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Journal

Frontiers in Immunology, 9(FEB)

ISSN

1664-3224

Authors

Kim, Girak
Gu, Min Jeong
Kim, Soo Ji
[et al.](#)

Publication Date

2018

DOI

10.3389/fimmu.2018.00196

Peer reviewed



Transcription Factor KLF10 Constrains IL-17-Committed $V\gamma 4^+$ $\gamma\delta$ T Cells

Girak Kim¹, Min Jeong Gu¹, Soo Ji Kim¹, Kwang Hyun Ko¹, Yoon-Chul Kye¹, Cheol Gyun Kim¹, Jae-Ho Cho², Woon-Kyu Lee³, Ki-Duk Song⁴, Hyuk Chu⁵, Yeong-Min Park⁶, Seung Hyun Han⁷ and Cheol-Heui Yun^{1,8,9*}

¹Department of Agricultural Biotechnology and Research Institute of Agriculture and Life Sciences, Seoul National University, Seoul, South Korea, ²Academy of Immunology and Microbiology, Institute for Basic Science, Pohang, South Korea, ³College of Medicine, Inha University, Incheon, South Korea, ⁴Department of Animal Biotechnology, Chonbuk National University, Jeonju, South Korea, ⁵Division of Bacterial Disease Research, Center for Infectious Disease Research, National Institute of Health, Korea Centers for Disease Control and Prevention, Osong, South Korea, ⁶Department of Immunology, Laboratory of Dendritic Cell Differentiation and Regulation, School of Medicine, Konkuk University, Chungju, South Korea, ⁷Department of Oral Microbiology and Immunology, DRI and BK21 Plus Program, School of Dentistry, Seoul National University, Seoul, South Korea, ⁸Center for Food Bioconvergence, Seoul National University, Seoul, South Korea, ⁹Institute of Green Bio Science Technology, Seoul National University, Pyeongchang, South Korea

OPEN ACCESS

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Center, United States

*Correspondence:

Cheol-Heui Yun
cyun@snu.ac.kr

Specialty section:

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

Received: 04 August 2017

Accepted: 23 January 2018

Published: 28 February 2018

Citation:

Kim G, Gu MJ, Kim SJ, Ko KH,
Kye Y-C, Kim CG, Cho J-H,
Lee W-K, Song K-D, Chu H,
Park Y-M, Han SH and Yun C-H
(2018) Transcription Factor KLF10
Constrains IL-17-Committed
 $V\gamma 4^+$ $\gamma\delta$ T Cells.
Front. Immunol. 9:196.
doi: 10.3389/fimmu.2018.00196

$\gamma\delta$ T cells, known to be an important source of innate IL-17 in mice, provide critical contributions to host immune responses. Development and function of $\gamma\delta$ T cells are directed by networks of diverse transcription factors (TFs). Here, we examine the role of the zinc finger TF, Kruppel-like factor 10 (KLF10), in the regulation of IL-17-committed CD27⁻ $\gamma\delta$ T ($\gamma\delta^{27-}$ -17) cells. We found selective augmentation of $V\gamma 4^+$ $\gamma\delta^{27-}$ cells with higher IL-17 production in KLF10-deficient mice. Surprisingly, KLF10-deficient CD127^{hi} $V\gamma 4^+$ $\gamma\delta^{27-}$ -17 cells expressed higher levels of CD5 than their wild-type counterparts, with hyper-responsiveness to cytokine, but not T-cell receptor, stimuli. Thymic maturation of $V\gamma 4^+$ $\gamma\delta^{27-}$ cells was enhanced in newborn mice deficient in KLF10. Finally, a mixed bone marrow chimera study indicates that intrinsic KLF10 signaling is requisite to limit $V\gamma 4^+$ $\gamma\delta^{27-}$ -17 cells. Collectively, these findings demonstrate that KLF10 regulates thymic development of $V\gamma 4^+$ $\gamma\delta^{27-}$ cells and their peripheral homeostasis at steady state.

Keywords: KLF10, $\gamma\delta$ T cells, IL-17, homeostasis, Innate-like $\gamma\delta$ -17

INTRODUCTION

Early studies on Kruppel-like factor 10 (KLF10), a transcription factor (TF) containing zinc finger DNA-binding domains, revealed its role in the induction of and balance between Foxp3⁺ regulatory T (Treg) cells and IL-17-producing T helper (Th17) cells (1–3). Stimulation of CD4⁺ T cells with T-cell receptor (TCR) or TGF- β transiently induces KLF10, which in turn suppresses TCR signaling or enhances TGF- β /Smad signaling, respectively. Therefore, KLF10-deficient CD4⁺ T cells that are hyper-activated by TCR stimuli are less differentiated into Treg cells than wild-type (WT) controls (1, 2), while Th17 cell differentiation is promoted (3). Nonetheless, the function of KLF10 *in vivo* is still unclear since the alteration of Treg cells in naïve KLF10-deficient mice is controversial (1–3) and the enrichment of Th17 cells in these mice has not been clearly reported. Most of all, the functions of KLF10 in other T lymphocytes producing IL-17, such as $\gamma\delta$ T cells, are largely unknown.

At steady state, $\gamma\delta$ T cells are only a minor subset of T lymphocytes but the major source of IL-17 (4–6). Innate-like IL-17-committed CD27⁻ $\gamma\delta$ T ($\gamma\delta^{27-}$ -17) cells are present in peripheral lymph nodes (pLN) as well as regional tissues, including dermis, lung, and peritoneal cavity (5, 7, 8). Most

peripheral $\gamma\delta^{27-}$ -17 cells stimulated by cytokines, for example, by IL-7 or by IL-1 β plus IL-23, can be enriched in the absence of TCR activation (8, 9), and thus respond rapidly to infection or tissue dysregulation. Although TCR signaling is involved in $\gamma\delta$ -lineage commitment and functional decision in the thymus (10–12), peripheral homeostasis and activity of $\gamma\delta^{27-}$ -17 cells is weakly dependent on TCR ligation, which triggers strong activation of $\gamma\delta^{27+}$ cells (8, 13–16). $\gamma\delta^{27-}$ -17 cells mainly consist of V γ 4 $^{+}$ and V γ 6 $^{+}$ subsets (Tonegawa nomenclature) (17) and phenotypically resemble effector memory cells (CD44 hi CD62L lo CD127 hi) (5, 9), mostly expressing a unique marker, CCR6 $^{+}$ NK1.1 $^{-}$ (18). It has been suggested that $\gamma\delta^{27-}$ -17 cells develop predominantly from early embryonic stage up to shortly after birth (19–21). However, whereas maturation of V γ 4 $^{+}$ $\gamma\delta^{27-}$ -17 cells occurs in the neonatal thymus (22), V γ 4 $^{+}$ $\gamma\delta^{27-}$ -17 cells can be still reconstituted by bone marrow (BM) cells (22, 23).

Thymic development of $\gamma\delta$ T cells is regulated by discrete TCR strengths and TCR-independent signaling modalities, which involve exogenous stimuli (TGF- β and IL-7) and/or intrinsic preprogramming of a gene regulatory network of diverse TFs (24–26). It is plausible that a weak TCR strength is required for the development of innate-like $\gamma\delta^{27-}$ -17 cells and, thus, IL-17-producing capacity is considered to emerge by default from uncommitted early thymocytes (10, 11, 27). However, other reports argue that innate-like $\gamma\delta$ -17 cells are dependent on strong TCR signals for their thymic development (13), leaving the role of TCR signaling in the generation of innate-like $\gamma\delta^{27-}$ -17 cells unclear. Moreover, TGF- β R or IL-7R signaling, as well as the TF Sox13, promote $\gamma\delta^{27-}$ -17 cell development through a TCR-independent signaling pathway (5, 9, 22); in particular, Sox13 selectively regulates V γ 4 $^{+}$ $\gamma\delta^{27-}$ -17 cell development (22).

Here, we identify KLF10 as a novel TF that negatively regulates the development and homeostasis of V γ 4 $^{+}$ $\gamma\delta^{27-}$ -17 cells. We found selective enlargement of IL-17-committed V γ 4 $^{+}$ $\gamma\delta^{27-}$ cells, but not of other IL-17-producing $\alpha\beta$ T cells, in KLF10-deficient mice. TCR or cytokine (IL-7 or IL-1 β plus IL-23) stimulation on $\gamma\delta$ T cells could induce KLF10, which in turn differently regulates $\gamma\delta$ T-cell responsiveness to these stimuli. Moreover, KLF10 deficiency affected the expression level of CD5, a stable indicator of TCR strength, on mature V γ 4 $^{+}$ $\gamma\delta^{27-}$ -17 cells within the neonatal thymus. These results suggest that the biology of V γ 4 $^{+}$ $\gamma\delta^{27-}$ -17 cells is dependent on transcriptional control by KLF10, which is differentially associated with TCR and cytokine signaling.

MATERIALS AND METHODS

Mice

KLF10-deficient mice with C57Bl/6 (B6) background were kindly provided by Dr. Woon Kyu Lee (Inha University, Incheon, South Korea) (28). B6.Rag1-deficient mice and B6.CD45.1 congenic mice were obtained from The Jackson Laboratory. All animals were bred and maintained under specific pathogen-free conditions at the Institute of Laboratory Animal Resource Seoul National University and treated in accordance with institutional guidelines that were approved by the Institutional Animal Care and Use Committee (SNU-140930-4-1).

Cell Preparation

Mouse peripheral lymph nodes (cervical, axillary, brachial, and inguinal), mesenteric lymph node, spleen, thymus, and lung were homogenized by mechanical disaggregation, strained through a 70- μ m strainer (BD Biosciences), and washed in RPMI 1640 medium containing 10% (vol/vol) fetal bovine serum (FBS). Peritoneal cells were obtained from peritoneal lavage in cold phosphate-buffered saline (PBS) containing 5% FBS.

Flow Cytometry

Single-cell suspensions were first blocked with anti-CD16/32 antibody (93; eBioscience) and then stained with antibodies at 4°C for 20 min in staining buffer (1 \times PBS containing 0.1% bovine serum albumin and 0.1% sodium azide). For intracellular cytokine staining, the cells were stimulated for 4 h with 50 ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) and 750 ng/ml ionomycin (Sigma-Aldrich) in the presence of brefeldin A (BD Biosciences). The cells were then fixed, permeabilized with a BD Cytofix/Cytoperm Kit according to the manufacturer's instructions (BD Biosciences), and stained for IL-17 and IFN- γ . Intracellular phosphorylated protein staining was performed as described (29) with modifications. For assays of intracellular Ca $^{2+}$ mobilization, cells from WT and knockout (KO) mice were first surface-stained with anti-CD45.2-PerCP-Cy5.5 and anti-CD45.2-Alexa 700, respectively, for subsequent identification of the two strains. Cells from both strains were mixed together at a ratio of 1:1 and loaded for 45 min at 37°C with the membrane-permeable fluorescent Ca $^{2+}$ indicator dye, Indo-1 AM (Invitrogen), at a concentration of 4 μ M in RPMI medium plus 5% (vol/vol) FBS. Cells were then stained for surface markers and were kept on ice. Before stimulation, cell aliquots were allowed to equilibrate to 37°C for 5 min and analyzed by flow cytometry. After acquisition of background intracellular Ca $^{2+}$ concentrations for 30 s, cells were stimulated with biotinylated anti-CD3 ϵ antibody and crosslinked by the addition of streptavidin. Samples were acquired on Canto II or Aria II (BD Biosciences) and data were analyzed with FlowJo software (TreeStar). Antibodies used for flow cytometric analyses were fluorochrome-labeled mAbs against mouse $\gamma\delta$ TCR (GL3), CD3 ϵ (145-2C11), CD27 (LG.3A10 or LG.7F9), CD25 (PC61), CD69 (H1.2F3), CD5 (53-7.3), CD24 (M1/69), CD103 (2E7), CD122 (TM-b1), CD132 (TUGm2), CD127 (eBioSB/199), Ly6C (HK1.4), CD28 (E18), CD44 (IM7), CD62L (MEL-14), CCR6 (29-2L17), NK1.1 (PK136), CD45.1 (A20), CD45.2 (104), V γ 1 (2.11), V γ 4 (UC3-10A6), IL-17 (TC11-18H10), IFN- γ (XMG1.2), CD4 (RM4-5), CD8 α (53-6.7), TCR β (H57-597), pErk1/2 (D13.14.4E), pZap70 (n3kbu5), pSTAT3 (D3A7), and pSTAT5 (47/Stat5) (from BD Biosciences, Biolegend, eBioscience, or Cell Signaling Technology).

Cell Sorting and Isolation

After blocking with anti-CD16/32, cells were negatively selected from pooled pLN and splenocytes using anti-TCR β biotin, anti-CD45R biotin (RA3-6B2), and anti-CD11b (M1/70) antibodies together with MagniSortTM Streptavidin Negative Selection Beads according to the manufacturer's instructions (eBioscience). The negatively selected cells were then stained

with fluorochrome-labeled anti- $\gamma\delta$ TCR, anti-CD27, and/or anti-V γ 4 antibodies. $\gamma\delta$ subsets (V γ 4⁻ $\gamma\delta$ 27⁺, V γ 4⁺ $\gamma\delta$ 27⁺, V γ 4⁻ $\gamma\delta$ 27⁻, and V γ 4⁺ $\gamma\delta$ 27⁻) were sorted using an Aria II with a purity of at least 98%. Alternatively, total $\gamma\delta$ cells were manually isolated by negative selection as described previously (30) with modification. Biotin-conjugated mAbs against the following proteins were used: TCR β , CD4, CD8 α , CD45R, CD11b (M1/70), CD11c, Ly-6G (RB6-8C5), TER-119 (TER-119), and CD49b (DX5) (all from eBioscience).

Cell Culture

Cells were cultured in RPMI 1640 containing 10% (vol/vol) FBS, 50 μ M 2-mercaptoethanol, 1% (vol/vol) non-essential amino acids, 10 mM HEPES, and 1% (vol/vol) antibiotics/antimycotic solution (all from Invitrogen). For TCR stimulation, the cells were incubated for the indicated times with plate-bound anti-CD3 ϵ mAb (0.1, 1, or 10 μ g/ml; PeproTech) only or in combination with plate-bound anti-CD28 mAb (1 or 10 μ g/ml; PeproTech). For cytokine stimulation, IL-7 (20 ng/ml; R&D Systems), TGF- β (10 ng/ml; R&D Systems), IL-6 (20 ng/ml; R&D Systems), IL-1 β (10 ng/ml; PeproTech), and IL-23 (20 ng/ml; PeproTech) were added to the medium.

Bone Marrow (BM) Chimeras

Bone marrow cells (5×10^6 cells) from B6.CD45.2 wild-type (WT) or B6.CD45.2 KLF10-deficient mice were intravenously transferred to B6.CD45.1 mice, which were irradiated at 900 cGy. For mixed BM chimeras, B6.CD45.1/2 mice were lethally irradiated at 900 cGy and intravenously administered 5×10^6 B6.CD45.1 WT cells mixed 1:1 with 5×10^6 B6.CD45.2 KLF10-deficient BM cells. Recipient mice were sacrificed and analyzed for reconstituted $\gamma\delta$ T cells after at least 12 weeks.

Homeostatic Expansion

Single-cell suspensions of pLN cells obtained from B6.CD45.1 WT and B6.KLF10 knockout (KO) mice were stained with Cell Trace Violet (CTV; Invitrogen) and injected intravenously with 2×10^6 cells into Rag-1-deficient mice. After 5 days, pLNs from the recipient mice were collected and examined for CTV dilution of the transferred T cells.

RNA Extraction and Real-time qPCR

RNA was purified from cells using a Qiagen RNeasy kit. After reverse transcription into cDNA, PCR was performed with a StepOnePlus real-time PCR system (Applied Biosystems) and iTaq SYBR Green Supermix (Applied Biosystems) and relative expression was displayed in arbitrary units or as a percent of maximum expression, normalized to *Eef1a1* (encoding eukaryotic translation elongation factor 1 α 1; called “*Efa1*” hereafter) via the $\Delta\Delta$ Ct method. The following primers were used: *Efa1* forward, 5'-TCCACCGAGCCACCATACA-3', reverse, 5'-CCAACCAGAAATTGGCACAA-3'; *Klf10* forward, 5'-ACCCAGGGTGTGGCAAGAC-3', reverse, 5'-AGCGAGCAAACCTCCTTTCA-3'; *Rorc* forward, 5'-TCAGCGCCCTGTGTTTTCT-3', reverse, 5'-CAAATTGTATTGCAGATGTTC CA-3'; *Sox13* forward, 5'-CTGCCACCTGGGTTACTTTGA-3', reverse, 5'-GAGTGGCGTGATGAACATGTG-3'.

Statistical Analyses

Prism software (GraphPad) was used for all statistical analyses. All quantitative data are shown as mean \pm standard deviation (SD) unless otherwise indicated. The two-tailed, paired *t*-test was used for BM chimeras. The two-tailed, unpaired *t*-test or two-way ANOVA followed by a Bonferroni *post hoc* test were used for all other data sets. Mean fluorescence intensity (MFI) indicates geometric MFI. Robust coefficient of variation (CV) defines $100 \times 1/2$ [intensity (at 84.13 percentile) – intensity (at 15.87 percentile)/Median] and was determined by FlowJo software. The robust CV is a normalized SD not as skewed by outlying values as the CV.

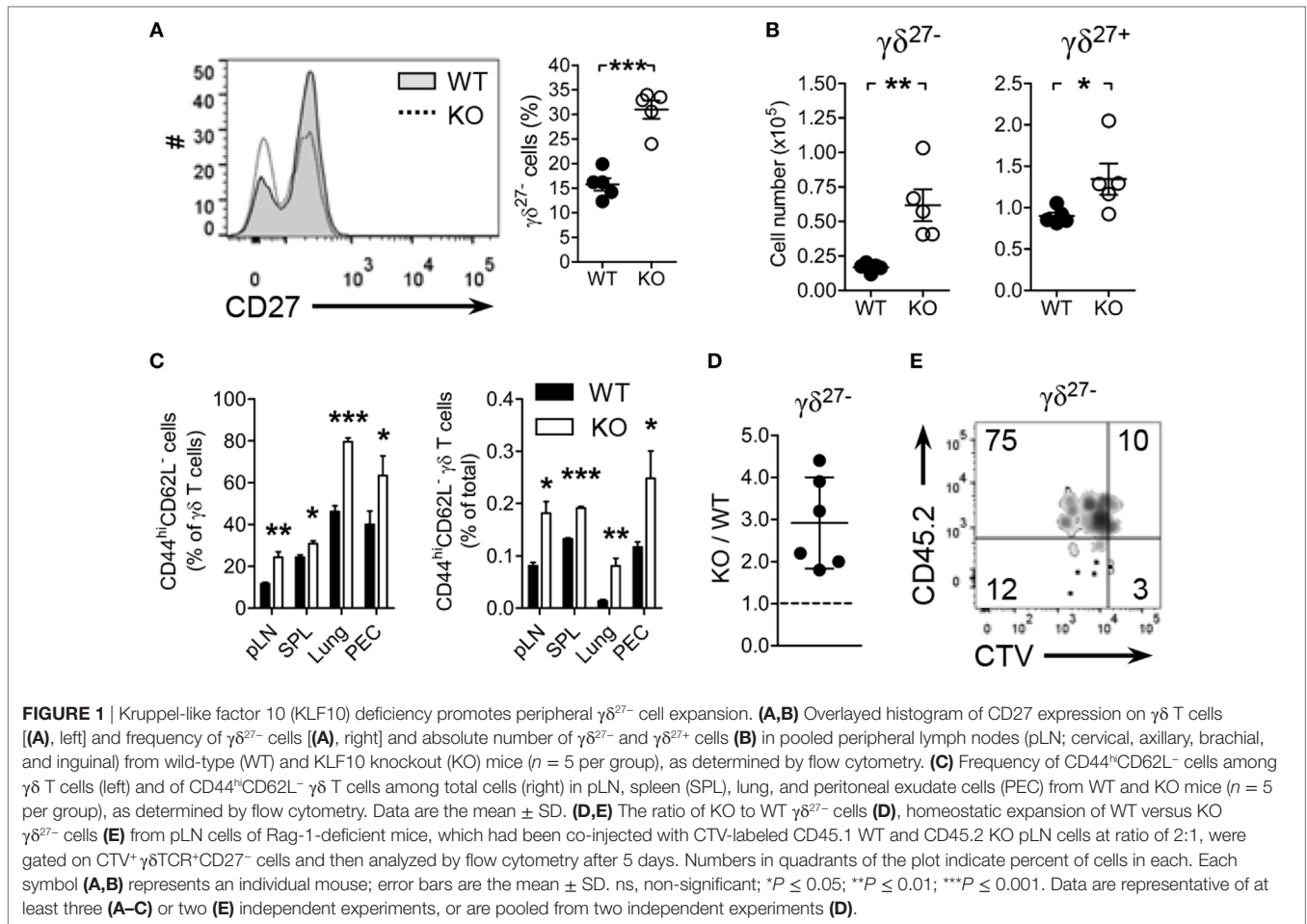
RESULTS

KLF10 Controls Homeostatic Proliferation of $\gamma\delta$ 27⁻ Cells

KLF10 has been reported to control Treg and Th17 cell induction (1, 3). Nonetheless, alterations of these cells in KLF10-deficient mice are still controversial (2, 3) and even the general T-cell status of the KO mice is not clearly defined (31). Therefore, we first examined the frequencies and absolute numbers of $\alpha\beta$ and $\gamma\delta$ T cells in the pLN, spleen, lung, and peritoneal cavity of KO mice under specific pathogen-free conditions. Compared with WT mice, levels of $\gamma\delta$ T cells, but not conventional CD4⁺ and CD8⁺ $\alpha\beta$ T cells, were significantly increased in pLN and lung of KO mice (Figures S1A,B in Supplementary Material). Analysis of discrete LNs (cervical, axillary, brachial, inguinal, and mesenteric; Figure S1C in Supplementary Material) confirmed the higher frequencies of $\gamma\delta$ T cells, with the exception of mesenteric LN (mLN). Interestingly, the increase in $\gamma\delta$ T cells was largely attributable to $\gamma\delta$ 27⁻ cell augmentation (Figures 1A,B). As expected, higher numbers of $\gamma\delta$ 27⁻ cells were observed in all LNs except for mLN (Figure S1D in Supplementary Material), in which innate-like $\gamma\delta$ -17 cells are reported to be absent (5).

CD27 expression discriminated $\gamma\delta$ subsets with CD44, CD62L, NK1.1, and CCR6; pLN $\gamma\delta$ 27⁻ cells were delineated as NK1.1⁻CCR6⁺CD44^{hi}CD62L⁻ effector memory-phenotype cells (11, 32). Consistent with the augmentation of $\gamma\delta$ 27⁻ cells in KO mice, there were considerably more effector memory-phenotype (CD44^{hi}CD62L⁻) $\gamma\delta$ T cells in the analyzed organs (Figure 1C). By contrast, similar frequencies of effector memory-phenotype CD4⁺ T and CD8⁺ T cells in these organs were observed from both strains (Figure S2A in Supplementary Material). Intriguingly, the frequencies of Tregs (Figure S2B in Supplementary Material) and Th17 cells (Figure S2C in Supplementary Material, left column) among CD4⁺ T cells were unchanged in KO mice relative to those in WT mice. In summary, these data suggested that the generation of $\gamma\delta$ 27⁻ cells was enhanced in KO mice.

To explore the nature of $\gamma\delta$ 27⁻ cell augmentation in KO mice, we investigated whether KLF10 deficiency affected homeostatic proliferation of $\gamma\delta$ 27⁻ cells. Considering that the absolute number of KO pLN $\gamma\delta$ 27⁻ cells was about twice higher than that in WT pLN cells (Figure 1B), we transferred CD45.1 WT and CD45.2 KO pLN cells together into lymphopenic mice at 2:1 ratio; we



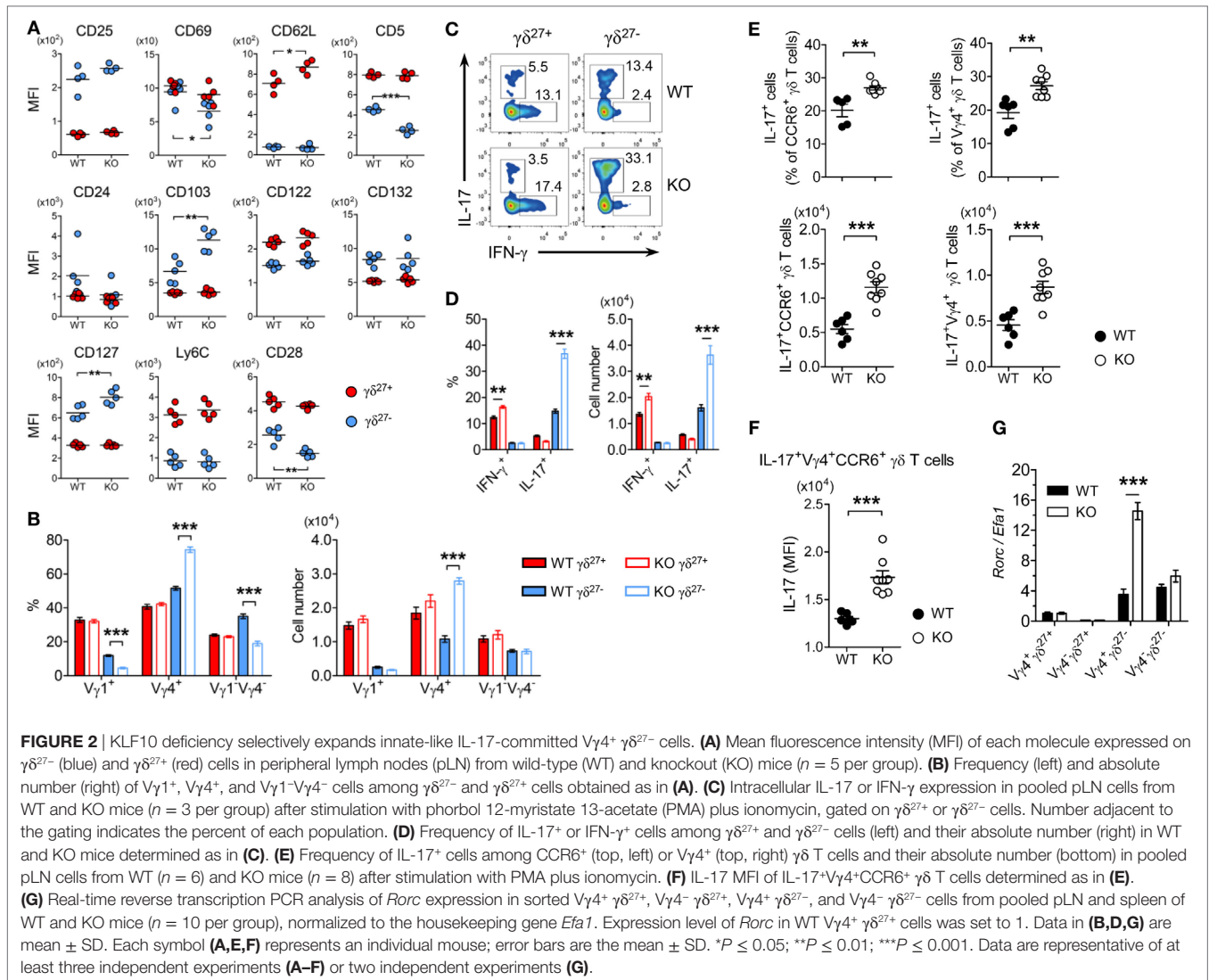
confirmed that there were the same number of $\gamma\delta^{27-}$ cells from both strains (data not shown). The homeostatic expansion of KO $\gamma\delta^{27-}$ cells was superior to that of WT $\gamma\delta^{27-}$ cells, with a ratio as great as threefold (**Figures 1D,E**). By contrast, we observed a similar expansion pattern of $\alpha\beta$ T cells from both strains in recipient mice transferred with the pLN mixture at 1:1 ratio (**Figures S2D-F** in Supplementary Material). Therefore, these data suggested that KLF10 specifically inhibited $\gamma\delta^{27-}$ cell expansion under lymphopenic conditions.

KLF10 Deficiency Preferentially Expands $V\gamma 4^+$ $\gamma\delta^{27-}$ -17 Cells

To determine phenotypic traits of $\gamma\delta^{27-}$ cells expanded in a KLF10-deficient condition (**Figures 1A-C**), we investigated the expression of surface molecules involved in innate and adaptive immune features of $\gamma\delta$ T cells. Interestingly, KO $\gamma\delta^{27-}$ cells showed lower expression of CD69, CD28, and CD5, but higher expression of CD103 and CD127, than their WT counterparts (**Figure 2A**). To understand these changes in the light of $V\gamma$ chains, we examined the $V\gamma$ usage and found a $V\gamma 4$ -biased composition of KO $\gamma\delta^{27-}$ cells (**Figure 2B, left**). Indeed, $V\gamma 4^+$ $\gamma\delta^{27-}$ cells expressed lower CD5 and CD28 but higher CD127 and CD103 than $V\gamma 4^-$ $\gamma\delta^{27-}$ cells (data not shown), which

demonstrated that the change in these surface molecules on $\gamma\delta^{27-}$ cells (**Figure 2A**) could be attributed to the preferential distribution of a $V\gamma 4$ subset. In addition, only $V\gamma 4^+$ $\gamma\delta^{27-}$ cells were considerably enhanced in pLN, whereas the abundance of $V\gamma 1^+$ $\gamma\delta^{27-}$ and $V\gamma 1^-V\gamma 4^-$ $\gamma\delta^{27-}$ cells was unchanged in KO mice compared with that in WT mice (**Figure 2B, right**). Collectively, our results indicated that $V\gamma 4^+$ $\gamma\delta^{27-}$ cells were selectively enriched under KLF10 deficiency.

As $\gamma\delta^{27-}$ cells are innate-like IL-17-producing $\gamma\delta$ T cells ($\gamma\delta$ -17) (6), we examined intracellular levels of IL-17A. Consistent with the greater abundance of $V\gamma 4^+$ $\gamma\delta^{27-}$ (**Figure 2B**) and $V\gamma 4^+$ CCR6⁺ cells (data not shown) in pLN of KO mice, considerably more IL-17⁺ cells were observed in $\gamma\delta^{27-}$ cells from KO pLN cells after stimulation with PMA plus ionomycin (**Figures 2C,D**). This was further confirmed by the increased number of IL-17⁺CCR6⁺ or IL-17⁺ $V\gamma 4^+$ $\gamma\delta$ T cells (**Figure 2E**). Of note, IL-17 production by IL-17⁺ $V\gamma 4^+$ CCR6⁺ $\gamma\delta$ T cells was higher in the KLF10-deficient condition than in the normal condition, as measured by the MFI of intracellular IL-17 (**Figure 2F**), indicating that KLF10 constrained IL-17 production by $V\gamma 4^+$ $\gamma\delta^{27-}$ cells. Finally, KO $V\gamma 4^+$ $\gamma\delta^{27-}$ cells contained considerably higher levels of *Rorc* than their WT counterparts (**Figure 2G**). These results collectively suggested that KLF10 impaired the size of the innate-like



$V\gamma 4^+$ $\gamma\delta^{27-}$ -17 population together with their IL-17-producing capacity.

KLF10 Differently Regulates $\gamma\delta^{27-}$ Cell Responsiveness to Cytokine and TCR Stimuli

Peripheral homeostasis and IL-17 production of innate-like $\gamma\delta$ -17 cells are mainly controlled by innate signaling triggered by cytokines, such as IL-7 and IL-1 β plus IL-23 (8, 9). Thus, we determined whether KLF10 was involved in cytokine-signaling on $V\gamma 4^+$ $\gamma\delta^{27-}$ cells. Under IL-7 treatment the absolute number of $V\gamma 4^+$ and $V\gamma 1^-V\gamma 4^- \gamma\delta^{27-}$ cells from KO mice was considerably increased compared with their WT counterparts (**Figure 3A**). Both $\gamma\delta^{27-}$ subsets of KO mice exhibited greater proliferation (**Figure 3B**) and there were higher frequencies of IL-17 $^+$ expanding cells among KO $\gamma\delta^{27-}$ subsets (**Figure 3C**). Because IL-7 enriches $\gamma\delta^{27-}$ -17 cells by activating STAT3 rather than STAT5 (9), we assessed STAT phosphorylation triggered

by IL-7. IL-7 substantially activated STAT5 in both $V\gamma 4^+$ and $V\gamma 4^- \gamma\delta^{27-}$ cells from WT mice, whereas the phosphorylation of STAT3 was slightly induced by IL-7 (**Figure 3D**), consistent with the previous report showing the capacity of IL-7 to activate STAT3 in $\gamma\delta^{27-}$ cells (9). Of note, compared to the normal condition, the KLF10 deficiency increased the level of phospho-STAT3 (pSTAT3), but not of pSTAT5, under IL-7 treatment (**Figure 3D**). Meanwhile, we observed the pSTAT3 induction in $V\gamma 4^- \gamma\delta^{27-}$ cells when treated with IL-6, but not in $V\gamma 4^+$ $\gamma\delta^{27-}$ cells (**Figure S3A** in Supplementary Material). These data suggested that STAT3 activation might be involved in the hyper-responsiveness of KO $\gamma\delta^{27-}$ cells to IL-7. On the other hand, contrary to the aforementioned *in vivo* $V\gamma 4^+$ $\gamma\delta^{27-}$ -17 cell-specific regulation of KLF10 (**Figure 2**), *in vitro* hyper-responsiveness to IL-7 was observed in KO $\gamma\delta^{27-}$ cells regardless of $V\gamma 4$ (**Figures 3A-C**). In addition, KLF10 deficiency promoted $\gamma\delta^{27-}$ cells, irrespective of $V\gamma 4$, providing an advantage for homeostatic expansion in lymphopenic conditions (**Figure 3E**). Collectively, these results suggest a

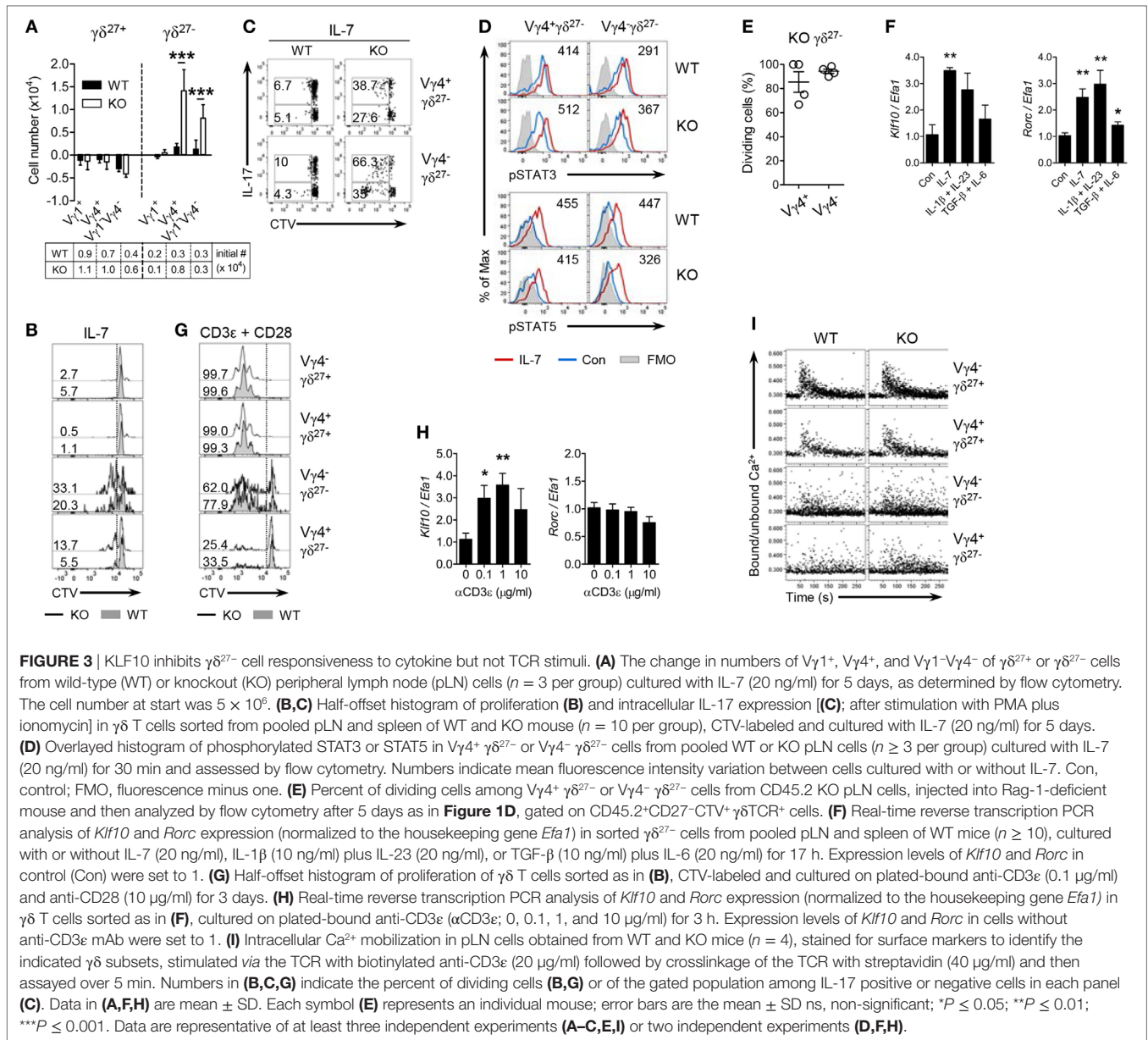


FIGURE 3 | KLF10 inhibits $\gamma\delta^{27-}$ cell responsiveness to cytokine but not TCR stimuli. **(A)** The change in numbers of V γ 1⁺, V γ 4⁺, and V γ 1⁻V γ 4⁻ of $\gamma\delta^{27+}$ or $\gamma\delta^{27-}$ cells from wild-type (WT) or knockout (KO) peripheral lymph node (pLN) cells ($n = 3$ per group) cultured with IL-7 (20 ng/ml) for 5 days, as determined by flow cytometry. The cell number at start was 5×10^6 . **(B,C)** Half-offset histogram of proliferation **(B)** and intracellular IL-17 expression **(C)**; after stimulation with PMA plus ionomycin in $\gamma\delta$ T cells sorted from pooled pLN and spleen of WT and KO mouse ($n = 10$ per group), CTV-labeled and cultured with IL-7 (20 ng/ml) for 5 days. **(D)** Overlaid histogram of phosphorylated STAT3 or STAT5 in V γ 4⁺ $\gamma\delta^{27-}$ or V γ 4⁻ $\gamma\delta^{27-}$ cells from pooled WT or KO pLN cells ($n \geq 3$ per group) cultured with IL-7 (20 ng/ml) for 30 min and assessed by flow cytometry. Numbers indicate mean fluorescence intensity variation between cells cultured with or without IL-7. Con, control; FMO, fluorescence minus one. **(E)** Percent of dividing cells among V γ 4⁺ $\gamma\delta^{27-}$ or V γ 4⁻ $\gamma\delta^{27-}$ cells from CD45.2 KO pLN cells, injected into Rag-1-deficient mouse and then analyzed by flow cytometry after 5 days as in **Figure 1D**, gated on CD45.2⁺CD27⁻CTV⁺ $\gamma\delta$ TCR⁺ cells. **(F)** Real-time reverse transcription PCR analysis of *Klf10* and *Rorc* expression (normalized to the housekeeping gene *Efa1*) in sorted $\gamma\delta^{27-}$ cells from pooled pLN and spleen of WT mice ($n \geq 10$), cultured with or without IL-7 (20 ng/ml), IL-1 β (10 ng/ml) plus IL-23 (20 ng/ml), or TGF- β (10 ng/ml) plus IL-6 (20 ng/ml) for 17 h. Expression levels of *Klf10* and *Rorc* in control (Con) were set to 1. **(G)** Half-offset histogram of proliferation of $\gamma\delta$ T cells sorted as in **(B)**, CTV-labeled and cultured on plated-bound anti-CD3 ϵ (0.1 μ g/ml) and anti-CD28 (10 μ g/ml) for 3 days. **(H)** Real-time reverse transcription PCR analysis of *Klf10* and *Rorc* expression (normalized to the housekeeping gene *Efa1*) in $\gamma\delta$ T cells sorted as in **(F)**, cultured on plated-bound anti-CD3 ϵ (α CD3 ϵ ; 0, 0.1, 1, and 10 μ g/ml) for 3 h. Expression levels of *Klf10* and *Rorc* in cells without anti-CD3 ϵ mAb were set to 1. **(I)** Intracellular Ca²⁺ mobilization in pLN cells obtained from WT and KO mice ($n = 4$), stained for surface markers to identify the indicated $\gamma\delta$ subsets, stimulated via the TCR with biotinylated anti-CD3 ϵ (20 μ g/ml) followed by crosslinkage of the TCR with streptavidin (40 μ g/ml) and then assayed over 5 min. Numbers in **(B,C,G)** indicate the percent of dividing cells **(B,G)** or of the gated population among IL-17 positive or negative cells in each panel **(C)**. Data in **(A,F,H)** are mean \pm SD. Each symbol **(E)** represents an individual mouse; error bars are the mean \pm SD, non-significant; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$. Data are representative of at least three independent experiments **(A-C,E,I)** or two independent experiments **(D,F,H)**.

critical role for KLF10 in IL-7 signaling-mediated homeostasis of innate-like $\gamma\delta^{27-}$ -17 cells.

We next examined whether KLF10 is also involved in the reactivity of $\gamma\delta^{27-}$ cells to inflammatory conditions such as IL-1 β plus IL-23 that are known to induce IL-17 production by $\gamma\delta$ T cells (8). Consistent with the results of IL-7 stimulation, $\gamma\delta^{27-}$ cells from KO mice were hyper-responsive to IL-1 β plus IL-23 (Figures S3B,C in Supplementary Material), which indicated that KLF10 also inhibited the activation of $\gamma\delta^{27-}$ cells triggered by inflammatory stimuli. Of note, *Klf10* expression was increased in $\gamma\delta^{27-}$ cells stimulated with IL-1 β plus IL-23, as well as those stimulated with IL-7 (**Figure 3F**). On the other hand, KLF10 deficiency did not lead to an alteration in the calcium fluxes directly triggered by stimulation with PMA plus ionomycin, ruling out the possibility that the hyper-responsiveness of KO $\gamma\delta^{27-}$ cells

might be qualitatively unspecific to cytokines (Figure S3D in Supplementary Material). Together, these data suggested that homeostatic and inflammatory cytokine signaling could induce KLF10, which in turn, as a negative feedback signaling factor, might impair $\gamma\delta^{27-}$ cell responsiveness to these innate stimuli.

Next, we sought to determine whether KLF10 might be involved in TCR-triggered activation of peripheral $\gamma\delta$ T cells (13, 32). $\gamma\delta^{27+}$ cells could readily expand under TCR/CD28 stimuli, whereas $\gamma\delta^{27-}$ cells showed different patterns of proliferation and, especially, V γ 4⁺ $\gamma\delta^{27-}$ cells hardly expanded (**Figure 3G**), supporting a preceding report that innate-like $\gamma\delta^{27-}$ cells display hypo-responsive TCR signaling (13). Although *Klf10* could be significantly induced by TCR activation in total $\gamma\delta$ T cells, in which $\gamma\delta^{27+}$ cells accounted for up to 85% of the cells (**Figure 3H**), there were similar expansions of each $\gamma\delta$ subset between WT and

KO mice (**Figure 3G**). As previously reported (13), a rapid and transient increase in cytosolic calcium concentration triggered by TCR engagement was readily detected in $\gamma\delta^{27+}$ cells, but not in $\gamma\delta^{27-}$ cells; however, there were no differences in calcium fluxes between the strains (**Figure 3I**). Most of all, a $V\gamma 4^+$ $\gamma\delta^{27-}$ subset almost completely failed to phosphorylate ERK after TCR stimulation (Figure S3E in Supplementary Material). Therefore, KLF10 has a minor role in TCR-triggered activation of peripheral $\gamma\delta$ subsets. To further investigate whether the level of engagement by the antigen or costimulatory receptor influenced KLF10 involvement in the TCR response, we treated the cells with different doses of agonist antibodies. $V\gamma 4^+$ $\gamma\delta^{27-}$ cells from both strains responded similarly to a high level of antigen engagement even in the absence of costimulatory signaling, but still showed an impaired proliferative response compared to that of $\gamma\delta^{27+}$ cells (Figure S3F in Supplementary Material); similar results were found with costimulatory stimuli. Therefore, neither the strong antigen receptor nor costimulatory signaling caused a substantial discrepancy in TCR response of peripheral $\gamma\delta$ subsets between the two strains.

CD5^{lo}CD127^{hi} $\gamma\delta^{27-}$ Subsets As Innate-Like $\gamma\delta$ -17 Cells

The expression level of CD5, a stable indicator of TCR strength, was lower in $\gamma\delta^{27-}$ cells than in $\gamma\delta^{27+}$ cells (**Figure 2A**), indicating that $\gamma\delta^{27-}$ cells might receive a relatively weak TCR strength compared with $\gamma\delta^{27+}$ cells; this is in line with the fact that a weak TCR-signal strength is required for thymic development of innate-like $\gamma\delta$ -17 cells (11, 33). Intriguingly, when we monitored CD5 expression (**Figure 4A**), $\gamma\delta^{27-}$ cells contained two different populations (CD5^{high} and CD5^{low}), meaning that they are a heterogeneous group receiving discrete TCR-signal strengths. Such a two-peak pattern was also observed for CD127 (IL-7 receptor- α , IL-7R α), one of the markers identifying innate-like $\gamma\delta$ -17 cells (**Figure 4A**) (9, 13), allowing us to distinguish CD5^{lo}CD127^{hi} and CD5^{hi}CD127^{hi} cells (**Figure 4B**) and further presume that CD5^{lo}CD127^{hi} $\gamma\delta^{27-}$ cells might represent the innate-like $\gamma\delta$ -17 cells. Indeed, the CD5^{lo}CD127^{hi} $\gamma\delta^{27-}$ subset was greater in KO mice, whereas the CD5^{hi}CD127^{lo} $\gamma\delta^{27-}$ subset was present in normal numbers (**Figure 4C**).

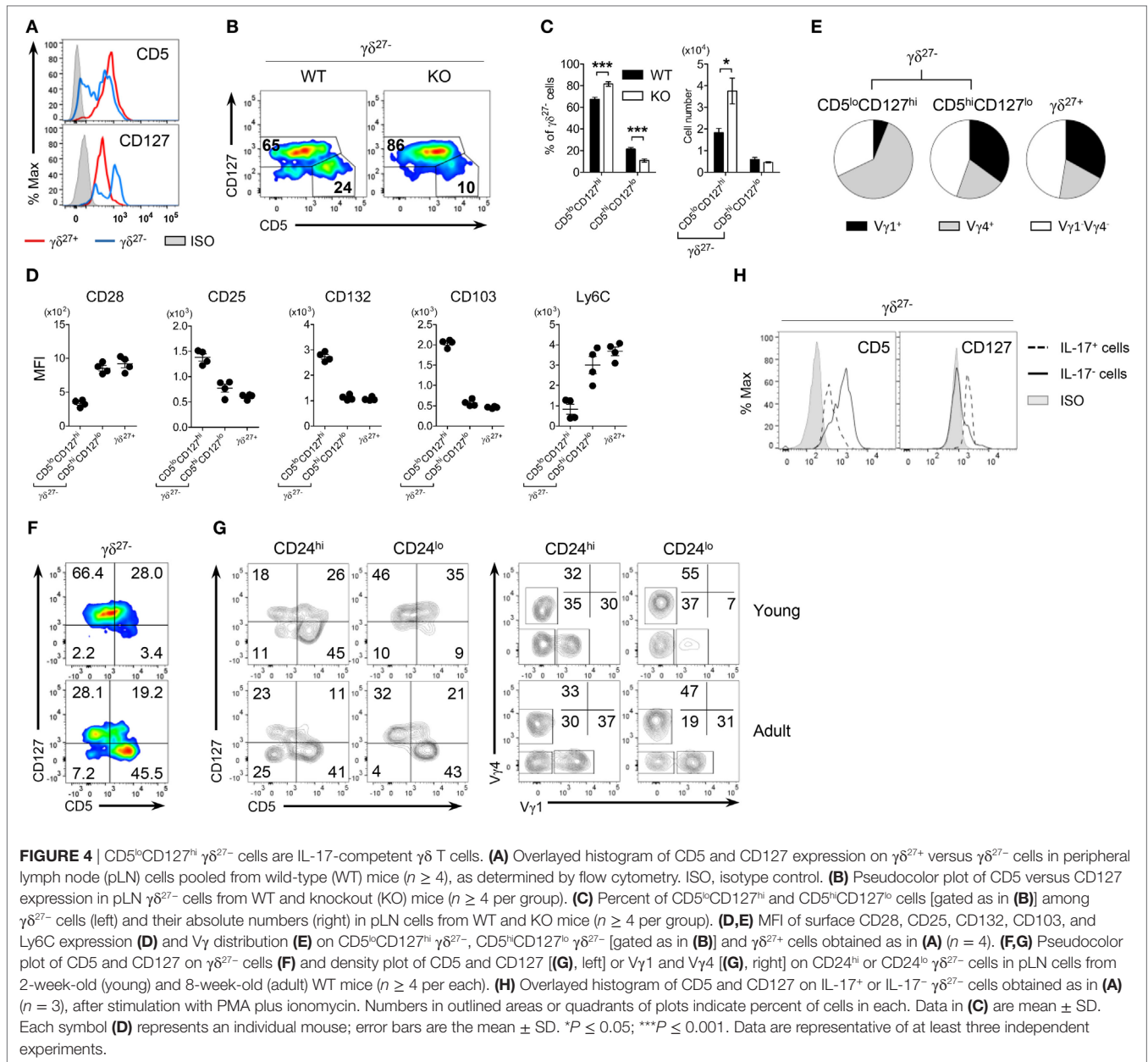
Interestingly, CD5^{hi}CD127^{lo} $\gamma\delta^{27-}$ cells closely resembled $\gamma\delta^{27+}$ cells rather than CD5^{lo}CD127^{hi} $\gamma\delta^{27-}$ cells in terms of surface protein expression (**Figure 4D**) and $V\gamma$ usage (**Figure 4E**). We noted that the frequency of CD5^{hi}CD127^{lo} cells among $\gamma\delta^{27-}$ cells was very low (3.4%) in young mice (2 weeks old) but greatly increased (45.5%) in the adult (8 weeks old) (**Figure 4F**). These cells mainly appeared within mature (CD24^{lo}) $\gamma\delta^{27-}$ cells of the thymus (Figure S4 in Supplementary Material) and pLN (**Figure 4G**), and were accompanied by higher levels of $V\gamma 1^+$ cells. In addition, CD5^{hi} $V\gamma 1^+$ cells emerged among immature (CD24^{hi}) $\gamma\delta^{27-}$ cells of the adult thymus (Figure S4 in Supplementary Material), in accordance with the previous report on sequential $V\gamma$ waves with age (34) and possibly indicating that CD5^{hi}CD127^{lo} $\gamma\delta^{27-}$ cells might be generated in the adult rather than the fetal/neonatal thymus.

In contrast to CD5^{hi}CD127^{lo} $\gamma\delta^{27-}$ and $\gamma\delta^{27+}$ cells, CD5^{lo}CD127^{hi} $\gamma\delta^{27-}$ cells had common γ chain receptor signal-dependent and epithelial-homing phenotypes with $V\gamma 4^+$ subset dominance (**Figures 4D,E**). In particular, IL-17⁺ $\gamma\delta^{27-}$ cells were CD5^{lo}CD127^{hi} (**Figure 4H**), which ultimately validated CD5^{lo}CD127^{hi} $\gamma\delta^{27-}$ cells as “virtually” innate-like $\gamma\delta$ -17 cells.

CD5^{int} $V\gamma 4^+$ CD127^{hi} $\gamma\delta^{27-}$ -17 Cell Development in KLF10-Deficient Mice

By scrutinizing the expression patterns of CD5 and CD127 on $\gamma\delta^{27-}$ cells, depicted in a pseudocolor plot (**Figure 4B**), we could recognize another peak of CD5 that was relatively high in CD5^{lo}CD127^{hi} $\gamma\delta^{27-}$ cells under KLF10 deficiency, but otherwise was barely detectable under normal conditions. Thus, we further distinguished CD5^{int} cells from CD5^{lo}CD127^{hi} $\gamma\delta^{27-}$ cells (**Figure 5A**) and found that the CD5^{int}CD127^{hi} subgroup was expanded in a KLF10-deficient condition (**Figure 5B**). This enriched sub-group skewed toward a $V\gamma 4$ chain (**Figure 5C**), consistent with the selective enrichment of a $V\gamma 4^+$ $\gamma\delta^{27-}$ subset in KO mice (**Figure 2B**). Indeed, IL-17 production was observed in both CD5^{lo} and CD5^{int} CD127^{hi} $\gamma\delta^{27-}$ cells, and the latter population (IL-17⁺CD5^{int}CD127^{lo}) was genuinely augmented by KLF10 deletion (**Figure 5D**). Taken together, these findings indicated that KLF10 deficiency selectively expanded innate-like IL-17-competent CD5^{int} $V\gamma 4^+$ CD127^{hi} $\gamma\delta^{27-}$ cells.

In normal conditions, CD5^{lo} and CD5^{int} CD127^{hi} $\gamma\delta^{27-}$ subgroups exhibited preferential distribution of $V\gamma 4^+$ and $V\gamma 1^+$ $V\gamma 4^-$ subsets, respectively (**Figure 5C**), raising the possibility that KLF10-deficient $V\gamma 4^+$ $\gamma\delta^{27-}$ cells might express higher amounts of surface CD5 than their WT counterparts. Indeed, we observed elevated CD5 expression on $V\gamma 4^+$ $\gamma\delta^{27-}$ cells of pLN from KO mice (**Figure 5E**; Figure S5 in Supplementary Material). It is noting that the considerable increase of CD5 was detected only in $V\gamma 4^+$ $\gamma\delta^{27-}$ cells, but not in other immune cells, including naive (CD44^{lo}CD62L^{hi}), effector (CD44^{lo}CD62L^{lo}), and memory (CD44^{hi}CD62L^{hi} as central, CD44^{hi}CD62L^{lo} as effector) phenotypes of CD4⁺ or CD8⁺ T cells (data not shown). Because CD5 is positively associated with the strength of TCR signaling that T cells received during their selection within the thymus (35), we next assessed the expression level of CD5 on thymic $\gamma\delta$ T cells in neonates in which $V\gamma 4^+$ $\gamma\delta^{27-}$ -17 cell maturation actively occurred (22). Interestingly, we observed considerably higher surface CD5 expression on mature (CD24^{lo}CD44^{hi}) thymic $V\gamma 4^+$ $\gamma\delta^{27-}$ cells under conditions of KLF10 deficiency, whereas the difference in CD5 was equivocal on the immature (CD24^{hi}CD44^{lo}) cells (**Figures 5F,G**). However, consistent with the peripheral observation (Figure S3E in Supplementary Material), TCR-triggered phosphorylation of ERK in neonatal thymic $V\gamma 4^+$ $\gamma\delta^{27-}$ cells was quite similar between both strains regardless of their maturation; ERK was hardly activated by TCR stimulation in $V\gamma 4^+$ $\gamma\delta^{27-}$ cells, contrary to the significant activation of ERK in $\gamma\delta^{27+}$ cells (**Figure 5H**). These data collectively suggested that KLF10 deficiency resulted into CD5^{int} $V\gamma 4^+$ CD127^{hi} $\gamma\delta^{27-}$ cell



development by controlling thymic maturation of V γ 4⁺ $\gamma\delta^{27-}$ cells in neonates.

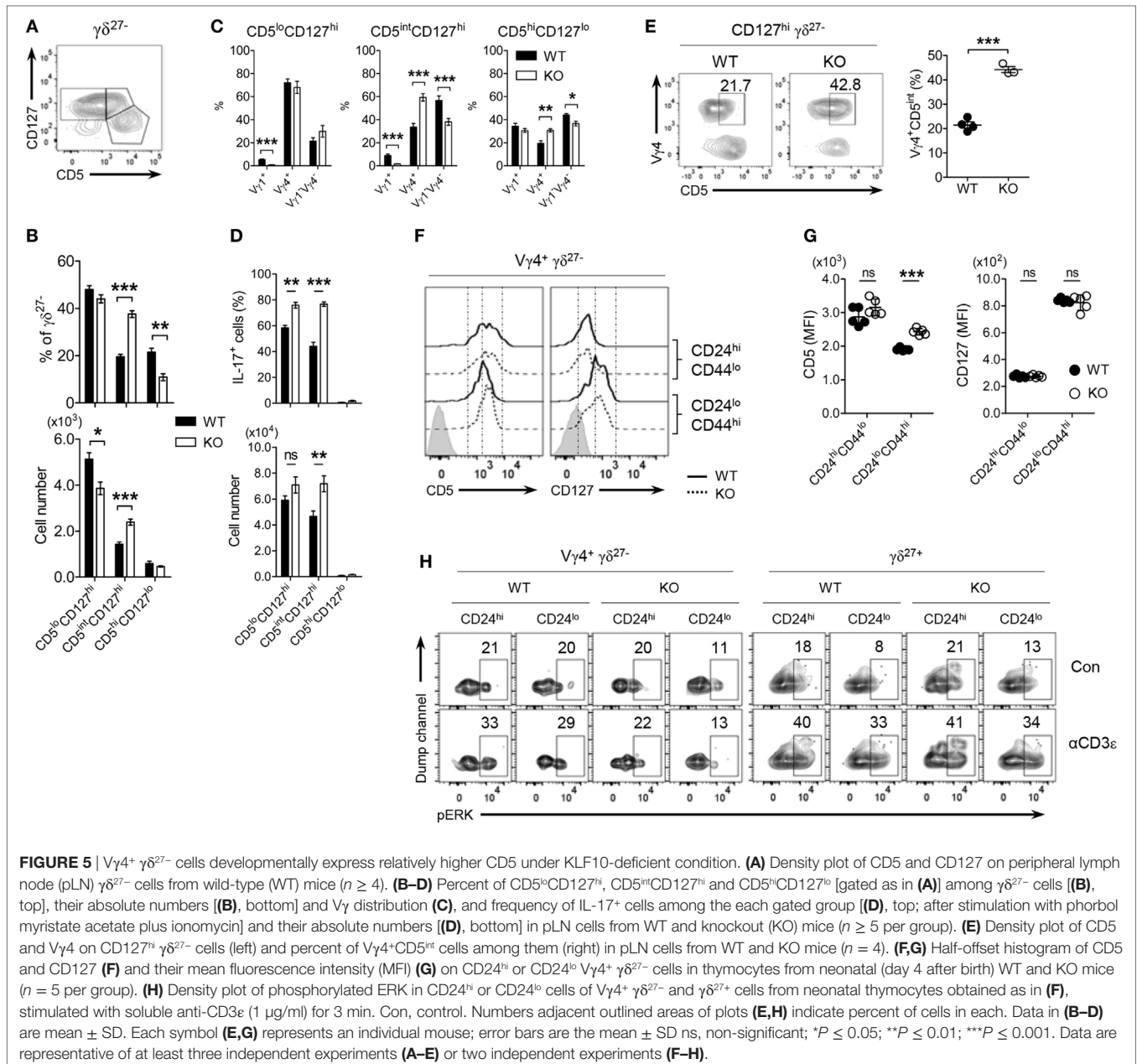
Enhanced Thymic Maturation of V γ 4⁺ $\gamma\delta^{27-}$ -17 Cells in KLF10-Deficient Neonates

Next, we determined whether the selective expansion of CD5^{int}V γ 4⁺ $\gamma\delta^{27-}$ -17 cells under KLF10 deficiency was developmental. As expected, V γ 4⁺ $\gamma\delta^{27-}$ cell maturation was enhanced in the neonatal thymus of KO mice, as measured by the frequency of mature (CD24^{lo}CD44^{hi}) cells among V γ 4⁺ $\gamma\delta^{27-}$ cells and their absolute number (**Figures 6A,C**). Intriguingly, frequencies of IL-17⁺ cells were higher in both V γ 1⁻V γ 4⁻ and V γ 4⁺ $\gamma\delta^{27-}$ cells of KO neonatal thymus (**Figure 6B**) and this abundance was even observed at the immature (CD24^{hi}CD44^{lo}) stage (**Figure 6D**),

indicating a general involvement of KLF10 in the IL-17-producing capacity of $\gamma\delta^{27-}$ cells before thymic maturation. However, only V γ 4⁺ $\gamma\delta^{27-}$ -17 cells were significantly more abundant in the KO neonatal thymus at both immature and mature stages (**Figure 6E**), confirming the preferential restraint of KLF10 on V γ 4⁺ $\gamma\delta^{27-}$ -17 cell development.

TCR-Dependent and -Independent Signaling Orchestrate KLF10 Execution Unique to V γ 4⁺ $\gamma\delta^{27-}$ Thymic Development

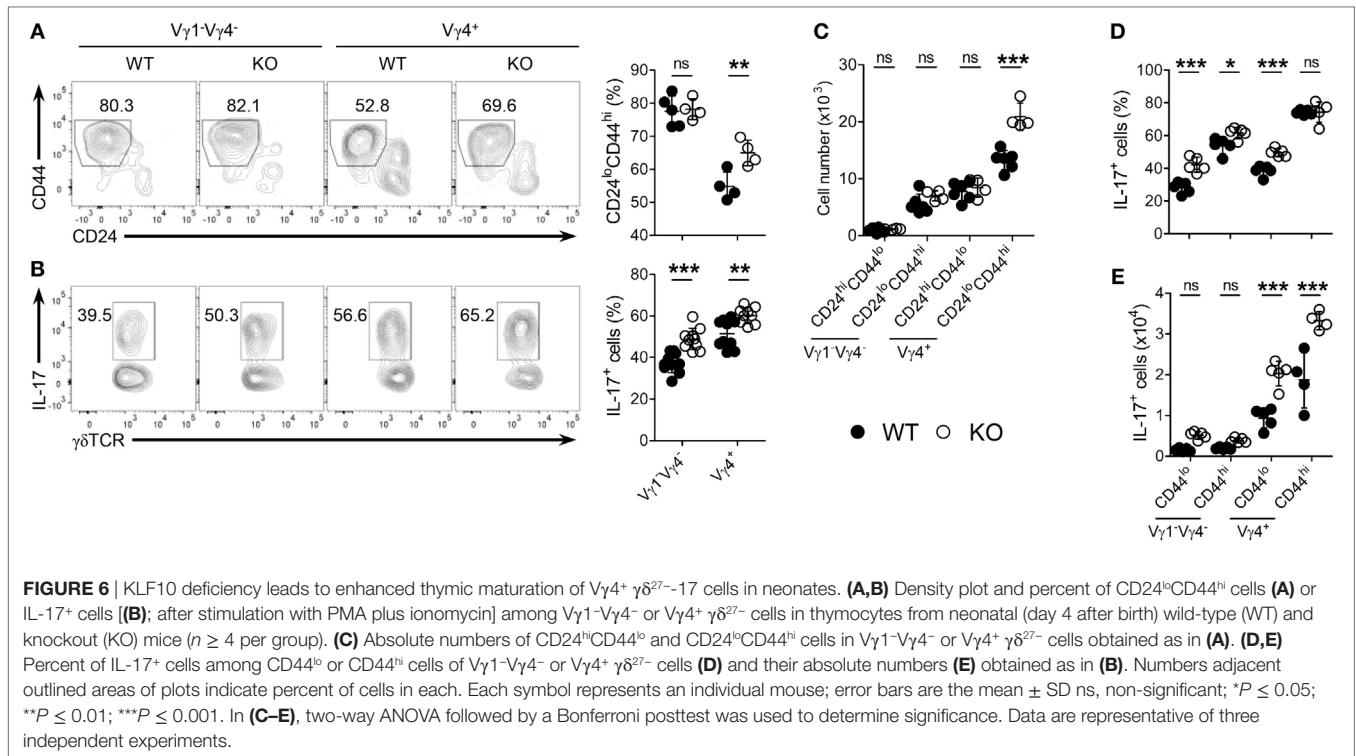
Consistent with the difference in CD5 expression on $\gamma\delta$ subsets in the periphery (**Figures 5C** and **7A**; **Figure S5** in Supplementary Material), CD5 expression on each $\gamma\delta$ thymic subset was unique and distinct based on the expression of V γ chains and CD27



(Figure 7B). These results might suggest that different strength of TCR signal is required for thymic development of $\gamma\delta$ subsets (11, 26). Regardless of CD27, the intensity of CD5 expression on immature $V\gamma 4^+$ subsets was at a low level and distinct from that of immature $V\gamma 1^+$ or $V\gamma 1^-V\gamma 4^-$ subsets, suggesting a requirement for weaker TCR signaling for thymic emergence of $V\gamma 4^+$ cells **(Figure 7B)**. We noted that immature $V\gamma 4^+ \gamma\delta^{27-}$ thymocytes expressed the lowest level of CD5, which was similarly maintained in the periphery **(Figure 7A)**.

We observed that thymic maturation induced a general decrease in CD5 expression on $\gamma\delta$ T cells **(Figure 7B)**. However, the CV of CD5 dramatically decreased in only $V\gamma 4^+$ thymocytes after maturation **(Figure 7C)**, suggesting their restricted

spectrum of TCR-signal mode. To determine the basal activity of TCR signaling, we directly assessed intracellular levels of pZap70 in each $\gamma\delta$ subset **(Figure 7D)**. Interestingly, in contrast to the $V\gamma$ chain-specific clustering of CD5 MFI **(Figure 7B)**, the level of pZap70 in immature $\gamma\delta$ subsets clustered according to the expression of CD27 **(Figure 7D)**, suggesting that each immature $V\gamma$ subset possessed basal TCR signaling that was influenced by CD27 costimulation (14, 32). Nonetheless, the level of pZap70 in immature $V\gamma 4^+ \gamma\delta^{27-}$ thymocytes was low, similar to that in immature $V\gamma 1^-V\gamma 4^- \gamma\delta^{27-}$ thymocytes, and then slightly decreased in the $V\gamma 4^+ \gamma\delta^{27-}$ subset and increased in the $V\gamma 1^-V\gamma 4^- \gamma\delta^{27-}$ subset after maturation; thus, a $V\gamma 4^+ \gamma\delta^{27-}$ subset could acquire a relatively lower activity of basal



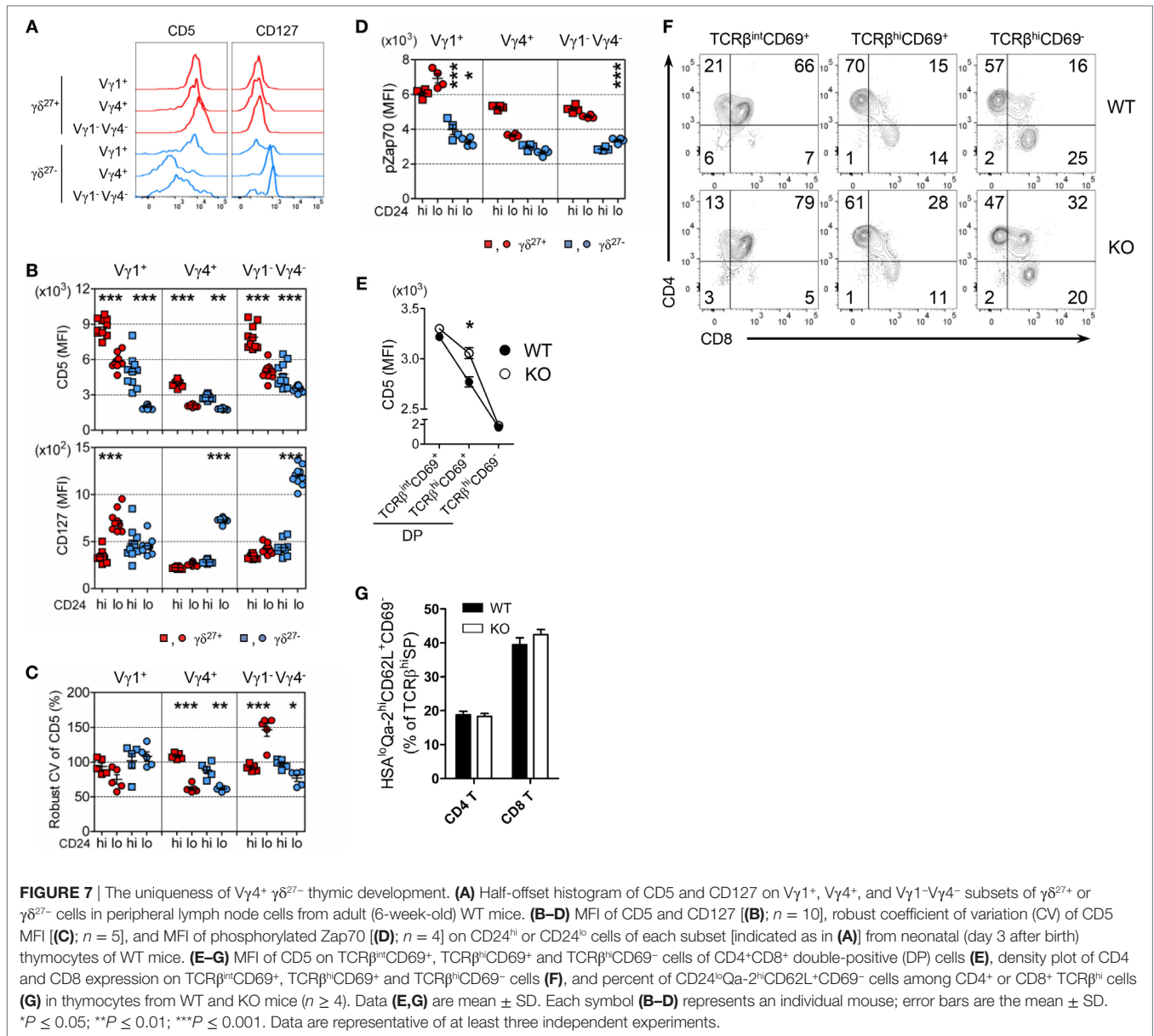
TCR signaling (**Figure 7D**). Together, these data emphasized a TCR-signaling modality unique to the emergence and maturational transition of a $V\gamma 4^+ \gamma\delta^{27-}$ subset, endorsing a weak TCR-signaling requirement for innate-like $\gamma\delta$ -17 differentiation (10, 36). It is important to note that *Klf10* could be induced by both TCR-dependent and -independent signaling pathways (**Figures 3E,H**), which were differently engaged in $\gamma\delta$ development (24). On the other hand, CD127 (IL-7R α) was abruptly induced in $V\gamma 4^+$ or $V\gamma 1-V\gamma 4^- \gamma\delta^{27-}$ subsets after thymic maturation (**Figure 7B**), consistent with the notion of mature stage-specific acquisition of cytokine receptor-mediated regulation of $\gamma\delta$ effector differentiation (25). Therefore, a series of weak TCR-signaling engagements with subsequent initiation of cytokine signaling seemingly cooperate for the function of KLF10 specific to $V\gamma 4^+ \gamma\delta^{27-}$ thymic development by primarily fine-tuning KLF10 transcription.

Transcriptional profiling of thymic $V\gamma$ subsets revealed that immature $V\gamma 4^+$ subsets are distinct from other immature $V\gamma$ subsets ($V\gamma 1^+$, $V\gamma 1.1+V\delta 6.3^+$, and $V\gamma 5^+$) but, interestingly, closely similar to $CD4^+CD8^+$ double-positive (DP) $CD69^+$ cells of the $\alpha\beta$ lineage, based on low expression of genes involved in metabolism and energy production (25). Of note, a gene constellation browser publicly provided by the Immunological Genome Project (ImmGen; www.immgen.org) reported that *Klf10* in $\gamma\delta$ T cells was closely correlated with genes encoding metabolic molecules, which showed lower expression in the immature $V\gamma 4^+$ subset than in other $V\gamma$ subsets (data not shown) (25, 37). When we assessed the expression of CD5 on DP cells, KO DP cells transiently displayed relatively higher CD5 expression than their WT counterparts at post-positive selection, but not at the

fully matured (TCR $\beta^{hi}CD69^-$) stage (**Figure 7E**), accompanied by a delayed CD4 lineage choice (**Figure 7F**). However, maturation of CD4 and CD8 T cells was normal in both strains, as measured by the percentage of $CD24^{lo}Qa-2^{hi}CD62L^+CD69^-$ cells among TCR $\beta^{hi}CD4^+$ or $CD8^+$ cells (**Figure 7G**). Thus, these data suggested that the putative mechanism involved in mature stage-specific alteration of surface CD5 on the $V\gamma 4^+ \gamma\delta^{27-}$ thymic subset by KLF10 deficiency (**Figures 5E,G**) might be related to a metabolic process that is common to both $V\gamma 4^+$ subsets and DP $CD69^+$ cells.

KLF10 Intrinsically Regulates the Development of $V\gamma 4^+ \gamma\delta^{27-17}$ Cells

Finally, we examined whether KLF10 extrinsically or intrinsically controlled homeostasis of $V\gamma 4^+ \gamma\delta^{27-17}$ cells. We reconstituted irradiated $CD45.2^+$ WT or KO mice with congenic WT BM cells and analyzed $CD45.1^+ \gamma\delta^{27-}$ cells after at least 12 wks (**Figure S6B** in Supplementary Material). Reconstitution of $V\gamma 4^+ \gamma\delta^{27-}$ cells and $CD5^{lo}CD127^{hi} \gamma\delta^{27-}$ cells in KO recipients was achieved at a level comparable to that in WT recipients, excluding the hematopoietic system-extrinsic effect of KLF10 on the homeostasis of $V\gamma 4^+ \gamma\delta^{27-17}$ cells (**Figure 8A**). By contrast, mixed BM chimera experiments in which a 1:1 mixture of $CD45.1^+$ WT and $CD45.2^+$ KO BM cells was injected into lethally irradiated $CD45.1/2^+$ WT mice (**Figure S8C** in Supplementary Material) showed a higher proportion of KO BM-derived cells among total $CD3e^+$ cells (data not shown). This suggested that KLF10-deficient BM cells outcompeted their WT counterparts during reconstitution of hematopoietic-derived cells, with the fact that KLF10 transcripts



are at high level in long- and short-term repopulating hematopoietic stem cells (www.immgen.org).

We next investigate whether $\gamma\delta$ cell-intrinsic KLF10 role is responsible for the restraint of $V\gamma 4^+$ $\gamma\delta^{27-}$ cells. In the mixed BM chimera setting, a proportion of $V\gamma 4^+$ $\gamma\delta^{27-}$ cells in KO BM-derived $\gamma\delta$ T cells was relatively higher than that of their WT counterpart **(Figure 8B)**. Although the total $\gamma\delta$ T cells from both WT and KO BM cells contained $\gamma\delta^{27-}$ cells similarly, the KO BM-derived $V\gamma 4^+$ cells generated $\gamma\delta^{27-}$ cells more than their WT counterpart did **(Figure 8B)**; as expected, there were comparable proportions of $\gamma\delta^{27-}$ cells in $V\gamma 4^-$ cells and of $V\gamma 4^+$ cells in $\gamma\delta^{27+}$ cells between the both origins (Figure S8D in Supplementary Material). Indeed, KO BM-derived $\gamma\delta^{27-}$ cells contained higher proportions of $V\gamma 4^+$ cells and $CD5^{lo}CD127^{hi}$ cells than their WT BM-derived counterparts **(Figure 8C)**. Of note, KO BM-derived $V\gamma 4^+$ $\gamma\delta^{27-}$ cells had

greater frequencies of IL-17⁺ cells **(Figure 8C)**. We also found a higher level of *Klf10* expression in $V\gamma 4^+$ $\gamma\delta^{27-}$ cells compared to the other $\gamma\delta$ subsets **(Figure 8D)**, possibly supporting the preferential engagement of KLF10 for the homeostasis of $V\gamma 4^+$ $\gamma\delta^{27-}$ cells. Collectively, these results suggest that KLF10 serves as an intrinsic negative regulator to constrain $V\gamma 4^+$ $\gamma\delta^{27-}$ cells and their production of IL-17.

DISCUSSION

Early studies revealed that KLF10-deficient mice had defective Treg cell generation under inflammatory conditions, emphasizing the role of KLF10 as a TF in the balance between Treg and Th17 cell differentiation (1–3). Although KLF10, previously named TIEG-1 (TGF- β -induced early gene-1), can be rapidly

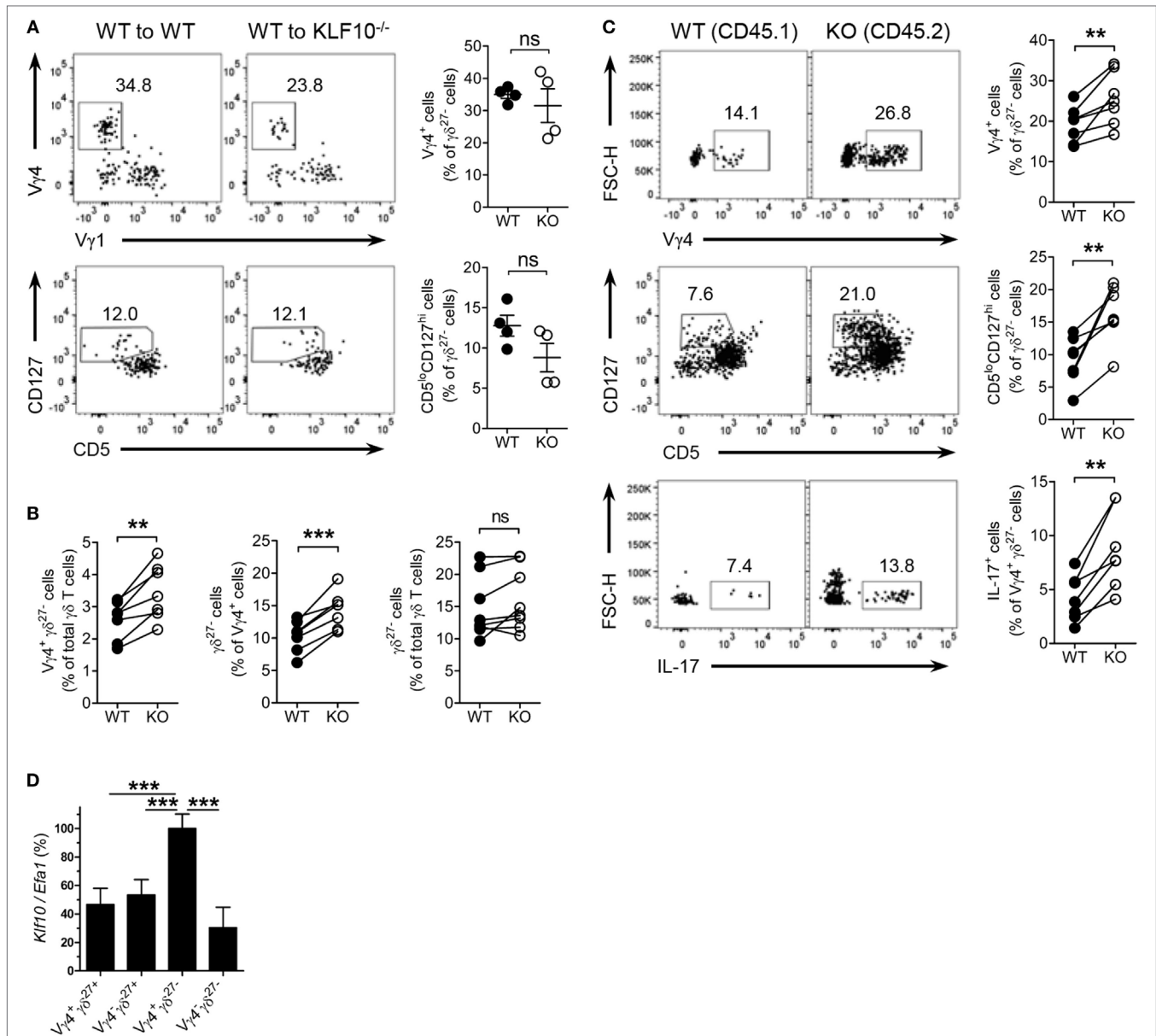


FIGURE 8 | KLF10 restraint of innate-like V γ 4⁺ $\gamma\delta^{27-}$ -17 cells is cell-intrinsic. **(A)** Dot plot and percent of V γ 4⁺V γ 1⁻ cells (top) and CD5^{lo}CD127^{hi} cells (bottom) among peripheral lymph node (pLN) $\gamma\delta^{27-}$ cells of CD45.2 WT or KO mice ($n = 4$), irradiated and reconstituted with CD45.1 WT bone marrow (BM) cells and then assessed by flow cytometry at least 12 weeks later, gated on CD45.1⁺ $\gamma\delta^{27-}$ cells. **(B,C)** CD45.1/2 WT mice ($n \geq 3$) were irradiated and reconstituted with a mixture of CD45.1 WT BM plus CD45.2 KO BM cells at ratio 1:1 and then pLN cells were analyzed by flow cytometry at least 12 weeks later. **(B)** Percent of V γ 4⁺ $\gamma\delta^{27-}$ cells (left) or $\gamma\delta^{27-}$ cells (right) among WT and KO total $\gamma\delta$ T cells or, $\gamma\delta^{27-}$ cells (middle) among WT and KO V γ 4⁺ cells. **(C)** Dot plot (left) and percent (right) of V γ 4⁺ (top) or CD5^{lo}CD127^{hi} cells (middle) among WT and KO $\gamma\delta^{27-}$ cells, or IL-17⁺ cells (bottom) among WT and KO V γ 4⁺ $\gamma\delta^{27-}$ cells. **(D)** Real-time reverse transcription PCR analysis of *Klf10* expression in sorted V γ 4⁺ $\gamma\delta^{27+}$, V γ 4⁻ $\gamma\delta^{27+}$, V γ 4⁺ $\gamma\delta^{27-}$, and V γ 4⁻ $\gamma\delta^{27-}$ cells from pooled pLN and spleen of WT mice ($n = 10$ per group), normalized to the housekeeping gene *Efa1* and presented as the percent of maximum expression of *Klf10*. Data **(A,D)** are mean \pm SD. Each symbol represents an individual recipient mouse. A line connects WT-derived cells to KO-derived cells that developed within the same recipient mouse. ns, non-significant; ** $P \leq 0.01$; *** $P \leq 0.001$. Data are representative of two independent experiments **(A,D)** or are pooled from two independent experiments **(B,C)**.

induced in CD4 T cells after TGF- β stimulation and then functions to maintain the activation of TGF- β /Smad signaling pathway (2), we found that KLF10 transcription did not respond to TGF- β in $\gamma\delta^{27-}$ cells (data not shown), indicating that KLF10 function in $\gamma\delta$ -17 cells was irrelevant to TGF- β /Smad signaling for $\gamma\delta$ -17 cell development (5). Intriguingly, by analyzing these KO

mice under steady-state conditions (unimmunized and specific pathogen-free), we have identified KLF10 as a critical negative regulator of development and homeostasis of V γ 4⁺ $\gamma\delta^{27-}$ cells. KLF10-deficient mice exhibited a spontaneous and selective augmentation of V γ 4⁺ $\gamma\delta$ -17 cells with normal frequencies of IL-17-producing TCR β ⁺ cells such as Th17, Tc-17 (IL-17⁺CD8⁺TCR β ⁺)

and *i*NKT-17 (IL-17⁺CD1d-tet⁺TCR β ⁺) cells, as well as Treg cells, suggesting a novel function of KLF10 unique to innate-like $\gamma\delta$ -17 cells. Meanwhile, the slight increase of IFN- γ ⁺ $\gamma\delta$ ²⁷⁺ cells in pLN of KO mice might demand further investigation.

Robust expansion of innate-like $\gamma\delta$ -17 cells under lymphopenic conditions is completely dependent on homeostatic cytokine IL-7 but not MHC recognition (15, 16). However, because of cellular competition for trophic cytokines or space (15), $\gamma\delta$ T cell homeostatic expansion was inhibited by $\alpha\beta$ T cells after transfer of pLN cells into lymphopenic mice. Interestingly, we found that KLF10 deficiency allowed $\gamma\delta$ ²⁷⁻ cells to overcome the competitive inhibition by $\alpha\beta$ T cells, reflecting the increased sensitivity of IL-7 signaling in KO $\gamma\delta$ ²⁷⁻ cells. This was confirmed by the hyper-responsiveness of KO $\gamma\delta$ ²⁷⁻ cells to exogenous IL-7 stimulation with increased STAT3 activation. KO $\gamma\delta$ ²⁷⁻ cells also hyper-responded to IL-1 β plus IL-23 stimuli. Of note, KLF10 transcription in $\gamma\delta$ ²⁷⁻ cells was dramatically induced by IL-6 (data not shown), IL-7, or IL-1 β plus IL-23, which are known to trigger STAT3 activation to induce RoR γ t expression (9, 38, 39), suggesting that KLF10 was a negative regulator of a STAT3-RoR γ t axis in $\gamma\delta$ -17 cells. Clearly, further studies are needed to determine the target genes, interacting signal proteins, and post-translational modifications of KLF10 to discern how KLF10 contributes to cytokine-signaling pathways in $\gamma\delta$ -17 cells. On the other hand, KLF10 was closely correlated with cell division control protein 42 homolog and Fas apoptotic inhibitory molecule in $\gamma\delta$ T cells according to a gene constellation view by ImmGen (data not shown) (37), suggesting a direct link between KLF10 and pro-survival proteins such as Bcl-2 and Bcl-xL, which are upregulated by IL-7 in $\gamma\delta$ -17 cells (16). Therefore, it is necessary to explore whether KLF10 directly engages in entry into the cell cycle and the intrinsic cell death pathway of $\gamma\delta$ -17 cells (16, 40).

The increased IL-7 signaling sensitivity of KO $\gamma\delta$ ²⁷⁻ cells was independent of V γ 4 under conditions of strong reliance on IL-7 (lymphopenic condition or direct treatment with IL-7), which is different from the V γ 4⁺ subset-specific $\gamma\delta$ ²⁷⁻ cell enrichment observed in KO mice. IL-7R α expression was not only similar between V γ 4⁺ and V γ 1-V γ 4⁻ $\gamma\delta$ ²⁷⁻ subsets, both of which are main producers of innate IL-17 among $\gamma\delta$ T cells, but was also unchanged by KLF10 deficiency (data not shown). These data suggest that V γ 4⁻ $\gamma\delta$ ²⁷⁻ cells, presumably V γ 1-V γ 4⁻ $\gamma\delta$ ²⁷⁻ subsets, are under homeostatic control by certain factors counteracting the effect of an active IL-7R-KLF10 signal axis (15). These factors could be directly or indirectly involved in the downstream of IL-7R signaling. Alternatively, considering that the steady-state level of KLF10 transcripts was preferentially higher in V γ 4⁺ $\gamma\delta$ ²⁷⁻ cells than in other $\gamma\delta$ subsets, we could postulate that V γ 1-V γ 4⁻ $\gamma\delta$ ²⁷⁻ subset-specific factors might dampen the inhibitory effect of KLF10 on IL-7-mediated homeostasis by downregulating KLF10 transcription.

Our analysis of surface CD5 expressed on emergent immature $\gamma\delta$ thymocytes clearly indicated discrete TCR-signal engagements for V γ chains. Immature V γ 4⁺ thymocytes adopted relatively low and narrow-ranged CD5 expression regardless of CD27 expression, seemingly consistent with the ligand-independent signaling of V γ 4⁺V δ 5⁺ TCR (10). This also suggests that V γ 4⁺ subsets may require relatively weak TCR-signal engagement for

thymic emergence compared with other V γ subsets, in support of the previous report that V γ 6⁺ thymocytes may depend on strong TCR signaling (11, 26, 41). Indeed, the surface CD5 expression of the peripheral V γ 4⁺ $\gamma\delta$ ²⁷⁻ subset was apparently lower than that of the V γ 1-V γ 4⁻ (and presumably V γ 6⁺) $\gamma\delta$ ²⁷⁻ subset (17). Most of all, we confirmed that innate-like $\gamma\delta$ -17 cells received relatively weak TCR-signal strength during thymic development by identifying IL-17-committed $\gamma\delta$ T cells as CD5^{lo}CD127^{hi} $\gamma\delta$ ²⁷⁻ cells predominantly composed of V γ 4⁺ and V γ 1-V γ 4⁻ subsets.

Seemingly, the lower expression of CD5 on immature V γ 4⁺ thymocytes and the subsequent super-induction of IL-7R α on $\gamma\delta$ ²⁷⁻ thymocytes upon maturation might indicate the initial engagement of weak TCR-signal strength for the V γ 4 wave, followed by heavy reliance upon IL-7 signaling for functional maturation during development (9, 25, 26). Considering that KLF10 transcription could be induced by either TCR or IL-7 signaling, we could anticipate that expression of the KLF10 transcript would be relatively low in immature V γ 4⁺ thymocytes compared with other thymic immature V γ subsets and then increase after maturation; indeed, the expected results were obtained from the public data resources of ImmGen (37). Interestingly, in contrast to KLF10, Sox13 transcription is suppressed by both TCR (36) and IL-7 stimuli (data not shown). Above all, Sox13 is highly expressed in thymic $\gamma\delta$ progenitors as a $\gamma\delta$ -lineage specific marker, but after maturation dramatically decreased with relatively higher expression in $\gamma\delta$ -17 thymocytes than in $\gamma\delta$ -IFN- γ thymocytes (36, 42). Of note, there is evidence that Sox13 is essential for the development of V γ 4⁺ $\gamma\delta$ -17 cells, as it is abundant in V γ 4⁺ rather than in V γ 4⁻ thymocytes at an immature stage (22, 37, 43). However, we found similar levels of Sox13 transcripts in the peripheral V γ 4⁺ $\gamma\delta$ ²⁷⁻ subset between WT and KO mice (data not shown), suggesting that Sox13 may not be associated with the selective effect of KLF10 deficiency on V γ 4⁺ $\gamma\delta$ ²⁷⁻ cell development (22, 43). This notwithstanding, the lower levels of KLF10 and higher levels of Sox13 in immature V γ 4⁺ thymocytes, with lower CD5 expression, imply engagement of weak-TCR-signal strength in V γ 4⁺ $\gamma\delta$ -17 thymic emergence. Furthermore, the more substantial involvement of KLF10 and Sox13 in V γ 4⁺ $\gamma\delta$ -17 cell development reinforced distinct developmental requirements for V γ 4⁺ and V γ 6⁺ $\gamma\delta$ -17 cells (11, 26, 43).

We showed that the intensity of surface CD5 was mildly but significantly increased in both thymic mature V γ 4⁺ $\gamma\delta$ ²⁷⁻ cells and TCR β ^{hi}CD69⁺ DP cells of KO mice. This analogous CD5 alteration by KLF10 deficiency could support the idea of close similarity between V γ 4⁺ cells and DP cells at an immature stage (25). The close relationship between KLF10 and genes involved in metabolic processes, whose expression was lower in these two populations, provided mechanistic insight into thymic maturation of CD5^{int}V γ 4⁺ $\gamma\delta$ -17 cells under KLF10 deficiency. Moreover, a recent report revealed that KLF10 binds to a nutritional regulatory element in the promoter region of SREBP-1c that is critical for glucose and lipid metabolism (44). On the other hand, maturational transition of $\gamma\delta$ T cells seems quite similar to “negative selection” in the light of the facts that surface CD5 is commonly reduced after maturation of $\gamma\delta$ T cells and the CD5 level correlates with the strength of TCR signal initially perceived (35, 45, 46).

We suggest that thymic programming for the generation of $V\gamma 4^+ \gamma\delta$ -17 cells is negatively regulated by KLF10, with many questions remaining to be answered. In particular, clear elucidation of the quantitatively and qualitatively distinct engagements of the TCR signal, whose role in $\gamma\delta$ -17 effector decisions is still controversial, will advance identification of the developmental mechanism of $V\gamma 4^+ \gamma\delta$ -17 cells under the transcriptional control of KLF10 (26); it is possible that $V\gamma$ chains transmit distinct TCR signals caused by differences in either intrinsic modality or extrinsic factors, such as selecting ligands or that a unique TCR signal (strength and duration) may trigger the expression of genes encoding certain $V\gamma$ chains (10). Moreover, determining whether KLF10 is associated with signal circuits of inherited (or intrinsic) TFs specific to $V\gamma 4^+ \gamma\delta$ -17 cell development and with the timing of generation of the $V\gamma 4$ wave will give insight into $\gamma\delta$ effector subset diversification (24, 47, 48).

ETHICS STATEMENT

All animals were bred and maintained under specific pathogen-free conditions at the Institute of Laboratory Animal Resource Seoul National University and treated in accordance with institutional guidelines that were approved by the Institutional Animal Care and Use Committee (SNU-140930-4-1).

AUTHOR CONTRIBUTIONS

C-HY conceived the idea. C-HY and GK designed the experiments and wrote the manuscript. GK, MG, SK, KK, Y-CK, and

CK performed the experiments. GK interpreted the data. J-HC, W-KL, K-DS, HC, Y-MP, and SH provided critical comments. All authors contributed to discussion of the results followed by writing and reviewing the manuscript.

ACKNOWLEDGMENTS

We thank the staffs at Institute of Laboratory Animal Resources, Seoul National University for outstanding experimental assistance.

FUNDING

This study was supported by the grants from NRF-2015R1D1A1A02061577, Next-Generation BioGreen 21 Program (PJ01112401), Rural Development Administration and SNU-Yonsei Research Cooperation Program through Seoul National University, and IBS-R005-D1 from the Institute for Basic Science (to J-HC), Korean Ministry of Science, Information/Communication Technology and Future Planning, Republic of Korea.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at <http://www.frontiersin.org/articles/10.3389/fimmu.2018.00196/full#supplementary-material>.

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Conflict of Interest Statement: 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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