Organ-specific features of human kidney lymphatics are disrupted in chronic transplant rejection

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SUPPLEMENTARY FIGURE LEGENDS

Fig.S1. Further characterization of kidney lymphatic molecular markers and cellular relationships

(A) Representative 3D reconstruction of three human cortical tissues stained for a PDPN monoclonal antibody (clone: D2-40) showing lymphatic vessels (white arrowheads) as compared to controls in which primary antibodies were omitted, resulting in no labelling of vessel structures. Some areas exhibited non-specific fluorescence / background immunoreactivity. Imaging is representative of three non-overlapping fields of view captured per donor kidney. Scale bars: 150 μm. (B) Representative *z*-section from confocal image stack as shown in Figure 1G, showing the extent to which $PDPN^+$ lymphatic vessels, shown with white arrowheads, extend towards the peripheries of the kidney cortex (shown with a white dashed line). (C) Representative 3D reconstruction of juxtamedullary regions from two human transplant donors labelled for PDPN and the thick ascending limb epithelial marker, uromodulin (UMOD). 3D reconstructions revealed that lymphatic vessels (LV) run adjacent to, but do not intermingle with medullary bundles of TAL tubules. Scale bar: 100 μm. (D) Analysis of non-lymphatic expression LYVE1 using 3D imaging within $CD31^+$ peritubular capillaries, shown with white arrowheads. Scale bar = 50 μm. (E) UMAP of the scRNA-seq dataset from control kidneys showing the expression of *LYVE1*. The UMAP corresponds to that in Figure 2A, and shows *LYVE1* expression in lymphatics, peritubular capillary endothelium and myeloid cells within the human kidney.

Fig.S2. Generating a human kidney single-cell transcriptomic atlas including alloimmune and non-alloimmune pathologies

(A) UMAP of the kidney cell atlas coloured by cell type and partitioned by dataset. Samples included non-rejection transplant biopsies (Control 1: 7,259 cells, Control 2: 9,785 cells, Control 4: 38,850 cells and Control 6: 22,592 cells), non-tumorous regions of tumour nephrectomies (Control 3: 58,934 cells, chronic kidney disease due to benign nephrosclerosis

(CKD): 58,934 cells), declined organ donor tissues (Control 5: 3,877 cells , Control 7: 9,741 cells) and surgically explanted allografts (Non-alloimmune graft injury 1: 6,063 cells, Non-alloimmune graft injury 2: 2,131 cells, Chronic rejection 1: 34,067 cells, Chronic rejection 2: 4,299 cells). (B) Stacked bar charts representing the relative proportions of each annotated cell type across the four study groups). (C) Stacked violin plot showing top 2 differentially expressed marker genes (*y* axis) by cell type (*x* axis), calculated using Seurat FindAllMarkers.

Fig.S3. Anatomical and organ-specific molecular heterogeneity within the human organ lymphatic atlas

(A) Integrated UMAP, as shown in Figure 3A, featuring 13,454 cells from a total of seven human organs, including kidney, skin, breast, heart lung, small intestine and large intestine. The cells here are grouped by the 19 anatomical regions from which the cells were acquired. (B) Dot plot showing marker genes of each LEC subtype. Expression of *PROX1* and *PDPN* confirms the lymphatic identity of all cells within the dataset. *CCL21* and *LYVE1* demarcate capillary lymphatic cells and are enriched in all clusters except for valve LECs, which instead are enriched for *FOXC2* and *ITGA9*. The remaining clusters have variable expression of the chemokines *CCL2, CXCL2*, *CXCL8,* and the peptide hormone *ADM.* (C) Heatmap showing predicted cell cluster-enriched transcription factor (TF) activity based on cluster-enriched DEGs, assessed using SCENIC analysis. Red indicates higher relative predicted activity whereas blue indicates lower relative predicted activity. TFs are cluster depending on whether the organ sampled was viscerally or superficially located. (D) Violin plot of cell type-specific expression of the 5 top differentially expressed genes by kidney lymphatics, across other cell types within the kidney scRNA-seq atlas (E) Heatmap showing top 10 marker genes of each non-kidney organ within the scRNA-seq dataset of human organ lymphatics.

Fig.S4. Identification of endothelial cell-cell junctions in three-dimensional imaging data of human kidney

3D reconstruction of confocal imaging of kidney cortical lymphatics (white arrowheads) in chronic transplant rejection tissues, representative of three images each acquired from *n* = 2 rejecting allografts. Endothelial cell junctions were labelled with CDH5. Arterioles in juxtaposition with lymphatics are shown with white asterisks. The 488nm channel was used to image autofluorescence and capture tissue architecture. Scale bar: 30 μm.

Fig.S5. Identification and characterization of high endothelial venules 3D imaging and scRNA-seq data of kidney transplant rejection

(A) Representative *z*-section of three confocal microscopy *z*-stacks acquired from *n* = 2 kidneys with chronic transplant rejection, stained for CD31 and the high endothelial venule (HEV) marker PNAd. Within the image, two HEV structures are shown, individually magnified on the panels on the right, with each panel labelled accordingly. $PNAd^+$ $CD31^+$ endothelium is shown with white arrowheads. Scale bars: 50 μm. (B) Dot plots demonstrating molecular characterisation of HEV endothelium with kidney transplant rejection scRNA-seq data. Lymphatic endothelial cells (LEC) are used as a comparison, and are enriched for *PROX1* and *CCL21*, whereas *NTAN1* and *PLVAP* are enriched in HEV cells. Compared to lymphatics, HEV endothelium has low expression of the Notch pathway molecules including *RBPJ* and *JAG1*. (C) Heatmap showing differentially expressed genes (DEG) between LECs and HEVs. Of note, a variety of MHC Class II encoding transcripts are upregulated in HEV endothelium as compared to lymphatics. (D) Stacked violin plot showing immunologically relevant differentially expressed genes between lymphatic endothelium and $CCL21^+$ $PNAd^+$ blood endothelium across rejecting allograft scRNA-seq data. Immune activating candidates (*CD40, IL32, TNFSF10, CX3CL1*), chemokines (*CX3CL1, CCL2, CCL23, CXCL16*) and immune cell adhesion molecules (*VCAM1, SELE, SELP, MCAM, VCAM1*) are all enriched in HEV cells.

Fig.S5. Cell-cell interaction analysis of lymphatics in human kidney single-cell RNA sequencing data

(A) Violin plot showing the total number of CellPhoneDB-computed cell-cell interactions between lymphatics and adaptive immune cell subsets within single-cell RNA sequencing (scRNA-seq) data of control, chronic kidney disease (CKD), and rejection tissues. (B) shows the interactions as A but are partitioned by the subtype of lymphocyte. (C) Statistically significant CellPhoneDB scores for lymphocyte-derived IFNγ interacting with lymphatics or other cell types within the dataset.

Fig.S6. Analysis of chemokine signalling mediated by kidney lymphatics in health and disease

(A) CellPhoneDB dot plot of scRNA-seq data demonstrating top 10 chemokine interactions between lymphatics and B cells or effector $CD4^+$ T cells, partitioned by disease aetiology. Dot size represents the scaled mean expression of the interaction, and those encircled with a red ring are deemed statistically significant by CellPhoneDB. (B) UMAP of all immune cell subsets with feature plots showing expression of ACKR2 ligands across these cell types.

Fig.S7. Visualization of immune-stimulatory and immune-inhibitory interactions mediated by kidney lymphatics in health and disease

(A-C) Circle plot of the transcriptional 'interactome', or putative cell-cell communication, between lymphatics and distinct immune cell subsets identified in the scRNA-seq dataset, computed between lymphatics and non-$CD4^+$ T cells in chronic rejection (A), lymphatics and $CD4^+$ cell subsets in CKD (B) and lymphatics and $CD4^+$ cell subsets in non-alloimmune graft injury (C). Each node represents a putative ligand or receptor, and each line represents an interaction, with stimulatory interactions coloured in red and inhibitory interactions coloured in blue. The size of the node represents the proportion of cells expressing the ligand or receptor, and the darkness of the line represents the strength of the CellPhoneDB interaction.

SUPPLEMENTARY VIDEO LEGENDS

Video S1. 3D confocal microscopy of the lymphatic vasculature in healthy human kidney

Three-dimensional reconstruction of healthy human kidney tissue stained with the D2-40 monoclonal antibody (green, PDPN) to label lymphatic vessels. Autofluorescent structures are shown in blue. Acquired using two-photon microscopy, the video begins with rotation, followed by high-magnification panning through the cortex, illustrating the architecture of the lymphatic plexus and its blind-ended capillary initiation.

Video S2. Arterial association and lymphatic vessel hierarchy in healthy human kidney

3D reconstruction of healthy human kidney tissue stained for PDPN (green) to label lymphatics. Autofluorescence (blue) highlights large arteries, revealing their close anatomical proximity to lymphatic vessels. The video rotates and pans to show lymphatic capillaries branching from arterial regions into the cortex, ending in blind tips.

Video S3. Cortical lymphatic initiation adjacent to proximal tubules in human kidney

3D reconstruction of healthy human kidney tissue co-stained for PDPN (green) and *Lotus tetragonolobus* lectin (LTL, orange), marking proximal tubules. The video alternates between channels and pans through at high magnification, highlighting the emergence of blind-ended lymphatic capillaries adjacent to proximal tubular epithelium.

Video S4. Lymphatic initiation near distal and connecting tubules in human kidney

3D reconstruction showing PDPN (green) for lymphatics and CALB1 (yellow) marking distal/connecting tubules in healthy human kidney. The video pans and rotates, demonstrating blind-ended lymphatic capillaries closely associated with distal nephron segments in the cortex.

Video S5. Spatial organization of lymphatics and collecting ducts in healthy kidney

3D reconstruction of healthy human kidney tissue stained with PDPN (green) for lymphatics and *Dolichos biflorus* agglutinin (DBA, red) for collecting ducts. The rotating image shows the anatomical arrangement between collecting ducts and surrounding lymphatic vessels.

Video S6. Lymphatic expansion in chronic kidney transplant rejection

3D reconstruction of kidney allograft tissue undergoing chronic rejection. PDPN (green) labels lymphatics, DBA (red) labels collecting ducts. The image rotates to show the pronounced expansion of lymphatics compared to healthy kidney (see Video S5), and their spatial relationship to collecting ducts.

Video S7. Junctional architecture of endothelial cells in human kidney

3D reconstruction of healthy human kidney tissue stained with CDH5 (red), highlighting endothelial cell-cell junctions of both blood and lymphatic vasculature. The video pans and zooms to display the junctional organization within the intact cortical microvasculature.

Video S8. Lymphatic–tertiary lymphoid structure interactions in transplant rejection

3D reconstruction of chronically rejecting human kidney transplant tissue stained with PDPN (green, lymphatics), CD21 (blue, follicular dendritic cells), and PNAd (high endothelial venules). The video rotates, zooms, and pans to show tertiary lymphoid structures (TLS) interconnected by lymphatic vessels.

Video S9. Lymphatic-lymphocyte spatial relationships in chronic transplant rejection

3D reconstruction of kidney allograft tissue stained with PDPN (green), CD4 (red), and CD20 (grey), marking lymphatics, T cells, and B cells, respectively. The video introduces each channel, then zooms and pans to demonstrate lymphocyte proximity to lymphatic vessels. Toward the end, extraluminal lymphocytes are subtracted, revealing cells located within lymphatic lumens.

Video S10. HLA-DR-expressing lymphatics exhibit adjacent T cell accumulation in chronic transplant rejection

3D reconstruction of kidney allograft tissue stained for PDPN (green), HLA-DR (red), and CD3 (grey). The video zooms in on a lymphatic vessel, cropping surrounding tissue to focus on HLA-DR$^+$ regions and adjacent CD3$^+$ T cells. 3D renderings reveal close spatial relationships between CD3$^+$ T cells and HLA-DR$^+$ regions of lymphatics.