농학박사학위논문

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Abstract

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γδ T cells, known to be an important source of innate IL-17 in mice, provide critical contributions to host immune responses. However, factors that control their development and homeostasis are poorly elucidated. Here I examine the role of the zinc finger transcription factor, Kruppel-like factor 10 (KLF10) in the development of IL-17-committed $CD27^-$ γδ T ($γδ^{27-}$-17) cells. I found a selective increase of $Vγ4^+$ $γδ^{27-}$ cells with IL-17 production in KLF10-deficient mice. Surprisingly, KLF10-deficient $CD127^{hi}Vγ4^+$ $γδ^{27-}$-17 cells expressed CD5 higher than their wild-type counterparts, with hyper-responsiveness to cytokine and T-

cell receptor stimuli. Maturation of $V\gamma4^{+}$ $\gamma\delta^{27-}$ cells was enhanced in newborn mice deficient in KLF10. Finally, mixed bone marrow chimera study indicated that intrinsic KLF10 signaling is mandatory to limit $V\gamma4^{+}$ $\gamma\delta^{27-}$-17 cells. Collectively, these findings demonstrate that KLF10 regulates thymic development of $V\gamma4^{+}$ $\gamma\delta^{27-}$ cells and their peripheral homeostasis at steady-state.

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Key words: KLF10, γδ T cells, IL-17

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List of Abbreviations

BM	Bone marrow
CD	Cluster of Differentiation
Erk	Extracellular signal-regulated kinases
Eef1a1	Eukaryotic translation elongation factor 1α1
Foxp3	Forkhead box P3
GATA3	Trans-acting T-cell-specific transcription factor 3
IFN-γ	Interferon-gamma
IL	Interleukin
IRF4	Interferon regulatory factor 4
KLF10	Kruppel-like factor 10
KO	Knock-out
LEF1	Lymphoid enhancer-binding factor 1
LN	Lymph node
LPS	Lipopolysaccharide
MHC	Major histocompatibility complex
Qa-2	H-2 class I histocompatibility antigen

Rag	Recombination activating gene 1
RoRγt	RAR-related orphan receptor gamma
SIAH1	Seven in absentia homolog 1
Sox13	Sex Determining Region Y-Box 13
STAT3	Signal transducer and activator of transcription 3
TCF1	T-cell specific transcription factor 1
Treg	Regulatory T cell
TGF-β	Transforming growth factor beta
T-bet	T cell-associated transcription factor
TCR	T-cell receptor
WT	Wild-type

I. Review of Literature

1. Kruppel-like factor 10

1.1. KLF10 as a zinc finger transcription factor

To date, there are at least 17 Krüppel-like factors (KLFs) identified in mammals, which are key *trans*-acting factors contributing to a numerous biological processes, especially in the context of lymphocyte lineage commitment, differentiation, and function (1, 2). All KLFs share a highly conserved set of three DNA-binding zinc fingers at or near C terminus where KLFs bind the CACCC boxes and GC-rich DNA regions (1, 2). However, each KLF is highly divergent and has non-DNA binding domains, which dictate regulatory function (*i.e.*, *trans*-activation or *trans*-repression) and binding specificity (1, 2). As such, KLF10 contains three corepressor motifs that spread from N terminus before zinc finger domains (3). Additionally, E3 ubiquitin ligase (Itch and SIAH1) binding domains (4, 5) and tyrosine kinase Tyk2-mediated phosphorylation site (6) are recently identified and thus these distinct features help us understanding how KLF10 functions and its expression is controlled for multiple immune responses (Figure. 1).

1.2. General function of KLF10

KLF10, originally termed TIEG-1 (TGF-β inducible early gene-1), was identified first in human fetal osteoblasts as a TGF-β-responsive gene, which is involved in osteoblast-mediated mineralization and osteoclast differentiation (7). In prostate cancer cells, KLF10 appeared to be homologous to EGRα (early growth response-α) where KLF10 and EGRα proteins differ in sequence only 12 amino acids at the N terminus (8, 9). However, the functional differences are extensive in which KLF10 expression is predominantly regulated by growth factors and cytokines such as TGF-β, bone morphogenetic protein-2, estrogen and epidermal growth factor. Subsequent studies demonstrated that KLF10 protein is expressed in epithelial cells (10) as well as breast cancer cells (11). Although the varied expressions and activities of KLF10 confound simple characterization of its role, function of KLF10 could be summarized as the regulation of growth, proliferation and apoptosis of cells, in which TGF-β signaling pathway is linked.

KLF10 increases the activity of TGF-β/Smad pathway by 1) relieving the negative feedback through repression of the inhibitory Smad7 and 2) enforcing the main signaling stream though induction of Smad2 expression

and phosphorylation (3, 12, 13). In addition, estrogen could induce KLF10 expression, which partially explains the estrogen-TGF-β/Smad crosstalk (14, 15). Experiments using breast cancer cell overexpressing KLF10 revealed that KLF10 plays a critical role in the TGF-β/Smad pathway mediated anti-proliferative responses (11). In addition, KLF10 induces apoptosis in epithelial cells (16, 17) as well as leukemic or melanoma cells (18, 19) through p53-induced mitochondrial apoptosis pathway, suggesting that KLF10 acts as a tumor suppressor transcription factor.

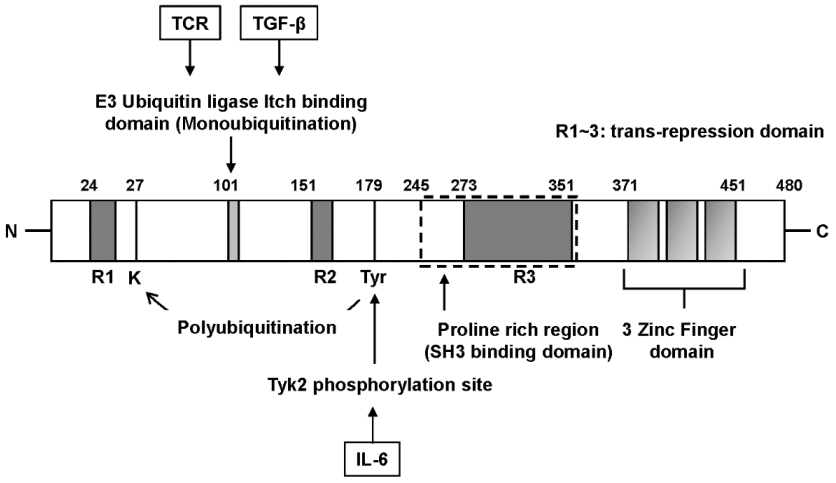

Figure 1. Schematic structure and functional domains of KLF10 protein.

1.3. Immunological roles of KLF10

1.3.1. Various functions of KLF10 in immune system

TGF-β is crucial for differentiation of $Foxp3^+$ regulatory $CD4^+$ T cells (Tregs) (4, 12) and KLF10 is a positive regulator of TGF-β signaling (3). The first evidence on immunological roles of KLF10 unveiled that it is important for the maturation and function of $Foxp3^+$ Tregs (4). Subsequent study showed that KLF10-deficient $CD4^+$ or $CD8^+$ T cells are hyperresponsive to T-cell receptor (TCR) stimulation as proliferation and cytokine production increased (12), suggesting inhibitory activity of KLF10 on TCR-triggered activation and differentiation of T cells. In addition, KLF10-deficient mice were reported to contain increased number of effector memory phenotype of $CD8^+$ T cells expressing TGF-βRII lower (20), which indicates regulatory role of KLF10 on $CD8^+$ T cell homeostasis. A study also found a novel role of KLF10 that IL-12p40 production was up-regulated in KLF10-deficient macrophages under LPS stimulation (21) and the colonic macrophage population was altered as they express a low level of TGF-βRII in KLF10-deficient mice (22). These findings collectively demonstrate that the roles of KLF10 on innate and adaptive immune system are mainly associated with TGF-βR signaling.

1.3.2. KLF10 as a Treg/Th17 balancer

It has been demonstrated that naïve $CD4^+$ T cells could differentiate into $Foxp3^+$ Treg cells under suboptimal TCR/CD28 activation plus TGF-β stimulation (23). KLF10 deficiency leads to a reduced differentiation of $Foxp3^+$ Tregs and impaired immuno-suppressive function of these cells. KLF10 activity is controlled by differential ubiquitination (4, 6). Monoubiquitination by E3 ligase, Itch is necessary for KLF10 to transactivate Foxp3 promoter to enhance Foxp3 transcription (4). On the other hand, polyubiquitination by tyrosine kinase, Tyk2 triggered by IL-6 stimulation block KLF10 nuclear translocation, resulting Th17 differentiation (6). These data suggest KLF10 as a regulatory transcription factor for Treg/Th17 differentiation.

It is, however, still controversial whether KLF10-deficient mice exhibit reduced $Foxp3^+$ Tregs (4, 6) and even, Th17 augmentation in these mice is unreported, questioning the role of KLF10 on Treg/Th17 differentiation. Therefore, exact role of KLF10 in immune cells, Th17 in particular, remains largely unclear and further investigation is in demand.

2. γδ T cells

2.1. General characteristics

αβ T and γδ T cells arise from a common T-cell precursor in the thymus but thymic development, functional differentiation, peripheral distribution and effector functions of γδ T cells are largely different from those of αβ T cells. First, γδ T cells arise before αβ T cells during thymic ontogeny and predominate at early stages of embryonic development with distinct temporal waves of different invariant γδ TCRs (24). Second, αβ T cells exit the thymus in a naïve state and acquire effector function in the periphery, whereas effector fates of a large number of γδ T cells are acquired in the thymus (25, 26). Third, γδ T cells make up a minor fraction of T cells in the secondary lymphoid organs, but are primarily localized and enriched at epithelial tissues such as lung, skin, intestine and genital tract (27, 28). Finally, γδ T cells recognize a broad range of antigens in unusual immunoglobulin-like recognition mode (29, 30). γδ T cells are categorized into innate-like T cells which could respond rapidly to antigens and produce functional cytokines (ex. IFN-γ and IL-17) without the requirement of clonal expansion (31). Moreover, their sensing ability, so called ‘stress-surveillance’, is supported by innate immune receptors such as toll-like receptors (TLRs) expressed on

γδ T cells, which provides unusual co-stimulatory signals (32).

2.2. γδ T cells as innate IL-17 producers

γδ T cells are only a minor subset of T lymphocytes but the major source of innate IL-17 at normal and infectious condition of mice (31, 33). γδ T cells could mature and differentiate to produce IL-17 after antigen encounter, whereas some of them are ready to produce IL-17 without explicit induction of an immune response (31). These 'innate-like' or 'natural' IL-17-producing γδ T cells are present in secondary lymphoid organs such as peripheral lymph node and spleen but preferentially localized in regional (barrier) tissues including dermis, lung and peritoneal cavity (29). Surprisingly, γδ T cells in spleen of wild-type C57BL/6 mice, stimulated with PMA/ionomycin, accounted for 63% of all the IL-17-producing cells (34). Moreover, these γδ T cells are found to make IL-17 within 24 hours after the infection in the absence of clonal expansion (Figure. 2) (35). Natural IL-17-committed γδ T (γδ-17) cells were shown to have characteristics of effector memory cells ($CD44^{hi}CD45RB^{lo}CD62L^{lo}$) with a unique phenotype of $CCR6^{+}NK1.1^{-}CD27^{-}$, consisting of $V\gamma4^{+}$ and $V\gamma6^{+}$ subsets (26, 36).

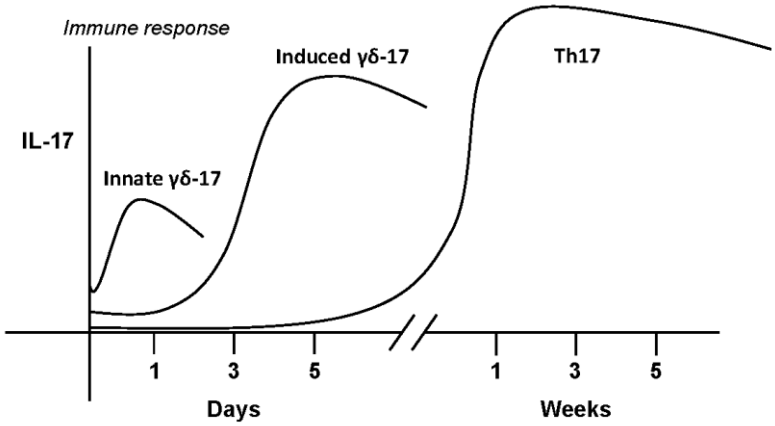

Figure 2. Kinetics of the development of innate and induced γδ-17 cells and of Th17 effector responses.

2.3. Peripheral homeostasis and activation of γδ T cells

Peripheral γδ T cell homeostasis seems to be mainly regulated via non-TCR stimuli consisting of cytokine receptor signals (ex. IL-7, IL-15, IL-12, IL-18, IL-1β and IL-23) by which γδ-17 cells receive homeostatic or activating signals differently (37, 38). Such innate stimuli are more important than TCR ligation for 'innate'-like γδ T cell homeostasis (37, 39).

Like as innate γδ-17 cells, innate or natural IFN-γ-producing γδ T (γδ-IFNγ) cells are also recently described and these natural γδ-IFNγ cells also produce their effector cytokines rapidly compared to conventional γδ T cells that display a phenotype more similar to αβ T cells (38). Recent report shows that natural γδ-17 cells rely exclusively on IL-7 for turnover and survival whereas natural γδ-IFNγ cells mainly depend on IL-15 with utilization of IL-7 if it is abundant (37). Such a homeostatic cytokine-dependency of natural γδ T cells could explain the robust expansion of these cells under lymphopenic condition. *In vitro* IL-7 treatment on γδ T cells was found to enrich for $CD27^-$ γδ-17 cells and promote their IL-17 production (40).

TCR signaling, however, is still necessary for IL-17 production by conventional γδ T cells (35, 41). By recognizing antigens, conventional naïve γδ T cells could produce IL-17 and also express IL-1R and IL-23R through inflammatory cytokine signals that further perpetuate IL-17 production by activated γδ T cells (41). Like as the natural γδ-17 cells, activated γδ-17 cells could produce cytokines rapidly without clonal expansion (31), meaning that 'inducible' γδ-17 cells also contribute the early stage of immune responses rather than inducible Th17 cells did.

2.4. Thymic development of γδ T cells

2.4.1. T-cell receptor signal strength

γδ and αβ T cells share a common thymic progenitor, $CD4^-CD8^-$ double negative (DN), in which TCRγ, TCRδ, and TCRβ rearrangements initiate (42, 43). Quantitative differences in TCR signal strength dictate αβ versus γδ fate decision, irrespective of TCR types. γδ lineage commitment requires strong TCR signaling compared to αβ lineage (Figure. 3) (44, 45). Strong TCR signals leads to inhibition of E3 protein activity through strong induction of Id3 via Erk-Egr1 axis (46, 47). In addition to strong strength of TCR, younger thymus and/or stage-specific commitment factors such as DN stage 2, Sox13 and IL-7R signaling also promote a γδ cell fate (24, 48, 49). It is recently suggested that γδ-17 cells are mainly of fetal and/or neonatal thymic origin (24).

As aforementioned, the effector fate of most γδ T cells is determined during thymic development in which TCR signaling plays a critical role, but the exact extent and mode of action is still unclear. Nevertheless, majority of previous reports demonstrated that thymic ligand encounter determines γδ T cell effector fate (29, 50). γδ thymocytes, that have not engaged ligand, develop into γδ-17 cells whereas ligand encounter drives those adopting into

γδ-IFNγ cells (25). Along these lines, the expression of CD27 on T cells is induced by TCR stimuli and $CD27^+$ γδ T cells produce IFN-γ, whereas $CD27^-$ γδ T cells make IL-17 (26).

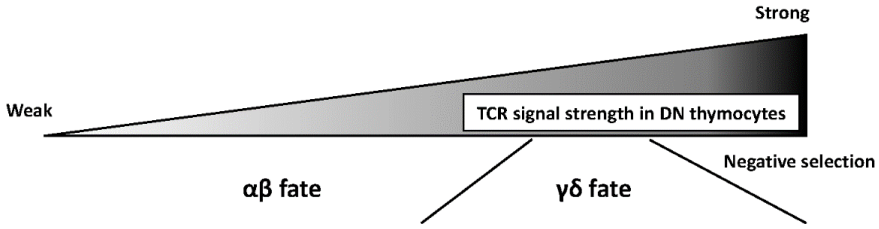

Figure 3. αβ versus γδ fate decision by TCR signal strength.

2.4.2. Cytokine-mediated signals

It is clear that Th17 differentiation requires TGF-β, IL-6, IL-1 and IL-23 in presence of TCR engagement (51). Similarly, γδ-17 cell development in the thymus is controlled by TGF-β (52). Mice deficient for TGF-β or Smad3 showed impaired γδ-17 cell development (52). However, a role of IL-

6 in γδ-17 cell generation is vague, since decrease or un-alteration of IL-17 production by γδ T cells were reported (52-54). Nevertheless, other reports in that signalings through Stat3 and IRF-4 are not required for γδ-17 cell development suggest IL-6 signaling is dispensable (55, 56). Moreover, IL-23 signaling is not required for γδ-17 cell development and maintenance (53), even though γδ-17 cells express IL-23R and this signaling triggers their expansion (41, 57).

2.4.3. Transcription factors

NFκB family members, RelA and RelB are differently implicated in lymphotoxin signaling-mediated regulation of γδ-17 cell development (58-60). RelB acts downstream of lymphotoxin-βR and enhance expression of RoRγt, a master transcription factor of IL-17 cytokine production (58, 59). Interestingly, mice deficient for RelB exhibit defected maturation of dendritic epithelial T cells, but IFN-γ production by γδ T cells in response to PMA and ionomycin was normal in these knockout mice (58, 59). These suggest the importance of NFκB-signaling in the development of γδ T cells.

Like as Th17 cells, γδ-17 cell development also requires RoRγt, confirmed by the absence of γδ-17 cells in RoRγt-deficient mice (24). γδ-17

cell development seems to be more dependent transcriptional regulation but less on conventional TCR signaling, which is consistent with ligand-independent TCR engagement for γδ-17 cells. It has been suggested that gene regulatory network consisting SOX4, SOX13, TCF1 and LEF1 programs IL-17 producing capacity of γδ T cells (61-63). Two requisite γδ-17 cell-specific genes *Rorc* and *Blk* are directly regulated by SOX4 and SOX13 which TCF1 and LEF1 inhibit, resulting into γδ-IFNγ cell-related genes (62).

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II. Introduction

Recent immunological studies on Kruppel-like factor 10 (KLF10) revealed a critical role as a transcription factor containing zinc finger DNA-binding domains in the induction of and balance between Foxp3^{+} regulatory T cells (Treg) and IL-17-producing T helper cells (Th17) (1-3). Stimulation with T-cell receptor (TCR) or TGF-β induces KLF10 in CD4 T cells transiently, which in turn suppresses TCR signaling but enhances TGF-β/Smad signaling. Therefore, KLF10-deficient CD4 T cells, hyper-activated by *in vitro* TCR stimuli, were less differentiated into Foxp3^{+} Tregs under Treg-polarizing condition (1, 2). Th17 cell differentiation, on the other hand, was promoted by KLF10 deletion under Th17-polarizing condition (3). Nonetheless, *in vivo* function of KLF10 in immune cells is unclear since the alteration of Tregs in naïve KLF10 KO mice is still controversial (1-3) and Th17 cells in these mice have not been reported. In addition, its function in other T cell subsets has been largely unknown.

At steady-state, γδ T cells are only a minor subset of T lymphocytes but the major source of IL-17 (4-6). These 'innate-like' IL-17-committed CD27^{-} γδ T (γδ^{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 $\gamma\delta^{27-}$ cells stimulated with cytokines such as IL-7 or IL-1β plus IL-23 rapidly produce IL-17 in the absence of TCR activation (8, 9). Although TCR signal is required for their thymic development (10-12), the peripheral homeostasis and activity of $\gamma\delta^{27-}$ cells is weakly dependent on TCR ligation while promoting a strong activation of $\gamma\delta^{27+}$ cells (8, 13-16). $\gamma\delta^{27-}$17 cells consist of $V\gamma4^{+}$ and $V\gamma6^{+}$ subsets (Tonegawa nomenclature) with a unique phenotype (17), mostly memory cell-like $CD44^{hi}CD62L^{lo}CD127^{hi}$ (5, 9) and $CCR6^{+}NK1.1^{-}$ (18). These γδ-17 cells develop predominantly from early embryonic stage up to shortly after the birth (19). Notably, maturation of $V\gamma4^{+}$ $\gamma\delta^{27-}$ cells occurs in the neonatal thymus (20). Evidences have shown that thymic $\gamma\delta^{27-}$ cell development is strictly regulated by TCR strength with both TCR-dependent and –independent pathways, in which exogenous stimuli and/or intrinsic programming of a gene regulatory network consisting of diverse transcription factors are involved (21, 22). It is plausible that a weak TCR strength is required for the IL-17-producing capacity of $\gamma\delta^{27-}$ cells, which seems a ‘default’ property of uncommitted early thymocytes (10, 23). It is also reported that SOX13 is an important transcription factor that positively regulates $V\gamma4^{+}$ $\gamma\delta^{27-}$ cell development (20). However, other regulatory factors of transcriptional network that control their development and function, especially response to TCR stimulation, still remain incomplete.

Here, I identify KLF10 as a critical transcription factor, of which $V\gamma4^+$ $\gamma\delta^{27-}$ cell development in neonatal thymus and their peripheral homeostasis in adulthood are negatively regulated. I found a selective enlargement of IL-17-committed $V\gamma4^+$ $\gamma\delta^{27-}$ cells, but not of other IL-17-producing αβ T cells in KLF10-deficient mice. KLF10 is induced either by TCR stimulation or by cytokines (IL-7 or IL-1β plus IL-23) independently of TCR, which in turn negatively regulates $\gamma\delta^{27-}$ cell responsiveness to these stimuli, suggesting that the development and function of $V\gamma4^+$ $\gamma\delta^{27-}$ cells are dependent on a transcriptional control by KLF10.

III. Materials and Methods

1. Mice

KLF10-deficient mice with C57Bl/6 (B6) background were kindly provided by Dr. Woon Kyu Lee (Inha University, Incheon, South Korea) (24). B6.Rag1-deficient mice and B6.CD45.1 congenic mice were all obtained from The Jackson Laboratory. All animals were bred and maintained under specific pathogen-free conditions at Institute of Laboratory Animal Resource Seoul National University. They were treated in accordance with institutional guidelines that approved by the Institutional Animal Care and Use Committee (SNU-140930-4-1).

2. Cell preparation

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

3. Flow cytometry

Single-cell suspensions were firstly blocked with anti-CD16/32 (93; eBioscience) and then stained with antibodies at 4 °C for 20 min in staining buffer (1 x PBS containing 0.1% bovine serum albumin and 0.1% sodium azide). For intracellular staining, the cell were stimulated for 4 h with 50 ng/ml phorbol myristate acetate (PMA; Sigma-Aldrich) and 750 ng/ml ionomycin (Sigma-Aldrich) in brefeldin A (BD Biosciences). The cells were then fixed, permeabilized with the BD Cytofix/Cytoperm Kit according to the manufacturer's instruction (BD Biosciences) and stained intracellularly for IL-17 and IFN-γ. The cells were examined using a FACSCantoII (BD Biosciences) and data were analyzed with FlowJo software (TreeStar). Antibodies used for flow cytometric analyses were as follows (all from BD Biosciences, Biolegend or eBioscience). Fluorochrome-labelled mAbs against mouse γδTCR (GL3), CD3ε (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), Vγ1 (2.11), Vγ4 (UC3-10A6), IL-17 (TC11-18H10), IFN-γ (XMG1.2), CD4 (RM4-5), CD8α (53-6.7), TCRβ (H57-597), Foxp3 (MF23), CD1d-tet (L363), and Qa-2 (695H1-9-9).

4. Cell sorting and isolation

Pooled pLN and splenocytes were firstly blocked with anti-CD16/32 and then negatively selected with anti-TCRβ biotin, anti-CD45R biotin (RA3-6B2) and anti-CD11b (M1/70) plus MagniSort^{TM} Streptavidin Negative Selection Beads according to the manufacturer's instruction (eBioscience). The negatively selected cells were then stained with fluorochrome-labelled anti-γδTCR, anti-CD27 and/or anti-Vγ4. Total γδ T cells or γδ T cell subsets ($V\gamma4^{-}\gamma\delta^{27+}$, $V\gamma4^{+}\gamma\delta^{27+}$, $V\gamma4^{-}\gamma\delta^{27-}$ and $V\gamma4^{+}\gamma\delta^{27-}$) were sorted by a FACSAriaII (BD Biosciences) with a purity of at least 98%. Alternatively, total γδ cells were manually isolated by negative selection as described previously (25) with modification. Biotin-conjugated mAbs (all from eBioscience) were used as following; TCRβ, CD4, CD8α, CD45R, CD11b (M1/70), CD11c, Ly-6G (RB6-8C5), TER-119 (TER-119), and CD49b (DX5). CD4 and CD8 αβ T cells were also negatively isolated by Mouse T lymphocyte Enrichment Set (BD Biosciences).

5. Cell culture

Cells were cultured in RPMI 1640 containing 10% (vol/vol) FBS, 50 μM 2-ME, 1% (vol/vol) nonessential amino acids, 10mM HEPES and 1% (vol/vol) antibiotics/antimycotic solution all from Invitrogen). For TCR stimulation, the

cells were incubated for 3 d on plate-bound anti-CD3ε antibody (0.1, 1, or 10 μg/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-1β (10 ng/ml; PeproTech) and IL-23 (20 ng/ml; PeproTech) were added to the medium.

6. Bone marrow chimeras

B6.CD45.1 mice were lethally irradiated with 900 cGy and intravenously transferred with $5x10^6$ of B6.CD45.2 wild-type or B6.CD45.2 KLF10-deficient bone marrow cells. For mixed bone marrow chimeras, B6.CD45.1/2 mice were lethally irradiated with 900 cGy and intravenously transferred with $5x10^6$ B6.CD45.1 wild-type mixed 1:1 with $5x10^6$ B6.CD45.2 KLF10-deficient bone marrow cells. Recipient mice were sacrificed and analyzed at least 12 wks later.

7. Homeostatic expansion

Single-cell suspension of pLN cells obtained from B6.CD45.1 wild-type and B6.KLF10 KO mice were stained with CTV (Cell Trace Violet; Invitrogen) and injected intravenously into Rag-1-deficient mice at 1:1 ratio ($1x\ 10^6$ cells each). After 5 d, pLNs from the recipient mice were collected and examined.

8. RNA extraction and real-time qPCR

RNAs from cells were isolated using a Qiagen Rneasy kit. After reverse transcription into cDNA, a StepOnePlus real-time PCR system (Applied Biosystems) with iTaq SYBR Green Supermix (Applied Biosystems) and relative expression is displayed in arbitrary units or as percent of maximum expression, normalized to *Eef1a1* (encoding eukaryotic translation elongation factor 1α1; called '*Efa1*' here) via ΔΔCt method. The following primers were used: *Efa1* forward, 5′-TCCACCGAGCCACCATACA-3′, reverse, 5′-CCAACCAGAAAT-TGGCACAA-3′; *Klf10* forward, 5′-ACCCAGGGTGTGGC-AAGAC-3′, reverse, 5′-AGCGAGCAAACCTCCTTTCA-3′; *T-bet* forward, 5′-TCGTGGAGGTGA-ATGATGGA-3′, reverse, 5′-TGAGTGATCTCTGCGTTCT-GGTA-3′; *RoRγt* forward, 5′-TCAGCGCCCTGTGTT TTTCT-3′, reverse, 5′-CA-AATTGTATT-GCAGATGTTCCA-3′; *Sox13* forward, 5′-CTGCCACCTGGGTT-ACTTTGA-3′, reverse, 5′-GAGTGGCGTGATGAACATGTG-3′.

9. Statistical analysis

Prism software (GraphPad) was used for all statistical analysis. All quantitative data are shown as mean ± standard deviation (s.d.) unless otherwise indicated. The two-tailed, paired *t*-test was used for bone marrow chimeras. The

two-tailed, unpaired *t*-test or two-way ANOVA followed by a Bonferroni posttest was used for all other data sets.

IV. Results

1. KLF10 deficiency induces not αβ but γδ T cell expansion

First, I investigated the frequencies and absolute numbers of γδ, $CD4^+$ and $CD8^+$ T cells in the pLN, spleen, lung and peritoneal cavity of B6.KLF10-deficient (KO) mice, and observed that γδ T cells, but not conventional $CD4^+$ and $CD8^+$ αβ T cells, are significantly increased in pLN and lung (Fig. S1). Interestingly, this increase was largely attributable to higher $CD27^-$ γδ T ($γδ^{27-}$) cells; The frequency of $γδ^{27-}$ cells among total γδ T cells (Fig. 1*A*) and their absolute nubmer in pLN of KO mice (Fig. 1*B*) were significantly increased. To make this observation clear, I examined γδ T cells in each LN (cervical, axillary, brachial and inguinal and mesenteric) and confirmed the same, except mesenteric LN (Fig. *S2A*) in which 'innate-like' IL-17 producing γδ T cells (γδ-17) are absent as reported (5). Consistent with previous findings that CD27 expression distinguishes two γδ T cell subsets with different expression of CD44, CD62L, NK1.1 and CCR6 (11, 26), pLN $γδ^{27-}$ cells showed (Fig. S2*B*; Blue contour) $CD44^{hi}CD62L^-$ effector memory phenotype with lower NK1.1 and higher CCR6 expression compared to $γδ^{27+}$ cells (red contour); However, such a distinct

phenotype bewteen $\gamma\delta^{27-}$ and $\gamma\delta^{27+}$ cells was not obvious within mLN (Fig. S2*B*). Apperently, KLF10 KO mice showed higher frequency of $CD44^{hi}CD62L^{-}$ γδ T cells in the pLN, spleen, lung and peritoneal cavity than wild type (WT) mice, whereas $CD44^{hi}CD62L^{-}$ $CD4^{+}$ T and $CD8^{+}$ T cells showed at normal frequencies (Fig. S2 *C* and *D*). These data suggested that $\gamma\delta^{27-}$ cells were present in high proportion and number in KO mice.

To deterimine whether KLF10 deletion influences homeostatic proliferation of γδ T cells, I transferred CTV-labeled pLN cells obtained from CD45.1 WT and CD45.2 KO mice into Rag-1-deficient mice and analyzed expansion of γδ T cells. Proliferation of KLF10 KO γδ T cells was superior to that of WT cells at which the ratio as high as 4 times (Fig. 1*C*). Furthermore, KLF10 KO $\gamma\delta^{27-}$ cells, but not $\gamma\delta^{27+}$ cells, had a competitive advantage of homeostatic expansion over their WT counterparts (Fig. 1*D*), whereas conventional αβ T cells from both strains showed similar proliferation pattern (Fig. S2 *E* and *F*). These data suggested that KLF10 deficiency promotes peripheral $\gamma\delta^{27-}$ cell expansion.

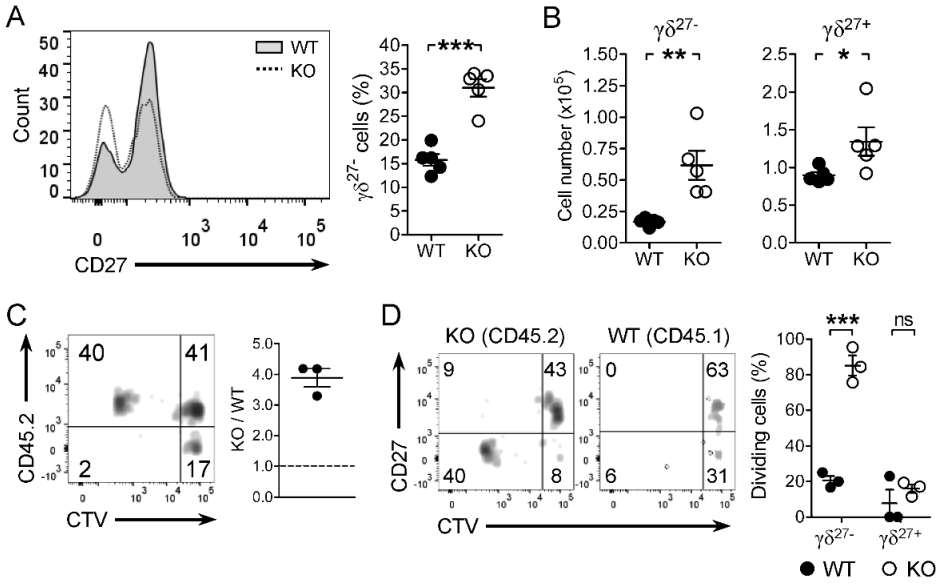


Figure 1. KLF10 deficiency promotes γδ^{27-} cell expansion. Single cells, prepared from pLN of WT and KO mice, were gated on total γδ T cells and examined by flow cytometry. (*A*) Overlayed histogram of CD27 expression on γδ T cells (left) and frequency of γδ^{27-} cells (right). (*B*) Absolute number of γδ^{27-} (left) and γδ^{27+} (right) cells are shown. (*C*) Homeostatic expansion (left) and the ratio (right) of CD45.1 WT or CD45.2 KO γδ T cells in pLN from Rag-1-deficient mice that had been intravenously administered with CTV-labeled WT or KO pLN cells at ratio of 1:1 for 5 d were examined using flow cytometry after gating on

CTV^+ γδ T cells. (*D*) Homeostatic expansion (left) and frequency of dividing cells (right) of $γδ^{27-}$ and $γδ^{27+}$ cells from CD45.1 WT or CD45.2 KO γδ T cells, obtained as in *C*. Numbers in quadrants of the plot (*C, D*) indicate percent of cells in population. (*B-D*) Each symbol represents an individual mouse and the data shown as the mean ±s.d. ns, non-significant; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$. Data are representative of (*A, B*) at least three independent experiments or (*C, D*) two independent experiments with three to four mice per group.

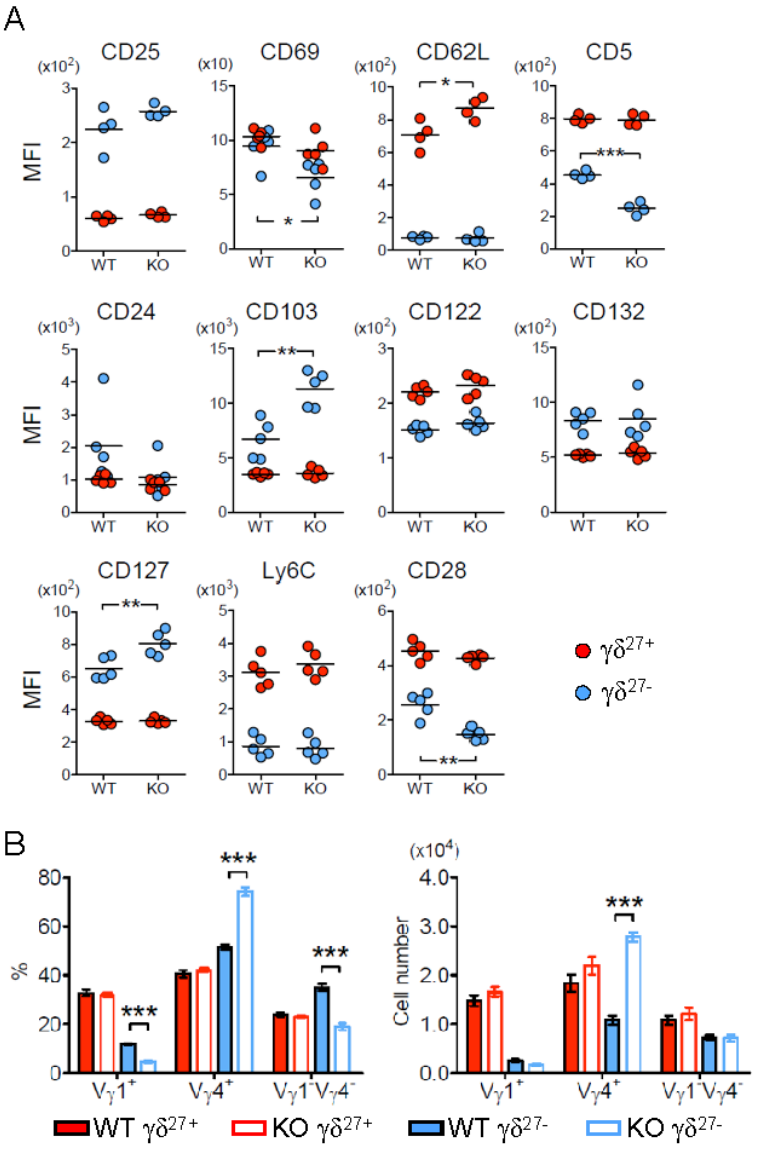
2. *KLF10 deficiency preferentially expands CD5^{int}CD127^{hi} Vγ4^{+} γδ^{27-} cells*

To characterize the phenotypic changes of γδ^{27-} cells in KLF10-deficient condition, I examined expression of surface proteins involved in innate and adaptive features of γδ T cells. As shown in Fig. 2*A*, KO γδ^{27-} cells showed lower expression of CD69, CD5 and CD28 but higher CD103 and CD127 than those of WT γδ^{27-} cells. I also examined Vγ1 and Vγ4 distribution of γδ^{27-} cells and found an increase in frequency of Vγ4^{+} cells among γδ^{27-} cells and their abolute number in KO mice (Fig. 2*B*). Given the fact that Vγ4^{+} γδ^{27-} cells had lower CD5 and CD28 but higher CD127 and CD103 expression than Vγ4^{-} γδ^{27-} cells did (Fig. S3*B*), the alteration of cell surface proteins on γδ^{27-} cells (Fig. 2*A*) is likely to attribute to the augmented percentage of Vγ4^{+} cells (Fig. 2*B*). These results indicated that Vγ4^{+} γδ^{27-} cells were selectively expanded by KLF10 deletion.

CD5, a stable indicator of TCR strength and CD127, IL-7 receptor-α (IL-7Rα) showed two peaks (high and low) of expression on γδ^{27-} cells (Fig. S3*A*), suggesting that γδ^{27-} cells are heterogenous populations dependant on TCR and IL-7 stimulation. To gain the insight into the γδ^{27-} cells, I investigated the expression pattern of these two molecules by which γδ^{27-} cells are segregated into two subsets; CD5^{lo}CD127^{hi} and CD5^{hi}CD127^{lo} (Fig. S4*A*). Intriguingly,

$CD5^{hi}CD127^{lo}$ $\gamma\delta^{27-}$ cells showed a similar expression of surface proteins with $\gamma\delta^{27+}$ cells, but not $CD5^{lo}CD127^{hi}$ cells (Fig. 4*B*). In addition, Vγ1 and Vγ4 distribution closely resembled that of $\gamma\delta^{27+}$ cells compared to $CD5^{lo}CD127^{hi}$ counterpart (Fig. S4*C*), suggesting that despite the $CD5^{hi}CD127^{lo}$ cells belong to $\gamma\delta^{27-}$ cell population, they seemingly have a very similar ontogeny and function with $\gamma\delta^{27+}$ cells. I also observed that the frequency of $CD5^{hi}CD127^{lo}$ among $\gamma\delta^{27-}$ cells was very low (3.4%) in young mice (2 wks old) but greatly increased (45.5%) in the adult (8 wks old; Fig. S4*D*). This augmentation appeared among mature ($CD24^{lo}$) $\gamma\delta^{27-}$ cells of pLN (Fig. S4*E*) and thymus (Fig. S4*F*) accompanying with higher fraction of $V\gamma1^{+}$ cells. There was an increase of $V\gamma1^{+}$ cells among immature ($CD24^{hi}$) $\gamma\delta^{27-}$ cells in adult thymus in accord with the prevous reports (27), possibly reflecting that the generation of $CD5^{hi}CD127^{lo}$ $\gamma\delta^{27-}$ cells is not restricted to embryonic and neonatal thymic waves. These data suggested that $CD5^{hi}CD127^{lo}$ cells, mainly originated from adult thymus and became predominant among pLN $\gamma\delta^{27-}$ cells, with skewed expansion of $V\gamma1^{+}$ cells. Moreover, the number of $CD5^{hi}CD127^{lo}$ $\gamma\delta^{27-}$ cells in pLN did not differ between WT and KO mice, whereas $CD5^{lo}CD127^{hi}$ $\gamma\delta^{27-}$ cells were significantly higher in KO mice (Fig. S4*A*). Collectively, these results suggested that KLF10 regulates $CD5^{lo}CD127^{hi}$ cells, which constitute the majority of pLN $\gamma\delta^{27-}$ cells in young mice.

Next, I further found that the number of $CD5^{int}CD127^{hi}$ $\gamma\delta^{27-}$ cells, distinguished from $CD5^{lo}CD127^{hi}$ $\gamma\delta^{27-}$ cells (Fig. 2*C*), was increased with a definite skewing toword $V\gamma4^{+}$ cells in KO mice (Fig. 2*D* and Fig. S5). This observation was confirmed by higher frequency of $CD5^{int}V\gamma4^{+}$ among $CD5^{lo}CD127^{hi}$ $\gamma\delta^{27-}$ cells in KO mice (Fig. 2*E*). $\Gamma\delta^{27-}$ cells expressed lower CD5 but higher CD127 than $\gamma\delta^{27+}$ cells did (Fig. 2*F*), consistent with the previous findings that $\gamma\delta^{27-}$ cells require relatively weak TCR signal strength for their thymic development and are expanded by IL-7 signaling (9, 10). It is noting that each Vγ sub-population has a differential expression of CD5 regardless of the level of CD27 (Fig. 2*F*), suggesting that different Vγ subsets have distinct requirement of TCR stregnth. Most of all, $V\gamma4^{+}$ $\gamma\delta^{27-}$ cells showed the lowest level of CD5 (Fig. 2*F*), which was augmented by KLF10 deficiency (Fig. 2*E*). Collectively, these results indicate that the absence of KLF10 spontaneously expanded relatively higher TCR strength ($CD5^{int}$) bearing $CD127^{hi}V\gamma4^{+}$ $\gamma\delta^{27-}$ cells that would be otherwise $CD5^{lo}$ in normal condition.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CD25
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CD24
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γδ27-
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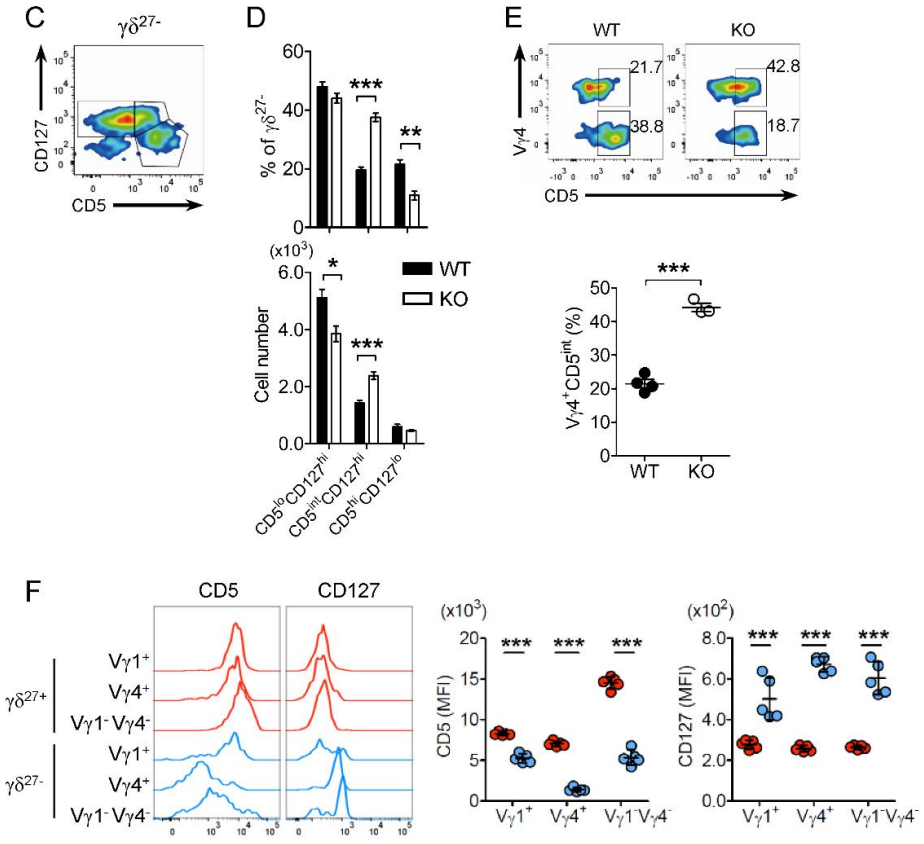


Figure 2. KLF10 deficiency preferentially expands CD5^{int}CD127^{hi} Vγ4^{+} γδ^{27-} cells. (*A*) Mean fluorescence intensity (MFI) of each molecule on γδ^{27-} (blue) and γδ^{27+} (red) cells in pLN from WT and KO mice, examined using flow cytometry. (*B*) Frequency (left) and absolute number (right) of Vγ1^{+}, Vγ4^{+}, Vγ1^{-}Vγ4^{-} cells

among $\gamma\delta^{27-}$ and $\gamma\delta^{27+}$ cells in pLN as in *A*. (*C*) Expression pattern of CD5 and CD127 on $\gamma\delta^{27-}$ cells in pLN from WT mice. $\gamma\delta^{27-}$ cells are gated into $CD5^{lo}CD127^{hi}$, $CD5^{int}CD127^{hi}$ and $CD5^{hi}CD127^{lo}$ subsets. (*D*) Frequency of three subsets (gated as in *C*) among total $\gamma\delta^{27-}$ cells (upper) and absolute number of those (below) in pLN. NAÏVE Representative plot showing pLN $V\gamma4^{+}CD5^{int}$ $\gamma\delta^{27-}$ cells (upper) and the frequency of $V\gamma4^{+}CD5^{int}$ cells among pLN $CD5^{lo}CD127^{hi}$ $\gamma\delta^{27-}$ cells (below). Number adjacent to the gating indicates the percent of each population. (*F*) Half-offset histogram (left) and MFI (right) of CD5 and CD127 on the each γδ subsets as indicated. (*A, E, F*) Each symbol represents an individual mouse. (*A, B, D, E* and *F*) Data indicate the mean ±s.d. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$. Data are representative of at least three independent experiments with four to five mice.

3. Vγ4^{+} γδ^{27-}17 cells are increased in KLF10-deficient mice

As reported previously that γδ^{27-} cells are the major source of innate IL-17 (4-6), I examined intracellular IL-17A level of γδ^{27-} cells from KO mice. Consistent with the abundance of Vγ4^{+} γδ^{27-} (Fig. 2*B*) and Vγ4^{+}CCR6^{+} cells (Fig. S3*C*), known as IL-17-committed γδ T cells, considerably greater γδ^{27-} cells expressing IL-17A were observed in the pLN after stimulation with PMA plus ionomycin (Fig. 3 *A* and *B*). This was further confirmed by the increased number of IL-17^{+}CCR6^{+} or IL-17^{+}Vγ4^{+} γδ T cells in pLN of KO mice (Fig. S6*A*). Moreover, KO mice exhibited high IL-17 production by IL-17^{+}Vγ4^{+}CCR6^{+} γδ T cells (Fig. 3*C*), indicating that KLF10 constrains IL-17A production in IL-17-committed Vγ4^{+} γδ^{27-} cells. Thus, our results suggest KLF10 negatively regulates the size of the innate-like Vγ4^{+} γδ^{27-}17 population and their IL-17 production.

I next examined the expression of CD5 and CD127 on γδ^{27-}17 cells and found that IL-17^{+} γδ^{27-} cells express low CD5 coincident with high CD127 when compared to IFN-γ^{+} γδ^{27-} cells (Fig. S6*B*). This result allows us to define CD5^{lo}CD127^{hi} γδ^{27-} cells as innate-like γδ-17 cells. By segregating γδ^{27-} cells with same manner in Fig. 2*C*, I confirmed that IL-17 production is restricted to CD5^{lo}CD127^{hi} and CD5^{int}CD127^{hi} cells (Fig. 3*D*), mainly consisting of Vγ4^{+} cells and Vγ1^{-}Vγ4^{-}, presumably Vγ6^{+} cells (11, 28), respectively (Fig. S5).

Furthermore, KO mice had a high IL-17^{+}CD5^{int}CD127^{hi} cells which are Vγ4^{+} cells dominantly (Fig. 3*D* and Fig. S5). Thus, our results indicate that KLF10 selectively constrains IL-17-committed CD5^{int}CD127^{hi}Vγ4^{+} $\gamma\delta^{27-}$ cells.

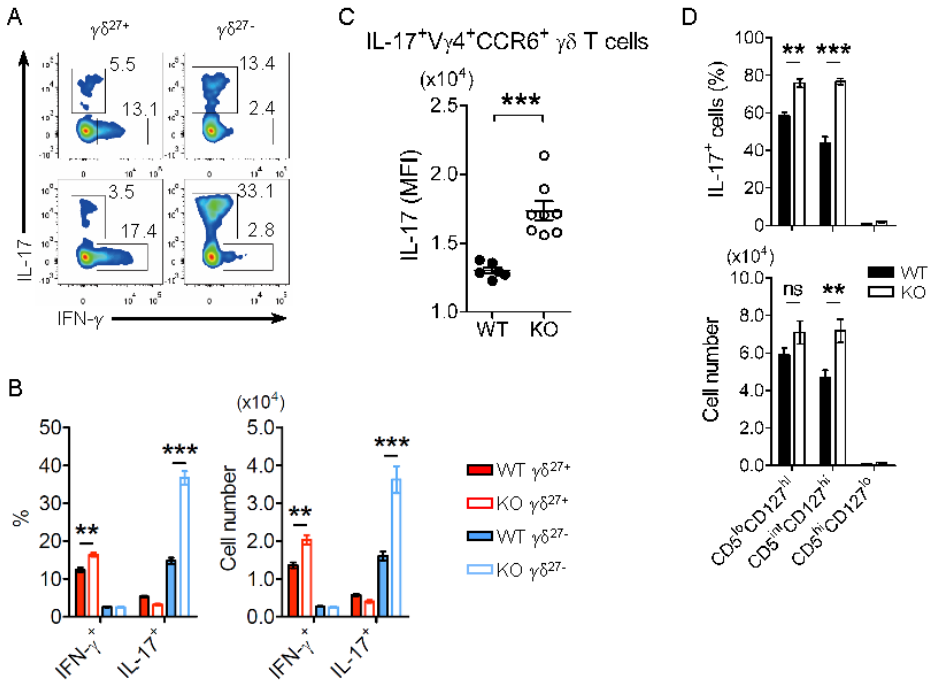


Figure 3. IL-17^{+}Vγ4^{+} γδ^{27-} cells are increased in KLF10-deficient mice. pLN cells, obtained from WT and KO mice, were stimulated with PMA plus ionomycin in the presence of brefeldin A for 5 h. (*A*) IL-17 or IFN-γ producing cells, gated on γδ^{27+} or γδ^{27-} cells, were examined after intracellular staining by using flow cytometry. Number adjacent to the gating indicates the percent of each population. (*B*) Frequency of IL-17^{+} or IFN-γ^{+} cells among γδ^{27+} or γδ^{27-} cells (left) and absolute number (right) are shown. (*C*) MFI of IL-17 in pLN IL-

$17^+V\gamma4^+CCR6^+$ γδ T cells. (*D*) Frequency of IL-17^+ cells among $CD5^{lo}CD127^{hi}$, $CD5^{int}CD127^{hi}$ and $CD5^{hi}CD127^{lo}$ $\gamma\delta^{27-}$ cells (upper) and absolute number (below) are shown. $\gamma\delta^{27-}$ cells are gated into $CD5^{lo}CD127^{hi}$, $CD5^{int}CD127^{hi}$ and $CD5^{hi}CD127^{lo}$ subsets as in Fig. 2*C*. (*C*) Each symbol represents an individual mouse, and (*B*-*D*) the data indicate the mean ±s.d. ns, non-significant; ** $P \leq 0.01$; *** $P \leq 0.001$. Data are representative of (*A*, *B*) at least three independent experiments with five mice or two independent experiments with (*C*) eight or (*D*) four mice.

4. KLF10 regulates γδ^{27-} cell responsiveness to cytokine and TCR stimuli

Given that IL-7R signaling is critical for γδ T cell homeostasis, especially γδ^{27-}17 cells (9), I sought to examine the response of pLN γδ T cells treated with IL-7 and observed that KO Vγ4^{+} and Vγ1^{-}Vγ4^{-} γδ^{27-} cells expanded more than their WT counterparts (Fig. 4*A*). This is confirmed by IL-7-mediated proliferation of γδ^{27-} cells (Fig. 4*B*), suggesting that KLF10 restricts γδ^{27-} cell responsiveness to IL-7, irrespective of Vγ4 expression and independently from its selective effects on Vγ4^{+} γδ^{27-} cell homeostasis (Fig. 2*B*). Next, I also examined γδ T cell responsiveness to IL-1β plus IL-23, inflammatory milieu, which are well known to induce IL-17 production from γδ-17 cells (8). Consistent with the results of IL-7 signaling, γδ^{27-} cells from KO mice stimulated with IL-1β plus IL-23 expanded better than their WT counterparts (Fig. 4*B*), indicating KLF10 also inhibits γδ^{27-} cell responsiveness to IL-1β plus IL-23.

I also assessed the production of IL-17 in γδ^{27-} cells under the same condition. In the same line with the enhanced proliferation (Fig. 4*B*), there was greater frequency of IL-17^{+} expanding cells among KO γδ^{27-} cells regardless of Vγ4 expression (Fig. 4*C*). Furthermore, *Klf10* expression in γδ T cells stimulated with IL-7 or IL-1β plus IL-23 was upregulated (Fig. 4*D*), indicating that IL-7 or IL-1β

plus IL-23 induce KLF10, which in turn could act as a negative feedback signal to limit $\gamma\delta^{27-}$ cell responsiveness. Collectively, these data suggest that KLF10 inhibits IL-17-committed $\gamma\delta^{27-}$ cell expansion and their IL-17 production in response to either homeostatic or inflammatory signaling.

In contrast to cytokine tested above, TCR stimulation induced a weak proliferative response of $\gamma\delta^{27-}$ cells, particularly $V\gamma4^{+}$ $\gamma\delta^{27-}$ cells compared to $\gamma\delta^{27+}$ cells (Fig. S7*A*). Most of all, IL-17 production by the expanding $\gamma\delta^{27-}$ cells was rarely induced by TCR stimulation (data not shown), indicating that it has a minor role on peripheral expansion and IL-17 production of $V\gamma4^{+}$ $\gamma\delta^{27-}$ cells. In the same line, although KLF10 was induced by TCR stimulation (Fig. S7*B*), there was only a slight increase of proliferation by KLF10 deficiency (Fig. S7*A*), indicating a negligible role of KFL10 for the TCR responsiveness of γδ T cells. Therefore, KLF10 differently regulates peripheral $\gamma\delta^{27-}$ cell responsiveness to cytokines and TCR stimuli.

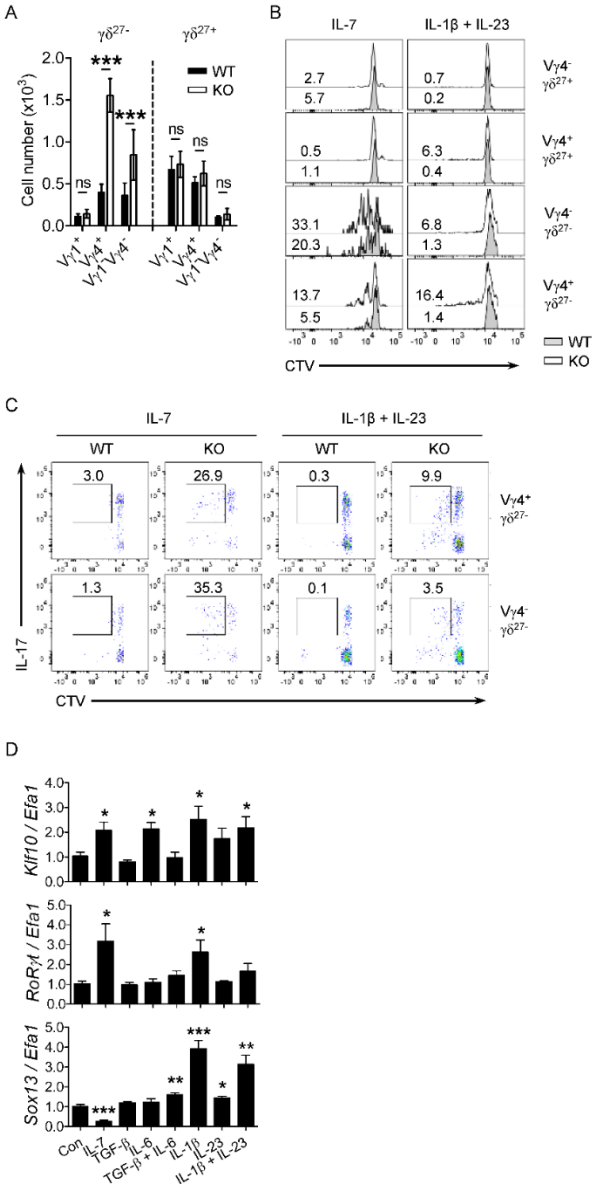

A
γδ27-
γδ27+
Cell number (x10^3)
WT
KO
***
ns
Vγ1+
Vγ4+
Vγ1Vγ4-
B
IL-7
IL-1β + IL-23
2.7
5.7
0.7
0.2
Vγ4-
γδ27+
0.5
1.1
6.3
0.4
Vγ4+
γδ27+
33.1
20.3
6.8
1.3
Vγ4-
γδ27-
13.7
5.5
16.4
1.4
Vγ4+
γδ27-
CTV
WT
KO
C
IL-7
IL-1β + IL-23
WT
KO
3.0
26.9
0.3
9.9
Vγ4+
γδ27-
1.3
35.3
0.1
3.5
Vγ4-
γδ27-
IL-17
CTV
D
Klf10 / Efa1
RoRγt / Efa1
Sox13 / Efa1
Con
IL-7
TGF-β
IL-6
TGF-β + IL-6
IL-1β
IL-23
IL-1β + IL-23

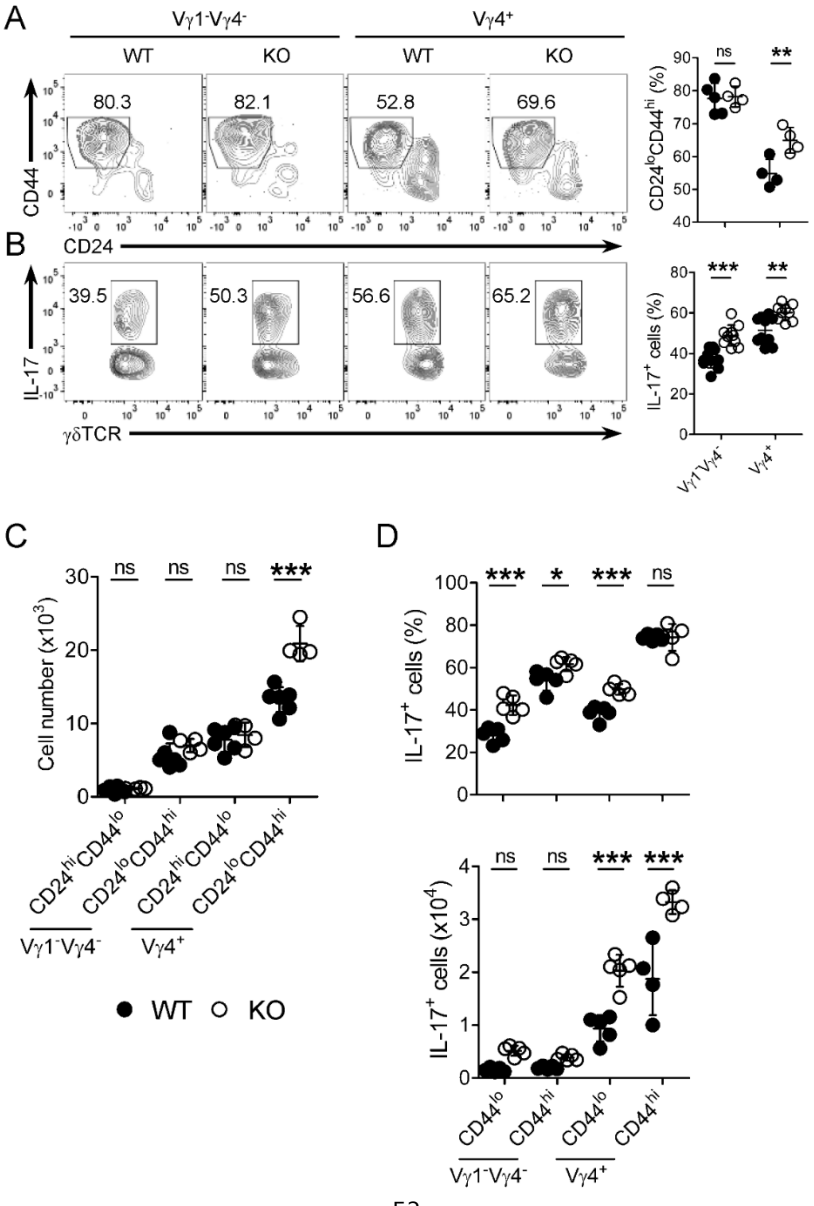
Figure 4. KLF10 inhibits IL-17 production in γδ^{27-} cells. (*A*) Absolute numbers of each γδ subset within WT or KO pLN cells cultured with IL-7 (20 ng/ml) for 5 d. (*B, C*) Purified γδ T cells from WT and KO mice, were labeled by CTV and cultured with IL-7 (20 ng/ml) for 5 d or with IL-1β (10 ng/ml) plus IL-23 (20 ng/ml) for 3 d and then analyzed by flow cytometry. (*B*) Half-offset histogram of each γδ subsets. Number indicates the percent of dividing cells. (*C*) Representative pseudo-dot plot showing IL-17 production by dividing cells within Vγ4^{+} γδ^{27-} and Vγ4^{-} γδ^{27-} cells. Number adjacent to the gating indicates the percent of cell population. (*D*) Real-time PCR analysis of *Klf10*, *RoRγt* and *Sox13* mRNA expression by purified γδ T cells cultured with IL-7 (20 ng/ml), TGF-β (10 ng/ml), IL-6 (20 ng/ml), IL-1β (10 ng/ml) and IL-23 (20 ng/ml) for 3 hours, normalized to *Efa1* expression. (*A*, *D*) the data indicate the mean ±s.d. ns, non-significant; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$. Data are representative of more than three independent experiments with (*A*) at least three mice or (*B-D*) ten to twenty mice.

5. Maturation of Vγ4^{+} γδ^{27-}17 cells was enhanced in neonatal thymus

Given the fact that there was an increased number of Vγ4^{+} γδ^{27-}17 cells in pLN of KO mice (Fig. 3 *A*-*D*), I sought to determine whether their development in the thymus was changed. As recently reported (20), thymic Vγ4^{+} γδ^{27-} cells matured during late neonatal stages (~ d 7 after the birth) (Fig. S8*A*) and approximately 50 % of neonatal thymic Vγ4^{+} γδ^{27-} cells from WT mice had a mature phenotype (CD24^{lo}CD44^{hi}). These mature Vγ4^{+} γδ^{27-} cells were increased in KO condition (Fig. 5 *A* and *C*), suggesting that KLF10 constrains thymic maturation of Vγ4^{+} γδ^{27-} cells. Notably, the frequencies of IL-17^{+} cells were higher in KO Vγ1^{-}Vγ4^{-} and Vγ4^{+} γδ^{27-} cells (Fig. 5*B*), which was also observed within their immature (CD24^{hi}CD44^{lo}) stage (Fig. 5*D*), suggesting that KLF10 is involved in IL-17 production of γδ^{27-} cells even before their maturation. In addition, KO neonatal thymus contained greater Vγ4^{+} γδ^{27-}17 cells at both stages than WT thymus did (Fig. 5*D*), confirming that KLF10 inhibits Vγ4^{+} γδ^{27-}17 cell development in the neonatal thymus.

KO pLN Vγ4^{+} γδ^{27-} cells had CD5^{int}CD127^{hi} phenotype (Fig. 2*E*), thus I also examined the expression of CD5 and CD127 on thymic Vγ4^{+} γδ^{27-} cells at both mature and immature stages (Fig. 5*E*). I found that the expression of CD5 was decreased but that of CD127 transiently increased from immature to mature stage.

This reciprocal change generally occurred during the γδ T cell maturation irrespective of Vγ sub-type (Fig. 5*F* and Fig. S8*B*). Notably, exceptional induction of CD127 was observed during $V\gamma4^{+}$ and $V\gamma1^{-}V\gamma4^{-}$ $\gamma\delta^{27-}$ maturation in contrary to a general reduction of CD5 (Fig. 5*F*), suggesting that thymic maturation of γδ-17 subsets is highly dependent on IL-7R signaling. Most of all, consistently with pLN $CD5^{int}CD127^{hi}$ $V\gamma4^{+}$ $\gamma\delta^{27-}$ cells (Fig. 2*E*), mature $V\gamma4^{+}$ $\gamma\delta^{27-}$ cells from KO neonatal thymus expressed CD5 higher than WT counterparts, implying that $CD5^{int}$ cells were 'selected' during $V\gamma4^{+}$ $\gamma\delta^{27-}$ maturation. In the same line with pLN $\gamma\delta^{27-}$ cells (Fig. 2*F*), thymic $V\gamma4^{+}$ cells steadily expressed CD5 lower than $V\gamma1^{-}V\gamma4^{-}$ cells (Fig. 5*F* and Fig. S8*B*), which indicates that each Vγ chain has a unique strength of TCR signal required for thymic wave. Therefore, these results collectively suggest that KLF10 influences thymic programming of γδ subsets and their effector function through regulating strength of TCR and IL-7R signaling.


A
Vγ1-Vγ4-
Vγ4+
WT
KO
WT
KO
80.3
82.1
52.8
69.6
CD44
CD24
ns
**
CD24loCD44hi (%)
B
39.5
50.3
56.6
65.2
IL-17
γδTCR
***
**
IL-17+ cells (%)
Vγ1-Vγ4-
Vγ4+
C
Cell number (x10³)
ns
ns
ns
***
CD24hiCD44lo
CD24loCD44hi
CD24hiCD44lo
CD24loCD44hi
Vγ1-Vγ4-
Vγ4+
WT
KO
D
***
*
***
ns
IL-17+ cells (%)
ns
ns
***
***
IL-17+ cells (x10⁴)
CD44lo
CD44hi
CD44lo
CD44hi
Vγ1-Vγ4-
Vγ4+

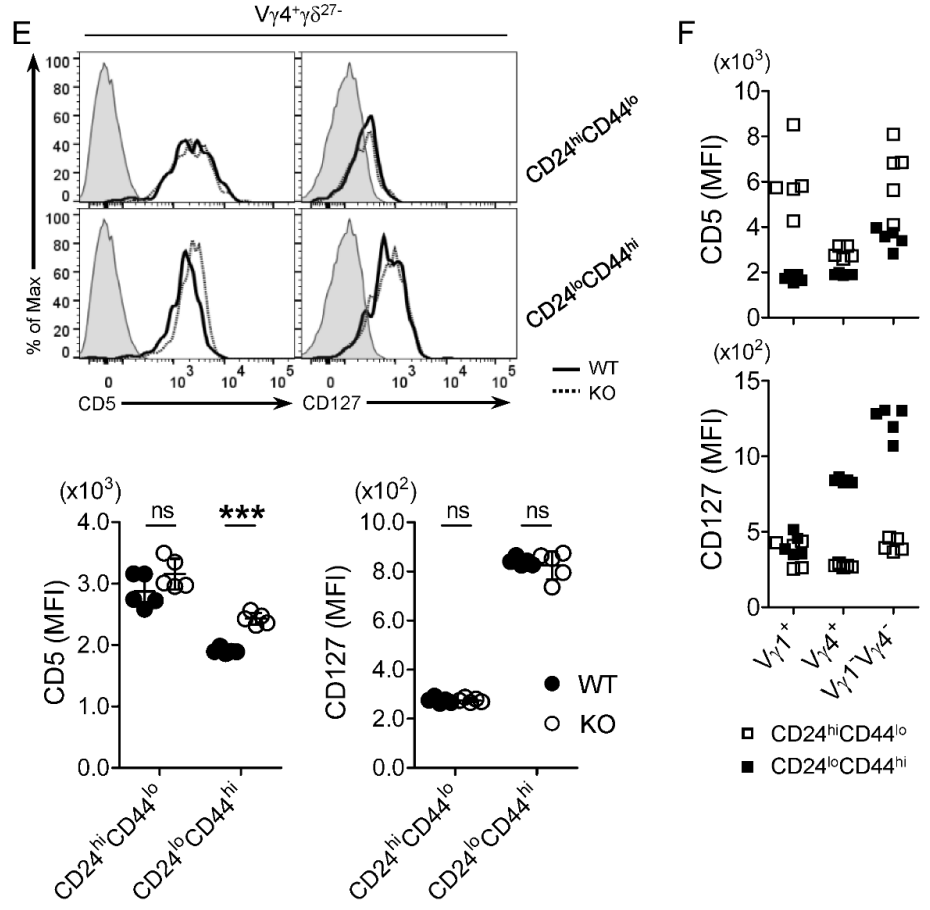


Figure 5. Development of IL-17^{+}Vγ4^{+} γδ^{27-} cells is advanced in neonatal thymus of KLF10 KO mice. Neonatal (d 4 after birth) thymocyte from WT and KO mice were examined using flow cytometry. (*A*, *B*) Frequencies of (*A*) mature

($CD24^{lo}CD44^{hi}$) cells or (*B*) IL-17^+ cells among $V\gamma4^+$ or $V\gamma1^-V\gamma4^-$ $\gamma\delta^{27-}$ cells. (*C*) Absolute number of mature or immature ($CD24^{hi}CD44^{lo}$) cells among $V\gamma4^+$ or $V\gamma1^-V\gamma4^-$ $\gamma\delta^{27-}$ cells. (*D*) Frequency of IL-17^+ cells among mature ($CD44^{hi}$) or immature ($CD44^{lo}$) $V\gamma4^+$ or $V\gamma1^-V\gamma4^-$ $\gamma\delta^{27-}$ cells (upper) and their absolute number (below). NAÏVE Overlayed histogram (left) and MFI (right) of CD5 and CD127 on mature or immature $V\gamma4^+$ $\gamma\delta^{27-}$ cells. (*F*) MFI of CD5 and CD127 on mature or immature each γδ subset as indicated. Each symbol represents an individual mouse. (*C*, *D*) Two-way ANOVA followed by a Bonferroni posttest was used to determine significance. Data indicate the mean ±s.d. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$. Data are representative of three independent experiments with at least four mice.

6. KLF10 intrinsically regulates the development of Vγ4^{+} γδ^{27-}17 cells

Next, I examined whether KLF10 intrinsically controlled Vγ4^{+} γδ^{27-}17 cell homeostasis. I reconstituted irradiated CD45.2^{+} WT or KO mice with congenic WT bone marrow (BM) and analyzed CD45.1^{+} γδ^{27-} cells after at least 12 weeks. Reconstitution of Vγ4^{+} γδ^{27-} cells and CD5^{lo}CD127^{hi} γδ^{27-} cells was identical between WT and KO mice, excluding a role of cell-extrinsic effect of KLF10 deletion (Fig. 6*A*). In contrast, BM chimera experiments in which a 1:1 mixture of CD45.1^{+} WT and CD45.2^{+} KO BM cells was injected to lethally irradiated CD45.1/2^{+} WT mice, showed increased proportion of KO BM-derived Vγ4^{+} γδ^{27-} cells, for both CD5^{lo}CD127^{hi} γδ^{27-} cells and IL-17^{+}Vγ4^{+} γδ^{27-} cells (Fig. 5*B*), supporting cell-intrinsic effect of KLF10-deficient progenitors outcompeting their WT counterparts during thymic development and peripheral maintenance. Moreover, I found a significant increase in percentage of Vγ4^{+} cells and CD5^{lo}CD127^{hi} cells among γδ^{27-} cells, derived dramatically from KO BM cells compared with WT (Fig. 5*C*). In addition, KO BM-derived Vγ4^{+} γδ^{27-} cells had greater frequencies of IL-17^{+} cells than WT BM-derived counterparts (Fig. 5*C*). Collectively, these results suggest that KLF10 serves as an intrinsic negative regulator to constrain Vγ4^{+} γδ^{27-}17 cells and their IL-17 production.

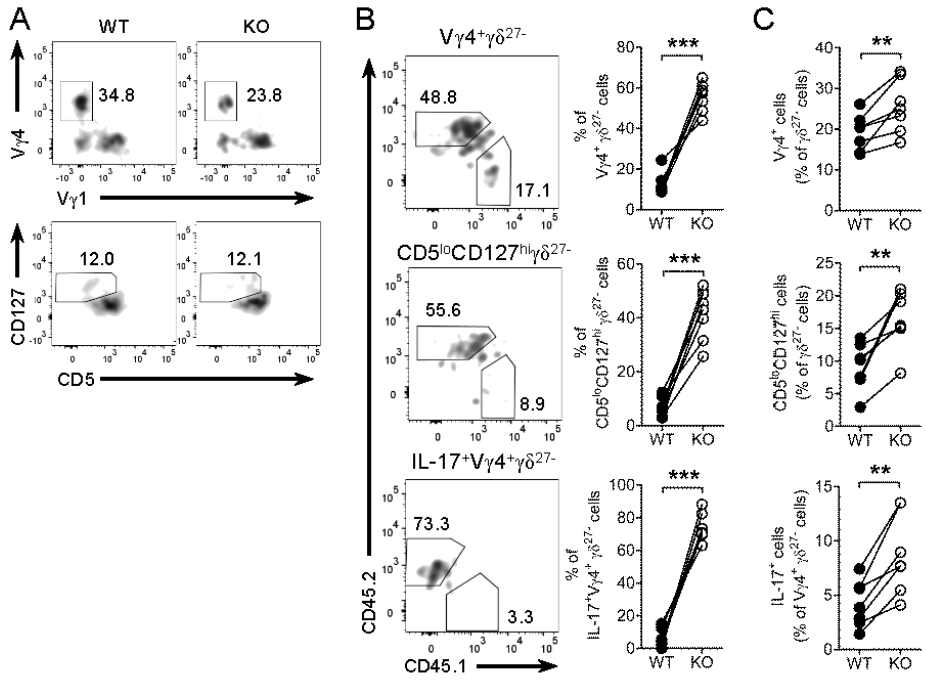
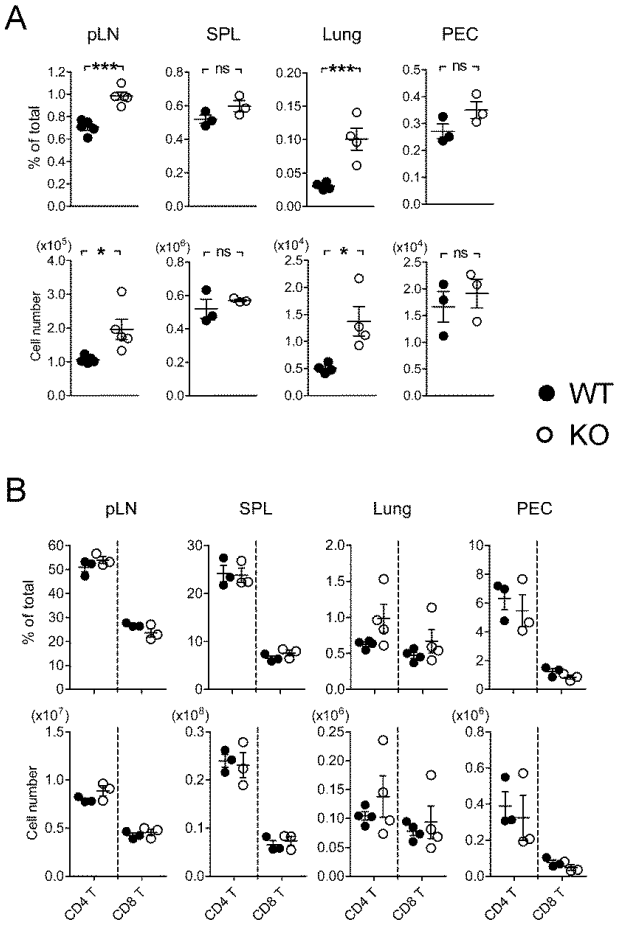


Figure 6. KLF10 intrinsically regulates the development of IL-17^{+}Vγ4^{+} γδ^{27-} cells. (*A*) Vγ4^{+}Vγ1^{-} γδ^{27-} cells (upper) and CD5^{lo}CD127^{hi} γδ^{27-} cells (below) in pLN of irradiated CD45.2 WT or KO mice reconstituted with CD45.1 wild-type bone marrow cells, gated on CD45.1^{+} γδ^{27-} cells. (*B, C*) Irradiated CD45.1/2 wild-type mice were reconstituted with a mixture of CD45.1 WT bone marrow plus CD45.2 KO bone marrow cells. A line connects WT-derived cells to KO-derived

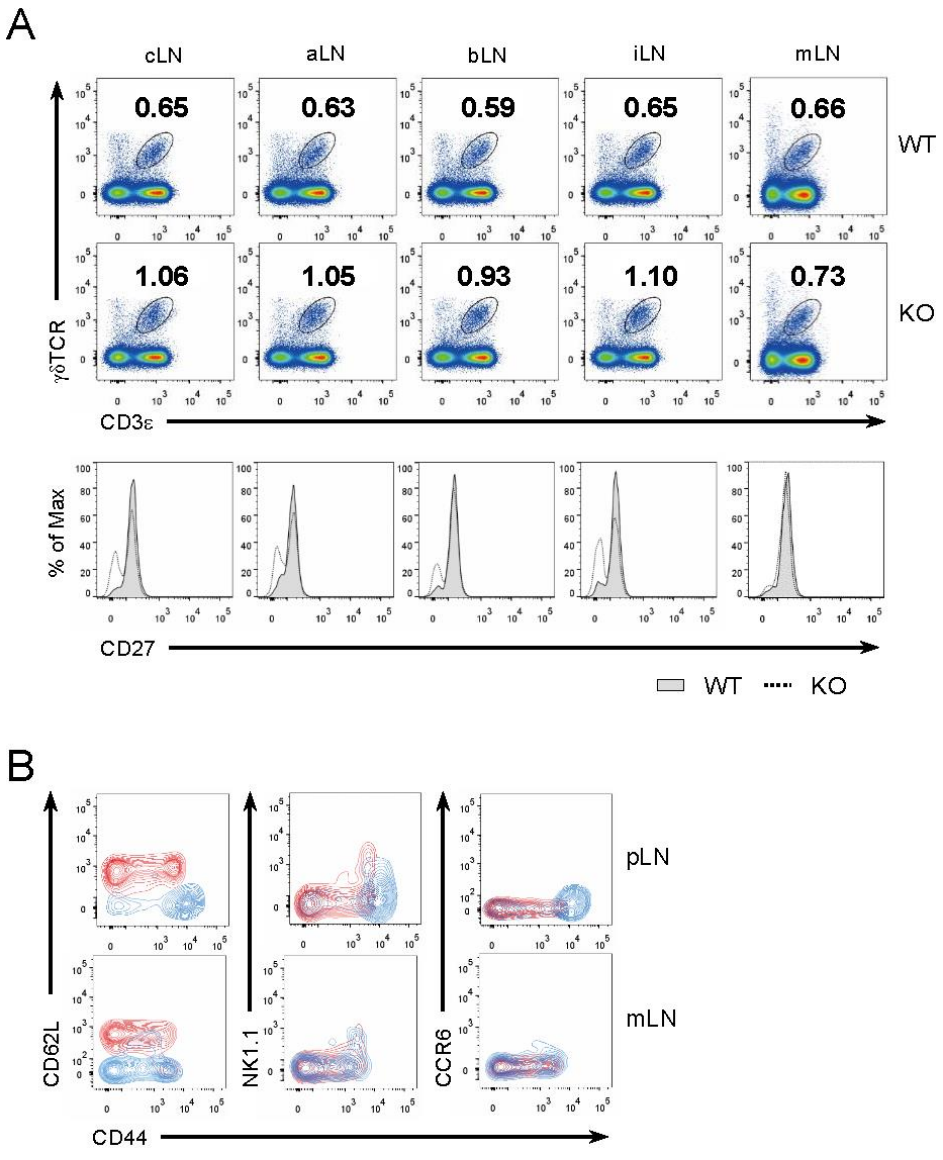
cells that developed within the same recipient mouse. The paired Student's *t*-test was used to determine significance. (*B*) Frequency of CD45.1 WT or CD45.2 KO-derived cells among pLN $V\gamma4^+$ $\gamma\delta^{27-}$ (upper), $CD5^{lo}CD127^{hi}$ $\gamma\delta^{27-}$ (middle), and IL-$17^+V\gamma4^+$ $\gamma\delta^{27-}$ (below). (*C*) Frequency of $V\gamma4^+$ cells (upper) and $CD5^{lo}CD127^{hi}$ cells (middle) among pLN WT and KO $\gamma\delta^{27-}$ cells and IL-17^+ cells (below) among pLN WT and KO $V\gamma4^+$ $\gamma\delta^{27-}$ cells. (*B, C*) Data indicate the mean ±s.d. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$. Data are representative of two independent experiments with (*A*) three to four mice or (*B, C*) five to ten mice.

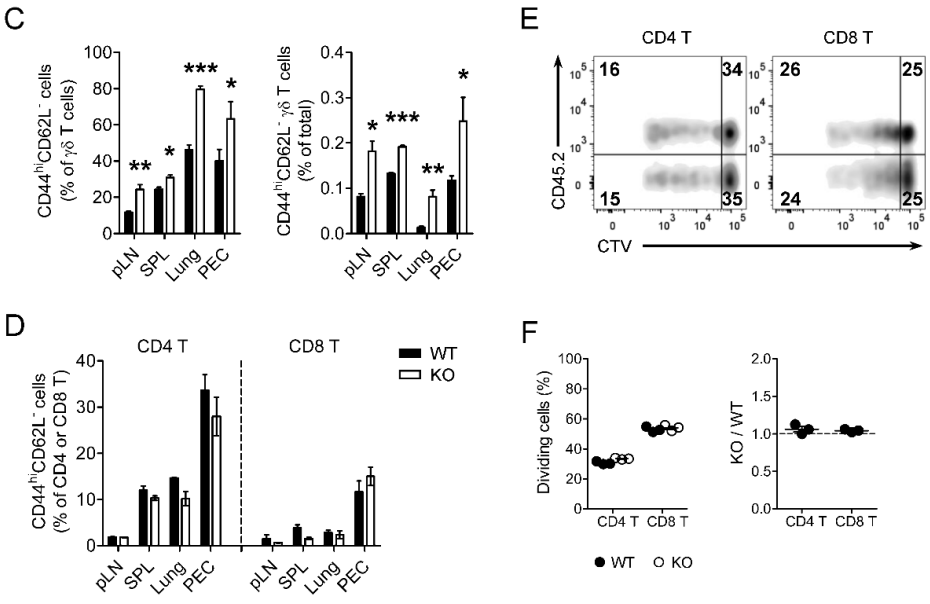
V. Supplementary Results



Supplementary Figure 1. Alteration of γδ T cells, but not of conventional T

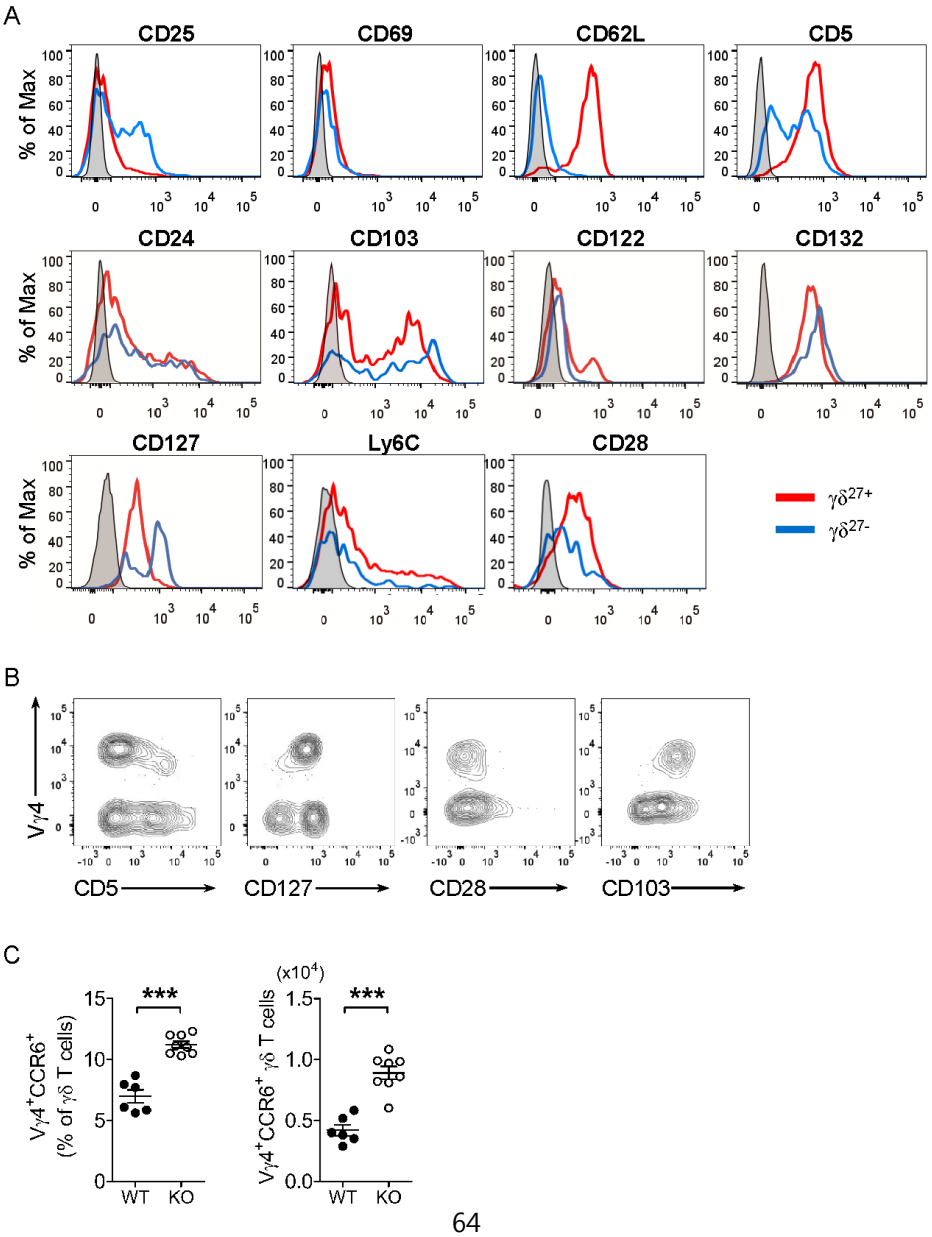
cells in KLF10 KO mice. (*A*) Frequency and absolute number of γδ T cells in pLN, spleen (SPL), lung and peritoneal exudate cells (PEC) obtained from WT and KO mice were examined by using flow cytometry. (*B*) Frequency and absolute number of $CD4^+$ or $CD8^+$ T cells as in *A*. Each symbol represents an individual mouse and the data shown as the mean ±s.d. ns, non-significant; * $P \leq 0.05$; *** $P \leq 0.001$. Data are representative of at least three independent experiments with three to four mice.


A
cLN
aLN
bLN
iLN
mLN
0.65
0.63
0.59
0.65
0.66
WT
1.06
1.05
0.93
1.10
0.73
KO
γδTCR
CD3ε
% of Max
CD27
WT
KO
B
pLN
mLN
CD62L
NK1.1
CCR6
CD44

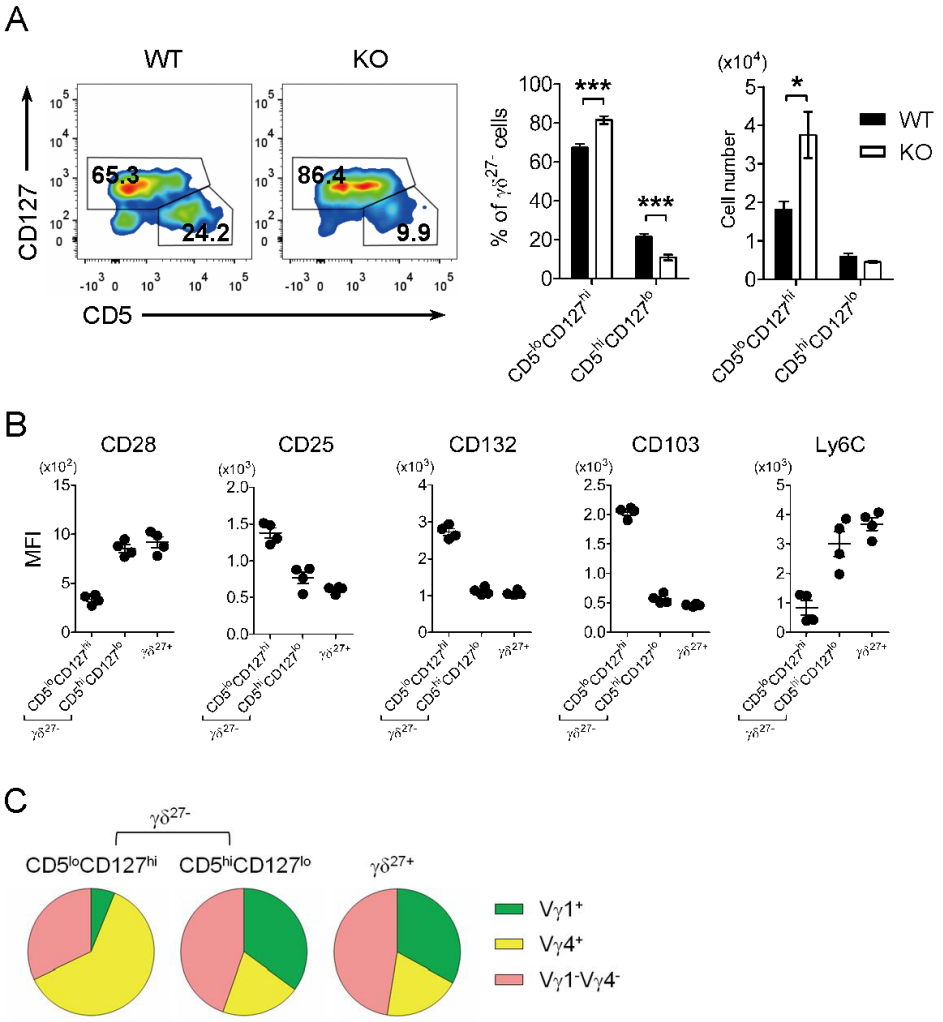


Supplementary Figure 2. Increased frequency of pLN CD44^{hi}CD62L^{-} γδ^{27-} cells in KLF10 KO mice. (*A*) Representative plot showing frequency of γδ T cells (upper) and overlayed histogram of CD27 expression on γδ T cells (below) in cervical (cLN), axillary (aLN), brachial (bLN), inguinal (iLN) and mesenteric (mLN) lymph nodes from WT and KO mice. Number in each box indicates the percent of cells. (*B*) Overlayed plot of CD44, CD62L, NK1.1 and CCR6 expression on γδ^{27-} (blue) or γδ^{27+} (red) cells in pLN, gated on γδ^{27-} or γδ^{27+} cells. (*C*) Frequency of CD44^{hi}CD62L^{-} cells among total γδ T cells (left) and total cells

(right) in the indicated organ. (*D*) Frequency of $CD44^{hi}CD62L^{-}$ cells among $CD4^{+}$ or $CD8^{+}$ T cells as in *C*. NAÏVE Homeostatic expansion of $CD4^{+}$ or $CD8^{+}$ T cells in pLN from Rag-1-deficient mice that had been intravenously administered with Cell Trace Violet (CTV)-labeled wild-type or KO pLN cells at ratio of 1:1 for 5 d were examined using flow cytometry with gating on CTV^{+} $CD4^{+}$ or $CD8^{+}$ T cells. (*F*) Frequency of dividing cells (left) and the ratio of KO to WT (right) among CTV^{+} $CD4^{+}$ or $CD8^{+}$ T cells as in *E*. Each symbol represents an individual mouse. (*C*, *D* and *F*) Data indicate the mean ±s.d. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$. Data are representative of (*A*-*D*) at least three independent experiments or (*E*, *F*) two independent experiments with three to four mice.


A
CD25
CD69
CD62L
CD5
CD24
CD103
CD122
CD132
CD127
Ly6C
CD28
% of Max
γδ27+
γδ27-
B
Vγ4
CD5
CD127
CD28
CD103
C
Vγ4+CCR6+ (% of γδ T cells)
Vγ4+CCR6+ γδ T cells
(x10^4)
***
WT
KO

Supplementary Figure 3. Expression pattern of cell surface proteins on $\gamma\delta^{27-}$ or $\gamma\delta^{27+}$ cells and augmentation of $V\gamma4^{+}CCR6^{+}$ γδ T cells. (*A*) Overlayed histogram for expression of indicated molecule on $\gamma\delta^{27-}$ (blue) and $\gamma\delta^{27+}$ (red) cells in pLN from WT mice. Filled area indicates isotype control. (*B*) Representative contour plot showing expression of the indicated molecules on $V\gamma4^{+}$ or $V\gamma4^{-}$ $\gamma\delta^{27-}$ cells in pLN from WT mice, gated on $\gamma\delta^{27-}$ cells. (*C*) Frequency of $V\gamma4^{+}CCR6^{+}$ cells among total γδ T cells (left) and absolute number of those (right) in pLN from WT and KO mice. Each symbol represents an individual mouse and data indicate the mean ±s.d. *** $P \leq 0.001$. Data are representative of (*A, B*) at least three independent experiments with four to five mice or (*C*) two independent experiments with six to seven mice.


A
WT
KO
CD127
CD5
65.3
24.2
86.4
9.9
% of γδ27- cells
***
***
(x10^4)
Cell number
*
WT
KO
CD5loCD127hi
CD5hiCD127lo
B
CD28
CD25
CD132
CD103
Ly6C
MFI
(x10^2)
(x10^3)
γδ27-
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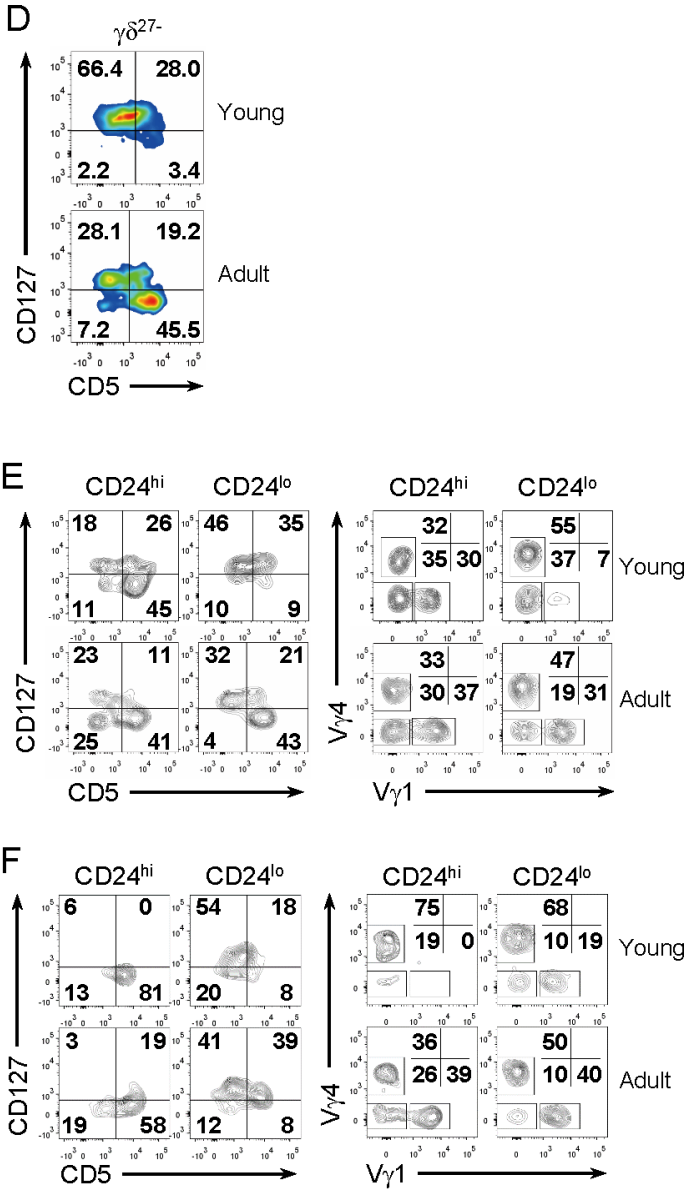
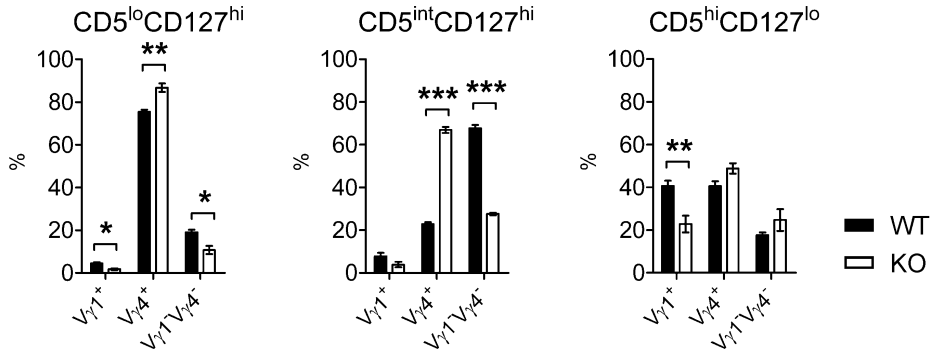
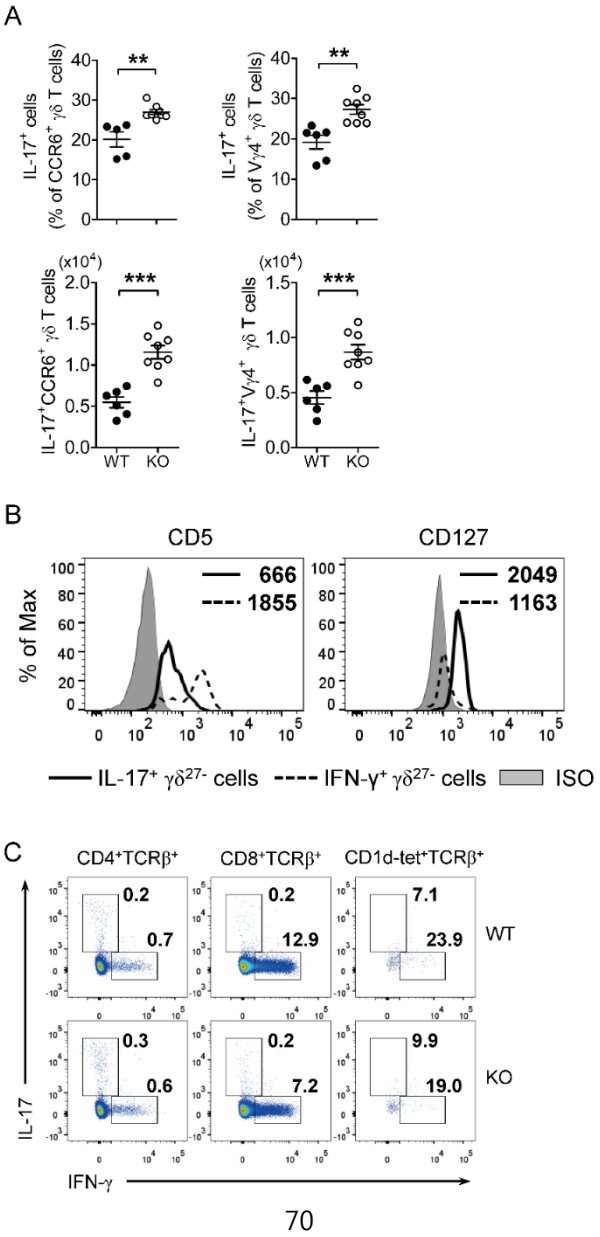
Vγ1+
Vγ4+
Vγ1-Vγ4-


D
γδ27-
66.4
28.0
2.2
3.4
Young
28.1
19.2
7.2
45.5
Adult
CD127
CD5
E
CD24hi
CD24lo
CD24hi
CD24lo
18
26
11
45
46
35
10
9
32
35
30
55
37
7
Young
23
11
25
41
32
21
4
43
33
30
37
47
19
31
Adult
CD127
CD5
Vγ4
Vγ1
F
CD24hi
CD24lo
CD24hi
CD24lo
6
0
13
81
54
18
20
8
75
19
0
68
10
19
Young
3
19
19
58
41
39
12
8
36
26
39
50
10
40
Adult
CD127
CD5
Vγ4
Vγ1

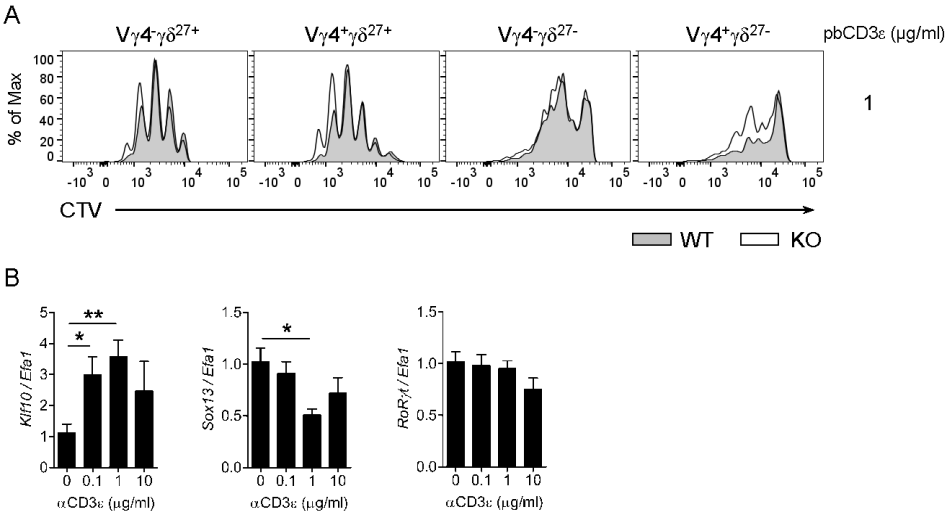
Supplementary Figure 4. Phenotypical characterization of $CD5^{lo}CD127^{hi}$ and $CD5^{hi}CD127^{lo}$ $\gamma\delta^{27-}$ cells. (*A*) Expression pattern of CD5 and CD127 on pLN $\gamma\delta^{27-}$ cells (upper) and the frequency of $CD5^{lo}CD127^{hi}$ or $CD5^{hi}CD127^{lo}$ cells (gated as in upper plot) among pLN $\gamma\delta^{27-}$ cells (below, left) and absolute number (below, right) from WT and KO mice, gated on $\gamma\delta^{27-}$ cells. (*B*) Mean fluorescence intensity (MFI) of CD28, CD25, CD132, CD103 and Ly6C on $\gamma\delta^{27-}$ subsets (as in *A*) and $\gamma\delta^{27+}$ cells in pLN from WT mice. Each symbol represents an individual mouse. (*A, B*) Data indicate the mean ±s.d. * $P \leq 0.05$; *** $P \leq 0.001$. (*C*) Representative pie chart showing Vγ1 and Vγ4 distribution of $\gamma\delta^{27-}$ subsets (as in *A*) and $\gamma\delta^{27+}$ cells obtained as in *B*. (*D*) Expression pattern of CD5 and CD127 of pLN $\gamma\delta^{27-}$ cells from young (2 wks old) and adult (8 wks old) WT mice, gated on $\gamma\delta^{27-}$ cells. (*E, F*) Expression pattern of CD5 and CD127 (left) or distribution of Vγ1 and Vγ4 (right) on $CD24^{hi}$ or $CD24^{lo}$ $\gamma\delta^{27-}$ cells in pLN NAÏVE and thymus (*F*), gated on $\gamma\delta^{27-}$ cells. Data are representative of (*A-D*) at least three independent experiments or (*E, F*) two independent experiments with four to five mice.



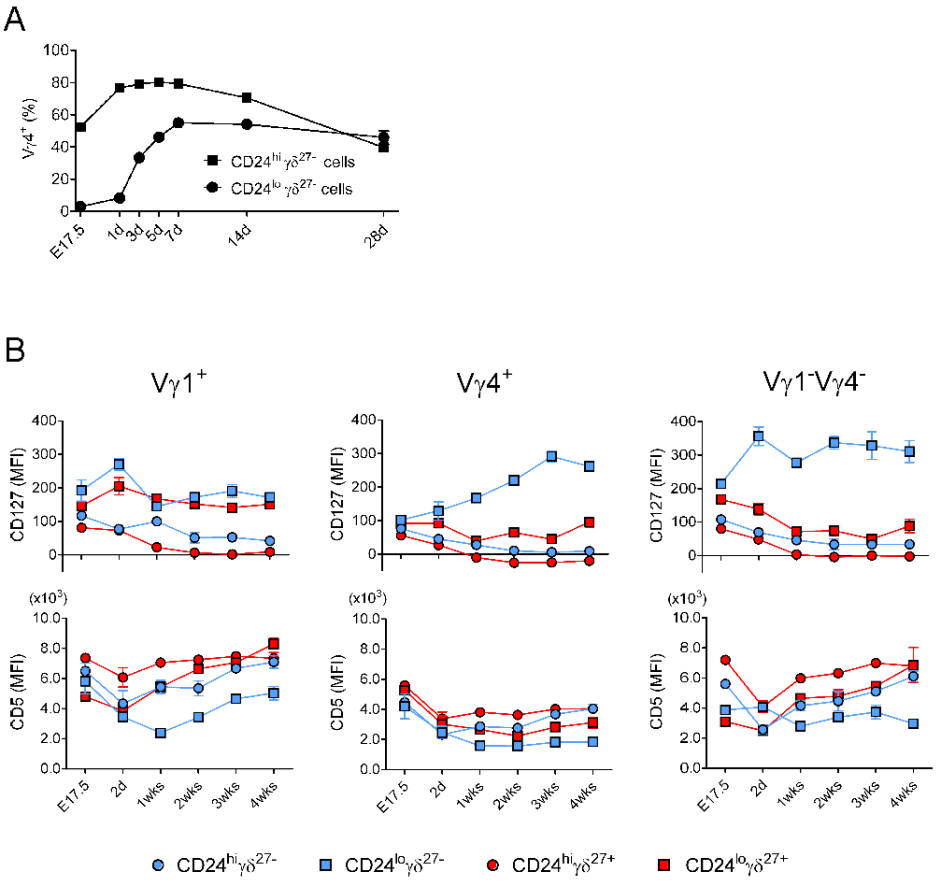
Supplementary Figure 5. Vγ1 and Vγ4 distribution on pLN γδ^{27-} subsets of wild-type and KLF10 KO mice. Vγ1 and Vγ4 distribution of CD5^{lo}CD127^{hi}, CD5^{int}CD127^{hi} and CD5^{hi}CD127^{lo} γδ^{27-} cells in pLN from WT and KO mice. γδ^{27-} cells are gated into CD5^{lo}CD127^{hi}, CD5^{int}CD127^{hi} and CD5^{hi}CD127^{lo} subsets as in Fig. 2*C*. Data indicate the mean ±s.d. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$. Data are representative of at least three independent experiments with four to five mice.


A
IL-17+ cells (% of CCR6+ γδ T cells)
IL-17+ cells (% of Vγ4+ γδ T cells)
IL-17+CCR6+ γδ T cells (x10^4)
IL-17+Vγ4+ γδ T cells (x10^4)
**
***
WT
KO
B
CD5
CD127
% of Max
666
1855
2049
1163
IL-17+ γδ27- cells
IFN-γ+ γδ27- cells
ISO
C
CD4+TCRβ+
CD8+TCRβ+
CD1d-tet+TCRβ+
0.2
0.7
0.2
12.9
7.1
23.9
WT
0.3
0.6
0.2
7.2
9.9
19.0
KO
IL-17
IFN-γ

Supplementary Figure 6. IL-17 production is augmented in Vγ4^{+} γδ^{27-} cells, but not in αβ T cells. pLN cells, obtained from WT and KO mice, were stimulated with PMA plus ionomycin in the presence of brefeldin A for 5 h. (*A*) Frequency of IL-17^{+} cells among CCR6^{+} or Vγ4^{+} γδ T cells (upper) and absolute number (below) are shown. (*B*) Overlayed histogram of CD5 or CD127 expression on IL-17^{+} or IFN-γ^{+} γδ^{27-} cells. Numbers in histogram indicate MFI. (*C*) IL-17 or IFN-γ producing cells among CD4^{+} T, CD8^{+} T or *i*NKT cells. (*A*) Each symbol represents an individual mouse and the data indicate the mean ±s.d. ** $P \leq 0.01$; *** $P \leq 0.001$. Data are representative of two independent experiments with (*A*) five to eight mice or (*B, C*) four mice.

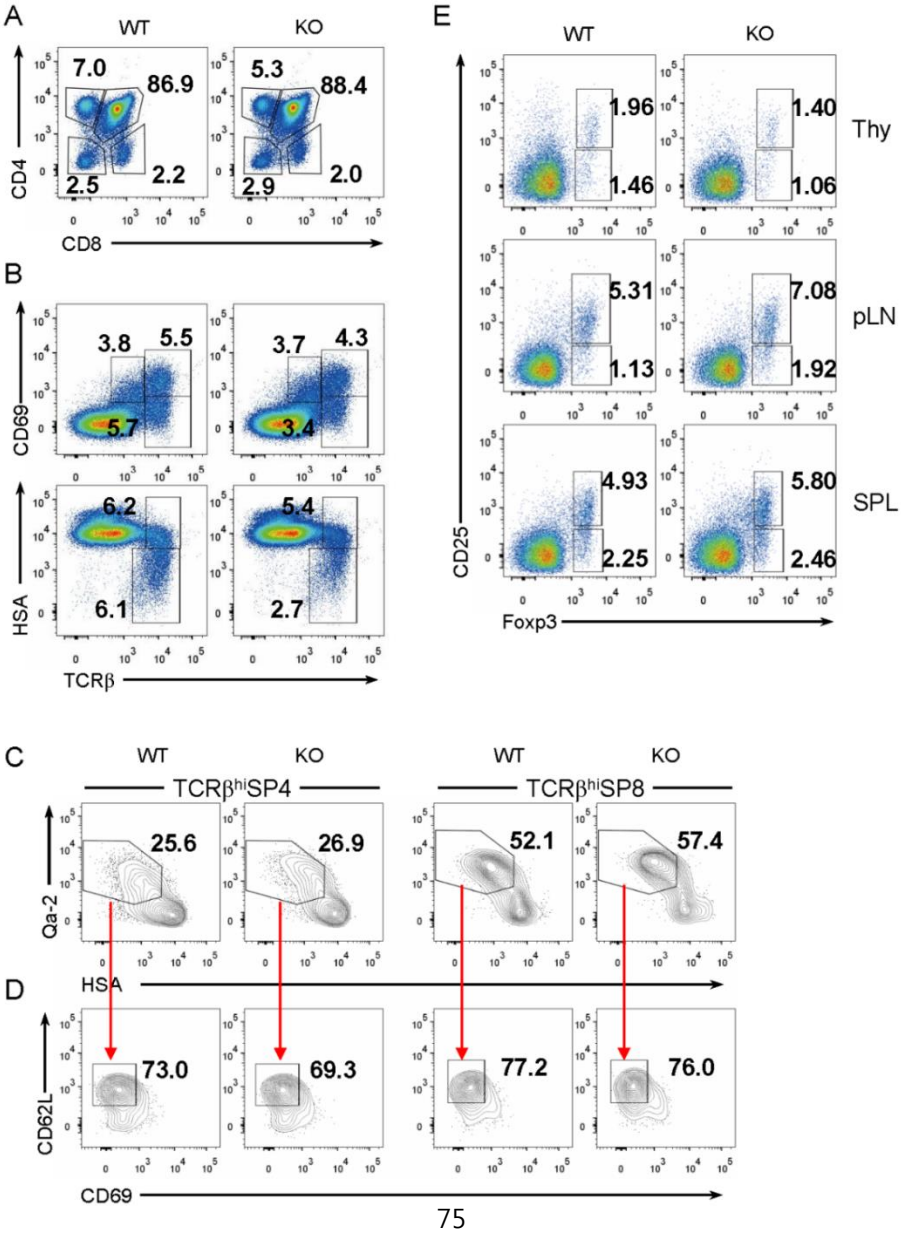


Supplementary Figure 7. KLF10 inhibits Vγ4^{+} γδ^{27-} cell proliferation triggered by TCR signal. (*A*) Purified γδ T cells from WT and KO mice, were labeled by CTV and cultured with pbCD3ε (1 μg/ml) for 3 d and then analyzed by flow cytometry. Filled and blank area indicate WT and KO γδ T cells, respectively. (*B*) Real-time PCR analysis of *Klf10, Sox13* and *RoRγt* mRNA expression by purified γδ T cells cultured with pbCD3ε (0.1, 1, 10 μg/ml) for 3 hours, normalized to *Efa1* expression. All data indicate the mean ±s.d. * $P \leq 0.05$; ** $P \leq 0.01$. Data are representative of at least three independent experiments with at least ten to twenty mice.

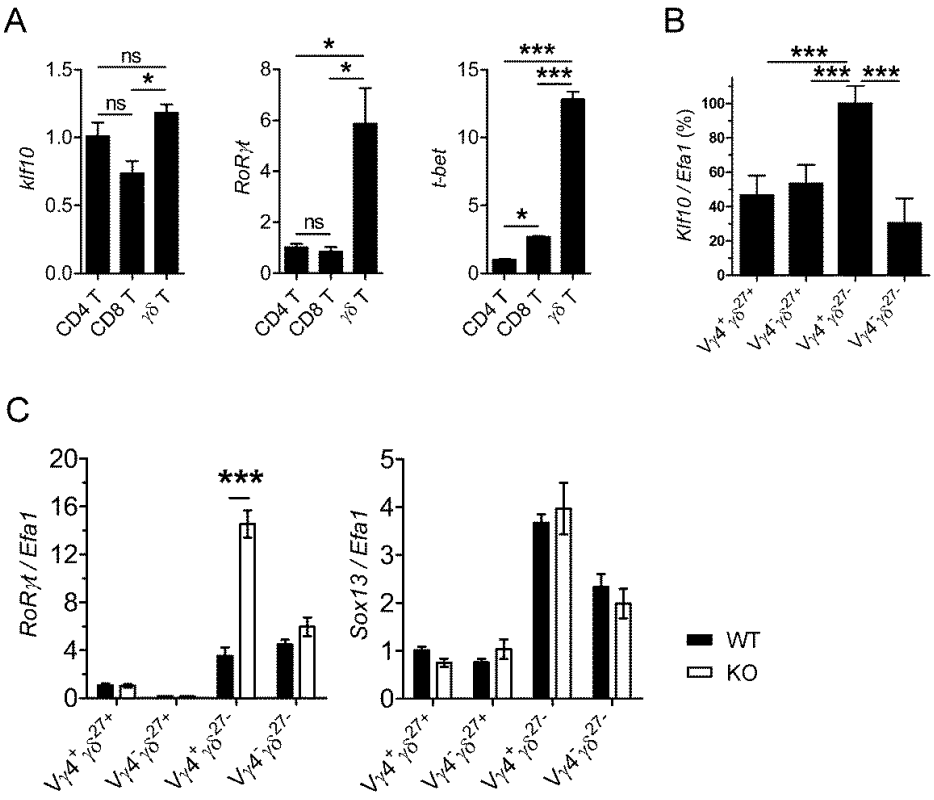


Supplementary Figure 8. Vγ4 wave and MFI of CD5 and CD127 on each γδ subset from late embryonic to adult thymus. (*A*) Frequency of Vγ4^{+} cells among immature (CD24^{hi}) and mature (CD24^{lo}) γδ^{27-} cells in thymus from WT mice at embryonic stage 17.5 (E17.5) and the indicated days after birth. (*B*) MFI

of CD5 and CD127 on each γδ subset obtained as in *A*. Data are representative of two independent experiments with at least four mice.


A
WT
KO
7.0
86.9
2.5
2.2
5.3
88.4
2.9
2.0
CD4
CD8
B
3.8
5.5
5.7
3.7
4.3
3.4
CD69
6.2
6.1
5.4
2.7
HSA
TCRβ
E
WT
KO
1.96
1.46
1.40
1.06
Thy
5.31
1.13
7.08
1.92
pLN
4.93
2.25
5.80
2.46
SPL
CD25
Foxp3
C
WT
KO
WT
KO
TCRβhiSP4
TCRβhiSP8
25.6
26.9
52.1
57.4
Qa-2
HSA
D
73.0
69.3
77.2
76.0
CD62L
CD69

Supplementary Figure 9. Normal development of thymic αβ T cell and distribution of regulatory T cells in KLF10 KO mice. (*A*) CD4 and CD8α expression and (*B*) CD69, HSA and TCRβ expression in total thymocytes. (*C*) Frequency of $HSA^{lo}Qa\text{-}2^{hi}$ cells among $TCR\beta^{hi}CD4^{+}CD8^{-}$ ($TCR\beta^{hi}SP4$) and $TCR\beta^{hi}CD4^{-}CD8^{+}$ ($TCR\beta^{hi}SP8$) cells. (*D*) Frequency of $CD69^{lo}CD62L^{hi}$ cells among $HSA^{lo}Qa\text{-}2^{hi}$ $TCR\beta^{hi}SP4$ or $TCR\beta^{hi}SP8$ cells. NAÏVE Frequency of $Foxp3^{+}$ regulatory T cells among $CD4^{+}$ T cells in thymus (Thy), pLN and SPL from WT and KO mice. Data are representative of at least two independent experiments with three mice.



Supplementary Figure 10. Preferential expression of *Klf10* mRNA in Vγ4^{+} γδ^{27-} cells. (*A*) Expression of *Klf10, RoRγt* and *t-bet* mRNA in CD4^{+} T, CD8^{+} T and γδ T cells purified from pooled pLN and SPL of WT mice. (*B*) Expression of *Klf10* mRNA in Vγ4^{+} γδ^{27+}, Vγ4^{-} γδ^{27+}, Vγ4^{+} γδ^{27-} and Vγ4^{-} γδ^{27-} cells sorted from

pooled pLN and SPL of WT mice, displayed as percent of maximum expression. (*C*) Expression of *RoRγt* and *Sox13* mRNA in each γδ subset from WT and KO mice, sorted as in *B*. Real-time PCR analysis of mRNA expression, normalized to *Efa1* expression. All data indicate the mean ±s.d. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$. Data are representative of three independent experiments with at least ten mice.

VI. Discussion

Previous studies with function of KLF10 had emphasized its role on balancing between Treg and Th17 cells (1-3). In the present study, I identified, for the first time of our knowledge, KLF10 and Th17 cells (1-3). In the present study, I identified, for the first time of our knowledge, KLF10 as a critical negative regulator of γδ T cell homeostasis. The most interesting finding with KLF10-deficient mice here was a significant augmentation of pLN Vγ4^{+} γδ^{27-}17 cells (Fig. 3 *A* and *B*, and Fig. S6*A*) with normal frequencies of other IL-17-producing cells such as Th17 and *i*NKT-17 cells (Fig. S6*C*). In marked contrast to γδ T cells, thymic αβ T cell development together with Foxp3^{+} Treg distribution was normal in these KO mice (Fig. S9 *A* – *C*). Therefore, our findings shed new light of a novel regulatory function of KLF10 specific for γδ T cells, exclusively Vγ4^{+} γδ^{27-}17 cells.

'Innate-like' γδ-17 cells transferred into a lymphopenic host exhibited a robust expansion, utterly dependent on homeostatic cytokine, IL-7 but not on MHC recognition (15, 16). In line with IL-7 dependency, such a homeostatic proliferation of γδ T cells is completely inhibited with co-transferred αβ T cells

(15), which enhance cellular competition for limiting amounts of IL-7, thereby rendering γδ T cells receiving insufficient IL-7 signal for inducing proliferation. Surprising finding, however, was that $\gamma\delta^{27-}$ cells, presumably 'innate-like' γδ-17 cells, from KO mice underwent dramatic homeostatic expansion, indicating that KLF10 deficiency allow $\gamma\delta^{27-}$ cells to overcome the competitive inhibition by αβ T cells for IL-7, presumably due to increased sensitivity of IL-7 signaling (Fig. 4*B*). Therefore, KLF10 selectively regulates peripheral 'innate-like' γδ-17 cell homeostasis where IL-7R signaling is mainly engaged.

Although *klf10* mRNA similarly expressed at a basal level among $CD4^+$, $CD8^+$ and γδ T cells (Fig. S10*A*), there was a preferential expression in $V\gamma4^+$ $\gamma\delta^{27-}$ cells compared to other γδ subtypes (Fig. S10*B*). This partially explained the selective increase of pLN $V\gamma4^+$ $\gamma\delta^{27-}$ cells observed in KLF10-deficient mice, further suggesting that –KLF10 actively functioned in $V\gamma4^+$ $\gamma\delta^{27-}$ cells. On the other hand, increase of *klf10* mRNA was observed in γδ T cells stimulated with TCR or cytokines (IL-7, IL-6, IL-1β and IL-23). These results suggested that the relatively higher basal level of *klf10* in $V\gamma4^+$ $\gamma\delta^{27-}$ cells is attributed to these signals and is consistent with previously reported role of TCR and IL-7 (9-11), which might differently engage and cooperate in its expression.

How KLF10 preferentially regulates γδ but not αβ T cells, especially Vγ4^{+} γδ^{27-} cells, in the periphery is still unclear, although our findings provided a partial clue that the steady-state function of KLF10 is dependent more on cytokine-mediated regulation (less on TCR). It is likely that KLF10 is linked to JAK-STAT signal pathways (3, 29). Further investigations mandate key intracellular pathways downstream of TCR and cytokine signaling which are regulated by KLF10 to control homeostasis of peripheral γδ T cells.

Transcriptional profiling of γδ thymocytes by the immunological Genome Project (http://www.immgen.org) revealed that *klf10* is upregulated during thymic maturation of Vγ4^{+} γδ T cells, which is consistent with our findings that KLF10-deficient neonates showed advanced maturation of thymic Vγ4^{+} γδ^{27-} cells (Fig. 5 *A* and *C*). The transition from immature to mature stage shaped CD5 expression by 'selecting' the cells expressing low level of CD5 as shown in our study, since CD5 level is correlated with the strength of TCR signal initially perceived during thymic selection (30-32). Each immature Vγ sub-type had a distinct level of CD5 and the 'negative selection' was also different, suggesting that a broad but specific range of TCR-signal strength for the thymic wave and maturation determines each Vγ sub-type (22). However, it is unclear that upregulation of KLF10 in Vγ4^{+} γδ T cells during the maturation is trigged solely

by TCR signaling. Furthermore, it is yet to be determined whether TCR signal engages the thymic γδ maturation. Notably, $V\gamma4^+$ cells require relatively weak TCR signal for their wave in contrast to other sub-types, consistent with the ligand-independent signaling of $V\gamma4^+V\delta5^+$ TCR (10). Taken together, KLF10 deficiency allowed $V\gamma4^+$ $\gamma\delta^{27-}$ cells to be 'selected' into $CD5^{int}$ cells – indicative of receiving relatively 'strong' TCR signal strength – in high frequency and absolute number when compared to WT (Fig. 5*E*).

Although IL-17 production is a 'default property' as immature $V\gamma4^+$ and $V\gamma1^-V\gamma4^-$ $\gamma\delta^{27-}$ cells already contained IL-17^+ cells (19, 23), I further showed that IL-17^+ cells are enriched more after the maturation. Notably, CD127 (IL-7Rα) expression was upregulated on these γδ-17 sub-types only during the maturation, suggesting that IL-7R signaling is exclusively associated with IL-17 production by mature γδ-17 cells. It is intriguing that the frequency of IL-17^+ cells was increased at immature, but not mature, stage in $V\gamma4^+$ and both stages in $V\gamma1^-V\gamma4^-$ $\gamma\delta^{27-}$ cells, suggesting that KLF10 is strongly associated with IL-17-producing property of γδ-17 cells even before the maturation regardless of Vγ sub-type.

Transcription factors which are controlled by external cues have been suggested as one possible programming for the generation of effector γδ subsets (20, 22, 33, 34). Notably, the high-mobility-group transcription factor *Sox13*

mRNA was normally expressed in KLF10-deficient $V\gamma4^{+}$ $\gamma\delta^{27-}$ cells (Fig. S10*C*), suggesting that the effect of KLF10 deletion on $\gamma\delta^{27-}$ cells is not related with SOX13 which are required for the differentiation of $V\gamma4^{+}$ γδ-17 cells (20, 34). In addition to transcription factors, differences on the TCR signal transmitted by genomically encoded Vγ chains and the generation timing of each Vγ wave could also determine γδ effector subset diversification (21, 35). Here, I introduced KLF10 as a novel transcription factor which appeared to be controlled by Vγ4-specific TCR strength and IL-7R signaling. However, I do not rule out the possibility that KLF10 is an intrinsic factor of the neonatal thymic Vγ4 wave. Elucidation of the KLF10-associated signal network with inherited or intrinsic factors specific to the Vγ chain will identify the mechanism for the thymic programming of γδ functional decision. Moreover, determining KLF10 function on thymic development and IL-17 production of other innate-like lymphocyte subsets will give an insight into the diversity of innate lymphoid effectors.

VII. Conclusion

IL-17-committed γδ T (γδ-17) cells contribute to host immune responses, but the transcriptional factors that regulate their thymic development and peripheral homeostasis are still being explored. Transcription factor, Kruppel-like factor 10 (KLF10) involves not only induction of $Foxp3^+$ regulatory T and Th-17 cells but also their balance, but its function at steady-state of mice is still unclear. Here, we demonstrate KLF10 as a novel negative regulator specific for γδ-17 cells, especially $V\gamma4^+$ γδ-17 cells, such that KLF10 deficiency promotes peripheral $V\gamma4^+$ γδ-17 cell expansion and their thymic maturation. Differential responsiveness of KLF10-deficient γδ cells to TCR and cytokine signals together with induction of KLF10 by these stimuli help us to discern how KLF10 selectively constrains $V\gamma4^+$ γδ-17 cells. This work provides an insight into the transcriptional programming in which TCR and cytokines cooperate to decide thymic γδ T cell effector fate.

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IX. Summary in Korean

전사인사 Kruppel-like factor 10 (KLF10)의 면역학적 기능에 관한 초기 연구들을 통해 KLF10이 Foxp3^{+} regulatory T (Treg) 세포와 Th17 세포의 유도를 조절한다는 사실이 밝혀졌다. KLF10이 결핍된 경우, T cell receptor (TCR)와 TGFβ 자극에 의한 CD4+ T 세포의 Treg 세포로의 분화는 약화되고, TCR + TGFβ + IL-6에 의한 Th17 세포 분화는 강화되는 것으로 보고되었다. 그러나, 이러한 *in vitro* 결과와는 반대로, KLF10이 결핍된 (KLF10 KO) 마우스가 Treg 세포를 적게 가지고 있는지는 의견이 분분하며 이들 마우스에서 Th17 세포의 절대 수에 대한 보고는 없기 때문에, Treg 세포와 Th17 세포의 조절에 대한 KLF10의 기능은 여전히 논쟁거리이다. 무엇보다도 KLF10이 IL-17을 생산하는 gamma delta ($\gamma\delta$) T 세포 ($\gamma\delta$-17)를 조절하는지에 대한 연구는 전무한 상태이다.

본 연구는 KLF10 KO 마우스를 사용하여, 전사인자 KLF10이 γδ-17를 억제한다는 사실을 처음으로 규명하였다. γδ-17 세포는 세포표면 단백질 CD27의 발현 유무와 Vγ chain에 기반하여 특정 표현형으로 정의될 수 있는데, γδ-17 세포는 대부분 CD27을 발현하지 않으며 Vγ4 또는 Vγ6 chain을 발현한다. 본 연구에서는 KLF10 KO 마우스에서 Vγ4^{+}CD27^{-} γδ-17 (Vγ4^{+} γδ^{27-}-17) 세포가 선택적으로 증가함을 확인하였다. 특히, KLF10 KO 마우스에서 증가한 Vγ4^{+} γδ^{27-}-17 세포는 wild type (WT) 마우스의 상응하는 세포에 비해 CD5의 발현이 상대적으로 높았다. CD5의 발현은 전구(precursor) T 세포가 흉선 내에서 TCR 자극 받으면 세포 표면에 발현되는 단백질로서 세포가 받은 TCR 자극의 강도를 안정적으로 지시해주는 지표로 여겨진다. 이러한 사실과 함께 KLF10 KO 신생(neonatal) 마우스의 흉선 내 Vγ4^{+} γδ^{27-}17 세포의 성숙이 증가한 결과에 근거하여 KLF10 결핍이 Vγ4^{+} γδ^{27-}-17 세포의 흉선 내 발달 과정에서 TCR 자극을 강하게 받은 세포들이 상대적으로 더 성숙되도록 하였음을 알 수 있다. 따라서, 본 연구는 KLF10 전사 인자가 강한 TCR 신호를 받은 Vγ4^{+} γδ^{27-}-

17 세포의 성숙을 억제한다는 것을 밝혔다. 더불어 기존 보고와 달리, Treg의 비율은 흉선, 비장과 말초 림프절 내에서 정상 마우스와 비슷했으며 Th17 (IL-17$^+$ CD4$^+$TCRβ^+), iNKT-17 (IL-17$^+$ CD1d-tet$^+$TCRβ^+), Tc17 (IL-17$^+$ CD8$^+$TCRβ^+) 세포의 비율 또한 정상 수준을 보였다.

나아가, TCR, IL-7 또는 IL-1β + IL-23 자극에 의한 Vγ4$^+$ $\gamma\delta^{27-}$-17 세포의 세포 분열(proliferation)과 IL-17 생성이 KLF10 결핍에 의해 증가하였으며, 이들 자극에 의해 *Klf10*이 유도됨을 확인할 수 있었다. 이러한 사실은 KLF10이 이들 주요 신호 체계의 'negative feedback loop'를 구성하고 있음을 시사해 준다. Mixed bone marrow chimera 실험에서는 KLF10이 Vγ4$^+$ $\gamma\delta^{27-}$-17 세포의 내재적 조절 인자로서 기능함을 증명하였다.

결론적으로, 본 연구는 전사 인자 KLF10을 Vγ4$^+$ $\gamma\delta^{27-}$-17 세포의 발달과 유지를 억제하는 내재적 조절인자로 제시함으로써 KLF10의 새로운 기능을 규명하였다. 향 후, $\gamma\delta$ T 세포와 유사한 특정을 지닌 다른 종류의 innate-like lymphocytes의 발달과

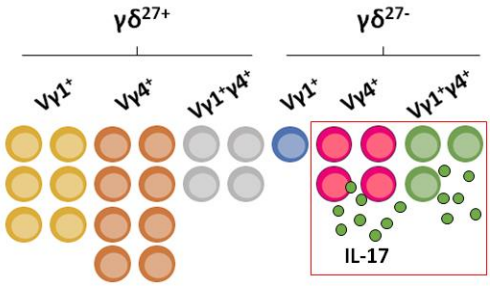
항상성에 관한 KLF10 기능 연구는 비정규 T 세포 연구에 의미 있는 통찰을 제시해 줄 것으로 기대된다.

주요어: KLF10, gamma delta T 세포, IL-17

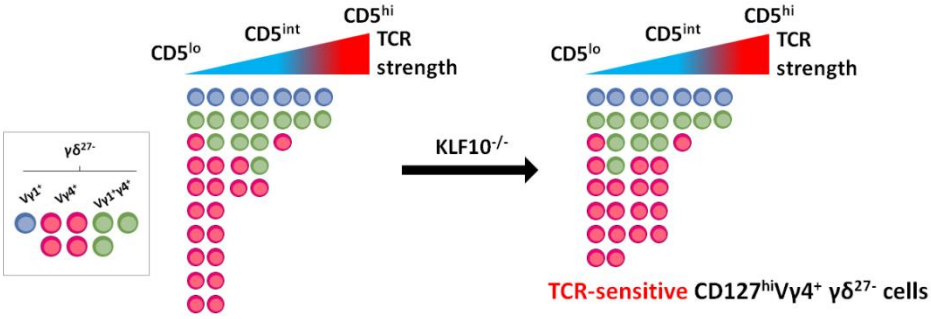
X. Appendix

Graphic summary

1. Phenotypic classification of innate γδ-17 cells

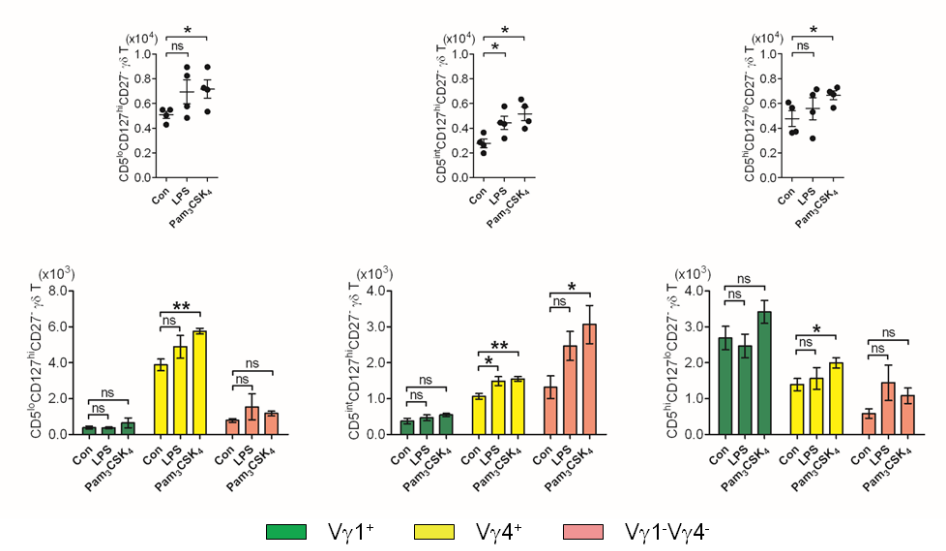


2. The change of thymic γδ T cell selection by KLF10 deficiency

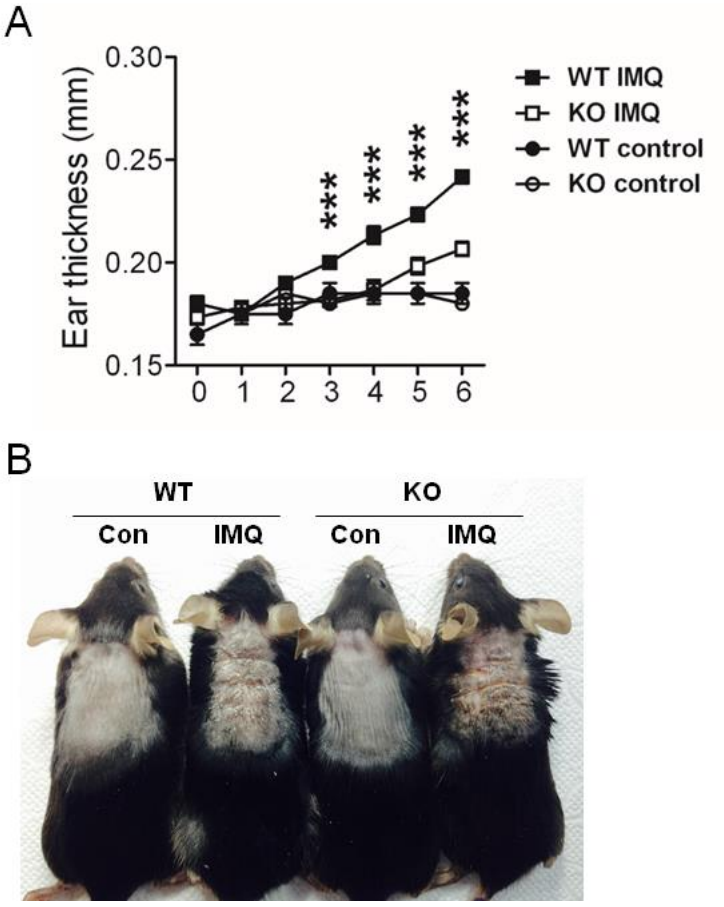


Data unpresented

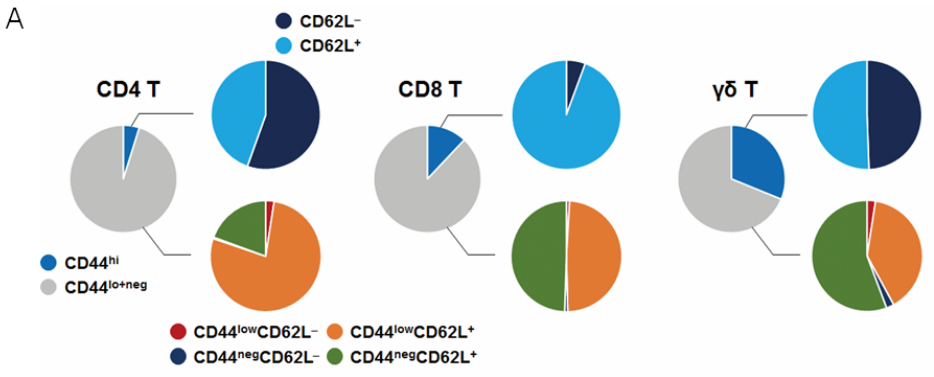
1. γδ T cell responses by LPS or Pam3CSK4 treatment

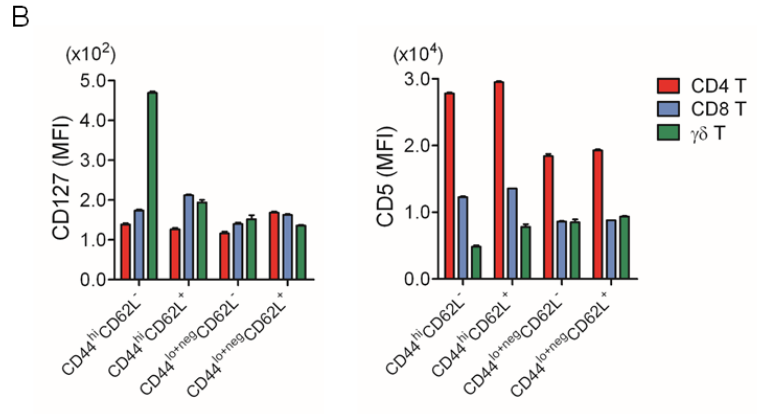


2. Imiquimod (IMQ)-induced psoriasis in KLF10-deficient (KO) mice

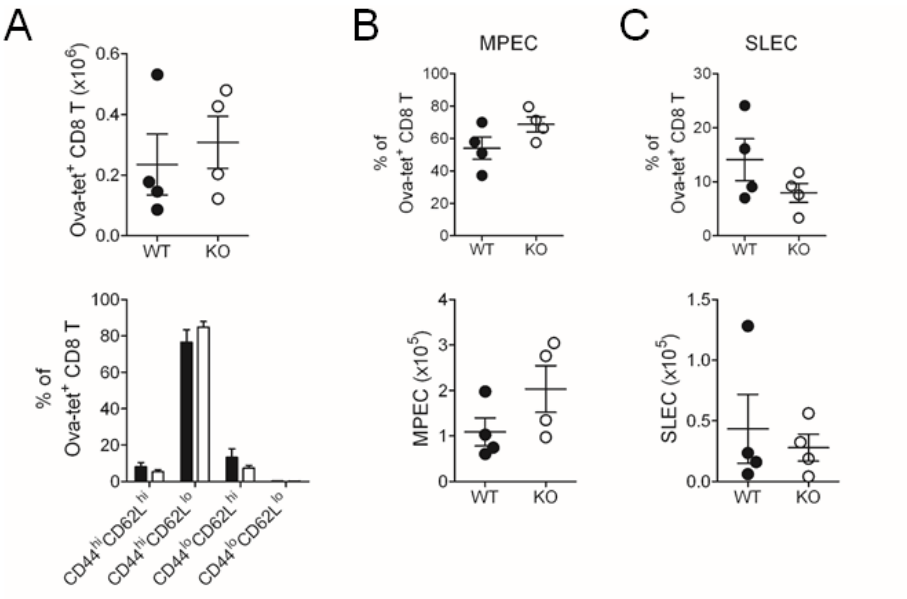


3. Expression comparison of CD44, CD62L, CD127 and CD5 among CD4, CD8 and γδ T cells

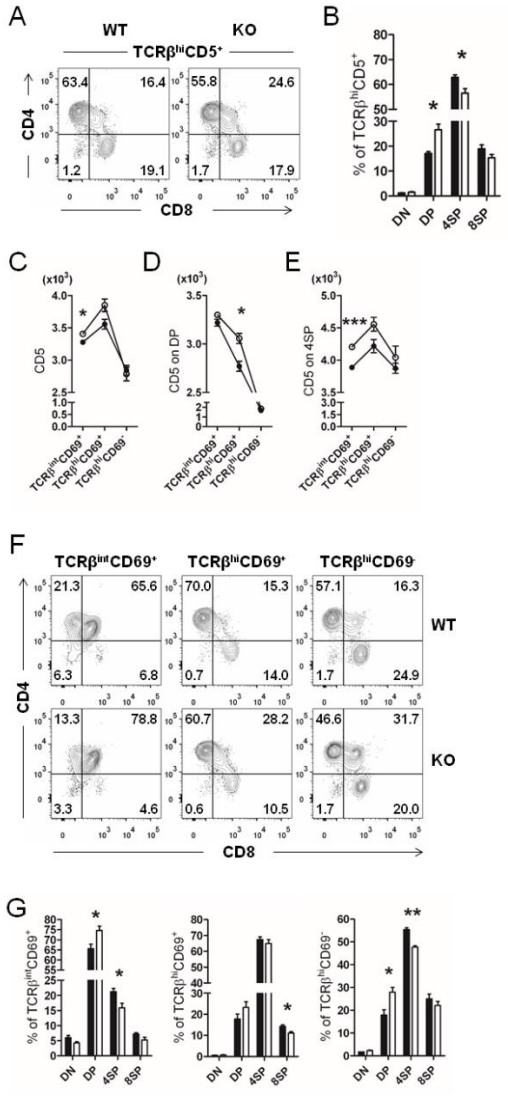




4. Ova-specific CD8 T cell generation in KLF10-deficient (KO) mice



5. CD5 augmentation during thymic αβ T cell development



Publication

1. **Stress, Nutrition, and Intestinal Immune Responses in Pigs - A Review**. Lee IK, Kye YC, **Kim G**, Kim HW, Gu MJ, Umboh J, Maaruf K, Kim SW, Yun CH. *Asian-Australas J Anim Sci*. 2016 Aug;29(8):1075-82.

2. **Systemic administration of RANKL overcomes the bottleneck of oral vaccine delivery through microfold cells in ileum.** Maharjan S, Singh B, Jiang T, Yoon SY, Li HS, **Kim G**, Gu MJ, Kim SJ, Park OJ, Han SH, Kang SK, Yun CH, Choi YJ, Cho CS. *Biomaterials*. 2016 Apr;84:286-300.

3. **A Bacterial Metabolite, Compound K, Induces Programmed Necrosis in MCF-7 Cells via GSK3β.** Kwak CW, Son YM, Gu MJ, **Kim G**, Lee IK, Kye YC, Kim HW, Song KD, Chu H, Park BC, Lee HK, Yang DC, Sprent J, Yun CH. *J Microbiol Biotechnol*. 2015 Jul;25(7):1170-6.

4. **Exploring the genetic signature of body size in Yucatan miniature pig.** Kim H, Song KD, Kim HJ, Park W, Kim J, Lee T, Shin DH, Kwak W, Kwon YJ, Sung S, Moon S, Lee KT, Kim N, Hong JK, Eo KY, Seo KS, **Kim G**, Park S, Yun CH, Kim H, Choi K, Kim J, Lee WK, Kim DK, Oh JD, Kim ES, Cho S, Lee HK, Kim TH, Kim H. *PLoS One*. 2015 Apr 17;10(4):e0121732.

5. **Curcumin inhibits CD4(+) T cell activation, but augments CD69 expression and TGF-β1-mediated generation of regulatory T cells at late phase. Kim G**, Jang MS, Son YM, Seo MJ, Ji SY, Han SH, Jung ID, Park YM, Jung HJ, Yun CH. *PLoS One*. 2013 Apr 26;8(4):e62300.

6. **Gene expression profile of human peripheral blood mononuclear cells induced by Staphylococcus aureus lipoteichoic acid.** Kang SS, Kim HJ, Jang MS, Moon S, In Lee S, Jeon JH, Baik JE, Park OJ, Son YM, **Kim GR**, Joo D, Kim H, Han JY, Yun CH, Han SH. *Int Immunopharmacol*. 2012 Aug;13(4):454-60.

7. **Interactions of dendritic cells with cancer cells and modulation of surface molecules affect functional properties of CD8+ T cells.** Seo MJ, **Kim GR**, Son YM, Yang DC, Chu H, Min TS, Jung ID, Park YM, Han SH, Yun CH. *Mol Immunol.* 2011 Sep;48(15-16):1744-52.

8. **Resistin enhances the expansion of regulatory T cells through modulation of dendritic cells.** Son YM, Ahn SM, **Kim GR**, Moon YS, Kim SH, Park YM, Lee WK, Min TS, Han SH, Yun CH. *BMC Immunol*. 2010 Jun 30;11:33.

9. **Synergistic production of interleukin-23 by dendritic cells derived from cord blood in response to costimulation with LPS and IL-12.** Jang MS,

Son YM, **Kim GR**, Lee YJ, Lee WK, Cha SH, Han SH, Yun CH. *J Leukoc Biol*. 2009 Sep;86(3):691-9.

Poster presentation

Gamma Delta Conference 2016. King's College London, London, UK. June 16-19, 2016

Kruppel-like Factor 10 Regulates IL-17-Committed CD27$^-$ γδ T Cell Homeostasis (Girak Kim, MinJeong Gu, Soo Ji Kim, Seung Hyun Han, Jae-Ho Cho, Cheol-Heui Yun)

The 2016 Spring Conference of the Korean Association of Immunobiologists. Kunkuk University, Seoul, Korea. April 14-15, 2016.

Kruppel-like Factor 10 Regulates IL-17 Producing γδ T Cell Homeostasis (Girak Kim, MinJeong Gu, Soo Ji Kim, Seung Hyun Han, Jae-Ho Cho, Cheol-Heui Yun)

Keystone Symposia: T Cells: Regulation and Effector Function. Snowbird Resort, Snowbird, Utah, USA. March 29-April 3, 2015.

Kruppel-like Factor 10 Regulates IL-17 Producing γδ T Cell Homeostasis (Girak Kim, MinJeong Gu, Soo Ji Kim, Jae-Ho Cho, Cheol-Heui Yun)