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

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Supplemental Materials and Methods

Sex as a biological variable

Due to the exploratory nature of 3D imaging and single-cell RNA sequencing performed in this study, and the limited kidneys available for 3D imaging analysis, sex was not considered as a biological variable.

Three-dimensional imaging and analysis of human kidney lymphatics

Acquisition, fixation and storage of human tissue for three-dimensional imaging

Human adult kidney tissue was derived from four deceased patients who had opted in for organ donation and undergone multi-organ procurement, but for whom the kidneys had ultimately been declined for implantation by recipient transplant centres. Kidneys were retrieved by a UK National Organ Retrieval Services teams. Following *in situ* flushing of the abdominal organs with University of Wisconsin (UW) solution, the kidneys were removed and stored in UW at 4^{o}C. Kidney allograft samples were obtained from three patients at Royal Free London NHS Trust undergoing nephrectomy for graft intolerance syndrome (n = 2) or graft malignancy (n = 1). All explants were performed by the transplant surgical team. Prior to acquisition, all patients were confirmed negative for COVID-19 by means of a qPCR test. After explant, pseudo-anonymised human adult kidney tissues were incubated overnight in Belzer University of Washington Cold Storage Solution (Bridge to Life Europe, London, UK) at 4^{o}C. Prior to fixation, human adult kidney was manually dissected into ~3mm full-thickness sub-regions containing cortex and outer medulla. These tissues were then incubated in 4% (w/v) paraformaldehyde (PFA, Sigma Aldrich), made up in 1 X phosphate buffered saline (PBS), at 4^{o} C overnight. After fixation, all biological tissues were washed and stored in 1 X PBS with 0.02% (w/v) sodium azide to prevent contamination. Randomly selected pieces of human adult kidney were transferred to and stored in 70% ethanol for histology.

Wholemount immunofluorescence

A modified version of the SHANEL protocol (1) was implemented for wholemount immunolabelling of kidney tissues. Unless otherwise stated, steps were performed at room temperature, and reagents purchased from Sigma Aldrich. Tissues were dehydrated in a methanol series (50, 70%) in double distilled (dd)H2O, for one hour per step, before bleaching in absolute methanol with 5% (v/v) of 30% hydrogen peroxide solution overnight at 4°C. Thereafter, tissues were rehydrated in the methanol series, followed by incubation in 1 x PBS for one hour. Overnight incubation of tissues was performed in a 0.5 M solution of acetic acid at 4°C, followed by five hours of incubation at 4°C with 4 M guanidine hydrochloride, 0.05 M sodium acetate and 2% (v/v) Triton X-100 made up in PBS. Tissues were then permeabilised with 5% (w/v) solution of 3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate (CHAPS) made up in ddH2O overnight. Then tissues were incubated for one day in blocking solution, comprising 1 x PBS with 0.2% Triton X-100, 5% (v/v) donkey or goat serum, 5% (v/v) pooled human plasma (Biowest, Nuaillé, France) and 10% (v/v) dimethyl sulfoxide (DMSO) before incubation in antibody solution (1 x PBS with 0.2% (v/v) Tween-20, 0.1% (v/v) of a 10mg/ml heparin solution in ddH2O, 0.1% (w/v) saponin, 2.5% donkey or goat serum, 2.5% pooled human plasma with primary antibodies at the appropriate concentration at 4°C. Blocking and antibody solutions were further supplemented with 1:150 Human TruStain FcX™ Fc Receptor Blocking Solution (BioLegend, London, UK), to reduce non-specific binding. Primary antibodies were incubated for 3-4 days, before replenishing the antibody solution and re-incubation for 3-4 days. Subsequently, tissues were washed in 1 x PBS with 0.2% Tween-20 four times for 1 hour per wash, before incubation in antibody solution with secondary antibodies at 1:200 at 4°C for four days. Tissues were then washed again in 1 x PBS with 0.2% Tween-20 four times for 1 hour each and stored until dehydration and clearing.

Primary antibodies, lectins and secondary antibodies

In order of appearance in the manuscript, the following primary antibodies or lectins were used in 1.5ml incubations at the indicated concentrations: mouse anti-PDPN monoclonal (clone:

D2-40, 1:100, M3619, Aligent), rabbit anti-PROX1 polyclonal (1:200, ABN278, Merck), goat anti-LYVE1 polyclonal (1:100, AF2089, R&D Systems), fluorescein-conjugated LTL (1:50, Vector Laboratories), rabbit anti-UMOD monoclonal (clone: EPR20071, 1:100, ab207170, Abcam), fluorescein-conjugated UAE-I (1:50, Vector Laboratories), rabbit anti-LRP2 polyclonal (1:50, ab76969, Abcam), rabbit anti-CALB1 monoclonal (clone: EP3478, 1:100, ab108404, Abcam), rhodamine-conjugated DBA (1:50, Vector Laboratories), mouse anti-CDH1 monoclonal (clone: HECD-1, 1:50, ab1416, Abcam), mouse anti-PECAM1 monoclonal (clone: JC70A, 1:50, M0823, Dako), mouse anti-CD68 monoclonal (clone: KP1, 1:100, ab955, Abcam), rabbit anti-αSMA polyclonal (1:50, ab5694, Abcam), rabbit anti-HLA-DR monoclonal (clone: EPR3692, 1:100, ab92511, Abcam), rabbit anti-C4d polyclonal (1:100, 0300-0230, Bio-Rad), rabbit anti-CD4 monoclonal (clone: EPR6855, 1:100, ab133616, Abcam), goat anti-CD20 polyclonal (1:100, ab194970, Abcam), goat anti-PVR polyclonal (1:100, AF2530, R&D Systems), rabbit anti-CD21 monoclonal (clone: EPR3093, 1:200, ab75985, Abcam), rat anti-PNAd monoclonal (clone: MECA-79, 1:100, MABF2050, Sigma), goat anti-CDH5 polyclonal (1:50, AF938, R&D Systems). All secondary antibodies were purchased from ThermoFisher Scientific, were conjugated to AlexaFluor fluorophores (488, 546, 568, 633 or 647) and were used at a concentration of 1:200 of the original secondary antibody stock. Controls for each panel involved omission of the primary antibody and including the secondary antibody only.

Solvent-based optical clearing

Tissues were dehydrated in a methanol series (50%, 70%, 100%) for 1 hour per step. BABB (benzyl alcohol and benzyl benzoate in a 1:2 ratio), was used for clearing, with all solutions containing BABB kept in glass scintillation vials (VWR International, Lutterworth, UK). Clearing was performed in glass scintillation vials, first using BABB:methanol in a 1:1 ratio, and thereafter BABB alone, until samples equilibrated and achieved transparency.

Confocal microscopy

We took advantage of the z-depth achievable by upright confocal microscopy whilst protecting the microscope objectives. All tissues were placed between a large coverslip and cover glass, supported by a O-Ring (Polymax Ltd, Bordon, UK) made from BABB-resistant rubber, as described previously (2). Confocal images were acquired on an LSM880 upright confocal microscope (Carl Zeiss Ltd.), with a 2.5x/numerical aperture (NA) 0.085 Pan-Neofluar Dry objective (working distance; WD = 8,800 µm) for low-resolution imaging, and 10×/NA 0.5 W-Plan Apochromat water dipping objective (working distance; WD = 3,700 µm) for high-resolution imaging. Gallium arsenide phosphide (GaAsP) internal and external detectors were used for high sensitivity. To obtain higher resolution imaging, an Airyscan setting (3), consisting of a 32-channel (GaAsP) photomultiplier tube area detector.

Lightsheet fluorescence microscopy

3D imaging of cleared tissues was performed using a custom-built mesoscale selective plane illumination microscope (mesoSPIM) (4). The cleared tissue was secured in a 3D-printed holder and immersed in BABB solutions inside a quartz cuvette (40 x 40 x 100 mm). Fluorescence images were acquired with an Olympus MVX-10 macroscope at 1x magnification, resulting in a voxel size of 6.55 x 6.55 x 5 μm^3. PDPN fluorescence signals were obtained using 638 nm laser excitation and 633nm long-pass optical filtering of emitted light, while autofluorescence was captured using 488 nm laser excitation and a 520/35 nm bandpass emission filter. Lightsheet illumination from both sides of the cuvette was carefully aligned after the sample was positioned at the centre of the macroscope's field of view and delivered simultaneously to capture a single *z*-stack image.

Post-acquisition image processing

All images were then exported to FIJI (NIH, Bethesda, US). Confocal image stacks were separated into individual fluorescence channels, and the Despeckle and Sharpen tools were used to reduce non-specific background fluorescence. Where maximum intensity *z*-

projections or optical *z*-sections were required, scale bars were applied and images and exported as TIFF files.

Image visualisation and binarization of three-dimensional imaging data

Visualisation of 3D reconstructions were performed by importing confocal images to the commercial software, Imaris (v8.2, Bitplane). The Isosurface Rendering tool in Imaris allows the extraction of surfaces based on fluorescence intensity. This was used to generate segmented images fluorescence masks to visualise expression patterns, or to generate binarized outputs for extraction of vessel branching metrics. LSFM data was imported into Amira (v2020.2, Fisher Scientific) and the vasculature segmented using intensity thresholding and region growing using the Magic Wand tool to generate a binarized network.

Extraction of vessel branching metrics from three-dimensional imaging data

Segmented and binarized confocal and LSFM images were imported as TIFF image stacks into Amira. The Filament Editor tool was used in Amira to generate spatial statistical parameters including vessel branch number, lengths, diameter and volumes from each segmented lymphatic plexus. The resulting values were exported these as CSV files.

Assessment of lymphatic cell-cell junctional architecture

Volumes of interest of kidney tissues co-labelled with PDPN and CDH5 were segmented in IMARIS, generating a mask of PDPN signal to discriminate lymphatic-derived $CDH5^+$ signal from non-lymphatic-derived $CDH5^+$ signal, the latter corresponding to the blood vasculature. The volume of the lymphatic network from each image was determined, and the number of segmented and rendered discontinuous $CDH5^+$ structures was counted. As the lymphatic network in rejecting allografts was significantly larger, each value of discontinuous $CDH5^+$ junctions was volume normalised according to the volume of interest.

178 ***Spatial statistical analysis of lymphatic-lymphocyte relationships***

179 Lymphatic 3D-skeletons were extracted from binarised confocal stacks using the BoneJ
180 Skeletonise3d function in FIJI (5). $CD4^+$ T cell and $CD20^+$ B cell counts, centroids and areas
181 were obtained using 3d-objectcounter with no further pre-processing (6). The mean distance
182 of each cell from the nearest point of the lymphatic network (*d*) was calculated using the cross-
183 product 3D point-line distance:

184 $$d = \frac{|(x_0 - x_1) \times (x_0 - x_2)|}{|(x_2 - x_1)|}$$

185

186 where x_1 and x_2 are the two closest adjacent nodes from the lymphatic 3D skeleton; found by
187 minimizing cross-nearest neighbor distances, and x_0 is the centroid of the cell of interest. To
188 evaluate whether the cell distances were different from what would be expected by chance,
189 within each region of interest, the $CD4^+$ T cell and $CD20^+$ B cell populations were randomly
190 redistributed under complete spatial randomness for twenty simulations. A comparison was
191 then made as to whether the measured mean cell-lymphatic distances fell within the 95%
192 confidence intervals obtained through the simulations under complete spatial randomness.

193

194 **Single-cell transcriptomic analysis of human kidney lymphatics**

195 ***Acquisition of material for single-cell transcriptomics and generation of a human***
196 ***kidney cell atlas***

197 The scRNA-seq dataset from this study consisted of previously published data and five new
198 samples. For the five new samples, kidney allografts undergoing graft nephrectomies were
199 collected at the time of surgery. Tissue was digested and processed to a single cell suspension
200 as described previously (7). Cells were counted using a haemocytometer and cell
201 concentrations adjusted using dilution in RPMI Medium (Sigma) to a concentration of 1000
202 cells/μL. Cells were loaded according to the protocol of the 10X Chromium single cell 5'v2 kit
203 to capture 10,000 cells per channel. Libraries were prepared according to manufacturer

instructions and sequencing was performed on an Ilumina Novaseq instrument with read lengths specified by 10X genomics. Sequencing data were mapped against GRCh38-3.0.0 using 10X Cellranger V6.0.2. These data were merged with previously published scRNA-seq data of the human kidney, including samples from non-tumorous regions of tumour nephrectomies with or without CKD (7, 8), live allograft biopsies with or without antibody-mediated rejection (9) and human kidney data from the Kidney Precision Medicine project (10). Data were merged using anndata (0.8.0), using a nuisance gene mask as previously described (7) to minimise the effect of technical noise on batch integration. Thereafter, the data were split into healthy and diseased datasets using publicly available metadata. Highly variable genes were calculated using scanpy (1.8.2) setting *n_top_genes* = 1500, *batch_key* = 'dataset', and flavour = '*seurat_v3'* and using raw counts. On the basis of these variable genes, we constructed a variational autoencoder model using scArches (11), using both dataset and the donor identity as categorical covariate keys, and a dissociation stress score calculated from aggregate dissociation-induced gene expression (12) as a continuous covariate. A single cell variational inference (scVI) model was trained using n_layers = 2, and n_latent = 15. We then used the latent space computed from this model as input to scanpy's neighbourhood graph calculation and UMAP computation functions. Data were clustered using Leiden clustering and annotated on the basis of marker genes. Immune cell annotations were further supported by prediction from a logistic regression model with the CellTypist python package. The data was then converted using seurat-disk before further analysis using the Seurat package in R. Unless otherwise stated, all downstream steps were performed in Seurat.

Analysis of kidney lymphatic transcriptional heterogeneity

To capture heterogeneity of lymphatic cells within the human kidney, the cell cluster corresponding to lymphatics was extracted from the control kidney dataset, and a raw count matrix was generated from all 295 lymphatic cells. We combined this with data from a recent study utilising scRNA-seq to examine nine human control kidneys after tumour nephrectomies

(13). From the latter dataset, a cluster of 157 lymphatic cells was identified and the raw count matrix merged with our dataset, giving a total of 452 lymphatic cells. A subset of newly generated count matrix was created to only include genes detected in both datasets, resulting in 15,316 genes. The data was pre-processed using the Seurat workflow including normalisation, scaling by all genes and principal component (PC) analysis. Integration of the two lymphatic cell datasets was achieved using the Harmony package, integrating by the study from which the cells were sourced. The *FindNeighbors, FindClusters* and RunUMAP algorithms were then computed, using 7 PCs, a resolution of 0.4 and utilising the embeddings generated by Harmony integration. Differential expression analysis was performed as below to discriminate subcluster-specific markers from the two transcriptionally distinct clusters detected.

Creation of a human organ lymphatic atlas

Count matrices of publically available datasets from the Human Cell Atlas, including skin (14), breast (15), heart (16, 17), lung (18) and small or large intestines (19), were downloaded. Lymphatic endothelium was identified and isolated from the metadata of each dataset and individually curated for the expression of *PROX1* and *PDPN*. We generated a count matrix which included each of these datasets, and the 452 lymphatic cells from control kidneys computationally isolated as aforementioned. Genes that not detected across all datasets or that were not represented in all count matrices were deleted to avoid artefactual clustering. The analysis workflow described as above, including Harmony integration, was then used, performing integration by individual donor. Predicted transcription factor activity was assessed using the SCENIC package in R.

Assessment of kidney lymphatic-enriched candidates in human kidney diseases

To assess the expression of *DNASE1L3* and *MDK* across a range of human kidney diseases, the NephroSeq database was used. We searched for RNAseq or microarray expression datasets containing these transcripts, only including datasets that assessed tubulointerstitial

gene expression. The datasets were compiled and log_2 expression values were extracted and plotting using Violin plots.

Differential expression analysis

The *FindAllMarkers* function was used for differential expression analysis of scRNAseq data. Wilcoxon Rank Sum tests were used to assess statistically significant (adjusted *p* value ≤ 0.05) between average log fold change values of expression. Selected differentially expressed genes were visually represented using the *VlnPlot* function, the *DoHeatMap* function or using the EnhancedVolcano (https://github.com/kevinblighe/EnhancedVolcano) package. For NephroSeq data, log_2 expression values were assessed using ANOVA tests with Tukey post-hoc correction for individual comparisons.

Gene ontology analysis

Gene ontology (GO) analysis was performed using the PANTHER tool for gene classification. Lists of differentially expressed genes, were exported and input into the PANTHER web tool (v16.0, http://www.pantherdb.org), using statistical overrepresentation tests to group genes using the GO biological processes complete database. Fisher's Exact tests were used to assess for statistical enrichment of genes for selected GO terms, and a false discovery rate (FDR) $p \leq 0.05$ was considered significant.

CellPhoneDB

To infer putative cell-cell interactions in single-cell RNA sequencing data, the CellPhoneDB resource (20) was used. Using normalised count and metadata files obtained from Seurat, CellPhoneDB was called by running appropriate commands, obtained from https://github.com/Teichlab/cellphonedb, in the command line through a Python virtual environment. The *statistical_analysis* method was used to assess predicted interactions, before functions in ktPlots (https://github.com/zktuong/ktplots) were used to generate custom dot plots or circle plots.

Lymphatic endothelial cell stimulation assays

Cell line and recombinant IFNγ treatment

Adult human dermal lymphatic endothelial cells (HDLECs; PromoCell GmbH) were cultured in Endothelial Cell Growth Medium MV2 (PromoCell) at 37 °C in a 5% CO_2 incubator. Media were refreshed twice weekly, and confluent cultures were passaged at a 1:3 ratio. cells (n = 2 independent lines) were seeded at a density of 1×10^5 in a 6-well plate, treated with either normal growth media (unstimulated group) or IFNγ (50ng/ml; Thermo Fisher Scientific,) for 24 hours, 48 hours and 72 hours.

Quantitative RT-PCR for LGALS9 expression by HDLECs

To assess *LGALS9* RNA expression, cells at each time point were harvested, lysed and total RNA extracted using the RNeasy Plus Mini Kit (Qiagen). cDNA was synthesised from 500 ng RNA using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories). Quantitative real-time PCR was performed on a CFX96 Real-Time PCR System (Bio-Rad) using qPCRBIO SyGreen Mix Lo-ROX (PCR Biosystems Ltd). Expression of *LGALS9* was normalised to the housekeeping gene *HPRT*, and fold-change was calculated using the $2^{-\Delta\Delta CT}$ method. Results were standardised to the mean expression in untreated controls (set to 1). The experiment was repeated three times and all assays performed in duplicate. The presented data shows the mean fold-change in gene expression measured in three independent cell lines. Primer sequences are available upon request.

Assessment and quantification of LGALS9 secretion by HDLECs

To assess LGALS9 protein secretion, conditioned media from HDLECs at each timepoint were collected and centrifuged to remove cell debris. Galectin-9 levels were quantified by ELISA

(DGAL90, R&D Systems) according to the manufacturer's instructions. Optical density was measured at 450 nm, and protein concentration was calculated using a standard curve derived from serial dilutions of recombinant LGALS9. The experiment was repeated three times and all assays performed in duplicate. Results were expressed relative to unstimulated wells, which were standardised to a value of 1.

Statistical analysis, data presentation and availability

Sample size estimation

In prior work examining the 3D architecture of lymphatic vessels in lymphangiomatous skin biopsies, conclusions were drawn based on the evaluation of three samples within the control group (21), and so a minimum of three patients per group were used to draw conclusions. For scRNA-seq, the number of samples and cells to be analysed was limited by the size of the dataset. The specific number of replicates used for each experiment and the number of regions images are indicated in the figure legends,

Reproducibility and data presentation

Descriptive conclusions are drawn based on a minimum of four imaging volumes of interest, each taken from samples from at least two different human kidneys. All confocal and brightfield images were exported and saved as TIFF files. Where brightness or contrast were adjusted, this was applied uniformly across all conditions within the same figure, and details are stated in figure legends. Cell culture experiments were performed in biological triplicates, with a minimum of two technical replicates for each assay. Graphs were generated in GraphPad PRISM and saved as TIFF format. Visualisations from scRNA-seq analysis were performed in RStudio and PNG screenshots were taken and saved. Figures were compiled in Microsoft PowerPoint (Microsoft, Redmond, US) and saved as PDF format.

Statistics

Except for scRNA-seq analysis and lymphatic-lymphocyte spatial relationships, all remaining statistical comparisons were performed using GraphPad PRISM. A two-tailed *p* value of less than 0.05 was considered statistically significant. For continuous data, Shapiro-Wilk tests were used to assess normality of distribution and Brown-Forsythe tests were used equality of variance. Where normal distribution and equality of variances were satisfied, data is presented as mean ± standard deviation. When graphed, error bars were used to represent the standard error of the mean. Student's *t*-test was used to compare two groups and ANOVA was used to compare more than two groups, applying post-hoc Bonferroni tests to provide adjusted *p* values for multiple comparisons. Statistics for scRNA-seq analysis were performed in RStudio and are as detailed above.

Study approval

For reference human kidney tissue, consent for the use of the organs for research was obtained from the donor family by Specialist Nurses in Organ Donation before organ retrieval and were then offered for research by NHS Blood & Transplant (NHSBT) if they were found to be unsuitable for transplantation by the surgical team. Ethical approval was granted by the National Research Ethics Committee in the UK (21/WA/0388) and was approved by The Royal Free London NHS Foundation Trust-UCL Biobank Ethical Review Committee (RFL B-ERC; NC.2018.010; IRAS 208955). Ethical approval to obtain explants with chronic rejection was covered by a prior agreement (NC.2018.007, UCL Biobank Ethical Review Committee, Royal Free London NHS Foundation Trust, B-ERC-RF). Human kidney tissues for scRNA-seq were explanted and processed at Cambridge University Hospitals NHS Foundation Trust under ethical approval (REC 16/EE/0014). Ethical approval for publicly available data for human lymphatic cells acquired from scRNA-seq experiments of kidney (22), skin (14), breast (15), heart (16, 17), lung (18) and intestines (19) are detailed in the original studies.

Data availability

Raw sequencing data for the five new human kidney scRNAseq samples have been made publicly accessible via the European Genome-phenome Archive (Accession number: EGAD00001015631). The annotated and processed Seurat objects and h5ad files are publicly available: https://doi.org/10.5281/zenodo.7566982. The code for analysis of the human kidney scRNA-seq atlas is available at https://github.com/daniyal-jafree1995/. The imaging data used is available from the lead contact upon request. All raw data used to plot graphs, except for scRNA-seq analyses, are provided within the Supporting Data Values document.

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