



DGK α and ζ Activities Control T_H1 and T_H17 Cell Differentiation

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Specialty section:

This article was submitted to
T Cell Biology,
a section of the journal
Frontiers in Immunology

Received: 26 April 2019

Accepted: 12 December 2019

Published: 15 January 2020

Citation:

Yang J, Wang H-X, Xie J, Li L,
Wang J, Wan ECK and Zhong X-P
(2020) DGK α and ζ Activities Control
 T_H1 and T_H17 Cell Differentiation.
Front. Immunol. 10:3048.
doi: 10.3389/fimmu.2019.03048

CD4⁺ T helper (T_H) cells are critical for protective adaptive immunity against pathogens, and they also contribute to the pathogenesis of autoimmune diseases. How T_H differentiation is regulated by the TCR's downstream signaling is still poorly understood. We describe here that diacylglycerol kinases (DGKs), which are enzymes that convert diacylglycerol (DAG) to phosphatidic acid, exert differential effects on T_H cell differentiation in a DGK dosage-dependent manner. A deficiency of either DGK α or ζ selectively impaired T_H1 differentiation without obviously affecting T_H2 and T_H17 differentiation. However, simultaneous ablation of both DGK α and ζ promoted T_H1 and T_H17 differentiation *in vitro* and *in vivo*, leading to exacerbated airway inflammation. Furthermore, we demonstrate that dysregulation of T_H17 differentiation of DGK α and ζ double-deficient CD4⁺ T cells was, at least in part, caused by increased mTOR complex 1/S6K1 signaling.

Keywords: Th differentiation, Th17, Th1, mTOR, DGK, airway inflammation

INTRODUCTION

CD4⁺ T helper (T_H) cells play a central role in orchestrating adaptive immune response to pathogens and also contribute to autoimmune diseases (1, 2). After antigen stimulation, naïve CD4⁺ T cells differentiate into discrete subsets of effector T_H cells with distinct functions and cytokine profiles. Interferon- γ (IFN- γ)-producing T_H1 cells, induced by IL-12 and directed by transcriptional factor T-bet, are critical for the clearance of intracellular pathogens (3, 4). T_H2 cells, which secrete IL-4, IL-5, and IL-13 and are controlled by GATA-3, are crucial for protection against parasites and extracellular pathogens (5, 6). T_H17 cells produce IL-17A, IL-17E, and IL-22, and play an important role in the control of specific pathogens such as fungi. T_H17 differentiation is driven by a combination of TGF- β and IL-6 and requires transcriptional factor ROR γ t and ROR α . IL-23 promotes T_H17 responses by enhancing their survival and stabilization (7–12).

Despite their importance in host immunity against pathogens, T_H cells can be pathogenic and contribute to various diseases. Both exaggerated and defective T_H1 response has been linked to the induction of autoimmune diseases (13–15). T_H2 cells contribute to allergies and asthma (16, 17). T_H17 cells are associated with many autoimmune and inflammatory diseases such as psoriasis, inflammatory bowel diseases, rheumatoid arthritis, type 1 diabetes, and multiple sclerosis (8, 11, 18–20). Thus, understanding how T_H responses are regulated is important to

manipulate immune responses, to improve host defense against microbial infection, and to treat autoimmune diseases.

Engagement of the TCR on naïve CD4⁺ T cells is essential for their activation and further differentiation to T_H cells (21, 22). Evidence has revealed that TCR signal strength and downstream signaling pathways as well as cytokine and costimulatory signals shape T_H lineage differentiation (23–26). A critical event after TCR engagement is the generation of the second messenger diacylglycerol (DAG) by activated PLCγ1. DAG associates with and allosterically activates RasGRP1 and PKCθ, leading to the activation of the Ras-Erk1/2-AP1 and PKCθ-IKK-NFκB signaling pathways, respectively, and is indispensable for T cell activation (27–30). Since it has been demonstrated that both Ras- and PKCθ-mediated signal cascades are involved in T_H differentiation (31–34), it is important to investigate if DAG concentrations should be tightly controlled during T_H differentiation.

DAG kinases (DGKs), a family of enzymes that catalyze phosphorylation of DAG to generate phosphatidic acid (PA), are employed to inhibit DAG-mediated signaling following TCR engagement in both thymocytes and peripheral T cells (28–30). DGKα and ζ, isoforms that express at high levels in T cells, have been demonstrated to inhibit the activation of both Ras-Erk and PKCθ-NFκB cascades as well as mTOR signaling (35–37). They regulate conventional αβT cell, iNKT cell, mucosal associated invariant T cell, and regulatory T cell development, negatively control T cell activation, regulate CD8 T cell mediated anti-viral responses and activation induced T cell death, promote T cell anergy, and inhibit anti-tumor responses (27, 38–55). However, the role of DGKs in T_H differentiation is unknown. We report here that a deficiency of either DGKα or ζ selectively impairs T_H1 cell differentiation, but the loss of both DGK isoforms enhances CD4⁺ naïve T cells differentiating into T_H1 and T_H17 *in vitro* and *in vivo*, establishing DGK activity as a critical regulator of effector CD4⁺ T cell differentiation.

MATERIALS AND METHODS

Mice

DGKα^{-/-}, DGKζ^{-/-}, and ERCCre mice were generated as previously described (38, 39, 56). DGKζ^{fl/fl} mice were generated by introducing two LoxP sites that flank exons 10–14 of the *Dgkz* locus (57). TCR transgenic OT2 mice were purchased from the Jackson Laboratory and were cross-bred with DGKα^{-/-}ζ^{fl/fl} ERCCre mice to generate DGKα^{-/-}ζ^{fl/fl} OT2 ERCCre 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. DGKα^{-/-}ζ^{fl/fl} or DGKα^{-/-}ζ^{fl/fl} OT2 ERCCre mice were intraperitoneally injected with tamoxifen (100 mg/kg body weight) on the first, second, and fifth day to delete DGKζ, and mice were then euthanized for experiments on the eighth day.

Reagents and Antibodies

Iscove's modified Dulbecco's medium (IMDM) was supplemented with 10% (vol/vol) FBS, penicillin/streptomycin,

and 50 μM 2-mercaptoethanol (IMDM-10). Fluorescence-conjugated anti-mouse antibodies CD4 (GK1.5), TCRVα2 (B20.1), CD44 (IM7), CD62L (MEL-14), Thy1.1 (OX-7), Thy1.2 (58-2.1), T-bet (4B10), IFN-γ (XMG1.2), IL-4 (11B11), IL-17A (TC11-18H10.1), and IL-17F (9D3.1C8) were purchased from BioLegend; anti-mouse antibodies for RORγt (AFKJS-9) and Foxp3 (FJK-16s) were purchased from eBioscience. Cell death was determined by Live/Dead Fixable Violet Dead Cell Stain (Invitrogen).

Flow Cytometry

Standard protocols were used to prepare single cell suspensions from the spleen and lymph nodes of mice (in IMDM containing 10% FBS and antibiotics). Red blood cells were lysed using an ACK buffer. Samples were subsequently stained with antibodies in PBS containing 2% FBS and collected on a BD FACSCanto II cytometer. Intracellular staining for T-bet and RORγt was performed using the eBioscience Foxp3 Staining Buffer Set. Intracellular staining for IFNγ, IL-4, IL-17A, and IL-17F was performed using the BD Biosciences Cytofix/Cytoperm and Perm/Wash solutions.

In vitro T_H Differentiation

CD4⁺ T cells were purified from the spleen and LN with anti-CD4 microbeads (Miltenyi Biotec) and then were further sorted as naïve CD4⁺CD62L^{hi}CD44^{lo}CD25⁻. Sorted cells were activated with plate-bound anti-CD3 (5 μg/ml, 1452C11, Bio Xcell) and soluble anti-CD28 (1 μg/ml, PV1, BioXcell) for 4–5 days with various combinations of cytokines and antibodies. For the non-polarizing (T_H0) condition, naïve cells were cultured in the presence of hIL-2 (100 U/ml, Peprotech). For the T_H1 condition, naïve cells were cultured with hIL-2 (100 U/ml), mIL-12 (20 ng/ml, Peprotech), and anti-mIL4 (10 μg/ml, 11B11, Bio Xcell) for 4 days. For the T_H2 condition, naïve cells were polarized in the presence of hIL-2 (100 U/ml), mIL-4 (20 ng/ml, Peprotech), and anti-IFNγ (10 μg/ml, XMG1.2, BioXcell) for 5 days. For the T_H17 condition, naïve cells were cultured with hTGF-β1 (5 ng/ml, Peprotech), mIL-6 (25 ng/ml, Peprotech), anti-mIL4 (10 μg/ml), and anti-IFNγ (10 μg/ml) for 4 days. For iTreg induction, 100 U/ml of hIL-2 and 1 ng/ml TGFβ (Peprotech) were included in the culture for 4 days, followed by intracellular Foxp3 staining. To assess proliferation, sorted naïve CD4⁺ T cells were labeled with CellTraceTM Violet (CTV, ThermoFisher) before cultured in different polarization conditions. For the inhibition assay, 10 μM S6K inhibitor (PF-4708671, Sigma) and 1 nM rapamycin were added to the T_H1 and T_H17 polarizing conditions at the beginning of culture, and cells were cultured for 4 days. At the end of polarizing, cells were stimulated with PMA (50 ng/ml) and ionomycin (500 ng/ml) in the presence of GolgiPlug (1 ng/ml) for 4–5 h. This was followed by cell surface and intracellular staining for appropriated cytokines.

Adoptive Transfer, Immunization, and Airway Inflammation

TCRVα2⁺ cells from splenocytes and LN cells for TCR OTII transgenic mice were enriched using MACS magnetic

beads and Miltenyi Biotec LS columns. About 100 million cells in 500 μ l of IMDM-10 were incubated with the PE-TCRV α 2 antibody (1:100 dilution) and then with anti-PE magnetic beads to isolate TCRV α 2⁺ cells according to the manufacturer's protocol. Enriched samples were stained with anti-CD4, -CD44, and -CD62L antibodies and sorted on a MoFlo Astrios sorter to obtain viable CD4⁺TCRV α 2⁺CD44⁻CD62L⁺ naïve OT2 T cells. Naïve WT or DGK α ^{-/-} ζ ^{fl/fl} OT2 cells (Thy1.1⁻Thy1.2⁺, 1.5×10^6 cell/mouse) were intravenously injected into sex-matched recipients (Thy1.1⁺Thy1.2⁺). Recipient mice were immunized by subcutaneous injection in the inguinal region with 100 μ g/mouse OVA₃₂₃₋₃₃₉ peptide emulsified in the CFA 24 h after adoptive transfer and were euthanized to harvest the spleen and drain inguinal lymph nodes on the seventh day after immunization. Splenocytes and dLN cells were stimulated with PMA and ionomycin in the presence of GolgiPlug for 4–5 h or stimulated with 10 μ g/ml OVA₃₂₃₋₃₃₉ for 2 days in the presence of 1 ng/ml GolgiPlug in the last 5 h. Cell surface and intracellular staining for appropriated cytokines were subsequently performed.

For airway inflammation, OTII T cell recipient mice were intranasally injected with 25 μ l of 2.5 mg/ml OVA₃₂₃₋₃₃₉ peptide in PBS daily for 3 consecutive days starting 24 h after adoptive transfer. Mice were euthanized on the eighth day after adoptive transfer for collection of BALF. Lungs were fixed in 10% formalin and thin-sectioned for hematoxylin and eosin (H&E) staining. Spleen and draining mediastinal LNs were harvested for cytokine analysis.

ELISA

Cultured supernatant or BALF samples were appropriately diluted and IFN γ , IL-4, and IL-17A concentrations were determined using Mouse ELISA max kits (BioLegend) according to the manufacturer's instructions.

Real-Time RT-PCR

Cells were lysed in Trizol for RNA preparation. The first strand cDNA was made using the iScript Select cDNA Synthesis Kit (Biorad). Real-time quantitative PCR was conducted using Eppendorf realplex². Expressed levels of target mRNAs were normalized with β -actin and calculated using the $2^{-\Delta\Delta CT}$ method. Primers used in this study are listed as following: DGK α Forward: GATGCAGGCACCCTGTACAAT, Reverse: GGACCCATAAGCATAGGCATCT; DGK ζ Forward: CGGCTGCCTGGTGTAGACA, Reverse: GCACCTCCAGAGATCCTTGATG; IFN- γ Forward: GCGTCATTGAATCACACCTG, Reverse: TGAGCTCATTGAATGCTTGG; IL-4 Forward: ACAGGAGAAGGGACGCCA, Reverse: GAAGCCCTACAGACGAGCTCA; IL-17A Forward: GCTCCAGAAGGCCCTCAGA, Reverse: CTTTCCCTCCGCATTGACA; Tbx21 Forward: GGTGTCTGGGAAGCTGAGAG, Reverse: GAAGGACAGGATGGGAACA; GATA-3 Forward: AACCACGTCCCGTCCCTACTA, Reverse: AGAGATCCGTGCAGCAGA; RORc Forward: CGACTGGAGGACCTTCTACG, Reverse: TTGGCAAACCTCCACCACATA; ROR α Forward: CCATGCAAGATCTGTGAGA, Reverse: CAGGAGTAGGTGGCATTGCT; β -actin

Forward: TGTCACCTTCCAGCAGATGT, Reverse: AGCTCA GTAACAGTCCGCCTAGA.

Western Blot Analysis

In vitro-cultured T_H cells were lysed in lysis buffer (1% Nonidet P-40, 150 mM NaCl, 50 mM Tris, pH 7.4) with freshly added protease and phosphatase inhibitors. Samples were subjected to immunoblotting analysis, and probed with anti-pS6 (S235/236), -pErk1/2, -total S6, -total Erk1/2, and β -actin antibodies (Cell Signaling Technology).

Statistical Analysis

Data are presented as mean \pm SEM, and statistical significance was determined by two-tailed Student's *t*-test. The *p*-values are defined as follows: **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

RESULTS

Deficiency of Either DGK α or ζ Impaired T_H1 Cell Differentiation

DGK α and ζ are dynamically regulated during T cell development and activation (27, 35, 39, 40). We found that DGK α mRNA was decreased in T_H0, T_H1, T_H2, T_H17, and iTregs compared with naïve CD4⁺ T cells. DGK ζ mRNA also was decreased in T_H0, T_H1, and T_H17 cells but not in T_H2 and iTregs compared with naïve CD4⁺ T cells (Figure 1A). Both DGK α and ζ appeared more significantly down-regulated in T_H1 and T_H17 conditions than in T_H0 condition. To examine the role of DGK α and ζ in T_H differentiation, WT, DGK α ^{-/-}, and DGK ζ ^{-/-} CD44⁻CD62L⁺ naïve CD4⁺ T cells were cultured in T_H1, T_H2, and T_H17 polarization conditions *in vitro* for 4–5 days. DGK α ^{-/-} or DGK ζ ^{-/-} CD4⁺ T cells displayed impaired differentiation to T_H1 cells, which was indicated by decreases of IFN- γ ⁺ cells in both percentages and numbers (Figures 1B,C), IFN- γ concentration in culture supernatants (Figure 1F), and IFN- γ mRNA levels (Figure 1G), accompanying the decreased expression of T-bet (Figure 1H). However, total CD4⁺ T cells numbers were increased in the absence of either DGK α or ζ during T_H1 polarization (Figure 1C), suggesting that impaired T_H1 differentiation of DGK α ^{-/-} or DGK ζ ^{-/-} CD4⁺ T cells did not result from decreased expansion. In contrast, T_H2 and T_H17 differentiation was not obviously affected by DGK α or ζ deficiency. This was reflected by similar percentages of IL-4⁺ or IL-17⁺ cells (Figures 1B,D,E) and similar levels of IL-4 or IL-17A proteins in culture supernatants (Figure 1F) and mRNAs (Figure 1G), which correlated with comparable expression of GATA-3 or ROR γ t (Figure 1H). Both DGK α ^{-/-} CD4⁺ T cells and DGK ζ ^{-/-} CD4⁺ T cells displayed slightly improved survival under the T_H1 condition and had similar survival rates under T_H2 and T_H17 conditions (Figure 1I), suggesting that their reduced T_H1 responses were not due increased cell death. Together, these data suggested individual DGK α and DGK ζ are required for T_H1 differentiation, but are dispensable for T_H2 and T_H17 development *in vitro*.

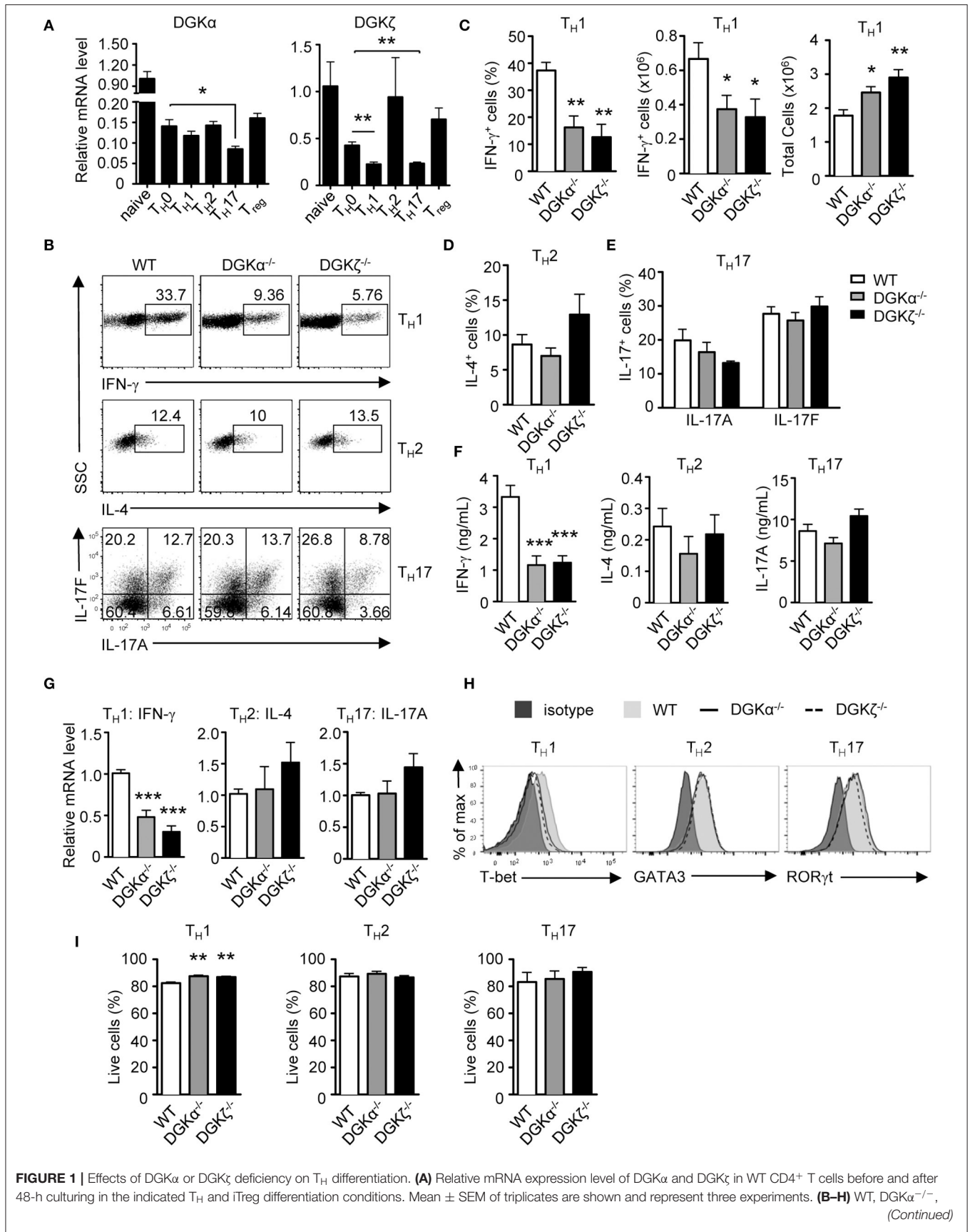
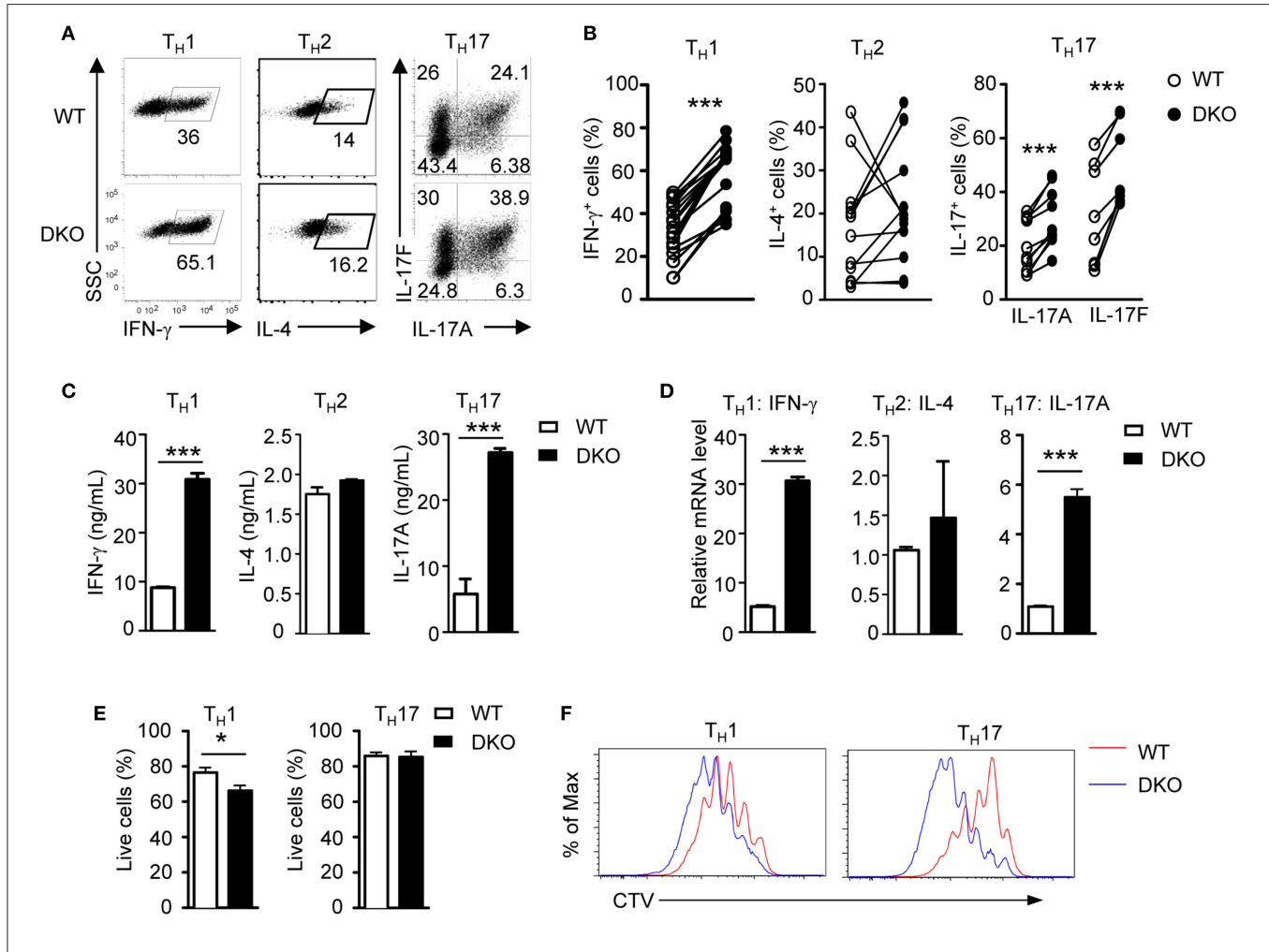


FIGURE 1 | Effects of DGK α or DGK ζ deficiency on T_H differentiation. **(A)** Relative mRNA expression level of DGK α and DGK ζ in WT CD4⁺ T cells before and after 48-h culturing in the indicated T_H and iTreg differentiation conditions. Mean \pm SEM of triplicates are shown and represent three experiments. **(B–H)** WT, DGK $\alpha^{-/-}$, (Continued)

FIGURE 1 | and DGK $\zeta^{-/-}$ naïve CD4⁺ T cells were cultured in T_H1, T_H2, and T_H17 conditions *in vitro* for 4–5 days. **(B)** Representative dot plots of cytokine-producing cells gated on CD4⁺ T cells after PMA and ionomycin stimulation for 4–5 h. **(C)** Bar graphs show mean \pm SEM of percentages and numbers of IFN γ ⁺ cells and total CD4⁺ T cells. **(D)** Bar graphs show mean \pm SEM of percentages IL-17A⁺ and IL-17F⁺ cells. **(E)** Bar graphs show mean \pm SEM of IL-17A and IL-17F concentrations in culture supernatants collected at 96 h. **(F)** Relative mRNA levels of cytokines in indicated T_H conditions after 48 h of incubation. **(G)** Overlaid histograms of intracellular staining of indicated transcription factors under indicated T_H conditions for 60 h. **(H)** Bar graphs show mean \pm SEM of survival rates of CD4⁺ T cells under different T_H conditions. Data shown are representative of or pooled from at least three independent experiments. **P* < 0.05; ***P* < 0.01; ****P* < 0.001 (Student *t*-test).



Deficiency of Both DGK α and ζ Promoted T_H1 and T_H17 Differentiation

DGK α and ζ promote T cell and iNKT cell maturation synergistically in the thymus (52, 54). To determine if DGK α and ζ exert a synergistic role during T_H differentiation, we generated DGK $\alpha^{-/-}\zeta^{fl/fl}$ -ERCre (DKO) mice so that both DGK α and ζ were ablated after tamoxifen-induced deletion of DGK ζ . In contrast to DGK α or ζ single-knockout T cells, DKO CD4⁺ naïve T cells showed enhanced capacity to differentiate into both T_H1 and

T_H17 cells but similar T_H2 differentiation compared with their WT counterparts (**Figures 2A,B**), coinciding with increased IFN- γ and IL-17A but not IL-4 concentration in culture supernatants (**Figure 2C**) and IFN- γ and IL-17A mRNA levels in these cells (**Figure 2D**). DKO CD4⁺ T cells displayed slightly decreased survival rate under T_H1 but similar survival rate under T_H17 polarization conditions, suggesting that their enhanced T_H1 and T_H17 responses were not due to improved survival (**Figure 2E**). However, under both T_H1 and T_H17 conditions, DKO CD4⁺

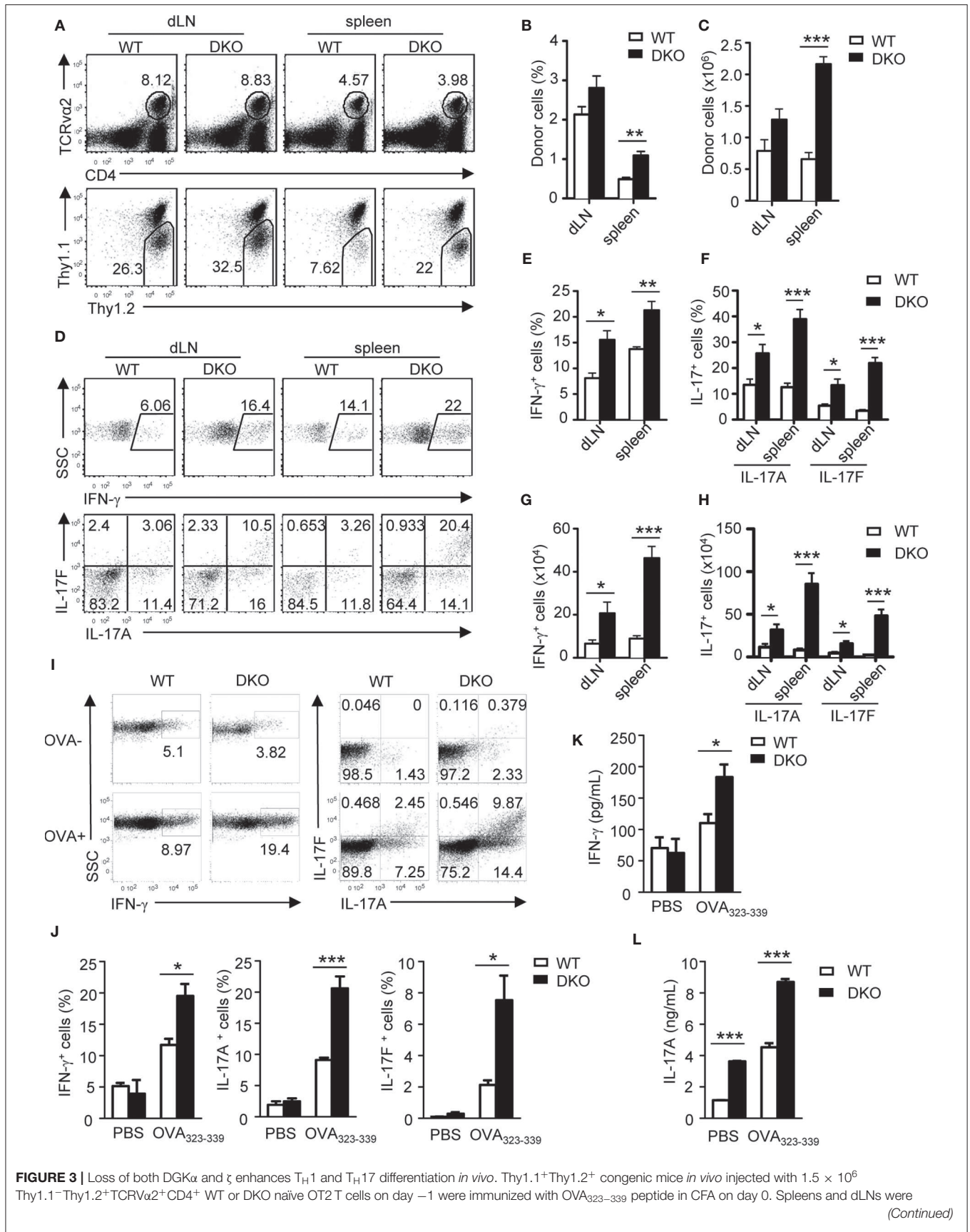


FIGURE 3 | Loss of both DGK α and ζ enhances T_H1 and T_H17 differentiation *in vivo*. Thy1.1⁺Thy1.2⁺ congenic mice *in vivo* injected with 1.5×10^6 Thy1.1⁻Thy1.2⁺TCR α 2⁺CD4⁺ WT or DKO naive OT2 T cells on day -1 were immunized with OVA₃₂₃₋₃₃₉ peptide in CFA on day 0. Splens and dLNs were (Continued)

FIGURE 3 | harvested on the seventh day after immunization. **(A)** Representative dot plots of dLN cells and splenocytes. Top panels: CD4 and TCRV α 2 staining. Bottom panels: Thy1.1 vs. Thy1.2 staining of the gated TCRV α 2⁺CD4⁺ population. **(B,C)** Mean \pm SEM of percentages **(B)** and number **(C)** of donor-derived OT2 T cells in dLNs and spleens ($n = 4$). **(D–H)** dLN cells and splenocytes were stimulated with PMA and ionomycin for 4–5 h in the presence of GolgiPlug, followed by cell surface and intracellular staining. **(D)** Representative dot plots of indicated cytokines in gated donor-derived OT2 cells. **(E–H)** Mean \pm SEM of percentages of IFN- γ -producing cells **(E)** and IL-17-producing cells **(F)** as well as total numbers of donor-derived IFN- γ -producing **(G)** and IL-17-producing **(H)** OT2 T cells. **(I–K)** Splenocytes and dLN cells were stimulated with (OVA+) or without (OVA-) OVA_{323–339} for 2 days, with the addition of GolgiPlug in the last 5 h, and then were cell surface and intracellular stained for OT2 T cells and cytokine expression. **(I)** Representative dot plots of indicated cytokine-producing cells in gated donor-derived OT2 cells. **(J)** Percentages of donor-derived cytokine-producing OT2 T cells ($n = 4$). **(K,L)** IFN- γ **(K)** and IL-17A **(L)** concentrations in culture supernatant harvested before adding GolgiPlug ($n = 3$). Data shown are representative of two independent experiments. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ as determined by the Student t -test.

T cells proliferated more vigorously than WT controls, which might contribute to their enhanced T_{H1} and T_{H17} responses (**Figure 2F**). In contrast to T_{H1} and T_{H17} differentiation, iTreg cell induction was not obviously different between WT and DKO naïve CD4⁺ T cells (WT iTreg percentages: 62.49 ± 9.186 $n = 7$; DKO iTreg percentages: 53.84 ± 8.465 $n = 7$; $P = 0.5022$). Together, these results indicated that deficiency of both DGK α and ζ promoted T_{H1} and T_{H17} differentiation with minimal effects on T_{H2} or iTreg cell differentiation *in vitro*.

Loss of Both DGK α and ζ Prompted T_{H1} and T_{H17} Differentiation *in vivo*

To further determine the impact of DGK α and ζ double deficiency on T_H differentiation *in vivo*, we generated DKO mice carrying the OT2 TCR transgene, which recognizes chicken ovalbumin peptide 323–339 (OVA_{323–339}) in the context of I-A^b (58) and adoptively transferred WT- or DKO-naïve OT2 T cells (Thy1.1⁻Thy1.2⁺CD4⁺TCRV α 2⁺) into congenic Thy1.1⁺Thy1.2⁺ recipients. Recipient mice were immunized with OVA_{323–339} peptide emulsified in complete Freund's adjuvant (CFA) 1 day after the transfer. Seven days after immunization, donor-derived DKO OT2 T cells were increased in both percentages and numbers in the spleen and draining lymph nodes (dLNs) compared with WT controls (**Figures 3A–C**). In addition, higher percentages of DKO OT2 T cells expressed IFN- γ , IL-17A, and IL-17F than WT controls following *in vitro* PMA and ionomycin stimulation for 4 h (**Figures 3D–F**). Because of increased DKO OT2 T cell numbers, DKO OT2 T_{H1} and T_{H17} cell numbers were much greater than WT controls in dLNs and particularly in the spleen (**Figures 3G,H**). Moreover, DKO OT2 T cells contained more IFN- γ -, IL-17A-, and IL-17F-positive cells, which was detected by intracellular staining (**Figures 3I,J**), and secreted more cytokines to culture supernatants, which was detected by ELISA (**Figures 3K,L**), than their WT controls following stimulation with OVA_{323–339} peptide for 2 days. Together, these results demonstrated that the deficiency of both DGK α - and ζ -enhanced T_{H1} and T_{H17} polarization and expansion *in vivo* via cell intrinsic mechanisms.

Accumulation of T_{H1} and T_{H17} Cells in the Absence of DGK α and ζ Caused Severe Airway Inflammation

T_{H17} cells promote airway inflammation and hyper-responsiveness via recruiting neutrophils and induce airway

smooth muscle contraction, which contributes to the severe form of asthma (59, 60). To determine if dysregulated T_H responses of DKO CD4⁺ T cells impact airway inflammation, we adoptively transferred naïve WT and DKO OT2 cells (Thy1.2⁺) into WT Thy1.1⁺Thy1.2⁺ congenic mice on day -1 and then intranasally injected OVA_{323–339} peptide into the recipient mice on days 0, 1, and 2. On the seventh day, we detected at least four-fold more DKO OT2 cells in both percentages and numbers in the draining mediastinal lymph nodes and spleen in recipient mice than their WT counterparts (**Figures 4A–C**). DKO donor-derived OT2 cells in both dLNs and spleens produced more IL-17A and IL-17F as well as IFN- γ in response to *in vitro* stimulation with PMA and ionomycin for 4 h (**Figures 4D–H**) or with OVA_{323–339} peptide for 2 days (**Figures 4I–M**). Concordantly, both IFN- γ and IL-17A levels in bronchoalveolar lavage fluid (BALF) were elevated in recipients with DKO OT2 T cells compared with those with WT OT2 T cells (**Figure 5A**). Moreover, DKO OT2 cell recipients contained more neutrophils and lymphocytes than those with WT control in BALF (**Figures 5B,C**) and in interstitial lung tissues that surround the bronchioles (**Figure 5D**). Together, these results demonstrated that DGK α and ζ deficiencies in CD4⁺ T cells exacerbated airway inflammation, likely as a result of enhanced T_{H17} responses to protein allergens.

Effects of DGK $\alpha\zeta$ Double Deficiency on Expression of Critical Lineage Transcription Factors

T-bet, GATA-3, ROR γ t, and ROR α are transcription factors that play critical roles in T_{H1}, T_{H2}, and T_{H17} differentiation, respectively. Under the T_{H1} polarization condition, DKO CD4⁺ T cells expressed higher levels of T-bet at both mRNA and protein levels than WT controls (**Figures 6A,B**), which was consistent with their elevated T_{H1} responses. In contrast, GATA-3 expression in DKO CD4⁺ T cells was not obviously different from WT controls under the T_{H2} polarization condition (**Figure 6C**), consistent with a minimal effect of DKO on T_{H2} responses as shown in **Figure 2**. Interestingly, *Rorc* (gene encoding ROR γ t) mRNA levels were obviously decreased in DKO CD4⁺ T cells under the T_{H17} polarization condition (**Figure 6D**), although ROR γ t protein was only slightly decreased (**Figure 6E**). In contrast, *RORA* mRNA levels were increased in DKO CD4⁺ T cells 24 and 36 h after polarization (**Figure 6F**). Both ROR α and ROR γ t are important for T_{H17}

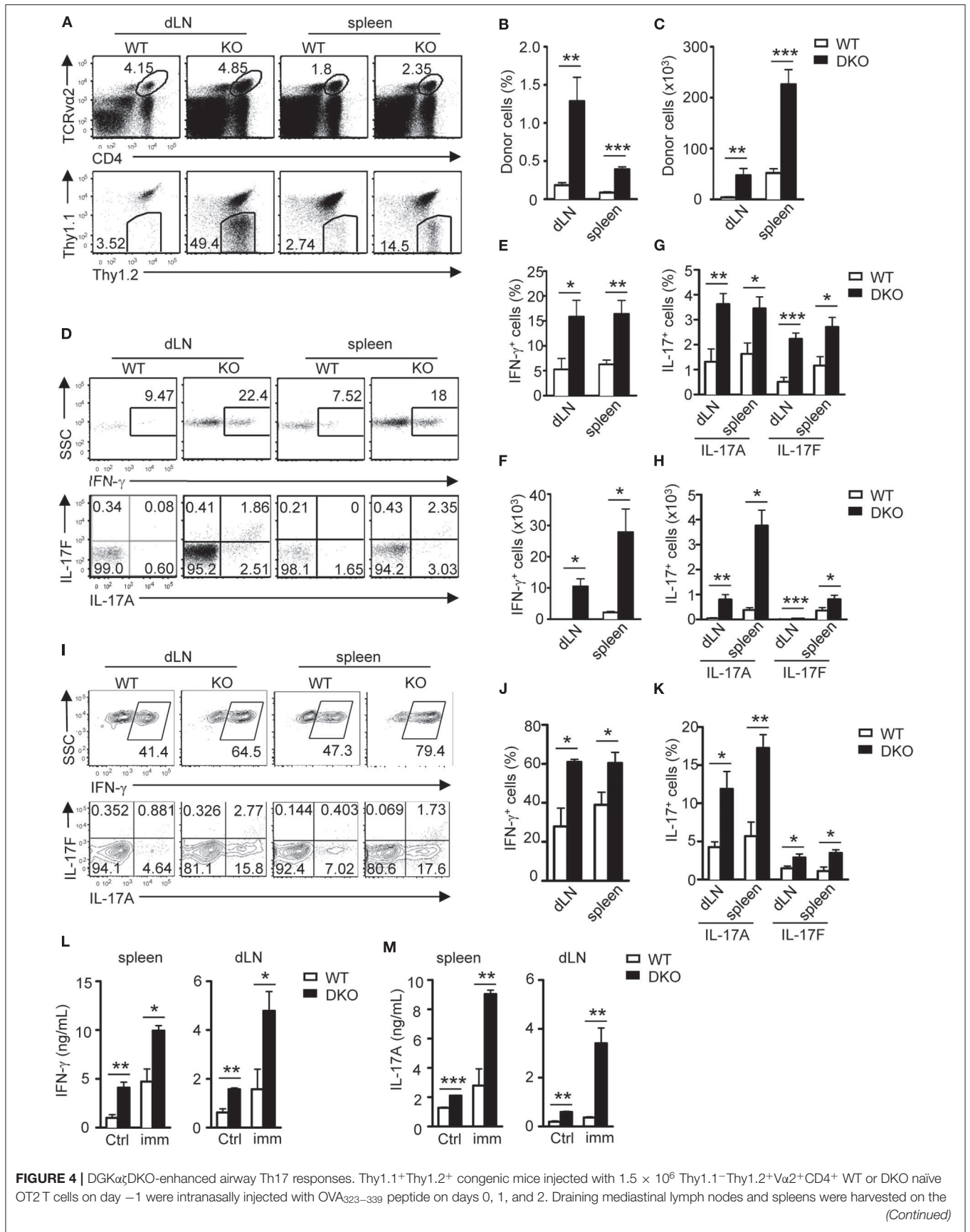
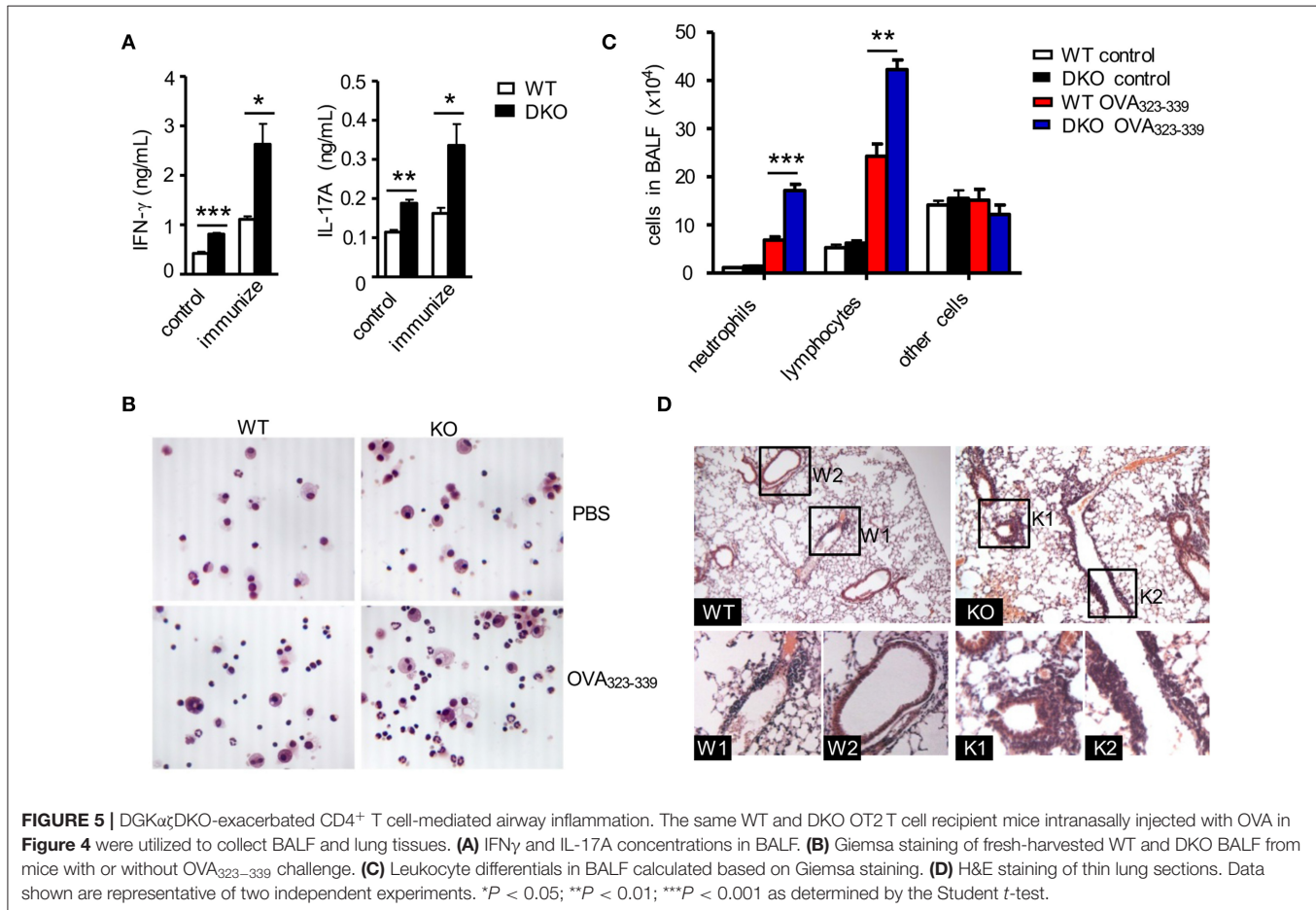


FIGURE 4 | DGK α DKO-enhanced airway Th17 responses. Thy1.1⁺Thy1.2⁺ congenic mice injected with 1.5×10^6 Thy1.1⁻Thy1.2⁺V α 2⁺CD4⁺ WT or DKO naive OT2 T cells on day -1 were intranasally injected with OVA₃₂₃₋₃₃₉ peptide on days 0, 1, and 2. Draining mediastinal lymph nodes and spleens were harvested on the (Continued)

FIGURE 4 | seventh day. **(A)** Representative dot plots of dLN cells and splenocytes. Top panels: CD4 vs. TCR α 2 staining. Bottom panels: Thy1.1 vs. Thy1.2 staining of the gated TCR α 2⁺CD4⁺ population. **(B,C)** Percentages **(B)** and number **(C)** of donor-derived OT2 T cells in dLNs and spleens. **(D–H)** Splenocytes and dLN cells from recipients were stimulated with PMA and ionomycin for 4–5 h, followed by cell surface and intracellular staining. **(D)** Representative dot plots of indicated cytokines in donor-derived OT2 T cells. **(E,F)** Percentages **(E)** and number **(F)** of donor-derived IFN- γ -producing OT2 T cells. **(G,H)** Percentages **(G)** and number **(H)** of donor-derived IL-17A- and IL-17F-producing OT2 T cells. **(I–M)** Splenocytes and dLN cells were stimulated with OVA_{323–339} for 2 days with GolgiPlug added in the last 5 h, followed by cell surface and intracellular staining. **(I)** Representative dot plots of indicated cytokine staining in gated donor-derived OT2 T cells. **(J,K)** Percentages of IFN- γ - **(J)** and IL-17-producing cells **(K)** in donor OT2 T cells. **(L,M)** IFN- γ **(L)** and IL-17A **(M)** concentrations in culture supernatants. Data shown are representative of or calculated from two independent experiments ($n = 8$). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ as determined by the Student t -test.



differentiation and ROR γ t is considered the master regulator of the Th17 lineage (61–63). It is intriguing that DGK α and ζ double deficiency enhanced Th17 differentiation yet downregulated ROR γ t expression. Increased ROR α expression in DKO CD4⁺ T cells might partially compensate for the decrease of ROR γ t. Additionally, DGK α ζ deficiency might alleviate the requirement of ROR γ t and promote T_H17 differentiation via other mechanisms.

Effects of DGK α and ζ Double Deficiency on mTORC1/S6K1 Signaling During T_H1 and T_H17 Cell Differentiation

DGK α and ζ negatively control DAG-mediated Ras-Erk1/2 activation in thymocytes and naïve T cells following TCR

engagement (36, 38, 54). We further examined how DGK α and ζ double deficiency might affect this pathway during T_H polarization. As shown in **Figure 7A**, Erk1/2 phosphorylation was obviously enhanced in DKO CD4⁺ T cells under T_H0, T_H1, T_H2, and T_H17 conditions, suggesting that DGK α and ζ negatively controlled Erk1/2 activation during effector CD4⁺ T cell differentiation. Previous studies have found that DAG-mediated RasGRP1-Ras-Erk, PI3K-Akt, and PKC θ -CARMA1 pathways participate in TCR-induced mTORC1 activation and DGK α and ζ double deficiency but not DGK α or ζ single deficiency leads to enhanced mTOR signaling in developing thymocytes (36, 64, 65) and that mTOR plays important roles in Th differentiation (65–69). Although, S6 phosphorylation, an mTORC1/S6K1-dependent event, in T_H1 cells appeared unaffected by DGK α and ζ double deficiency, it was obviously

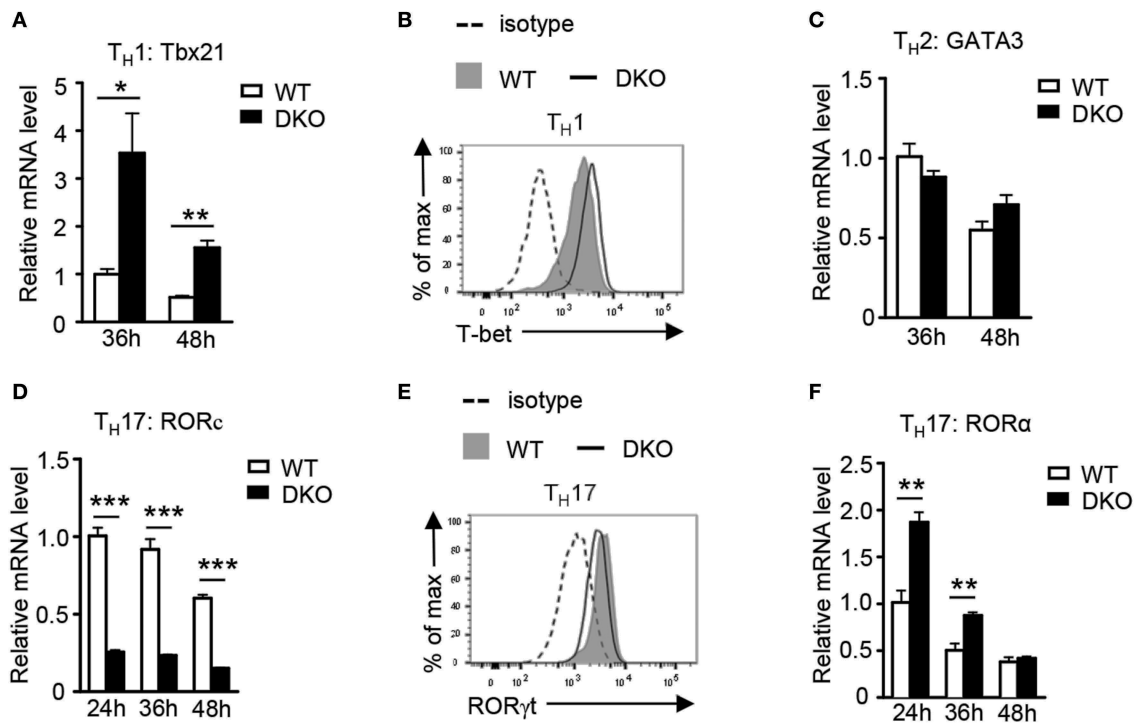


FIGURE 6 | Effects of DGK α and ζ double deficiency on T_H lineage-specific transcription factors. **(A,B)** *Tbx21* mRNA **(A)** and T-bet protein **(B)** levels in CD4⁺ T cells during T_H1 polarization. **(C)** Relative mRNA level of GATA-3 in CD4⁺ T cells during T_H2 polarization. **(D,E)** *RORc* mRNA **(D)** and ROR γ t protein **(E)** levels in CD4⁺ T cells during T_H17 polarization. **(F)** Relative *ROR α* mRNA levels in CD4⁺ T cells during T_H17 polarization. Data shown are representative of five independent experiments. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ determined by the Student *t*-test.

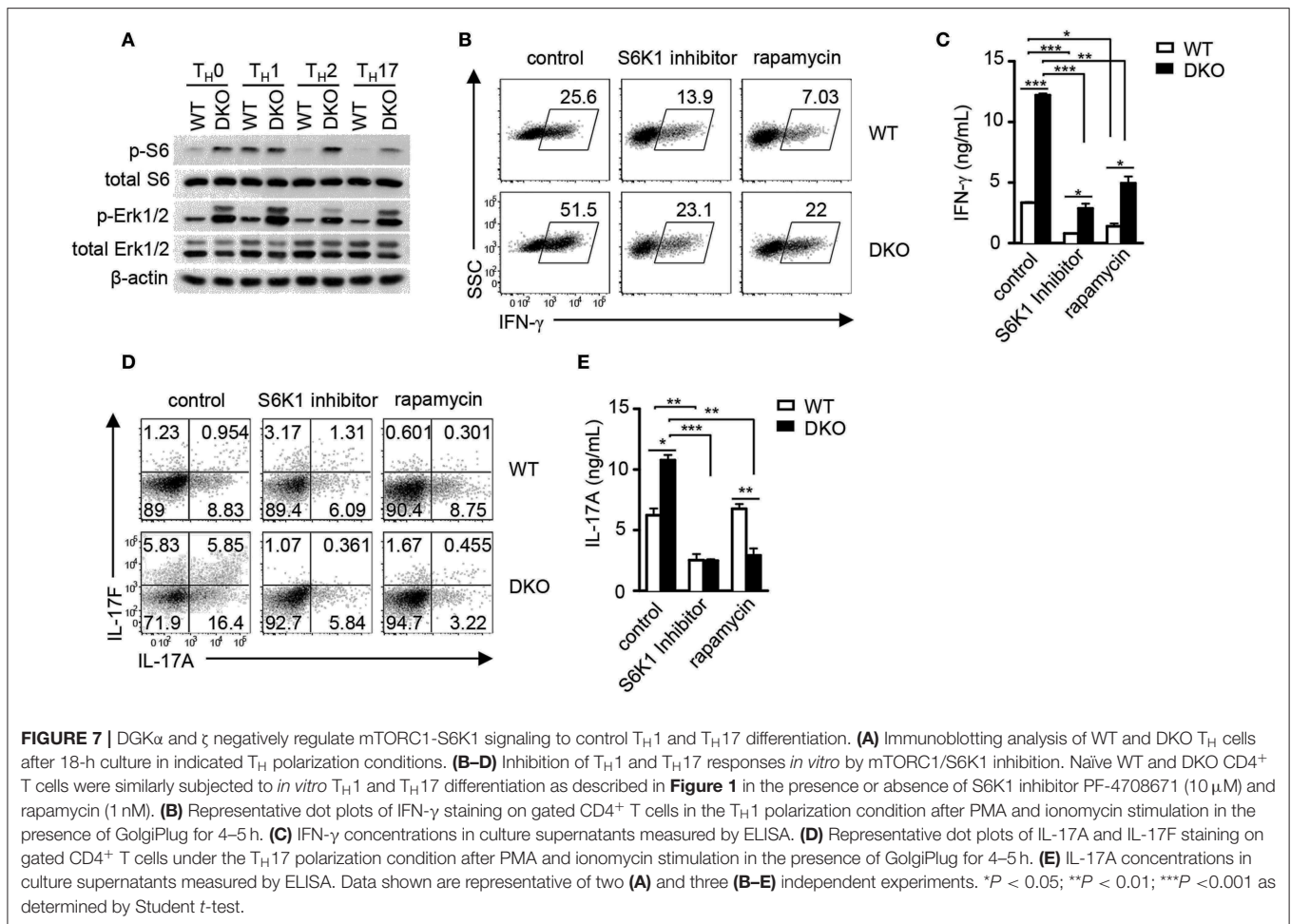
increased in DKO CD4⁺ T cells under T_H0, T_H2, and T_H17 polarization conditions, suggesting that DGK α and ζ negatively controlled mTORC1 signaling in T_H0, T_H2, and T_H17 cells. Treatment of WT and DKO CD4⁺ T cells with either rapamycin or the S6K1 inhibitor PF-4708671 caused about 50% reduction of IFN γ ⁺ cells in both cell types but DKO CD4⁺ T cells still contained higher percentages of IFN γ ⁺ cells than WT controls. Thus, DKO CD4⁺ T cells were partially sensitive to mTORC1/S6K1 inhibition (**Figures 7B,C**), suggesting that additional mechanisms might contribute to enhanced T_H1 differentiation in these cells. In contrast, T_H17 differentiation of both DKO and WT CD4⁺ T cells was potently inhibited by either rapamycin or PF-4708671 (**Figures 7D,E**). Although, we could not rule out potential off-target effects of PF-4708671 and rapamycin, our data suggested that enhanced mTORC1/S6K1 signaling might contribute to the elevated T_H17 responses of DKO CD4⁺ T cells.

DISCUSSION

Previous studies have demonstrated that DGK α and ζ play crucial roles in T cell development, activation, anergy, and survival, and CD8 T cell-mediated anti-viral immune responses, iNKT cell development, regulatory T cell differentiation, and

anti-tumor immune responses (27, 38–54). Additionally, DGK ζ has been found to regulate B cell development (70), mast cell activation (71), TLR-mediated innate immunity (72), and NK cells (73). In this study, we have demonstrated that graded DGK activities differentially control CD4⁺ T_H differentiation. Although, the absence of either DGK α or ζ selectively impairs T_H1 differentiation, simultaneous ablation of both DGK α and ζ enhances both T_H1 and T_H17 responses *in vitro* and *in vivo*.

Recent studies have demonstrated that mTOR signaling plays a critical role in T cell activation and T_H differentiation. mTORC1 promotes T_H1, T_H2, and T_H17 differentiation while mTORC2 activity is indispensable for T_H2 cells development (65–67). Among different effector CD4⁺ T cells, T_H1 cells appear to possess the highest S6 phosphorylation and, thus, mTORC1 activity. Although, S6 phosphorylation is not increased in DKO T_H1 cells, elevated DKO T_H1 response is substantially decreased when mTORC1-S6K1 signaling is inhibited, suggesting that enhanced DKO T_H1 response is at least in part via enhanced mTORC1-S6K1 signaling. Different from T_H1 cells, DKO T_H0, T_H2, and T_H17 cells contain elevated S6 phosphorylation, and inhibition of either mTORC1 or S6K1 reverts their elevated T_H17 responses. Our study suggested a linkage between DGKs and mTORC1/S6K1 in the regulation of T_H17 cell differentiation. In thymocytes, T cell line models, and primary T cells, both



RasGRP1-Ras-Erk1/2 and PKC θ -CARMA1 pathways signal to promote mTORC1 activation (36, 64). Although, it remains to be defined, DGK α and ζ may inhibit mTORC1/S6K1 signaling via modulating these DAG-mediated signaling pathways during effector CD4⁺ T cell differentiation. In addition to S6K1, many other molecules and pathways that play important roles in T_H differentiation are regulated by mTOR (65, 68, 69, 74–77). Future studies should investigate whether DGK α and ζ may regulate T_H differentiation through other mechanisms.

Dysregulated T_H1 and T_H17 responses contribute to the pathogenesis of numerous autoimmune diseases, including psoriasis, inflammatory bowel disease, rheumatoid arthritis, type 1 diabetes, multiple sclerosis, experimental autoimmune encephalomyelitis, and neutrophil-related airway inflammation (8, 11, 13–15, 18–20). We have shown that dysregulated T_H1 and T_H17 responses in the absence of DGK α and ζ are pathogenic, indicated by exacerbated neutrophil-related airway inflammation. Interestingly, DGK α and ζ double deficiency leads to a loss of T cell tolerance and the development of autoimmune diseases in mice (manuscript in preparation). Enhanced CD4⁺ T cell effector function might be an important contributor to

the development of autoimmune diseases in these mice. Thus, modulating DGK α and ζ activity could be a potential strategy to shape immune responses. Of note, although DGK α and ζ double deficiency does not obviously affect iTreg induction *in vitro*, our data do not rule out a potential role of DGK activity in peripheral Treg induction from naïve CD4⁺ T cells *in vivo*. Additional studies are needed to determine whether DGK α and ζ play a redundant role in Treg cells.

In summary, DGK activity plays selective roles in T_H cell differentiation. A single knockout of DGK α or ζ impaired T_H1 cell differentiation whereas a deficiency of both DGK α and ζ promoted T_H1 and T_H17 cell differentiation *in vitro* and *in vivo*. Such dysregulated expansion of both T_H cells in the absence of DGK α and ζ caused severe airway inflammation. DGK α and ζ double deficiency led to enhanced mTORC1-S6K1 activation during T_H17 cell differentiation, which may contribute to enhanced T_H17 cell differentiation. Our study demonstrated the role of DGKs in T_H cell differentiation and provides useful evidence for these enzymes as potential targets for therapeutic approaches of autoimmune diseases associated with the dysregulation of T_H1 and T_H17 cells.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

ETHICS STATEMENT

The experiments in this study were performed according to a protocol approved by the Institutional Animal Care and Usage Committee of Duke University.

AUTHOR CONTRIBUTIONS

JY designed and performed experiments, analyzed data, and wrote the paper. H-XW, JX, LL, and JW performed experiments

and analyzed data. EW generated critical reagents. X-PZ conceived the project, designed experiments, participated in data analysis, and wrote the paper.

FUNDING

This study was supported by the National Institutes of Health (R01AI079088, R01AI101206, and R56AG060984).

ACKNOWLEDGMENTS

We thank the Transgenic and Knockout Mice Facility and the Flow Cytometry Core Facility at Duke Cancer Institute for generating DGK $\zeta^{f/+}$ mice and providing sorting service, respectively.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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