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A single-cell immune atlas of primary and secondary lymphoid organs in pigs

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Single-cell RNA sequencing (scRNA-seq) has revolutionized understandings of cellular identities and functions due to the ability to study transcriptome-wide gene expression within individual cells. Multi-tissue scRNA-seq atlases have generated holistic understandings of body-wide cell dynamics and serve as key foundational resources for further scientific studies across a variety of species. Pigs are a valuable biomedical model, and pork is an essential global food source, but minimal understanding of immune cell identities and functions across anatomical locations limits agricultural and health advancements in pigs. To address current limitations, we apply scRNA-seq to create an atlas of immune cells recovered from key immune tissues including primary lymphoid organs (bone marrow and thymus) and secondary lymphoid organs (lymph node and spleen). Thymus data was compared to a previously published scRNA-seq dataset of pig thymus and shared a general consensus while also identifying several new thymic cell populations. Comparison of spleen to a human splenic scRNA-seq dataset also revealed conserved features, including two subsets of innate lymphoid cells conserved between pigs and humans. Inference of lymph node cell interactions and proximities from scRNA-seq data resembled some features of follicular organization and conventional germinal center reaction processes. To expand accessibility of the scRNA-seq atlas for biological query, we deploy an interactive application and demonstrate its use for non-computational exploration of diverse cell populations recovered from bone marrow. Overall, results expand current foundational understandings of immune cell identities and functions in pig lymphoid organs and demonstrate pig-to-human immune similarities to consider for future research applications. Materials associated with this work are made readily accessible for others to investigate individual queries requiring foundational knowledge pertaining to pig immunity.

KEYWORDS

atlas, cell annotation, FAANG, immune, lymphoid, pig, scRNA-seq, single-cell

1 Introduction

The immune system is a complex orchestration of coordinating cells, vessels, and molecules that coalesce to prevent or control damages caused by pathogens, toxins, and other harmful insults. Lymphoid organs are the central hubs providing strategic localizations and specialized microenvironments needed to bolster immunity through production and activation of immune cells and immune mediators. Primary lymphoid organs serve as sites for immune cell production and development, while secondary lymphoid organs are sites where immune reactions are initiated and deployed. However, different primary and secondary lymphoid organs serve unique functions that are supported by distinct cell types (1). As cells are the building blocks of immune reactions, an understanding of cellular heterogeneity across primary and secondary lymphoid organs is key to gaining a holistic interpretation of immune cell function and coordination. In this work, pigs were used to study cellular heterogeneity across lymphoid organs due to their agricultural importance as a food source (2) and their suitability as a biomedical model (3, 4).

To best survey the plethora of immune cells found across primary and secondary lymphoid organs in pigs, a high-resolution, omics-level technology is required. Single-cell RNA sequencing (scRNA-seq) is a technology that can achieve a transcriptome-wide survey of gene expression within individual cells, thus revolutionizing the functional annotation of individual cells, cell types, and cell states across various organisms, tissues, organ systems, disease states, and a varied multitude of other biological systems and scenarios. scRNA-seq is a suitable technology for discovery-based cell analysis, as previous phenotypic knowledge of cells is not required, and protein-based immunoreagent availability is not a limiting factor (5, 6).

Annotation of cell types is crucial to accurately interpret scRNA-seq data. The annotation process requires both an accurately annotated genome for correct assignment of sequence data to expressed genes within each cell and cell-level transcriptomes from many different tissues, ages, and biological states. The porcine genome is sufficiently contiguous (7), and substantial bulk RNA-seq (8) and scRNA-seq transcriptomic resources are available and accumulating, respectively, to provide sufficient information for porcine cell type annotation. Wang and colleagues recently published a multi-tissue atlas of 11 adult pig tissues, with the brain subdivided into another nine components (9). While these tissues contained diverse cell types, this study was limited in immune tissue coverage, with only spleen and peripheral blood mononuclear cells (PBMCs) represented. Another recent report by Chen et al. created a scRNA-seq atlas of seven pig tissues, including two types of fat and many organs overlapping with the work by Wang et al. (10). However, this work again was not immunologically focused, with spleen being the only lymphoid organ analyzed. Another recent pig scRNA-seq atlas by Zhang et al. was immunologically focused (spleen, lymph node, Peyer's patches), but work was performed in germ-free and specific pathogen-free miniature pigs that aren't representative of conventional livestock, and no primary lymphoid organs were analyzed in the work (11). Others have focused on important

porcine immune tissues such as thymus (12), gut-associated organized lymphoid tissues (i.e. Peyer's patches) (13–15), spleen (16), and PBMCs (5, 17, 18). While multi-tissue atlases are extremely useful for understanding the full embodiment of swine cell biology, and current individual immune tissue surveys have lent higher-resolution immune understandings in pigs, an immune-focused, more comprehensive multi-tissue assessment of pig leukocytes is still lacking but needed to gain comprehensive insights into immune coordination across lymphoid organs.

In this work, we aim to address important gaps in understanding the porcine immune cell landscape by establishing an immune organ-centric, multi-tissue scRNA-seq atlas. Tissues from both primary and secondary lymphoid organs were utilized to understand immune cellular dynamics at primary lymphoid sites of immune cell production and development (bone marrow, thymus) and at secondary lymphoid effector sites where immune cells routinely function to activate versus regulate immune responses (spleen, lymph node). In this work, we establish functionally- and phenotypically-relevant annotations for immune cells identified in each lymphoid organ, demonstrate applicable ways each tissue dataset can be utilized to further resolve immunological functions in swine, and draw several similarities of our findings to human immune components, thus suggesting potential suitability of pigs for several areas of biomedical study.

2 Materials and methods

2.1 Animals and sample collection

Four-week-old Yorkshire piglets were delivered from a Michigan State University facility to the National Animal Disease Center (NADC) in Ames, Iowa and housed in a biosecurity level (BSL)-2 room. Pigs were initially given commercial starter feed and then transitioned to a commercial grower/finisher feed after approximately two weeks. Pigs remained healthy throughout the housing period, showing no clinical signs of disease nor requiring any antibiotic treatment. At approximately six months of age, two non-castrated male pigs were euthanized with an intravenous bolus of FatalPlus (pentobarbital solution at 390 mg/mL; Vortech Pharmaceuticals) administered via the auricle vein at label dosage of 1 mL per 10 pounds body weight (1 mL per 4.53 kg body weight) to effect. Immediately postmortem, four rib bones and sections of spleen, thymus, and ileocecal lymph node were collected. Ribs were used to isolate bone marrow cells. Other tissues were collected for cell isolations and *in situ* staining as described in subsequent sections. Upon gross examination at necropsy, the major organs appeared healthy and free of disease. Animal experiments were performed according to procedures approved by the Institutional Animal Care and Use Committee at the NADC.

2.2 Cell isolations and cryopreservation

Collected spleen, thymus, and ileocecal lymph node tissues (~2 g) were transported back to the lab in 10 mL tissue buffer (2 mM EDTA [Invitrogen AM9260G], 2mM L-glutamine

[Gibco 25-030], 0.5% bovine serum albumin [BSA; Sigma-Aldrich A9418] in Hank's Balanced Salt Solution [HBSS; Gibco 14175]) in a gentleMACS C Tube (Miltenyi 130-093-237). In the lab, tissues were mechanically homogenized using a gentleMACS Octo Dissociator (Miltenyi 130-095-937) with the programed spleen – cells protocol. Cell suspensions were passed through a 100-micron nylon mesh cell strainer, and strainers were washed with 10 mL tissue buffer. Cells were pelleted by centrifugation at 300 xg for 5 min room temperature (RT). Cell pellets were resuspended in residual volume (<1 mL) and then incubated with 20 mL ACK lysis buffer (ThermoFisher A1049201) for 3 min to lyse red blood cells and centrifuged again at 300 xg for 5 min RT. Spleen cells were treated with ACK lysis buffer a second time. Cells were resuspended in 10 mL tissue buffer and passed through a 70-micron nylon mesh cell strainer. Cells were centrifuged 300 xg for 5 min RT and resuspended in tissue buffer.

Bone marrow cells were isolated by flushing ~30 mL tissue buffer through two rib bones using an 18 gauge needle with 10 mL syringe and collecting cells into a 50 mL conical as flushed out. Recovered cells were pelleted by centrifuging 300 xg for 5 min RT. Cells were then passed through cell strainers and treated with ACK lysis buffer as described above.

Cell quantity and viability was assessed using the Muse Count & Viability Assay Kit (Luminex MCH100102) with a Muse Cell Analyzer (Luminex 0500-3115). To further enrich for live cells, cell suspensions were processed through a Dead Cell Removal Kit (Miltenyi 130-090-101) according to manufacturer's protocol with a starting quantity of 5×10^7 total cells as previously described (14). Recovered cells were cryopreserved according to the 10X Genomics Sample Preparation Demonstrated Protocol in multiple aliquots of 1×10^7 cells per vial.

2.3 Single-cell RNA library preparation sequencing

Cells were thawed according to the 10X Genomics Sample Preparation Demonstrated protocols, then processed twice (spleen, thymus, lymph node) or once (bone marrow) through the Dead Cell Removal Kit. Cell quantity and viability was assessed as described above and deemed adequate for scRNA-seq (>75% live cells per sample). Partitioning and library preparation were performed according to the Chromium Single Cell 3' Reagent Kits v2/3 User Guide (10X Genomics CG00052), and 100 base paired-end sequencing was performed on a HiSeq3000 (Illumina) at the Iowa State University DNA Facility. Aliquots of bone marrow cells from both pigs and lymph node cells from one pig were thawed and processed for an additional scRNA-seq submission as described above. Library preparation reagents used v2 or v3 chemistry as follows: bone marrow samples and the lymph node sample processed for an additional submission used v2 chemistry in the first run and v3 chemistry in the second run; thymus cells used v3 chemistry; the lymph node sample processed only once used v3 chemistry; spleen samples used v2 chemistry. Cells derived from separate animals were not pooled together for partitioning but were processed as individual sample libraries. While 10,000 cells per partitioning event were targeted, both bone marrow and spleen had

lower numbers of partitioned cells. Data integration (described in subsequent methods) was used to alleviate effects of submission batch and library chemistry. Sequencing data were deposited as.fastq files for both forward and reverse strands following image analysis, basecalling, and demultiplexing.

2.4 Data analysis

2.4.1 Initial data processing

Initial data processing was performed similar to previous work (5). Briefly, reads were aligned to the *Sus scrofa* 11.1 reference genome and v97 annotation file (19) with Cell Ranger v4.0 (10X Genomics). Single-cell gene counts were next analyzed with SoupX v1.4.5 (20) to calculate and remove contaminating ambient RNA. Genes with sum-zero expression across all samples were removed from the dataset, as were cells with ≤ 300 total genes, ≤ 500 UMIs, and/or $\geq 10\%$ mitochondrial reads detected. While 10,000 cells per partitioning event was the target, both bone marrow and spleen had lower number of recovered cells for unknown reasons but was likely due to limited accuracy of enumeration method used prior to partitioning on instrument. Scrublet v0.2 (21) was used to estimate doublet probabilities, and cells with doublet probabilities ≥ 0.25 were removed. Filtered data were then further analyzed with Seurat v4.3.0.1 (22, 23) to perform data normalization (SCT and log transformations), integration, dimensionality reductions (PCA, t-SNE, UMAP), nearest neighbor calculations, and clustering as previously described (5), treating each tissue as an individual dataset. SCT-normalized data were used for data integration, and integrated data were used for PCA. The number of principal components used for additional dimensionality reductions (t-SNE, UMAP), finding nearest neighbors, and hierarchical clustering were calculated as previously described (5). Filtering metrics are available as described in the Data availability statement.

2.4.2 Cell annotation

Clustering was performed in Seurat as previously described (5). Clustering resolutions were tested in intervals of 0.5 for each tissue dataset until sufficient segregation of cell types was achieved for annotation. Cell clusters were annotated by assessing canonical gene expression and lists of differentially expressed genes (differential gene expression analysis described below). At times, multiple clusters were converged into a single cell type annotation due to overlapping transcriptional profiles used to describe cell types at a biological level. In bone marrow, a single cluster containing progenitors, B cells, and myeloid cells could not be segregated into distinct cell lineages by increasing clustering resolution (clustering resolution tested from 0.5 to 10 at 0.5 intervals), so a data subset of cells from the single cluster was created and re-processed as a new dataset (normalization, integration, dimensionality reduction, nearest neighbor calculation, clustering) to identify cell types, and cell identities for the single cluster were incorporated back into the original bone marrow dataset containing all cells. In bone marrow and spleen datasets, a cluster containing high levels of hemoglobin genes

(*HBA¹**, *HBB*) and conflicting expression profiles of lineage-specific markers was identified. Creating subsets of data containing cells with high hemoglobin expression and performing re-clustering did not further indicate cell identity, segregate conflicting lineage-specific markers, or suggest an alternative cell identity (e.g. progenitor cells). We therefore removed these cells from further analyses and propose these clusters may be aggregates of cells adhered to erythrocytes, enclosed in the same droplet as erythrocytes during cell partitioning, or another form of unknown technical artifact.

2.4.3 Data merging of cell lineages

From each tissue dataset, data subsets were created for each of myeloid, B, and T/innate lymphoid cell (ILC) lineage cells similar to previous work (14). Cell lineage data subsets from each tissue were then merged into a new Seurat object to create new datasets containing all cells of each single lineage. Cells were processed through data normalization, integration, PCA calculations, and dimensionality reduction as described in methods for initial data processing.

2.4.4 Reference-based cell type prediction and mapping

Reference-based cell type prediction and mapping was performed with defined query and reference datasets as described in previous works (13, 14, 24). Thymus and spleen datasets were treated as query datasets and mapped separately to previously published datasets of porcine thymus (12) or human spleen (25), respectively, that were treated as reference datasets. For reference mapping to human data, pig single-cell data was humanized as previously described (14) prior to performing reference-based cell type prediction and mapping. A Seurat object of reference data for previously published porcine thymus was obtained by requesting data from the corresponding author (12). A Seurat object of reference data for previously published human spleen was obtained as outlined in the article's data availability statement (25). Canonical correlation analysis (CCA) reduction methods were used to identify prediction/mapping anchors.

2.4.5 Data merging of innate lymphoid cells from human and pig spleen

Cells annotated as cytotoxic ILCs or *NCR1+EOMES+* ILCs in our humanized spleen dataset and *CD160+* natural killer (NK) or *FCGR3A+* NK cells in the human spleen dataset (25) were extracted and merged into a single Seurat object. Cells were processed through data normalization, integration, PCA calculations,

dimensionality reduction, and hierarchical clustering as described in methods for initial data processing.

2.4.6 Cell-cell interaction network analysis

CellChat v2.1.2 (26) was used to identify cellular communication networks as previously described (13). The human CellChat database was used to identify ligand- and receptor-encoding genes after porcine data from our lymph node dataset was humanized as previously described (14). Only cell-cell interactions were used for analyses.

2.4.7 Three-dimensional tissue organization reconstruction

CSOmapR v1.0 (27) was applied to the humanized lymph node tissue dataset used for cell-cell interaction analysis above. The ligand-receptor pairs identified in the human CellChat database (available through the CSOmapR package) were used to identify orthologous ligand- and receptor-encoding genes that were applied to create a 3D reconstruction of lymph node cell organization based on receptor and ligand expression profiles.

2.5 Shiny app development

The Shiny R package for immune tissue cell data visualization is a collection of components that facilitates the interactive representation and viewing of R analysis results (28). The Shiny-PIGGI (<https://shinypiggi.ansci.iastate.edu>) is implemented completely in R, runs on any modern web browser, and requires no programming. Our main goal was to develop an interactive web application that allows users such as animal scientists and immunologists to visualize biological datasets. We utilized the R package Seurat and several other R packages (shiny, shiny dashboard, shiny themes, uwot, DT shinycssloaders, shinydisconnect, shiny alert, HTML tools, and HTML widgets) to design the user interactive interface. For differential expression, we used Wilcoxon rank sum test via the presto package (29). Users can select any two cell types across tissues for further tissue-based cell type comparisons. The results are displayed in a table and can be downloaded based on filtering within the app. For gene expression visualization, we used feature and violin plot to visualize expression of a query gene across four tissues. Next, Seurat based reference mapping was implemented, using bone marrow as a reference tissue, and the other three tissues serves as query datasets. For each predicted cell type, mapping scores, prediction scores, and predicted cell annotations were computed and visualized. Additionally, we provided a link to downloadable .cloupe and .h5seurat files converted from the Seurat objects and available through Ag Data Commons .cloupe files are compatible with interactive data query features available from Loupe Cell Browser (10X Genomics) to facilitate easy visualization/analyses outside the Shiny-PIGGI app. .h5seurat files maintain all features of Seurat objects used for data analysis herein and are compatible with upload into R environments using SeuratDisk but are also convertible to data object formats compatible with other computing environments via additional file conversions.

1 * Ensembl ID used for data analysis was replaced with corresponding gene symbol in results and figures. Gene symbols were not present in the genome annotation file version used for this analysis but were recoverable from gene descriptions of Ensembl IDs, updated gene symbols in future Ensembl version releases, or multiple Ensembl IDs that corresponded to a single gene symbol name.

2.6 *In situ* staining

Sections of lymph node tissues were cut to appropriate size, placed into a cassette, fixed in 10% neutral-buffered formalin (3.7% formaldehyde) for ~48 h RT, transferred to 70% ethanol, and embedded in paraffin blocks within a week of collection. Embedded tissues were cut into four-micron thick sections and adhered to Superfrost Plus charged microscope slides (Fisherbrand 12-550-15). RNA *in situ* hybridization to detect T receptor delta constant (*TRDC*) transcript was performed using a *Sus scrofa TRDC* probe (Advanced Cell Diagnostics 553141) and RNAscope 2.5 HD Detection Kit (Advanced Cell Diagnostics 322360) as previously described (30). Four regions of *TRDC* labeled lymph node with an emphasis on follicle inclusion were assessed at 20X magnification for presence of *TRDC* labelling in the follicle and extrafollicular regions. Immunohistochemistry to detect CD3ε protein was performed using a polyclonal rabbit anti-human CD3ε antibody (Dako A0452) as previously described (31). Serial sections were used for *TRDC* and CD3ε labeling of lymph node tissue.

3 Results

3.1 An immune atlas of lymphoid organs in pigs

Single-cell RNA sequencing (scRNA-seq) was performed using cell fractions isolated from primary lymphoid organs (bone marrow, thymus) and secondary lymphoid organs (spleen, ileocecal lymph node) of two ~6-month-old intact male Yorkshire pigs. Following quality control processing, final single-cell datasets included 5,899 bone marrow-derived cells (Figure 1A), 17,940 thymus-derived cells (Figure 1B), 20,210 lymph node-derived cells (Figure 1C), and 5,621 spleen-derived cells (Figure 1D), totaling overall at 49,670 cells that were further analyzed and annotated. Canonical gene expression patterns were used to annotate cell types (Figures 1E–H, 2A–C). Progenitor cells were identified in primary lymphoid organs by *KIT* expression (32) and lack of detected lineage commitment genes (Figures 1E, F), with the majority identified in bone marrow (Figure 1A). Lineage-committed and/or developmentally mature immune cells belonging to T/innate lymphoid cell (ILC), B/antibody-secreting cell (ASC), and myeloid lineages were also identified in all tissues by expression of lineage-specific/lineage-enriched genes (*CD3E*, *CD3G*, *CD3D*, *CD247*, *ZAP70* for T/ILC; *CD79B*, *CD19*, *PAX5*, *JCHAIN* for B/ASC; *AIF1*, *CST3* for myeloid (5, 14) (Figures 1E–H, Figures 2A–C). Stromal and epithelial cells were not identified, likely due to cell isolation methods catering to leukocyte rather than stromal/epithelial cell isolation (lack of enzymatic tissue digestion (33) and cryopreservation protocols (34)). Granulocytes were also not recovered, likely due to lack of cell isolation and data analysis methods that cater to their recovery (35). Overall, scRNA-seq analysis revealed both conserved and distinct transcriptional features of leukocytes derived from primary lymphoid organs (bone marrow, thymus) and secondary lymphoid organs (lymph node, spleen) of pigs.

3.1.1 Myeloid lineage leukocytes

The majority of all recovered myeloid lineage leukocytes were derived from bone marrow (Figure 1A), and smaller populations were also identified in thymus, spleen, and lymph node (Figures 1B–D). Canonical gene expression used to identify myeloid lineage cells is shown in Figure 2A and discussed below. Though pDCs are derived from lymphoid progenitors (36), these cells also share transcriptional and functional commonalities with myeloid cells and are included in Figure 2A.

AIF1 and *CST3* were used as myeloid lineage identifiers constitutively expressed across all pDC, cDC, and monocyte/macrophage (monocyte/MΦ) populations with the exception of a population of osteoclasts (lacking detectable *AIF1*) and cycling early myeloid cells (lacking detectable *AIF1* and *CST3* expression), both located in bone marrow. Osteoclasts notably expressed several highly specific and previously-reported genes, including *ATP6V0D2*, *NFATC1*, *MMP9*, *CALCR*, *SIGLEC15*, *ACP5*, *DCSTAMP*, *OCSTAMP*, *TNFRSF11A* (37–39). Strong expression of *KIT* indicated cycling early myeloid cells were recently committed to the myeloid lineage, likely associated with hematopoietic development in the bone marrow (32, 40, 41). Cycling early myeloid cells also had strong expression of cell cycle markers, such as *PCLAF*, *BIRC5*, *TOP2A*, *STMN1* (42, 43), indicating further that they were a cycling cell population.

cDCs were identified in all tissues by expression of *FLT3* and high relative expression of MHC-II-encoding genes (*SLA-DQB1*, *SLA-DRA*) (5, 14). The only cycling population of cDCs was identified in bone marrow and expressed cell cycle genes such as *PCLAF*, *BIRC5*, *TOP2A*, *STMN1*. cDC populations likely contained a combination of subsets that were not further annotated due to limitations in clustering granularity associated with low recovered cell numbers. However, cDCs did express genes that were indicative of cDC1s (expressing *XCRI*, *BATF3*, *RAB7B*, *ANPEP*), cDC2s (expressing *FCER1A*, *CD1.1*, *CD207*, *SIRPA*), DC3s (transcriptional intermediates of cDC2s and monocytes) and transitional DCs (tDCs; transcriptional intermediates of cDC2s and pDCs) that have been recently reported as circulating cDC populations in pigs (44). In thymus and lymph node, both cDC and monocyte/MΦ populations were small and therefore combined into a single annotation, also due to limitations of clustering granularity associated with low cell number recovery.

Monocyte/MΦ populations had strong expression of *LYZ* and mixed expression of *FCGR3A**, *CD163*, *CD14*, *CD68*, *NLRP3*, *TLR4*, *CSF1R*, *CCR2*. The majority of monocyte/MΦ populations were identified in bone marrow, where all populations constitutively expressed *FCGR3A** (encoding CD16) but had variable detected expression of *CD163* and *CD14* that was used to discriminate populations further. Use of *CD163* as a marker for discrimination of bone marrow monocyte/MΦ populations is similar to previous work, where *CD163* expression was used as a discriminate between resident and non-resident bone marrow MΦs (45). Expression of cell cycle genes (*PCLAF*, *BIRC5*, *TOP2A*, *STMN1*) was used to further identify cycling *CD163*+ and *CD163*- bone marrow monocyte/MΦ cells.

pDCs were identified by expression of *TCF4*, *IRF8*, *CD93*, *CLEC12A*, *XBP1*, *CD4*, *CD8B* (5, 14) in only thymus and bone

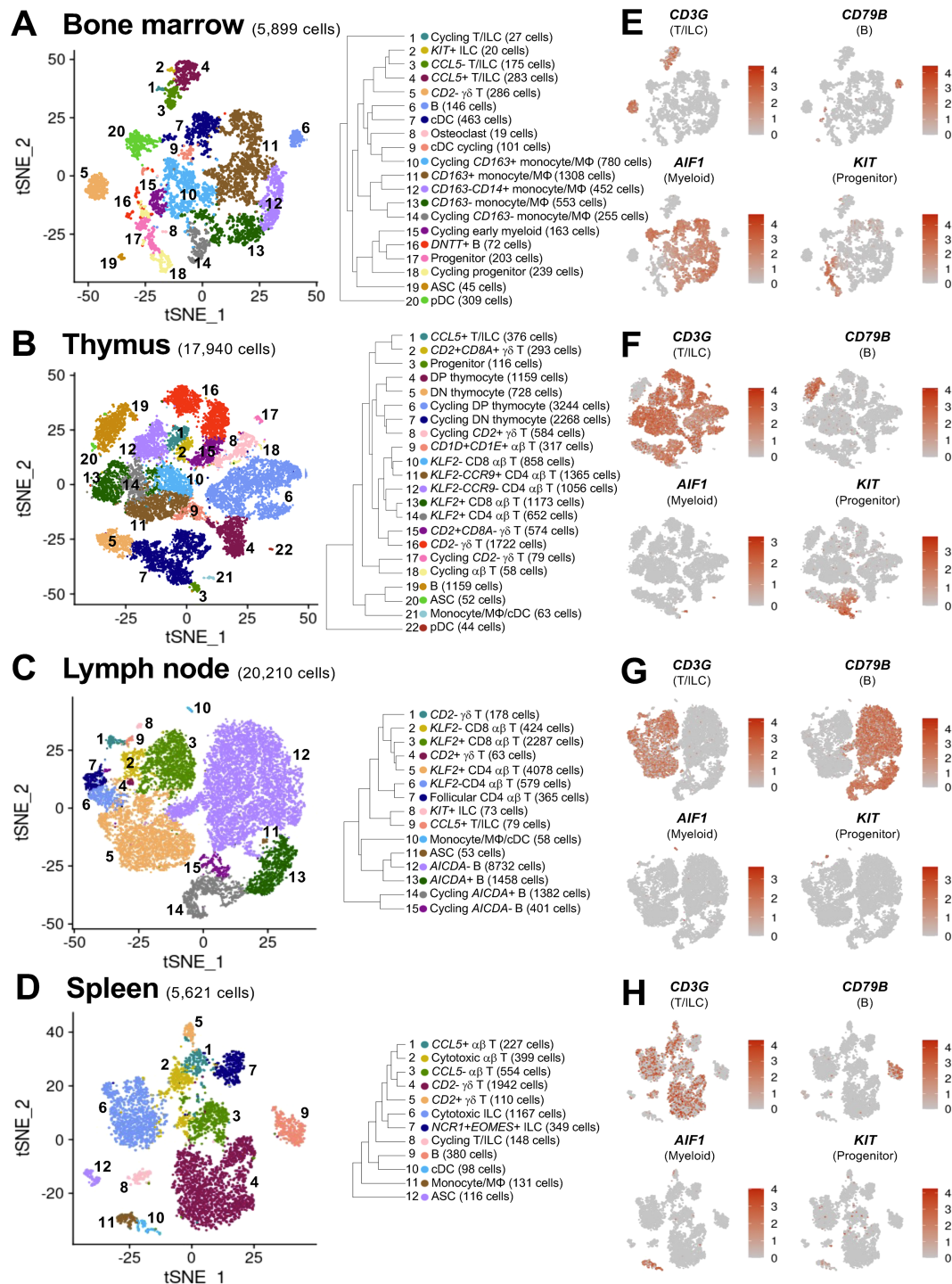


FIGURE 1

Cell types identified in porcine primary and secondary lymphoid tissues via scRNA-seq. (A–D) t-SNE plots of cell types identified in scRNA-seq datasets of porcine bone marrow (A), thymus (B), lymph node (C), and spleen (D). Each individual point represents one cell, and the color of each point corresponds to annotated cell identity. On the right of each t-SNE plot is a hierarchical tree indicating relatedness of annotated cell types in each dataset. (E–H) t-SNE plots of expression levels for selected canonical genes in scRNA-seq datasets of porcine bone marrow (A), thymus (B), lymph node (C), and spleen (D). Each individual point represents one cell, and the color of each point corresponds to relative expression level of the indicated gene. ASC (antibody-secreting cell); cDC (conventional dendritic cell); DN (double negative); DP (double positive); ILC (innate lymphoid cell); M Φ (macrophage); pDC (plasmacytoid dendritic cell); scRNA-seq (single-cell RNA sequencing); t-SNE (t-distributed stochastic neighbor embedding).

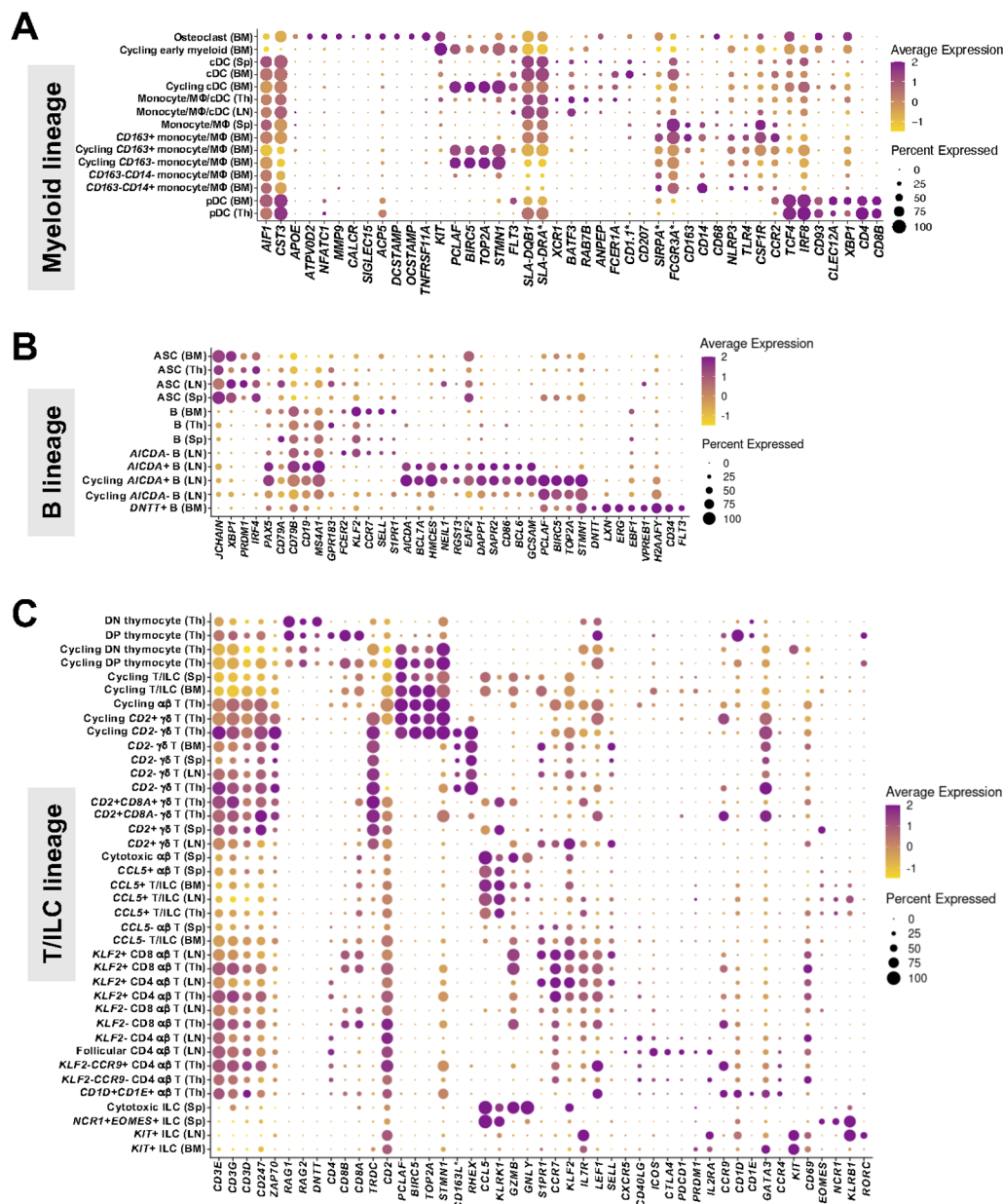


FIGURE 2

Canonical gene expression in myeloid lineage, B lineage, and T/ILC lineage cells recovered from porcine primary and secondary lymphoid tissues via scRNA-seq. (A–C) Dot plots of canonical gene expression (x-axes) in annotated cell types (y-axes) of myeloid lineage (A), B lineage (B), and T/ILC lineage (C) cells recovered from porcine bone marrow, thymus, lymph node, or spleen (cells shown in Figures 1A–D). Dot size indicates the percentage of an annotated cell type expressing a gene. Dot color indicates average gene expression level for those cells expressing a gene within an annotated cell type relative to all other cells in the same lineage. ASC (antibody-secreting cell); BM (bone marrow); cDC (conventional dendritic cell); DN (double negative); DP (double positive); ILC (innate lymphoid cell); LN (lymph node); MΦ (macrophage); pDC (plasmacytoid dendritic cell); scRNA-seq (single-cell RNA sequencing); Sp (spleen); Th (thymus).

marrow. Similar to monocyte/MΦ and cDCs, pDCs also expressed *AIF1* and *CST3*.

3.1.2 B cells and antibody-secreting cells

ASCs expressing *JCHAIN*, *XBP1*, *PRDM1*, *IRF4* and B cells expressing *PAX5*, *CD79A*, *CD79B*, *CD19*, *MS4A1* (5, 14) were recovered in all tissues (Figures 1A–D), with the largest and most diverse B cell populations being found in lymph node (Figure 1C).

Canonical gene expression used to identify B/ASC lineage cells is shown in Figure 2B and further discussed below.

Within lymph node, the majority of B cells expressed genes indicative of a resting state, including *GPR183*, *FCER2*, *KLF2*, and circulatory genes *CCR7*, *SELL*, *S1PR1* (13, 14). These cells also lacked detectable expression of *AICDA*, which encodes for the activation-induced cytidine deaminase enzyme that introduces mutations for B cell somatic hypermutation and class switching, along with other activation-associated genes. Activation-associated

genes (*AICDA*, *BCL7A*, *HMCES*, *NEIL1*, *RGS13*, *EAF2*, *DAPPI*, *S1PR2*, *CD86*) were indicative of somatic hypermutation, class switching, localization to germinal centers, and apoptosis (13, 46–50) and occurred in lymph node *AICDA*+ B cell populations. Expression of germinal center-associated genes (*BCL6*, *GCSAM*) (13, 51, 52) further suggested *AICDA*+ cells likely originated from active germinal centers. Cycling *AICDA*+ B cells were further annotated by expression of cell cycle genes (*PCLAF*, *BIRC5*, *TOP2A*, *STMN1*) that indicated probable locations within germinal center light and dark zones associated with stages of B cell activation and cycling (13). An additional population of cycling *AICDA*- B cells lacking detectable expression of many activation- and germinal center-associated genes was also identified in lymph node.

In bone marrow, a unique population of pro-/pre-B cells was also recovered, termed *DNTT*+ B cells. *DNTT*+ B cells expressed genes associated with B cell lineage commitment and development (*DNTT*, *LXN*, *ERG*, *EBF1*, *VPREB1*, *H2AFY*, *CD34*, *FLT3*), indicating this was a population of recently committed B lineage cells undergoing development in the bone marrow, the primary site of B cell development (46, 53–56).

Remaining B cell populations in bone marrow, thymus, and spleen lacked detectable expression of genes indicating B cell activation, germinal center localization, or development but did express genes in common with lymph node *AICDA*- B cells presumed to be in a resting state (*GPR183*, *CCR7*, *KLF2*, *SELL*, *FCER2*, *S1PR1*). High activation marker expression and prevalence of *AICDA*+ B cells in lymph nodes from healthy animals may be attributed to persistent microbial stimulation experienced in gut-draining lymph nodes (57), which was expected to be present in the animals utilized in this work.

ASCs were also identified across all four tissues, and ASCs in bone marrow and spleen had higher expression of cell cycle genes (*PCLAF*, *BIRC5*, *TOP2A*, *STMN1*), suggesting a higher occurrence of plasmablasts.

3.1.3 Thymocytes, T cells, and innate lymphoid cells

T cells expressed pan-T cell marker *CD3E* and other T cell receptor (TCR)-related genes (*CD3D*, *CD3G*, *CD247*, *ZAP70*), while ILCs were transcriptionally similar to T cells but lacked detectable expression of *CD3E* (5, 14). T cells and ILCs were identified in all analyzed tissues (Figures 1A–D). Canonical gene expression used to identify T/ILC lineage cells is shown in Figure 2C and discussed below.

Thymocytes were unsurprisingly unique to the thymus, the primary site of T cell development. All thymocyte populations expressed *RAG1*, *RAG2*, and *DNTT*, indicative of TCR rearrangement occurring during thymic T cell development (12, 58). Double-positive (DP) thymocytes expressed *CD4*, *CD8A*, and *CD8B*, indicative of both CD4 and CD8 co-receptor expression, while double-negative (DN) thymocytes lacked such detectable expression (12, 58). Variable detected expression of *TRDC*, encoding for a portion of the TCR expressed by $\gamma\delta$ T cells, was also noted in both DN and DP thymocyte populations. DP and DN

thymocytes were further divided into cycling and non-cycling populations by detected expression of cell cycle genes (*PCLAF*, *BIRC5*, *TOP2A*, *STMN1*), leaving a total of four thymocyte populations identified.

Mature $\alpha\beta$ T cells, $\gamma\delta$ T cells, and ILCs were found in all tissues and lacked detectable expression of genes associated with TCR rearrangement (*RAG1*, *RAG2*, *DNTT*). Clusters of cycling T cells and/or ILCs were identified in all samples except lymph node by expression of genes associated with cellular replication and division (*PCLAF*, *BIRC5*, *TOP2A*, *STMN1*). In bone marrow and spleen, cycling T cells and ILCs were grouped into single populations, while in thymus, where the largest number of total T/ILC populations were defined, cycling T cells were divided into $\alpha\beta$ T cells and two subsets of cycling $\gamma\delta$ T cells discussed subsequently.

$\gamma\delta$ T cells (*TRDC*+) were separated into subsets based on detected *CD2* expression, which is often used as a phenotypic marker to classify porcine $\gamma\delta$ T cells in previous scRNA-seq work (5, 14). *CD2*- $\gamma\delta$ T cells were found in every tissue, and five total *CD2*- $\gamma\delta$ T cell populations were annotated, including a population of cycling *CD2*- $\gamma\delta$ T cells in thymus and populations of non-cycling *CD2*- $\gamma\delta$ T cells in each of thymus, bone marrow, lymph node, and spleen. Similar to previous works (5, 14), porcine *CD2*- $\gamma\delta$ T cells had strong expression of genes *CD163L** and *RHEX* compared to other T cells across all tissues, suggesting these genes as a conserved expression signature for *CD2*- $\gamma\delta$ T cells throughout anatomical locations of the pig.

Five populations of *CD2*+ $\gamma\delta$ T cells were also identified, including three populations in thymus and single populations in lymph node and spleen. In thymus, two populations were comprised of non-cycling *CD2*+ $\gamma\delta$ T cells that were further divided into *CD8A*+ and *CD8A*- populations based on detected *CD8A* expression, corresponding to conventional porcine $\gamma\delta$ T cell classifications also used in previous porcine-specific scRNA-seq work (5). Thymic *CD2*+*CD8A*+ $\gamma\delta$ T cells had increased expression of cell activation and/or effector-associated genes previously reported in PBMCs (5), including *KLRK1*, *CCL5*, and *GZMB*. Cycling *CD2*+ $\gamma\delta$ T cells in thymus were also *CD8A*- and lacked expression of *KLRK1*, *CCL5*, and *GZMB*. However, we emphasize caution in inferring thymic $\gamma\delta$ T cell effector functions due to the potential for recovery of relatively immature $\gamma\delta$ T cells in the thymus, where T cell development is occurring. *CD2*+ $\gamma\delta$ T cell populations found outside of the thymus included splenic *CD2*+ $\gamma\delta$ T cells that had strong expression of activation/effector genes *KLRK1*, *CCL5*, and *GZMB* and lymph node *CD2*+ $\gamma\delta$ T cells that lacked such detectable expression. Of note, very few $\gamma\delta$ T cells were detected in the lymph node overall, with less than 1.5% of the total lymph node cells identified as $\gamma\delta$ T cells.

CCL5, an inferred marker of effector cell status (14, 59), was used to further discriminate populations of non-cycling $\alpha\beta$ T cells (lacking detectable *TRDC* expression) and ILCs (lacking detectable *CD3E* and other TCR-related gene expression) in all tissues as *CCL5*+ or *CCL5*- populations. In bone marrow, $\alpha\beta$ T cells and ILCs were included in the same populations due to the low number of total $\alpha\beta$ T cells and ILCs identified and included clusters of *CCL5*+ T/ILCs and *CCL5*- T/ILCs in addition to the cycling T/ILC cluster and $\gamma\delta$ T cell clusters mentioned above. In spleen, two populations of non-cycling $\alpha\beta$ T

cells were *CCL5*⁺ and further divided by expression (cytotoxic $\alpha\beta$ T cells) or absence in detected expression (*CCL5*⁺ $\alpha\beta$ T cells) of cytotoxicity-related genes (*GZMB*, *GNLY*). A third non-cycling $\alpha\beta$ T cell population without *CCL5* expression detected was also identified in spleen, termed *CCL5*⁻ $\alpha\beta$ T cells. Compared to *CCL5*⁺ counterparts (inferred to be effector cells) in spleen or bone marrow, *CCL5*⁻ $\alpha\beta$ T cells in spleen and *CCL5*⁻ T/ILCs in bone marrow each had higher expression of genes associated with migratory circulation patterns characteristic of naïve and central memory T cells, including expression of lymph node homing gene, *CCR7*, and lymph node egress gene, *S1PR1* (5, 14). CD4 and CD8 $\alpha\beta$ T cell subsets were not differentiated from one another in bone marrow due to the low number of total $\alpha\beta$ T cells identified, and detectable gene expression of *CD4*, *CD8A*, and *CD8B* expression was too sparse in the spleen dataset to accurately define the two $\alpha\beta$ T cell subsets.

Though the majority of $\alpha\beta$ T cells and ILCs were *CCL5*⁻ in thymus and lymph node, small populations of combined T cells and ILCs that were *CCL5*⁺ were identified, termed *CCL5*⁺ T/ILCs in each of thymus and lymph node. Remaining non-cycling *CCL5*⁻ $\alpha\beta$ T cell populations in thymus and lymph node were also segregated into CD4 (expressing detectable *CD4*) and CD8 (expressing detectable *CD8A* and *CD8B*) $\alpha\beta$ T cell subsets. Within CD4 and CD8 $\alpha\beta$ T cell subsets, further differentiation was performed based on expression of the migratory regulator gene, *KLF2*, which has decreased expression in activated lymphocytes (60). In both thymus and lymph node, *KLF2*⁺ CD4 and CD8 $\alpha\beta$ T cell populations were presumed to be able to egress from tissues and circulate, often in conjunction with markers characteristic of naïve or central memory T cells, including *IL7R* and *CCR7* in both tissues, as well as *SELL*, *S1PR1*, and *LEF1* in lymph node (5, 14). In total, a population of *KLF2*⁺ CD4 $\alpha\beta$ T cells and *KLF2*⁺ CD8 $\alpha\beta$ T cells was identified in each of thymus and lymph node, totaling four *KLF2*⁺ $\alpha\beta$ T cell populations identified. A total of seven *KLF2*⁻ $\alpha\beta$ T cell populations were identified in thymus and lymph node. In lymph node, a population of *KLF2*⁻ CD8 $\alpha\beta$ T cells was identified along with two populations of CD4 $\alpha\beta$ T cells lacking detectable *KLF2* expression, termed *KLF2*⁻ CD4 $\alpha\beta$ T cells and follicular CD4 $\alpha\beta$ T cells. Though both *KLF2*⁻ CD4 $\alpha\beta$ T cells and follicular CD4 $\alpha\beta$ T cells in lymph node expressed genes such as *CXCR5* and *CD40LG* that may be associated with a follicular location, follicular CD4 $\alpha\beta$ T cells had higher expression of additional genes indicating follicular T cell signaling, including *PDCD1*, *CTLA4*, *ICOS*, *PRDM1*, and *IL2RA* (14). In thymus, *KLF2*⁻ $\alpha\beta$ T cells included a population of *KLF2*⁻ CD8 $\alpha\beta$ T cells, two populations of *KLF2*⁻ CD4 $\alpha\beta$ T cells further divided by *CCR9* expression, and a population of $\alpha\beta$ T cells with high expression of *CD1D* and *CD1E*. Detectable expression of *CCR9* on *KLF2*⁻ $\alpha\beta$ T cells in thymus suggested a role in thymocyte development or gut homing potential (12, 61, 62). The final population of *KLF2*⁻ $\alpha\beta$ T cells in thymus was termed *CD1D*⁺*CD1E*⁺ $\alpha\beta$ T cells, but such cells could not be easily discerned as CD4 or CD8 $\alpha\beta$ T cells. These cells also had high expression of genes such as *CD1D*, *CD1E*, *CCR4*, *GATA3* that were not typically expressed at high levels by other $\alpha\beta$ T cell populations.

Similar to $\alpha\beta$ T cells, populations comprised solely of ILCs could be divided by *CCL5* expression. Two *CCL5*⁺ ILC populations were identified in spleen, termed cytotoxic ILCs and *NCR1*⁺*EOMES*⁺

ILCs. Cytotoxic ILCs in spleen expressed cytotoxicity-related genes *GZMB* and *GNLY*. Conversely, *NCR1*⁺*EOMES*⁺ ILCs in spleen lacked detectable expression of cytotoxicity genes and instead had strong expression of genes characteristic of porcine natural killer (NK) cells (5, 14), including *NCR1*, *EOMES*, and *KLRB1*. Lastly, two populations of *CCL5*⁻ ILCs, identified in each of bone marrow and lymph node, had high expression of *KIT*, *IL7R*, and *CD69* and were termed *KIT*⁺ ILCs. *KIT*⁺ ILCs in lymph node also expressed *RORC* and *KLRB1*, suggesting a population of group 3 ILCs similar to those identified in porcine intestine that also have similar expression patterns and represent a population of putative lymphoid tissue inducer (LTi) cells (13, 14).

3.2 Two independent porcine thymus scRNA-seq datasets yield similar cell type identities

One recent publication (12) defines porcine thymic cell populations with a high level of detail in defining immune cell populations. We compared the manual annotation of our thymic dataset to that of previous work (12) to establish a general consensus of cell types annotated in porcine thymus across different published datasets. Comparisons were performed using reference-based mapping and cell label predictions (see methods), where the previously published work was treated as a reference dataset (Figure 3A), and our thymic dataset was treated as a query dataset (Figure 3B) projected onto the reference.

Mapping scores were recovered as a metric for each query cell (Figure 3C) and indicated the degree of representation each query cell had in the reference dataset, with higher mapping scores indicating better representation. Predicted reference dataset identities for each cell of the query data were also calculated (Figure 3D) by identifying the reference identity with the highest prediction score (Figure 3E) for each query cell. Results indicated a general consensus of annotation cell groupings between reference and query datasets with some exceptions (Figure 3F). General annotation consensus was found across datasets for thymocytes, $\gamma\delta$ T cells, mature $\alpha\beta$ T cell populations, and B lineage cells. Largest discrepancies arose for query populations of progenitor cells, monocyte/M Φ /cDCs, and pDCs, as similarly annotated populations were not identified in the reference data. Query progenitor cells were most closely predicted as DN thymocytes, though with low mapping scores indicating a similar cell type was not represented in the reference data. Monocyte/M Φ /cDCs and pDCs in the query dataset were predicted as most similar to reference B cells, though mapping scores were particularly low for pDCs, again indicating lack of a similar cell type in the reference data. Another interesting finding was *CD1D*⁺*CD1E*⁺ $\alpha\beta$ T and *KLF2*⁻*CCR9*⁺ CD4 $\alpha\beta$ T cells in the query data being predicted as T entry cells in the reference, which were documented as a T cell population with high *CCR9* and low *CCR7* expression that occur early in the T cell lineage commitment pathway (12). High mapping scores were also recovered for the two populations highly predicted as T entry cells, indicating representation of very similar cell populations across both datasets. An additional population of *KLF2*⁻*CCR9*⁻ CD4 $\alpha\beta$ T cells in the query dataset were predicted

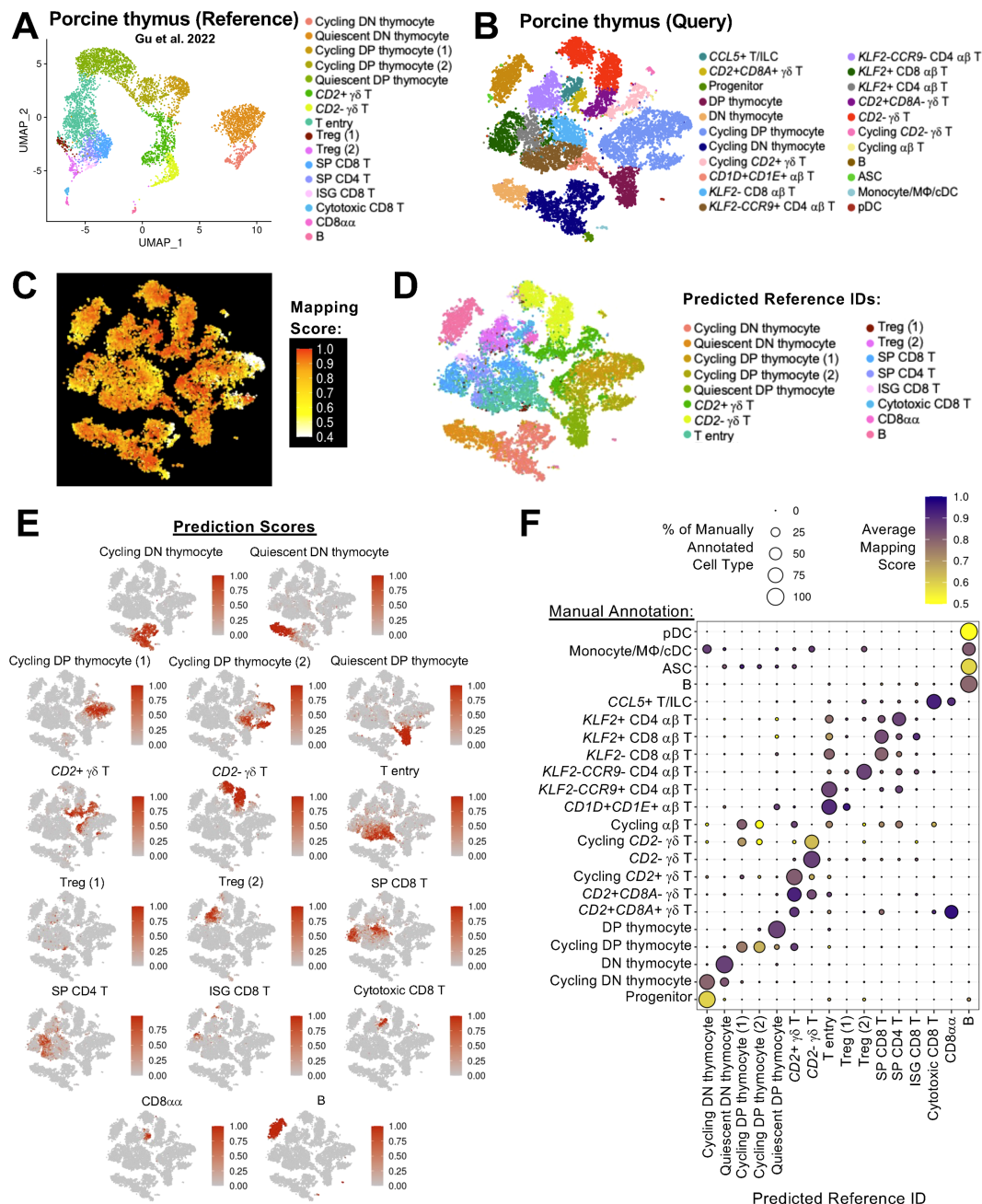


FIGURE 3

Annotation of porcine thymic cells across scRNA-seq datasets. (A, B) UMAP plot of cell types identified in a previously published dataset of porcine thymus, treated as a reference dataset (A), and t-SNE plot of cell types identified in porcine thymus, treated as a query dataset (B). Each individual point represents one cell, and the color of each point corresponds to annotated cell identity. (C) t-SNE plot of mapping scores (range 0 to 1) obtained from projecting query data in (B) onto the reference dataset in (A). Each individual point represents one cell of the query dataset, and the color of each point corresponds to mapping score, where a higher mapping score indicates better representation of a query cell in the reference dataset. (D) t-SNE plot of predicted reference cell identity obtained from projecting query data in (B) onto the reference dataset in (A). Predicted annotation was assigned as the reference cell type with the highest prediction score. Each individual point represents one cell of the query dataset, and the color of each point corresponds to the predicted reference annotation. (E) t-SNE plots of prediction scores (range 0 to 1) obtained from projecting query data in (B) onto the reference dataset in (A). Each individual point represents one cell of the query dataset, and the color of each point corresponds to the prediction score for cell type annotations found in the reference dataset. (F) Dot plot showing predicted reference cell type (x-axis) for cell types of query data (y-axis). Predicted annotation was assigned as the reference cell type with the highest prediction score. Dot size indicates the percentage of a query cell type (y-axis) predicted to be a reference cell type (x-axis). Dot color indicates average mapping score for those cells predicted as a reference cell type within an annotated query cell type. A higher mapping score indicates better representation of query cells in the reference dataset. ASC (antibody-secreting cell); cDC (conventional dendritic cell); DN (double negative); DP (double positive); ILC (innate lymphoid cell); ISG (interferon-stimulated gene); M Φ (macrophage); pDC (plasmacytoid dendritic cell); scRNA-seq (single-cell RNA sequencing); SP (single positive); t-SNE (t-distributed stochastic neighbor embedding); Treg (T regulatory); UMAP (uniform manifold approximation and projection).

as Treg cells in the reference, also with high mapping scores that indicated similar representations across datasets. Results thus indicated a general consensus for most cell types similarly annotated across the two datasets, though populations such as progenitors, monocyte/MΦ/cDCs, and pDCs may be unique to our newly-established thymus scRNA-seq dataset, which contains a greater number of cells than previously-published work. Comparison to the previously-published thymus data also suggested populations of T entry cells were differentiated into two distinct populations in our work, including *CD1D+CD1E+* αβ T and *KLF2-CCR9+* CD4 αβ T cells, and *KLF2-CCR9-* CD4 αβ T cells are a probable Treg cell population.

3.3 Two populations of splenic innate lymphoid cells are transcriptionally similar in pigs and humans

Reference-based mapping and prediction is also a useful tool for comparative immunology, where cell types can be compared across single-cell datasets of different species, including between pigs and humans (5, 14, 18, 63, 64). To perform comparative analysis of splenic cells from pigs and humans, our spleen dataset was mapped to a previously-published scRNA-seq dataset of human spleen (25). Comparisons were performed using reference-based mapping and cell label prediction (see methods), where the previously published work was treated as a reference dataset (Figure 4A), and our splenic dataset was treated as a query dataset (Figure 4B) projected onto the reference. Mapping scores were generally high for cell types annotated in our pig query data (Figure 4C), indicating pig cells had transcriptionally similar human counterparts for most splenic cell types, including ILCs. ILCs are not as well-studied in pigs as other species such as humans or mice (14, 65–68), and we characterized two newly-defined splenic populations of ILCs in porcine spleen (Figures 1D, 2C), including splenic cytotoxic ILCs and *NCRI+EOMES+* ILCs. Prediction of porcine splenic ILCs to human splenic cell annotations indicated porcine cytotoxic ILCs were primarily predicted as human *FCGR3A+* NK cells, and porcine *NCRI+EOMES+* ILCs were primarily predicted as *CD160+* NK cells (Figures 4D, E), with high mapping scores indicating representation of highly similar ILCs and NK cells across datasets (Figure 4F). In a new dataset, porcine and human ILC/NK cells were subsetted and integrated, revealing hierarchical clustering of annotated cells resulted in species intermixing, with one node containing porcine cytotoxic ILCs and human *FCGR3A+* NK cells that were most closely related to one another, while the other node included porcine *NCRI+EOMES+* ILCs and human *CD160+* NK cells that were most closely related to one another (Figure 4G). Visualization with dimensionality reduction supported these relationships, again indicating pig-to-human similarities of porcine cytotoxic ILCs to human *FCGR3A+* NK cells and porcine *NCRI+EOMES+* ILCs to human *CD160+* NK cells (Figure 4H). Collectively, comparison of pig and human ILC/NK cell populations revealed conservation across species, with two primary populations identified in spleen belonging to the ILC lineage that encompasses both ILC subsets and NK cells (69, 70). Porcine splenic ILCs did not meet the traditional classification of porcine NK cells due to lack of specific markers

such as *CD8A* but did have gene expression best represented by the group 1 ILC lineage, reminiscent of previous work in porcine ileum (14). Specific classification of species-conserved populations of ILCs remains complicated due to tissue-specific features, as well as transcriptional and functional overlapping of subsets, especially between ILC1s and NK cells (69–71). However, cross-species comparisons reveal high levels of transcriptional conservation for splenic ILCs in pigs and humans regardless of ILC1 versus NK classification.

3.4 *KLF2*- T cells and ILCs but not γδ T cells are likely key contributors to conventional germinal center immune inductive processes in porcine lymph nodes

Lymph nodes are critical tissue sites for immune induction where immune responses are dependent on the intricate organization of follicles and surrounding interfollicular zones that foster cellular interactions required for immune processes. To better understand cellular organization in the porcine lymph node, communication networks were calculated for annotated cell types of the lymph node scRNA-seq dataset (20,210 cells) and used to construct an inferred organization of cells based on expression of genes encoding ligands and receptors of known signaling pathways, where cells with complementary ligand-receptor genes were inferred to be located in closer proximity to one another. Based on reconstructed cellular organization patterns, significant inferred interactions (indicating close proximity and complementary receptor-ligand gene expression) and non-interactions (indicating more distant proximity and lack of complementary receptor-ligand gene expression) were found for all cell type combinations (Figure 5A). The inferred structure resembled a sphere, with cell type prevalence observed to vary based on distance from the structure's center. Hence, distances of each cell type from the center of the inferred structure were calculated to understand cell type distributions (Figure 5B). The structure was reminiscent of some features of cellular distributions in a lymph node follicle, where cell density was highest at the structure center that contained the largest numbers of B cells (though distinct germinal center light zones and dark zones were absent), and distributions of cell distances from the center were differently skewed across T/ILC subsets (Figure 5C). Loss of *KLF2* expression is associated with T cell activation, tissue retention, and lymph node follicle entry (60), and lymph node T cell and ILC populations with low/no detectable expression of *KLF2* (*KLF2*- CD8 αβ T, *KLF2*- CD4 αβ T, follicular CD4 αβ T, *KIT+* ILC, *CCL5+* T/ILC; Figure 2C) generally had increased numbers of significant interactions with other cell types (Figure 5A) and had locations skewed closer to the structure center (Figures 5B, C), suggesting organizational transitions reminiscent of a follicle (though clear demarcations of T cell and B cell zones were lacking) and important roles of *KLF2*- T/ILC populations in germinal center signaling interactions. Several of the strongest recovered cell-cell signaling pathways (CD45, MHC-II, CD40, CD86, CD80, PDL2, PD-L1) are involved with germinal center immune processes (72–76) (Figure 5D). Monocyte/MΦ/cDCs alongside subsets of CD4 αβ T cells and B cells were identified as

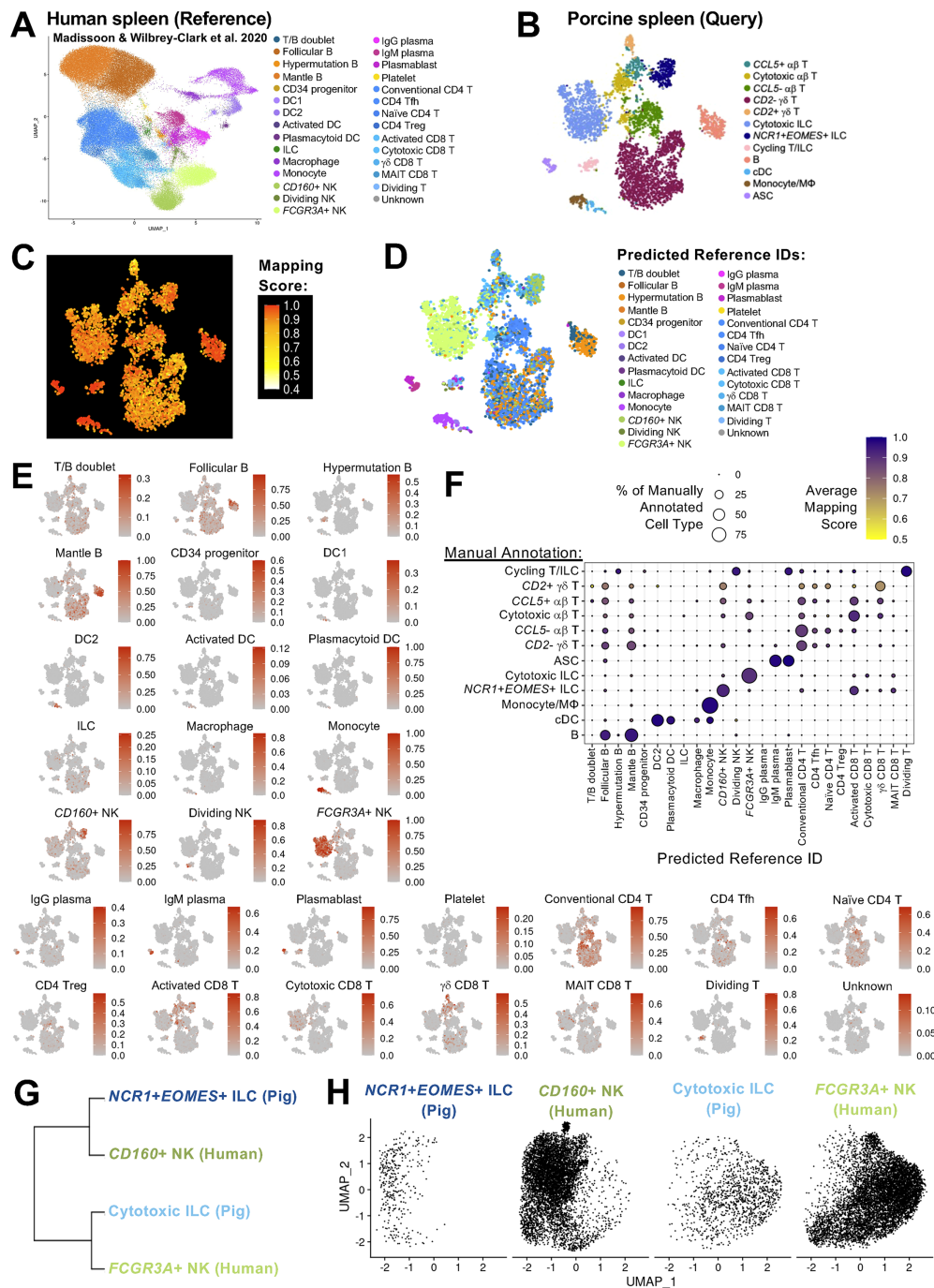


FIGURE 4

Porcine splenic ILCs share transcriptional similarities with NK cells in human spleen. (A, B) UMAP plot of cell types identified in a previously published scRNA-seq dataset of human spleen, treated as a reference dataset (A), and t-SNE plot of cell types identified in porcine spleen of this work, treated as a query dataset (B). Each individual point represents one cell, and the color of each point corresponds to annotated cell identity. (C) t-SNE plot of mapping scores (range 0 to 1) obtained from projecting query data in (B) onto the reference dataset in (A). Each individual point represents one cell of the query dataset, and the color of each point corresponds to mapping score, where a higher mapping score indicates better representation of a query cell in the reference dataset. (D) t-SNE plot of predicted reference cell identity obtained from projecting query data in (B) onto the reference dataset in (A). Predicted annotation was assigned as the reference cell type with the highest prediction score. Each individual point represents one cell of the query dataset, and the color of each point corresponds to the predicted reference annotation. (E) t-SNE plots of prediction scores (range 0 to 1) obtained from projecting query data in (B) onto the reference dataset in (A). Each individual point represents one cell of the query dataset, and the color of each point corresponds to the prediction score for cell type annotations found in the reference dataset. (F) Dot plot showing predicted reference cell type (x-axis) for cell types of query data (y-axis). Predicted annotation was assigned as the reference cell type with the highest prediction score. Dot size indicates the percentage of a query cell type (y-axis) predicted to be a reference cell type (x-axis). Dot color indicates average mapping score for those cells predicted as a reference cell type within an annotated query cell type. A higher mapping score indicates better representation of query cells in the reference dataset. (G) Hierarchical clustering of indicated porcine ILC and human NK cell types when established as a merged dataset. (H) UMAP plot of indicated porcine ILC and human NK cell types when established as a merged dataset. Cells for each cell type are shown in independent panels where each point represents one cell. ASC (antibody-secreting cell); cDC (conventional dendritic cell); DC (dendritic cell); DN (double negative); DP (double positive); ILC (innate lymphoid cell); MΦ (macrophage); MAIT (mucosal-associated invariant T); NK (natural killer); scRNA-seq (single-cell RNA sequencing); t-SNE (t-distributed stochastic neighbor embedding); Tfh (T follicular helper); Treg (T regulatory); UMAP (uniform manifold approximation and projection).

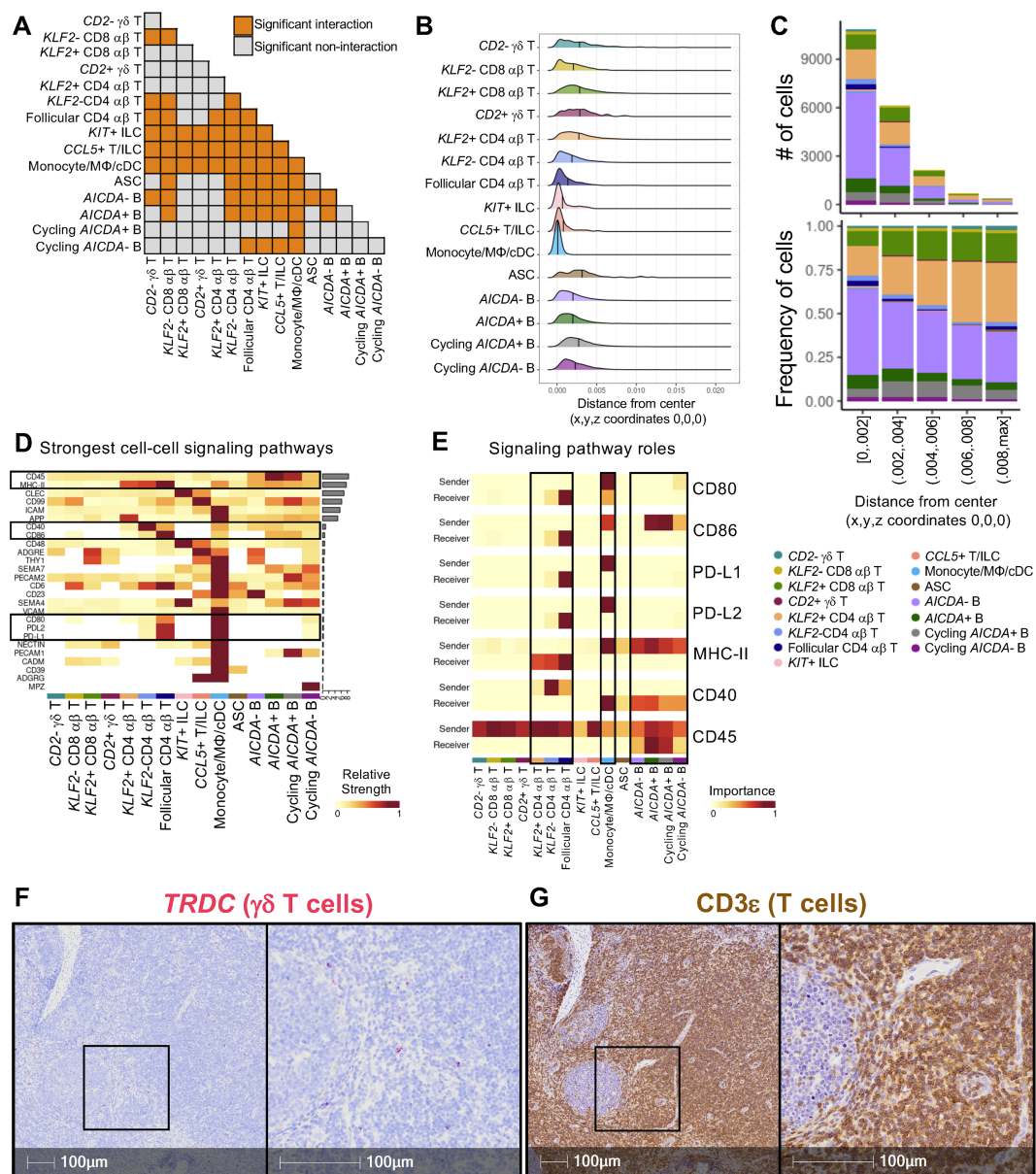


FIGURE 5

Inferred reconstruction of lymph node signaling and organization suggests porcine lymph nodes undertake conventional germinal center immune induction signaling processes with minimal contributions from $\gamma\delta$ T cells. (A) Table of inferred significant interaction (orange) or non-interaction (grey) between lymph node cell types. Significant interaction or non-interaction were inferred based on significant high or low proximity, respectively, of cells in a three-dimensional structure inferred based on ligand-receptor gene expression across cells. No non-significant interactions were inferred for any combination of annotated cell types. (B) Ridge plot showing the distribution of cell distances from the central coordinates (0,0,0) of the inferred three-dimensional structure. Distributions are plotted for each individual cell type, and mean distance from central coordinates is noted with a vertical line. (C) Stacked bar plots showing the number of cells (top) or frequency of cells (bottom) (y-axes) occurring at different ranges of distance from the central 0,0,0 coordinates of the structure (x-axes). Bar color corresponds to an annotated cell type. (D) Heatmap of the strongest cell-cell signaling pathways (y-axis) inferred amongst cell types (x-axis) in porcine lymph node scRNA-seq data. Fill color corresponds to the relative strength a corresponding cell type has in a corresponding signaling pathway. To the right of the heatmap, bar plots indicate the overall strength of each signaling pathway. (E) Heatmaps indicating roles of annotated lymph node cell types (x-axes) as senders (expressing ligand-encoding genes) or receivers (expressing receptor-encoding genes) of signals (y-axes) for a subset of signaling pathways taken from (D). Fill color indicated the importance of a corresponding cell type in acting as a sender or receiver for an indicated pathway. (F, G) Microscopy images of TRDC RNA staining (red) to indicate presence of $\gamma\delta$ T cells (F) and CD3 ϵ protein staining (brown) to indicate presence of total T cells (G) in a section of lymph node also taken and processed for scRNA-seq. ASC (antibody-secreting cell); cDC (conventional dendritic cell); ILC (innate lymphoid cell); M Φ (macrophage).

particularly important senders (expressing ligand) or receivers (expressing receptor) of signaling pathways associated with germinal center processes (Figure 5E), though annotating all myeloid lineage cells into a single cell population due to limitations of their low recovery likely generalized interaction

patterns that may be unique to more specific subsets of monocytes, M Φ s, or cDCs that cannot be appreciated in this analysis. Regardless, general cell groupings of myeloid lineage cells, CD4 $\alpha\beta$ T cells, and B cells are traditionally defined to interact via ligand-receptor bindings that activate an antigen-

specific adaptive immune response in lymph node germinal centers (72–76).

Within CD4 $\alpha\beta$ T cell subsets, follicular CD4 $\alpha\beta$ T cells were generally the most important recipients of signals from several pathways, including CD80, CD86, PD-L1, PD-L2, and MHC-II signaling followed by *KLF2*- CD4 $\alpha\beta$ T cells, while *KLF2*+ CD4 $\alpha\beta$ T cells had little to no role as receivers for most pathway signals (Figure 5E). Greater importance of follicular CD4 $\alpha\beta$ T cells, followed by *KLF2*- CD4 $\alpha\beta$ T cells, in several germinal center signaling processes (Figure 5E) coincided with increased numbers of significant interactions with other cell types (Figure 5A) and increased association with the structure center where other known follicle-associated cell types were located (Figures 5B, C) compared to *KLF2*+ CD4 $\alpha\beta$ T cells that had more significant non-interactions (Figure 5A) and were more often identified further from the structure center (Figures 5B, C). Results suggest cells annotated as follicular CD4 $\alpha\beta$ T cells are located in close proximity to other known follicle-associated cell types and express ligand-receptor genes that are critical to signaling processes required for germinal center processes. Results also suggest *KLF2*- CD4 $\alpha\beta$ T cells have closer proximities to follicle-associated cells and associated signaling pathways compared to their *KLF2*+ counterparts, suggesting *KLF2* may be a marker that can be targeted for functional inference of porcine CD4 T cells in future work.

Though many of the interactions and signaling pathways defined in porcine lymph node were similar to those defined in other species (72–76), a peculiarity of pigs is an increased number of circulating $\gamma\delta$ T cells compared to other species and presence in lymph node tissue (77, 78). Annotated $\gamma\delta$ T cell populations in our porcine lymph node scRNA-seq data (241 cells) had mostly non-interactions with B lineage cell types (Figure 5A), were generally located outside of the structure center (similar to other *KLF2*+ T/ILC populations; Figures 5B, C), and were negligible contributors to most lymph node cell-cell signaling pathways (Figures 5D, E). *In situ* RNA staining for $\gamma\delta$ T cell marker, *TRDC*, verified localization of $\gamma\delta$ T cells primarily outside of germinal centers (Figure 5F), and $\gamma\delta$ T cells made up a minor fraction of total T cells (indicated by CD3 ϵ protein immunolabeling) in lymph node tissue (Figure 5G) (77, 79). Collectively, *in situ* staining and scRNA-seq analysis results suggested $\gamma\delta$ T cells are minimal direct contributors to germinal center immune responses in pig lymph nodes. While the cell interaction database used is derived from human cell interactions and may not fully represent interactions between $\gamma\delta$ T cells and other leukocytes in pigs, the sparsity of $\gamma\delta$ T cells in the follicle of porcine lymph nodes and inference of significant non-interactions with follicular cell populations studied herein suggests porcine $\gamma\delta$ T cells have little direct influence on germinal center processes.

3.5 Using an open-source application interface of the porcine single-cell immune atlas to explore immune cells in pig bone marrow

To promote usability of the multi-tissue immune atlas created in this work, we developed an open-source application interface available to perform gene expression query and cell type population

comparisons across pig primary and secondary lymphoid organs captured in our work. The web application, called Shiny-PIGGI will be an important tool for exploration of porcine immune genes and cell types in these tissues.

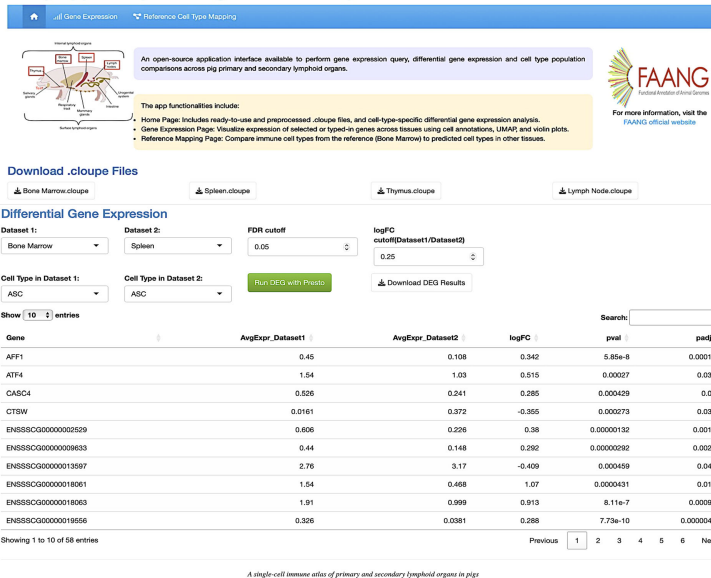
As a demonstration, we utilize the application to interrogate immune cell types recovered from bone marrow and to compare them to cells in other tissues of our immune atlas. In Figures 6A, we show the home page for the Shiny-PIGGI web application, along with key functionalities of each tab, including gene expression query and reference cell type mapping. The homepage also includes a link to downloadable data files for easy downstream analysis and visualization and a “Differential Gene Expression” section for performing user-specified cell type-specific differential gene expression analysis.

Under the “Gene Expression” tab, we demonstrate how the application interface can be used to assess detected expression of user-specified genes across all four dataset tissues, such as key marker gene *CD163* that was used to annotate unique monocyte/M Φ populations in bone marrow. Violin plots of detected *CD163* expression within cell types of each tissue that were generated in the web application are shown in Figure 6B and indicate *CD163* was not detected at high expression levels in myeloid populations of thymus or lymph node; however, cycling and non-cycling *CD163*+ monocyte/M Φ populations were recovered in bone marrow, and some splenic monocytes/M Φ s also expressed detectable *CD163*. Further comparison of cell types recovered from bone marrow and spleen datasets was performed using mapping procedures similar to those used in Figures 3, 4 under the “Reference Cell Type Mapping” tab in the web application, where bone marrow was considered as a reference dataset, and spleen was considered as a query dataset. Figure 6C shows a dot plot obtained from the application interface results of spleen-to-bone marrow reference mapping. Splenic cells had generally high mapping scores to the bone marrow dataset, indicating close counterparts could be identified in bone marrow. Similarly annotated cell types were generally in consensus based on cell type predictions, including B cells, ASCs, monocytes/M Φ s, and cDCs. Remaining splenic cell populations belonged to the T/ILC lineage and were also largely predicted as types of T/ILCs in bone marrow data; however, cycling T/ILCs in spleen were predicted as mostly cycling *CD163*+ monocytes/M Φ s from bone marrow, likely due to shared expression of many cell cycle genes that drive transcriptional profiles in both populations. Other effector populations of T cells and ILCs described in spleen were predicted mostly as *CCL5*+ T/ILCs from bone marrow, coinciding with *CCL5*+ T/ILCs being a likely mature effector cell population in bone marrow. Overall, the application is an open-source resource that can be used to query tissue datasets for forming important biological conclusions and inferences, such as examples shown above. Access to the query interface is described in the Data availability statement of this work.

4 Discussion

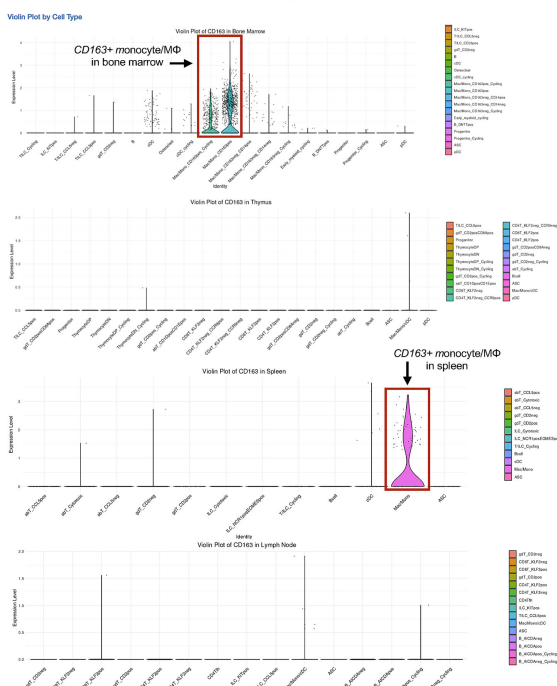
Results described above demonstrate the vast heterogeneity of the immune landscape across primary and secondary lymphoid

A Shiny App to Explore Single Cell RNAseq Tissues



A single-cell immune atlas of primary and secondary lymphoid organs in pigs

B CD163 expression across cell types in each tissue



C Spleen (query) to bone marrow (reference) mapping and cell type prediction

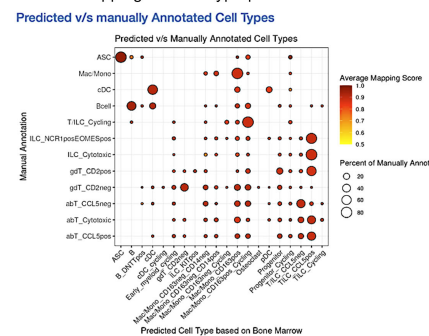


FIGURE 6

Utilization of an interactive application for further data query of monocyte/macrophage heterogeneity in pig bone marrow. **(A)** Home page for the web application created to query the pig single-cell multi-organ immune atlas. **(B)** Violin plots created to assess *CD163* expression in cell types of all tissues under the "Gene Expression" tab of the web application. **(C)** Dot plot showing predicted bone marrow reference cell type (x-axis) for cell types of spleen query data (y-axis) as created under the "Reference Cell Type Mapping" tab of the web application. Predicted annotation was assigned as the reference cell type with the highest prediction score. Dot color indicates the percentage of a query cell type (y-axis) predicted to be a reference cell type (x-axis). Dot size indicates average mapping score for those cells predicted as a reference cell type within an annotated query cell type. A higher mapping score indicates better representation of query cells in the reference dataset.

organs in pigs and include important insights into cell phenotypes, functions, and implications for comparisons across species. Thymus and bone marrow, the two primary lymphoid organs analyzed, were home to progenitor cells, cells undergoing recent lineage commitment and development, as well as fully differentiated cells and potential tissue-resident cell populations. Comparison of thymus cells to another porcine thymus scRNA-seq dataset

revealed further functional implications for several cell subsets, including high degrees of validity, functionally-implicating annotations across datasets along with novel cell populations captured in our larger thymus scRNA-seq dataset, including progenitors, monocytes/MΦ/cDCs, and pDCs that may play important roles in thymic developmental processes that can be further studied through scRNA-seq. In bone marrow, we

demonstrate similarities and differences to cells captured at other tissue sites through interactive query with a newly developed application interface, revealing exceptional myeloid lineage cell diversity that has previously been unable to be documented in pigs without scRNA-seq applications. In the two secondary lymphoid organs analyzed, spleen and lymph node, diverse effector cell subsets were discovered and were highly distinct across the two immune tissues, though cells from both tissues shared similarities with human cells from corresponding lymphoid organs. In spleen, several highly activated, effector T and ILC populations were characterized, including novel ILC subsets highly similar to those in human spleen. Discovery of diverse ILC subsets in the spleen dataset also complements existing works that have expanded upon NK cell diversity in porcine spleen via antibody immunophenotyping (66, 80, 81). Though it remains to be established if protein labeling strategies can identify ILC subsets corresponding to those identified in our work, general parallels may be drawn between documented protein and RNA expression for splenic ILCs in pigs, such as a general lack of detectable *CD8 α /CD8A* expression and expression of *NKp46/NCR1* and *CD16/FCGR3A** in a large number of cells. Identification of ILC subset-specific markers will also aid in more accurate identification rather than relying on annotation methods based solely on lack of expression for particular markers, such as lack of detected *CD3E* expression that was used in our work. In lymph node, conventional leukocyte populations that facilitate germinal center immune reactions were characterized and had highly similar signaling and organizational dynamics to those in human lymph nodes, despite pigs having an inverted lymph node structure comprised of an internal cortex and peripheral medulla, unlike humans (82, 83). Though $\gamma\delta$ T cells are more prevalent in pigs compared to humans, our lymph node dataset and *in situ* staining suggested this species-specific peculiarity did not result in a major role of $\gamma\delta$ T cells in contributing to germinal center responses, though further investigations may still be warranted. Overall, our work provides an expanded overview of the immune landscape in pigs across primary and secondary lymphoid organs, and each tissue was critically analyzed in a different manner to demonstrate the utility of the single-cell immune atlas as a resource that can be wielded to address further research queries through a variety of methodologies. Furthermore, an interactive web application for data query was created and is publicly available to enable better accessibility and usage of our dataset as a resource, including for non-computational viewership and query (e.g. investigating expression of additional gene markers and differentially expressed genes not mentioned in this work). Shiny-PIGGI may be utilized to expand current knowledge in other fields applying scRNA-seq to identify and explore cell types in immune tissues.

Though our work successfully captures and analyzes a diverse array of immune cells across multiple immunologically-critical anatomical locations of pigs, the atlas is by no means comprehensive. Cells were captured from two animals and are not reflective of the full spectrum of pig life stages and biological scenarios, including age, rearing environment, disease state, gender, or genetic predispositions. Some cell types were captured at low abundances (e.g. myeloid lineage cells) and other populations may

have potentially been excluded (e.g. granulocytes) due to rarity or biased capture from cell isolation processes used [e.g. mechanical rather than enzymatic digestion, cryopreservation, or on-column dead cell removal methods that were utilized in our samples (34, 84, 85)]. Further work utilizing methods to enrich for rare cell types may be required to more fully understand the role of lowly-captured cells. We also emphasize that spatial organization of cells in tissue is lost in scRNA-seq data, and spatial transcriptomics technologies are best utilized to more fully understand native cell organizations (86, 87). Though scRNA-seq data was analyzed to suggest general patterns of cell proximities and signaling interactions in the lymph node dataset, the recreated structure lacked discretely identifiable B cell and T cell zones and differentiation of light and dark zones of germinal centers, suggesting the limitations in scRNA-seq in defining tissue organization. Lastly, additional anatomical locations likely include expanded immune cell diversity, such as mucosal effector sites lacking organized primary or secondary lymphoid structures (e.g. lungs, intestinal tract), additional lymph nodes draining unique effector sites, or even neurological tissues. Regardless, our work presents an immunologically-focused multi-tissue, single-cell atlas in pigs and serves as an initial data resource to expand upon for understanding the comprehensiveness of the porcine immune system. Further, this work expands the number of tissues interrogated through single-cell analyses for the pig, increasing the value of large scale integration of such data for accurate cell annotations and foundation models of biology at the cell level (88).

In that vein, this work was completed as part of the Functional Annotation of Animal Genomes (FAANG) research initiative to annotate the functional components of domesticated species through detecting RNA expression and epigenetic status in tissues (89). Describing the transcriptome of important cell types within tissues and under conditions relevant to understanding immune response and function is a first step to develop immune system gene regulatory knowledge, which has been initiated in this work. The second step is understanding the interactions of components of the system by linking genes with their regulators through these regulatory elements; a network describing such interactions as an “intermediate phenotype” is a paradigm for understanding complex disease and predicting biological outcomes (90, 91). Global transcriptomic studies in FAANG research are expected to reveal co-expression of transcriptional regulators with their target genes in specific tissues or cells (92), providing useful annotation of livestock genomes, and this work may be further applied in future as a resource to reach such goals. The datasets generated herein may also serve as resources for various other research queries such as those related to pig immunology and health or potential biomedical applications of pigs for human health advancement.

Data availability statement

Scripts used for data analysis, Cell Ranger output file summaries, and filtering metrics (recorded within scripts) are available at https://github.com/SwiVi/FAANG_MultiTissue_

Immune_scRNAseq. The interactive query application, Shiny-PIGGI, can be accessed at <https://shinyiggi.ansci.iastate.edu/> or downloaded from https://github.com/kapoormuskan/Pig_Immune_Tissue_ShinyApp. Raw sequencing data are available at the European Nucleotide Archive (ENA) under project PRJEB97326 (<https://www.ebi.ac.uk/ena/browser/view/PRJEB97326>). Additional files of processed datasets (tissue-specific and cell lineage-specific data subsets) used for computational analysis and files compatible with interactive data query through Loupe Browser (10X Genomics; .cloupe files) are available at Ag Data Commons (<https://www.doi.org/10.15482/USDA.ADC/29492726.v1>).

Ethics statement

The animal study was approved by National Animal Disease Center Institutional Animal Care and Use Committee. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

JW: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Software, Visualization, Writing – original draft, Writing – review & editing. MK: Data curation, Methodology, Software, Visualization, Writing – original draft, Writing – review & editing. SS: Data curation, Software, Writing – review & editing. KB: Investigation, Methodology, Writing – review & editing. CL: Conceptualization, Data curation, Investigation, Methodology, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing. CT: Conceptualization, Funding acquisition, Investigation, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing.

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Conflict of interest

The author(s) declared that this work was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Generative AI statement

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