Distal *Lck* Promoter–Driven Cre Shows Cell Type–Specific Function in Innate-like T Cells

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Abstract

Innate-like T cells, including invariant NKT cells, mucosal-associated invariant T (MAIT) cells, and γ δ T (γδT) cells, are groups of unconventional T lymphocytes. They play important roles in the immune system. Because of the lack of Cre recombinase lines that are specific for innate-like T cells, pan–T cell Cre lines are often used to study innate-like T cells. In this study, we found that distal Lck promoter–driven Cre (dLckCre) in which the distal *Lck* gene promoter drives Cre expression in the late stage of thymocyte development has limited function in the innate-like T cells using *ROSA26$^{floxed-Stop-tdTomato}$* reporter. Innate-like T cells differentiate into mature functional subsets comparable to the CD4$^+$ Th subsets under homeostatic conditions. We further showed that dLckCre-expressing γδT cells are strongly biased toward γδT1 phenotype. Interestingly, the γδT cells residing in the epidermis and comprising the vast majority of dendritic epidermal T cells nearly all express dLckCre, indicating dLckCre is a useful tool for studying dendritic epidermal T cells. Taken together, these data suggest that *Lck* distal promoter has different activity in the conventional and unconventional T cells. The use of dLCKcre transgenic mice in the innate-like T cells needs to be guided by a reporter for the dLckCre function.

INTRODUCTION

Innate-like T cells (invariant NKT [iNKT] cells, mucosal-associated invariant T [MAIT] cells, and γδT cells) constitute a significant fraction of the T cells in mice and humans, and they have nonredundant functions in many responses. Whereas conventional αβT cells express diverse repertoire of TCRs and differentiate from naive cells into effector cells when

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activated by cognate peptide Ags, innate-like T cells express limited repertoire of TCRs, undergo effector differentiation in the thymus, and exhibit a "memory-like" phenotype under steady state. These unconventional T cells recognize nonpeptide Ags such as lipids, vitamin B metabolites, and phosphoantigens, respectively (1), and are restricted by nonclassical MHC molecules, for example, CD1d for iNKT cells and MR1 for MAIT cells.

To understand the processes of the development and activation of innate-like T cells, genetic tools especially the conditional knockout mice are critical. There are three major T cell–specific Cre lines active in early to late stages of thymocytes: proximal lymphocyte protein tyrosine kinase (*Lck*) promoter–driven Cre (*pLckCre*), active in the αβ-lineage starting at the CD4$^-$CD8$^-$ double-negative (DN) stage in the thymocyte development (2–6), but with limited effect in γδT cell lineage (7); CD4 enhancer, promoter and silencer sequences driven Cre (CD4Cre), active at the CD4$^+$CD8$^+$ double-positive (DP) stage in the αβ-linage (8, 9); and distal *Lck* promoter–driven Cre (*dLckCre*), active at or after the upregulation of TCR during the positive selection (10, 11). It has been shown that many signaling molecules, transcription factors as well as cytokines are specifically important for the development of innate-like T cells (1, 12). Deletion of these molecules early during development often result in abolishment of these cells, making it impossible to understand the functions of these genes during inflammation. Given the delayed onset of dLckCre activity, it has been generally used to analyze the effect of gene deletions in mature αβT cells (13, 14). However, the function of dLckCre in innate-like T cells has not been shown.

In this study, we examined the function of dLckCre using *ROSA26$^{floxed-Stop-tdTomato}$* reporter in 6- to 8-wk-old C57BL/6 mice. We found that tdTomato reporter expression is limited to a minor population of innate-like T cells in lymphoid and nonlymphoid organs. Analysis of the developmental stages in the thymus revealed that cells at more mature stages showed higher percentage of reporter expression. Further analysis of the effector subsets in the peripheral organs revealed that although dLckCre was active in MAIT cell subsets at similar levels, its activity was higher in NKT17 cells than in NKT1 and NKT2 cells. In γδT cells dLckCre activity was skewed toward γδT1 cells, and was excluded in γδT17 cells as evidenced by the expression of signature transcription factors, cytokines, TCR γ-chains and other surface markers. Lastly, dLckCre showed a near complete penetrance in γδT cells in the epidermis (dendritic epidermal T cell [DETC]), making it a good tool for gene deletion in DETCs.

MATERIALS AND METHODS

Mice

dLckCre (11) [B6.Cg-Tg(Lck-icre)3779Nik/J; The Jackson Laboratory stock no: 012837], *ROSA26$^{flox-stop-tdTomato}$* [B6.Cg *Gt(ROSA)26Sor$^{tm14(CAG-tdTomato)Hze}$*/J; The Jackson Laboratory stock no: 007914] mice were purchased from The Jackson Laboratory. Mice were bred and housed at the Oklahoma Medical Research Foundation vivarium under specific pathogen–free conditions. All experiments were conducted in accordance with the protocols approved by the Institutional Animal Care and Use Committee of Oklahoma Medical Research Foundation.

Flow cytometry and Abs

Single-cell suspensions were prepared from moues organs following standard procedure. Before Ab staining cells were preincubated with Fc block (BD Biosciences). CD1d-αGalCer tetramers and MR1–5OPRU tetramers were obtained from the National Institutes of Health Tetramer Facility, and used at a dilution of 1:200 and 1:300, respectively. Staining for transcription factors was performed using reagents and protocols from the Transcription Factor Buffer Set (BD Biosciences). Staining for intracellular cytokines was performed using reagents and protocols from BD Cytofix/Cytoperm Fixation/Permeablization Kit. The complete list of other Abs and reagents used is as follows: Live/Dead-blue (Thermo Fisher Scientific), anti–TCR β (H57–597), anti-CD3ε (145–2C11), anti–TCR δ (GL-3), anti-CD4 (RM4–5), anti-CD8α (53–6.7), anti-CD19 (6D5), anti-CD24 (M1/69), anti-CD44 (IM7) and anti-CD73 (TY/11.8), anti-CD49a (Ha31/8), anti–IFN-γ (XMG1.2), anti-IL-4 (11B11), anti–IL-17A (TC11–18H10.1), anti–TCR Vγ1 (2.11), anti–TCR Vγ4 (UC3–10A6), anti–TCR Vγ5 (536), anti-CD27 (LG.3A10), anti-CD45RB (C363–16A), anti-PLZF (R17–809), anti–T-bet (O4–46), anti-RORγt (Q31–378 or B2D). Stained samples are analyzed using Cytek Aurora and FlowJo software (Treestar).

In vitro and in vivo cytokine production

For in vitro stimulation with PMA and ionomycin, cells were incubated with PMA (phorbol 12-myristate 13-acetate; 50 ng/ml) and ionomycin (1.5 μM) for 4 h with brefeldin A (Sigma-Aldrich) added for the final 2 h in T cell culture medium (RPMI medium supplemented with 10% [vol/vol] FBS, 50 μM β-mercaptoethanol, 50 μg/ml penicillin/streptomycin/glutamine mix, 10 μM HEPES, 1× MEM nonessential amino acids, 1 μM sodium pyruvate), and intracellular cytokines were analyzed by flow cytometry. For in vivo stimulation of iNKT cells, mice were injected i.v. with 1 μg αGC (α-galactosylceramide) and spleen cells were analyzed 1.5 h later.

Epidermis preparation

Epidermal sheets were taken from abdomen and processed into single-cell suspension as previously described (15, 16). Cells were then stained for surface markers and transcription factors following standard procedures as above, and analyzed using Cytek Aurora and FlowJo software (Treestar).

Statistical analysis

All statistical analysis was performed using the Prism9 software.

RESULTS

dLckCre-mediated tdTomato reporter expression is limited in the innate-like T cells

To assess the activity of dLckCre, we crossed the *cre* transgenic line to mice carrying an *loxP*-Stop-*loxP*-tdTomato element knocked in the ROSA26 locus (17) (dLckCre[tdTomato]). Although Rosa26 locus supports strong and ubiquitous expression of inserted sequences, the Stop element prevents the transcription of the red fluorescence protein variant tdTomato. DNA recombination mediated by dLckCre results in the excision of the *loxP* flanked "Stop"

cassette and allows for robust expression of tdTomato, which reports the activity of dLckCre within the cells.

Innate-like T cells reside in the lymphoid (thymus, spleen, and lymph nodes [LNs]) and nonlymphoid (lung and liver) organs in dLckCre^{tdTomato} mice with different abundance as γδT cells (CD3ε$^+$TCRδ$^+$), MAIT cells (CD3ε^{int} MR1–5OPRU-tetramer$^+$), and iNKT cells (CD3ε^{int} CD1d-αGC-tetramer$^+$) (Fig. 1A). Although dLckCre was shown to be active at or after TCR upregulation during thymic development (11), tdTomato reporter was observed in a minority (30.2 ± 4.2%) of CD4$^+$ T cells (CD4$^+$TCRβ^{hi}) in dLckCre^{tdTomato} mice. Consistent with higher penetrance of dLckCre in CD8$^+$ T cells (11), 86.8 ± 2.8% of CD8$^+$ T cells (CD8$^+$TCRβ^{hi}) were tdTomato$^+$. In contrast, a minor fraction of γδT cells (3.8 ± 1.2%), MAIT cells (31.8 ± 6.4%), and iNKT cells (26.5 ± 4.7%) were tdTomato$^+$ (Fig. 1B). In the peripheral organs, dLckCre was active in the majority (>70%) of CD4$^+$ T cells (CD4$^+$TCRβ^{hi}) and CD8$^+$ T cells (CD8$^+$TCRβ^{hi}) as reflected by the percentage of tdTomato$^+$ cells, and dLckCre remained low penetrance in innate-like T cells (Fig. 1B).

dLckCre-mediated reporter expression is higher in the mature stages during the thymic development

Like conventional T cells, innate-like T cells develop in the thymus. Consistent with the onset of dLckCre activity postpositive selection (11), tdTomato reporter was barely detectable in CD4$^-$ CD8a$^-$ (DN) and CD4$^+$CD8a$^+$ (DP) cells (data not shown). CD24/ Heat-stable Ag is a marker for immature T cells at the precursor stages. Immature γδT cells (CD24$^+$CD73$^-$) gradually upregulate CD73 (CD24$^+$CD73$^+$) and downregulate CD24 (CD24$^-$CD73$^+$) as they mature (18–20). Although tdTomato reporter was only expressed in a very minor population of total γδT cells in the thymus (Fig. 1), reporter expression was the lowest in the immature stage (CD24$^+$CD73$^-$, 3.5 ± 1.1%), intermediate in CD24$^+$CD73$^+$ (7.1 ± 2.7%) and significantly higher in mature CD24$^-$CD73$^+$ (11.1 ± 4.9%) γδT cells (Fig. 2A–D). The similar trend of increasing tdTomato expression in percentage from immature stage to mature stage was also observed in MAIT cells (CD24$^+$CD44$^-$, CD24$^-$CD44$^-$, CD24$^-$CD44$^+$) (Fig. 2E–H) and iNKT cells (St0-CD24$^+$CD44$^-$, St1-CD24$^-$CD44$^-$, St2-CD24$^-$CD44$^+$, St3-CD24$^-$CD44$^+$CD49a$^+$) (Fig. 2I–M). This indicates that dLckCre is active in a limited fraction of developing innate-like T cells in the thymus, and further skews toward mature cells.

dLckCre-mediated tdTomato expression in the functional subsets of MAIT cells and iNKT cells

For conventional CD4$^+$ and CD8$^+$ T cells, it takes days to differentiate into mature effector cells after activation. Innate-like T cells carry out rapid effector functions within hours upon stimulation. This is mediated by functional subsets of these cells, which develop under homeostatic conditions. Signature transcription factors are shared among functional counterparts of innate-like T cell subsets and CD4$^+$ helper T cells. For example, T-bet is expressed in Th1, NKT1, MAIT1, and γδT1 cells capable of IFN-γ production, whereas RORγt is expressed in Th17, NKT17, MAIT17, and γδT17 cells producing IL-17 when stimulated. To assess the activity of dLckCre in different subsets of innate-like T cells, we defined the subsets based on transcription factor expression and examined the level

of tdTomato expression in each subset. In splenic MAIT cells (CD3ε^{int}MR1–5PORU-tetramer$^+$) (Fig. 3A), MAIT1 (T-bet$^+$RORγt$^-$), MAIT17 (T-bet$^-$RORγt$^+$), and MAIT1/17 (T-bet$^+$RORγt$^+$) had comparable proportions of tdTomato$^+$ cells (Fig. 3B–D), indicating that dLckCre has similar activity in different MAIT cell subsets. Consistently, when stimulated by PMA and ionomycin, the percentages of IFN-γ$^+$ and IL-17A$^+$ cells, as well as the expression of activation marker CD69, in tdTomato$^-$ and tdTomato$^+$ MAIT cells were similar (Supplemental Fig. 1A–D). Furthermore, in thymic MAIT cells, MAIT17 (RORγt$^+$T-bet$^-$) was the predominant subset and we did not detect MAIT expressing both T-bet and RORγt (MAIT1/17) as in the spleen. MAIT1 and MAIT17 cells in the thymus contained comparable percentages of tdTomato$^+$ cells (Supplemental Fig. 1E–H).

Similar to MAIT cells, iNKT cell subset distribution is organ specific (21). NKT17 cells (RORγt$^+$PLZF^{int}) made a significant proportion of iNKT cells in the LN (Fig. 3F), whereas in the liver and spleen, NKT1 (PLZF^{lo}RORγt$^-$) cells predominate and NKT17 is a minor subset (21). Interestingly, compared with NKT1 and NKT2 (PLZF^{hi}RORγt$^-$) cells, NKT17 cells in the LN had significantly higher percentage of tdTomato$^+$ cells (Fig. 3E–H). Higher percentage of tdTomato$^+$ was also observed in thymic NKT17 cells compared with NKT1 and NKT2 cells (Supplemental Fig. 2A–D). This suggests that dLckCre has higher activity in NKT17 cells. α-galactosyl ceramide (αGC), the prototypic iNKT cell agonist, induces immediate cytokine productions (IFN-γ, TNF-α, and IL-4) from splenic and liver iNKT cells when injected i.v. Consistent with low NKT17 presence in the spleen and liver, IL-17A was not detected. In agreement with similar tdTomato$^+$ percentage in NKT1 and NKT2 cells (Fig. 3, Supplemental Fig. 2), IFN-γ, TNF-α, and IL-4 expressions as well as the activation marker CD69 in tdTomato$^+$ splenic iNKT cells were comparable to those in tdTomato$^-$ cells (Supplemental Fig. 2E–H).

γδT1 cells are enriched and γδT17 cells are excluded in tdTomato$^+$ γδT cells

Based on the expression of T-bet and RORγt, splenic γδT cells (CD3ε$^+$TCRδ$^+$) were separated into γδT1 (T-bet$^+$RORγt$^-$), γδT17 (T-bet$^-$RORγt$^+$) and γδT-DN (T-bet$^-$RORγt$^-$) (Fig. 4A, 4B). Interestingly, whereas γδT1 and γδT-DN had similar percentage of tdTomato$^+$, tdTomato was absent in γδT17 (Fig. 4C, 4D). RORγt expression was exclusively in the tdTomato$^-$ γδT cells (Fig. 4E, 4G), while T-bet was expressed in both tdTomato$^-$ and tdTomato$^+$ populations with a slightly but significantly higher percentage in tdTomato$^+$ γδT cells (Fig. 4E, 4F). Consistent with the transcription factor expression, after PMA and ionomycin stimulation ex vivo, IL-17A production in γδT cells came exclusively from tdTomato$^-$ cells. Both tdTomato$^-$ and tdTomato$^+$ cells produce IFN-γ; however, IFN-γ$^+$ percentage was significantly higher in tdTomato$^+$ cells. To determine if the subset-specific expression of tdTomato is a general phenomenon, we examined γδT cells in other peripheral organs including LN, lung, and liver. As shown in Supplemental Fig. 3, although various tdTomato$^+$ percentage was observed in γδT cells from different organs, γδT17 (RORγt$^+$) cells were exclusively within tdTomato$^-$ population (Supplemental Fig. 3A), whereas γδT1 (T-bet$^+$) cells were not (Supplemental Fig. 3B). Therefore, tdTomato expression reporting the activity of dLckCre indicates that dLckCre may have limited function in γδT1 and γδT-DN cells, but is futile in γδT17 cells.

γδT cells in the adult lymphoid organs are mostly Vγ1$^+$ or Vγ4$^+$ (22). We examined the Vγ-chain usage by γδT1 (T-bet$^+$) and γδT17 (RORγt$^+$) cells in the LNs (Fig. 5A–C). Although γδT1 cells used both Vγ1 and Vγ4 chain, γδT17 cells preferably used Vγ4 (Fig. 5C). We then analyzed the Vγ1 and Vγ4 usage by tdTomato$^−$ and tdTomato$^+$ γδT cells. Although both Vγ-chains were used, tdTomato$^+$ γδT cells preferentially expressed Vγ1, whereas tdTomato$^−$ γδT cells skewed toward Vγ4$^+$. Next, we examined other surface markers. γδT1 (T-bet$^+$) cells are mostly CD27$^+$ CD45RB$^+$ and γδT17 (RORγt$^+$) cells are CD27$^−$ CD45RB$^−$ in the LNs (Fig. 5G). Consistent with the lack of γδT17 cells in tdTomato$^+$ γδT cells, CD27$^−$ CD45RB$^−$ population was significantly lower in tdTomato$^+$ than in tdTomato$^−$ γδT cells (Fig. 5H, 5I). Conversely, CD27$^+$CD45RB$^+$ population was significantly higher in tdTomato$^+$. Biased expressions of Vγ-chains and surface markers were also seen in splenic γδT cells (data not shown). These data indicate that dLCKcre is selectively active in part of γδT1 cells, but not γδT17 cells.

DETCs are tdTomato$^+$

DETCs are a unique group of γδT cells that develop first in the mouse embryonic thymus and home to the epidermis of the skin and are programmed to produce IFN-γ rather than IL-17 (22, 23). The preferential expression of dLckCre-driven tdTomato in IFN-γ–producing γδT cells (Fig. 4J) led us to hypothesize dLckCre may have higher penetrance in DETCs. We first confirmed that DETCs (CD3ε^{hi}TCRδ$^+$) comprised the majority of all CD3ε$^+$ cells in the epidermis (Fig. 6A, 6B), expressed higher level of CD3e (Supplemental Fig. 4A, 4B) than αβT cells, and used exclusively Vγ5 chain not Vγ1 or Vγ4 (Supplemental Fig. 4C, 4D). Neither αβT nor γδT cells in the epidermis expressed RORγt (Supplemental Fig. 4E, 4F). Although both T cell populations expressed T-bet, αβT cells expressed significantly higher T-bet level than γδT cells (Supplemental Fig. 4G, 4H). Consistent with T-bet expression, both αβT and γδT cells were CD45RB$^+$ (Fig. 6C, 6D), which is a γδT1 marker (Fig. 5G) (22). In striking contrast to γδT cells residing in other peripheral organs, nearly all DETCs (97.8 ± 1.1%) expressed tdTomato, and vast majority of αβT cells (92.9 ± 3.6%) were also tdTomato$^+$ (Fig. 6E–G). This indicates that dLckCre can be a useful tool to mediate gene deletion in DETCs.

DISCUSSION

The specific timing of dLckCre at or after the TCR^{high} stage of thymocyte development (11) makes it useful to study the effect of gene knockout in mature T cell functions without the potential impairment of thymic development. Using *ROSA26$^{floxed-stop-tdTomato}$* reporter and dLckCre strain from The Jackson Laboratory, we found dLckCre is active in a minor fraction of innate-like T cells, including γδT cells, MAIT cells, and iNKT cells, from both lymphoid and nonlymphoid organs in C57BL/6 mice (Fig. 1). A similar reporter system has been used to show the specific expression of dLckCre and pLckCre in T cells (7, 11). We further found dLckCre was preferentially active in mature stages of innate-like T cells during thymic development (Fig. 2). Interestingly, we observed distinct patterns for the subset distribution of dLckCre activity in MAIT cells, iNKT cells, and γδT cells (Figs. 3–5). Although MAIT cell subsets had comparable levels of tdTomato expression, higher percentage of NKT17 cells was tdTomato$^+$ than NKT1 and NKT2 cells. Conversely, γδT17

cells were excluded from tdTomato$^+$ γδT cells in all the organs tested. Reporter-positive γδT cells showed higher level of Vγ1 and other surface markers preferentially expressed by γδT1 cells (Figs. 4, 5). Consistent with the enrichment of IFN-γ–producing cells within tdTomato$^+$ γδT cells in spleen (Fig. 4), Vγ5$^+$ DETCs that are committed IFN-γ producers in the epidermis, showed complete penetrance of dLckCre function (Fig. 6), indicating that dLckCre is a useful tool to target epidermis resident γδT cells.

Low activity of dLckCre in innate-like T cells indicates the distal promoter of *Lck* gene is not functional in the majority of these unconventional T cells. The proximal promoter of Lck gene drives pLckCre expression in all iNKT cells and MAIT cells in the thymus (7), suggesting pLck promoter is preferentially used for expressing endogenous *Lck* gene in iNKT cells and MAIT cells. Comparable levels of tdTomato expression among iNKT cell subsets (with slightly higher level in NKT17 cells), as well as among MAIT cell subsets suggest that dLck promoter may be turned on in a fraction of the common precursor cells where all functional subsets derive from, although it remains unknown what determines the activity of dLck promoter in individual iNKT and MAIT cells.

In contrast to αβT cells, pLckCre is ineffective in adult γδT cells, as reflected by the low percentage of tdTomato$^+$ γδT cells in different organs as well as the low proximal Lck transcript level in γδT cells (7). pLckCre is transiently active perinatally in γδT cells, although it is not penetrant in all cells (7). dLckCre-driven tdTomato expression in adult γδT cells shown in this study (Fig. 1) was higher than pLckCre driven tdTomato (7), consistent with higher distal Lck transcript level than proximal ones (7). However, tdTomato$^+$ cells were still a minor population in dLckCre^{tdTomato} mice. Further studies should address if unidentified *cis*-acting elements regulate the transcription of Lck gene in γδT cells.

The striking difference in dLckcre activity in different γδT cell subsets, high in DETC and low in γδT17 cells for example, is consistent with the different Vγ-chain usage in these subpopulations. DETCs exclusively express Vγ5 (24), whereas γδT17 are mainly Vγ6$^+$ during fetal development and preferentially express Vγ4 in adults (25), and Vγ1 is highly expressed by IFN-γ–producing γδT1 cells (26). As TCR signal strength has been proposed to play an important role in the differentiation of γδT cell subsets (19, 25–31), it is intriguing to understand if dLck promoter activity reflects the distinct TCR signaling requirement in γδT cell subsets. However, this is a complex question given the involvement of other regulatory elements for Lck expression including pLck promoter, and further the contradictory data supporting either stronger or weaker TCR signals promote different γδT cell subset differentiation. In addition, signaling cascades downstream of TCR activation are not the only modulator for γδT cell differentiation, costimula-tory pathways and cytokine pathways may also contribute (1).

In conclusion, our study revealed the cell type specific function of a commonly used pan–T cell Cre recombinase driven by distal *Lck* promoter in innate-like T cells. The overall limited dLckCre activity in these cells requires that a reporter system (e.g., *ROSA26$^{floxed-stop-tdTomato}$*) is present when dLckCre is used to mediate gene deletion in innate-like T cells in order to identify bona fide knockout cells. Subset-skewed dLckCre

activity in iNKT cells and γδT cells also makes it necessary to use dLckCre⁺reporter⁺ mice as control instead of dLckCre⁻reporter⁻ ones. Furthermore, the results suggest that unconventional T cells may depend on unique regulatory mechanisms for important T cell signaling molecules distinct from that in mainstream T cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

Abbreviations used in this article:

DETC	dendritic epidermal T cell
dLckCre	distal Lck promoter–driven Cre
DN	double-negative
DP	double-positive
iNKT	invariant NKT
LN	lymph node
MAIT	mucosal-associated invariant T

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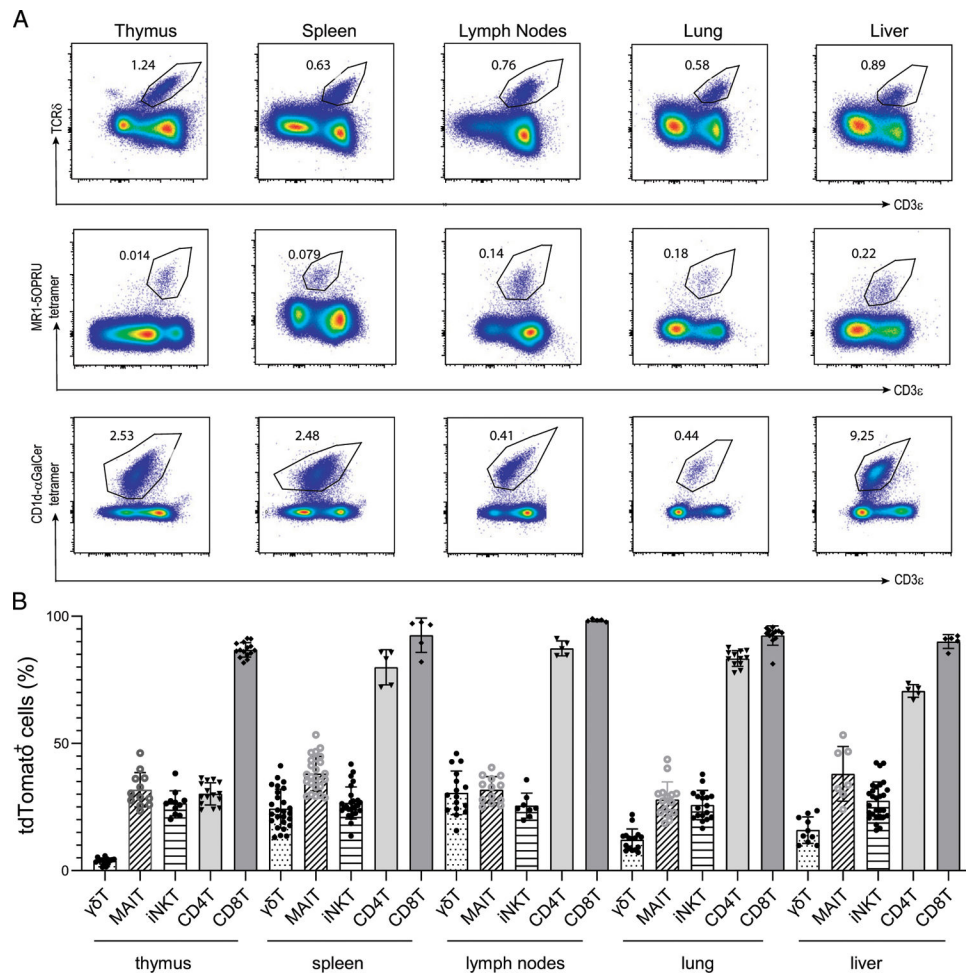
FIGURE 1. Innate-like T cells inefficiently express the dLckCre-mediated tdTomato reporter gene in lymphoid and nonlymphoid organs.

(**A**) Percentage of γδT cells (CD19⁻CD3ε⁺TCRδ⁺), MAIT

cells (CD19⁻TCRδ⁻CD3ε⁺MR1–5OPRU-tetramer⁺) and iNKT cells

(CD19⁻CD8α⁻CD3ε⁺CD1d-αGalCer-tetramer⁺) among live non-DP cells in thymus, or among total live cells in spleen, LNs, lung and liver. (**B**) the percentages of tdTomato⁺ cells in different T cell populations across tissues in dLckCre^tdTomato mice. Graphs represent mean ± SD with symbols representing individual mouse samples.
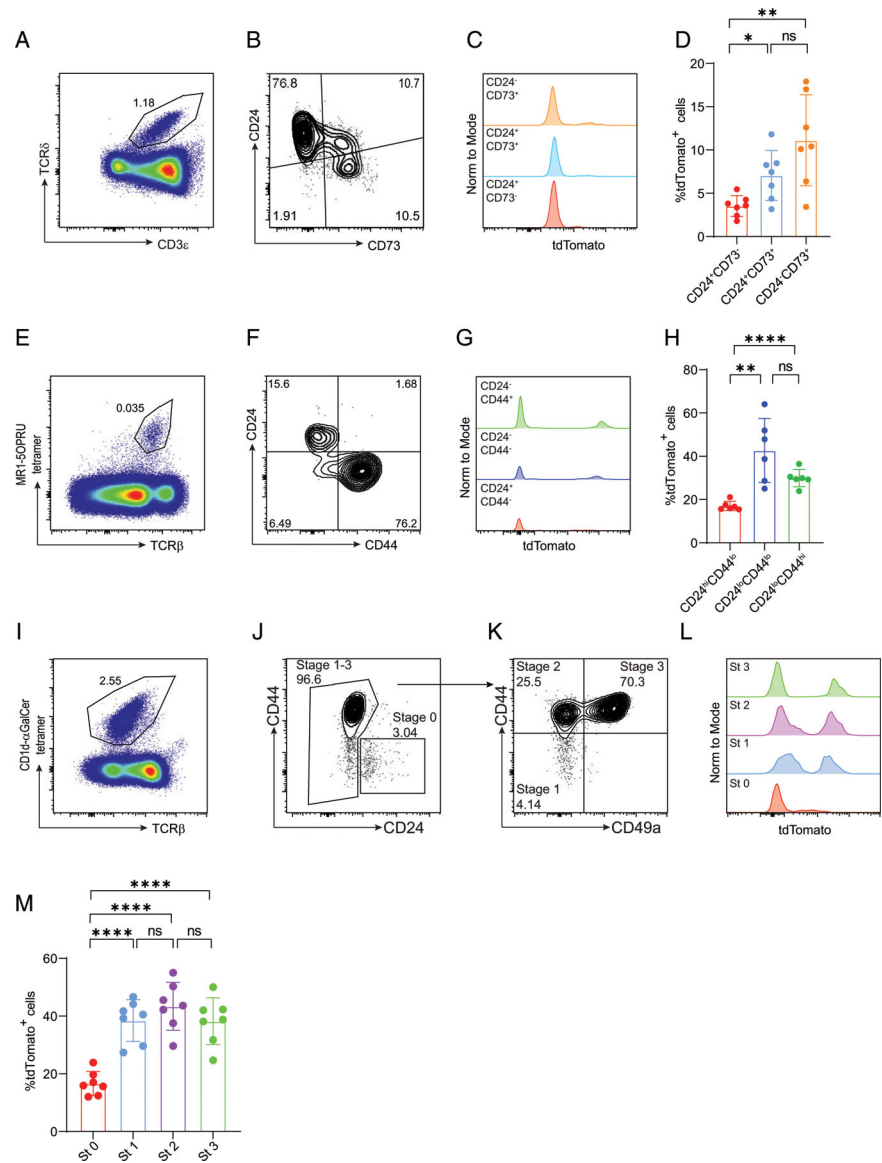
FIGURE 2. dLckCre-mediated reporter expression in innate-like T cells in the thymus is skewed toward mature stages.

(**A** and **B**) Thymic γδT cells (CD3ε⁺TCRδ⁺) develop from immature CD24⁺CD73⁻ stage into mature CD24⁻CD73⁺ cells. (**C** and **D**) The percentage of tdTomato⁺ in different stages of γδT cells are shown. (**E**–**H**) tdTomato expression in different stages of thymic MAIT cells (CD3ε⁺MR1–5OPRU-tetramer⁺) based on CD24 and CD44 expression. (**I**–**M**) tdTomato expression in different stages (stage 0–3) of thymic iNKT cells (CD3ε⁺CD1d-αGC-tetramer⁺). Graphs represent mean ± SD with symbols representing individual mouse samples. $*p < 0.05$, $**p < 0.01$, $****p < 0.0001$, paired two-tailed Student t test. n.s., not significant.
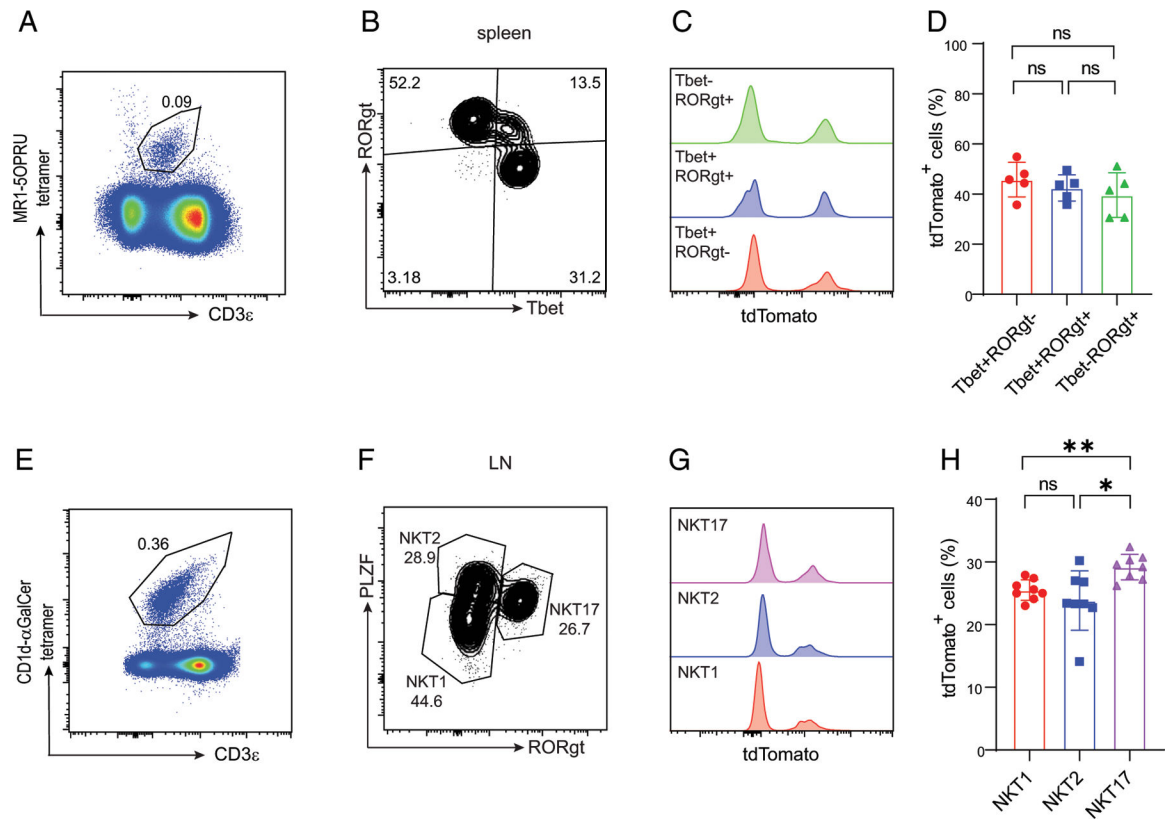
FIGURE 3. dLckCre-mediated reporter expression in the functional subsets of MAIT cells and iNKT cells.

(**A**–**D**) Splenic MAIT cells are separated into functional subsets based on the expression of transcription factors T-bet and RORγt. (C and D) Percentage of tdTomato⁺ cells in different subsets are shown. (**E**–**H**) tdTomato expression in the functional subsets of iNKT cells in the LNs. Graphs represent mean ± SD with symbols representing individual mouse samples. $*p < 0.05$, $**p < 0.01$, paired two-tailed Student t test. n.s., not significant.
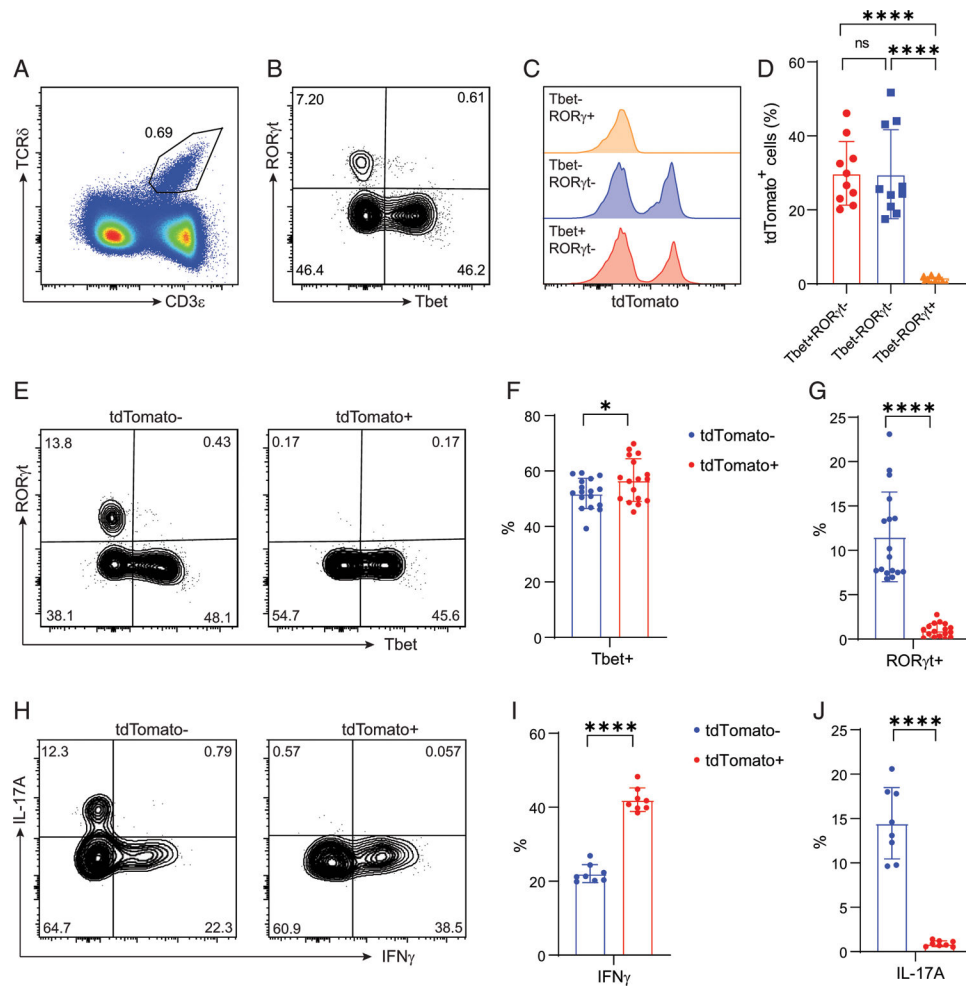
FIGURE 4. dLckCre-mediated reporter expression is skewed in the subsets of γδT cells.
(A–E) Splenic γδT cells are separated into γδT1 (T-bet⁺RORγt⁻), γδT17 (T-bet⁻RORγt⁺),
and γδT-DN (T-bet⁻RORγt⁻) subsets, and the percentages of tdTomato⁺ cells in each subset
are shown (C and D). (E–J) γδT1 and γδT17 cells in tdTomato⁻ and tdTomato⁺ splenic
γδT cells. (H–J) IFN-γ and IL-17A production from tdTomato⁻ and tdTomato⁺ splenic
γδT cells after PMA and ionomycin stimulation ex vivo. Graphs represent mean ± SD
with symbols representing individual mouse samples. *$p < 0.05$, ****$p < 0.0001$, paired
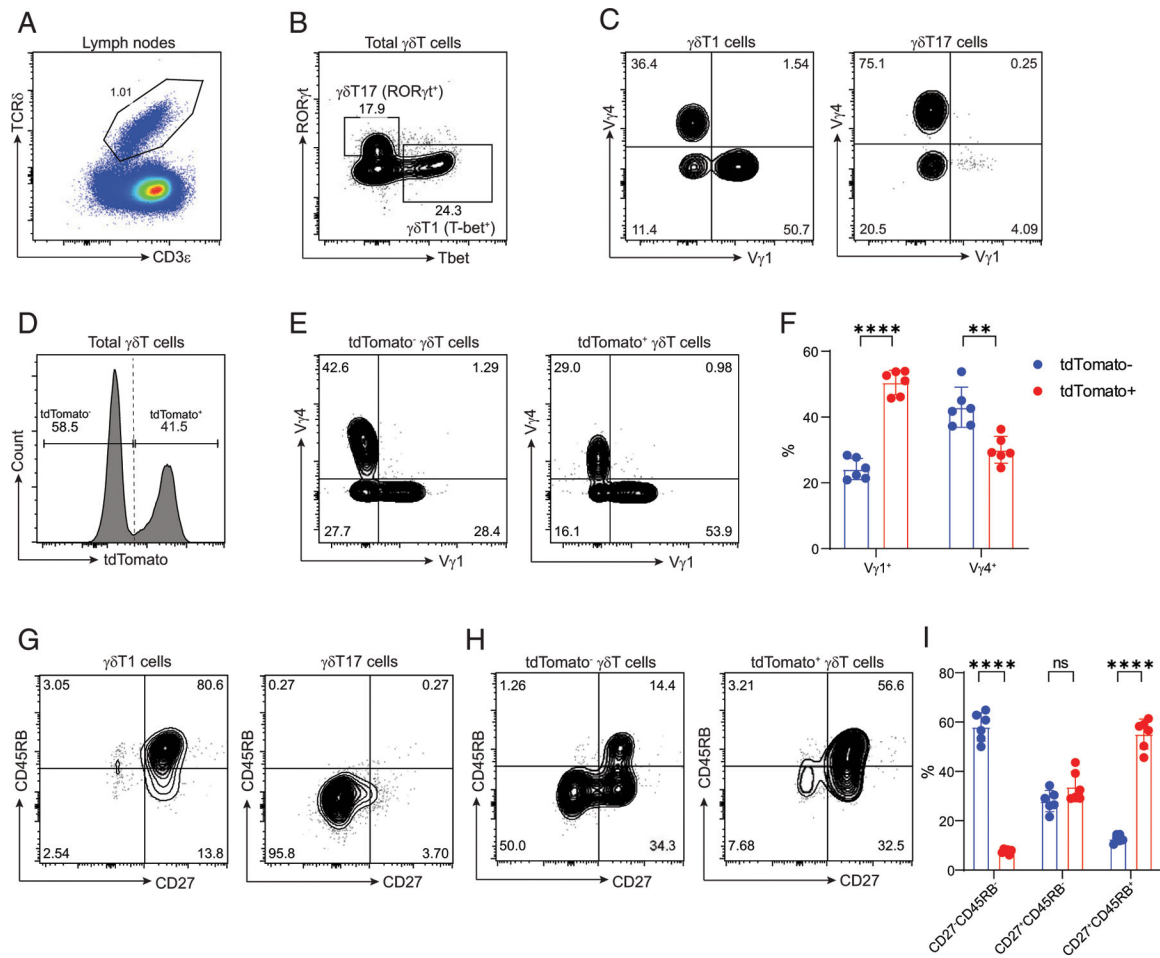two-tailed Student t test. n.s., not significant.

FIGURE 5. Biased Vγ-chain usage and surface marker expression in tdTomato⁺ γδT cells.
(**A–C**) Vγ1 and Vγ4 usage in γδT1 and γδT17 cells from LNs. (**D–F**) differential Vγ1
and Vγ4 usage in tdTomato⁻ and tdTomato⁺ γδT cells from LNs. (**G**) CD27 and CD45RB
expression in γδT1 and γδT17 cells. (**H** and **I**) Differential CD27 and CD45RB expression
in tdTomato⁻ and tdTomato⁺ γδT cells from LNs. Graphs represent mean ± SD with
symbols representing individual mouse samples. **$p < 0.01$, ****$p < 0.0001$, paired two-
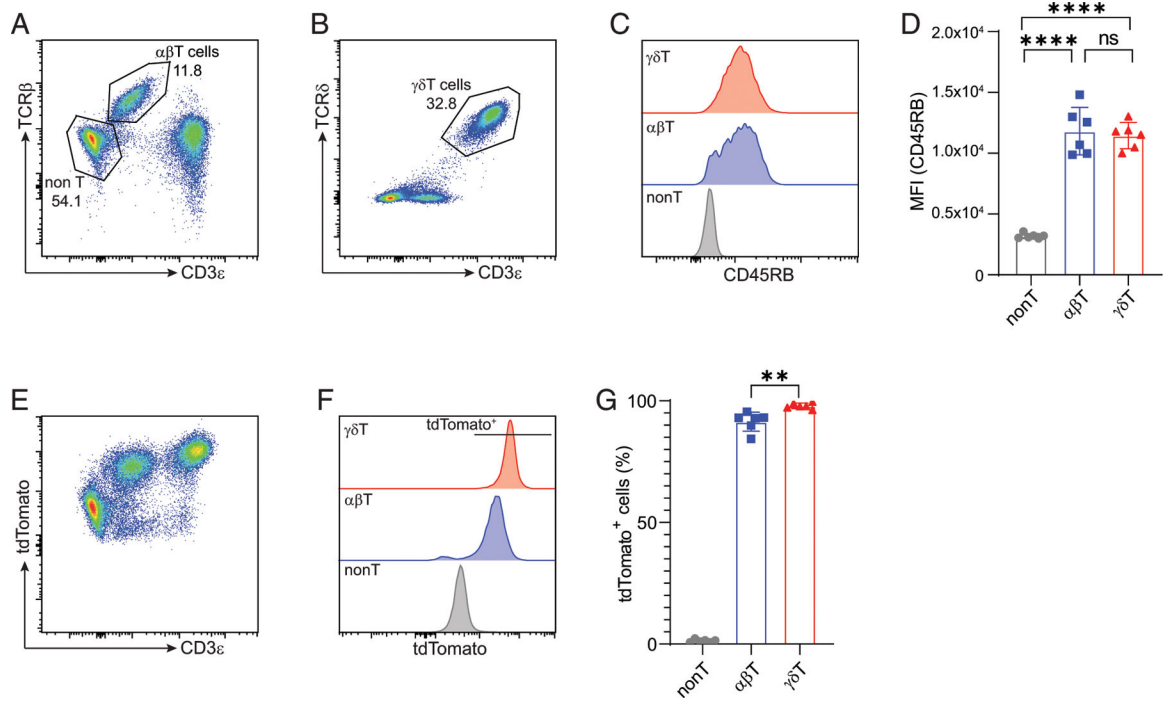tailed Student t test. n.s., not significant.

FIGURE 6. dLckCre-mediated reporter expression is shown in all DETCs.
(**A**) αβT cells (CD3ε$^+$TCRβ$^+$) and (**B**) γδT cells (CD3ε^{hi}TCRδ$^+$) in live CD45$^+$ epidermal cells. Expression of CD45RB (**C** and **D**) in T and non–T cells in epidermis. (**E–G**) expression of tdTomato in T and non–T cells in the epidermis. Graphs represent mean ± SD with symbols representing individual mouse samples. **$p < 0.01$, ****$p < 0.0001$, paired two-tailed Student t test. n.s., not significant.