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Research Paper

Diacylglycerol Kinase ζ Limits Cytokine-dependent Expansion of CD8$^+$ T Cells with Broad Antitumor Capacity

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ABSTRACT

Interleukin-2 and -15 drive expansion/differentiation of cytotoxic CD8$^+$ T cells that eliminate targets *via* antigen-independent killing. This property is clinically relevant for the improvement of T cell-based antitumor therapies. Diacylglycerol kinase α and ζ (DGKα/ζ) metabolize the diacylglycerol generated following antigen recognition by T lymphocytes. Enhanced expression of these two lipid kinases in tumor-infiltrating CD8$^+$ T cells promotes a hyporesponsive state that contributes to tumor immune escape. Inhibition of these two enzymes might thus be of interest for potentiating conventional antigen-directed tumor elimination. In this study, we sought to characterize the contribution of DGKα and ζ to antigen-independent cytotoxic functions of CD8$^+$ T cells. Analysis of DGKζ-deficient mice showed an increase in bystander memory-like CD8$^+$ T cell populations not observed in DGKα-deficient mice. We demonstrate that DGKζ limits cytokine responses in an antigen-independent manner. Cytokine-specific expansion of DGKζ-deficient CD8$^+$ T cells promoted enhanced differentiation of innate-like cytotoxic cells *in vitro*, and correlated with the more potent *in vivo* anti-tumor responses of DGKζ-deficient mice engrafted with the murine A20 lymphoma. Our studies reveal a isoform-specific function for DGKζ downstream of IL-2/IL-15-mediated expansion of innate-like cytotoxic T cells, Pharmacological manipulation of DGKζ activity is of therapeutic interest for cytokine-directed anti-tumor treatments.

1. Introduction

CD8$^+$ T cells respond to pathogens and tumors following T cell receptor (TCR) recognition of specific peptides presented by the major histocompatibility complex (MHC). TCR-activated cells secrete interleukin 2 (IL-2), which promotes expression of CD25, the IL-2 receptor α chain that forms the high-affinity IL-2 receptor (IL2-R) together with the constitutively expressed β (CD122) and common γ chains (γc; CD132). Antigen recognition by the TCR thus ensures IL-2-dependent clonal expansion of cytotoxic T cell populations (Cantrell and Smith, 1984). Following their expansion and differentiation phase, most antigen-specific effector T cells die, and the few surviving cells develop into memory T cells. At this stage, memory cells depend largely on IL-15 (Schluns et al., 2002; Becker et al., 2002; Goldrath et al., 2002), a cytokine that shares CD122 and CD132 chains with IL-2 (Grabstein et al., 1994). CD8$^+$ memory T cells, characterized by a CD44^{hi}CD122^{hi} phenotype, respond more rapidly to antigen and produce larger amounts of cytokines after antigenic challenge.

In addition to conventional memory CD8$^+$ T cells, experimental evidence has identified a preexisting pool of CD8$^+$ T cells with a CD44^{hi}CD122^{hi} phenotype (Dubois et al., 2006). This population is found at high frequency in mice with impaired differentiation of conventional CD44^{lo}CD122^{lo} CD8$^+$ T cells, suggesting a distinct origin (Atherly et al., 2006; Broussard et al., 2006). Non-conventional CD44^{hi}CD122^{hi} CD8$^+$ T cells need IL-15 to expand (Dubois et al., 2006) and are absent in IL-15$^{-/-}$ mice (Judge et al., 2002). They express NK (natural killer) receptors such as NKG2D, NKG2A/C/E, CD94 and Ly46, which recognize NK ligands in an MHC class I context (Dubois et al., 2006). Cytokine-mediated expansion of memory-like CD8$^+$ T cells provides them with innate-like (non-antigen-dependent) abilities for target recognition and concurs with the capacity of IL-2 or IL-15 to generate potent CD8$^+$ T cell-dependent anti-tumor responses (Murphy et al., 2003).

Diacylglycerol kinases (DGK) α and ζ are lipid kinases that limit diacylglycerol (DAG)-dependent activation pathways downstream of the TCR (Merida et al., 2015). Their upregulation in tumor infiltrating lymphocytes (TIL) has been linked to generation of hyporesponsive states that contribute to immune evasion by tumors (Riese et al., 2013). DGKζ-deficient mice show stronger anti-tumor effects in antigen-dependent models, and mouse models of chimeric antigen receptor-engineered (CAR). T cell-infiltrating solid tumors show high expression of both DGK isoforms (Riese et al., 2011; Riese et al., 2013). DGKζ-deficient mice have greater numbers of CD44^{hi}CD122^{hi} CD8$^+$ T cells (Riese et al., 2011) and DGKζ deficiency enhances homeostatic

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expansion of $CD8^+$ T cells in lymphopenic hosts (Zhong et al., 2003). Whether this is the result of an intrinsic advantage in response to cytokines caused by selective DGKζ deficiency has not been addressed.

Here we sought to characterize the contribution of DGKα and DGKζ to the generation of non-conventional bystander memory-like $CD8^+$ T cell populations. We show that DGKζ deficiency, but not that of DGKα leads to increased output of $CD44^{hi}CD122^{hi}$ $CD8^+$ T cells and facilitates IL-2 and IL-15 responses. Cytokine-specific expansion of DGKζ-deficient $CD44^{hi}CD122^{hi}$ $CD8^+$ T cells correlates with enhanced anti-tumor effects observed *in vivo*. Our studies suggest a unique function for DGKζ in the control of innate-like cytotoxic T cell populations, and suggest that pharmacological manipulation of DGKζ activity could be of therapeutic interest for cytokine-directed anti-tumor therapies.

2. Material and Methods

2.1. Mice and Cell Lines

Mice were maintained in pathogen-free conditions and handled in accordance with Spanish and European directives. DGK$\alpha^{-/-}$ C57BL/6 mice were kindly donated by Dr. Xiao-Ping Zhong (Duke University Medical Center, Durham NC) and DGK$\zeta^{-/-}$ C57BL/6 mice were kindly donated by Dr. Gary Koreztky (University of Pennsylvania, Philadelphia PA). For A20 xenograft experiments, DGK$\zeta^{-/-}$ C57BL/6 mice were backcrossed with BALB/c mice for 10 generations. Mouse experiments in which genetic background is not indicated were performed on the C57BL/6 background; for experiments in which age is not indicated, we used 12- to 16-week-old mice.

2.2. Tumor Experiments

All mouse work was carried out in accordance with a protocol approved by the CNB/CSIC Ethics Committee for Animal Experimentation (RD53/2013). Mice were assigned at random to experimental groups. For tumor experiments, A20 cells (5×10^6) were injected subcutaneously (s.c.) into one flank of female BALB/c WT or DGK$\zeta^{-/-}$ mice. Tumor growth was monitored in a blind manner with calipers, and volume was estimated according to the formula: volume $= (a^2 \times b)/2$, where a = tumor width and b = tumor length in mm. Mice were sacrificed at the end of the experiment.

For analysis of cytokine-induced cytotoxic cells, A20 cells (10^7) were injected s.c. into one flank of female BALB/c WT mice. After eight days, mice were divided into two groups and inoculated intratumorally with IL-2-differentiated cells from BALB/c WT or DGK$\zeta^{-/-}$ mice.

2.3. Cytokine-differentiated Cells

Splenocytes from BALB/c WT or DGK$\zeta^{-/-}$ mice were cultured in RPMI complete medium containing IL-2 (200 U/ml) or IL-15 (100 ng/ml) for 6–7 days. Cells were divided every 2–3 days and used for flow cytometry analysis, real-time RT-PCR, degranulation or cytolytic assays on days 6–8.

2.4. Flow Cytometry and Antibodies

Antibodies used were anti-CD8-PeCy7, -CD3-PERCP Cy5.5, -CD44-PeCy5, -CD25-PeCy7, -CD335-APC (NKP46) (BioLegend), -CD8-eFluor450, CD3-Pecy7, -CD122-FITC, -IL15-receptorα-PE, -CD279-eFluor780 (PD-1), -CD314-PE (NKG2D) (eBioscience), -CD4-PE, -CD3-FITC (Beckman Coulter), and -CD69-PE (Pharmingen). Samples were collected on a LSRII (BD Biosciences) or a Cytomics FC 500 (Beckman Coulter) and analyzed with FlowJo software (Tree Star, Ashland, OR).

For analysis of phosphorylated proteins, splenocytes were incubated with IL-2 (200 U/ml) or IL-15 (100 ng/ml) (20 min), fixed in 1% paraformaldehyde (10 min, room temperature (RT)) and permeabilized using Phosflow Perm Buffer III (BD Bioesciences; 30 min, 4 °C). Cells were stained for surface markers (15 min, RT), washed, incubated with anti-pS6 (Ser235/236, D57.2.2E) or -pSTAT5 (Tyr694, D47E7) (both from Cell Signaling; 1 h, RT), washed and stained with secondary antibody (goat F(ab′)2 anti-rabbit IgG (H + L)-PE; Beckman Coulter).

For proliferation experiments, cells were stained with CellTrace Violet (Molecular Probes) and cultured with IL-2 (200 U/ml) or IL-15 (100 ng/ml). After 72 h, cells were harvested, stained for surface markers and analyzed.

2.5. Cell Activation

For TCR or cytokine activation, splenocytes were cultured in antibody-coated plates (anti-mouse CD3e; BD PharMingen, 5 μg/ml) or in complete RPMI medium containing IL-2 (200 U/ml) or IL-15 (100 ng/ml) for 48 h. Cells were harvested, stained for surface markers and analyzed by flow cytometry. For incubation with tumor cells, BALB/c WT or DGK$\zeta^{-/-}$ splenocytes were co-cultured with A20 cells (1:1) for 14 h, stained for surface markers and analyzed by flow cytometry.

For degranulation assays, IL-2-differentiated cells from BALB/c WT or DGK$\zeta^{-/-}$ mice were incubated with A20 cells (1:1 proportion) during 4 h in presence of anti-CD107a-PE (BD Pharmigen). Then cells were washed, stained for surface markers and analyzed by flow cytometry.

2.6. RNA Preparation and Real-time RT-PCR

Total RNA was reverse-transcribed using the High Capacity cDNA Archive Kit (PN4368813; Applied Biosystems). Real-time PCR reactions were performed in triplicate with HOT FIREPol EvaGreen qPCR Mix Plus (ROX) (Solis BioDyne) in an Applied Biosystems ABI PRISM 7900HT machine with SDS v2.4 software, using a standard protocol. Results were analyzed by the comparative Ct method (ΔΔCt). Expression was normalized using the β-actin housekeeping gene for each sample. Primers used were β-actin 5′-GGCTCCTAGCACCATGAAGA-3′; 5′-CCACCGATCCACACAGAGTA-3′; RAET-1 5′-TGAAGAGGAAATATTATA CCCAAGGA-3′; 5′-CTGTAACTCCAGTTCCACA GGAT-3′; H60a 5′-ATGCAGGTCTCCCCTAGCTT-3′; 5′-TCACACAGACTCAATGC AGGT-3′; MULT-1 5′-TGAAGTCACCTGTGTTTATGCAG-3′; 5′-GGCACTGTCAAAG AGTCATCC-3′; IL15 5′-CAGAGGCCAACTGGATAGATG-3′; 5′-ACTGTC AGTGTATA AAGTGGTGTCAAT-3′. Primers for IFNγ and perforin (Macintyre et al., 2011), IL-10 and IL-2 (Fontenot et al., 2003), and granzyme A, B and C (Janas et al., 2005) were reported previously.

2.7. Statistical Analysis

Data were analyzed using GraphPad Prism 5 and SPSS software. An unpaired two-tailed *t*-test with 95% confidence intervals was used for data with normal distribution and equal variances, and an unpaired *t*-test with Welch's correction for data sets with different variances. The Mann-Whitney test was used for data with non-normal distribution. Normality was analyzed by the Kolmogorov-Smirnov test. For multiple comparisons, ANOVA or two-way ANOVA and Bonferroni post-tests were performed. In the case of ANOVA with non-normal data, Dunn's multiple comparisons test was used; in the case of two categorical independent variables and non-normal data, the Mann-Whitney test with Bonferroni correction was used. In Fig. 4b, percentages were calculated for all data from the independent experiments in each case (as a result, graphs lack error bars). Data for each day were analyzed by the Chi-square test. Data in Fig. 6b were analyzed by the Gehan-Breslow-Wilcoxon test. Differences were considered non-significant (ns) when $p > 0.05$, significant (*) when $p < 0.05$, very significant (**) when $p < 0.01$ and extremely significant (***) when $p < 0.001$.