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Differential regulation of primary and memory CD8 T cell immune responses by diacylglycerol kinases

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Abstract

The manipulation of signals downstream of the T cell receptor (TCR) can have profound consequences for T cell development, function, and homeostasis. Diacylglycerol (DAG) produced following TCR stimulation functions as a secondary messenger and mediates the signaling to Ras-MEK-Erk and NF-κB pathways in T cells. DAG kinases (DGKs) convert DAG into phosphatidic acid (PA), resulting in termination of DAG signaling. In this study, we demonstrate that DAG metabolism by DGKs can serve a crucial function in viral clearance upon lymphocytic choriomeningitis virus (LCMV) infection. Antigen-specific $CD8^+$ T cells from $DGK\alpha^{-/-}$ and $DGK\zeta^{-/-}$ mice show enhanced expansion and increased cytokine production following LCMV infection, yet DGK-deficient memory $CD8^+$ T cells exhibit impaired expansion after re-challenge. Thus, DGK activity plays opposing roles in the expansion of $CD8^+$ T cells during the primary and memory phases of the immune response, while consistently inhibiting anti-viral cytokine production.

Introduction

Effective control of viral replication is dependent upon the expansion of antigen specific $CD8^+$ T cells that are poised to directly kill virally infected cells, and are capable of producing cytokines that will help to shape the developing immune response. After infection, virally derived proteins that have been processed by the infected cells are presented in complex with MHC class I (MHCI) molecules to the naïve $CD8^+$ T cell pool. Clones with the appropriate T cell receptor (TCR) will engage the peptide:MHCI complex and become activated. Once activated, the reactive $CD8^+$ T cell pool differentiates into effector cytotoxic T lymphocytes (CTLs) and will migrate to the sites of infection. The effector phase is characterized by the aggressive expansion of the specific $CD8^+$ T cell pool. Once the infection is resolved, the reacting T cell pool undergoes a contraction phase, where only 5%–10% of the expanded effectors will persist. Finally, the formation and maintenance of the long-lived antigen specific memory $CD8^+$ T cell pool is formed, which will protect the host against subsequent infections by the same virus (1, 2).

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Competing Interest Statement
The authors declare that they have no competing financial interests.

The initiation of the T cell immune response begins with the recognition of foreign peptides, presented on MHC molecules, to the TCR. This immediate interaction, along with signals proximal to the TCR, has profound effects on the ensuing T cell response to bacterial and viral antigens (3–9). Additionally, many extracellular determinants, along with intracellular signaling molecules and enzymes have been identified as key regulators in the formation, function, and maintenance of the ensuing T cell response (10–15).

TCR signaling results in the production of secondary messengers, which serve to amplify and direct unique signaling pathways in activated T cells. The initiation of TCR signaling results in the activation of the Syk and Src family kinases. These proteins relay their signal by phosphorylating the adaptor proteins SLP-76 and LAT, which serve as docking sites for additional molecules in the TCR signaling cascade (16, 17). PLC-γ1 is then recruited to the SLP-76/LAT complex, where it becomes activated, and subsequently hydrolyzes PIP_2 into IP_3 and diacylglycerol (DAG) (18, 19). Serving as a potent secondary messenger, IP_3 initiates calcium release from the endoplasmic reticulum, activating the calcineurin pathway, and ultimately leading the nuclear translocation of NFAT (20, 21). DAG binds to and activates both RasGRP1 and PKC-θ via their cysteine-rich C1 domains. The result of DAG binding to RasGRP1 and PKC-θ is the activation of the Ras-ERK-AP1 and NF-κB pathways, respectively (22–27). The proximity of DAG production to the signaling events immediately downstream of the TCR, and its ability to activate multiple signaling pathways simultaneously, led investigators to hypothesize that the dys-regulation of DAG signaling and metabolism might perturb normal T cell homeostasis and function.

As a potent positive regulator of T cell activation, the termination of DAG signaling is necessary to limit damage mediated by perpetually activated T cells or the development of an autoreactive T cell pool. A family of enzymes, the DAG kinases (DGKs), converts DAG to phosphatidic acid (PA), effectively terminating DAG mediated signaling (28–30). While ten DGK isoforms have been identified in mammalian cells, DGKα and DGKζ are expressed in T cells (31–35). In order to assess the functional relevance of DAG signaling in T cells *in vivo*, DGKα- and ζ-deficient mice were created. Analysis of DGK-deficient T cells revealed a variety of abnormalities. Enhanced activation of the Ras-MEK-Erk pathway and increased expression of CD25 and CD69 following anti-CD3 stimulation was observed in DGK-deficient T cells in comparison to their WT counterparts. Additionally, DGKζ-deficient T cells were observed to be hyperproliferative. In an *in vivo* model, DGKζ-deficient mice infected with lymphocytic choriomeningitis virus (LCMV) exhibited an enhanced ability to control infection (9, 36). In $CD4^+$ T cells, deficiency of DGKα and DGKζ results in the resistance to the induction of T cell anergy both *in vitro* and *in vivo* (36). These observations clearly demonstrate a vital role for DAG metabolism in T cell mediated immunity.

In this study, we examine the $CD8^+$ T cell response to LCMV infection in DGK-deficient mice. The development of MHC class I tetramers specific for LCMV specific $CD8^+$ T cell clones, as well as synthetic peptides that mimic virally derived antigens, have allowed us to enumerate the expansion of antigen specific T cells on a single cell scale in germ-line knockout models. Our data demonstrate that DGKα and ζ repress primary anti-viral immune response by dampening $CD8^+$ T cell expansion and cytokine production, but promote memory $CD8^+$ T cell responses.

Materials and Methods

Mice

$DGK\alpha^{-/-}$ (aKO) and $DGK\zeta^{-/-}$ (zKO) mice were generated as previously described (9, 36). TCR transgenic LCMV-specific P14 mice were purchased from Taconic and were bred to

aKO or zKO to generate aKO-P14 and zKO-P14 mice in specific pathogen free facilities at Duke University Medical Center. The experiments in this study were performed according to a protocol approved by the Institutional Animal Care and Usage Committee of Duke University. Eight to twelve weeks old mice were used in this study.

Virus and infection

LCMV-Armstrong (Arm) stocks were propagated on BHK-21 cells and quantitated as described previously (37). 2×10^5 plaque forming units (PFU) of LCMV-Arm were administered to mice by intraperitoneal injection (i.p.) for the acute viral infection.

Adoptive Transfers

To assess the primary immune response, 1×10^4 $CD8^+V\alpha2^+CD44^{lo}$ cells were sorted from the spleen of WT-P14, aKO-P14, and zKO-P14 mice and were transferred intravenously into WT congenic hosts, which were infected the next day. For the memory response, 5×10^3 $CD8^+CD44^{hi}$ cells were sorted from the spleen and lymph nodes of WT, aKO, and zKO mice 4 months post infection with LCMV-Arm, and were transferred intravenously into WT congenic hosts. The next day, these hosts were infected with LCMV-Arm. Peripheral blood and splenocytes from infected mice were collected for analysis at the indicated times.

Flow cytometry

Cells from peripheral blood (PB) and spleen (SP) were harvested, stimulated and stained using appropriate antibodies directly conjugated with fluorochrome. Tetramers of H-2D^{b}GP33-41 (TetG) and H-2D^{b}NP396-404 (TetN) specific for LCMV were conjugated with allophycocyanin. The construction and purification of H-2D^{b}GP33-41 (TetG) and H-2D^{b}NP396-404 (TetN) have been described previously (38). For intracellular cytokine staining, splenocytes were stimulated with indicated peptides (10 μg/ml) in the presence of Golgi-Plug protein transport inhibitor (1:1000, BD Biosciences). After 5 h stimulation, the cells were harvested and stained using Cytofix/Cytoperm Fixation/Permeabilization Kit (BD Biosciences). The peptides used for stimulation were GP33-41 (KAVYNFATM), GP276-286 (SGVENPGGYCL) and NP396-404 (FQPQNGQFI). To measure the mTOR activity, cells were stained with a rabbit anti-phospho-S6 (Ser240/244) followed by FITC-conjugated anti-rabbit secondary antibodies (Cell Signaling Technology). All of the data were collected using BD FACSCanto II and were analyzed with FlowJo software (Tree Star). Geometric mean fluorescence intensity (gMFI) is used to represent the amount of cytokine produced per cell (39).

Quantititive real-time PCR

To determine viral clearance, RNAs from spleen were extracted using Trizol Reagent and were reversely transcribed to cDNAs by Superscript III (Invitrogen). qRT-PCR was performed with Mastercycler realplex and SYBR Green master mix (Eppendorf). For LCMV glycoprotein (GP) RNA expression, forward 5'-TGC CTG ACC AAA TGG ATG ATT-3' and reverse 5'-CTG CTG TGT TCC CGA AAC ACT-3' primer pairs were used according to a published protocol (40). Relative expression of LCMV-GP mRNA were normalized with β-actin control and were presented as arbitrary unit (a.u.) of fold change using $2^{-\Delta\Delta CT}$ method (41).

Statistics

Two-tailed unpaired Student *t*-tests were performed for determining *p* values. Absolute numbers of gated groups such as $CD8^+$ and $CD4^+$ splenocytes were calculated with the percentages multiplied by total splenocyte number. All the graphs represent mean ± SEM. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

Results

Increased CD8$^+$ T cell expansion in DGKα and DGKζ–deficient mice infected with LCMV

In order to assess the physiological role for DAG metabolism during T cell expansion *in vivo*, we infected WT, aKO, and zKO mice with LCMV-Arm. Seven days after infection, peripheral blood (PB) and spleens (SP) were harvested for analysis. Similar to wild-type (WT) mice, splenic cellularities were increased in aKO and zKO mice following LCMV infection (Fig. 1*A*). The expansion of CD4$^+$ T cells was not obviously different between WT and aKO or zKO mice (Fig. 1*B*, 1*C*). Although the percentages of CD8$^+$ T cells in the peripheral blood were similarly increased between WT and DGK-deficient mice, the increase of the percentages and absolute numbers of CD8$^+$ cells in aKO or zKO spleens were 2 fold greater than the WT control (Fig. 1*B*, 1*D*). Thus, deficiency of either DGKα or ζ results in enhanced expansion of CD8$^+$ T cells following LCMV infection.

One feature of CD8$^+$ T cell mediated responses to viral infection during the effector phase is the expansion of viral specific T cell pool. Using MHC class I tetramers we were able to determine the frequency of LCMV specific T cell clones within the bulk CD8$^+$ T cell population. We observed a small increase in the frequency of CD8$^+$ T cells reactive to the NP396-404 epitope and equivalent frequencies for the GP33-41 epitope in DGK-deficient mice as compared with WT mice (Fig. 1*E*). However, because of the increase in total CD8$^+$ T cells in the spleen, the number of Tetramer$^+$ CD8$^+$ T cells is higher in the spleen of DGK-deficient mice infected with LCMV (Fig. 1*F*). Collectively, these data reveal that deficiency of either DGKα or ζ promotes the expansion of LCMV specific CD8$^+$ T cell clones *in vivo*.

Enhanced functionality of LCMV-reactive CD8$^+$ T cells in the absence of DGKα or ζ

Another feature of CD8$^+$ T cell-mediated anti-viral immune responses is differentiation of naïve CD8$^+$ T cells into viral specific cytokine producing CTLs. In response to LCMV infection, CD8$^+$ T cells are known to produce large amounts of IFNγ and TNFα that are important for control of the infection (42). In order to determine if LCMV specific DGK-deficient CD8$^+$ T cells developed into cytokine producing effector cells after infection, we harvested splenocytes from WT and DGK-deficient mice 7 days after immunization, and cultured them with MHC class I restricted LCMV specific peptides GP33-41, GP276-286, and NP396-404. IFNγ and TNFα production was determined by intracellular staining and FACS analysis. The percentages of cytokine producing cells and geometric mean fluorescence intensities (gMFI) of produced IFNγ, but not TNFα, were increased in DGK-deficient CD8$^+$ T cells (Fig. 2*A*, 2*B*). Because the total number of CD8$^+$ T cells as well as the proportion of overall number of cytokine producing CD8$^+$ T cells were increased in the DGK-deficient mice, the overall number of cytokine producing effector T cells was dramatically enhanced (Fig. 2*C*). Consistent with increased cytokine production upon LCMV infection, lower amount of LCMV transcript was detected in DGKα or ζ deficient mice as compared to WT controls, indicating that viral clearance was more effective in the absence of DGKα or ζ (Fig. 2*D*).

Intrinsic role for DGKζ-mediated DAG metabolism in anti-viral CD8$^+$ T cell expansion

The infection of aKO and zKO mice revealed a crucial role for DAG metabolism in regulating the CD8$^+$ T cell response to viral infection. Because these mice are germ-line knockout models, we cannot rule out the contribution of extrinsic factors (i.e. cytokines, growth factors, and co-stimulatory molecules) produced by cells other than T cells that may influence CD8$^+$ T cells during LCMV infection. To investigate whether the enhanced anti-viral response due to DGKα or ζ deficiency involves a T cell intrinsic mechanism, we bred aKO and zKO mice with P14 TCR transgenic mice to generate aKO-P14 and zKO-P14 mice. P14 mice have an MHC class I restricted Vα2$^+$Vβ8.1$^+$ TCR specific to the GP33-41

epitope derived from LCMV (43). Sorted transgenic $CD8^+V\alpha2^+CD44^{lo}$ naïve T cells from WT (C57B6/J)-P14, aKO-P14, and zKO-P14 mice ($Thy1.2^+$) were adoptively transferred into congenic $Thy1.1^+$ or $Thy1.1^+Thy1.2^+$ WT hosts. The recipient mice were infected with LCMV-Arm one day after adoptive transfer and analyzed 7 days after the infection (Fig. 3*A*). Comparing recipients injected with zKO-P14 or WT-P14 $CD8^+$ T cells, there was no obvious difference of total number of splenocytes between these two groups (Fig. 3*B*). However, the percentage of donor-P14 cells within the $CD8^+V\alpha2^+$ population was increased in recipient mice receiving zKO-P14 T cells as compared to WT-P14 recipient mice (Fig. 3*C*). The absolute number of splenic zKO-P14 donor cells was about seven-fold higher than the WT control (Fig. 3*D*). Together, these observations suggest that zKO-P14 T cells possess an advantage in clonal expansion over WT-P14 T cells in competing with the endogenous LCMV-specific T cells. In contrast to zKO-P14 cells, aKO-P14 cells did not show enhanced clonal expansion in the recipients as compared with WT-P14 cells (Fig. 3*E*–*G*). When stimulated with peptide *in vitro*, both aKO- and zKO-P14 cells produced slightly more IFNγ than WT-P14 cells (Fig. 3*H*). However, only aKO-P14 cells produced more TNFα than WT-P14 cells. Collectively, data from the adoptive transfer experiments demonstrate that the enhanced anti-viral responses of DGKζ deficient, but not DGKα deficient T cells, are cell intrinsic.

DGK-deficient mice contain fewer antigen-specific memory $CD8^+$ T cells following LCMV infection

The experiments described above have revealed important roles for DGKα and ζ as negative regulators in $CD8^+$ T cells during primary antiviral immune responses. We further investigated whether DGK-deficient $CD8^+$ T cells can differentiate into long-lived antigen specific memory cells. Four months after infection, the total numbers of splenocytes in each group were similar (Fig. 4A). However, the percentages and total numbers of splenic $CD8^+TetG^+$ cells were decreased about 50% in DGK-deficient mice. (Fig. 4*B top panels* and Fig 4*C*). It has been reported that central memory T cells expand more effectively than effector memory T cells (44), however, the ratios of $CD44^{hi}CD62L^{hi}$ central memory and $CD44^{hi}CD62L^{lo}$ effector memory within the $CD8^+TetG^+$ population were similar among these three groups (Fig. 4*B bottom panels*). The proportion of $KLRG1^{lo}IL7R\alpha^{hi}$ long-lived memory precursor cells (MPECs) within the LCMV-specific $CD8^+$ T cell pool was comparable among the WT, aKO, and zKO mice (Fig. 4*D*). Cumulatively, these data suggest that the generation and/or maintenance of an antigen specific memory $CD8^+$ T cells is impaired by DGK deficiency.

Impaired expansion of DGK-deficient memory $CD8^+$ T cells after re-exposure to antigen

To assess the function of DGK activity in the memory $CD8^+$ T cell response, we sorted $CD8^+CD44^{hi}$ T cells from the mice ($Thy1.2^+$) infected with LCMV-Arm 4 months prior, normalized the numbers of LCMV-specific memory T cells using TetG, and adoptively transferred 5×10^3 TetG-reactive $CD8^+$ memory cells into naïve hosts ($Thy1.1^+$). Recipient mice were infected with LCMV-Arm one day later and analyzed on 3, 5 and 7 days after infection. The overall splenic cellularities in recipients of DGK-deficient memory $CD8^+$ T cells were slightly reduced over a three day time course as compared to recipients of WT control cells (Fig. 5*A*). Furthermore, the recipient mice receiving DGK-deficient memory T cells showed reduction in the numbers of $CD8^+Thy1.2^+$ cells as well as TetG-reactive $CD8^+Thy1.2^+$ cells (Fig. 5*B*, 5*C*). Importantly, the recipients of DGK-deficient memory cells were less efficient in viral clearance than those received WT memory cells (Fig. 5*D*). Furthermore, the detection of higher virus transcript at days 3 and 5 in mice receiving DGK-deficient memory T cells.indicates that the reduced number of reacting memory T cells at day 7 is due to impaired T cell expansion and not the result of enhanced viral clearance

coupled with the quicker initiation of T cell contraction. Together, these data indicated that DGK activity promotes memory $CD8^+$ T cell expansion during recall responses.

The mechanisms that control the formation and function of memory T cells have been intensively investigated. Recent reports demonstrated that mTOR inhibition promotes the generation of memory T cells (45–48) and our study revealed that DGKs function as negative regulators of mTOR activation through the inhibition of the DAG-RasGRP1-Ras-Erk1/2 pathway (49). Consistent with previous reports, we observed higher level of phospho-S6 (a marker of mTOR activation) in DGK-deficient memory $CD8^+$ T cells than in WT control (Fig. 5*E*), suggesting that DGK activity may promote memory CD8 T cell responses by inhibiting mTOR signaling.

DGK-deficiency does not impair cytokine production by memory $CD8^+$ T cells during recall response

The impaired proliferation of DGK-deficient memory $CD8^+$ T cells after LCMV infection led us to investigate whether DGKs may also regulate the production of anti-viral cytokines by re-activated memory T cells. In contrast to decreased expansion of memory $CD8^+$ T cells, DGK-deficient memory cells produced more cytokines than the WT control cells following *in vitro* stimulation with LCMV-derived peptides (Fig. 6). Although we could not rule out that the increased cytokine production was influenced by delayed viral clearance in the recipient mice, our data suggest that DGKα and ζ exert differential effects on memory $CD8^+$ T cells expansion and effector cytokine production.

Discussion

$CD8^+$ T cells play crucial roles in anti-viral immunity. Understanding how $CD8^+$ T cells are regulated during viral infection is instrumental for treatment of infectious diseases and for enhancing the efficacy of vaccination. Engagement of $CD8^+$ T cells with antigen presenting cells through the interaction between viral specific TCRs and viral peptide:MHC-I complex is critical for the initiation of $CD8^+$ T cell mediated anti-viral immunity. The signal strength from viral specific TCRs can influence the effector and memory $CD8^+$ T cell differentiation, maintenance, and activation (3–5, 7, 50). We demonstrate that both DGKα and ζ are important regulators for $CD8^+$ T cell-mediated anti-viral immune responses and that DGK activity plays differential roles in primary and memory $CD8^+$ T cell responses.

Our data has revealed that DGKα and ζ negatively control $CD8^+$ T cell-mediated anti-viral responses during primary infection. These observations are consistent with the role of DGKs as negative regulators of T cell activation *in vitro* by terminating DAG-mediated signaling. As a potent secondary messenger, DAG promotes signaling in both the NFκB and Ras-Erk pathways. In the absence of either DGKα or ζ, TCR induced activations of Ras-Erk1/2 and PKC-NFκB pathways are enhanced (9, 51), which likely contribute to enhanced anti-viral responses of DGK-deficient T cells during primary LCMV infection. Under the control of T cell specific promoter, a dominant IκB severely impairs T cell proliferation (52). Furthermore, $RelB^{-/-}$ (a component of NFκB signaling complex) mice show increased susceptibility to LCMV infection and impaired expansion of the $CD8^+$ T cell pool. RelB has also been shown to associate with the TNFα promoter (53). In addition, proper signaling through the Ras-Erk pathway has also been shown to be critical during an immune response. RasGRP1 mice show impaired antigen specific $CD8^+$ T cell expansion, and fail to generate LCMV specific IFNγ producing $CD8^+$ T cells in response to LCMV infection. This impaired response results in higher viral titers in the spleen of infected mice (54). The increased expansion and cytokine production observed in DGK-deficient $CD8^+$ T cells after LCMV infection could be directly related to the accumulation of DAG and enhanced activation of the Ras-Erk1/2 and NFκB signaling *in vivo*. Although these data support that

proper regulation of the signaling cascades downstream of DGK activity is crucial for the generation of an effective adaptive immune response, we cannot rule out that DGKs may regulate $CD8^+$ T cell mediated immune responses through the generation of PA. Similar to DAG, PA binds to and regulates multiple signal molecules. DGK-derived PA has been implicated in several signaling pathways to modulate cellular function and development of different cell lineages. Furthermore, while our study focuses on the role of DGKs in mature T cells after infection, it is worth noting that DGKs and the Ras-Erk and NFκB pathways are critical to immune cell development (55, 56). Although we could not rule out that DGKα or ζ deficiency may directly or indirectly affect the generation of LCMV specific T cells in thymus, we found that they are involved in regulating mature $CD8^+$ T cells during viral infection. The P14 adoptive transfer experiment suggests DGKζ functions in mature T cells to dampen anti-viral immune responses, and does not function in development to profoundly distort the T cell receptor repertoire.

DGKα and ζ play a redundant role for T cell maturation in thymus (56). Although both DGKα and ζ exert similar roles in $CD8^+$ T cells during anti-LCMV responses, some differences between these two DGK isoforms have been noted. DGKζ appears to play a stronger role than DGKα, because the enhanced anti-LCMV response seen in aKO mice appears to rely, at least partially, on T cell extrinsic factors. The different anti-viral effectiveness between DGKα and DGKζ deficiency is not surprising since there are important differences in the structure and activation of these proteins. DGKα, which contains two EF hand motifs, requires the binding of Ca^{++} to achieve full enzymatic activity, while DGKζ is not sensitive to such regulation. Furthermore, DGKζ contains a nuclear localization sequence within its myristoylated alanine-rich C-kinase substrate domain, which may give DGKζ regulatory functions not associated with DGKα. Finally, these enzymes may serve important non-enzymatic functions by acting as protein docking substrates, where differences in primary and tertiary protein structure determine the components of multi-protein signaling complexes (57).

Our data indicates that naive and memory $CD8^+$ T cells can be differentially regulated by signaling molecules modulated by DGK activity during anti-viral immune responses. In contrast to inhibiting primary anti-viral immune responses, DGKα and ζ appear to promote expansion of viral specific memory $CD8^+$ T cells during secondary infection. The mechanisms underlying such differential roles of DGK activity in primary and memory anti-viral immunity are unclear at present. It is known that the mammalian target of rapamycin (mTOR) promotes primary, but inhibits memory $CD8^+$ T cell responses during LCMV infection (45). Recently, we have reported that DGKα and ζ synergistically inhibit TCR-induced mTOR activation by downregulating the DAG-RasGRP1-Ras-Erk1/2 pathway (49). Consistent with these observations, we have found increased phosphorylation of S6 in DGK-deficient memory $CD8^+$ T cells. Thus, the opposing roles of DGKs and mTOR in primary and memory $CD8^+$ T cells during LCMV infection suggests that in addition to regulating Ras-Erk1/2 and NFκB signaling, DGKα and ζ may differentially control primary and memory $CD8^+$ T cell responses during LCMV infection by inhibiting mTOR activation.

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Abbreviations

LCMV lymphocytic choriomeningitis virus

DGK	diacylglycerol kinase
Tet	tetramer

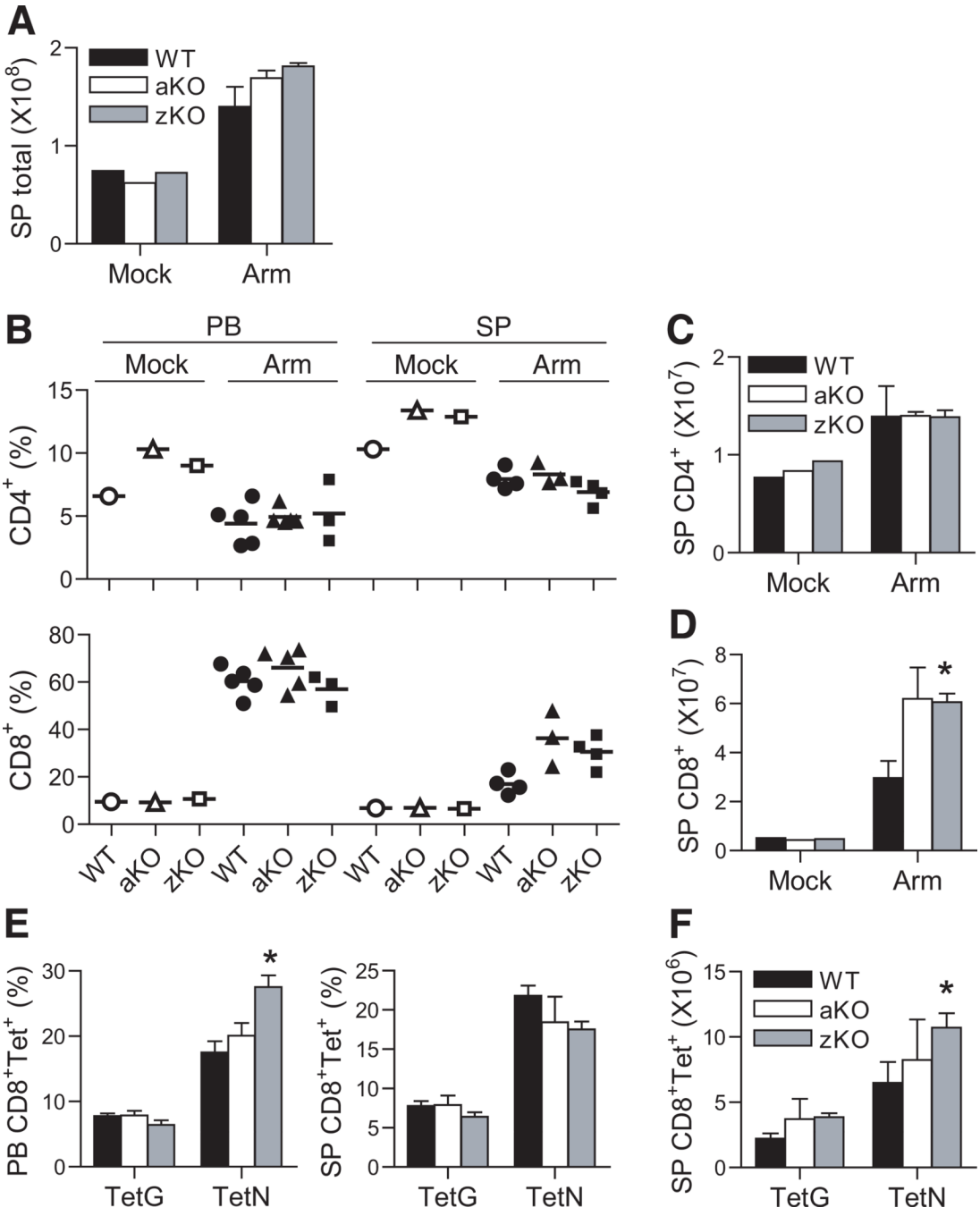

FIGURE 1.

LCMV-induced $CD8^+$ T cell expansion in DGKα and DGKζ KO mice. Peripheral blood (PB) and spleen (SP) were collected from WT, aKO or zKO mice on day 7 after LCMV-Arm infection and were analyzed by flow cytometry. *A*, Total splenocyte number (mean ± SEM). *B*, Percentages of $CD4^+$ and $CD8^+$ splenocytes were calculated by flow cytometry. Each data point in scatter plots represented individual mice. *C* and *D*, Total $CD4^+$ (*C*) and $CD8^+$ (*D*) T cell numbers (mean ± SEM). *E*, Percentages of $CD8^+$ and $TetG^+$ or $TetN^+$ T cells in the blood and spleen after infection (mean ± SEM). *F*, Total cell number of $CD8^+$ and $TetG^+$ or $TetN^+$ from spleen (mean ± SEM). Data shown were from one experiment with five (PB) or four (SP) WT, five (PB) or three (SP) aKO, and three (PB) or four (SP)

zKO mice after infection and were representative of three experiments. *, $p<0.05$ as determined by Student *t*-test. All statistical significance is in comparison to WT controls.

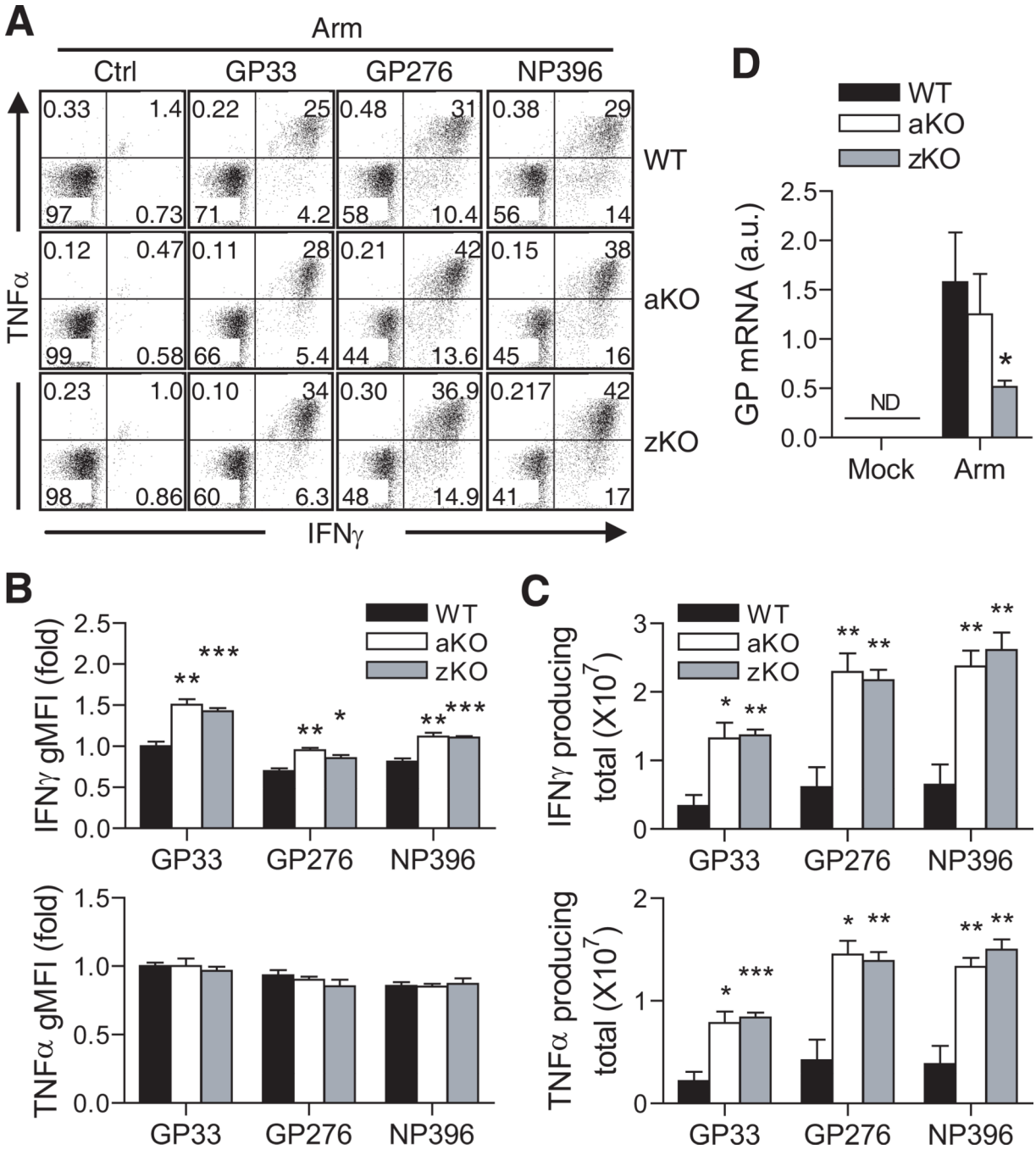

FIGURE 2.

Increased cytokine production of $CD8^+$ T cells and enhanced viral clearance in the absence of DGKα or DGKζ. *A–C*, Measurement of IFNγ and TNFα production by splenocytes following LCMV infection. Virus specific splenocytes were isolated from mock or Arm-infected mice on day 7 post-infection and were incubated with the indicated MHC-I restricted LCMV derived-peptides (GP33, GP276 and NP396) in the presence of Golgi plug. After 5 h, the cells were stained, fixed and analyzed by flow cytometry for intracellular cytokine production. *A*, Representative dot plots showed IFNγ and TNFα producing cells gated on $CD8^+$ T cells. *B*, Bar graphs demonstrate geometric mean fluorescence intensity (gMFI) of IFNγ (*top panel*) and TNFα (*bottom panel*) into the indicated peptide-stimulated

$CD8^+$ T cells. black, WT; white, aKO; gray, zKO. *C*, Absolute numbers of IFNγ (*top panel*) and TNFα (*bottom panel*) producing $CD8^+$ T cells were calculated through multiplying percentages (*A*) by total $CD8^+$T cells in the spleen (mean ± SEM). *D*, Enhanced viral clearance in the absence of either DGKα or ζ. RNAs were extracted from splenocytes on day 7 post-infection and reversely transcribed. The levels of LCMV GP mRNA were measured by qRT-PCR (mean ± SEM). Data shown are from experiment with at least 3 mice per group (n= 4 WT, 3 aKO, and 4 zKO) and are representative of three experiments. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ determined by Student *t*-test. All statistical significance is in comparison to WT controls. ND, no detection.

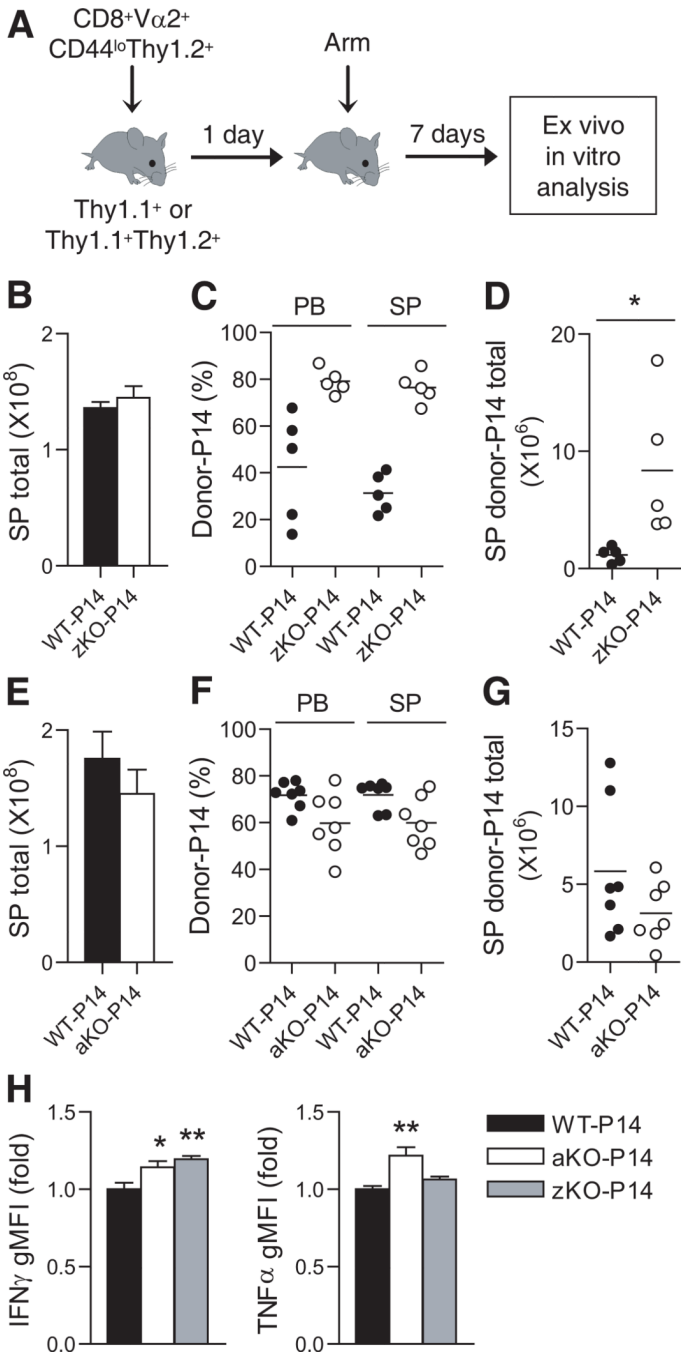

FIGURE 3.

Effects of DGKα or ζ deficiency in T cells on anti-LCMV immune responses. *A*, Schematic diagram of adoptive transfer experiment. $CD8^+$ naïve T cells from WT-P14, aKO-P14, or zKO-P14 mice ($Thy1.2^+$) were transferred into WT recipient mice ($Thy1.1^+$ or $Thy1.1^+1.2^+$) at 1 day before infection and were analyzed on day 7 post-infection. *B–D*, Comparison between WT and zKO donor P14 T cells. *B*, Total splenocyte numbers. *C*, Percentage of donor-P14 ($CD8^+V\alpha2^+Thy1.2^+$) cells from total $CD8^+V\alpha2^+$ splenocytes. *D*, Total number of SP donor-P14 cells. *E–G*, Comparison between WT and aKO donor P14 T cells. *E*, Total splenocyte numbers. *F*, Percentage of donor-P14 cells from total $CD8^+V\alpha2^+$ splenocytes in recipient mice. *G*, Total number of SP donor-P14 cells. *H*, Analysis of gMFI of IFNγ and

TNFα production in GP33-peptide stimulated WT, aKO and zKO-P14 cells (mean ± SEM). Each circle represents one mouse of the indicated genotype. Data are representative of three experiments. *, $p<0.05$,; **, $p<0.01$ determined by the Student *t*-test.

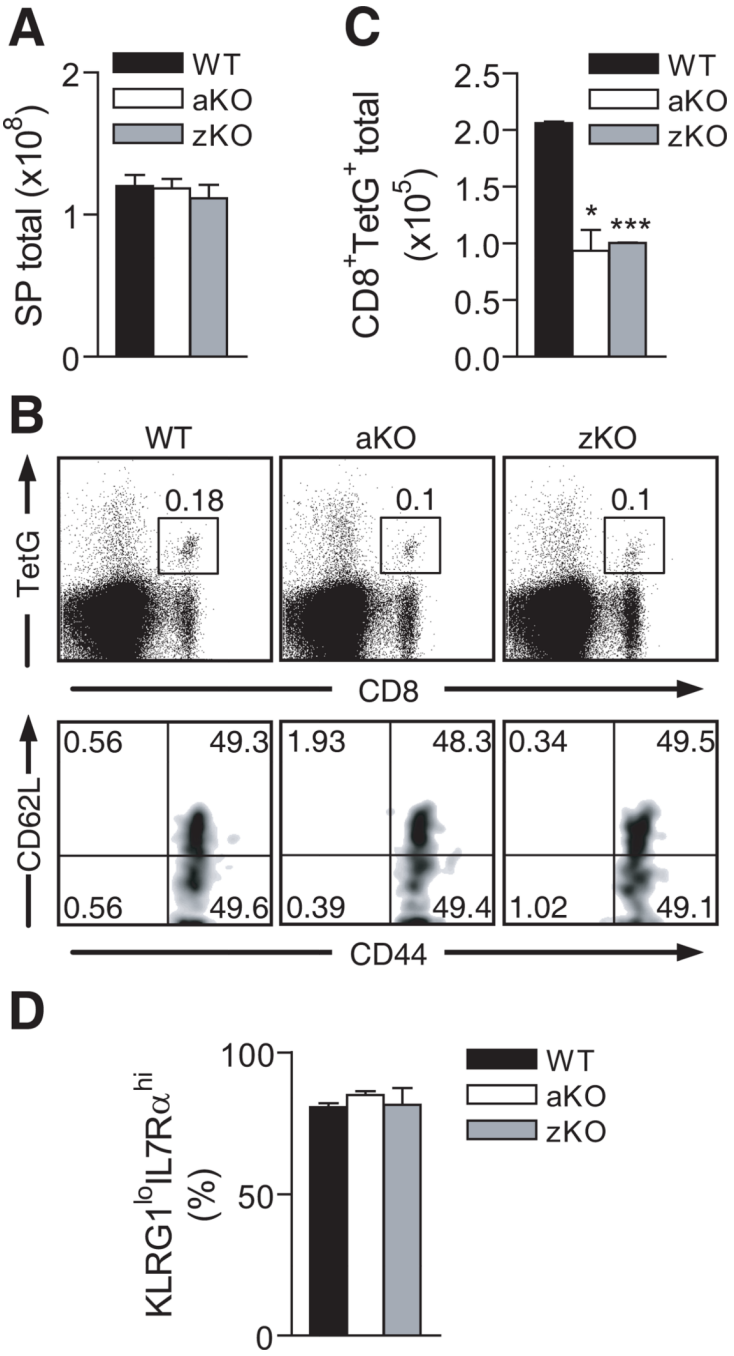

FIGURE 4.

Memory $CD8^+$ T cell subsets in DGK-deficiency. WT, aKO and zKO mice were infected with LCMV-Arm and were analyzed 4 month post-infection. *A*, Total numbers of splenocytes. *B*, Harvested splenocytes were stained with TetG and anti-CD8 Abs to identify LCMV-specific T cells (*top panel*). Gated $CD8^+TetG^+$ T cells were analyzed for CD44 and CD62L expression (*bottom panel*). *C*, Total numbers of $CD8^+TetG^+$ T cells in the spleen. *D*, The percentages of $KLRG1^{lo}IL7R\alpha^{hi}$ memory cells within $CD8^+TetG^+$ T cells. Data are representative of at least 2 mice per group and of three experiments. *, $p<0.05$; ***, $p<0.001$ determined by Student *t*-test. All statistical significance is in comparison to WT controls.

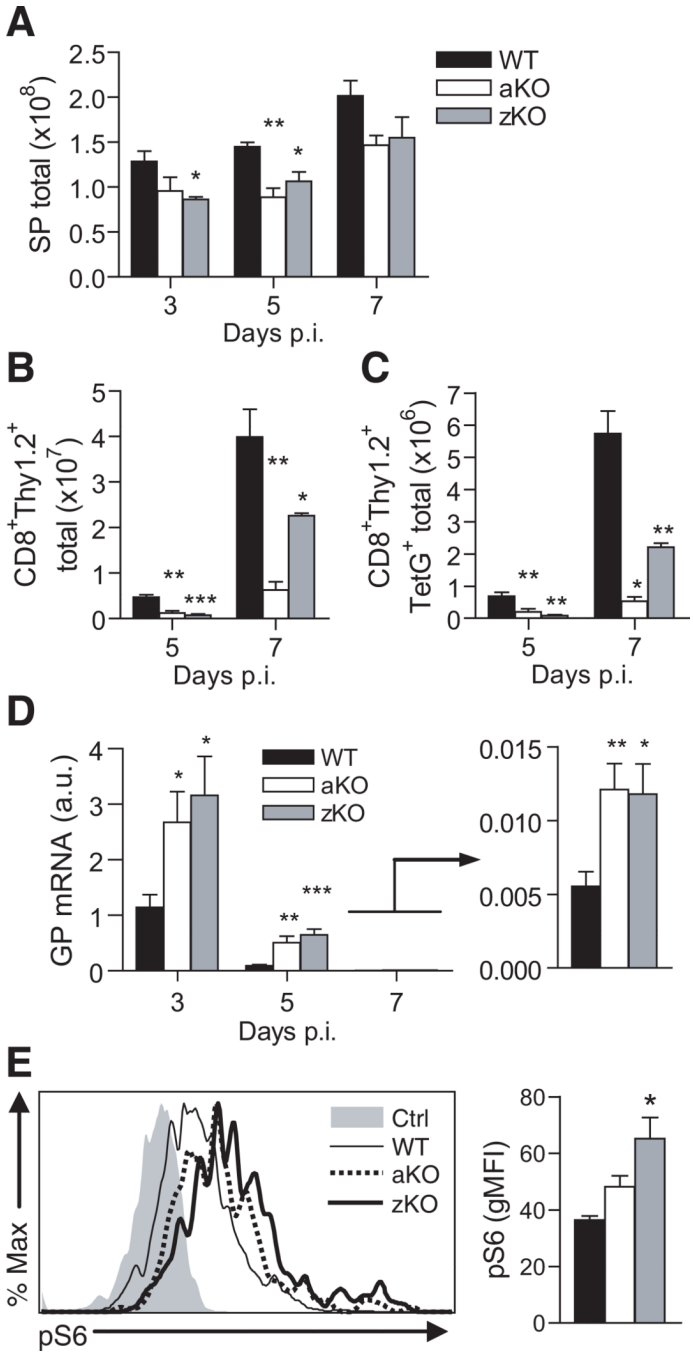

FIGURE 5.

Impaired expansion and LCMV clearance of DGK-deficient memory $CD8^+$ T cells. WT, aKO, and zKO mice ($Thy1.2^+$) were infected i.p. with LCMV-Arm. 4 months later $CD8^+CD44^{hi}$ T cells were sorted from the spleen and lymph nodes from infected mice. Sorted $CD8^+CD44^{hi}$ T cells were injected i.v. into naïve recipient hosts ($Thy1.1^+$) after normalizing by TetG staining. Hosts were then infected i.p. with LCMV-Arm one day after adoptive transfer and analyzed 3, 5, and 7 days post infection. Splenocytes were stained with anti-CD8, anti-Thy1.1, anti-Thy1.2 Abs, and TetG. *A*, Total splenocyte numbers on the indicated days post-infection. *B*, The numbers of donor-derived $CD8^+Thy1.2^+$ T cells in spleen. *C*, Total numbers of donor-derived $Thy1.2^+CD8^+TetG^+$ cells in the spleen of

recipient mice 5 and 7 days post-infection. *D*, Viral titers were determined using LCMV GP mRNA by qRT-PCR on the indicated days. *E*, S6 phosphorylation in adoptively transferred memory $TetG^+$ cells on day 5 after viral infection. Splenocytes were cell surface-stained with anti-CD8, anti-Thy1.2, and TetG, fixed, permeabilized, and intracellularly stained with an anti-phospho-S6 (Ser240/244). Bound primary anti-phospho-S6 antibody was detected with a FITC-conjugated anti-rabbit secondary antibody and analyzed by flow cytometry. Secontary antibody staining without primary antibody was used for control (Ctrl). Bar graphs showed mean ± SEM (n = 3 for WT, 4 for aKO, and 4 for zKO mice) and data are representative of three experiments. . p.i., post-infection. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$. All statistical significance is in comparison to WT controls.

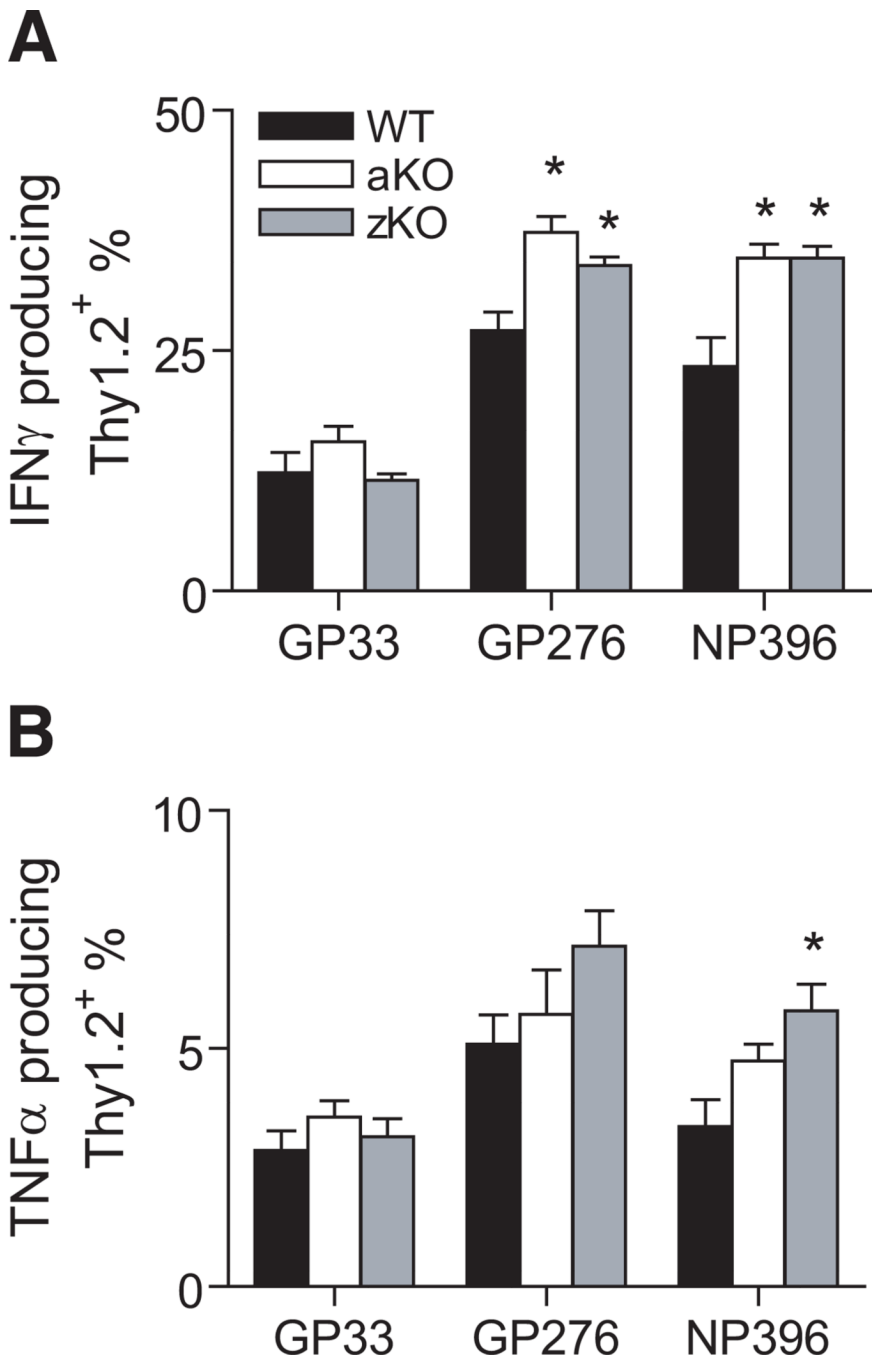

FIGURE 6.

DGK-deficient memory $CD8^+$ T cells exhibit enhanced cytokine production after re-infection. Spleens from recipient mice ($Thy1.1^+$) adoptively transferred with WT and DGK-deficient $CD8^+Thy1.2^+CD44^{hi}$ memory T cells ($Thy1.2^+$) were harvested 7 days post-infection. Splenocytes were stimulated with the indicated peptides in the presence of Golgi Plug *in vitro* for 6 hours before cell surface stained with anti-CD4, anti-CD8, anti-Thy1.1, and anti-Thy1.2 Abs and intracellular stained with anti-IFNγ and anti-TNFα Abs. *A*, Percentage of $IFN\gamma^+$ cells within the $CD8^+Thy1.2^+$ cells determined by flow cytometry. *B*, Percentage of $TNF\alpha^+$ cells within the $CD8^+Thy1.2^+$ cells determined by flow cytometry. *, $p<0.05$. All statistical significance is in comparison to WT controls.