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Using single cell technologies to map the human immune system – implications for nephrology

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Abstract

Advances in single cell technologies are transforming our understanding of cellular identity. For instance, the application of single cell RNA sequencing and mass cytometry technologies to the study of immune cell populations in blood, secondary lymphoid organs and the renal tract is helping researchers to map the complex immune landscape within the kidney, define cell ontogeny and understand the relationship of kidney-resident immune cells with their circulating counterparts. These studies also provide insights into the interactions of immune cell populations with neighbouring epithelial and endothelial cells in health, and across a range of kidney diseases and cancer. These data have translational potential and will aid the identification of drug targets and enable a better prediction of off-target effects. The application of single cell technologies to clinical renal biopsy samples, or even cells within urine, will improve diagnostic accuracy and assist with personalised prognostication for patients with various kidney diseases. A comparison of immune cell types in peripheral blood and secondary lymphoid organs in healthy individuals and in patients with systemic autoimmune diseases that affect the kidney will also help unravel the mechanisms that underpin the breakdown in self-tolerance and propagation of autoimmune responses. Together, these immune cell atlases have the potential to transform nephrology.

[H1] Introduction

Advances in single cell technologies are revolutionising our understanding of cellular identity across biological disciplines. In the field of immunology, single cell transcriptomic studies have revealed an unappreciated complexity of immune cells subsets, identified new cell types, redefined cellular ontogeny, and enabled inference of cell fate trajectories and function [1,2]. In parallel, a heightened awareness that some immune cell populations exist almost exclusively within non-lymphoid organs[3] (**Fig. 1**), highlights an obvious application for single cell RNA sequencing (scRNAseq) to study the identity and function of tissue-resident immune cells. These cells are found in small numbers relative to the overall cell composition of individual organs, presenting a challenge to standard methods of transcriptional characterisation. In kidneys, this issue is particularly problematic due to the limited availability of samples for study, which is in part related to the risks associated with percutaneous biopsy. Hence, our understanding of kidney-resident immune cells in humans is still fairly rudimentary. This knowledge gap has disease relevance, since almost all conditions that affect the kidney involve some level of activation of the immune system, either systemically or locally. For example, systemic autoimmune diseases, such as systemic lupus erythematosus (SLE), involve peripheral immune dysfunction with the generation of autoantibodies, as well as local inflammation in response to the deposition of IgG immune complexes in the kidney. Similarly, pyelonephritis and primary glomerulonephritides, such as IgA nephropathy, involve overt activation of the immune system. However, tissue-resident cells within the kidney can respond to tissue damage that arises from any insult, including ischaemia, toxins and renal calculi, and the outcome of this response, in terms of injury resolution or progression to chronic inflammation, is likely to determine whether progression to fibrosis occurs[4,5]

In this Review, we focus on scRNAseq, reflecting the relative abundance of available publications that have used this technology, but also discuss how scATACseq **[G]**, and mass cytometry are being applied to study immune cell populations in blood, secondary lymphoid organs, and tissue-resident cells. These efforts are beginning to map the complex immune landscape within organs, and reveal the relationship of tissue-resident immune cells with their circulating counterparts and their interaction with neighbouring epithelial and endothelial cells under physiologic conditions and across a range of diseases including inflammatory diseases and cancer. These studies are relatively new, and many efforts to date lack orthogonal experimental validation. However, the application of single cell technologies to the study of immune cells in the kidney holds promise to enable better understanding of the role of the immune system in kidney health and in disease pathogenesis, as well as facilitate the identification of novel treatment strategies.

[H1] Single cell technologies

The immune system has evolved to provide defence against microbial pathogens but can also respond to tissue damage and to cells that have undergone malignant transformation [6-8]. It comprises an innate and adaptive system, each with cellular and soluble components. The innate arm provides an immediate response with a range of cell types functioning as sentinels and rapid effectors distributed throughout the tissues of the body. These cells, including dendritic cells, macrophages, neutrophils, and a range of innate lymphocytes have key roles in defence, but also in tissue homeostasis and repair. The adaptive arm of the immune system takes time to develop and can generate highly specific responses following antigen recognition. Central to these responses are B and T lymphocyte receptors that are extremely diverse, and in the case of the B cell receptor, can evolve a higher affinity for antigen during the course of an immune response[9,10]. Adaptive immune responses take place in a network of specialised secondary lymphoid organs (that is, spleen, tonsils, and lymph nodes), and require the migration of immune cells into these structures from blood and lymph[11] (**Fig. 1**).

The earliest efforts of immunologists to understand this complex system involved categorisation of different immune cell subsets by virtue of their morphological characteristics, and as technology enabled, their expression of different molecular markers. These phenotypically and molecularly defined cell subsets were then assigned specific functions and anatomical locations on the basis of imaging studies and on *in vitro* and *in vivo* perturbation studies — the latter frequently using mice as a model system. Flow cytometry, the simplest single cell technology, has been used for several decades to assess the molecular composition of immune cells by measuring a handful of markers (typically less than 15) on individual cells, with extremely high throughput. Before the widespread availability of single cell transcriptomics techniques, transcriptomic studies of immune cells largely relied on the measurement of RNA (using gene microarrays or RNA sequencing[12,13] (**Box 1**) in heterogeneous, mixed cell populations, for example, in circulating peripheral blood mononuclear cells (PBMCs), or in aggregated subsets isolated by flow cytometry, magnetic bead selection or density gradient centrifugation. Developments in single cell technologies over the past decade have ushered a revolution in our ability to assess immune cell heterogeneity and function in a marker-free, unbiased manner[1,2]. Such approaches generate high-dimensional data **[G]** that enables cells to be grouped according to their expression of a large number of surface markers (>40 in mass cytometry, **Box 2**), or transcriptional signatures (via scRNAseq), or chromatin accessibility profiles (via scATACseq, **Box 3**). Emerging single cell technologies permit multiple sources of biological information to be uncovered in parallel, for example, simultaneous high-dimensional measures of transcript and protein abundance[14], or simultaneous measurement of transcript abundance and DNA sequence[15,16], or chromatin accessibility[17]. These approaches allow regulatory interactions to be probed and enable data-rich definitions of cell type and state.

Around 5 years ago, scRNAseq experiments began to probe the cellular constituents of tissues. These studies using cell sorting and droplet microfluidic-based methods allowed transcriptionally distinct murine splenic immune cell populations and lipopolysaccharide-induced transcriptional changes to be delineated[18], and highlighted the capability of this technique to characterise the temporal development of epithelial cell lineages in murine lung[19]. The evolution of droplet microfluidics **[G]** and cellular barcoding **[G]** methods enabled the population structure of developing and mature murine tissues to be dissected in an unbiased manner[20,21]. In the past couple of years these methods have paved the way for massive throughput scRNAseq experiments capable of generating cell atlas **[G]**-scale datasets of human and murine tissues containing in excess of 100,000 cells[22-25]. Currently available scRNA sequencing methodologies enable cellular or nuclear RNA of single cells to be isolated and profiled using multi-well plates or microfluidics (**Fig. 2**), and have been reviewed in depth elsewhere [23,26].

In addition to facilitating higher throughout scRNAseq methodologies, technological advances have enabled simultaneous single-cell surface protein and cellular transcriptome measurements using protocols such as cellular indexing of transcriptomes and epitopes by sequencing (CITE-seq)[14] or RNA expression and protein sequencing (REAP-Seq)[27]. These methods label cells with antibodies, in an analogous way to flow cytometry, however, the antibody is labelled with a DNA barcode rather than a fluorophore. This DNA barcode is captured at the same time as the RNA from the target cell, separated following the reverse transcription step, and used to prepare a separate cell surface protein-specific library. The DNA barcode of the antibody is captured along with the barcode that uniquely identifies each cell, enabling single cell quantification of cell surface protein expression. The major advantage of this technique over flow cytometry is that it can measure a much larger number of proteins. In flow cyometery, the spectral overlap of fluorophores limits the number of markers to around 15 proteins; mass cytometry experiments are limited by isotope availability to around 40 markers; however, a DNA barcode composed of 8 base pairs, yields up to 65,536 unique combinations, theoretically permitting thousands of proteins to be measured. To date, around a hundred proteins have been measured in a single experiment, and current experience suggests that the combined assessment of surface protein and cellular transcriptome data can facilitate cell clustering **[G]**[14]. Future advances should see the development of barcoded epitopes, to enable investigation of antigen-specific B and T cells.

These high-throughput approaches require tissue samples to be dissociated into single cell suspensions, often using both physical disaggregation and enzymatic digestion. This approach has a number of disadvantages. Firstly, the dissociation process can change the transcriptome and proteome of cells, for example, by upregulating stress response genes such as those that encode heat shock proteins [28]. Secondly, disaggregation of an organ results in loss of information relating to the spatial arrangement of cells and their anatomical localisation. The spatial arrangement of

cells is uniquely adapted to enable each organ to optimally function. For example, the cortex and medulla of the kidney contain different segments of the nephron, and have differing tissue environments, with the medulla providing both a hypersaline and hypoxic milieu for resident immune cells [29]. Therefore, the precise anatomical location of each cell in a single cell transcriptomic study must be considered in order to fully appreciate the effect of the local tissue environment. Two broad approaches can be used to marry spatial and transcriptional information in single-cell analyses (**Fig. 2**). The first is to link a cellular barcode to the spatial position of the cell using spatial transcriptomic protocols[30] (**Box 4**). Alternatively, standard confocal microscopy can be used to identify transcripts on tissue sections using RNA probes[31] (**Box 5**).

Alongside developments in spatial and high-throughput methodologies, the field has also seen rapid advances in the development of powerful and scalable computational methods to enable analysis of large single cell data sets generated by mass cytometry[32,33] and scRNAseq experiments[34]. These methods include tools to cluster data, computationally reconstruct developmental trajectories, and infer cell-cell communication networks[35]. Methods have also been developed to integrate analysis of single cell transcriptomic datasets generated in distinct experimental batches or using differing protocols[36-39]. Such methods will allow faithful alignment of cell types through removal of technical batch effects, and permit sensible comparisons of distinct experimental conditions and biological replicates.

Immunologists have been quick to apply medium and high-throughput scRNAseq approaches to human samples to address questions around immune cell heterogeneity, ontogeny, polarisation, and fate as well as to identify rare, previously unidentified cell subsets[1,40,41]. Although these experiments have focused on the most easily accessible immune cells —–those within the circulation — improvements in tissue processing are now enabling investigators to extend their remit to relatively rare tissue-resident immune cells[42], and to the study of developmental and disease states[43,44]. In the following sections we discuss studies that have used single-cell technologies to define immune cells across different cellular compartments, with a focus on human studies. These experiments illustrate the potential of these methodologies to address specific questions about the immune system and its role in kidney homeostasis and disease.

[H1] Mapping immune cells in blood

Peripheral blood represents the most easily accessible immune compartment in humans. Most immune cells, with the exception of T lymphocytes, develop in the bone marrow and migrate into the circulation once mature. As such, blood provides a readily available but incomplete view of whole organism immune status. As outlined in the below sections, the application of single-cell technologies to study peripheral blood leukocytes has the potential to deliver a number of insights into immune cell biology (**Fig. 3**). Moreover, since many kidney diseases arise from perturbations in systemic immune cell populations, better profiling of circulating immune cells might aid disease

diagnosis and prognostication, improve understanding of disease mechanisms and facilitate the development of biomarkers of disease activity.

[H2] Unbiased mapping of blood

Before the advent of scRNAseq, efforts to probe heterogeneity within circulating cell subsets relied on the use of known markers to identify and profile cell frequency, cell transcripts, and cell function. The availability of scRNAseq approaches allows researchers to profile circulating cells in an unbiased manner — an approach that has the potential to improve our understanding of the heterogeneity and relationships of circulating cells. One example is a study that performed scRNAseq of 68,000 PBMCs to delineate the global structure of lymphoid and myeloid cell populations in peripheral blood, highlighting the ability of this approach to characterise rare subsets, and providing a useful reference dataset to enable comparisons with disease states[22].

[H2] Identification of novel cell types

In addition to its ability to profile large volumes of cells in an unbiased manner, scRNAseq can provide insights into cellular heterogeneity within the circulating compartment, and identify novel cell subsets (Figure 3a). If the novel subsets are rare their identification may require pre-enrichment of a specific immune cell population of interest using known markers to ensure a reasonable number of cells are available for analysis. Once enriched, scRNAseq can be applied to a cell population to assess whether additional diversity, or indeed, rare novel immune cell subsets, exist. Much of the effort to date in this regard has focused on conventional dendritic cells (cDCs), which are important antigen presenting cells (APCs) that have a critical role in initiating adaptive immune responses by activating $CD4^+$ T cells[45]. Two major subsets of cDCs exist: cDC1 express CLEC9A and CD141 and cross-present antigen, whereas cDC2 express CD1c and function as classical APCs. A study that performed plate-based scRNAseq on 2,400 sorted mononuclear phagocytes (MNPs), including monocytes, cDCs and plasmacytoid dendritic cells (pDCs), to better understand cDC heterogeneity without relying on surface marker expression, identified dendritic cells corresponding to the previously described cDC2 and cDC1 subsets, as well as an additional inflammatory population of cDC2 cells[46]. This approach enabled the researchers to distinguish pDCs from a novel population of dendritic cells that expressed *AXL* and *SIGLEC6* (termed 'AS' dendritic cells). These AS dendritic cells were functionally and morphologically distinct from pDCs,.underscoring the ability of single cell technologies to identify rare novel immune cell subsets.

[H2] Characterisation of cell precursors

Immune cell precursors can be identified by assessing transcriptional similarities between immature immune cells in bone marrow and mature immune cells in blood, and by plotting transcriptional trajectories between developmental stages (Figure 3b). Most studies that have used

scRNAseq in this context have attempted to characterise the precursors of cDCs (pre-cDCs). cDCs arise from a bone marrow-derived progenitor cell — the common DC progenitor — that differentiates into a pre-cDC, which is the direct precursor of cDCs. A study that sought to address whether pre-cDCs were already polarized towards a cDC1 or cDC2 fate by performing scRNAseq on isolated cDC1 and cDC2 from peripheral blood and pre-cDCs from cord blood found that pre-cDCs consist of two distinct precursor populations that are committed to becoming either cDC1 or cDC2 [47]. A separate study that performed scRNAseq of flow cytometry-sorted cDCs identified a rare cDC progenitor in adult peripheral blood, which was proposed to give rise to pre-DC1 and pre-DC2[46]. The nature of circulating DC precursors was further refined by another study[48], which combined mass cytometry **[G]** (CyTOF) and scRNAseq to identify a human blood DC precursor, characterized by the markers CD123, CD33 and CD45RA. These pre-DCs shared surface markers with pDCs and had distinct functional properties that were previously attributed to pDCs. Moreover, tracing the differentiation of DCs from bone marrow to peripheral blood led to the identification of distinct lineage-committed subpopulations of pre-DCs[48].

[H2] Ex vivo perturbation studies

It is well established that immune cells within a specific subset do not respond homeogeneously to stimuli. Single cell technologies are ideal tools with which to delineate the mechanisms that underlie this heterogeneous response (Figure 3c). For example, transcriptional changes that occur in response to a stimulus can be analysed in single cells, as has been done in studies of lipopolysaccharide-stimulated murine splenocytes[18]. A more sophisticated approach used an innovative droplet-based microfluidic platform that combined single-cell cytokine analysis with scRNA-seq profiling to investigate the production of type I interferon by human peripheral blood-derived pDCs in response to stimulation with toll-like receptor ligands [49]. By modulating the droplet microenvironment, the researchers showed that production of type I interferon was limited to a small subpopulation of individually stimulated pDCs, and that this function was controlled by stochastic gene regulation. In fact, the pDC cytokine response was driven by a cell-autonomous type I interferon amplification loop[49].

[H2] Understanding cellular function

Early attempts to understand the nature and mechanisms of immune cell dysfunction in patients with systemic autoimmune diseases, including those that affect the kidney, predominantly involved transcriptional profiling of bulk PBMCs. These studies provided some insights into the molecular changes that occur in the context of immune cell dysfunction, for example, with the identification of a type 1 interferon signature in patients with systemic lupus erythematosus [50]. Further insights into the exact cell subset(s) that mediate disease-associated transcriptional changes were achieved by profiling populations in bulk after they had been isolated using flow cytometry. For example, one 2015 study showed that a CD8$^+$ T cell exhaustion signature identified patients with a better

prognosis and lower rate of relapse across multiple autoimmune diseases[51]. These studies generally included hundreds of cases and controls — a scale that currently prohibits the application of scRNAseq technologies to study peripheral blood samples, owing to the expense of this approach. However, as the cost of these technologies fall and throughput increases, it will become feasible to perform scRNAseq studies of peripheral blood samples from patients and healthy controls, with the potential to further resolve causative cell subsets and pathways (Figure 3d).

The first steps in this direction have already been taken. For example, a high throughput scRNASeq study of PBMCs leveraged the sequence variation present in transcriptomic data to differentiate host-derived and donor-derived cells amongst bone marrow mononuclear cells before and after allogeneic bone marrow transplantation[22]. This example highlights the potential of this approach to improve our understanding of the fates of host and donor-derived cells in the context of stem cell and bone marrow transplantation, or in the context of solid organ transplantation.

[H1] Immune cells in secondary lymphoid organs

Secondary lymphoid organs represent the sites at which adaptive immune responses are generated. This process requires precise spatial localisation of B and T lymphocytes and APCs in a dynamic manner that is orchestrated by stromal cells within the lymph nodes and spleen[11]. High dimensional single cell studies in secondary lymphoid organs in humans are sparse, although studies in mice illustrate the potential utility of these technologies to track antigen-specific B and T cell clones; to study immune cell subsets that do not circulate, but are limited to peripheral organs and secondary lymphoid tissues; and to probe interactions between the immune and stromal compartments of lymph node (**Fig. 4**).

[H2] Antigen-specific lymphocyte clones

B and T lymphocyte receptors are central to the generation of adaptive immune responses. The genomic regions that encode the antigen recognition domains of these receptors undergo extensive reorganisation events in a process termed V(D)J recombination, to generate extremely diverse repertoires of receptors that are capable of specific binding to self and non-self antigen. Over the course of an adaptive immune response, selection and expansion of antigen-specific lymphocytes ensures that the response is robust and specific. Specificity in antibody responses are further refined by the evolution of increased antigen affinity of B cell receptors and their secreted antibodies over the course of an immune response[10]. Clonal diversity at the single cell level can be assessed by RNA sequencing (Figure 4a)[40,52,53], and has been used to track the fates of distinct T lymphocyte clones through the time-course of salmonella and malaria infection in

murine models[40,54]. These methods have also gained traction for the study of immune system development. For example, T memory lymphocytes in human fetal gut exhibit clonal expansion when compared to their naïve counterparts, suggesting the existence of a tissue-resident adaptive immune architecture that is primed for post-natal colonisation[55]. Further application of these techniques is likely to assist our understanding of the clonal landscape of tissue-resident lymphocytes in immune development, homeostasis, infection, and in the context of allograft tolerance and rejection.

[H2] Profiling non-circulating immune cells

Some immune cell subsets are present in very small numbers within the circulation, or do not circulate, remaining within tissues or secondary lymphoid organs. Therefore, studying secondary lymphoid organs directly represents a useful strategy to better understand the biology of these cells (Figure 4b). These non-circulating immune cells include subsets of resident macrophages that have specialised functions within lymph nodes and spleen, such as lymph node subcapsular sinus macrophages **[G]**, splenic red pulp macrophages **[G]** and marginal zone macrophages **[G]**. In addition, many innate lymphocytes have a very limited presence in the circulation, which has prompted investigators to focus their efforts on lymphoid organs. One study mapped the transcriptional profile of natural killer (NK) cells and the three canonical subsets of non-cytotoxic innate lymphoid cells **[G]** (ILCs) among flow-sorted lymphocytes that expressed CD127 but not antigen-specific T cell receptors from human tonsils,.and demonstrated heterogeneity amongst ILC3 cells[56]. Another study that applied scRNAseq to splenic and blood NK cells reported substantial transcriptional heterogeneity within blood and splenic NK cell populations from both mice and humans, but identified two broad NK cell subsets that were conserved across organs and species[57]. These NK populations exhibited divergent functional profiles, with 'NK1' cells demonstrating enrichment for a cytotoxic profile, and 'NK2' cells enriching for a chemokine expression profile, expressing the conserved dendritic cell chemokine gene *XCL1* [57].

[H2] Immune-stromal cell interactions

Stromal cells within lymph nodes and spleen have a critical role in organising immune cells into specific niches to enable the sequential immune cell interactions that are required to generate an effective immune response[58]. The positioning of B cells within follicles is orchestrated by follicular dendritic cells and marginal reticular cells — a stromal cell subset that produces B cell-attracting chemokines such as CXCL13, and cytokines such as BAFF, which promote B cell survival[59]. In the T cell zone, CCL21-expressing and CCL19-expressing stromal cells, termed T-zone reticular cells (TRCs), attract CCR7-expressing lymphocytes[60]. One study that used droplet-based scRNAseq to study sorted CD45 and CD31 double-negative murine lymph node stromal cells under homeostatic conditions and following viral challenge, identified nine clusters of stromal cells[61]. These clusters included known subsets, but also included several novel subsets. Specifically, the researchers

noted heterogeneity within the TRCs, with a population of CCL19^{low} TRCs located at the perimeter of the T cell zone and CXCL9-positive TRCs within the T-zone and interfollicular region, as well as CD34-expressing stromal cells in the capsule and medullary vessels, indolethylamine N-methyltransferase-positive stromal cells in the medullary cords, and a population of *Nr4a1*-positive stromal cells that were more broadly distributed. Their work further refines our understanding of stromal cells within the lymph node, and suggests that some subsets seem to be in an activated state, even in homeostasis (Figure 4b)[61]. Extending these studies to human lymph node and spleen will improve our understanding of how to promote or inhibit different elements of the adaptive immune response, potentially those that contribute to autoimmune or allo-immune responses.

[H1] Immune cells in non-lymphoid organs

All organs, including the kidney, contain a network of immune cells that mediate responses to damaged tissue and microbial challenge and contribute to the maintenance of organ homeostasis. Much of our knowledge of tissue-resident immune cells has been obtained from studies in mice[62]; much less is known about human tissue-resident immune cells owing to the limited availability of fresh tissue samples. An additional challenge is presented by the small number of tissue-resident immune cells relative to those in epithelial and endothelial compartments. Therefore studies that measure the transcriptome of bulk tissue samples are likely to miss important immune cell-specific transcriptional signatures, as these are dominated by the strong signal obtained from the more numerous epithelial cells. scRNAseq can overcome this problem. Studies in mice indicate that the non-immune compartment of organs that interface with the environment, such as gut and skin, might also contribute to pathogen sensing and tissue defense[63]. Moreover, cross-talk also occurs between epithelial cells and immune cells, for example, to orchestrate the specific anatomical positioning of immune cells via chemokine production, as observed in the kidney[29]. Indeed, local immune responses and subsequent tissue repair are the consequence of the coordinated interaction between immune and non-immune cells[64]. scRNAseq studies of organs, such as the kidney, under homeostatic conditions have potential to reveal the true heterogeneity of immune cell populations within non-lymphoid organs, aid our understanding of immune cell ontogeny and adaptation to tissue environment, and their interactions with non-immune cells within the organ environment (**Fig. 5**).

[H2] Immune cell heterogeneity

Studies of immune cells in human organs have frequently relied on the application of microscopy to biopsy or post-mortem samples. However, this approach is limited in its ability to assess the heterogeneity of immune cells within a sample by the number of markers (<4) that can be simultaneously assessed. Microscopy also relies on the availability of specific antibodies for known

immune cell markers that bind to fixed tissue sections. Inevitably, this limitation has resulted in an incomplete appreciation of the heterogeneity of immune cell subsets that are resident within human organs. scRNAseq has the potential to deliver an unbiased assessment of tissue-resident immune cells, but might require a selection step, for example, using flow sorting, magnetic bead selection, or density gradient centrifugation to enrich for rarer populations within a sample (Figure 5a).

A good example of the power of this technology to generate a comprehensive map of immune cells within an organ is provided by a 2019 study of human lungs[65]. By performing scRNAseq on biopsy samples from different parts of the airways, the researchers were able to identify neutrophils, mast cells, macrophages, dendritic cells, NK cells and B and T lymphocytes, as well as a novel subset of migratory CD4^{+} T cell with features of both circulating memory cells and tissue-resident memory cells. The study also confirmed the presence of the pulmonary ionocyte, a lung epithelial cell population previously identified by scRNAseq studies that uniquely expresses the *CFTR* gene (that is, the gene mutated in cystic fibrosis)[66,67]. This small population of cells are now thought to represent the major source of physiological CFTR activity in the airway epithelium, with important implications for cystic fibrosis research.

Similarly, a study of five healthy human liver samples that sequenced 8,444 cells led to the identification of 20 discrete cell populations of hepatocytes, endothelial cells, cholangiocytes, hepatic stellate cells, B cells, plasma cells, conventional $\alpha\beta$ CD4^{+} and CD8^{+} T cells, $\gamma\delta$ T cells, NK-like cells, and distinct intrahepatic mononuclear phagocyte populations[68]. The latter included two CD68-expressing macrophage populations, one with a more pro-inflammatory and the other a more tolerogenic transcriptional identity.

Together these studies illustrate the potential of scRNAseq to describe immune cell heterogeneity in healthy tissues, and provide a platform to investigate how these cells contribute to organ homeostasis, and how they change in disease.

[H2] Immune cell ontogeny

Fate-mapping studies in mice have established that tissue macrophages can originate from yolk-sac or fetal liver progenitors that are seeded pre-natally or from haematopoetic stem cells and are continually replenished from the circulating monocyte pool[69,70]. As described for circulating cells, quantification of transcriptional similarities and trajectories between potential precursors and differentiated tissue macrophages based on scRNAseq data can enable macrophage ontogeny to be inferred (Figure 5b).

[H2] Tissue specification

The environment generated by the homeostatic function of each organ in the body is unique, and affects the basal activation state and transcriptome of immune cells resident within the organ. For example, immune cells in the gut must adapt to their close proximity to trillions of microbes within

the intestinal lumen, whereas those within the medulla of the kidney are exposed to a markedly hypersaline environment. Studies of mouse macrophages show that tissue-specific transcriptional signatures are adopted by macrophages residing within different organs as a consequence of cues from the local cellular neighbourhood and by sampling of the surrounding milieu[71]. A comparison of immune cell subsets in different organs using scRNAseq will provide even greater insights into the granularity of organ-specific signatures of tissue-resident immune cells (Figure 5c). Moreover, the ability to perform such studies on cells from human organs is now a realistic prospect, since the infrastructure to take multiple samples from organ donors has been established in a number of centres[72].

[H2] Immune cell interactions

Tissue-resident immune cells occupy a specific niche, in which surrounding cells produce the chemokines required to attract and contain them, as well as the survival factors needed to stay viable. Immune cells that arrive from the circulation must also interact closely with the vascular endothelial cells within the organ. In addition, murine studies over the last few years have shown that some tissue resident immune cells interact closely, and may even directly connect, with electrically excitable tissue. For example, cardiac macrophages form gap junctions with the specialised cardiomyocytes of the cardiac conducting system to facilitate electrical conduction [73]. In the gut, macrophages interact with enteric neurons to promote peristalsis **[G]**[74,75], with some transcriptionally distinct macrophage subsets in close apposition with the tissue vasculature[75]. scRNAseq provides a means to interrogate the basis for these interactions, since a catalogue of receptor and ligand expression can be generated for every cell type, and interactions inferred from these data (Figure 5d). A number of investigators have now used this approach to study the nature of immune cell interactions within tissues. For example, use of scRNAseq to explore ligand–receptor cross-talk within developing mouse lung tissue demonstrated that lung-resident basophils are primed by signals (IL-33 and GM-CSF) produced within the lung environment, whereupon the basophils adopt a distinct resident transcriptional signature, which enables them to support alveolar macrophage development[76]. Another study that used using scRNAseq to characterise the murine intestine epithelium within the gastrointestinal tract demonstrated a variety of subtypes of cells and shed light on how these cells maintain homeostasis and interact with pathogens [77]. Furthermore, this study identified two specific subsets of tuft cells, one of which expressed *Tslp*, a key molecule that promotes T helper (Th) 2 responses by Th2 CD4^{+} T cells and ILC2s[77], which mediate epithelial-immune cross-talk.

Other large-scale profiling studies are poised to deliver analogous insights into the signalling networks that operate other organs. To date, the most comprehensive effort to interrogate cell-cell communication in a human organ system was done using human placental tissue. Use of scRNAseq to map signalling networks within human placenta at single cell resolution distinguished an array of maternal and fetal, as well as immune and non-immune cell types [35]. The researchers

involved in that study also generated a tool, called CellPhoneDB (https://www.cellphonedb.org/), to interrogate the expression of ligand-receptor pairs on different cell types to facilitate the analysis of immune cell interactions with other tissue-resident cell types. . This tool is now publically available for application to scRNAseq datasets.

[H2] Anatomical localisation

As detailed earlier, high-throughput scRNAseq and mass cytometry experiments utilise disaggregated tissue samples, with inevitable loss of information regarding the anatomical location of cells within organs (Figure 5e). Numerous examples show that specific positioning of immune cells within organs is needed to optimise organ defence and function. In the skin, for example, DCs are located adjacent to hair follicles to enable optimal sampling of skin commensals [78] whilst kidney macrophages with a high phagocytic capability are located in the medulla and pelvis, poised to deal with bacteria ascending from the bladder[29]. Experiments using dissociated tissue samples can be resolved at the macro-anatomical scale through reference to the biopsy position; however, spatial transcriptomic methods will be required to understand how specific cell populations interact in three dimensional space.

[H2] Immunity in ageing and disease

scRNAseq also has the potential to address a number of important questions about how immune cells within tissues change during ageing and disease. Several examples exist in which investigators have compared the transcriptome of single cells from diseased or aged tissues with that of cells from healthy or young tissues. A 2019 study that used paired mass cytometry and scRNAseq to explore the cellular landscape of inflammed and non-inflamed ileum from patients with Crohn's disease demonstrated an enrichment of inflammatory macrophages and mature dendritic cells in inflamed tissue, with organisation of a module of pathogenic cells through putative signalling from inflammatory macrophages[79]. The expression of genes associated with this cellular module was associated with resistance to anti-TNF therapy, potentially providing a framework for stratifying patients at diagnosis[79]. A separate study of patients with rheumatoid arthritis performed scRNAseq on more than 20,000 synovial cells taken at the time of arthoplasty. This approach identified 13 cell clusters, including $CD4^{+}$ and $CD8^{+}$ T cells, B cells, plasma cells, NK cells, macrophages, dendritic cells and mast cells, as well as several different types of fibroblasts. Inflammatory cytokine gene modules were enriched in immune cells, and a subcluster of NK cells was found to have high expression of XCL1 and XCL2 — chemokines that have previously been shown to regulate matrix metalloproteinase secretion by fibroblasts[43], suggestive of cell-cell interactions that potentially drive disease.

In contrast to tissues with mucosal, external facing environments, the central nervous system (CNS) represents a sterile, immune privileged site. Microglia represent the resident mononuclear phagocyte population in the human CNS, and are thought to have a variety of roles in health and

disease. scRNAseq has been used to profile mouse and human brain cells, allowing the identification and characterisation of microglia and perivascular macrophages[25,80,81]. This approach has been extended to a mouse model of Alzheimer's disease, enabling the identification of a distinct population of microglia associated with neurodegeneration termed 'disease associated microglia' (DAM)[82]. These cells are postulated to sense and respond to neurodegeneration, and elicit a protective response [82]. Interestingly, scRNAseq of immune cells within cerebrospinal fluid of patients with HIV infection identified a rare population of microglia that were enriched for the same DAM signature identified in mice with Alzheimer's disease [44]. Efforts to better characterise the diverse microglial populations within the human brain, and their association with disease are on-going. For instance, one study used massively parallel scRNAseq **[G]**, single-molecule fluorescence in-situ hybridization, and immunohistochemistry to interrogate the transcriptional and protein profile of microglia in mice and humans, in development, health and disease[83]. In an analysis of cortical microglia from healthy human brain and from the brains of patients with multiple sclerosis, researchers identified seven clusters of microglia, including two that were enriched in, and one that was exclusive to, the brains of patients with multiple sclerosis[83]. These data show that even in health, there is heterogeneity within the microglial compartment in humans as well as transcriptionally distinct disease-associated subsets. A separate study, published in preprint form, that profiled microglial populations from healthy brains and from brains of patients affected by a number of diseases including Alzhiemer's disease and multiple sclerosis, demonstrated disease-associated diversity in microglial transcriptomes, consistent with the DAM expression profiles identified in mice[82,84]. The same research group also examined microglia from elderly individuals and identified a set of genes that are preferentially expressed by microglia in the aged human brain and enriched in susceptibility genes for Alzheimer's disease and multiple sclerosis [42].

Tumours represent unique disease states and alter the tissue milieu to create an environment that suppresses immune cell activation. There is a great deal of interest in using single cell technologies to better understand the mechanisms that underpin this phenomenon. For instance, a number of groups have combined scRNAseq with T cell receptor reconstruction **[G]** to better understand the mechanisms that regulate tumour-associated T cell clonality and dysfunction in various cancers, including in hepatocellular carcinoma, ovarian and colorectal cancers[85-87].

These studies together illustrate the potential of single cell technologies to provide important insights into tissue immunity in health and disease, and provide interesting concepts that can be applied to the study of human kidney immunity.

[H1] Kidney immunity and disease

[H2] Kidney immune populations in health

The kidneys have a critical role in the maintenance of whole-body homeostasis, by excreting waste and acid and maintaining electrolyte and water balance. They also produce a hormone (erythropoietin) that stimulates the production of red blood cells to prevent anaemia, and an enzyme (1α-hydroxylase) that generates the active form of vitamin D to preserve levels of calcium and phosphate, and bone health. A variety of cells in the kidney — including epithelial, mesangial, endothelial and neuronal cells, as well as a network of immune cells — interact to maintain normal kidney function. Within the kidney tissue environment,.substantial regional differences exist as a result of its homeostatic functions (**Fig. 5E**). In particular, a high interstitial sodium concentration is established within the medulla and inner regions of the kidney, primarily as a result of ion transport within the loop of Henle, which is required for the kidney to achieve its homeostatic function of water reabsorption[88]. The kidney is also a dynamic environment, with modulation of the intrarenal sodium gradient depending on physiological need. For example, in response to dehydration and elevated serum osmolality, vasopressin secreted by the posterior pituitary increases the reabsorption of free water by the kidney and generates a further increase in interstitial sodium concentration in the medulla[89,90]. As well as differences in salinity, marked regional differences in oxygenation are also evident. The blood supply to the distal nephron varies according the degree of vasoconstriction in the afferent glomerular arteriole, and this supply, together with the high metabolic demands of the tubular cells as they transport electrolytes, glucose and amino acids from the tubular space to the interstitium, leads to varying levels of hypoxia in the medulla. These environmental conditions are likely to have powerful roles in shaping the immune architecture of the organ, either through direct effects on immune cells, or mediated by epithelial, endothelial, or stroma cells.

The first attempt to provide a transcriptional map of cells within the kidney was performed in healthy mouse kidneys. Using unbiased scRNAseq approach, Park and colleagues identified major subtypes of nephron epithelial cells, including podocytes, proximal tubule epithelial cells, Loop of Henle, distal tubule, and collecting duct cells[91]. The collecting duct cell populations contained a novel transitional cell type that was capable of interconverting between intercalated and principal cells, confirming findings in a preceding small-scale scRNAseq study of murine collecting duct cells[92]. Importantly this study highlighted the diversity of immune cells present in the murine kidney, including resident macrophages, neutrophils, B and T lymphocytes, and natural killer cells[91]. These findings were complemented by a further study that used scATACseq to assess the chromatin accessibility profiles of approximately 100,000 single cells from 13 murine tissues, including epithelial, endothelial and immune cells in the kidney[93]. This study showed that tissue macrophages from kidney, heart, and liver demonstrated common patterns of chromatin accessibility, which were distinct from those of alveolar macrophages and microglia.

To date, efforts to comprehensively map immune populations in the kidney in health are limited. One study published in preprint form, that aimed to provide a census of cell types in the human kidney by generating transcriptional data from 22,000 cells, reported a heterogeneous set of immune cells, including B cells and plasma cells, two subsets of myeloid cells, T cell, NK cells, and mast cells[94]. Our group has also undertaken a large-scale study of human kidneys, using scRNAseq to profile more than 67,000 cells from 14 kidneys across the human lifespan, as well as 6 fetal human kidneys [95]. Our findings confirm the presence of a complex immune landscape within the human kidney, with dominant representation of MNPs, NK cells and T cells. Fine clustering of the myeloid compartment demonstrated two populations of monocyte-derived macrophages — one with transcriptional similarity to $CD14^+$ classical monocytes, and the other to $CD16^+$ non-classical monocytes, as well as a distinct tissue-macrophage population, which is enriched for an anti-inflammatory M2 transcriptome expressing CD206. DCs in the human kidney predominantly expressed markers consistent with a cDC2 phenotype, such as CD1C. We also considered the macro-anatomical localisation of immune cells within the kidney by referencing it to publically available bulk RNAseq data generated from biopsy samples taken from known regions of the kidney. This analysis showed a differential distribution of immune cell subsets between cortex versus the medulla and pelvis. Analysis of ligand–receptor interactions predicted pelvic enrichment of neutrophils and anti-bacterial mononuclear phagocytes due to the pattern of chemokine expression in the pelvic epithelial cells. Using a mouse model, we confirmed that these interactions specifically localised neutrophils to the region of the kidney first encountered by ascending bacterial infection[95]. Thus, our study shows that the human kidney represents a highly organised immune environment, with different regions demonstrating functional specialisation, which are likely adapted to the dominant immunological challenge.

Another group used mass cytometry to profile immune cells in human kidney samples [96]. Although this study focused on renal cell carcinoma (RCC), the researchers also performed mass cytometry on healthy renal tissue as a comparator. Use of a complementary set of marker panels enabled dissection of T cell and myeloid cell heterogeneity. The study findings demonstrated an enrichment of CD4+ central memory T cells, CD4+ and CD8+ effector memory T cells in normal kidney samples; however regulatory T cells and T cells that expressing the exhaustion marker PD-1 were absent from normal samples, suggesting an enhanced degree of immunosuppression in the cancer microenvironment. The researchers also observed classical and non-classical monocytes in normal kidney tissue, and mirroring our single cell transcriptional census, a population of CD206-expressing, M2 polarised tissue-resident macrophages in healthy tissue [96].

Studies of the human developing kidney have revealed not only patterns of epithelial cell development, but also populations of tissue resident immune cells — which likely represent mononuclear phagocytes — with high expression of MHCII genes, at 87-132 days gestation[97].

Consistent with studies showing early colonisation of the fetal kidney with macrophages, studies in human fetal kidneys show a cluster of immune cells that express the MHC class II genes, *IL1B*, and *FCER1G*[98,99]*.* Our scRNAseq study shows that macrophages dominate the resident immune cell compartment of human fetal kidneys in the first trimester, but that from post-conception week 9 onwards, other immune cell subsets, including DCs and lymphocytes, increase[95]. These findings are consistent with data from mouse studies, which suggest that tissue-resident macrophages in the kidney do not solely originate from colonisation by bone marrow-derived monocytes, but might be seeded early in embryonic development from erythromyeloid precursors in the fetal yolk sac or liver [69,70,100].

[H2] Kidney immune populations in disease

The kidneys can be affected by a number of prevalent and serious conditions including acute kidney injury (AKI), glomerulonephritis, ascending infection (pyelonephritis), and cancer. In each of these conditions, recognition and response by the immune system to pathogenic or danger signals, or to malignant cells is critical. Furthermore, in the context of kidney transplantation, donor-derived tissue-resident immune cell populations in the kidney can be replaced by recipient cells, particularly during rejection [101]. Maintenance immunosuppression can also affect the phenotype, transcriptome and function of resident immune cells.

Renal biopsies play a central part in nephrology diagnostics, and represent a potentially useful source of tissue with which to interrogate intra-parenchymal populations of immune cells and determine how cell states and frequencies change with disease onset and progression. One challenge presented by this approach is that percutaneous biopsy obtains only a relatively small sample of tissue and only the outer kidney cortex is routinely sampled. Despite these caveats, a number of groups have begun to optimise methodologies to enable scRNAseq to be performed on clinical biopsy samples (summarised elsewhere[102]).

[H3] Lupus nephritis

One early effort to perform scRNAseq on kidney biopsy samples used kidney (n=16) and skin (n=12) biopsy samples from patients with lupus nephritis, as well as 5 skin biopsy samples from healthy individuals, generating data on 899 cells[103]. In the epithelial compartment, the researchers uncovered the expression of a type 1 interferon signature in both renal tubular cells and skin keratinocytes from patients with active lupus nephritis, highlighting the potential utility of using more easily accessible skin samples to assess systemic disease activity[103]. The number of immune cells identified in these kidney samples was very limited, but included a handful of T cells and myeloid cells.

Efforts are now underway to create a detailed map of immune infiltrates in kidneys of patients with lupus nephritis, using strategies to enrich for immune cells prior to sequencing. Data from the

Accelerating Medicines Partnership network, suggest that kidneys of patients with lupus nephritis contain a rich landscape of immune cells, including inflammatory and phagocytic macrophages, DCs, NK cells and a range of memory T cells[104,105]. A subset of B cells and plasma cells – the sources of autoantibody – expressed a type 1 interferon signature, implicating these cells as central players of disease pathogenesis[104]. Further studies may enable stratification of patients based on the dominant features of their single-cell transcriptomic profiles, and provide insights into whether particular B cell clones become expanded at the site of tissue injury, which would affect the ability to target these cells therapeutically.

The Accelerating Medicines Partnership network has also demonstrated the feasibility of performing scRNAseq on cells, including immune cells, from urine samples[104], providing a potentially attractive means of assaying kidney immune cells in a non-invasive manner. Mirroring the methodological approach used by Park *et al.*[91], the Accelerating Medicines Partnership network have attempted to integrate information on disease susceptibility genes identified from genome wide association studies (GWAS) of patients with SLE, with information on the expression of these genes in cells obtained through scRNAseq of biopsy samples from patients with lupus nephritis[104]. Similarly, Park and colleagues mapped susceptibility genes identified from GWAS and other genetic studies to gene expression profiles of single cell clusters obtained by scRNAseq of mouse kidneys, finding cell type-specific expression of disease-associated genes[91]. This approach therefore provides an additional means by which scRNAseq data can be leveraged to better understand the cell subsets that underlie disease pathogenesis.

[H3] Transplantation

A different study compared the efficacy of two high-throughput scRNAseq systems, Drop-Seq **[G]**[20] and inDrop **[G]** [21], to generate single-cell transcriptomes from human kidney biopsy samples. Superior results were ultimately generated with inDrop, which the researchers then used to perform scRNAseq of 4,487 single cells obtained from a biopsy sample of a kidney allograft with acute antibody-mediated rejection (ABMR), and nuclear RNA sequencing of 4,259 single cells obtained from a healthy control kidney[106]. In the allograft sample, the researchers identified three distinct clusters of endothelial cells, two of which seemed to be activated, with upregulation of Fc receptor pathway **[G]** components consistent with the diagnosis of ABMR. Monocytes, B cells, plasma cells and T cells were evident in the immune compartment, with a gene expression pattern in CD16-negative monocytes suggestive of dendritic cell maturation[106]. The presence of plasma cells is compatible with the local generation of donor-specific antibodies, which has therapeutic implications. Lymphoid aggregates or tertiary lymphoid follicles have been previously described in transplant biopsy samples in the context of AMBR[107], and these immune cell aggregates might be refractory to systemically-administered monoclonal antibodies due to poor accessibility into the local niche, and the presence of local pro-survival factors[108]. Notably, immune cells were not

identified in the healthy control kidney sample used in this study; the absence of these cells could relate to the differing platforms (nuclear sequencing versus cell sequencing) used to generate these data, and the relatively modest number of nuclei sampled.

[H3] Kidney cancer

Immune infiltrates are also present within kidney tumours, but their phenotype and function might be altered by the tumour microenvironment. The extent of immune cell infiltration is an independent predictor of poor prognosis in patients with RCC[109], emphasising the importance of tumour-immune cell interactions. One study used a mass cytometry approach to identify exhausted and regulatory T cells within RCCs, as well as an assortment of macrophages that expressed markers associated with pro-tumour and anti-tumour phenotypes[96]. The frequencies of exhausted T cells correlated with the frequencies of CD38+ tumour-associated macrophages, and associated with progression-free survival, highlighting the opportunity to use single cell technologies to further understand how tumours manipulate immunity in progression and metastasis. We have used scRNAseq to compare the transcriptional profiles of cells within kidney tumours to those of tubular epithelial cells in normal paediatric, adult and fetal kidneys, and identified the likely cells of origin that lead to development of Wilm's tumour and RCC[110]. This study also revealed a population of RCC-associated macrophages that express *VEGFA*, the protein product of which promotes and is a target of modern treatment regimens for this malignancy[110]. This study illustrates the ability of scRNAseq to address questions of tumour ontogeny, as well as identify putative pathophysiological mechanisms and cell signalling networks that may be amenable to pharmacological treatment.

[H1] Conclusions

Single cell technologies are poised to transform our understanding of immunology and of human biology more broadly. The data generated by studying human blood, secondary lymphoid organs and tissues throughout the body, including the renal tract, could potentially form part of the Human Cell Atlas, an international effort that aims to produce a comprehensive and systematic reference map of human cells of the human body, across age, in health and disease, that will be freely accessible to researchers [111]. Ultimately, such a resource will provide a reference of cell states that the biomedical research community can apply to a range of biological questions. In nephrology, the translational potential is obvious. A better understanding of kidney cell heterogeneity and how this heterogeneity changes through development and disease will assist with drug target identification, and improve prediction of off-target effects. The application of single cell technologies to clinical renal biopsy samples, or to cells in urine, will improve diagnostic accuracy in nephrology and transplantation and assist with personalised prognostication. In systemic autoimmune diseases that affect the kidney, analysis of peripheral blood and secondary lymphoid organs will yield similar insights and help unravel the cause and nature of the breakdown in self-tolerance. Finally, in

regenerative medicine, single cell analyses will enable a better understanding of how *in vitro* cell differentiation compares to the state of cells *in vivo*, facilitating the development of replacement tissues or even organs. Together, these data will have the potential to transform biology and medicine in an analogous way to the Human Genome Project, ushering in a new era of understanding of the processes underlying physiology and disease.

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Related links:

CellPhoneDB: https://www.cellphonedb.org/

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Author contributions

The authors researched data for the article, contributed to discussion of the article's content, writing and review/editing of the manuscript before submission.

Competing interests

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Key points

- Single cell technologies have enabled the mapping of immune cell populations in the kidney, the circulation, and secondary lymphoid tissues in unprecedented detail.
- A variety of single cell technologies have become mainstream over the last 5 years, including high-throughput single-cell RNA sequencing (scRNAseq), single cell chromatin accessibility assays, and mass cytometry.
- scRNAseq has enabled researchers to interrogate the transcriptional diversity present in specific cell populations, for example in circulating dendritic cells and monocytes, and create large scale atlases profiling the landscape of tissues.
- Using trajectory analysis, single cell methods can reveal snapshots of dynamic processes such as cellular differentiation and responses to different immune stimuli.
- Analysis of scRNAseq data enables an assessment of how antigen-specific B and T lymphocyte clones expand in vivo in different tissue and disease states.

- scRNAseq data also enables ligand–receptor interactions to be explored in an unbiased manner, allowing novel cell signalling networks to be identified.
- Single cell studies have also uncovered disease-associated cell states and gene expression profiles, deepening our understanding of disease mechanisms and potential identifying therapeutic targets.

Box 1 – Principles of RNA sequencing

There are over 20,000 genes in the human and mouse genome, but not all are transcribed in any given cell. RNA sequencing (RNA-Seq) estimates the expression levels of different genes by measuring the abundance of RNA transcripts. This is achieved by first converting the RNA to cDNA. This RNA can be derived from whole tissue, sorted cell subsets or even single cells. Adapters are added to the cDNA to enable sequencing and indexes added for multiplexing of samples during the preparation of the cDNA library. The resulting cDNA is amplified by PCR before being pooled and sequenced on high throughput platforms such as the Illumina platform. These systems use the ‘sequencing by synthesis’ method, whereby complementary fluorescently tagged base pairs are added to the target to be sequenced during the sequencing reaction. After each round of addition, the base pair that has been incorporated is revealed by its fluorescent tag, and over many cycles the sequence of the cDNA is identified. The addition of the index barcodes and the fixation of each cDNA library to a flow cell within the sequencer enables multiple sequences to be read simultaneously. This process generates millions of short sequencing reads (50–250 basepairs), which are then computationally aligned to the genome. These aligned reads are counted with the number of reads for each gene corresponding to the original level of expression of RNA for that gene within a given sample (see figure).

RNASeq studies of tissues or sorted cell populations usually generate data on around 20,000 gene transcripts, but due to the lower amount of RNA available and the reduced transcriptional complexity of single cells, single cell RNASeq (scRNAseq) technologies detect only 500-5000 transcripts per cell. In addition, all current droplet-based single cell sequencing approaches do not sequence the full length of the transcript so alternative transcripts cannot be measured. Full length transcript sequencing can be performed in plate-based single cell protocols such as smart-seq2; however, these protocols have a lower throughput than droplet-based approaches. Finally, due to the lower RNA abundance within a single cell compared to larger cell numbers or whole tissue and the stochastic nature of RNASeq, scRNAseq does not capture all the available transcripts for each cell, resulting in different transcripts being present or absent — a phenomenon known as drop-out. As scRNAseq technologies continue to develop, the number of genes detected per cell is increasing.

Box 2 – Principles of mass cytometry

Mass cytometery, or CyTOF, replaces the fluorophore tag present on antibodies bound to target proteins on the cells of interest with a heavy metal tag. The labelled single cells are then segregated into droplets using a nebulizer and ionised and atomised using an inductively coupled

plasma (ICP) torch to yield a mix of heavy reporter ions and lighter ions derived from the cell and contaminants. These lighter ions are removed using a quadrupole before being passed to a time of flight mass spectrometer. Here, dependent on the time of flight, both the heavy reporter ion tags from the labelled antibodies along with a heavy tagged DNA intercalation agent (used to identify single cells) are identified. The abundance of each tag within each collection of heavy ions originating from a single cell is used to measure the expression of each target protein (see figure). This readout is used for downstream analysis taking both a classic analysis approach similar to low dimensional flow cytometer but also a higher dimensional approach similar to that used for single cell RNA sequencing.

Advantages:

The use of heavy metal tags eliminates the problem of spectral overlap, greatly increasing the number of parameters that can be simultaneous measured in a single cell (40-50 markers, theoretically, up to 100), compared with flow cytometry (5-15 markers).

Disadvantages:

The mass spectrometer is expensive, requires specialist maintenance, and allows lower throughput than a flow cytometer.

Box 3 – Principles of ATAC sequencing

ATAC-sequencing identifies accessible regions in the genome. DNA is packed in a complex three-dimensional structure, wrapped around nucleosomes, and condensed to form chromatin. The genome in any given cell is organized into regions accessible to transcription machinery, and condensed, inaccessible regions.

Initially nuclei are isolated, with chromatin structure intact. ATAC-seq utilizes the prokaryotic Tn5 transposase to insert sequencing adapters and fragment DNA at accessible regions. Fragments of DNA, which are ligated to adapters are then isolated, undergo PCR amplification, and are sequenced. Sequencing data is then aligned to a reference genome, enabling peaks to be called at discrete sites in the genome.

In a similar fashion to scRNAseq, single-cell ATAC-seq (scATACseq) can be performed in small volume reaction chambers (plate or microfluidic devices), or using a barcode-droplet microfluidics approach. These methods generate barcoded DNA fragments allowing the cellular origin of each read to be ascertained (see figure).

Box 4 – High dimensionality spatial transcriptomics

Frozen tissue is sectioned and placed onto a barcode matrix. Each barcoded area measures 10x10 µm, which will include several cells. The slide is imaged and then cells are lysed onto this matrix and the barcodes incorporated into the cDNA. Following further library preparation and sequencing, the resulting data can then be tracked back to the original image thus providing the spatial data.

Advantages: Transcriptional artefacts introduced by the tissue disaggregation process are avoided.

Limitations: This technology is not yet at a single cell resolution, but provides transcriptional information on 100 $µm^2$ regions. Due to the lysis of the tissue onto the slide the transcriptome is only viewed in 2 dimensions and any z-information is lost.

Box 5 – Low dimensionality spatial transcriptomics

These methods use standard confocal microscopy to identify RNA transcripts on tissue sections using RNA probes. This can be achieved through fluorescence in-situ hybridisation (FISH) based methods, where multiple probes bind to a target RNA of interest along its length. Multiple methods are suitable for low dimensional spatial transcriptomics such as RNAScope, which can detect 3-6 transcripts simultaneously, or ‘strip and probe’ methods including osm-FISH. In the latter method, around 4 different RNA transcripts can be detected after each round of probe application, and multiple rounds can be performed, increasing the potential number of RNA targets that can be detected. The initial paper describing osm-FISH used the strip and probe methods to sequentially detect 33 different transcripts in in a single image compilation of the mouse somatosensory cortex[31].

Advantages: By combining a probe-based approach with high throughput scRNA methods, specific cell markers identified by the latter can be interrogated on sections to determine the physical location of cells using unique marker genes. This approach also provides information of cellular or even subcellular resolution in three dimensions.

Limitations: Limited number of markers and low throughput.

Figure 1 - Localisation of immune cells in the circulatory system, secondary lymphoid organs and non-lymphoid organs. Leukocytes comprising cells from the innate and adaptive arms of the immune system circulate in the blood and lymph, and traffic to secondary lymphoid organs (that is, the spleen and lymph node). Tissue-resident leukocytes are found in non-immune organs such as the kidney where they have roles in organ defense, homeostasis, and disease. These cells may infiltrate tissues from the circulation, self-renew *in situ*, and traffic to secondary lymphoid organs on activation.

Figure 2 - Single cell RNA sequencing technologies. Samples of tissue (for example, kidney biopsy samples), can be disaggregated into a suspension of single cells or single nuclei before being subjected to scRNAseq by either a droplet-based approach in which single cells or nuclei are encapsulated within droplets that contain barcoded beads and the reaction mix, prior to RT-PCR and sequencing, or a plate-based sequencing approach whereby isolated cells or nuclei undergo reactions within the wells of a large microwell plate. Single cells can either undergo a reaction in each well, or a split-pool barcoding approach is used (eg SPLIT-seq). Disaggregation of tissue destroys the three-dimensional tissue architecture. High or low dimensional spatial transcriptomics methods can be used to assay the transcriptional heterogeneity of cells while preserving their spatial information. High dimensional spatial transcriptomics approaches assay a large number of genes across small spatially associated aggregates of cells, whereas low dimensional spatial transcriptomics can generate smaller scale data on single cells.

Figure 3 - Mapping immune cells in peripheral blood. a. scRNAseq has been used to identify new or novel subsets of cells in peripheral blood. For example, plate-based scRNAseq of sorted myeloid populations has been used to identify a novel subset of dendritic cell (DC5; see upper approach)[46]. Another study used massively parallel droplet-based scRNAseq to obtain data on >68,000 peripheral blood mononuclear cells, demonstrating the capacity of this method to uncover rare cell populations (coloured)[22]. Such data can be used to generate heatmaps that show cell type-specific marker genes. **b.** scRNAseq can also be used to reconstruct developmental trajectories, for example to assess changes that occur in the transcriptional profile of progenitor cells during haematopoeisis. **c.** scRNAseq can also reveal transcriptome-wide divergent responses of leukocytes to perturbations or stimuli such as lipopolysaccharide (LPS) or IFNγ **d.** Ultimately scRNAseq will be an important tool for investigating cellular disease states at high resolution, identifying disease specific cell subsets or activation states, and cell-type specific disease signatures.

Figure 4 - Mapping immune cells in lymphoid organs. a. Using paired-end single cell sequencing data in which both the 3' and 5' end of a cDNA fragment is sequenced, the clonotype of a T or B lymphocyte can be inferred in parallel with the transcriptome. Through the course of an immune response, lymphocytes undergo clonal selection and expansion, alongside the reshaping of the transcriptome through differentiation. **b.** Various immune cells in the lymph node have specific anatomical arrangements. For example, under homeostatic conditions (left-hand panel) subcapsular sinus macrophages, natural killer (NK) and innate lymphoid (ILC) cells are arranged in the interfollicular space. These cells are found exclusively or predominantly within secondary lymphoid organs. Spatial transcriptomics techniques may aid our understanding of this spatial patterning. During the course of an immune response (right-hand panel), leukocytes undergo compartmentalized interactions with stromal cells, for example marginal reticular cells and T zone reticular cells. Single cell technologies will enable the dynamic heterogeneity of these important subsets to be probed.

Figure 5 - Mapping immune cells in the kidney. a. scRNAseq can be used to dissect the transcriptional identities of kidney cell types in a marker-free manner. Cells from human or mouse kidney samples are initially dissociated before scRNAseq is performed. Resulting data can be clustered to identify the landscape of cell types, and to identify their defining transcriptional profiles. **b.** Tissue-resident macrophages arise from either common myeloid precursors (CMP) in the mature bone marrow or from erythromyeloid progenitors (EMP) in the fetal yolk sac and liver, with seeding early in life. Single cell transcriptional profiles may reflect developmental heterogeneity among kidney-resident immune cells. **c.** Resident leukocytes in different organs adapt to their tissue environment, and have tissue-specific roles. This heterogeneity is reflected in the transcriptional circuitry of the cells, and a comparison of cells from different organs may uncover shared and specific residency signatures. **d.** Cell–cell signaling is essential for proper organ function, and crosstalk between immune cells and non-immune cells contributes to host defense. Such signaling networks can be inferred using scRNAseq data, for example using the CellphoneDB tool. **e.** Solid organs such as the kidney contain distinct microenvironments; for example, the hypoxic and hypersaline environment of the medulla and the pelvis, which is exposed to ascending pathogens. Local cues guide leukocyte positioning and can be interrogated using single cell technologies.

Glossary

scATACseq
Cell assay for transposase accessible chromatin with high-throughput sequencing is a sequencing-based assay that detects open regions of chromatin.

High dimensional data

Data characterised by a high number of simultaneous measurements (dimensions) measured for each sample. In the case of scRNAseq, a large number of genes is measured for each cell.

Droplet microfluidics
Formation of individual droplets though combination or reagents within an oil suspension to form individual barcoded reaction vessels.

Cellular barcoding
Labelling the cDNA or RNA originating from a single cell with a DNA barcode which once sequenced enables the tracing back of each individual sequenced transcript to the cell of origin.

Cell atlas
A large scale census of cell types and states found in a tissue, or a collection of tissues. Typically, such datasets contain tens or hundreds of thousands of cells and are powered to detect minority populations (<1% of total).

Cell clustering
An approach to partition sets of cells into communities with similar gene or protein expression profiles.

Mass cytometry
Use of a modified mass spectrometer to measure the binding of heavy metal tagged antibodies attached to target cells to infer protein expression levels at single cell resolution.

Subcapsular sinus macrophages
A layer of macrophages positioned in the subcapsular sinus of the lymph node, where they are poised to sample antigens in lymph.

Splenic red pulp macrophages
Macrophages within the red pulp regions of the spleen with specialised roles in phagocytosis of senescent and damaged erythrocytes, and iron recycling.

Marginal zone macrophages
Macrophages positioned within the marginal zone of the spleen, where they are poised to sample antigens in the blood.

Innate lymphoid cells
Lymphocytes that lack somatically rearranged antigen specific receptors.

Peristalsis
Rhythmic contraction and relaxation of the smooth muscle lining a viscus, resulting in wave like propulsion of its contents.

Massively parallel scRNAseq
A method of scRNAseq in which cells are first sorted into individual wells, before undergoing lysis and reverse transcription.

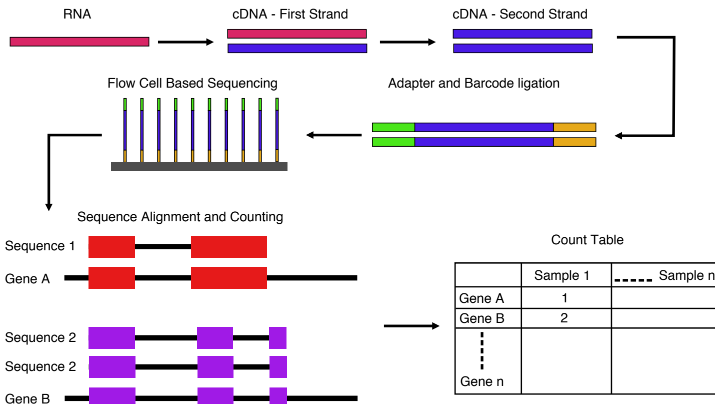
T cell receptor reconstruction
A method for identifying the specific rearranged sequences of T cell receptors in scRNAseq data.

DropSeq
Early microfluidics-based droplet sequencing method where the microfluidics were assembled by the end user.

inDrop
A droplet microfluidics scRNAseq approach in which cells are encapsulated into droplets and combined with oligonucleotide labelled hydrogel microspheres.

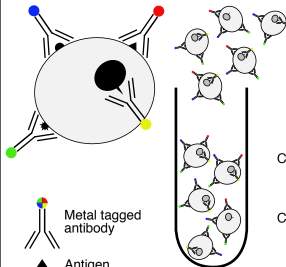
Fc receptor pathway
Intracellular signaling cascade downstream of ligation of Fc receptors by the Fc portion of immunoglobulin.

Blurb:
In this Review, Stewart and colleagues describe how single cell technologies, in particular single cell RNA sequencing, can be used to map the complex immune landscape within organs, and how such technologies might provide insights into the role of the immune system in kidney health and disease pathogenesis.

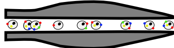

Box 1 RNA Sequencing
RNA
cDNA - First Strand
cDNA - Second Strand
Flow Cell Based Sequencing
Adapter and Barcode ligation
Sequence Alignment and Counting
Sequence 1
Gene A
Sequence 2
Sequence 2
Gene B
Count Table
Sample 1
Sample n
Gene A
1
Gene B
2
Gene n

Box 2 - Mass Cytometry

Cell Staining and loading



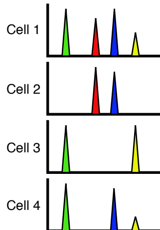
Nebulizer



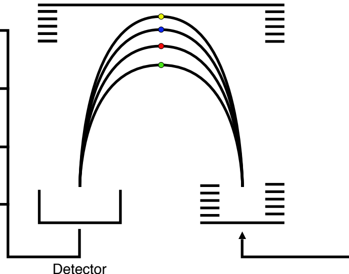
ICP Torch

Quadrupole

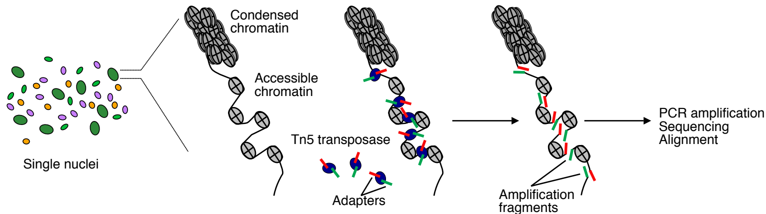
Analysis

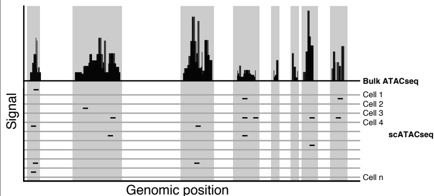


Time of Flight Mass Spectrometer



Box 3 - scATAC-seq





	Cell 1	Cell 2	Cell 3	----- Cell n
Peak A	1	0	0	
Peak B	0	0	1	
⋮ Site n				

Peak matrix

Fig 1

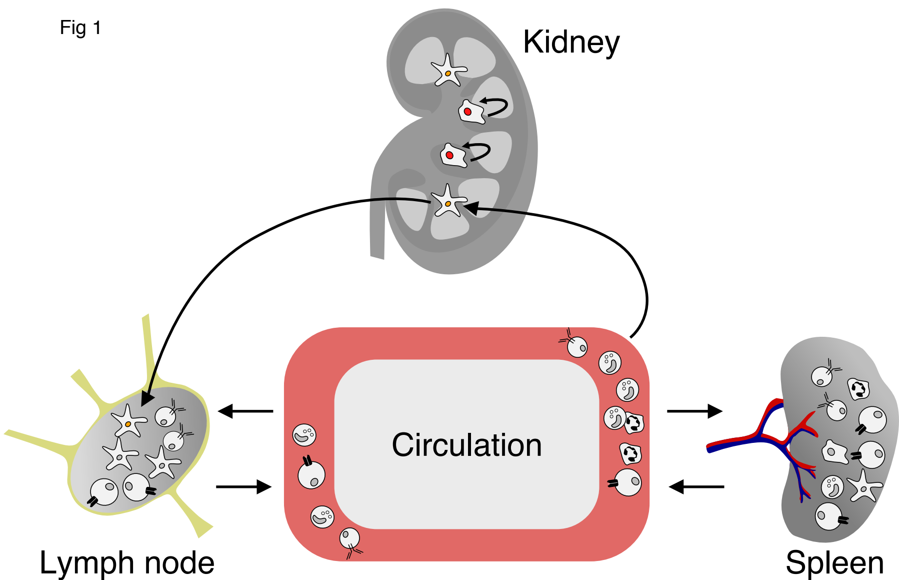
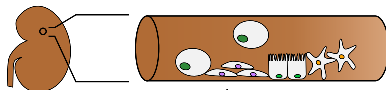
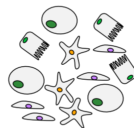


Fig 2

Tissue Biopsy

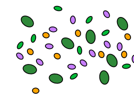


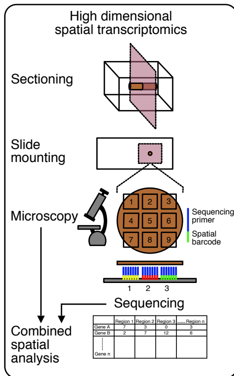
Single cell disaggregation

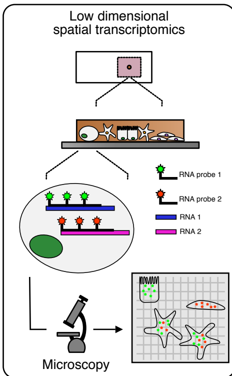


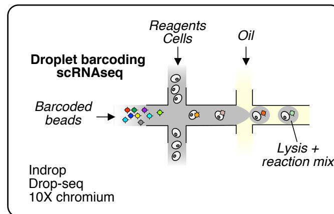
Spatial transcriptomics

Single nucleus disaggregation









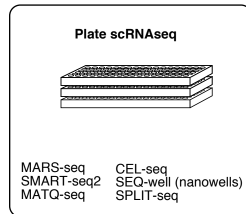
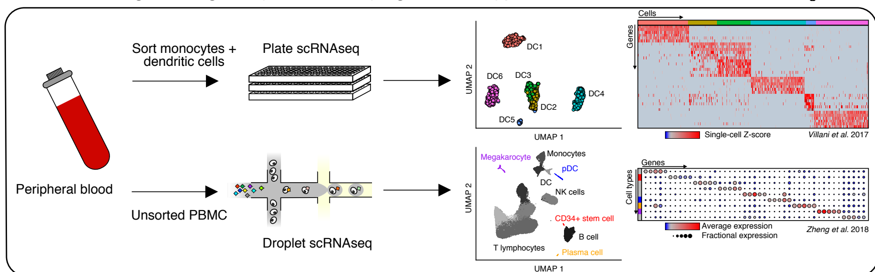
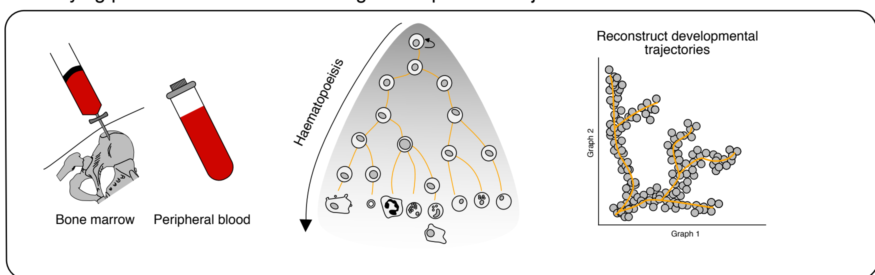
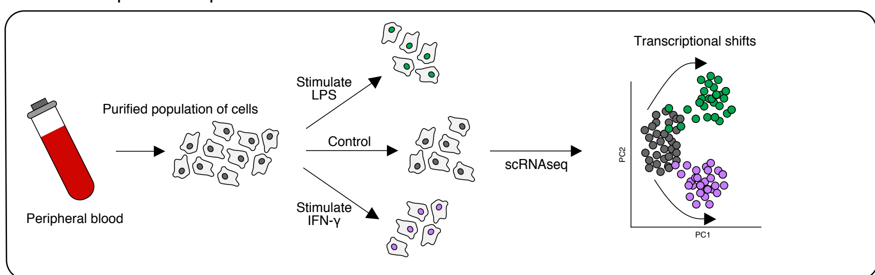


Fig 3







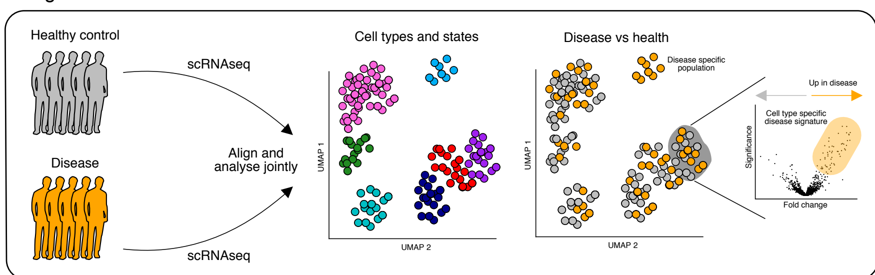
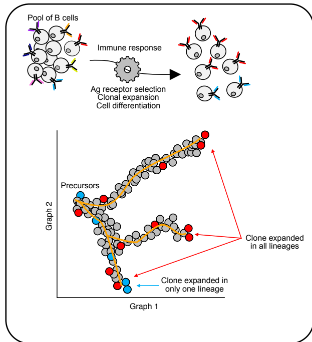
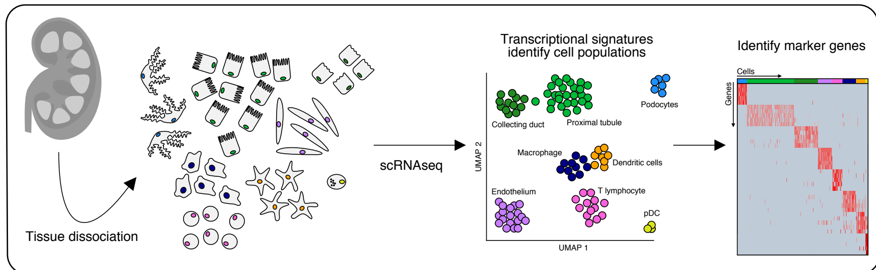


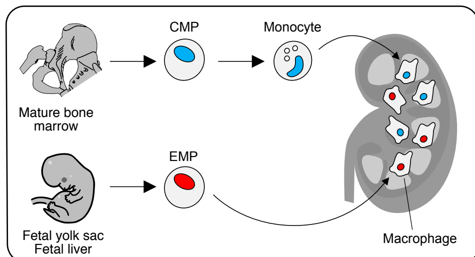
Fig 4

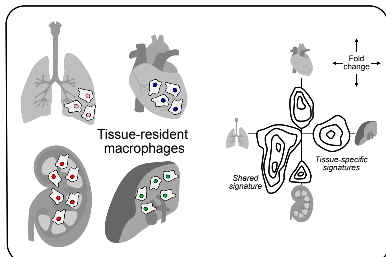
A Coupling transciptome and clonotype

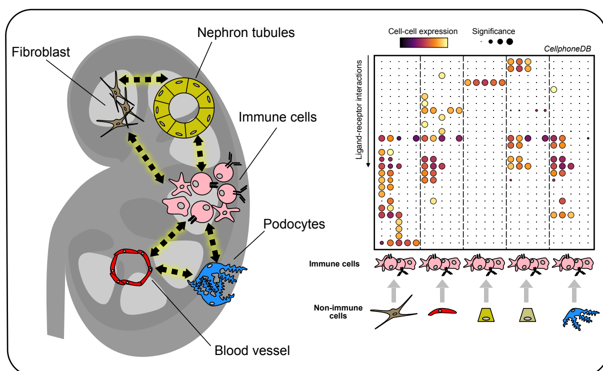


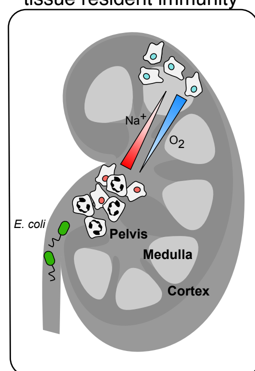
B Transcriptional and spatial organisation of adaptive immunity


A Probe cellular heterogeneity in the kidney
Tissue dissociation
scRNAseq
Transcriptional signatures identify cell populations
Collecting duct
Proximal tubule
Podocytes
Macrophage
Dendritic cells
Endothelium
T lymphocyte
pDC
UMAP 2
UMAP 1
Identify marker genes
Cells
Genes


B Predict cellular ontogeny
Mature bone marrow
CMP
Monocyte
Fetal yolk sac
Fetal liver
EMP
Macrophage


C Uncover tissue residency signatures
Tissue-resident macrophages
Fold change
Shared signature
Tissue-specific signatures


D Putative cell-cell signalling networks
Fibroblast
Nephron tubules
Immune cells
Podocytes
Blood vessel
Cell-cell expression
Significance
CellphoneDB
Ligand-receptor interactions
Immune cells
Non-immune cells


E Spatial organisation of tissue resident immunity
Na+
O2
E. coli
Pelvis
Medulla
Cortex