated, perhaps resulting from a feedback loop involving DGKζ.

To probe more rigorously the nature of ERK phosphorylation after TCR engagement, we evaluated DGKζ-deficient CD8+ T cell responses over a spectrum of stimulus quality. Toward that goal, we took advantage of altered peptide ligands of ovalbumin that elicit different degrees of functional responses from ova-specific CD8+ T cells (22). After generating DGKζ-deficient mice expressing a CD8+ TCR specific for the immunodominant peptide of ovalbumin (OT-I mice) (23), we stimulated splenic CD8+ T cells with cognate peptide SIINFEKL (OVAp) or the altered peptide ligands SAINFEKL (A2), SIYNEFKL (Y3), SIQFKEKL (Q4), SIITFKEKL (T4), or SIIVFKEKL (V4). Similar to anti-CD3, we observed that stimulation with any of these peptides resulted in an increase in the percentage of pERK-positive DGKζ-deficient OT-I cells relative to wild type OT-I cells (Fig. 2C, left graph) but with only modest changes, if any, in the mean fluorescent intensity of pERK (Fig. 2C, right graph). These findings are also consistent with model predictions.

**Increased Number of CD8+ T Cells from DGKζ-deficient Mice Express High Levels of CD44**—We also observed that DGKζ-deficient mice have relatively increased numbers of splenic CD8+ T cells that express high levels of the cell surface marker CD44 (CD44high) (Fig. 2B, left panel, flow cytometry plots, and Fig. 3A, lower panels). Evaluation of other T cell surface markers identified high levels of CD122 on DGKζ-deficient CD8+ T cells when compared with wild type CD8+ T cells (data not shown) but unchanged levels of CD62L (Fig. 3A, lower panels), CD3, TCR, CD25, CD69, or the IL7 receptor CD127 (data not shown). Expression of high levels of CD44 on lymphocytes marks cells that are capable of generating rapid functional responses, such as cytokine production, in response to TCR stimulation (24). The changes that confer rapid functional responsiveness in CD44high cells are primarily at the level of gene transcription, downstream of the TCR signal machinery (25). Consistent with this, we observed no difference in enhanced ERK phosphorylation in the setting of DGKζ deficiency between CD44high and CD44low T cells when compared with wild type CD8+ T cells (22). After generating DGKζ-deficient OT-I mice, as the expression of CD44high by DGKζ-deficient OT-I cells relative to wild type controls as evidenced by an increased number of cells incorporating BrdU over the course of a 12-day BrdU treatment (Fig. 3E), although this finding may relate either to enhanced proliferation secondary to lymphopenia or an intrinsic cellular difference in DGKζ-deficient cells. Regardless,
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because the differences in CD44 expression might impact the interpretation of our results, for each of our experiments we routinely compare wild type and DGKζ-deficient CD8+ T cells with similar levels of surface CD44. DGKζ-deficient CD8+ T Cells Demonstrate Enhanced Cytokine Production after TCR Stimulation—To determine whether deletion of DGKζ impacts the function of CD8+ T cells, we evaluated cytokine production after TCR stimulation. As depicted in Fig. 4A, we observed an enhanced percentage of DGKζ-deficient CD8+ (CD44high) T cells producing cytokine in response to α-CD3 (top row), including IFNγ (left column), TNFa (middle column), or IL2 (right column), compared with wild type CD8+ (CD44hi) T cells. As with ERK phosphorylation, we found that the dose-response curves were similar between DGKζ-deficient and wild type CD8+ cells after α-CD3 stimulation for each of the three cytokines (Fig. 4A, middle row). Somewhat different from ERK phosphorylation, the amount of cytokine produced per positive cell was not enhanced in DGKζ-deficient CD8+ T cells compared with wild type CD8+ T cells (Fig. 4A, bottom row). Similar results were obtained upon titration of functional avidity of the stimulating antigen using altered peptide ligands in the OT-1 system in that an increased percentage of DGKζ-deficient CD8+ CD44high OT-1 cells became positive for cytokine secretion (Fig. 4B, top row), but the amount of cytokine produced per positive cell (mean fluorescent intensity, bottom row) was similar between the two groups. As we found for ERK activation, the EC50 of each peptide (middle row) was similar for wild type and DGKζ-deficient cells. Differences between DGKζ-deficient CD8+ T cells and wild type CD8+ T cells were also observed after restimulation of cells that had been cultured with α-CD3 and α-CD28 for 3 days, such that 25 and 60.4% of DGKζ-deficient cells produced IL2 and IFNγ, respectively, whereas 10 and 42.9% of wild type CD8+ T cells produced IL2 and IFNγ (data not shown, p values < 0.01, n = 3 replicate samples in each group from a representative experiment).

DGKζ-deficient CD8+ T Cells Demonstrate Enhanced Proliferation but Similar Cytotoxicity of Target Cell when Compared with WT CD8+ T Cells—In response to stimulation, CD8+ T cells proliferate and develop the capacity to lyse target cells. We next sought to examine whether altering Ras activation thresholds impacted these cellular functions. To determine the impact of DGKζ deletion on proliferation, we incubated purified CD44low or CD44high CD8+ T cells with plate-bound α-CD3 for 3 days. 500A2, the α-CD3 clone used in the OT-I system in that an increased percentage of DGKζ-avidity of the stimulating antigen using altered peptide ligands was observed in DGKζ- deficient CD8+ T cells compared with wild type CD8+ T cells. Similar results were obtained upon titration of functional avidity of the stimulating antigen using altered peptide ligands.
in our other experiments, fails to elicit proliferation in wild type cells; thus, we instead stimulated cells with a stronger agonist antibody against CD3, 2C11. As shown, DGKζ/H9256-deficient CD8/H11001CD44high and CD8/H11001CD44low T cells proliferated more robustly than their wild type counterparts (Fig. 5A, top panel). Because we had previously observed enhanced cytokine production in DGKζ/H9256-deficient CD8/H11001T cells (Fig. 4), we wondered whether the enhanced proliferation of these cells could be related to increased IL2 production. To evaluate this possibility, we added saturating doses of IL2 to cell cultures to determine whether this could correct the observed differences between DGKζ-deficient and wild type CD8+ T cells. As expected, administration of exogenous IL2 enhanced the proliferation of wild type cells to levels near that of DGKζ-deficient CD8+ T cells (Fig. 5A, upper panel). Furthermore, measurement of the amount of IL2 from culture supernatants of α-CD3-stimulated cells demonstrated a substantially increased amount of IL2 produced by DGKζ-deficient CD8+ T cells when compared with wild type cells (Fig. 5A, lower panel). Together, these results demonstrate that proliferation is enhanced in DGKζ-deficient CD8+ T cells, likely because of their increased production of IL2.

The capacity of activated DGKζ-deficient CD8+ T cells to lyse effector cells was also evaluated. Alteration of the cytotoxic effector function of activated CD8+ lymphocytes would be a surprising finding in DGKζ-deficient CD8+ T cells, because other mouse models of enhanced TCR signaling, such as cbl-b/H11002/H11002 or Shp-1/H11002/H11002 mice, do not demonstrate enhanced CD8+ T cell-mediated lysis (26, 27). Cell suspensions from OT-I or DGKζ-deficient OT-I splenocytes were incubated in the presence of OVAp and IL2 for 3 days, and the number of effector cells was determined by calculating the percentage of CD8+Vβ5+ cells within the total cell mixture. An approximate 10-fold expansion of CD8+Vβ5+ cells was observed over the course of incubation relative to other cell types (data not shown). Chromium-labeled target cells were provided to the effectors with and without OVAp at various effector to target ratios. As depicted, we observed no difference in the lysis of OVAp-labeled target cells when incubated with OT-I or DGKζ-deficient OT-I effector cells (Fig. 5B). These results

![Figures and graphs](https://example.com/figures.png)
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A

|       | IFNγ | TNFα | IL2 |
|-------|------|------|-----|
| %positive cells (of CD8+ CD44hi) | ![Graph](image1) | ![Graph](image2) | ![Graph](image3) |
| %maximum pos.                     | ![Graph](image4) | ![Graph](image5) | ![Graph](image6) |
| mean fluorescent intensity        | ![Graph](image7) | ![Graph](image8) | ![Graph](image9) |

B

|       | IFNγ | TNFα | IL2 |
|-------|------|------|-----|
| maximum %positive cells (of CD8+ CD44hi) | ![Graph](image10) | ![Graph](image11) | ![Graph](image12) |
| EC50 (x10⁻¹³)                           | ![Graph](image13) | ![Graph](image14) | ![Graph](image15) |
| mean fluorescent intensity            | ![Graph](image16) | ![Graph](image17) | ![Graph](image18) |
indicate that some but not all functional outputs of CD8+ T cells are affected by deletion of DGKζ.

**DGKζ-deficient Mice Develop Smaller Subcutaneously Implanted EL4-ova Tumors**—We next sought to determine whether the enhanced Ras signaling and in vitro functional effects observed in the DGKζ-deficient CD8+ T cells translate into improved in vivo immune responses by testing how DGKζ-deficient mice respond in a lymphoma model that engages a CD8+ T cell response. For these studies, we made use of subcutaneously implanted EL4-ova lymphomas, a well-described system to assess in vivo CD8+ T cell function (28). EL4-ova cells are derived from a clone of EL4 cells that have been genetically manipulated to stably express ovalbumin (29). One major advantage of the EL4-ova model is that these tumors are able to grow in immunocompetent mice. Additionally, it has been shown that a robust CD8+ T cell response directed against ova can abrogate tumor growth (30). For our initial studies, 25,000 EL4-ova cells were implanted subcutaneously on the right flank of wild type or DGKζ-deficient mice. Twenty-one days later, mice were sacrificed, and the tumors and spleens of host mice were dissected. We observed significantly smaller tumors in the DGKζ-deficient mice when compared with wild type (Fig. 6A). Moreover, DGKζ-deficient mice generated enhanced CD8+ T cell responses after inoculation with EL4-ova cells, when compared with wild type mice, as evidenced by decreased expression of the naive T cell surface marker CD62L on CD44high expressing CD8+ T cells (Fig. 6B). Additionally, a subset of the DGKζ-deficient mice generated robust CD8+ T cell responses specific against ovalbumin as evidenced by staining with fluorochrome-conjugated OVAp tetramer (Fig. 6C).

Intratumoral CD8+ T cells were also evaluated in tumor-bearing mice. Although tumors could not be identified in all DGKζ-deficient mice, when present, a greater percentage of intratumoral DGKζ-deficient CD8+ T cells were found to be dividing, as determined by positive staining for the cell proliferation marker Ki-67 (Fig. 6D). In contrast, both wild type and DGKζ-deficient mice revealed comparable percentages of dividing CD8+ T cells harvested from the spleen (Fig. 6D). These results suggest that DGKζ-deficient CD8+ T cells demonstrate enhanced activity toward the implanted EL4-ova tumors.

**Anti-tumor Effect of DGKζ-deficient Mice Is Intrinsic to CD8+ T Cells**—The decreased tumor growth of EL4-ova cells and the enhanced tumor activity of CD8+ T cells that were observed in DGKζ-deficient mice could have resulted from intrinsically enhanced CD8+ T cell function due to DGKζ deficiency or could have resulted from an indirect effect on this lineage due to genomic deletion of the DGKζ gene. To determine whether DGKζ−/− CD8+ T cells possess enhanced intrinsic anti-tumor activity, naive (CD44-low) DGKζ-deficient or wild type CD8+ CD45.2+ OT-I T cells were intravenously injected into congenically marked (CD45.1) recipient mice. Two days later, 5 × 10⁶ EL4-ova cells were subcutaneously injected into the right flank of recipient mice. One week later, mice were euthanized, tumors and spleens were dissected, and adoptively transferred cells (CD45.1 negative) were evaluated. Mice that received tumor-specific DGKζ-deficient T cells demonstrated smaller tumor growth than mice receiving wild type tumor-specific T cells (Fig. 7, top panel). Furthermore, DGKζ-deficient transferred cells demonstrated enhanced activation after transfer, as evidenced by up-regulation of CD44 (Fig. 7, middle panel), and retained their enhanced intrinsic capacity to secrete IL2 (Fig. 7, bottom panel). Collectively, these results indicate that loss of DGKζ enhances the anti-tumor effects of CD8+ T cells, even in the setting of a mouse that is otherwise replete for this enzyme.

**Enhanced Function of DGKζ-deficient CD8+ T Cells**

A two-component model of Ras signal transduction has been proposed to explain the analog to digital conversion of TCR signal transduction (5). This model suggests that TCR signaling activates RasGRP proteins, which leads to the conversion of some Ras to its GTP-bound active state. This Ras-GTP is then available for binding to the allosteric pocket of SOS, thus priming SOS to activate Ras much faster than the basal rate. This positive feedback loop results in digital Ras signaling and hysteresis. According to this model, weak and abortive TCR signals, such as might occur in peripheral lymphoid tissues in the absence of appropriate antigen, would fail to generate sufficient RasGRP1 complexes to prime the system, whereas strong signals, such as those following TCR binding to cognate antigen-MHC complexes, would create sufficient RasGRP1 to prime SOS and fully activate Ras leading to the large amount of phosphorylated ERK observed during productive T cell activation (5).

In the studies presented herein, we tested this model of Ras/ERK activation through the use of DGKζ-deficient mice. DGKζ co-localizes with DAG-activated proteins, such as RasGRP1, shortly after nucletation of the proximal TCR signaling complex and thus limits the amount of DAG-mediated signaling through metabolism of this second messenger to phosphatidic acid (16). We reasoned that interfering with the metabolism of DAG (through DGKζ deficiency) would create a situation such that once the TCR is appropriately triggered, a larger amount of RasGRP1 would become activated, resulting in more robust Ras/ERK signaling. Under these conditions, we predicted that T cell activation through the TCR...
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A

\[
\text{\%CFSE diluted} \quad \begin{array}{cccc}
\text{Dgkζ-/-} & \text{WT} & \text{Dgkζ-/-} & \text{WT} \\
\text{hi} & \text{hi} & \text{hi} & \text{hi} \\
\text{lo} & \text{lo} & \text{lo} & \text{lo}
\end{array}
\]

B

\[
\text{Relative IL2 concentration} \quad \begin{array}{cccc}
\text{Dgkζ-/-} & \text{WT} & \text{Dgkζ-/-} & \text{WT} \\
\text{CD44hi} & \text{CD44hi} & \text{CD44lo} & \text{CD44lo}
\end{array}
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FIGURE 5. Proliferation but not target cell lysis is enhanced in DGKζ-deficient mice. A. Top panel, 1 × 10^6 CFSE-labeled flow-sorted DGKζ-/- or wild type (WT) CD8+ CD44^hi or CD8+ CD44^lo T cells were incubated with plate-bound α-CD3 (2C11, 2.5 μg/ml) for 3 days in the absence or presence of 100 units/ml of IL2, and dilution of CFSE was determined by flow cytometry. Bottom panel, media from cells stimulated with α-CD3 (without IL2) used in the upper panel were evaluated for the presence of IL2 by ELISA. A representative experiment from three iterations is shown, n = 2 each group. B, splenocytes from OT-I (WT) or DGKζ-/- OT-I (DGKζ-/-) mice were cultured with IL2 (100 units/ml) and OVAp (1 μg/ml) for 3 days. Effector cells were then incubated at the indicated ratios with 35Cr-labeled target splenocytes loaded with media or OVAp. Percent cell lysis was calculated from release of 35Cr as described under “Experimental Procedures.”

would result in more frequent triggering of the digital response, resulting in a higher percentage of phospho-ERK-positive cells for any effective stimulus but similar amounts of phospho-ERK per cell (resulting in similar mean fluorescent intensities of phospho-ERK). A refinement of the model originally published predicts these effects (Fig. 1B). In addition, the model predicts that enhanced RasGRP activity should decrease the amount of antigen needed to induce ERK phosphorylation.

Using purified CD8+ T cells from wild type mice or mice deficient in DGKζ, we found that most predictions of the model are supported. A greater percentage of DGKζ-deficient CD8+ T cells was triggered to phosphorylate ERK when compared with wild type CD8+ T cells for any concentration of anti-CD3 capable of eliciting responses in wild type cells, whereas the amount of ERK phosphorylated per cell was comparable (Fig. 2B). This observation is consistent with the predicted enhanced digital signaling due to increased RasGRP activity. Additionally, this pattern was maintained over a titration of stimulant quality, because stimulation of DGKζ-deficient OT-I cells led to a higher percentage of cells generating an ERK response relative to wild type OT-I cells for either wild type (SIINFEKL) OVAp or altered OVAp ligands known to induce suboptimal functional responses (Fig. 2C) (22).

One prediction of the model, which assumes that DGK deficiency affects only active RasGRP levels, was not supported. Different from the prediction that the EC50 should be reduced for enhanced RasGRP activity, we found that DGKζ deficiency does not affect the EC50 values. The cause of this discrepancy is uncertain, but it suggests that DGKζ deficiency may affect signaling in ways other than just increasing RasGRP activity, as is assumed in the model (Fig. 1A). For example, feedback could exist between an isoform of PKC and RasGRP, an association known to occur in some mammalian cell types (31, 32). Alternatively, if the activation of DGKζ occurs through a component of TCR signaling independent of Ras activation, then wild type CD8+ T cells receiving stimuli strength incapable of activating DGKζ would appear similar to CD8+ T cells deficient in DGKζ. Differentiating between these possibilities is an area of ongoing investigation.

As noted previously (12), we found that DGKζ-deficient mice had decreased numbers of splenic CD8+ T cells relative to wild type controls (Fig. 3, A, upper flow cytometry plots, and B). Possible explanations for this finding include the following: (i) decreased formation of CD8+ T cells due to increased negative selection in the thymus (resulting from enhanced strength of TCR signaling during development); (ii) increased destruction of CD8+ T cells receiving stimuli strength incapable of activating DGKζ; (iii) increased destruction of CD8+ T cells secondary to impairments in IL7 or IL15 signal transduction; or (iv) effects related to impaired production of phosphatidic acid, the reaction product of diacylglycerol kinases. The finding that a higher number of DGKζ-deficient CD8+ T cells express high levels of CD44 winnows the list of possible explanations. For instance, mice deficient in the high affinity receptors for IL7 or IL15 tend to have decreased numbers of CD44^hi expressing T cells (33). In contrast, mice with radiation or chemically induced lymphopenia express higher percentages of CD44^hi CD8+ T cells (34–38). The phenotype of the DGKζ-deficient peripheral lymphocyte compartment is strikingly similar to that observed in mice deficient in the negative regulatory membrane protein SIT, which, like DGKζ-deficient mice, demonstrate decreased numbers of CD8+ T cells, increased percentages of CD44^hi CD8+ T
cells, and enhanced rates of homeostatic proliferation (39). In SIT-deficient mice, these changes appear to result from a decrease in the number of mature T cells as a result of increased thymic negative selection (39). We are currently assessing the cause of decreased numbers of CD8<sup>+</sup>/H11001 T cells in DGK<sup>-/-</sup>/H9256-deficient mice through the generation of bone marrow chimeric mice with marrow reconstituted from a mixture of wild type and DGK<sup>-/-</sup>/H9256 hematopoietic stem cells, with the expectation that CD44 expression levels will be similar between DGK<sup>-/-</sup>/H9256-deficient and wild type CD8<sup>+</sup>/H11001 T cells in the peripheral lymphoid organs of adult mice. Experiments with this system will also help to determine whether the enhanced incorporation of BrdU observed in DGK<sup>-/-</sup>/H9256-deficient mice is related to environmental factors or is cell intrinsic.

Beyond probing the relationship between altered DAG metabolism and Ras activation, we aimed to establish the functional ramifications of manipulating the CD8<sup>+</sup>/H11001 T cell Ras activation threshold. To this end, we evaluated well established in vitro aspects of CD8<sup>+</sup>/H11001 T cell function such as cytokine production, proliferation, and cytotoxicity along with in vivo responses to EL4-ova lymphomas. As shown in Fig. 4, DGK<sup>-/-</sup>/H9256-deficient CD8<sup>+</sup>/H11545 T cells demonstrated enhanced cytokine production in a manner strikingly similar to activation of ERK, in that a greater percentage of DGK<sup>-/-</sup>/H9256-deficient CD8<sup>+</sup>/H11001 T cells produced cytokine than wild type CD8<sup>+</sup>/H11001 T cells with similar dose responsiveness and quantity of cytokine produced per positive cell between the two groups. Moreover, as demonstrated previously for CD4<sup>+</sup>/H11001 T cells (12), DGK<sup>-/-</sup>/H9256-deficient CD8<sup>+</sup>/H11545 cells demonstrated enhanced proliferation in response to TCR stimulation, when compared with wild type CD8<sup>+</sup>/H11545 T cells, likely secondary to their enhanced probability of producing IL2 after stimulation. As with other models of enhanced strength of TCR signaling (26, 27), cytotoxic capacity was not affected by deletion of DGK<sup>+</sup>/H11545. To determine whether the Ras activation threshold represents a physiologically meaningful point in which to manipulate TCR signaling in vivo, we tested CD8<sup>+</sup>/H11545 T cell responses to tumor in the setting of DGK<sup>-/-</sup> deficiency. DGK<sup>-/-</sup>/H9256-deficient mice were found to grow smaller tumors than wild type mice after injection of EL4-ova cells and generated tumor-specific CD8<sup>+</sup>/H11001 T cells, as

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**FIGURE 6. Enhanced immune response to tumor in DGK<sup>-/-</sup>/H9256-deficient mice.** 2.5 × 10<sup>6</sup> EL4-ova cells were subcutaneously injected into the right flank of DGK<sup>-/-</sup>/H9256-deficient or wild type (WT) mice. Three weeks later, tumors were excised and weighed (A), and the number of activated CD8<sup>+</sup>/H11001 splenocytes (CD62<sub>L</sub>low, CD44<sup>high</sup>) was determined by flow cytometry (B). C, ova-specific CD8<sup>+</sup>/H11001 T cells were identified by flow cytometry after staining with APC-conjugated Kb-ovAp (Kb-SIINFEKL), with representative flow cytometry plots (top panels) demonstrating tetramer (y axis) versus CD44-Pacific Blue (x axis), with pooled data shown below. D, flow cytometry of the percentage of CD8<sup>+</sup>/H11001 T cells staining positively for the cell division marker Ki-67 within tumors or spleens (spl) of wild type or DGK<sup>-/-</sup>/H9256-deficient mice (p = 0.042 between tumor bars). Data are pooled from four experiments (A and B) or two experiments (C and D).
Enhanced Function of DGKζ-deficient CD8+ T Cells

One additional means to probe the relationship between DAG-RasGRP1 units and the Ras activation threshold would be to overexpress RasGRP1. Although one may predict that DAG and not RasGRP1 levels define the rate-limiting step in Ras activation, mice that overexpress RasGRP1 in thymocytes do appear to have enhanced TCR signaling, as evidenced by the ability of RAG-deficient T cells to bypass impaired positive selection within the thymus (40). Comparing Ras activation in these mice with DGKζ-deficient mice might represent an interesting additional avenue of investigation.

Two of the best-studied TCR signaling proteins known to play a role in the murine response to tumor are Shp-1 and cbl-b. Both Shp-1−/− and cbl-b−/− CD8+ T cells demonstrate enhanced anti-tumor activity (3, 4, 41). Shp-1−/− is a tyrosine phosphatase that acts to oppose proximal signaling components of TCR such as the tyrosine kinases Zap-70 and Lck (42). cbl-b is an E3 ubiquitin ligase that limits TCR signaling by degrading several key signaling proteins, including Vav and phosphoinositol 3 kinase (43). The quality of altered functions is different among Shp-1−/−, cbl-b−/−, and DGKζ−/− CD8+ T cells. For instance, Shp-1−/− TCR transgenic CD8+ T cells produce similar amounts of cytokine per responding cell but respond to peptide concentrations several orders of magnitude lower than wild type cells and are capable of responding to altered peptide ligands that fail to elicit responses in wild type cells (27). In contrast, cbl-b−/− TCR-fixed CD8+ T cells demonstrate enhanced cytokine production on a per cell basis but do not respond to concentrations of peptide lower than those capable of eliciting cytokine production in wild type cells (26). Physiologically, the manner of enhanced activation of CD8+ T cells may be important, because Shp-1−/− and cbl-b−/− mice both develop significant spontaneous autoimmune disease (44, 45), whereas DGKζ−/− mice do not develop overt signs of autoimmune disease. This observation suggests that selective inhibition of DGKζ could represent a unique means to enhance CD8+ T cell tumor responses without significantly compromising immune self-recognition. Although deletion of DGKζ alone confers enhanced ERK activation and tumor activity to CD8+ T cells, the magnitude of this effect is likely dampened by the presence of the other DGK isoform present in T cells, DGKζ. An ideal determination of the role of DGK globally in tumor responses would have used mice deficient in both DGKζ and DGKα; however, this approach is not currently feasible, because mice deficient in both DGKs fail to undergo maturation within the thymus due to enhanced negative selection (46).

We found that cytokine production in DGKζ-deficient CD8+ T cells in the presence of an inhibitor of DGKζ (R59022, Sigma) results in an augmented percentage of cells producing cytokine in response to TCR stimulation, as compared with DGKζ-deficient CD8+ T cells in the absence of inhibitor (data not shown). This finding indicates that the remaining DGK activity within DGKζ-deficient T cells does play a functional role and suggests that mice deleted of both DGKζ and DGKα could generate anti-tumor immune responses superior to mice deleted of DGKζ alone. However, deletion of both isoforms of DGK may result in an unacceptable degree of autoimmunity, resulting in a requirement for a high degree of specificity in therapeutic attempts.

evidenced by expression of activated (CD44high) T cells that expressed TCR specific for Kb-OVAp tetramer. Furthermore, CD8+ T cells were capable of autonomously demonstrating enhanced anti-tumor effects, because prior transfer of naive (CD44low) DGKζ-deficient OT-1 CD8+ T cells relative to wild type counterparts resulted in smaller EL4-ova tumors 1 week after the subcutaneous inoculation of EL4-ova cells, along with evidence of activation, as determined by the up-regulation of CD44. Transferred DGKζ-deficient or wild type naive CD8+ T cells were not identified in significant numbers after 7 days in host mice that did not receive an inoculum of EL4-ova cells (data not shown). This represents the first study to evaluate the effect of altering the analog to digital TCR signal threshold within a tumor microenvironment and identifies DGKζ as a novel target for enhancing antitumor immunity.

FIGURE 7. Enhanced tumor responsiveness of DGKζ−/− CD8+ T cells. 2 × 10^6 naive (CD44low), CD45.1− OT-1, or DGKζ−/− OT-1 cells were adoptively transferred into CD45.1-positive mice. Forty-eight hours later, 5 × 10^6 EL4-ova cells were injected subcutaneously into the right flanks of host mice. Seven days later, mice were sacrificed, and tumors were excised and weighed (top panel). Splenocytes were prepared and donor lymphocytes were assessed for the up-regulation of CD44 on donor CD8+ T cells (middle panel), or stimulated for 5 h with OVAp and assessed for the production of IL2 in donor cells as described for Fig. 4 (bottom panel).
to manipulate this pathway. Future experiments will extend our observations into mice deficient in both T cell DGK isoforms and evaluate the effects of DGKζ deficiency alone in more physiologic models of tumorigenesis.

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