Single-Cell Transcriptomics of a Human Kidney Allograft Biopsy Specimen Defines a Diverse Inflammatory Response

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ABSTRACT

Background Single-cell genomics techniques are revolutionizing our ability to characterize complex tissues. By contrast, the techniques used to analyze renal biopsy specimens have changed little over several decades. We tested the hypothesis that single-cell RNA-sequencing can comprehensively describe cell types and states in a human kidney biopsy specimen.

Methods We generated 8746 single-cell transcriptomes from a healthy adult kidney and a single kidney transplant biopsy core by single-cell RNA-sequencing. Unsupervised clustering analysis of the biopsy specimen was performed to identify 16 distinct cell types, including all of the major immune cell types and most native kidney cell types, in this biopsy specimen, for which the histologic read was mixed rejection.

Results Monocytes formed two subclusters representing a nonclassical CD16+ group and a classic CD16- group expressing dendritic cell maturation markers. The presence of both monocyte cell subtypes was validated by staining of independent transplant biopsy specimens. Comparison of healthy kidney epithelial transcriptomes with biopsy specimen counterparts identified novel segment-specific proinflammatory responses in rejection. Endothelial cells formed three distinct subclusters: resting cells and two activated endothelial cell groups. One activated endothelial cell group expressed Fc receptor pathway activation and Ig internalization genes, consistent with the pathologic diagnosis of antibody-mediated rejection. We mapped previously defined genes that associate with rejection outcomes to single cell types and generated a searchable online gene expression database.

Conclusions We present the first step toward incorporation of single-cell transcriptomics into kidney biopsy specimen interpretation, describe a heterogeneous immune response in mixed rejection, and provide a searchable resource for the scientific community.

The renal biopsy provides critical diagnostic and prognostic information, but interpretation rests on a limited number of techniques perfected decades ago. With few exceptions, there have been no substantive changes in the way that biopsies are read in 25 years. Recent techniques for massively parallel single-cell RNA-sequencing (scRNA-seq) have been developed, in which the expression of thousands of genes in thousands of individual cells can be measured rapidly, simultaneously, and quan-

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titatively. These approaches offer an unprecedented opportunity to define cell types and states comprehensively with molecular precision. Several international efforts, including the Kidney Precision Medicine Project, are underway using these strategies and other tissue interrogation strategies to generate kidney cellular expression atlases in health and disease. Recently, the first comprehensive mouse scRNA-seq dataset was published.

Hurdles limit application of scRNA-seq to human kidney biopsies. Tissue availability is unpredictable, requiring access to equipment, such as fluorescence-activated sorting machines, with little notice. Additionally, the kidney biopsy consists of a very small sample of about 10×2 mm, but low-input samples pose challenges in scRNA-seq, which relies on double-Poisson loading of cells and oligobearing beads, leading to loss of 95% of cellular input, for example, with the DropSeq approach. Despite these limitations, we hypothesized that scRNA-seq can be successfully applied to a single human kidney biopsy and that doing so would reveal novel insights about disease pathogenesis. Successful application of scRNA-seq to human biopsies will enable evaluation of this powerful tool as an adjunct to traditional renal biopsy interpretation.

We chose to focus on renal allograft biopsies, because a substantial body of data has already established bulk transcriptional profiling to be predictive of outcomes in transplantation. These analyses, however, are limited, because population-averaged measurements mask single-cell transcriptomic heterogeneity, particularly with rare cell types. Moreover, rejection in all of its forms involves the complex interaction of many different immune and parenchymal cells. scRNA-seq is uniquely well suited to dissecting a complex tissue into multiple cellular subpopulations, because it can be easily scaled to tens of thousands of cells. Longitudinal clinicopathologic studies, such as the DeKAF Study, and bulk transcriptional analysis using microarrays suggest that antibody-mediated immune injury is the most common driver of late allograft loss. Our understanding of the molecular and cellular mechanisms of rejection, in particular, antibody-mediated rejection (ABMR), is poor. Analyses of allograft biopsy transcriptional profiles by microarray have classified patterns of gene expression that can define the different clinical phenotypes of allograft rejection or predict the development of IFTA using biopsy tissue. From these studies, a large number of genes have been described that play a role in allograft rejection and failure. Whether resolution of these bulk transcriptional profiles to individual cell types might improve our understanding of rejection or provide for better disease subclassification is unknown.

In this study, we present the first scRNA-seq analysis of a single human kidney allograft biopsy. We used unsupervised computational approaches to identify 16 different cell types and novel cell states within endothelium. We compared tubular cell transcriptomes with healthy adult kidney and thereby, identified proinflammatory parenchymal responses in the rejecting kidney. We mapped genes with expression that is known to associate with allograft pathologies to specific kidney cell types, in many cases finding expression in unexpected cells. Our data prove the feasibility of applying comprehensive scRNA-seq to human kidney biopsies and identify complex immune cell infiltrates and proinflammatory parenchymal responses.

METHODS

Clinical Sample
The patient described in this study consented under the Washington University Kidney Translational Research Core (WUKTRC) Institutional Review Board protocol (identification 201102312). The donor and recipient HLA haplotypes, the clinical biopsy Banff scores, and follow-up clinical outcome data were received in a deidentified manner through the WUKTRC. The healthy kidney tissue was from a discarded human donor kidney where donor anonymity was preserved. The donor was a 70-year-old white man with a serum creatinine of 1.1 mg/dl.

Tissue Processing and Single-Cell Dissociation
The renal biopsy was minced into small pieces with a razor blade and incubated at 37°C in freshly prepared dissociation buffer containing 0.25% trypsin and 40 U/ml DNase I. Dissociated cells were harvested every 10 minutes by filtering the cell suspension through a 70-µm cell strainer (pluriSelect) into 10% FBS buffer on ice. The residual biopsy tissue trapped on the cell strainer was dissociated once again with 1 ml dissociation buffer for 10 minutes and passed through the cell strainer into the same FBS buffer from the first collection. We repeated this dissociation procedure three times until most of the tissue had been dissociated into single cells (total dissociation time was 30 minutes). Finally, cells were collected by centrifugation at 400×g for 5 minutes, resuspended in drops cell suspension buffer (9% Optiprep), and strained through a 40-µm cell mesh (pluriSelect) to further remove cell clumps and large fragments. Cell viability was approximately 90% for the biopsy used in this study as assessed by Trypan Blue staining.
RESULTS

A clinically indicated kidney allograft biopsy was performed on a 21-year-old white man with ESRD secondary to hemolytic uremic syndrome. He received a deceased donor kidney transplant 2 years prior (HLA mismatch 1A, 2B, 1DR) with thymoglobulin induction (Supplemental Table 1). At the time of the biopsy, his creatinine was 6.1 mg/dl, and it had increased from a baseline of 1.2 mg/dl 4 months prior. Maintenance immunosuppression was tacrolimus (trough levels 4–8 ng/ml), mycophenolic acid, and prednisone. He failed to get laboratory testing in the 4 months before biopsy, raising suspicion of medication noncompliance.

The histologic diagnosis was acute T cell–mediated rejection with plasma cells graded Banff 1B and acute C4d-negative ABMR (Figure 1A, Supplemental Table 2). Donor-specific antibodies (DSAs) were positive at the time of biopsy (Supplemental Table 3). Treatment consisted of high-dose corticosteroids (750 mg methylprednisone), thymoglobulin (3.5 mg/kg), IVIG (1 g/kg), and rituximab (200 mg). Allograft function did not improve, and he returned to dialysis 2 months later.
scRNA-seq Identifies Kidney Allograft Biopsy Cell Types
In preliminary experiments, we used a 30-minute 0.25% trypsin cell dissociation procedure to compare the cell yield and viability from four human kidney biopsies. Biopsy length ranged from 10 to 15 mm, cell yield ranged from 44,000 to 75,000 cells, and viability was between 88% and 96% (Figure 1A). We next assessed the ability of two common droplet-based techniques, DropSeq10 and inDrops,11 to generate single-cell transcriptomes from human kidney (Supplemental Figure 1). We consistently failed to generate libraries using DropSeq lysis buffer and beads, but we were successful using inDrops reagents (Supplemental Figure 1B). Of note, the DropSeq lysis buffer includes the detergent sarcosyl, whereas the inDrops lysis buffer includes NP40, suggesting the possibility that incomplete cell lysis might explain our failure to generate libraries using DropSeq.

We enzymatically dissociated one 16G biopsy core (Figure 1B) into a single-cell suspension, performed droplet-based microfluidic cell separation using InDrops, generated libraries, and sequenced the cells to a read depth of approximately 50,000 reads per cell. A cell separation using InDrops, generated libraries, and sequenced supervised clustering analysis using Seurat identified an average, 1481 transcripts from 827 different genes per cell. Unsupervised clustering analysis using Seurat identified 16 distinct cell clusters (Figure 1C). A similar number of unique clusters were identified using an independent clustering approach12 (Supplemental Figure 2). We confirmed that there was no batch effect by projecting the cells from different tubes onto the same tSNE (Supplemental Figure 3).

We annotated clusters on the basis of anchor gene expression and the literature. We independently validated the clusters by computing Pearson correlation on averaged expression profiles derived from a recently generated mouse P1 kidney scRNA-seq dataset and a PBMC dataset from a healthy donor (http://support.10xgenomics.com/single-cell/datasets) (Figure 2A, Supplemental Figure 4A).13 We could, therefore, confidently annotate four tubular cell types (38.0%), three leukocyte populations (21.4%), four types of lymphocytes (19.8%), three stromal cell types (8.2%), endothelial cells (ECs; 11.5%), and an actively proliferating cell population (1.1%) (Supplemental Figure 4B). The expression of unique marker genes in each cell population confirmed the robustness of our cell type classification approach (Figure 1D). Full lists of differentially expressed genes in each cluster are provided in Supplemental Table 4. A searchable database for this dataset, including gene expression projected onto the tSNE diagram, is available at http://humphreyslab.com/SingleCell/.

Comparative Analysis Reveals Monocyte Cell-State Transition and Cell-Cell Interactions between Many Cell Types
Monocyte infiltration is quantitatively associated with kidney dysfunction during allograft rejection.14,15 By mapping our dataset to a published single-cell PBMC dataset,16 we identified two distinct monocyte clusters (Figure 2A). Monocyte 1 strongly expressed FCGR3A (CD16) and was most similar to CD16-positive, proinflammatory, nonclassic monocytes.17 Of note, CD16++ cells are strongly associated with allograft rejection.16,19

Monocyte 2 seems to be a classic or intermediate monocyte population (Figure 2A, Supplemental Table 4). Interestingly, ABCA1, which mediates sterol efflux in activated dendritic cells (DCs),20,21 was uniquely detected in monocyte 1 (Figure 2D), likely indicating a phenotypic transition toward a DC fate by monocytes in allograft rejection. In support of this interpretation, direct comparison of monocyte 1 with the monocyte_CD16+_C1qa cluster from the PBMC dataset revealed a clear separation of monocyte 1 and the PBMC monocytes, although they were highly correlated on the basis of averaged expression (Figure 2C). Although both cells clusters expressed typical monocyte markers (Figure 2D), monocyte 1 from the biopsy dataset highly expressed two receptors (SDC3 and ABCA1) (Figure 2D) and a panel of DC maturation markers, including APOE,22 PDE3A,16 IGKC,23 LGMN, and iCD83,24 suggesting differentiation into DC in situ (Figure 2D).

We identified unique marker genes for each monocyte cluster (Figure 2E) and performed immunohistochemistry on independent transplant biopsies, with histologic diagnoses of no disease, mixed rejection, or ABMR. There was sparse interstitial staining for both the monocyte 1 marker (FCGR3A or CD16) and the monocyte 2 marker (FCN1) in biopsies with no disease. By contrast, there was strong staining for both monocyte subsets in mixed rejection, with lesser infiltration in pure ABMR (Figure 2F, Supplemental Figure 5). Costaining by immunofluorescence analysis confirmed that the monocyte subtypes are separate populations (Figure 2G). The presence of these monocyte subsets in all six independent biopsies with mixed rejection or ABMR validates the use of scRNA-seq to identify novel cell types associated with kidney rejection.

Ligand-receptor analysis revealed expression of 14 receptors (excluding collagens) for which we could detect expression of their cognate ligands (Figure 2B). These ligands were detected in all cell types, emphasizing the integration of signals between multiple kidney and leukocyte cell types. Perciytes, fibroblasts, and myofibroblasts expressed the chemoki CXCL12, which promotes lymphocyte and monocyte chemotaxis through its cognate receptor CXCR4, which itself is expressed in T cells, monocytes, and mast cells in our dataset. Further evidence for parenchymal cell regulation of the inflammatory response comes from the observation that collecting duct epithelia express KITLG, also known as stem cell factor, which binds the transmembrane receptor encoded by KIT and is expressed on mast cells in the biopsy (Figure 2B).25 Stem cell factor promotes mast cell migration, adhesion, proliferation, and survival. Mast cells transcripts correlate strongly with allograft biopsy fibrosis.26 These results suggest the unexpected hypothesis that collecting duct epithelia actively coordinate mast cell infiltration during rejection. Consistent with an important role for mast cells in kidney injury, a recent study showed that mast cell ablation in the early phases of renal injury is sufficient to reduce subsequent fibrosis by decreasing the inflammatory response.27,28
Activation of Epithelial, Endothelial, and Stromal Cells in Allograft Rejection

We next compared epithelial transcriptomes from the biopsy with their healthy counterparts. Multiple attempts at scRNA-seq of healthy nephrectomy tissue failed to generate libraries; however, we were successful in generating adult human kidney single-nucleus RNA-sequencing (RNA-seq) data. We sequenced 4259 nuclei to a similar depth as the biopsy and identified six distinct epithelial cell clusters, including podocytes, proximal tubule, loop of Henle, distal tubule, principal cells, and intercalated cells (Figure 3, A–C). The absence of stromal or leukocyte populations presumably reflects either dissociation bias and/or a cell frequency below our limit of detection.

Figure 2. Annotation of leukocyte subsets. (A) Heat map indicating Pearson correlations of the averaged transcriptional profiles between human PBMCs and leukocyte clusters from the kidney biopsy. (B) Ligand-receptor pair expression according to cell type. Ligands are indicated in the left panel, and receptors are indicated in the right panel. Straight lines indicate ligand-receptor pairs. (C) The biopsy monocyte 1 cluster is most similar to CD16+ peripheral blood monocytes on the basis of average expression; however, these cells form different clusters on the basis of tSNE, indicating that they are different cell types. (D) The monocyte 1 cluster is differentiating toward an acquired dendritic cell phenotype on the basis of expression of marker genes. (E) Violin plot showing that FCGR3A (CD16) distinguishes monocyte 1 from monocyte 2, whereas FCN1 is expressed in monocyte 2 but not monocyte 1. MSR1 is expressed in both clusters. (F) Immunohistochemistry for FCGR3A or FCN1 on normal, mixed rejected, or pure antibody-mediated rejection (ABMR) transplant kidney biopsies. Distinct monocyte 1 and 2 cell types can be seen. Upper and lower panels are serial sections. Scale bar, 50 μm. (G) Immunofluorescence analysis of mixed rejection. FCN1+ cells (arrowheads) and FCGR3A+ cells (arrows) are separate cell types. CD, collecting duct; DAPI, 4′,6-diamidino-2-phenylindole; EC, endothelial cell; LOH (AL), loop of Henle, ascending limb; LOH (DL), loop of Henle, distal limb; Mono, monocyte; PT, proximal tubule. Scale bar, 10 μm.
Comparison of scRNA-seq and single-nucleus RNA-seq datasets has been shown to be valid after normalizing to reduce method-specific differences. Using a computational approach allowing for integrated analysis of multiple datasets across techniques, we compared proximal tubule, loop of Henle, and collecting duct cells from our biopsy dataset with the same cell types from our healthy adult kidney single-nucleus RNA-seq dataset. Unsupervised analysis of combined cells from all six clusters (three healthy and three rejection) by tSNE resulted in the expected three cell types, indicating that rejection did not fundamentally alter tubular cell identity (Figure 3D, Supplemental Figure 6). Consistent with this interpretation, projecting the source of cells onto this tSNE confirmed that healthy proximal tubule was overlaid with rejecting proximal tubule as was loop of Henle and collecting duct (Figure 3E).

Despite the overall similarity between healthy and rejecting proximal tubule, loop of Henle, and collecting duct, differential gene analysis revealed downregulation of terminal differentiation markers and upregulation of proinflammatory genes in biopsy epithelia (Figure 3F). The proximal tubule in particular expressed proinflammatory cytokine genes, such as CXCL14, which induces monocyte chemotaxis, and IL32, which induces expression of TNFα by macrophages (Figure 3F). ITGB6, a gene previously shown to be expressed in distal tubule of the diseased kidney transplant, was specifically induced in the loop of Henle in our biopsy dataset. Within collecting duct epithelia, the top statistically significant canonical signature by GSEA that distinguished the biopsy collecting duct from the healthy collecting duct was the TGF-β/BMP signaling signature (Supplemental Figure 7). Collectively,

**Figure 3.** Comparison of epithelia from single-cell RNA-sequencing of healthy adult kidney with transplant biopsy reveals activated and proinflammatory cell states. (A) Unsupervised clustering identified six distinct cell types in human adult kidney. These types include three tubular cell types (proximal tubule [PT], loop of Henle [LOH], and distal tubule [DT]), two collecting duct (CD) cell populations principal cells (PC) and intercalated cells (IC), and one podocyte population (P). (B) The heat map showed that putative molecular signature marks the identity of each cluster. (C) The violin plot further confirmed the clean expression of well-known cell type–specific markers in each cell population, which makes it suitable for use in benchmarked comparison analysis. (D) tSNE analysis of PT, loop of Henle (LOH), and CD cells from the allograft biopsy and healthy adult human kidney cluster together indicating that cell identity is maintained despite allograft inflammation. (E) Collecting duct cells from the biopsy (CD-bx) coproject by tSNE onto the collecting duct cluster from healthy kidney (CD-h). The same is true of PT and LOH. (F) A dot plot comparing expression of terminal differentiation or inflammatory genes in epithelial cells from the biopsy or healthy kidney. (G) Ordering of healthy and activated PT cells along pseudotime using Monocle. (H) Selected transcription factors that are upregulated during PT activation. LOH-bx, loop of Henle from the biopsy; LOH-h, loop of Henle from healthy kidney; PT-bx, proximal tubule from the biopsy; PT-h, proximal tubule from healthy kidney.
these results highlight the degree to which epithelial damage during rejection amplifies proinflammatory and profibrotic responses.

To further investigate proximal tubule injury responses, we performed cell trajectory analysis by reconstituting the healthy and biopsy proximal tubule clusters by pseudotime analysis. We found a smooth transition between healthy and activated proximal tubule cells (Figure 3G and F). We next asked what genes might be regulating proximal tubule activation by plotting differentially expressed transcription factors across pseudotime. Among others, we observed strong upregulation of Sox9, which has recently been identified as a master regulator of proximal tubule repair after injury, validating our approach.30

Donor ECs are the primary targets of the recipients’ humoral immune response in ABMR. Reclustering of the ECs revealed three separate cell states in rejection (Figure 4, A and B). Differential gene expression analysis suggested that the predominant EC cluster was in the resting state, because it expressed EC markers (PECAM1, VWF, and ENG) but no transcripts previously associated with ABMR or angiogenesis (Figure 4C). We compared average gene expression of the three clusters with healthy human brain and pancreas ECs, and the resting cluster correlated best (Figure 4D). A smaller subcluster expressed the angiogenic program,31 with several of these genes previously described in association with ABMR (Figure 4, C and E).33,34 A third EC cluster expressed markers of endoplasmic stress (XBP1) and the cold shock gene RBM3, suggesting cell stress and activation. This cluster was also actively transcribing Ig, such as has been observed in vitro (Figure 4C).36 The src family kinases have been shown to be activated on ingestion of IgG. Consistent with this, FYN is highly expressed in this EC subcluster.37 Thus, these ECs maybe internalizing bound IgG, consistent with DSA-EC binding in ABMR. Studies have shown that this process occurs in EC via Fc receptor–mediated phagocytosis.38 In line with

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Figure 4. Analysis of endothelial cell (EC) subsets. (A) tSNE plot of endothelial subclusters from the kidney biopsy. (B) Heat map showing selected marker genes for the three EC subclusters. (C) Violin plots showing conserved expression of endothelial markers across all three subclusters. The genes SEMA3G, IGFBP3, SERPINE2, and AQP1 indicate activation of an angiogenic program. A separate EC subcluster expresses lgs as well as markers of endoplasmic reticulum stress (XBP1) and cold shock (RBM3). (D) Pearson correlation of all three EC subsets shows best correlation between the resting EC cluster and healthy brain or pancreas ECs. (E) Markers of antibody-mediated rejection are detected in the angiogenic cluster.
this notion, our GO analysis data showed that the top GO terms for this cluster were all related to phagocytosis (Supplemental Figure 8).

We identified three separate stromal clusters (Supplemental Table 4). Cluster 1 highly expresses RSG5, a pericyte marker, as well as CACNA1C, which is expressed in a pericyte pattern. Stromal cluster 2 expresses MoxD1, a gene upregulated in early kidney transplant biopsies and associated with subsequent fibrosis. This cluster likely represents a fibroblast cell type. The third stromal cluster uniquely expressed COL8A1 and COL12A1, two genes that we have previously shown to be strongly upregulated in kidney myofibroblasts during fibrotic injury, suggesting a myofibroblast identity for this cluster. The interstitial expression patterns for marker genes from each stromal cluster are shown in Figure 5.

Antigen Expression and Antibody Production
Because alloantigens play a central role in ABMR, we profiled the expression of HLA transcripts. Appropriately, class 2HLA transcripts were predominantly expressed in the antigen-presenting cell clusters (Supplemental Figure 9A). Seurat analysis of our biopsy revealed two plasma cell clusters and one B cell cluster. The B cell cluster expressed few Ig genes but did express genes involved in antigen presentation (CD86) and T cell regulation (indoleamine 2,3-dioxygenase, IDO1), thus behaving like a naïve B cell. Plasma cell clusters highly express Ig genes; however, cluster 1 was κ-chain restricted, suggesting that these cells are monoclonal. Plasma cell cluster 2 was not light-chain restricted, and it highly expressed both κ- and λ-gene types (Supplemental Figure 9B). These clusters were labeled plasma cells on the basis of the correlation with a PBMC reference dataset; however, these cells could also represent mature B cells.

Reassessing the Molecular Signature for Disease Classification at the Single-Cell Level
We next assigned expression of genes previously associated with allograft pathologies to single-cell types. When we mapped putative EC-associated transcripts, we found that most of these genes were actually not specifically expressed in ECs. Some of them were even uniquely expressed in non-EC types, such as ENPEP (PT), ANGPT1 (fibroblast), MGP (myofibroblast), and FOSB (mast cells) (Figure 6A). Over one half of the transcripts were expressed in multiple cell types. Whether disease outcome correlates best with expression of these genes in one cell type and not others will require further...
investigation. By contrast, the 30 genes with expression that is associated with T cell–mediated rejection are predominantly expressed in immune cell types, with the majority expressed in T cells and almost no expression in kidney parenchyma (Figure 6B).43 Finally, most of the transcripts reported to be enriched in ABMR biopsies35 were mainly detected in the EC cluster in our biopsy dataset (Figure 6C), substantiating the critical role of endothelium in the pathogenesis of ABMR.44

DISCUSSION

This study establishes the feasibility of using scRNA-seq to comprehensively measure gene expression from thousands of cells from a single human kidney biopsy. We were able to identify 16 separate cell types from just under 5000 single-cell libraries. We are confident that future improvements of our basic workflow will enable the generation of ten times as many single-cell libraries from a single human biopsy, which should allow for much finer cell type separation and deeper transcript detection. Our experience suggests that standard DropSeq approaches are poorly suited for this application, however, potentially due to incomplete cell lysis as well as inefficient cell capture rates. By contrast, InDrops, as we used here, or the 10× Chromium system are much better suited to analyze single human kidney biopsy.

Our study is limited to a single biopsy, and therefore, results cannot be generalized. However, our findings do reveal several novel insights into human allograft rejection. Anti-HLA antibodies are known to play important roles in rejection, with much attention focused on EC responses to antibody binding. Previous studies suggest that antibody binding causes a number of EC responses, including cell proliferation,45–48 induction of secondary factors (FGFR and VEGF),49,50 and leukocyte recruitment (including vWF and P-selectin expression),51–53 as well as cell survival responses. Our data show two distinct pathologic EC responses in mixed rejection: a resting state and two ABMR response states consisting of an angiogenic state or an Ig phagocytosis state. The resting-state cells probably elicit the initial humoral response, because they express donor-specific HLA and leukocyte adhesion molecules (PECAM1). Evaluating the EC responses to different antibodies in the setting of ABMR will help us better understand why DSAs confer variable degrees of pathogenicity. Therapeutically, the B cell-plasma cell lineage is an attractive target for therapy, because inhibition of alloantibody production will likely improve outcomes in ABMR. To this end, we identified B cells that are likely naïve, polyclonal plasma cells (or mature
We have additionally provided the first single-nucleus RNA-seq dataset from healthy adult human kidney. The reasons for our failure to generate scRNA-seq libraries from healthy adult kidney compared with our success with a diseased transplant biopsy are unclear. We speculate that the robust inflammation present in the transplant biopsy made the tissue more amenable to dissociation. However, this points to a major current challenge and limitation in the application of single-cell technologies to human kidney—the difficulty of successful dissociation without RNA degradation and the associated problem of dissociation bias. For example, we could not identify podocytes in the transplant biopsy, whereas they were present in the healthy kidney single-nucleus dataset, although similar numbers of cells/nuclei were analyzed. However, the healthy human kidney dataset contained only epithelial cell types without stroma, endothelium, or leukocytes. These striking differences indicate that dissociation methods selectively enrich for certain cell types, while losing others—either because they die during dissociation or because they remain in clumps. Clearly, future work is needed to define the optimal cell/nucleus dissociation approach for human kidney. It is worth considering that nuclear dissociation can be carried out entirely on ice—which eliminates the artefactual transcriptional stress responses that are known to arise on incubation of kidney tissue with proteases at 37°C.13

An additional important consideration is that information concerning cell positioning is lost with scRNA-seq. Such information may be critical to properly interpret results. For example, a ligand-receptor analysis between podocytes and intercalated cells does not make sense biologically, because they are positioned so far apart, whereas the same comparison between principle cells and intercalated cells is biologically meaningful. New experimental and computational tools are in development to address the loss of spatial context with scRNA-seq. One approach, fluorescent in situ sequencing,54 allows for genome-wide expression profiling in fixed tissues—preserving tissue architecture for RNA localization studies. In addition, computational approaches can integrate scRNA-seq data with in situ hybridization RNA expression patterns, allowing inference of spatial expression patterns by virtual in situ hybridization.55

Despite current challenges, we envision a time in the future when kidney biopsies are routinely sent for scRNA-seq as part of a molecular diagnostic workup. scRNA-seq can unmask the transcriptional heterogeneity that is hidden in population-averaged measurements. We illustrate the power of this approach by analyzing paracrine signaling pathways between infiltrating leukocytes and kidney parenchyma and by mapping predictive gene expression to single-cell types. We expect that application of scRNA-seq technologies to human native and allograft biopsies will ultimately generate clinically useful datasets to improve diagnostic and prognostic accuracy, enable disease subphenotyping, and accelerate adoption of molecular biopsy interpretation.

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DISCLOSURES

None.

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Supplemental Information

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Inventory of Supplemental Information

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Figure S1. Bioanalyzer traces of library preparations resulting from the two microfluidic-based single cell techniques, Dropseq and inDrop. (A) cDNA trace for a library prepared using the Dropseq method. This method was unsuccessful in making quality libraries from a human biopsy tissue core sample. (B) cDNA trace for a library prepared using the inDrop method. Using this method we were able to create a quality cDNA library from a human biopsy tissue core.
Figure S2. Consensus clustering of our single cell RNA-seq data using SC3. The SC3 method is another unsupervised method used to cluster cells. This method is highly accurate and robust and combines multiple clustering approaches into one. Using SC3 we found that our initial clustering using Seurat was replicated using SC3.
Figure S3. Minimal batch effects observed between sequencing platforms and library preparations. Cells processed using the inDrops method on one biopsy sample were collected sequentially into 4 tubes. Libraries prepared from tubes 1 to 3 were sequenced using the Illumina HiSeq 2500 platform and the library prepared from tube 4 was sequenced using the Illumina NextSeq platform. Projecting cells into one tSNE map shows that all clusters from each tube overlap.
Figure S4. Comparison of cell-type specific gene expression from a P1 mouse kidney and a human PBMC RNAseq dataset. (A) Heatmap comparing cell-type specific gene expression from a P1 mouse kidney RNAseq dataset (y-axis) with our cell-type specific gene expression (x-axis). The results confirm our cluster annotations. Color key denotes the Pearson correlation score. (B) Proportion of cell types within our biopsy sample.
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Figure S5. Low power views of independent transplant biopsies stained for monocyte#1 and monocyte#2 subsets. FCGR3A identifies the monocyte#1 subset, and staining was absent from transplant biopsies with a histologic diagnosis of no rejection, but strong staining was present in biopsies with a histologic diagnosis of mixed rejection, and intermediate staining in ABMR biopsies. The monocyte#2 marker FCN1 exhibited a very similar pattern. The top and bottom slides were from serial sections. Scale bar 100 µm.
Figure S6. Confirmation of proximal tubule, loop of Henle and collecting duct cell clusters. Clustering analysis of epithelial cell types from the combined biopsy and normal human samples show proximal tubular cell markers CUBN and LRP2 in the proximal tubule cluster, UMOD and SLC12A1 in the loop of Henle cluster and AQP2 and AQP3 in the collecting duct cluster.
Figure S7. Upregulated TGF-β/BMP signaling signature in collecting duct cells from biopsy. (A) GSEA enrichment plot of the TGF-β receptor signaling pathway, one of the top pathways upregulated in biopsy CD when compared with the healthy CD. (B) Expression of selected TGF-β/BMP pathway genes were mapped to healthy vs. biopsy epithelial cell types. Unexpectedly, a very strong TGF-β/BMP signature was observed in collecting duct epithelia from the biopsy kidney sample (CD-bx) compared to healthy collecting duct (CD-h). Receptor-ligand pairs were identified from human the Database of Ligand–Receptor Partners (DLRP), IUPHAR and Human Plasma Membrane Receptome (HPMR).
Figure S8. GO analysis on the EC subgroup that expressed immunoglobulins. The results were obtained from the ToppGene suite using differentially expressed genes in the activated EC subcluster (p value < 0.01) as the input gene list. Note the enrichment for immunoglobulin binding and phagocytosis, suggesting antibody-mediated activation of endothelial cells in this biopsy with antibody-mediated rejection.
Figure S9. Distribution of HLA and light chain transcript expression across cell types. (A) The class I HLA transcripts are expressed equally across all cell types. The Class II HLA transcripts are predominantly expressed in the professional antigen presenting cells (APCs), monocyte cluster 1 and 2 and B cells. The class II transcripts corresponding to the donor specific antibodies circulating at the highest concentration (anti-HLA-DRB1/DRA and anti-HLA-DRB5/DRA) are expressed highly in the APCs. (B) Light chain transcript expression in lymphocytes and plasma cells. Plasma cell cluster number 1 is expressing only kappa light chains (IGKC) and is thus light chain restricted. This suggests that this cluster represents a single clone. Plasma cell cluster number 2 expresses kappa and lambda light chain constant regions (IGKC and IGLC) and is therefore not light chain restricted is thus expresses polyclonal.
Supplementary Methods

**InDrops single cell RNA-seq**
InDrops was performed as described\(^1\). In brief, cells were diluted into 60,000 cells/mL in 9% Optiprep buffer. Single cell encapsulation was carried out using an inDrops instrument and microfluidic chip manufactured by 1CellBio. In total, four tubes of cells with about 1,000 cells/tube were collected. Library preparation was performed according to the protocol provided by the manufacturer. Of note, we used low PCR cycles (8-9 cycles) to amplify the libraries from this sample as our previous trials with higher PCR cycles (12-13 cycles) resulted in an overamplification of the libraries where averaged transcripts/cell detected was less than 300. Libraries were sequenced by HiSeq 2500 and NextSeq with a sequencing depth of 50K mapped reads/cell.

**InDrops single nucleus RNA-seq**
Nuclei were isolated with Nuclei EZ Lysis buffer (Sigma #NUC-101) supplemented with protease inhibitor (Roche #5892791001) and RNase inhibitor (Promega #N2615, Life Technologies #AM2696). Samples were cut into <2 mm pieces and homogenized using a Dounce homogenizer (Kimble Chase #885302-0002) in 2ml of ice-cold Nuclei EZ Lysis buffer and incubated on ice for 5 min with an additional 2ml of lysis buffer. The homogenate was filtered through a 40-µm cell strainer (pluriSelect #43-50040-51) and then centrifuged at 500 x for 5 min at 4 ºC. The pellet was resuspended and washed with 4 ml of the buffer and incubated on ice for 5 min. After another centrifugation, the pellet was resuspended with Nuclei Suspension Buffer (1x PBS, 0.07% BSA, 0.1% RNase inhibitor), filtered through a 20-µm cell strainer (pluriSelect 43-50020-50) and counted. RNA from single nucleus was encapsulated, barcoded and reversed transcribed using an InDrop microfluidics system (1CellBio). The library was sequenced in HiSeq2500 with custom primers.

**InDrops data preprocessing**
We used a recently developed inDrops computational pipeline, dropEst,\(^1\) to process the single cell and single nuclei InDrops data. In brief, cell barcodes and UMIs from the library were extracted from read1 by the dropTag program and added to the names of the transcript reads, resulting in a new fastq file for read alignment. We used STAR (version 2.5.3a) to map the high quality reads to the human genome (GRCh38). Only reads that were uniquely mapped to the genome (~70% of the total reads) were used for UMIs counts. We next ran the dropEst program to estimate the accurate molecular counts, which generated a UMI count matrix for each gene in each cell.

**Unsupervised clustering and cell type identification**
UMI count matrices from four tubes were combined and loaded into the R package Seurat. For normalization, the DGE matrix was scaled by total UMI counts, multiplied by 10,000 and transformed to log space. Only genes found to be expressed in >10 cells were retained. Cells with a relatively high percentage of UMIs mapped to mitochondrial genes (≥0.3) were discarded. Moreover, cells with fewer than 300 or more than 4,000 detected genes were omitted, resulting in 4,487 cells. We also regressed out the variants arising from library size and percentage of mitochondrial genes using the function RegressOut in R package Seurat. The highly variable genes were identified using the function MeanVarPlot with the parameters: x.low.cutoff = 0.0125, x.high.cutoff = 6 and y.cutoff = 1, resulting in an output of 2,404 highly variable genes. The expression level of highly variable genes in the cells was scaled and centered along each gene, and was conducted to principal component analysis. We then assessed the number of PCs to be included in downstream analysis by (1) plotting the cumulative standard deviations accounted for each PC using the function PCElbowPlot in Seurat to identify the ‘knee’ point at a PC number after which successive PCs explain diminishing degrees of variance, and (2) by exploring primary sources of heterogeneity in the datasets using the PCTHeatmap function in Seurat. Based on these two methods, we selected first 20 PCs for two-dimensional t-distributed stochastic neighbor embedding (tSNE), implemented by the Seurat software with the default parameters. Based on the tSNE map, sixteen clusters were identified using the function FindCluster in Seurat with the resolution
parameter set to 0.6. Alternatively, we used SC3\(^2\) to validate the clusters identified by Seurat with cluster number set to 14 (ks=14). We found that the cells clustered by Seurat as a cell type were also grouped together by SC3. Differentially expressed genes that were expressed at least in 25% cells within the cluster and with a fold change more than 0.25 (log scale) were considered to be marker genes. In total, 2,837 marker genes were identified for the clusters in the biopsy dataset. A heatmap of selected marker gene expression across clusters was plotted using a Python plotting library Matplotlib. We applied the same unsupervised clustering analysis on the single nucleus dataset. First, we generated the digital expression matrix using the same dropEst pipeline that utilizes both exonic and intronic reads. After filtering low quality nuclei, 4,259 nuclei with > 400 genes expressed were imported into Seurat for clustering analysis. In total, we identified six kidney cell types in the single nucleus dataset, including PT, LOH, intercalated cells (IC), principal cells (PC), DCT and podocytes.

Comparison of immune cell types to a published PBMC single cell dataset
Cell-type-specific expression patterns of the cell clusters identified in our dataset were compared to signatures previously defined in a PBMC dataset by calculating the pairwise Pearson correlations coefficients between each pair of cell types for the same set of genes. First, a precomputed Seurat object containing cell cluster information for 33K human PBMCs was downloaded from the Satija lab (http://satijalab.org/seurat/get_started.html). Only genes detected in both our dataset and the PBMC dataset were used for downstream correlation analysis. Second, pearson correlation was computed between the cell clusters in our dataset and the cell clusters identified in the PBMC dataset, using the previously defined cell-type annotations and normalized average gene expression values for each cell type. Data was shown by pheatmap R package.

To compare monocyte transcriptomes between the biopsy and PBMC datasets, we extracted the expression profiles for the cluster we annotated as monocyte#1 and the corresponding monocyte cluster (mono_CD16+_C1qa) in the PBMC dataset. We then clustered the cells after removing cell-cell variations driven by the number of detected molecules and mitochondrial gene contents. Cells in tSNE were colored by the original dataset where they are extracted, or the cell cluster where they are assigned based on unsupervised clustering analysis. To compare the marker gene expression in the monocyte from different sources, we extracted the scaled UMI expression values for the selected genes that are known as monocyte markers, receptors and DC differentiation markers. Average expression level of these selected genes was visualized as the violin plot using ggplot2 package in R.

Correlation analysis of the kidney cell types from allograft biopsy and P1 mouse kidney
To assess the similarity between biopsy cell types and embryonic kidney, we re-analyzed a recently published Dropseq dataset from P1 kidney (GSE94333).\(^3\) We used the Seurat clustering parameters described by the authors and reproduced the same cell types from the datasets. We calculated the Pearson correlation based on the expression patterns of highly variable genes between cell populations within the mouse embryonic kidney dataset against the cell types identified in our biopsy dataset. Correlation matrix were visualized by R package pheatmap. Color keys represent the range of the coefficients of determination (r\(^2\)) in this analysis.

Ligand-receptor interaction analysis
To study ligand-receptor interactions across the cell types identified from the transplant biopsy, we used a human ligand–receptor list comprising 2,557 ligand–receptor pairs curated by the Database of Ligand–Receptor Partners (DLRP), IUPHAR and Human Plasma Membrane Receptome (HPMR).\(^4, 5\) We selected the receptors that were only differentially expressed in each cell type from the biopsy dataset. To determine the ligand-receptor pairs to plot on the heatmap, we required (i) the receptors are uniquely expressed in each cell type (q-val<0.05 and logFC>0.6); (ii) Each receptor should have at least one corresponding ligand to pair with. We used heatmap.2 function from gplots package to visualize the ligand-receptor pairs in each cell type.
Comparison of healthy kidney and allograft kidney
To compare the transcriptional difference across each tubular cell type in healthy and disease state, we extracted the expression profiles for PT, LOH, and PC (CD) from the healthy kidney single nucleus dataset and the biopsy single cell dataset. We then performed integrated analysis on both datasets using a recently developed computational strategy (implemented in Seurat v2.0). First, we selected the union of the top 2,000 genes with the highest dispersion from both datasets for a canonical correlation analysis (CCA) to identify common sources of variation between the two datasets. Then we aligned the CCA subspaces using the first 15 dimensions of the CCA. After CCA alignment, we performed clustering analysis on the nuclei and cells with the resolution parameter set to 0.6. We visualized the cells by their original identity or by their cluster identity classified by this integrated analysis. Differential gene analysis was performed on the nuclei and cells from different datasets but grouped in the same cluster by the alignment analysis. Differential genes were visualized using DotPlot function in Seurat.

Pseudotemporal ordering of PT single cells and TF analysis
We used Monocle2 to draw a minimal spanning tree connecting the PT cells collected from healthy and diseased kidneys. As input into Monocle2, we selected the highly variable genes for cell ordering as described in the Monocle2 tutorial (http://cole-trapnell-lab.github.io/monocle-release/docs_mobile/). We then reduced the data space to two dimensions using the reduceDimension function with ‘DDRTree’ method and ordered the cells using the orderCells function in Monocle2. Individual cells were color-coded based on the kidneys where they were collected. To identify the transcription factors whose expression are dynamically changing across the trajectory from healthy PT to diseased PT, we first identified the genes that were differentially expressed across pseudotime using the differentialGeneTest function in Monocle2 with the fullModelFormulaStr parameter set to ‘Psudeotime’. To identify the TFs, we crossed the differential genes to the human TF list downloaded from AnimalTFDB (http://bioinfo.life.hust.edu.cn/AnimalTFDB/). We selected some of the important TFs that have been reported by the literature as key regulators of proximal tubule injury and repair and visualized them by R package ggplot2.

Analysis of heterogeneity in the EC population
We performed unsupervised clustering analysis on the endothelial cells extracted from the biopsy dataset using Seurat with similar parameters as described above. Top markers in each subcluster were visualized with the DoHeatmap function in Seurat. We used a stacked violin plot to show the expression of endothelial markers, angiogenic markers, and immunoglobulin genes across the three EC subgroups. Marker genes were selected based on literature search, and were differentially expressed among the EC subclusters. GO analysis was performed by uploading the differential genes from the EC subclusters to ToppGene Suite (http://toppgene.cchmc.org). Top 4 GO terms from categories of biological process, molecular function and cellular component were visualized using ggplot2 package.

Re-assessing disease-associated gene lists from the public datasets
To map the disease-associated genes to single cell, we selected the top genes from three public datasets where the authors identified EC enriched transcripts, TCMR enriched genes, and ABMR enriched markers by comparing the microarray data from the biopsies with different rejection patterns. Expression values of these genes in our single cell dataset were normalized, z-scored and visualized the heatmap.2 in gplots R package.

DropSeq single-cell RNA sequencing
Dropseq was performed on the dissociated biopsy cells as previously described. Briefly, biopsy was dissociated into single cell using the same methods as being used in inDrops. Single cell suspension was visually inspected under a microscope, counted by hemocytometer (INCYTO C-chip) and resuspended in PBS + 0.01% BSA. Single cells were coencapsulated in droplets with barcoded beads purchased from Chemgenes (MACOSKO-
cDNA libraries were constructed according to an online Dropseq protocol (McCarroll’s lab: http://mccarrolllab.com/dropseq/). cDNA quality was determined by BioAnalyzer (Agilent) with a high sensitivity DNA chip.

**GSEA pathway analysis**

GSEA (http://software.broadinstitute.org/gsea/index.jsp) was used to estimate the enriched pathways in the collecting duct subgroups from biopsy and healthy kidney. We used the normalized UMI count matrix generated by Seurat (NormalizeData and ScaleData) as an expression dataset, and a gene set containing all human pathways downloaded from Bader Lab (http://download.baderlab.org/EM_Genesets/current_release/Human/Entrezgene/) as gene set file input to GSEA. We used 1,000 gene label permutations and identified significantly enriched pathways defined by adjusted p value < 0.05 between the collecting duct cells from biopsy and healthy kidney.

**Supplementary Tables**

**Table S1**

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**Tables S1-3: Clinical Features of Study Biopsy.**

(1) Haplotype matching of donor a recipient at class I and class II loci. (2) Full Banff criteria scoring for the study biopsy showed histologic features consistent with Banff 1B T-cell mediated rejection and acute C4d-negative antibody mediated rejection. (3) Donor specific antibody (DSA) found in the patients serum at the time of biopsy. mfi- mean fluorescent intensity, t = tubulitis, i = interstitial lymphocyte infiltration, v = intimal arteritis, ptc = peritubular capillaritis, g = glomerulitis, cg = transplant glomerulopathy, ci = interstitial fibrosis, ct = tubular atrophy, mm = mesangial matrix expansion, ah = arteriolar hyalinosis, cv = vascular intimal fibrosis.
Supplementary Table S4.
Link to data set .xls

Supplementary References

SIGNIFICANCE STATEMENT

The renal biopsy provides critical diagnostic and prognostic information for clinicians when patients develop kidney disease. Today, biopsies are read using a combination of light microscopy, electron microscopy, and indirect immunofluorescence with a limited number of antibodies. These techniques were all perfected decades ago. More recently, new techniques in single-cell genomics have been transforming scientists’ ability to characterize cells. Rather than measure expression of several genes at a time by immunofluorescence, it is now possible to simultaneously measure expression of thousands of genes in thousands of single cells by single-cell RNA-sequencing (scRNA-seq). We show that comprehensive scRNA-seq of a single human kidney transplant biopsy is feasible and that it allows the molecular characterization of this heterogeneous tissue.