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Original Research

The heterogeneity and sexual-dimorphism of human $\text{TCR}\alpha\beta^+\text{CD4}^-\text{CD8}^-$ double negative T cells

Dan Tian (田丹)^{a, b, c, 1}, Fengcui Qian (钱凤翠)^{d, 1}, Lehan Pan (潘乐涵)^{a, b, c, 1}, Mingxue Yin (尹明雪)^d, Bihan Liu (刘碧茵)^{a, b, c}, Yuxi Zhang (张玉喜)^{a, b, c}, Xiaobao Yang (杨小宝)^{a, b, c}, Lu Yang (杨璐)^{a, b, c}, Guangyong Sun (孙广永)^{e, f}, Zhongtao Zhang (张忠涛)^{a, b, c}, Songlin Wang (王松灵)^g, Patrick SC. Leung^h, M. Eric Gershwin^{h, *}, Chunquan Li (李春权)^{d, *}, Dong Zhang (张栋)^{e, f, g, *}

^a Department of General Surgery, Beijing Friendship Hospital, Capital Medical University, Beijing 100050, China^b State Key Lab of Digestive Health, Beijing Friendship Hospital, Capital Medical University, Beijing 100050, China^c National Clinical Research Center for Digestive Diseases, Beijing 100050, China^d The First Affiliated Hospital, Cardiovascular Lab of Big Data and Imaging Artificial Intelligence, Hengyang Medical School, University of South China, Changsha, Hunan 421001, China^e Medical Research Center, Beijing Institute of Respiratory Medicine and Beijing Chao-Yang Hospital, Capital Medical University, Beijing 100020, China^f Department of Gastroenterology, Beijing Chao-Yang Hospital, Capital Medical University, Beijing 100020, China^g Beijing Laboratory of Oral Health, Capital Medical University School of Basic Medicine, Beijing 100069, China^h Division of Rheumatology, Allergy, and Clinical Immunology, University of California Davis, Davis, CA 95616, USA

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ABSTRACT

$\text{TCR}\alpha\beta^+$ double negative T (DNT) cells are increasingly being recognized for their critical roles in the progression of various diseases. However, the molecular and functional signatures remain poorly understood and controversial. In this study, we characterize the single-cell transcriptome, TCR repertoire, and chromatin accessibility of peripheral DNT cells from 10 healthy donors. Naïve, regulatory, cytotoxic, antibody-dependent cell-mediated cytotoxicity, and helper-like DNT cell subsets exhibit specific gene expression, transcriptome regulation, development signature, and sexual heterogeneity, respectively. Using publicly available data, we observe changes in the proportion and gene expression of DNT cell subsets, especially the helper subset, in patients with ulcerative colitis and type 1 diabetes. Overall, human DNT cells exhibit phenotypic and functional diversity, providing a valuable framework for understanding the development and progression of immune-related diseases.

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Introduction

$\text{TCR}\alpha\beta^+$ double negative T (DNT) cells are a subset of unconventional T cells found in human blood and tissues (Zhang et al., 2000). DNT cells express CD3 and $\text{TCR}\alpha\beta$ but do not express canonical T cell surface markers CD4 and CD8 or other unconventional T cell markers like $\text{TCR}\gamma\delta$, CD161, and CD56. DNT cells make up only 1%–3% of peripheral T cells in the blood, spleen, and lymph nodes.

However, they are abundant in local tissues, particularly the mucosa and liver (Carrasco et al., 2016; Li et al., 2022).

Recent studies have highlighted the critical and diverse role of DNT cells in the progression of diseases. However, there is still no consensus regarding the core functional molecules of DNT cells or their pro-inflammatory versus anti-inflammatory roles in disease contexts. Most existing studies have focused on alterations in DNT cell abundance or phenotype in disease settings, whereas relatively few have demonstrated the functional roles of DNT cells or their specific impact on human disease. In systemic lupus erythematosus and primary Sjogren's syndrome, peripheral DNT cells expand and secrete pro-inflammatory cytokine IL-17 in the peripheral blood and inflamed organs (Anand et al., 2002; Crispin and Tsokos, 2009; Alunno et al., 2013; Li et al., 2020). Additionally, DNT cells have

* Corresponding author.

E-mail addresses: megershwin@ucdavis.edu (M.E. Gershwin), lcqbio@163.com (C. Li), zhangd@ccmu.edu.cn (D. Zhang).¹ These authors contributed equally to this work.<https://doi.org/10.1016/j.jgg.2026.04.008>

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demonstrated potent anti-tumor activity in sarcoma, pancreatic cancer, and lung cancer (Hundeyin et al., 2019; Ponzetta et al., 2019; Yao et al., 2019). The chimeric antigen receptor (CAR)-T cell strategy has been applied to DNT cell therapy in T-cell acute lymphoblastic leukemia (Fang et al., 2023). Other studies suggest that DNT cells exert immune regulatory functions via cytotoxic and anti-inflammatory pathways in allograft rejection (Zhang et al., 2000; Chen et al., 2005). Our previous studies demonstrated the immune regulatory role of DNT cells in mouse models of allergic asthma, nonalcoholic steatohepatitis, and type 1 diabetes, as well as the heterogeneity of mouse DNT cells (Zhang et al., 2011; Tian et al., 2019; Sun et al., 2021; Yang et al., 2021). These findings suggest the versatile functions of DNT cells, which exhibit both pro-inflammatory and anti-inflammatory characteristics. The underlying mechanisms of human DNT cells in immunological diseases still remain elusive.

Unsolved questions remain about DNT cell development and TCR repertoires, including variability across genders. The origin of peripheral DNT cells remains controversial. During conventional T cell development, T cells undergo a “double negative” stage followed by a “double positive” (DP) stage in the thymus. DNT cells, which lack both CD4 and CD8 co-receptors, may bypass negative selection and migrate to peripheral tissues (Pobezinsky et al., 2012). Adoptive transfer assays have shown that peripheral DNT cells primarily derive from CD8⁺ Thymocytes in mice (Rodríguez-Rodríguez et al., 2020). DNT cells may also derive from activated peripheral CD8 or CD4 T cells in vitro (Zhang et al., 2007; Crispin and Tsokos, 2009). The differentiation pathways of peripheral DNT cells appear to be complex.

The activation and metabolism of peripheral immune cells influence a range of physiological processes (Zhao et al., 2024), and with the participation of DNT cells. To address these fundamental questions about DNT cells, we applied multi-omics single-cell sequencing to generate a comprehensive profile of human peripheral DNT cells from both male and female donors. We combined scRNA-seq with single-cell chromatin accessibility and TCR repertoire analysis of DNT cells. PBMC data from the same donors revealed DNT cell differentiation and potential interactions in peripheral blood. Finally, we used flow cytometry to verify the function and surface markers of the DNT cells.

Results

Cellular composition of the human peripheral DNT cells

To investigate the heterogeneity, differentiation, and interaction of human DNT cells, we performed multi-omic single-cell sequencing on peripheral blood mononuclear cells (PBMCs) and peripheral DNT cells from 10 healthy donors (Figs. 1A and S1A). To ensure the purity of DNT cells, we performed FACS sorting of DNT cells with the surface marker CD3⁺ CD56⁺ CD161⁺ TCR α 7.2⁺ CD1d-tetramer⁺ TCR α β ⁺ CD4⁺ CD8 α ⁺, thereby excluding natural killer T (NKT) cells, mucosal-associated invariant T (MAIT) cells, TCR γ δ T cells, CD4 T cells, and CD8 T cells (Fig. 1B). A total of 94,901 cells were captured, with 93,074 DNT cells retained after filtering. DNT cells comprised five clusters, which were consistently distributed across both RNA sequencing strategies and donors (Figs. 1C, S1B, S1C; Table S1).

The heatmap of the top 10 differentially expressed genes (DEGs) indicated substantial transcriptional diversity and distinct functional gene expression across the clusters (Fig. 1D and 1E). All cells exhibited high levels of *CD3E* and low levels of *CD4* and *CD8A* gene, confirming the purity of DNT cells (Fig. 1E). Cells in cluster 0 expressed high levels of naïve T cell-associated genes (*LEF1*, *SELL*, *CCR7*, and *TCF7*), indicating a naïve state. In comparison, cells in cluster 2 showed similar levels of naïve genes, but also

displayed markers of regulatory function, such as *HAVCR2* and *IL10*. Clusters 1 and 3 primarily comprised cytotoxic cells, expressing genes associated with cell-killing functions (*PRF1*, *NKG7*, *KLRG1*, and *ZEB2*). Moreover, cells in cluster 3 exhibited higher levels of Fc receptor *FCGR3A* than those in cluster 1, suggesting a potential role in antibody-dependent cell-mediated cytotoxicity (ADCC). A series of pro-inflammatory genes (*LTB*, *CCR6*, *RORC*, and *IL4I1*) were uniquely expressed in cluster 4, indicating a helper function within this cluster. Uniform manifold approximation and projection (UMAP) plots showed the expression of some of these representative marker genes across the DNT clusters (Fig. 1F). Furthermore, we extracted putative DNT cells from publicly available healthy PBMC scRNA-seq datasets (GSE158055 and E-MTAB-10026) by applying expression-based criteria corresponding to canonical DNT markers (*CD3E* and *TRAC* > 0, *CD4*, *CD8*, *TRDC* = 0). As shown in Fig. S2, these extracted DNT-like cells could also be categorized into naïve, ADCC-like, cytotoxic-like, helper-like, and regulatory-like DNT subsets. These findings further support the robustness and generalizability of our DNT cell classification.

To validate the classification and function of DNT subsets, we investigated the surface markers and functional protein expression of human peripheral DNT cells using flow cytometry. Surface markers, CCR7, TIM3, CCR6, and CD16 clearly distinguished naïve, regulatory, helper, and ADCC-like DNT subsets, respectively (Fig. 1G). Intracellular staining revealed high granzyme B levels in the CD16⁺ ADCC-like DNT cells and all (four marker-negative) DNT cells, indicating two distinct cytotoxic subsets (Fig. 1H and 1I). The pro-inflammatory cytokine IL-17A was prominently expressed in CCR6⁺ helper-like DNT cells, while the anti-inflammatory cytokine IL-10 was approximately expressed in CCR7⁺ naïve DNT cells, TIM3⁺ regulatory-like DNT cells, and CCR6⁺ helper-like DNT cells (Fig. 1H and 1I). These data suggest distinct protein expression patterns among human DNT subsets, providing insight for further purification and potential applications of these subsets.

Human peripheral DNT cells exhibit functional diversity and similarity with mouse peripheral DNT cells

To further delineate the primary function of DNT cell clusters, we performed a gene set enrichment analysis (GSEA), confirming the upregulation of diverse functional pathways across different clusters (Fig. 2A). Clusters 1 (cytotoxic-like DNT cells) and 3 (ADCC-like DNT cells) were enriched for pathways related to cell killing and cytotoxicity, while clusters 2 (regulatory-like DNT cells) and 4 (helper-like DNT cells) showed enrichment for immune regulation and pro-inflammatory pathways, respectively. Cells in cluster 0 (naïve DNT cells) exhibited downregulation of functional and cell growth pathways, indicating that this cluster represents a naïve state. These findings were corroborated by critical gene expression data and gene set variation analysis (GSVA) scores for the aforementioned pathways (Fig. 2B and 2C). Collectively, these results identified five subsets of human peripheral DNT cells with distinct functional roles, reflecting the heterogeneity of DNT cell populations.

In our previous study, we identified five clusters of mouse DNT (mDNT) cells using single-cell sequencing (Yang et al., 2021). By applying the gene signatures of mouse DNT cell subsets, we observed similar high expression of helper, intermediate, and cytotoxic mDNT genes in their corresponding human counterparts (Fig. 2D). However, we were unable to identify human counterparts for the mouse clusters associated with resting and innate mDNT cells. Consistent with single gene expression data, the marker gene indices suggested that the human ADCC, cytotoxic, regulatory, and helper-like DNT cells displayed high functional similarity with their respective mouse DNT cell clusters (Fig. 2E). Additionally, genes highly expressed in resting mDNT cells were upregulated in human

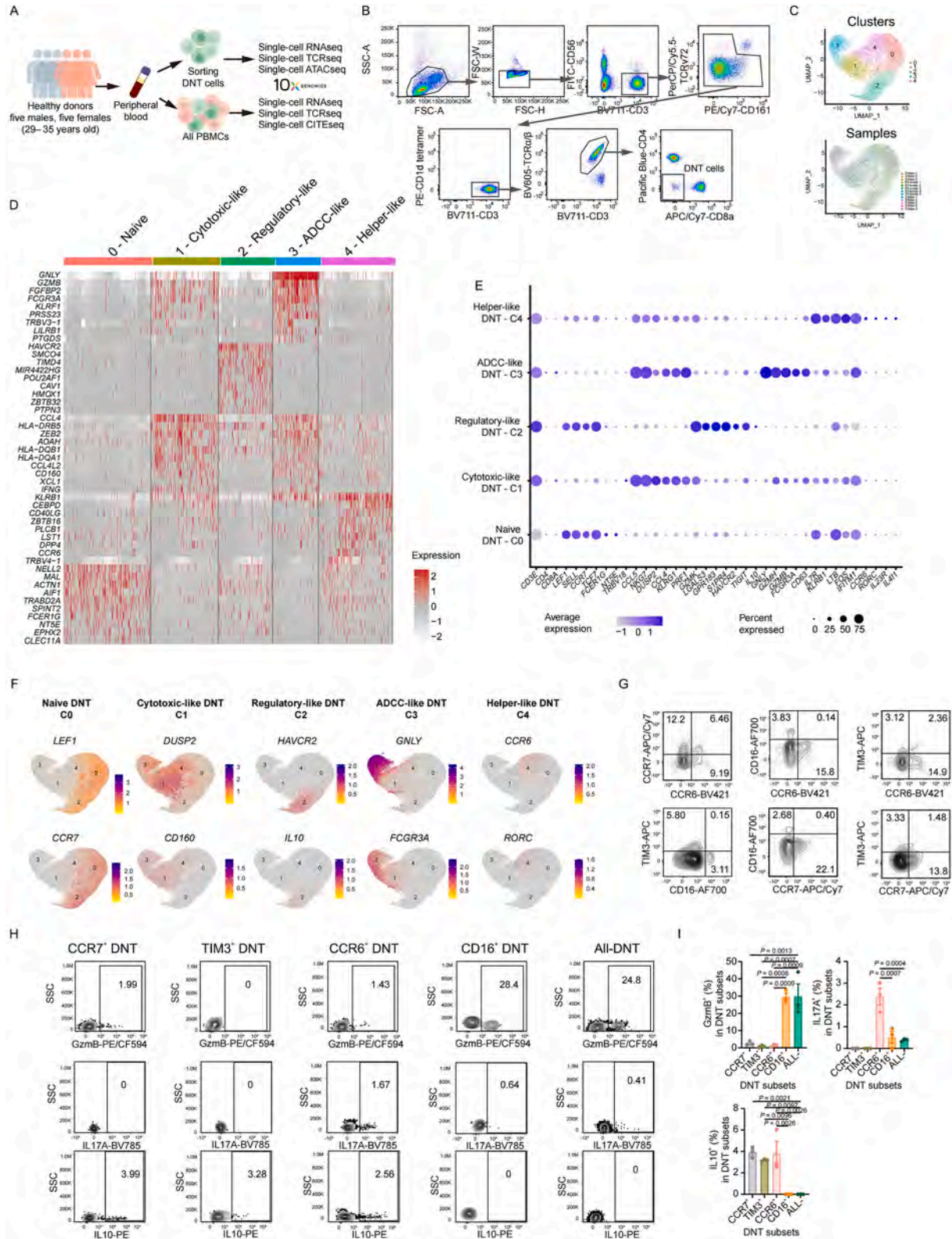


Fig. 1. Human peripheral DNT cells exhibit transcriptional heterogeneity. **A:** Experimental design of single-cell sequencing of peripheral DNT cells and matched PBMCs from healthy donors. **B:** Gating strategy of FACS sorting of DNT cells. **C:** UMAP of peripheral DNT cells colored by the five identified clusters (upper panel) or by samples (lower panel). **D:** Heatmap showing row-scaled top 10 differentially expressed genes for each cluster. **E:** Expression of marker genes in DNT cell clusters. Dot color indicates the average gene expression, and dot size indicates the percentage of cells expressing the gene. **F:** UMAP colored by expression of differentially expressed marker genes. **G:** Typical images of surface markers of DNT cells that were measured using flow cytometry. **H** and **I:** Typical flow cytometry plots (**H**) and statistical analysis (**I**) of intracellular markers in DNT subsets. All-, CCR7, TIM3, CCR6, and CD16 negative ($n = 3$; Student's t -test). DNT, double negative T; PBMCs, peripheral blood mononuclear cells; FACS, fluorescence-activated cell sorting; UMAP, uniform manifold approximation and projection; SSC, side scatter.

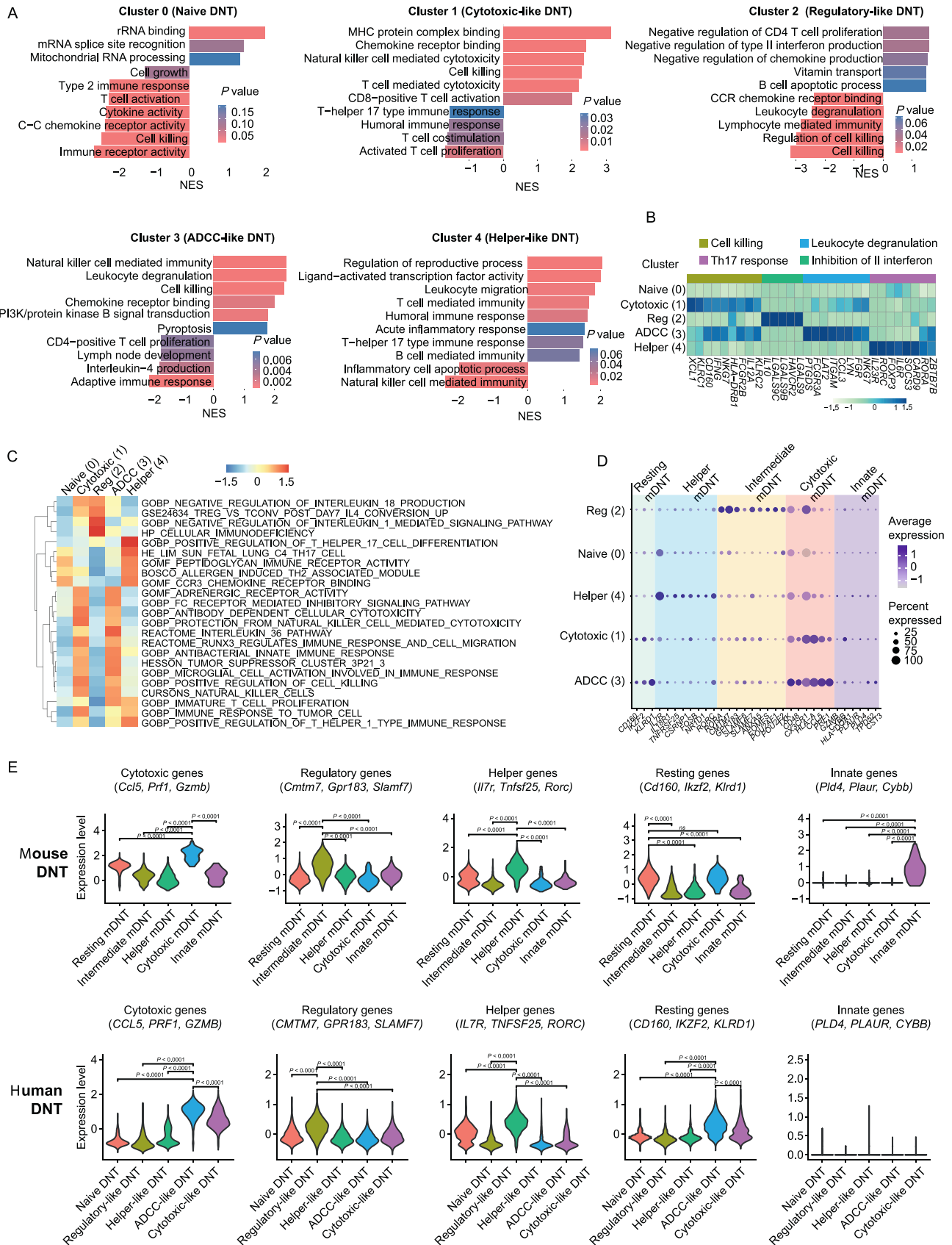


Fig. 2. Human peripheral DNT cells exhibit functional diversity and similarity with mouse peripheral DNT cells. **A:** GSEA of clusters of human peripheral DNT cells. **B:** Heatmap of the expression of enriched GSEA pathway-related genes in clusters of human peripheral DNT cells. **C:** Heatmap showing the pathway activities in clusters of human DNT cells as scored by GSVA. **D:** Expression of corresponding marker genes of mouse DNT cell clusters in human DNT cell clusters. **E:** Violin plots showing integrated gene expression in mouse DNT cell

ADCC and cytotoxic-like DNT cells, while marker genes characteristic of mouse innate DNT cells were not observed in any human DNT subsets.

Lineage analysis indicated the differential pathway of human DNT cells

To further delineate the development of human DNT cells across the different clusters, we predicted pseudo-time ordering overlaid on UMAPs (Fig. 3A). Naïve DNT cells started the pseudotime trajectory and were divided into two branches: one leading to regulatory-like DNT cells and another leading to helper, cytotoxic, and ADCC-like DNT cells. Pseudotime scores confirmed the early developmental state of naïve DNT cells, along with the developmental sequence of other DNT cell clusters (Fig. 3B). Consistent with overall cluster states, the expression of marker genes across pseudotime distinctly reflected the developmental stages of each DNT cell cluster (Fig. 3C).

To further investigate the cell state, we performed single-cell TCR sequencing (scTCR-seq) of peripheral human DNT cells. The TCR repertoire analysis revealed variable degrees of clonal expansion and sharing across clusters (Fig. 3D–3G). Naïve and regulatory-like DNT cells exhibited the least clonal expansion, whereas ADCC and cytotoxic-like DNT cells showed substantial hyper-expansion, indicating a high activation and proliferation state (Fig. 3D and 3E). Moreover, TCR clonal overlap was observed across DNT clusters (Fig. 3F and 3G). Naïve DNT cells shared clones with effector DNT clusters, corroborating their less differentiated state. Most inter-effector DNT cell clonal overlaps occurred among helper, cytotoxic, and ADCC-like DNT cell clusters (Fig. 3F and 3G). These findings align with the pseudotime analysis and provide a detailed understanding of the developmental relationships among human DNT cell clusters.

Considering that DNT cells are a subset of peripheral T cells, previous studies have indicated a transition from peripheral T cell subsets to DNT cells (Zhang et al., 2007; Crispin and Tsokos, 2009; Rodríguez-Rodríguez et al., 2020). In this study, we performed a multi-modal assay combining scRNA-seq, scTCR-seq, and scCITE-seq on PBMCs from the same donors. This enabled precise identification of peripheral T cell subsets and active status at both the transcriptional and protein levels (Fig. S3A–S3C). The shared clonotype heatmap revealed that ADCC-like DNT cell clones were predominantly linked to peripheral CD8 T cells, while helper-like DNT cells were exclusively associated with peripheral MAIT cells (Fig. 3H). The shared TCR clones were located within an intensive cluster on the UMAP plot (Figs. 3I, S3D, S3E). ADCC-like DNT cells and CD8 T cells exhibited substantial TCR clonotype sharing, with 52 conserved clonotypes representing 13.6% of their combined repertoires (Fig. 3J). Helper-like DNT cells and MAIT cells also displayed notable overlap, sharing 43 clonotypes that accounted for 0.93% of their respective repertoires. Permutation testing confirmed that the observed overlaps were significantly greater than expected by chance, indicating a robust clonal relationship among these subsets (Fig. 3K). These TCR clonotypes suggest a developmental connection between helper-like DNT cells and MAIT cells and between ADCC-like DNT cells and CD8 T cells.

Chromatin accessibility profiles distinguish transcriptional regulations of human DNT cells

Chromatin accessibility is the initial step in transcription and plays a critical role in cell development. To epigenetically characterize

human DNT cells, we performed the single-cell assay for transposase accessible chromatin sequencing (scATAC-seq) and label-transferred the DNT cell transcriptional subclusters onto seven chromatin accessibility clusters (Figs. 4A and S4A). Differential accessibility analysis revealed distinct chromatin accessibility between naïve-1 and naïve-2 DNT cells, which corresponded to non-regulatory and regulatory-like DNT cells, respectively (Fig. 4B). This suggests that effector DNT cell fate transitions may originate from different subsets of naïve DNT cells. Using the SCENIC algorithm (Aibar et al., 2017), we identified transcription factor (TF) activity unique to each DNT cell cluster based on scRNA-seq data (Figs. 4C and S4B). Venn diagram suggested that several TFs, including LEF1, SNAI1, SOX4, STAT1, FOSL2, and NFE2L2, play critical roles at both the epigenetic and transcriptomic levels in DNT cell development (Fig. 4D). Many of these TFs ranked highly in SCENIC analysis and were differentially activated in DNT clusters (Fig. 4E and 4F). The enrichment of these TFs in DNT clusters was further confirmed through TF motif activity in the scATAC-seq data (Fig. 4G). TF footprinting analysis from scATAC-seq revealed upregulated activity of LEF1 and ETS1 in naïve-2 and regulatory-like DNT clusters, while NFE2 and FOSL2 activities were elevated in the ADCC-like DNT cluster (Fig. 4H). Chromatin accessibility signals also suggested a highly accessible *LEF1* locus in naïve-2 and regulatory-like DNT cells, as well as notable accessibility of *RORC* locus in helper-like DNT cells (Fig. 4I). Together, these results showed intricate epigenetic regulation and identified several TFs as potential key regulators in the development of human peripheral DNT cells.

Potential communications between DNT cells and other peripheral immune cells

To explore the interactions between DNT cells and other peripheral immune cells, we performed CellChat (Jin et al., 2021) analysis using scRNA-seq data from DNT cells and PBMCs of the same donors. We recorded 53,587 PBMCs and assigned them into 11 clusters through both protein and RNA expression data from single-cell levels (Fig. S5A; Table S2). Together with DNT cell subsets, several pathways are observed to mediate communication between DNT cells and PBMCs (Fig. S5B). A scatterplot further revealed the functional similarity between regulatory-like DNT cells and unconventional T cells, as well as between helper, cytotoxic, and ADCC-like DNT cells (Fig. S5C). Specifically, DCs received a FLT3 and CD40 signal from helper-like DNT cells. Additionally, ADCC-like DNT cells and cytotoxic-like DNT cells send CCL signal to DC and CD14⁺ monocytes (Fig. S5C and S5D). DNT cells also receive SIRP1 signals from CD4 T cells, CD8 T cells, and NKT cells, and CD90 signals from various PBMCs (Fig. S5E). These interactions may play a key role in the development and differentiation of peripheral DNT cells and PBMCs, particularly during autoimmune diseases and infections.

Sexual-dimorphism in the composition and function of human DNT subsets

Recent studies have highlighted sex differences in immune cells, which are tightly regulated by sex hormones (Li et al., 2024). In this study, we recruited both male and female donors to explore potential sexual-dimorphism in human DNT cells. We compared DEGs between female and male DNT cell clusters (Fig. 5A). Female DNT cells exhibited a greater number of upregulated genes across all clusters compared to male DNT cells. Notably, the proportion of helper-like DNT cells was fivefold higher in females (Figs. 6B and S1C). The estrogen receptor gene *ESR1* was highly expressed in helper-like

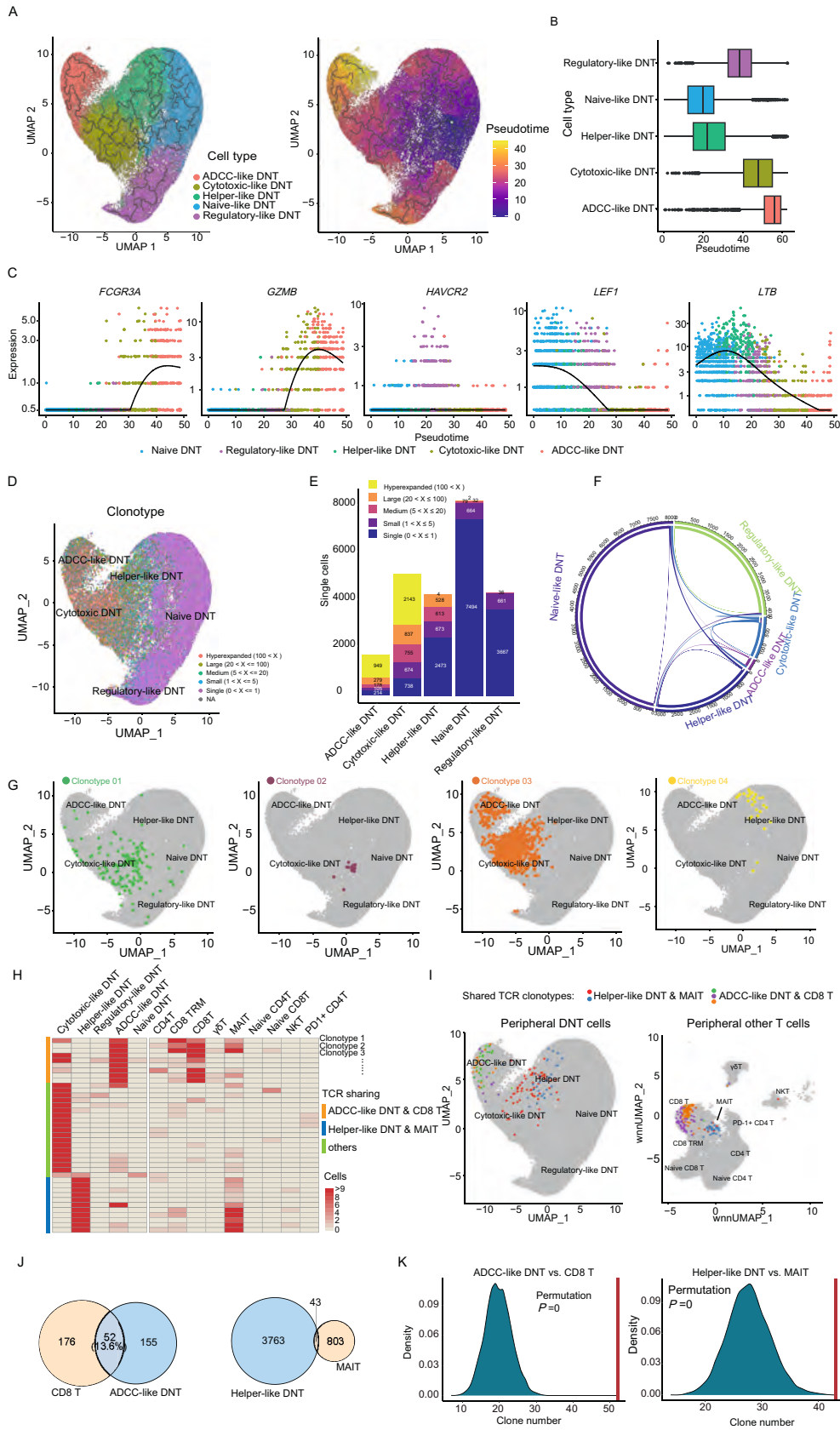


Fig. 3. Lineage analysis indicated the differential pathways of human DNT cells. **A:** Single-cell trajectories of human DNT cells. **B:** Boxplot showing the average pseudotime values in human DNT cell clusters. **C:** Dotplots showing expression of marker genes along the pseudotime in human DNT cell clusters. **D:** UMAP of human DNT cells colored by the same clonotype frequency. **E:** Barplot showing the cell numbers sharing the same clonotype numbers among human DNT clusters. **F:** Circos plot of shared TCR clonotypes. Links denote clonal sharing between clusters. **G:** Cell differentiation of human DNT cell clusters inferred by shared TCRs. Each color represents a distinct TCR clonotype. **H:** Heatmap showing the

DNT cells, with extreme upregulation in female helper-like DNT cells (Fig. 5C). GSEA revealed that pathways related to sex differentiation and cell growth were upregulated in female helper-like DNT cells (Fig. 5D and 5E). In contrast, pathways associated with oxidative phosphorylation, immune-regulatory interleukin-10, and cytotoxicity were significantly downregulated in female helper-like DNT cells. These findings were supported by the expression of key genes in these pathways (Fig. 5F). Additionally, female helper-like DNT cells showed significant upregulation of the transcription factor *RORC* and the effector gene *IL4I1* (Fig. 5G). Consistent with this, publicly available ChIP-seq data from breast cancer cells showed a notable increase in chromatin accessibility at the *RORC* locus following estrogen (17 β -estradiol [E2]) stimulation (Fig. 5H). To address this point, we stimulated peripheral human DNT cells with E2. Estrogen significantly promotes the IL-17A production and ROR γ t expression of human DNT cells ex vivo, supporting the critical role of estrogen in the differentiation of helper-like DNT cells (Fig. 5I and J). These data suggest pronounced sexual-dimorphism in human DNT clusters, with the relative accumulation of female helper-like DNT cells likely driven by estrogen-induced *RORC* activation.

Peripheral helper-like DNT cells accumulated and activated during autoimmune diseases

Compared to males, females experience more serious complications from autoimmune diseases, including ulcerative colitis (UC) and type 1 diabetes (T1D) (Ehrmann et al., 2020; Cumming et al., 2025). To investigate changes of DNT cells in autoimmune diseases, we analyzed the publicly available scRNA-seq datasets of PBMCs from UC and T1D patients. DNT cells were identified from PBMCs based on the positive expression of *CD3E* and *TRAC*, and negative expression of *CD4*, *CD8A*, *CD8B*, and *TRDC*. In UC patient datasets (GSE125527) (Boland et al., 2020), we identified four DNT clusters with functional gene expression profiles similar to our characterized human DNT cell clusters (Fig. 6A and B). Compared to healthy donors, UC patients showed an increased proportion of helper-like DNT cells (Fig. 6C). Moreover, the pro-inflammatory gene *LTB* was upregulated, while naïve genes *IL7R* and *LEF1* were downregulated in helper-like DNT cells from UC patients (Fig. 6D). Similarly, in T1D patient datasets (syn53641849) (Honardoost et al., 2024), peripheral DNT cells comprised five clusters with distinct marker gene expression (Fig. 6E and 6F). T1D patients also exhibited an increased proportion and *LTB* expression of helper-like DNT cells (Fig. 6G and 6H). These findings suggest a potential relation between peripheral DNT cells and the development of autoimmune diseases.

Discussion

Although peripheral DNT cells were discovered over three decades ago (Yachie et al., 1989; Abraham et al., 1992), their heterogeneity and differentiation pathways remain poorly understood, contributing to ongoing debates about the exact function of DNT cells. Here, our multiomics single-cell data on human peripheral DNT cells suggest that these cells comprise distinct subsets with versatile functions and differentiation trajectories. We identified naïve, regulatory, cytotoxic, and ADCC subsets of human DNT cells and their gene expression heterogeneity. ScTCR-seq revealed shared clonotypes between different DNT cell subsets and between DNT cell subsets and other peripheral T cells. Combined with scRNA-seq, scATAC-seq revealed two differentiation branches of naïve DNT

cells and key transcription factors, such as *LEF1* and *RORC*, involved in DNT cell development. As a part of PBMCs, DNT cell subsets exhibited potential interactions with other peripheral immune cells and sexual-dimorphism related to estrogen receptor activation.

The cytotoxic, regulatory, and pro-inflammatory functions of DNT cells have been validated in various diseases and animal models in recent years. Broadly defined DNT cells include NKT cells, TCR $\gamma\delta^+$ DNT cells, and TCR $\alpha\beta^+$ DNT cells. Recent studies using single-cell sequencing have provided detailed characterization of the subpopulations and functions of these broadly defined DNT subsets (Tin et al., 2025). These data show that TCR $\alpha\beta^+$ DNT cells display a transcriptional profile distinct from all other DNT populations, with elevated signaling pathways related to IL-2 and IL-12 responses, T-cell differentiation, monocyte differentiation, and immune cytotoxicity, suggesting the potential presence of additional subclusters within this population. DNT cells have also been reported to express high levels of both inhibitory checkpoints and cytotoxic molecules (Parthasarathy et al., 2023). Jin et al. (2025) further demonstrated that murine and human DNT cells highly express the immune checkpoint CD39. In addition, costimulatory molecule CD28 and the immune checkpoint CTLA-4 were shown to be abundantly expressed on DNT cells from patients with SLE (Anand et al., 2002). Okamura et al. identified a significant enrichment of DNT cells in colorectal cancer tissues, characterized by high expression of the cytotoxic gene *GZMK* (Okamura et al., 2024). Collectively, these studies highlight the markedly heterogeneous and context-dependent pro-inflammatory or regulatory features of human DNT cells in health and disease. In line with these findings, our data identified helper-like, regulatory, cytotoxic, and ADCC-like DNT subsets, which help resolve prior uncertainties regarding the precise functional spectrum of human DNT cells.

The cytotoxic function of DNT cells suggests potential applications in anti-tumor therapy and further improvement to CAR-T strategies (Vasic et al., 2022; Fang et al., 2023). Our study identified two DNT cell subsets with cytotoxic functions, cytotoxic-like DNT cells and ADCC-like DNT cells. The ADCC function of DNT cells may enhance anti-tumor therapy when combined with ADCC antibodies, such as trastuzumab and elotuzumab (Wang et al., 2016; Augustin et al., 2022). Our study also exhibited that the surface markers TIGIT and CCR6 were expressed on regulatory-like and helper-like DNT cells, respectively. These surface markers may facilitate the purification of specific subsets and enhance the efficacy of DNT cell therapies.

Single-cell trajectory analysis and scTCRseq revealed the potential differentiation pathways of peripheral DNT cell subsets. Similar to other T cell subsets, naïve DNT cells exhibited early pseudotime scores and shared TCR clones with effector DNT subsets. Previous studies have suggested diverse origins for DNT cells. Megan S. Ford et al. demonstrated that regulatory-like DNT cells may preferentially develop from non-CD8 T cells outside the thymus (Ford et al., 2006). In contrast, other groups traced DNT cells back to CD8 T cells (Crispin and Tsokos, 2009; Li et al., 2020; Rodríguez-Rodríguez et al., 2020). Data from Inna V. Grishkan et al. and our group demonstrated both in vivo and in vitro origins of DNT cells from CD4 T cells (Zhang et al., 2007; Grishkan et al., 2013). In this study, TCR clonal sharing revealed connections between specific DNT subsets and conventional T cell populations. Cytotoxic and ADCC-like DNT cells shared clones with CD8 T cell and CD8 TRM cells, whereas helper-like DNT cells shared clones with MAIT cells, indicating a simultaneous model of diverse origin and differentiation among DNT

distribution of shared clonal clonotypes in DNT clusters and other peripheral T cell subsets. I: Shared clonotypes between DNT and peripheral T cell clusters are color-coded as in (G). J: Venn diagram showing unique and overlapping TCR clonotypes among CD8 T, ADCC-like DNT, Helper-like DNT, and MAIT cells. K: Permutation test assesses the significance of the observed clonal overlap, where the red vertical line marks the actual number of conservative clonotypes (permutation $P = 0$). DNT, double negative T; TCRs, T cell receptors; UMAP, uniform manifold approximation and projection; ADCC, antibody-dependent cell-mediated cytotoxicity; MAIT, mucosal-associated invariant T.

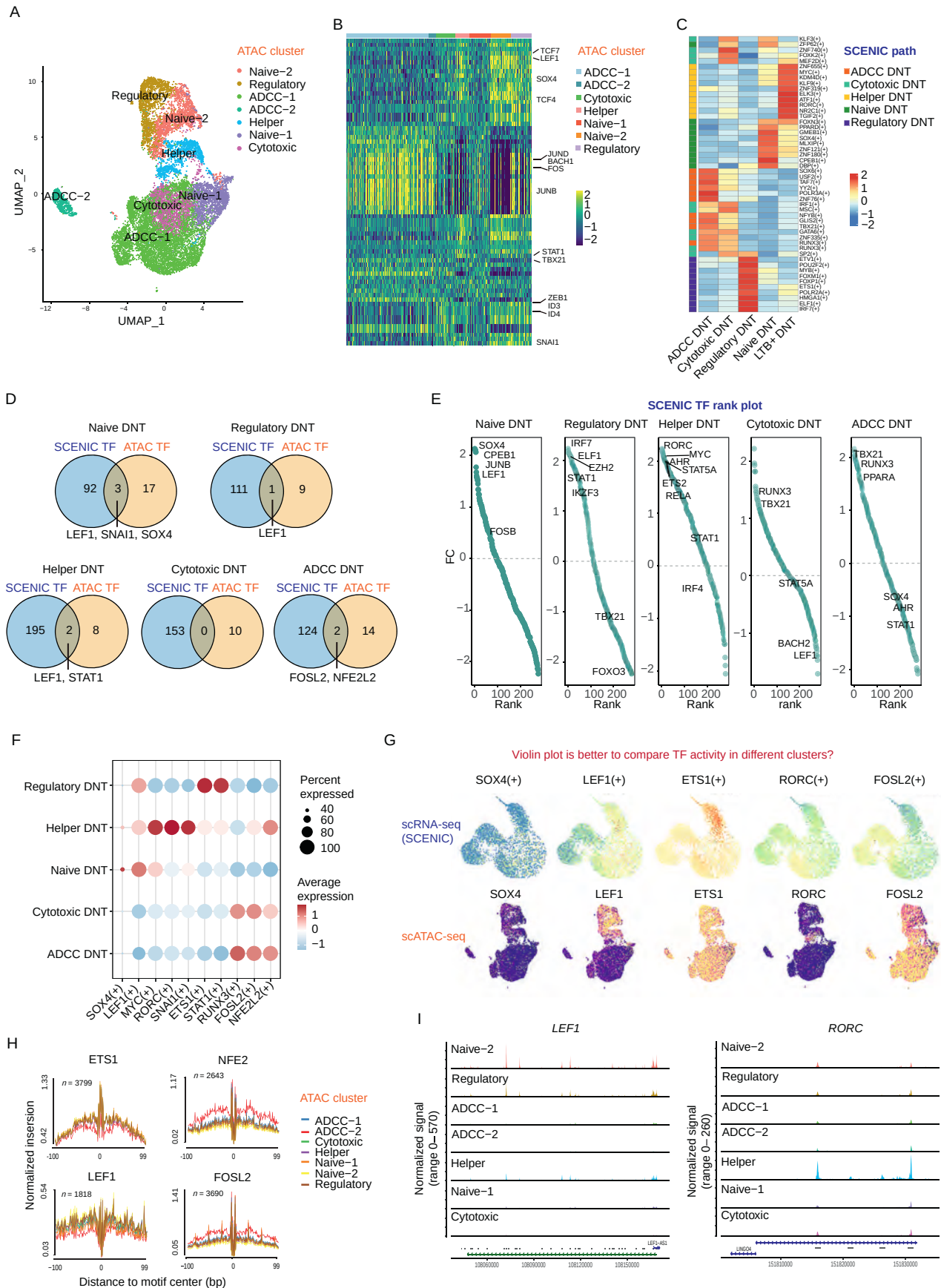


Fig. 4. Chromatin accessibility profiles distinguish transcriptional regulations of human DNT cells. **A:** UMAP plot displaying human peripheral DNT cells colored by scATAC-seq clusters. **B:** Heatmap showing row-scaled TF activity in scATAC-seq clusters. **C:** Heatmap showing TF activity by scRNA-seq using the SCENIC algorithm. **D:** Venn diagram showing the number

subsets. Interestingly, the cytotoxic or helper functions of clone-sharing DNT subsets were consistent with those of related T cell subsets. Elucidating these mechanisms could facilitate the development of precise strategies to modulate the equilibrium between effector DNT subsets.

The retinoic acid receptor-related orphan nuclear receptor (ROR γ t) is a key transcription factor in Th17 cells, which secrete the pro-inflammatory cytokine IL-17A and play critical roles in autoimmune diseases (Ivanov et al., 2006). In our study, we observed upregulated *RORC* (which encodes ROR γ t) in helper-like DNT cells, along with increased expression of *IL4I1* and *LTB*. Increased IL-4 and IL-17 production by DNT cells has been observed in patients with systemic lupus erythematosus (SLE) and autoantibody-associated vasculitis (AAV) (Lai et al., 2013; Qin et al., 2021). Lymphotoxin, a member of the tumor necrosis factor (TNF) superfamily, is essential for the development of secondary lymphoid tissues and effector functions (Upadhyay and Fu, 2013). Activation of the mTOR signaling pathway in DNT cells is associated with lupus-prone mice and elevated circulating DNT cells, together with IL-17 secretion, also correlated with disease activity in patients with SLE (Li et al., 2024). T-cell-derived lymphotoxins exacerbate experimental autoimmune encephalomyelitis (EAE) (Gogoleva et al., 2024). Our data suggested exclusive *LTB* expression in helper-like DNT cells. Moreover, *LTB* expression in helper-like DNT cells is upregulated in patients with UC and T1D. These data suggest a potent pro-inflammatory role of helper-like DNT cells in immune tissue development and immune cell activation.

Sex differences have been demonstrated in the immune system of both healthy and diseased individuals. Females are more susceptible to autoimmune diseases, while males tend to be more susceptible to infections and tumors (Fairweather et al., 2008; Klein and Flanagan, 2016; Dias et al., 2022). Mouse models of lupus-like disease also exhibit a sex bias (Jivrajka et al., 2023). Sexual dimorphism has been observed in immune cell transcriptome profiling of male and female C56BL/6J mice (Gal-Oz et al., 2019). In healthy individuals, females exhibit higher proportions of circulating CD4 T cells and B cells, and lower proportions of peripheral monocytes and natural killer (NK) cells (Patin et al., 2018; Bongen et al., 2019). In this study, we also observed sex-dimorphism in DNT cells, with a higher proportion of helper-like DNT cells in females than in males. Similar to the high level of estrogen receptor ESR1 in helper-like DNT cells, the proportion and ESR1 expression in helper-like DNT cells are notably increased in female peripheral blood. Sex hormone receptors are extensively expressed in diverse tissues and cells, indicating their direct role in physiological activities (Morselli et al., 2017; Li et al., 2024). Estrogen accelerates symptoms in spontaneous autoimmune models (Verheul et al., 1995). The high expression of *RORC* in female helper-like DNT cells, along with the *RORC* locus opening in cell lines after estrogen stimulation, suggests a potential mechanism for estrogen-induced differentiation and proliferation of helper-like DNT cells. A deep understanding of this process in helper-like DNT cells could be beneficial for the treatment of autoimmune diseases. Interestingly, we also noted a higher number of upregulated genes in all DNT clusters in females than in males, together with differences in upregulated pathways between sexes, implicating the potential impact of sex-related differences in treating immunological diseases.

This study also underscored the significance of differential DNT clusters in autoimmune diseases, as exemplified by elevated helper-

like DNT in UC and elevated regulatory-like DNT in T1D patients. Recently, Yasumizu et al. (2024) examined peripheral CD4 T cell populations from autoimmune patients (myasthenia gravis, multiple sclerosis, and lupus) and healthy individuals by scRNA-seq, TCR-seq, and meta-analysis of publicly available single-cell data sets. They identified twelve gene programs in autoimmune disease. Clearly, there are distinct variations in peripheral blood T cell clusters between healthy and diseased individuals. In this study, we also explored the communications of DNT with other immune cells and identified several signaling pathways involved. Hence, further comprehensive interpretation of alterations in T cell landscape and their interactions with other immune cells in immunological diseases is highly warranted.

Mouse models are reliable tools in immunological research. In this study, we identified similar transcriptomic profiles for cytotoxic, regulatory, and helper subsets between mouse and human DNT cells (Yang et al., 2021). However, we did not observe similar gene expression patterns for resting and innate mouse DNT cells in human DNT cells. This discrepancy might stem from the years of foreign antigen and microbiota exposure in adult humans, as mice in a specified pathogen-free environment are more similar to neonates than adult humans (Beura et al., 2016). On the other hand, our group and others have suggested both cytotoxic and regulatory functions of DNT cells in mouse models (Zhang et al., 2000; Zhao et al., 2016; Tian et al., 2019, 2023; Sun et al., 2021). Other studies have also suggested a pro-inflammatory role for DNT cells in mouse models of autoimmune diseases (Rodriguez-Rodriguez et al., 2016; Li et al., 2020). These similarities and differences underscore the importance of further understanding to reveal the role of DNT cells in health and disease progression.

This study has several limitations. We analyzed samples from only ten individuals (five males and five females) of Asian ancestry, all of whom were young to middle-aged adults. Therefore, our findings may represent only a subset of the healthy population. Although our multi-omics approach provides a detailed molecular map within this cohort, the absence of an independent validation dataset limits the generalizability and reproducibility of the findings. Future studies involving larger cohorts with greater diversity in ethnicity, age, and disease status are warranted to further elucidate the phenotypic and functional heterogeneity of DNT cells.

Materials and methods

Ethics statements

This study was approved by the Ethics Committee of Beijing Friendship Hospital, Capital Medical University (No. 2020-P2-174/DR20200174) and complied with all relevant ethical regulations. All donors provided written, informed consent before consent.

Isolation of peripheral blood mononuclear cells (PBMC)

PBMCs were isolated from fresh whole blood by density gradient centrifugation (Dakewe) at 800 $\times$ g for 25 min with no brake. Cells were washed with PBS (Corning) and resuspended in FACS buffer (PBS containing 0.5% BSA (Sigma-Aldrich) and 2 mM EDTA (Thermo Fisher Scientific)).

of hyper-activated TF based on SCENIC of scRNA-seq (blue) and scATAC-seq (orange). **E:** Bubble-rank plot of differential TF activity of scRNA-seq in human DNT clusters. **F:** TF activity in DNT cell clusters by scRNA-seq with SCENIC algorithm. Dot color indicates the average TF activity, and dot size indicates the percentage of cells activating the TF. **G:** Scaled activity for indicated transcription factor motifs shown on UMAP plots generated by scRNA-seq (SCENIC) and scATAC-seq. **H:** TF footprinting for hyper-activated TFs by scATAC-seq clusters. **I:** Genome browser showing average scaled chromatin accessibility signals at *LEF1* and *RORC* for human DNT clusters. TF, transcriptional factor; UMAP, uniform manifold approximation and projection; DNT, double negative T; ADCC, antibody-dependent cell-mediated cytotoxicity.

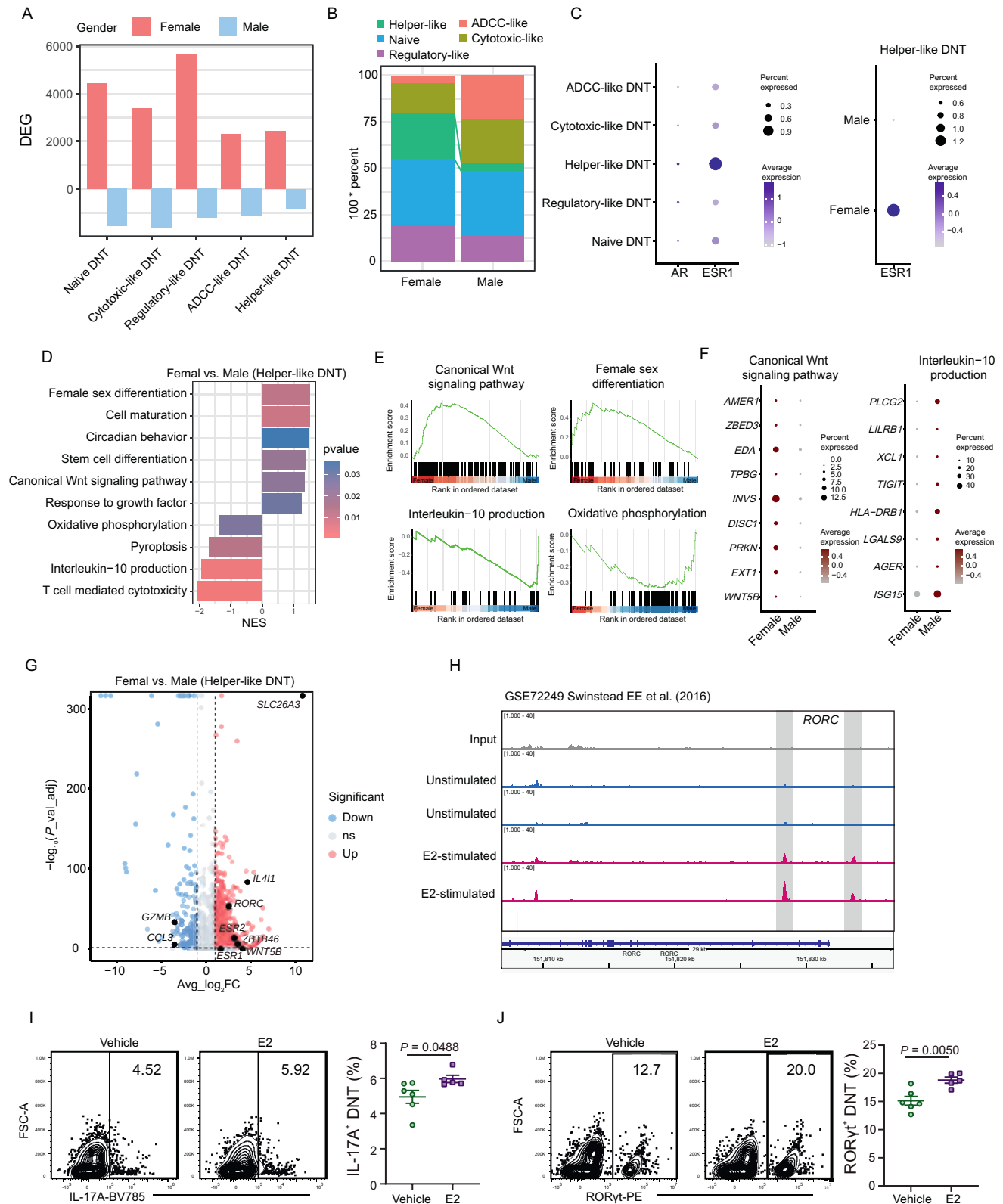


Fig. 5. Sexual-dimorphism in the composition and function of human DNT subsets. **A:** Bar plot showing the number of DEGs upregulated in female or male DNT cell clusters. Adjust P value < 0.01, average $\log_2$ Fold change > 0. **B:** Stacked bar plot showing the proportions of DNT cell clusters between females and males. **C:** Expression of sex hormone receptors in DNT clusters. **D:** GSEA of helper-like DNT cells between females and males. **E:** GSEA of differential gene sets in female and male helper-like DNT cells. **F:** DEG of GSEA enriched pathways in female and male helper-like DNT cells. **G:** Volcano plot showing the expression of genes in female versus male helper-like DNT cells, plotted against P values. **H:** Visualization of chromatin accessibility in the *RORC* locus with or without stimulation of estrogen E2 in breast cancer cells. **I** and **J:** Flow cytometry results illustrating the IL-17A⁺ (I) and RORγt⁺ (J) expression of sorted human peripheral DNT cells after being stimulated by vehicle or E2 for 48 h ($n = 5-6$; Student's t -test). DEGs, differentially expressed genes; DNT, double negative T; GSEA, gene set enrichment analysis; NES, normalized enrichment score; RORγt, retinoic acid receptor-related orphan nuclear receptor; E2, 17 β -estradiol.

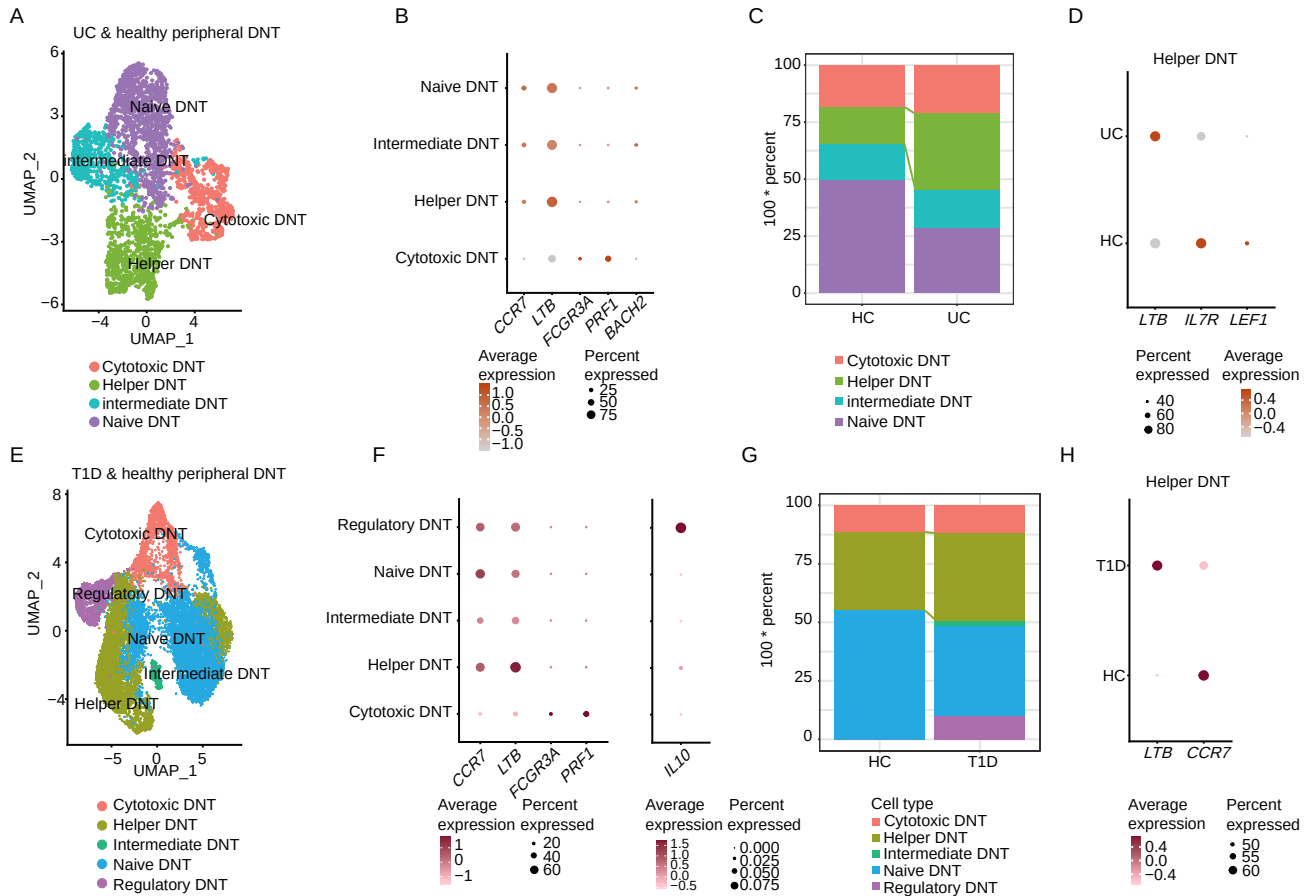


Fig. 6. Peripheral helper-like DNT cells accumulated and activated during autoimmune disease. **A–D:** Analysis of scRNA-seq data of peripheral DNT cells of UC patients and HC donors from publicly released dataset GSE125527. **A:** UMAP plot colored by scRNA-seq clusters. **B:** Expression of marker genes in DNT cell clusters. Dot color indicates the average gene expression, and dot size indicates the percentage of cells expressing the gene. **C:** Stacked bar plot showing the proportions of DNT cell clusters between HC and UC patients. **D:** Expression of helper-like DNT cell effector gene *LTB* and naive genes in helper-like DNT cells from HC and UC patients. **E–H:** Analysis of scRNA-seq data of peripheral DNT cells of T1D patients and HC donors from publicly released dataset syn53641849. **E:** UMAP plot colored by scRNA-seq clusters. **F:** Expression of marker genes in DNT cell clusters. **G:** Stacked bar plot showing the proportions of DNT cell clusters between HC and T1D patients. **H:** Expression of helper-like DNT cell effector gene *LTB* and naive gene *CCR7* in helper-like DNT cells from HC and T1D patients. UMAP, uniform manifold approximation and projection; DNT, double negative T; HC, healthy control; UC, ulcerative colitis; T1D, type 1 diabetes.

Surface staining and DNT cell sorting

PBMCs were incubated in human TruStain FcX (Biolegend) for 10 min at 4°C in FACS buffer. Human CD1d Tetramer-PE (a-GalCer loaded, MBL) and DimerX I: Recombinant Soluble Dimeric Human CD1d (LPC loaded, BD) staining was performed for 1 h at 4°C in FACS buffer. Cells were washed twice and incubated with PE Rat Anti-Mouse IgG1 (BD) for 30 min at 4°C in FACS buffer. Suspended PBMCs were stained with a panel of anti-human surface antibodies (CD56 [clone: HCD56], CD3 [clone: SK7], CD161 [clone: HP-3G10], TCR $\alpha\beta$ 7.2 [clone: 3C10], TCR α/β [clone: IP26], CD4 [clone: RPA-T4], and CD8 [clone: RPA-T8] purchased from Biolegend) for 15 min at 4°C after washing twice. DNT cells were sorted as the phenotype of CD3⁺ CD56⁺ TCR $\alpha\beta$ 7.2⁺ CD161⁺ CD1d⁺ TCR β ⁺ CD4⁺ CD8⁺ by Aria III Flow Cytometer (BD). Cell viability and number were checked by LUNA-FL Automated Fluorescence Cell Counter (Dichbio) after sorting.

Intracellular marker staining for flow cytometry

Fresh isolated PBMCs or DNT cells were incubated in 10% FBS 1640 media with Cell Activation Cocktail (with Brefeldin A, Biolegend) for 5 h. Cells were stained for surface markers as described above. We performed intracellular staining using Foxp3/Transcription Factor

Staining Buffer Set (Thermo) following the manufacturer's instructions. Anti-human intracellular marker antibodies (Granzyme B [clone: QA16A02], IL-17A [clone: BL168], IL-10 [clone: JES3-9D7], and ROR γ t [clone: Q21-559] are purchased from Biolegend). Flow cytometry data were acquired on a LongCyte™ flow cytometer (Challenbio) and analyzed using FlowJo software (Tristar).

Single-cell seq library preparation and sequencing

DNT cells were immediately subjected to library preparation after viability counting. The scRNA-seq, scTCR-seq, scATAC-seq, and scCITE-seq libraries were prepared using the Library & Gel Bead Kit (v3.1), Chromium Next GEM Single Cell 5' Kit (v2), and Chromium Next GEM Chip K Single Cell Kit by ANNOROAD Gene Technology Company. The cDNA concentration and quality were assessed using Qubit Fluorometer (Thermo Scientific) and bioanalyzer 2100 (Agilent), respectively. The scRNA, scTCR, and scATAC libraries sequencing was performed on an Illumina Novaseq 6000 using a PE150 sequencing strategy by ANNOROAD Gene Technology company.

scCITE-seq

Human PBMC CITE-seq was carried out together with scRNAseq. After PBMC isolation, cells were stained with a mix of 130 surface

antibodies labeled with oligonucleotides (TotalSeq-C Human Universal Cocktail, V1.0, Biolegend) as the manufacturer recommended. Briefly, 1 million cells were suspended in 45 μ L of Cell Staining Buffer (Biolegend) and blocked by 5 μ L of Human TruStain FcX™ Fc Blocking reagent (Biolegend) for 10 min at 4°C. Cells were incubated with TotalSeq antibody cocktail (Table S2) for 30 min at 4°C. PBMCs are ready for library preparation after washing twice and verification of cell concentration and viability. ScRNA-seq and scCITE-seq libraries were generated according to the instructions of the manufacturer.

Data processing of scRNA-seq and scTCR-seq

Raw fastq files derived from our libraries were processed with cellranger count (v3.1.0) using the 10 $\times$ Genomics prebuilt human reference. Our libraries were processed independently and merged into a single experiment for analysis using Seurat (v5.1.0). Quality control was performed by removing cells with high (>15%) mitochondrial UMI content. Cells with more than 6000 or fewer than 500 genes were excluded. Paired TCR clones were defined by the V, (D), J, and CDR3 nucleotide composition. TCR clonal overlap was assigned to cells expressing identical V, (D), J, and CDR3 nucleotide and amino acid sequences.

Normalization, dimensionality reduction, batch correction, and clustering

The raw UMI counts were normalized by SCTransform function in the Seurat (v5.1.0) package. The cells were first dimensionally reduced by PCA using the scaled data. Harmony package was used to correct the batch effects across different samples. The first seven principal components were further used for visualization using the UMAP. Marker genes were detected by comparing each cluster against all other cells through the Wilcoxon rank sum test.

Gene set enrichment analysis (GSEA) and gene set variation analysis (GSVA)

Marker genes belonging to every cluster were ranked by log₂FC and used to perform GSEA analysis using the clusterProfiler (4.10.1) package. Gene set variation analysis was performed using the GSVA (1.50.5) package and the KEGG, GO, and MsigDB gene sets.

Trajectory inference analysis

Monocle 3 (v.1.3.4) was used to generate the pseudotime trajectory analysis in human DNT cells (Cao et al., 2019). Specifically, we transformed a Seurat object into a cell_data_set using SeuratWrappers function. The cells were then clustered to identify distinct cellular populations, enabling the construction of a trajectory graph using the learn_graph function. Subsequently, cells were ordered along this trajectory to assess pseudotime. Further, we performed a differential expression analysis using the graph_test function to identify genes with significant spatial correlation in pseudotime. Top genes were selected based on Moran's I statistic, highlighting those with the most pronounced changes along the trajectory.

Cell-cell interaction analysis

CellChat (v2.1.2) tool was used to predict the cell-cell interaction between subsets of DNTs and PBMCs. The ligand-receptor interactions in CellChat database for human (CellChatDB.human) were applied to assess the likelihood of communication. The PBMC single-cell objects of the same donors were merged with the DNT

scRNAseq object with clusters renamed previously. We followed the methodologies of the original article described by Jin et al. (2021). The plots were generated using the built-in functions within CellChat.

SCENIC analysis

Transcription factor (TF) activity from scRNA-seq was analyzed using SCENIC (v1.3.1). The DNT single-cell RNAseq object was down-sampled to 1000 cells per cell type before proceeding with the downstream analysis. The regulons and TF activity (AUC) for each cell were calculated by the pySCENIC (v0.12.1) pipeline with default parameters. The differentially activated TFs of each subcluster were identified by the average difference in deviation score.

scATAC-seq analysis

scATAC-seq data from three DNT samples were pre-processed using the Cell Ranger ATAC software, aligning to GRCh38 human reference genome and generating peak-barcode matrices. The output files from multiple samples were combined using the cellranger aggr command with read depth normalization to generate a single peak-barcode matrix for all the data. Then, the Seurat and Signac packages were used for further analysis. Cells were filtered according to the following criteria: the number of fragments in peaks >3000, total fragments < the 95th percentile, a blacklist ratio <0.05, a nucleosome signal strength <4, and a TSS enrichment score greater than the 5th percentile. For the filtered matrix, a singular value decomposition (SVD) was performed using the RunSVD function after adjusting for sequencing depth variations among cells via the RunTFIDF function. Dimensionality reduction and clustering were then performed on the corrected LSI components by the Harmony method. For each cluster, we calculated differential chromatin accessibility regions using FindMarkers, with a threshold P_{val_adj} <0.05. To quantify the chromatin accessibility at the gene levels, the fragments for each cell that intersect the gene and promoter (2000 bp upstream) regions were counted. Single-cell TF motif activity in a single cell is measured using run_chromVAR function in scATAC-pro software. Differential TF activity between cell types was then calculated by scATAC-pro's do_DA_motif function.

Estradiol stimulation of DNT cells in vitro

PBMCs were isolated from fresh whole blood of healthy volunteers by density gradient centrifugation (Dakewe) at 800 $\times$ g (acceleration 3, deceleration 2) for 25 min. Cells were washed with PBS and resuspended in FACS buffer (PBS containing 0.5% BSA [Sigma-Aldrich] and 2 mM EDTA [Thermo Fisher Scientific]). After 10 min blocking with TruStain FcX (BioLegend, 422301) at 4°C, cells were stained with PE-conjugated anti-CD4, -CD8 α , -CD19, and -CD56 for 15 min at 4°C in the dark. CD4⁺CD8 α ⁺CD19⁺CD56⁺ cells were enriched by negative selection using anti-PE MicroBeads (Miltenyi, 130-048-801). The negatively selected cell population was further stained with a cocktail of antibodies against human CD3, CD161, TCR α 7.2, TCR α / β , and TCR γ δ at 4°C in the dark for 15 min. After two washes, DNT cells, defined as CD3⁺TCR β ⁺CD4⁺CD8 α ⁺CD19⁺CD56⁺TCR α 7.2⁺TCR γ δ ⁺CD161⁺, were sorted using a BD FACSAria III Flow Cytometer.

The sorted DNT cells were expanded for 28 days under anti-CD3 stimulation in the presence of recombinant human IL-2. Following expansion, the cells were treated with 10 nM E2 (MCE, HY-B0141) for 48 h and then analyzed by flow cytometry.

Statistical analyses

Differentially expressed genes from single-cell data were determined by a Wilcoxon rank sum test and corrected with the Bonferroni method, as implemented in Seurat. Statistical analysis was performed using Prism Graphpad software (9.0.0). Results are shown as bar plots with the mean and SEM. Statistical significance was determined using one-way repeated measure analysis of variance (ANOVA) with multiple comparison testing or Student's *t*-test for two-group comparison. *N* value and *P* value are exhibited in figure legends and figures.

Data availability

The sequencing data from this study are publicly accessible at China National Center for Bioinformation (CNCB) under accession numbers HRA008611 (DNT cell data) and HRA008610 (PBM data). Single-cell DNT data from four healthy individuals are additionally available under HRA015334. The publicly available scRNA-seq datasets of healthy peripheral PBMCs were obtained from the European Genome-Phenome Archive (E-MTAB-10026) and the NCBI Gene Expression Omnibus (GSE158055). The scRNA-seq datasets of PBMCs from individuals with T1D and UC were accessed through Synapse (syn53641849) and the NCBI Gene Expression Omnibus (GSE125527), respectively.

CRediT authorship contribution statement

Dan Tian: Conceptualization, Methodology, Project administration, Writing - Original draft. **Fengcui Qian:** Methodology, Writing - Original draft. **Lehan Pan:** Methodology, Investigation, Writing - Review & Editing. **Mingxue Yin:** Methodology, Investigation. **Bihan Liu, Yuxi Zhang, Xiaobao Yang, Lu Yang, Guangyong Sun:** Methodology. **Zhongtao Zhang, Songlin Wang, Patrick SC Leung:** Writing - Review & editing. **M. Eric Gershwin:** Conceptualization, Writing - Review & Editing. **Chunquan Li:** Conceptualization, Supervision, Writing - Review & Editing. **Dong Zhang:** Conceptualization, Project administration, Supervision, Writing - Review & Editing.

Conflict of interest

The authors declare that they have no competing interests.

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Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jgg.2026.04.008>.

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