

RESEARCH ARTICLE

A yolk sac-derived hepatic $\gamma\delta$ T cell subpopulation develops in a hematopoietic stem cell- and thymus-independent mannerYuzhe Chen^{1,†}, Chaojie Wang^{2,†}, Kangyu Huang^{1,†}, Guoju You^{2,†}, Bing Liu^{1,3,*}, Yu Lan^{1,2,4,*}¹State Key Laboratory of Experimental Hematology, Institute of Hematology, School of Medicine, Jinan University, Guangzhou 510632, China²State Key Laboratory of Experimental Hematology, National Clinical Research Center for Blood Diseases, Haihe Laboratory of Cell Ecosystem, Institute of Hematology & Blood Diseases Hospital, Chinese Academy of Medical Sciences & Peking Union Medical College, Tianjin 300020, China³State Key Laboratory of Experimental Hematology, Haihe Laboratory of Cell Ecosystem, Senior Department of Hematology, Fifth Medical Center, Medical Innovation Research Department, Chinese PLA General Hospital, Beijing 100071, China⁴Tianjin Institutes of Health Science, Tianjin 301600, China[†]These authors contributed equally to this work.^{*}Correspondence: bingliu17@126.com (B. Liu), rainyblue_1999@126.com (Y. Lan)

Abstract

$\gamma\delta$ T cells are a highly heterogeneous T cell lineage with diverse immunological functions; however, the developmental origins underlying this heterogeneity, particularly the existence and physiological relevance of extrathymic pathways, remain incompletely defined. Our previously generated transcriptomic datasets indicate that *Stab2* is specifically expressed in the yolk sac but not the aortic endothelial cells. Leveraging this spatially restricted expression pattern, we generated a *Stab2-CrexERT2* inducible genetic lineage tracing mouse model and demonstrated its ability to label yolk sac-derived hematopoietic cells *in vivo*. Importantly, we detected the contribution of the yolk sac endothelial cells labeled at embryonic day 9.5 to a subset of V γ 1 $\gamma\delta$ T cells in the adult liver but hardly to any hematopoietic progenitors in the thymus and bone marrow. Single-cell transcriptomic profiling combined with functional evaluation revealed that the *Stab2*-labeled yolk sac endothelial cell-derived hepatic $\gamma\delta$ T cells preferentially enrich IFN- γ -producing $\gamma\delta$ T1 cells and overrepresent characteristics associated with tissue homeostasis and repair. Collectively, our findings provide direct *in vivo* evidence for the existence of a bone marrow hematopoietic stem cell-independent and thymus-independent developmental pathway of liver $\gamma\delta$ T cells originating from yolk sac endothelial cells, and suggest that different origins and developmental paths may confer distinct functional properties to $\gamma\delta$ T cells.

Keywords yolk sac endothelial cells, $\gamma\delta$ T cells, liver, extrathymic development, hematopoietic stem cell-independent hematopoiesis

Introduction

$\gamma\delta$ T cells are a distinct lineage of T lymphocytes defined by T cell receptors (TCRs) composed of γ and δ chains. These cells exhibit a broad functional spectrum that encompasses both innate and adaptive immune activities, along with diversified mechanisms of antigen recognition and presentation (Hu et al., 2023). Although representing a minor fraction of circulating T cells, $\gamma\delta$ T cells play nonredundant roles

in tissue surveillance, early immune responses, and maintenance of physiological homeostasis. *Tcrd*-deficient mice display increased epidermal keratinocyte apoptosis and reduced intestinal epithelial turnover, establishing a critical role for $\gamma\delta$ T cells in epithelial barrier maintenance (Komano et al., 1995; Sharp et al., 2005). In addition, $\gamma\delta$ T cell deficiency has been associated with persistent periodontal inflammation and impaired short-term memory, suggesting broader involvement in tissue homeostasis and neuroimmune

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regulation (Krishnan et al., 2018; Reisel et al., 2002). By virtue of their MHC-independent recognition and diversified cytotoxic programs, $\gamma\delta$ T cells may mitigate tumor immune escape and limit alloreactive complications, including graft-versus-host disease, associated with conventional CAR-T cell therapy. Despite growing appreciation of their functional importance, the developmental origins and lineage diversification of $\gamma\delta$ T cells remain incompletely defined.

$\gamma\delta$ T cells follow a developmental trajectory distinct from that of $\alpha\beta$ T cells. Generally, the thymus serves as the primary site for their development, differentiation, and maturation. Beginning around embryonic day (E) 15.0, the murine fetal thymus generates $\gamma\delta$ T cells with distinct molecular and functional characteristics through successive developmental waves. These cells subsequently migrate to peripheral tissues, where they establish long-lived, self-renewing resident populations (Fiala et al., 2020; Ribot et al., 2021). In brief, monoclonal V γ 5V δ 1 $\gamma\delta$ T cells emerge around E15.0 and preferentially home to the epidermis; oligoclonal V γ 6V δ 1 and V γ 4 subsets arise several days later; V γ 7 $\gamma\delta$ T cells are produced during the perinatal period and predominantly localize to the intestine. Postnatally, polyclonal V γ 1 and V γ 4 $\gamma\delta$ T cells are continuously generated from hematopoietic stem cells (HSCs)-derived thymic progenitors and enter the peripheral circulation (Haas et al., 2012). Continuous colonization of the thymus by hematopoietic progenitors is essential for sustained T cell production. Although multi-omics studies indicate that $\gamma\delta$ T cells originate from distinct double-negative (DN) progenitors (Sagar, 2024), lineage tracing evidence defining their hematopoietic origins remains limited. Apart from epidermal dendritic epidermal T cells, which derive from embryonic yolk sac hematopoiesis and are maintained through *in situ* self-renewal (Gentek et al., 2018), the developmental origins of most $\gamma\delta$ T cell subsets remain poorly characterized.

Emerging evidence also supports the existence of extrathymic developmental pathways for $\gamma\delta$ T cells. In nude mice, intestinal $\gamma\delta$ T cell populations are largely preserved despite the absence of a functional thymus, suggesting that the intestinal crypt microenvironment can support their local maturation (Bandeira et al., 1991). In addition, transplantation of adult liver hematopoietic progenitor (Lin-Sca-1⁺Mac-1⁺, LSM) into thymectomized mice results in the generation of IFN- γ -producing $\gamma\delta$ T cells in the liver, implicating extrathymic development for a subset of hepatic $\gamma\delta$ T cells (Hu et al., 2021). However, whether these thymus-independent hepatic $\gamma\delta$ T cells originate from HSCs or the extraembryonic yolk sac remains unknown. Collectively, due to the limitations of currently available experimental approaches, the development and biological characteristics of extrathymically derived $\gamma\delta$ T cells under physiological conditions require further investigation.

The continued refinement of lineage tracing models has demonstrated that multiple adult immune cell populations arise independently of HSCs. These include tissue-resident macrophages—such as microglia in the

brain and Kupffer cells in the liver, as well as peritoneal B-1a cells and certain circulating lymphocyte subsets (Hoeffel et al., 2015; Kobayashi et al., 2023). Despite their utility, existing lineage tracing models for studying HSC-independent hematopoiesis (e.g., *Cdh5-CreERT2*, *Csf1r-CreERT2*, and *Runx1-CreERT2*) remain limited by incomplete discrimination between intraembryonic and extraembryonic hematopoietic sources. Single-cell transcriptomic profiling from our laboratory revealed specific expression of *Stab2* in yolk sac endothelial cells (Hou et al., 2022), supporting its potential utility as a marker for yolk sac-derived hematopoiesis. Leveraging this observation, we established an inducible *Stab2-CrexERT2* lineage tracing model to delineate the contribution of yolk sac-derived progenitors to $\gamma\delta$ T cell populations across adult tissues.

Results

Generation of an inducible *Stab2-CrexERT2* mouse model that specifically labels yolk sac-derived hematopoiesis

By reanalyzing our previously generated single-cell RNA sequencing datasets from E8.0 to E10.0 yolk sac and embryo proper (Hou et al., 2022), we identified the five genes most highly enriched in yolk sac endothelial cells but minimally expressed in embryo proper endothelial populations (Fig. 1A). *Mrc1*, encoding CD206, is predominantly expressed on macrophages, immature dendritic cells, and liver sinusoidal endothelial cells (Sheikh et al., 2000). This expression pattern introduces potential bias and therefore limits its suitability as a marker for unbiased lineage tracing applications. In contrast, *Stab2*, a member of the scavenger receptor family, is predominantly expressed by liver sinusoidal endothelial cells (Schledzewski et al., 2011). Its endothelial-restricted expression pattern makes *Stab2* a suitable candidate for tracing yolk sac-derived hematopoiesis. Accordingly, we generated a *Stab2-CrexERT2* knockin mouse model using a CRISPR/Cas9-mediated gene editing strategy, in which an inducible *CrexERT2* cassette was inserted between exon 69 and the 3' untranslated region (3'UTR) of the endogenous *Stab2* locus (Fig. 1B). *Stab2-CrexERT2* mice were intercrossed with CX40-EGFP arterial reporter mice to enable the identification of arterial endothelial populations. Immunofluorescence staining was conducted to assess estrogen receptor (ER) expression in the yolk sac and embryo proper at E8.5, E9.5, and E10.5. ER expression was evident in yolk sac endothelial cells at E8.5 but was undetectable in the dorsal aorta of the embryo proper at the same developmental stage (Fig. 1C). ER remained undetectable in the dorsal aortic endothelial cells while being robustly expressed in the yolk sac vasculature at E9.5 and E10.5 (Fig. 1D and 1E). Furthermore, ER expression was absent from hematopoietic cells in both the yolk sac and the embryo proper at E8.5, E9.5, and E10.5. Collectively, these data corroborate our transcriptomic results, demonstrating that *Stab2* expression is preferentially confined

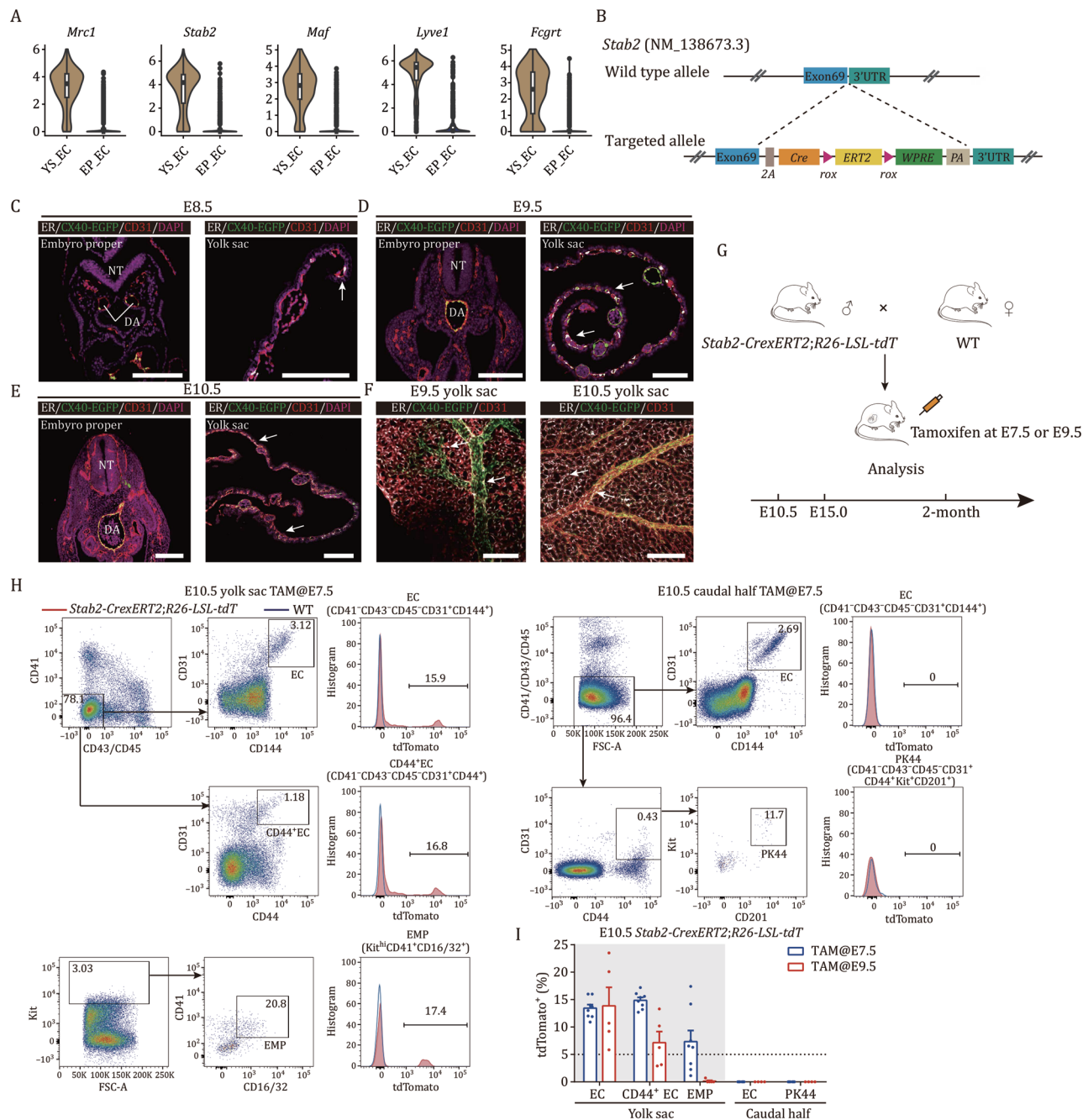


Figure 1. An inducible *Stab2-CreERT2* mouse model labels yolk sac-derived hematopoiesis. (A) Violin plots depicting the top five genes significantly enriched in E8.0–E10.0 yolk sac endothelial cells, with minimal expression detected in embryonic endothelial populations. (B) Schematic representation of the CRISPR/Cas9-mediated gene-targeting strategy used to generate the *Stab2-CreERT2* lineage tracing mouse line. (C–E) Representative immunofluorescence staining of E8.5 (C), E9.5 (D), and E10.5 (E). AGM and yolk sac sections from *Stab2-CreERT2*; *CX40-EGFP* embryos. Arrows indicate ER⁺ endothelial cells. Scale bars, 200 μ m. (F) Whole-mount immunostaining of E9.5 and E10.5 yolk sac from *Stab2-CreERT2*; *CX40-EGFP* embryos. The two white lines point to cavities that represent the dorsal aorta. Arrows indicate ER⁺ endothelial cells. (G) Schematic overview of the experimental design for genetic lineage tracing using *Stab2-CreERT2*; *R26-LSL-tdT* mice. (H) Representative flow cytometry profiling of YS and CH regions from E10.5 *Stab2-CreERT2*; *R26-LSL-tdT* embryos after temporal induction with tamoxifen at E7.5. (I) Statistical quantification of the flow cytometry data shown in (H). Data are shown as means $\pm$ SEM from three independent experiments. The tdTomato⁺ cells below 5% were considered to have negligible contribution. Scale bars, 200 μ m. YS: yolk sac, EP: embryo proper, EC: endothelial cell, DA: dorsal aorta, NT: neural tube, CH: caudal half, EMP: erythro-myeloid progenitor.

to the yolk sac endothelial cells and excluded from the dorsal aortic endothelial cells where HSCs are generated. Detailed spatial analysis revealed that, at E9.5 and E10.5, ER expression was enriched within the yolk sac venous

plexus, with only minor expression detected in arterial endothelial structures (Fig. 1F).

To determine whether the *Stab2-CreERT2* specifically fate-maps yolk sac-derived hematopoiesis, we generated

Stab2-CrexERT2; *R26-LSL-tdT* mice. Wild-type females were crossed with *Stab2-CrexERT2*; *R26-LSL-tdT* males, and temporal recombination was induced by a single dose of tamoxifen administered at E7.5 or E9.5. Embryos were harvested at E10.5, and recombination patterns were analyzed in the yolk sac and in the caudal half region (Fig. 1G). Induction at E7.5 or E9.5 led to robust labeling of yolk sac endothelial cells (CD41⁺CD43⁺CD45⁺CD31⁺CD144⁺), while recombination in endothelial cells of the caudal half region was negligible, confirming the selective expression of *Stab2* in extraembryonic yolk sac endothelial cells (Fig. 1H and 1I). Hematopoietic stem and progenitor cells arise in both the yolk sac and the AGM region via endothelial-to-hematopoietic transition (EHT) (Chen et al., 2009; Kissa and Herbomel, 2010). In the AGM, hemogenic endothelium is enriched within the PK44 (CD201⁺CD44⁺Kit⁺) population (Hou et al., 2020, 2024), whereas in the yolk sac, hematopoietic competence progressively consolidates within CD44⁺ endothelial cells from approximately E10.0 (Wang et al., 2021a). Consistent with the overall endothelial labeling pattern, tamoxifen administration at either E7.5 or E9.5 resulted in recombination in a subset of yolk sac CD44⁺ endothelial cells, whereas hemogenic endothelium within the caudal half region was minimally labeled (Fig. 1H and 1I). Notably, induction at E7.5, but not E9.5, led to efficient labeling of the erythro-myeloid progenitors (EMPs) in the yolk sac (Fig. 1H and 1I). This temporal restriction aligns with the known dynamics of yolk sac-derived hematopoiesis. Collectively, these findings demonstrate that temporally controlled activation of the *Stab2-CrexERT2* at E7.5 or E9.5 selectively marks yolk sac-derived hematopoietic output while largely excluding contributions from the caudal half region.

Adult liver contains yolk sac endothelial cell-derived V γ 1 $\gamma\delta$ T cells

To assess the contribution of *Stab2*-labeled yolk sac endothelial cells to tissue-resident immune cell populations, we administered a single dose of tamoxifen at E7.5 or E9.5 and subsequently traced the contribution to microglia and B-1 cells in 2-month-old mice. Consistent with their early yolk sac origin, microglia (CD45^{int}Ly6C⁺CD11b⁺F4/80⁺) exhibited robust labeling upon E7.5 induction, while E9.5 administration resulted in only minimal recombination (Fig. 2A–D). Neither E7.5 nor E9.5 recombination marked B-1 cells in the peritoneal cavity and spleen (Fig. 2B–D), indicating minimal contribution from *Stab2*-expressing yolk sac endothelial cells to these compartments. $\gamma\delta$ T cells across multiple tissues, including the liver, lung, skin, spleen, and lymph nodes, also exhibit tissue-resident properties (Du Halgouet et al., 2024; Qu et al., 2022; Wang et al., 2021b). We next examined $\gamma\delta$ T cells in five tissues from 2-month-old mice, including liver, lung, small intestine, spleen, and thymus. Notably, E9.5, but not E7.5 induction labeled a discrete fraction of hepatic $\gamma\delta$ T cells (CD45⁺CD3⁺ $\gamma\delta$ TCR⁺), whereas $\gamma\delta$ T cells in the lung, small

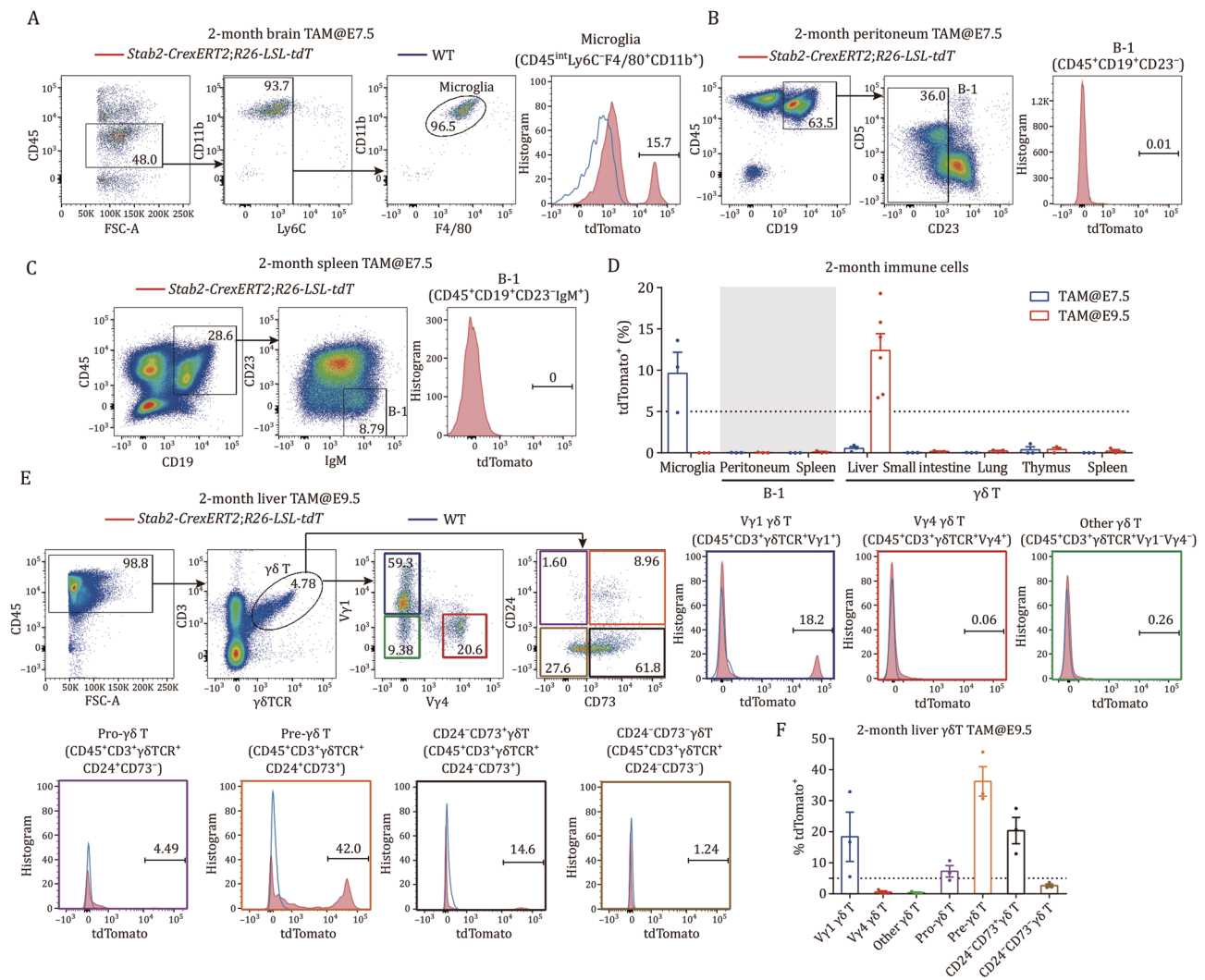
intestine, spleen, and thymus remained unlabeled at both time points (Fig. 2E–D), suggesting a temporally restricted and tissue-specific yolk sac contribution to the liver $\gamma\delta$ T cell pool. We next analyzed the V γ chain usage of this labeled $\gamma\delta$ T cell subset and found that the E9.5-labeled $\gamma\delta$ T cells predominantly expressed V γ 1 (Fig. 2E and 2F). To define their developmental maturation state, we analyzed CD24 and CD73 expression, established markers of $\gamma\delta$ T cell ontogeny (Coffey et al., 2014). TdTomato⁺ cells were enriched within both the CD24⁺CD73⁺ progenitor and CD24⁺CD73⁺ precursor populations, indicating that E9.5-labeled yolk sac-derived cells contribute to early developmental stages of the hepatic $\gamma\delta$ T cell compartment (Fig. 2E and 2F).

E9.5-labeled yolk sac-derived $\gamma\delta$ T cells originate in a HSC-independent and thymus-independent manner

To delineate the ontogeny of the E9.5 yolk sac endothelial cell-derived hepatic $\gamma\delta$ T cells, we examined the labeling in adult bone marrow hematopoietic stem and progenitor cells, including long-term hematopoietic stem cells (LT-HSCs, LIN-Kit⁺Sca-1⁺CD135⁺CD34⁺), short-term hematopoietic stem cells (ST-HSCs, LIN-Kit⁺Sca-1⁺CD135⁺CD34⁺), multipotent progenitors (MPPs, LIN-Kit⁺Sca-1⁺CD135⁺CD34⁺), common myeloid progenitors (CMPs, LIN-Kit⁺Sca-1⁺CD34⁺CD16/32⁺), granulocyte-macrophage progenitors (GMPs, LIN-Kit⁺Sca-1⁺CD34⁺CD16/32⁺), and megakaryocyte-erythroid progenitors (MEPs, LIN-Kit⁺Sca-1⁺CD34⁺CD16/32⁺) (Cheng et al., 2020). No labeling was detected following E9.5 induction (Fig. 3A), supporting an adult HSC-independent developmental origin.

As immature $\gamma\delta$ T cells are known to exit in the thymus prior to full maturation and differentiate in peripheral tissues (Joannou et al., 2022), we evaluated the labeling in thymic progenitor compartments across embryonic (E12.0, E15.0, and E18.0) and adult (2-month) stages. E9.5 induction failed to label ETPs (LIN-CD45⁺CD44⁺CD25⁺Kit⁺), any DN subsets, or CD45⁺ cells at all time points analyzed (Fig. 3B), providing compelling evidence that the E9.5 yolk sac-derived $\gamma\delta$ T cells develop independent of the thymus.

The robust labeling of immature $\gamma\delta$ T cells in the adult liver suggested that the liver may serve as a developmental niche for this subset. The fetal liver serves as the principal hematopoietic organ during murine embryogenesis, and mouse adult liver also contains hematopoietic stem cells derived from the fetal liver (Bai et al., 2021). We further examined LSK (LIN⁺Sca-1⁺Kit⁺) compartments, which enrich hematopoietic stem progenitor cells, in 2-month-old mice liver and fetal liver. E9.5 induction resulted in the labeling of LSK in both fetal and adult liver (Fig. 3C). Furthermore, E9.5 induction selectively labeled the common lymphoid progenitors (CLPs, LIN-CD127⁺CD135⁺ $\alpha\beta$ 7⁺) population in the E15.0 fetal liver, whereas other progenitor subsets, including lymphoid-primed multipotent progenitors (LMPPs, LIN-CD127⁺Kit⁺Sca-1⁺CD135⁺), CMPs,



GMPs, and MEPs, remained unlabeled (Fig. 3D). This selective labeling suggests the emergence of a yolk sac-derived progenitor population with lymphoid-restricted potential in the fetal liver, which may be retained within the hepatic niche and serve as a direct source of hepatic $\gamma\delta$ T cells.

Characterizing E9.5 yolk sac-derived $\gamma\delta$ T cells by single-cell transcriptomic analysis

To delineate the molecular features of E9.5-labeled $\gamma\delta$ T cells, we conducted single-cell RNA sequencing on tdTomato⁺ cells in 2-month-old mice liver isolated by flow cytometry. This population encompassed all $\gamma\delta$ T cells and a minor proportion of other cell types, with tdTomato-counterparts analyzed in parallel as controls (Fig. 4A). Unsupervised clustering resolved 13 transcriptionally distinct cell populations in the liver, which were further

refined and annotated according to their characteristic gene expression profiles, including natural killer cells (*Ncr1*, *Klr1d1*), innate lymphoid cells (*Il7r*, *Gata3*), $\alpha\beta$ T cells (*Cd3e*, *Trac*), $\gamma\delta$ T cells (*Cd3e*, *Trdc*), B cells (*Cd79a*, *Cd19*), dendritic cells (*H2-Aa*, *Irf8*), monocytes (*Lyz2*, *Ctss*), macrophages (*Cd68*, *C1qa*), neutrophils (*S100a8*, *S100a9*), endothelial cells (*Kdr*, *Pecam1*), mesenchymal cells (*Dcn*, *Col3a1*), hepatocytes (*Apoc3*, *Ttr*), and cholangiocytes (*Cldn3*, *Spp1*) (Fig. 4B and 4C; Table S1). Notably, six $\gamma\delta$ T cell populations were identified, characterized by the expression of *Trdc*, *Trgc1*, *Trgv1*, and distinct TCR repertoires (Fig. 4C and 4D). We next focused on the $\gamma\delta$ T cell populations to investigate their molecular characteristics and developmental dynamics (Fig. 4E–G; Table S2). The gdT_Sell cluster corresponds to a naïve $\gamma\delta$ T cell population in the liver, defined by elevated expression of

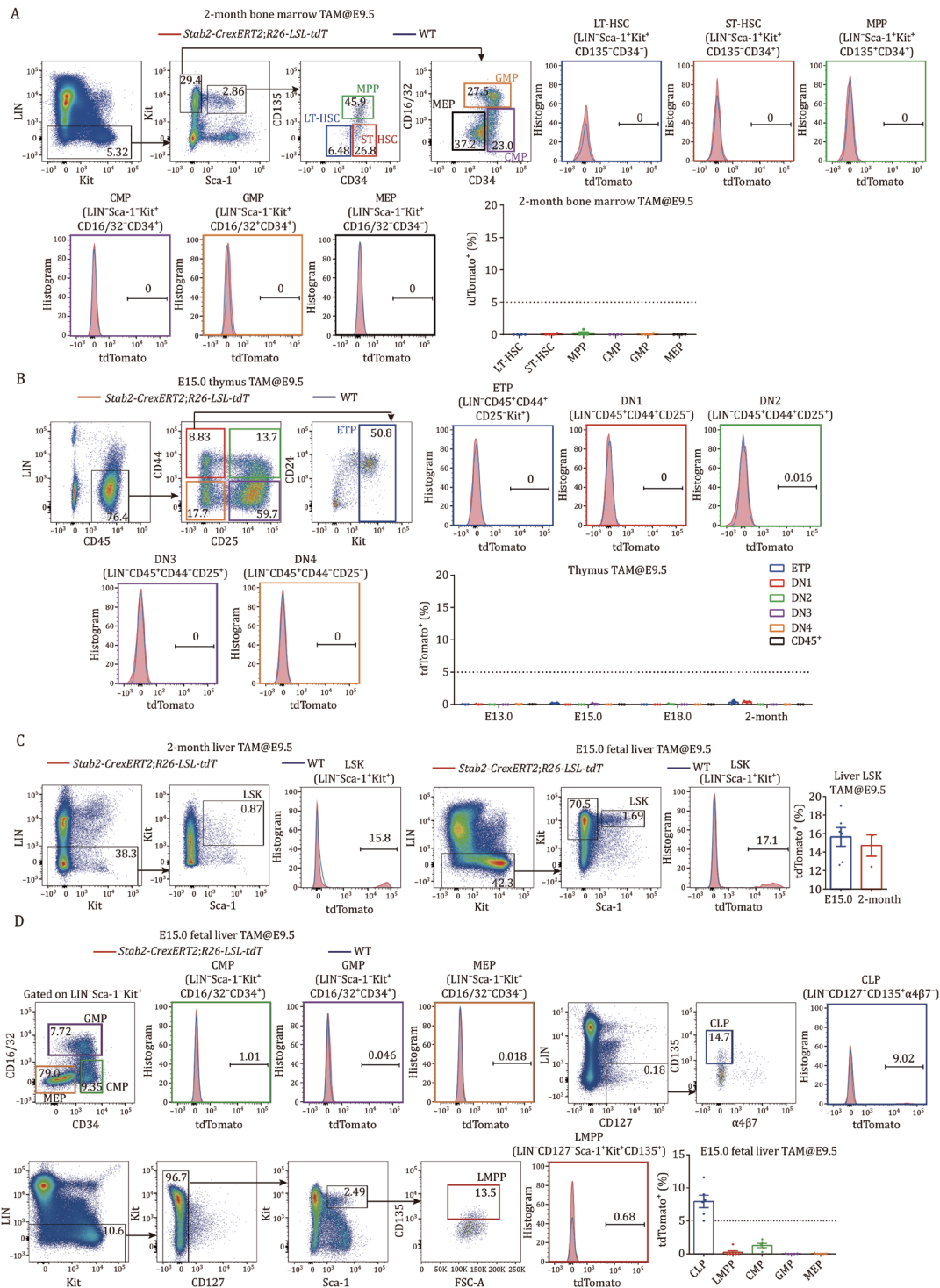


Figure 3. E9.5 yolk sac-derived $\gamma\delta$ T cells originate independent of the thymus. (A) Representative flow cytometric analysis and statistical quantification of hematopoietic stem and progenitor cells in 2-month-old *Stab2-CrexERT2; R26-LSL-tdT* mice bone marrow following tamoxifen induction at E9.5. LIN: TER119, Gr-1, CD11c, CD19, CD127, CD11b, CD3, CD4, CD8 α , NK1.1. (B) Representative flow cytometric analysis and statistical quantification of ETP and DNs in embryonic and adult *Stab2-CrexERT2; R26-LSL-tdT* mice thymus following tamoxifen induction at E9.5. LIN: TER119, Gr-1, B220, CD4, CD8 α , NK1.1. (C) Representative flow cytometric analysis and statistical quantification of LSK (LIN⁺Sca-1⁺Kit⁺) in embryonic and adult *Stab2-CrexERT2; R26-LSL-tdT* mice liver following tamoxifen induction at E9.5. LIN: TER119, Gr-1, CD11c, CD19, CD3, NK1.1. (D) Representative flow cytometric analysis and statistical quantification of hematopoietic progenitors in fetal liver following tamoxifen induction at E9.5. LIN (CMP, GMP, and MEP): Ter119, Gr-1, CD11c, CD19, CD3, NK1.1. LIN (LMPP, CLP): Ter119, Gr-1, CD11c, CD19, NK1.1, CD3e, CD4, CD8. Data are shown as means $\pm$ SEM from three independent experiments. The tdTomato⁺ cells below 5% were considered to have negligible contribution. LT-HSC: Long-term HSC, ST-HSC: Short-term HSC, MPP: multipotent progenitor, CMP: common myeloid progenitor, LMPP: lymphoid-primed multipotent progenitor, CLP: common lymphoid progenitor, MEP: megakaryocyte-erythroid progenitor, GMP: granulocyte-monocyte progenitor, ETP: early thymic progenitor, DN: double negative.

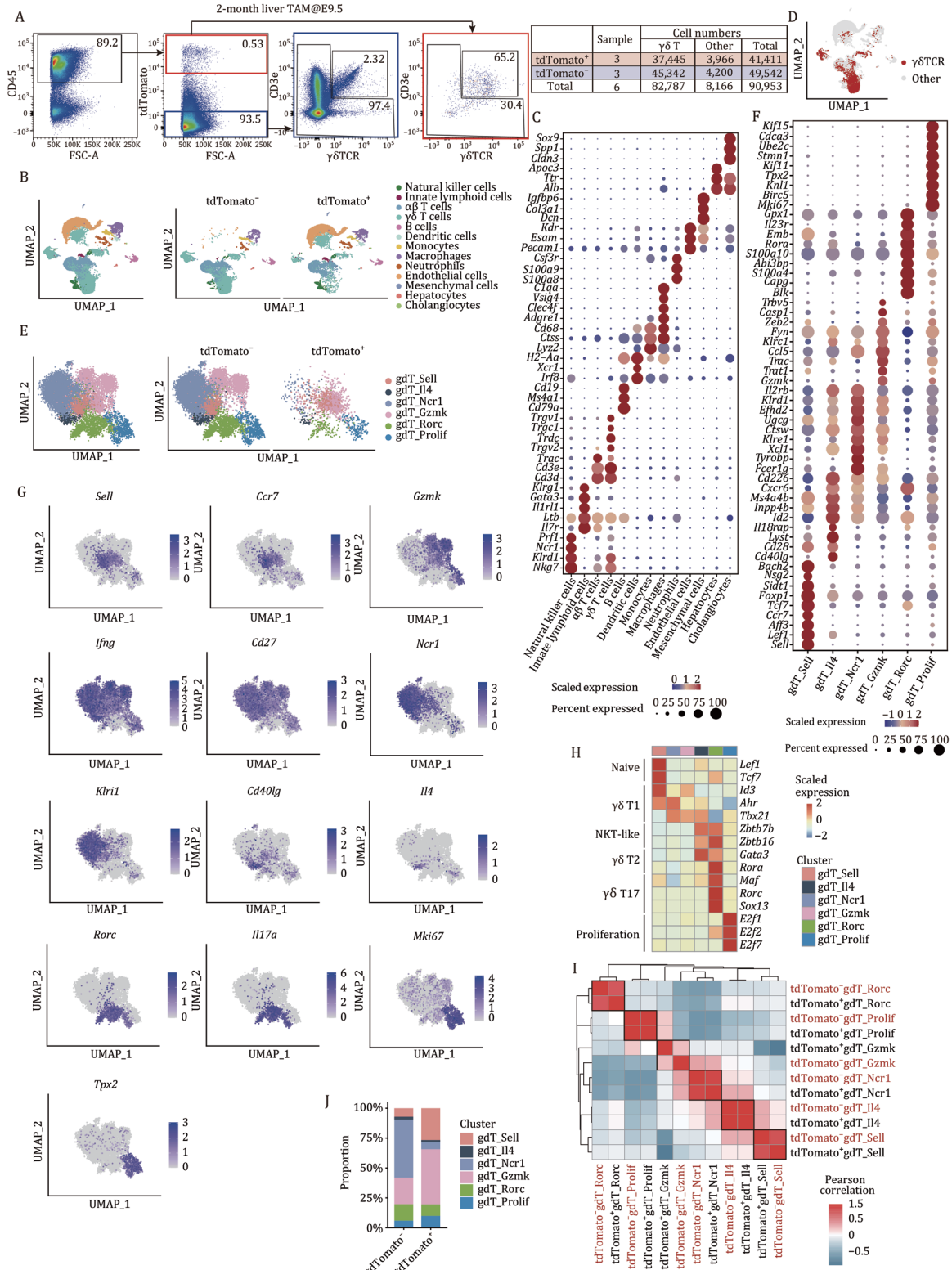


Figure 4. Features of E9.5 yolk sac-derived $\gamma\delta$ T cells based on single-cell transcriptomic analysis. (A) Flow cytometry gating strategy for single-cell RNA sequencing in 2-month-old *Stab2-CreERT2*; *R26-LSL-tdT* mice liver following tamoxifen induction at E9.5. The table displaying FACS-purified cell information. (B) Visualization of Uniform Manifold Approximation and Projection (UMAP) for liver cells from 2-month-old mice, with cells colored by types. (C) Dot plot showing the scaled expression of signature genes in each cell cluster. (D) UMAP showing TCR repertoire distribution in hepatic cells, as defined in panel B. (E) UMAP visualization of distinct subclusters of hepatic $\gamma\delta$ T cells. (F) Dot plot showing the scaled expression of differentially expressed genes in each $\gamma\delta$ T subset. (G) UMAP plot showing the expression of signature genes for $\gamma\delta$ T subsets. (H) Heatmap showing the scaled expression of representative transcription factor genes across hepatic $\gamma\delta$ T cell clusters. (I) Heatmap showing correlation analysis of tdTomato⁺ and tdTomato⁻ cells within each $\gamma\delta$ T cell cluster based on principal component analysis embeddings. (J) Bar plot presenting the proportion of subclusters with tdTomato⁻ and tdTomato⁺ $\gamma\delta$ T cells.

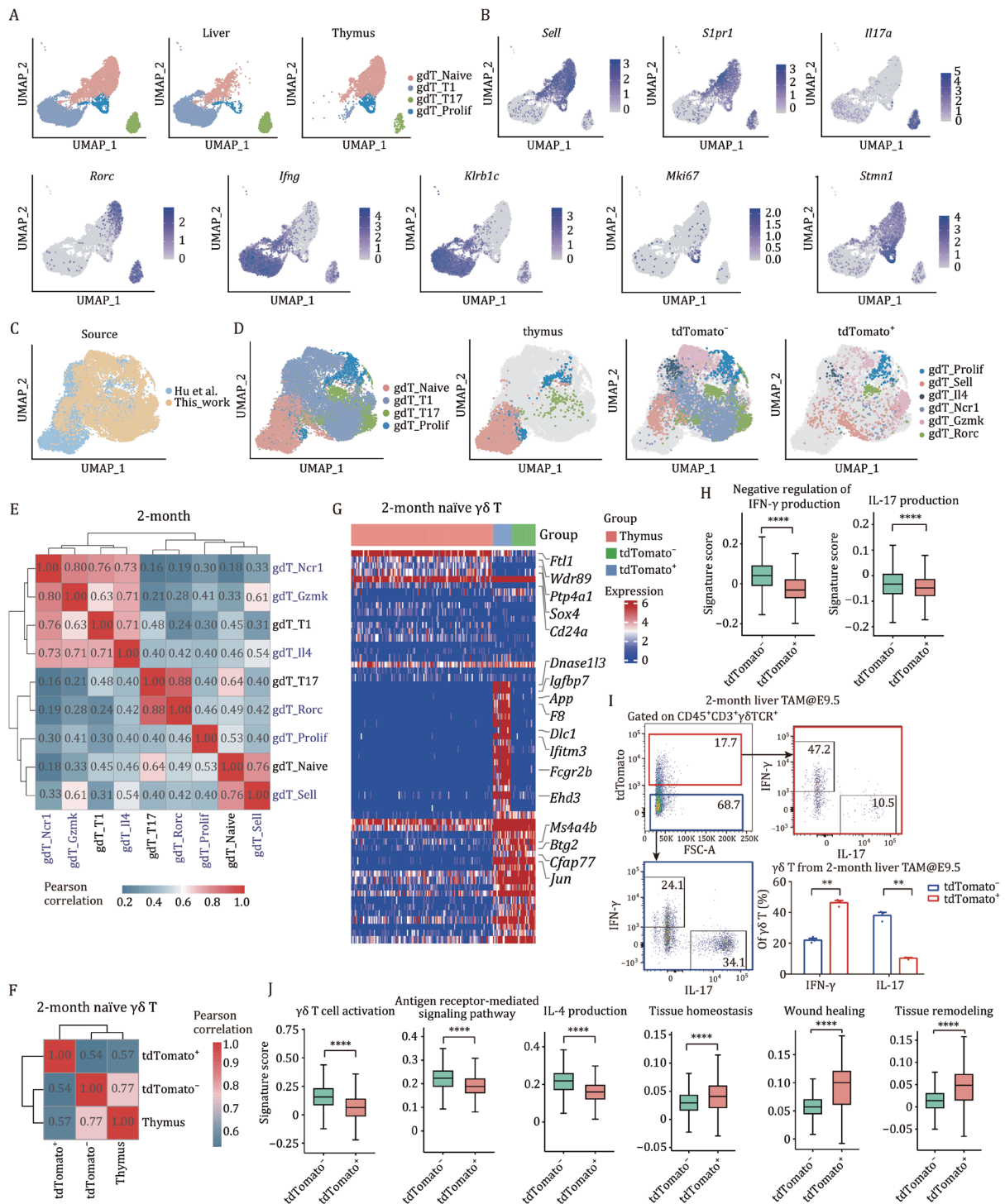


Figure 5. Comparative analysis of the $\gamma\delta$ T cells with distinct origins. (A) UMAP plot showing hepatic and thymic $\gamma\delta$ T subclusters from 2-month-old mice, with cells colored by different sources. Public data was downloaded from GSE164106. (B) UMAP plot showing the expression of signature genes for each cell type, as defined in panel A. (C) UMAP representing integrated $\gamma\delta$ T subclusters from public and in-house data, with cells colored by datasets. (D) UMAP plot showing $\gamma\delta$ T subclusters from different sources. (E) Heatmap shows the correlation across different $\gamma\delta$ T clusters. (F) Heatmap showing the correlation among thymic, tdTomato⁻, and tdTomato⁺ naive $\gamma\delta$ T cell populations. (G) Heatmap showing the expression of differentially expressed genes among thymic, tdTomato⁻, and tdTomato⁺ naive $\gamma\delta$ T cell populations. (H) Box plots showing Gene Ontology (GO) signature analysis of IFN- γ and IL-17 production signaling in tdTomato⁺ and tdTomato⁻ hepatic $\gamma\delta$ T cells from 2-month-old mice (**** $P < 0.0001$). (I) Representative flow cytometry plots and quantification of IFN- γ and IL-17 expression by tdTomato⁺ and tdTomato⁻ liver $\gamma\delta$ T cells from 2-month-old *Stab2-CreERT2*; R26-LSL-tdT mice treated with tamoxifen at E9.5. tdTomato⁺ $\gamma\delta$ T cells show significantly higher IFN- γ and lower IL-17 production compared to their tdTomato⁻ counterparts (** $P < 0.01$, $n = 3$). (J) Box plots showing GO signature analysis of representative biological processes in tdTomato⁺ and tdTomato⁻ hepatic $\gamma\delta$ T cells from 2-month-old mice (**** $P < 0.0001$).

migration-associated genes (*Sell* and *S1pr1*) and maturation-related transcription factor genes (*Tcf7* and *Lef1*) (Arbones et al., 1994; Okamura et al., 1998). The *gdT_GzmK*, *gdT_IL4*, and *gdT_Ncr1* clusters both exhibited high expression of *Ifng*, *Cd27*, and *Il2rb*, suggesting that they represent IFN- γ -producing $\gamma\delta$ T1 cells (Hu et al., 2021; Zhong and Zhu, 2017). Additionally, *gdT_GzmK* displayed elevated levels of *Casp1* and *GzmK*, consistent with a pro-inflammatory phenotype, whereas *gdT_Ncr1* and *gdT_IL4* were enriched for *Il4*, *Fcer1g*, *Tyrbp*, and *Klre1*, indicative of an NK-like functional program. The *gdT_Rorc* cluster represents $\gamma\delta$ T17 cells, characterized by high expression of *Rorc*, *Ccr6*, *Itgae*, and *Il17a* (Hu et al., 2021; Ivanov et al., 2006). The *gdT_Prolif* cluster exhibited elevated expression of proliferation-associated genes, including *Mki67*, *Tpx2*, and *Cdk1* (Fig. 4F and 4G). Supportively, the expression pattern of several known transcriptional regulators in the identified $\gamma\delta$ T subsets is consistent with established $\gamma\delta$ T cell differentiation programs (Pinheiro and Silva-Santos, 2025) (Fig. 4H). Correlation analysis of *tdTomato*⁺ and *tdTomato*⁻ cells showed that same annotated $\gamma\delta$ T cell populations essentially clustered together, indicating the largely preserved $\gamma\delta$ T cell identities regardless of *tdTomato* labeling (Fig. 4I). Notably, *gdT_Sell* and *gdT_GzmK* subsets were enriched in *tdTomato*⁺ $\gamma\delta$ T cell clusters (Fig. 4J), in line with the results that E9.5 induction sufficiently labeled early developmental stages of the hepatic $\gamma\delta$ T cell compartments.

Our lineage tracing data suggested that, among the liver $\gamma\delta$ T cell populations we identified, the yolk sac-derived *tdTomato*⁺ subset is less likely to originate from the thymus. To further compare the molecular features of the $\gamma\delta$ T cells with different origins, we used published single-cell datasets of thymic and hepatic $\gamma\delta$ T cells from 2-month-old mice as a reference dataset (Hu et al., 2021) and integrated them with our own dataset (Fig. 5A–C). The published thymic and hepatic $\gamma\delta$ T cells were reannotated as naive (*Sell* and *S1pr1*), T1-like (*Ifng* and *Klrb1c*), T17-like (*Il17a* and *Rorc*), and proliferating (*Mki67* and *Stmn1*) sub-clusters (Fig. 5A and 5B). As expected, integrative analysis showed that our *gdT_Sell* aligned with naive $\gamma\delta$ T cells in the reference dataset; *gdT_GzmK*/*gdT_IL4*/*gdT_Ncr1* with $\gamma\delta$ T1; *gdT_Rorc* with $\gamma\delta$ T17; and *gdT_Prolif* with the corresponding proliferating population, respectively (Fig. 5D). Notably, *tdTomato*⁻ but not *tdTomato*⁺ naive $\gamma\delta$ T cells clustered in closer proximity to thymic naive $\gamma\delta$ T cells on UMAP plot (Fig. 5D). This relationship was supported by transcriptomic similarity, showing that *tdTomato*⁻ naive $\gamma\delta$ T cells are more closely aligned with thymic naive $\gamma\delta$ T cells (Fig. 5E and 5F). We next compared gene expression profiles between naive $\gamma\delta$ T cell clusters from the thymus and liver (Fig. 5G; Table S3). Proliferation-associated genes, including *Cd24a* and *Sox4*, were more highly expressed in the thymic naive $\gamma\delta$ T cell cluster, suggesting an earlier developmental

stage, consistent with previous reports (Hu et al., 2021). In contrast, the *tdTomato*⁺ hepatic naive $\gamma\delta$ T cell cluster was enriched for genes associated with tissue residency and functional priming, including *Ehd3*, *Igf1bp7*, *Ifitm3*, *Fcgr2b*, *Dlc1*, suggesting a bias toward differentiation into IFN- γ -producing $\gamma\delta$ T1 cells and enhanced granzyme-mediated activity (Kumar et al., 2025; Sun et al., 2024). Consistent with the computational prediction, intracellular cytokine staining following *ex vivo* PMA and ionomycin stimulation showed that *tdTomato*⁺ hepatic $\gamma\delta$ T cells produced significantly more IFN- γ and less IL-17 than *tdTomato*⁻ counterparts (Fig. 5H and 5I). Collectively, these findings indicate that *Stab2*-labeled yolk sac-derived hepatic $\gamma\delta$ T cells are biased toward the IFN- γ -producing $\gamma\delta$ T1 cells.

Previous studies have shown that murine *V γ 1*⁺ $\gamma\delta$ T cells exhibit diverse context-dependent functions. They participate in microbial antigen recognition and phosphoantigen sensing (Born et al., 2013; Happ et al., 1989), supporting their involvement in early immune surveillance. Furthermore, they are implicated in the interleukin-4-mediated negative regulation of antitumor immunity (Hao et al., 2011), the promotion of airway inflammation (Hahn et al., 2004), and the tissue-protective responses in brain injury (Abou-El-Hassan et al., 2023) and myocarditis (Huber and Sartini, 2005). By comparison, the *tdTomato*⁻ $\gamma\delta$ T cells in adult liver showed enrichment of $\gamma\delta$ T cell activation, antigen receptor-mediated signaling pathway, and interleukin-4 production, whereas the *tdTomato*⁺ counterparts were characterized by higher scores for tissue homeostasis, wound healing, and tissue remodeling (Fig. 5J). These characteristics are consistent with previously reported features of yolk sac-derived macrophages (Elhag et al., 2021). Moreover, yolk sac-derived dendritic epidermal T cells have also been reported to exhibit tissue repair capacity (Gentek et al., 2018). These findings collectively suggest that yolk sac-derived immune cells possess a low pro-inflammatory and high repair-oriented functional propensity, thereby might playing a distinctive role in adult tissue homeostasis and injury repair.

Taken together, this study revealed the existence—under physiological conditions—of a distinct developmental pathway for a subset of $\gamma\delta$ T cells, which originate in the extraembryonic yolk sac, develop in the liver, and bypassing both hematopoietic stem cells and the thymus. These yolk sac-derived hepatic *V γ 1* $\gamma\delta$ T cells preferentially enrich $\gamma\delta$ T1-like subset, showing relatively high capacity to produce IFN- γ (Fig. 6).

Discussion

A major obstacle in developmental hematology has been the lack of tools to definitively resolve the site origin and long-term contribution of successive hematopoietic waves *in vivo*, thereby obscuring their presumed distinct

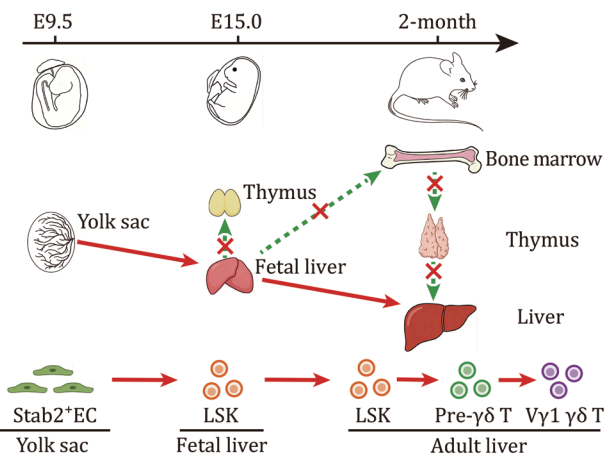


Figure 6. Schematic diagram summarizing the development pathway of the yolk sac-derived hepatic $\gamma\delta$ T cell subpopulation.

Solid arrows indicate the suggested developmental pathway revealed in this study. Dashed arrows indicate the classic pathways for hematopoietic stem and progenitor cell colonization and T cell development, which is not the manner for the development of the yolk sac-derived specific subtype of hepatic $\gamma\delta$ T cells. EC, endothelial cells; LSK, Lineage-Sca-1⁺Kit⁺ hematopoietic stem progenitor cells.

functional roles in embryogenesis and adult tissue homeostasis. Here, we establish a *Stab2-CrexERT2* inducible genetic lineage tracing mouse model that enables the specific fate mapping of yolk sac-derived hematopoiesis *in vivo*. Temporal induction at E7.5 or E9.5 preferentially targets yolk sac endothelial cells without marking aortic endothelial cells, allowing the *Stab2-CrexERT2* to dissect yolk sac-derived hematopoiesis despite the partially overlapping time windows of different hematopoietic waves. Furthermore, *Stab2*-labeled yolk sac endothelial cells do not contribute to bone marrow HSCs, supporting its utility as a tool for investigating HSC-independent hematopoiesis in adult mice. This refined specificity substantially improves the *in vivo* resolution of hematopoietic lineage tracing.

$\gamma\delta$ T cells were initially identified in 1984 (Saito et al., 1984), and subsequent studies have established them as a distinct T cell lineage with potent immunoregulatory and cytotoxic functions, positioning them as promising targets for immunotherapeutic intervention. Canonically, $\gamma\delta$ T cells are thought to arise in the thymus, where strong TCR signaling in double-negative thymocytes instructs commitment to the $\gamma\delta$ lineage (Munoz-Ruiz et al., 2017). While the existence of extrathymic $\gamma\delta$ T cells in the intestine and liver has been described, the supporting evidence primarily stems from thymectomy models. Consequently, these observations may be confounded by compensatory adaptations to thymic removal and may not accurately represent $\gamma\delta$ T cell development under steady-state physiological conditions. By employing a newly established inducible genetic lineage tracing mouse model that permits physiological scenario-based tracking of the yolk

sac-derived progeny, we uncover a distinct population of hepatic $\gamma\delta$ T cells that originates from the yolk sac and develops independently of the thymus.

Collectively, our findings refine current understanding of extra-thymic $\gamma\delta$ T cell ontogeny and establish a foundation for systematically interrogating the origin-dependent functional specialization of $\gamma\delta$ T cells. Our research has opened up new directions for future studies, including the upstream transcriptional circuitry and signaling networks orchestrating the ontogeny and hepatic residency of this extra-thymic $\gamma\delta$ T cell subset, and the specific functions and mechanisms of the yolk sac-derived hepatic $\gamma\delta$ T cells in homeostasis and diseases. These studies will be of profound significance for understanding the functional diversification of immune cells.

Supplementary data

Supplementary data is available at *Protein & Cell* Journal online.

Conflict of interest

The authors declare no conflicts of interest.

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Abbreviations

AGM, aorta-gonad-mesonephros; CLP, common lymphoid progenitor; CMP, common myeloid progenitor; DN, double-negative; E, embryonic day; EMP, erythro-myeloid progenitor; ER, estrogen receptor; GMP, granulocyte-macrophage progenitor; HSC, hematopoietic stem cell; LMPP, lymphoid-primed multipotent progenitor; MEP, megakaryocyte-erythroid progenitor; MPP, multipotent progenitor; TCR, T cell receptor

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Data availability

All raw and processed data underlying this article have been deposited in the Gene Expression Omnibus (GEO)

database under accession number GSE329257. Public datasets of hepatic and thymic $\gamma\delta$ T cells were obtained from GSE164106 (Hu et al., 2023).

Code availability

This paper does not report original code. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

Author contributions

Y.L. and B.L.: designed and supervised the study; Y.C. and C.W.: performed the cell sorting and immunostaining assays with help from K.H.; Y.C. and K.H.: performed scRNA-seq; G.Y. and K.H.: performed bioinformatics analysis; Y.C., C.W., and Y.L.: wrote the manuscript. All authors reviewed the manuscript.

Ethics approval

The experimental manipulations of mice were approved by the Animal Care and Use Committee of the Academy of Military Medical Sciences.

Artificial intelligence (AI)

No AI-assisted technologies were used in the preparation of this manuscript.

References

- Abou-El-Hassan H, Rezende RM, Izzy S et al. Vgamma1 and Vgamma4 gamma-delta T cells play opposing roles in the immunopathology of traumatic brain injury in males. *Nat Commun* 2023;**14**:4286.
- Arbones ML, Ord DC, Ley K et al. Lymphocyte homing and leukocyte rolling and migration are impaired in L-selectin-deficient mice. *Immunity* 1994;**1**:247–260.
- Bai L, Vienne M, Tang L et al. Liver type 1 innate lymphoid cells develop locally via an interferon-gamma-dependent loop. *Science* 2021;**371**:eaba4177.
- Bandeira A, Itohara S, Bonneville M et al. Extrathymic origin of intestinal intraepithelial lymphocytes bearing T-cell antigen receptor gamma delta. *Proc Natl Acad Sci U S A* 1991;**88**:43–47.
- Born WK, Kemal Aydinoglu M, O'Brien RL. Diversity of gamma-delta T-cell antigens. *Cell Mol Immunol* 2013;**10**:13–20.
- Chen MJ, Yokomizo T, Zeigler BM et al. Runx1 is required for the endothelial to haematopoietic cell transition but not thereafter. *Nature* 2009;**457**:887–891.
- Cheng H, Zheng Z, Cheng T. New paradigms on hematopoietic stem cell differentiation. *Protein Cell* 2020;**11**:34–44.
- Coffey F, Lee SY, Buus TB et al. The TCR ligand-inducible expression of CD73 marks gamma-delta lineage commitment and a metastable intermediate in effector specification. *J Exp Med* 2014;**211**:329–343.
- Du Halgouet A, Bruder K, Peltokangas N et al. Multimodal profiling reveals site-specific adaptation and tissue residency hallmarks of gamma-delta T cells across organs in mice. *Nat Immunol* 2024;**25**:343–356.
- Elhag S, Stremmel C, Zehrer A et al. Differences in cell-intrinsic inflammatory programs of yolk sac and bone marrow macrophages. *Cells* 2021;**10**:3564.
- Fiala GJ, Gomes AQ, Silva-Santos B. From thymus to periphery: molecular basis of effector gamma-delta-T cell differentiation. *Immunol Rev* 2020;**298**:47–60.
- Gentek R, Ghigo C, Hoeffel G et al. Epidermal gamma-delta T cells originate from yolk sac hematopoiesis and clonally self-renew in the adult. *J Exp Med* 2018;**215**:2994–3005.
- Haas JD, Ravens S, Düber S et al. Development of interleukin-17-producing gamma-delta T cells is restricted to a functional embryonic wave. *Immunity* 2012;**37**:48–59.
- Hahn Y-S, Taube C, Jin N et al. Different potentials of gamma Delta T cell subsets in regulating airway responsiveness: V gamma 1+ cells, but not V gamma 4+ cells, promote airway hyperreactivity, Th2 cytokines, and airway inflammation. *J Immunol* 2004;**172**:2894–2902.
- Hao J, Dong S, Xia S et al. Regulatory role of Vgamma1 gamma-delta T cells in tumor immunity through IL-4 production. *J Immunol* 2011;**187**:4979–4986.
- Happ MP, Kubo RT, Palmer E et al. Limited receptor repertoire in a mycobacteria-reactive subset of gamma Delta T lymphocytes. *Nature* 1989;**342**:696–698.
- Hoeffel G, Chen J, Lavin Y et al. C-Myb(+) erythro-myeloid progenitor-derived fetal monocytes give rise to adult tissue-resident macrophages. *Immunity* 2015;**42**:665–678.
- Hou S, Guo X, Du J et al. New insights into the endothelial origin of hematopoietic system inspired by “TIF” approaches. *Blood Sci* 2024;**6**:e00199.
- Hou S, Li Z, Dong J et al. Heterogeneity in endothelial cells and widespread venous arterialization during early vascular development in mammals. *Cell Res* 2022;**32**:333–348.
- Hou S, Li Z, Zheng X et al. Embryonic endothelial evolution towards first hematopoietic stem cells revealed by single-cell transcriptomic and functional analyses. *Cell Res* 2020;**30**:376–392.
- Hu Y, Fang K, Wang Y et al. Single-cell analysis reveals the origins and intrahepatic development of liver-resident IFN-gamma-producing gamma-delta T cells. *Cell Mol Immunol* 2021;**18**:954–968.
- Hu Y, Hu Q, Li Y et al. Gamma-delta T cells: origin and fate, subsets, diseases and immunotherapy. *Signal Transduct Target Ther* 2023;**8**:434.
- Huber S, Sartini D. T cells expressing the Vgamma1 T-cell receptor enhance virus-neutralizing antibody response during coxsackievirus B3 infection of BALB/c mice: differences in male and female mice. *Viral Immunol* 2005;**18**:730–739.
- Ivanov II, McKenzie BS, Zhou L et al. The orphan nuclear receptor RORgamma directs the differentiation program of proinflammatory IL-17+ T helper cells. *Cell* 2006;**126**:1121–1133.
- Joannou K, Golec DP, Wang H et al. Gamma-delta thymocyte maturation and emigration in adult mice. *J Immunol* 2022;**208**:2131–2140.
- Kissa K, Herbolme P. Blood stem cells emerge from aortic endothelium by a novel type of cell transition. *Nature* 2010;**464**:112–115.

- Kobayashi M, Wei H, Yamanashi T *et al.* HSC-independent definitive hematopoiesis persists into adult life. *Cell Rep* 2023;**42**:112239.
- Komano H, Fujiura Y, Kawaguchi M *et al.* Homeostatic regulation of intestinal epithelia by intraepithelial gamma Delta T cells. *Proc Natl Acad Sci U S A* 1995;**92**:6147–6151.
- Krishnan S, Prise IE, Wemyss K *et al.* Amphiregulin-producing gammadelta T cells are vital for safeguarding oral barrier immune homeostasis. *Proc Natl Acad Sci U S A* 2018;**115**:10738–10743.
- Kumar A, O'Brien M, Young VB *et al.* Single-cell RNA sequencing identifies accumulation of Fcgr2b + virtual memory-like CD8 T cells with cytotoxic and inflammatory potential in aged mouse white adipose tissue. *Aging Cell* 2025;**24**:e70278.
- Munoz-Ruiz M, Sumaria N, Pennington DJ *et al.* Thymic determinants of gammadelta T cell differentiation. *Trends Immunol* 2017;**38**:336–344.
- Okamura RM, Sigvardsson M, Galceran J *et al.* Redundant regulation of T cell differentiation and TCRalpha gene expression by the transcription factors LEF-1 and TCF-1. *Immunity* 1998;**8**:11–20.
- Pinheiro RGR, Silva-Santos B. Reaching maturity: thymic gammadelta T cell differentiation in mice and humans. *Sci Immunol* 2025;**10**:eadn2093.
- Qu G, Wang S, Zhou Z *et al.* Comparing mouse and human tissue-resident gammadelta T cells. *Front Immunol* 2022;**13**:891687.
- Reisel D, Bannerman DM, Schmitt WB *et al.* Spatial memory dissociations in mice lacking GluR1. *Nat Neurosci* 2002;**5**:868–873.
- Ribot JC, Lopes N, Silva-Santos B. Gammadelta T cells in tissue physiology and surveillance. *Nat Rev Immunol* 2021;**21**:221–232.
- Sagar. Unraveling the secrets of gammadelta T cells with single-cell biology. *J Leukoc Biol* 2024;**115**:47–56.
- Saito H, Kranz DM, Takagaki Y *et al.* Complete primary structure of a heterodimeric T-cell receptor deduced from cDNA sequences. *Nature* 1984;**309**:757–762.
- Schledzewski K, Géraud C, Arnold B *et al.* Deficiency of liver sinusoidal scavenger receptors stabilin-1 and -2 in mice causes glomerulofibrotic nephropathy via impaired hepatic clearance of noxious blood factors. *J Clin Invest* 2011;**121**:703–714.
- Sharp LL, Jameson JM, Cauvi G *et al.* Dendritic epidermal T cells regulate skin homeostasis through local production of insulin-like growth factor 1. *Nat Immunol* 2005;**6**:73–79.
- Sheikh H, Yarwood H, Ashworth A *et al.* Endo180, an endocytic recycling glycoprotein related to the macrophage mannose receptor is expressed on fibroblasts, endothelial cells and macrophages and functions as a lectin receptor. *J Cell Sci* 2000;**113**:1021–1032.
- Sun W, Zhu Y, Zou Z *et al.* An advanced comprehensive multi-cell-type-specific model for predicting anti-PD-1 therapeutic effect in melanoma. *Theranostics* 2024;**14**:2127–2150.
- Wang C, Gong Y, Wei A *et al.* Adult-repopulating lymphoid potential of yolk sac blood vessels is not confined to arterial endothelial cells. *Sci China Life Sci* 2021a;**64**:2073–2087.
- Wang Y, Guan Y, Hu Y *et al.* Murine CXCR3(+)/CXCR6(+)gammadeltaT cells reside in the liver and provide protection against HBV infection. *Front Immunol* 2021b;**12**:757379.
- Zhong C, Zhu J. Transcriptional regulators dictate innate lymphoid cell fates. *Protein Cell* 2017;**8**:242–254.