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## Resident Macrophage Subpopulations Occupy Distinct Microenvironments in the Kidney

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**Abstract:**

The kidney contains a population of resident macrophages from birth that expands as it grows and forms a contiguous network throughout the tissue. Kidney resident macrophages (KRM) are important in homeostasis and the response to acute kidney injury (AKI). While the kidney contains many microenvironments, it is unknown whether KRM are a heterogeneous population differentiated by function and location. We combined single-cell RNA sequencing (scRNAseq), spatial transcriptomics, flow cytometry, and immunofluorescence imaging to localize, characterize, and validate KRM populations during quiescence and following 19 minutes of bilateral ischemic kidney injury. scRNAseq and spatial transcriptomics revealed seven distinct KRM subpopulations, which are organized into zones corresponding to regions of the nephron. Each subpopulation was identifiable by a unique transcriptomic signature suggesting distinct functions. Specific protein markers were identified for two clusters allowing analysis by flow cytometry or immunofluorescence imaging. Following injury, the original localization of each subpopulation is lost, either from changing locations or transcriptomic signatures. The original spatial distribution of KRM is not fully restored for at least 28 days post-injury. The change in KRM localization confirms a long hypothesized dysregulation of the local immune system following acute injury and may explain the increased risk for chronic kidney disease.

## **Introduction:**

Macrophages are ubiquitous in the kidney (1). They perform numerous functions within the kidney, including integration of signals related to tissue health, phagocytosis of debris and apoptotic cells, defense against infectious agents entering via the urinary tract, and responses to physical injury or damage by pharmacologic agents. In a normal murine kidney, macrophages constitute approximately 50-60% of CD45<sup>+</sup> immune cells and of those, the majority are tissue-resident, having seeded into the kidney in embryonic and early life from the fetal yolk sac, fetal liver, and bone marrow (2). Kidney resident macrophages (KRM) are defined as such because they are replenished primarily through self-renewal and receive no further input from the peripheral circulation (3, 4). The KRM population can be distinguished from infiltrating macrophages by their high expression of F4/80 (also known as Emr1 and Ly71) and intermediate expression of CD11b (integrin  $\alpha_m\beta_2$ , also known as Mac-1) (5). Infiltrating macrophages, which are exclusively bone marrow-derived, turnover within two weeks (3), and express lower levels of the F4/80 antigen and higher levels of CD11b (F4/80<sup>INT</sup>, CD11b<sup>HI</sup>). Parabiosis studies following an ischemic event show that these characteristics do not change after kidney injury (3).

Kidney macrophages are known to be important in acute kidney injury (AKI), but their conflicting roles in the pathogenesis and recovery from injury are poorly understood. In models of AKI such as kidney ischemia-reperfusion injury in mice, the extent and timing of macrophage depletion affect the outcome of injury (6, 7).

KRMs are distributed throughout the kidney which contains distinct physiological microenvironments along the nephron. Macrophage location has been shown to affect function in other organs/tissues (8–12). Therefore, KRMs may have specialized functions tailored to the local cellular environment and possess transcriptional programs to respond distinctly to acute injury. We have previously shown in an ischemia-reperfusion injury model that there are changes in the expression of selected surface molecules, such as MHCII, and in transcriptional

programming, as measured by bulk mRNA sequencing (3). It is not clear if the observed transcriptomic and phenotypic shifts reflect alterations in global macrophage programming or previously indistinguishable subpopulations.

We used single-cell RNA sequencing (scRNAseq) and spatial transcriptomics to demonstrate that KRMs consist of at least seven major subpopulations localized to defined zones within the kidney. The distinct transcriptomic programs of each subpopulation suggest specialized functions including type I interferon responses, heme/iron metabolism, inflammation, or anti-bacterial responses. Following acute ischemic injury, the transcriptomic programming is altered, and the delineation of the subpopulations within specific zones is lost and does not return to normal by 28 days post-injury, possibly reflecting chronic immune dysregulation.

## **Results:**

### **Generation of a comprehensive single-cell kidney resident macrophage dataset**

We performed scRNAseq on CD45<sup>+</sup>TCRb<sup>-</sup>CD19<sup>-</sup>NK1.1<sup>-</sup>Gr-1<sup>-</sup>CD11b<sup>INT</sup>F4/80<sup>HI</sup> KRMs identified and sorted by flow cytometry as described previously (Figure S1) (3). The KRMs were obtained from the kidneys of quiescent (uninjured) mice and those subjected to 19 minutes of bilateral warm ischemia (37± 1°C rectal temperature) under ketamine-xylazine anesthesia (Figure 1A). Histologic changes and serum creatinine confirmed the initial injury on day one post-ischemia with observed hypercellularity and an average increase of 0.21 mg/dL in serum creatinine with subsequent recovery of function from days six to 28 post-injury (Figure 1B-C). Samples from three quiescent mice and three mice each at 12 hours, days one, six, and 28 days post-injury were sequenced using the 10X Genomics 3' Chromium platform. In total, 58,304 KRMs were sequenced and retained based on the expression of *Adgre1* (F4/80), *Ilgam* (CD11b), and *C1qa* (3, 13). The data from all time points were integrated using Harmony to facilitate a direct comparison of the analyses at each time point by minimizing batch effects and nonbiologic variation. After quality control, 3000 variable genes were identified for all groups, and after normalization and scaling, the cells were clustered. Dimensional reduction using uniform manifold approximation and projection (UMAP) resolved thirteen clusters (Figure S2A). Elimination of contaminating kidney parenchymal cells and clusters constituting ≤ 1% of the total number of cells, left seven clusters, indicating that the KRM population is composed of at least seven major distinct subpopulations with unique transcriptomic signatures (Figure 2A, 2SB). KRMs were distinguished from parenchymal cells by expression of canonical markers such as of *C1qa* (Figure 2B) (13).

### **Delineation of transcriptionally distinct kidney resident macrophages in quiescent mice**

We initially analyzed the quiescent samples to characterize the seven major subpopulations in the uninjured state (Figure 2A). A heatmap of the top five significant differentially expressed

genes (DEGs) in each cluster illustrates distinct transcriptional profiles and provides potential markers for identification (Figure 2C). Very few transcripts were uniformly expressed at high levels in Cluster 0, although *Ccr2* and *Ptprc* (CD45) were among the DEGs that defined this cluster (Figure 2C). The top DEGs in the remaining six clusters indicated at least one specific function. Cluster 1 expressed heat shock protein transcripts and the *Fos* and *Btg2* transcription factor/cofactors, which are associated with the immediate-early response (Figure 2C). These transcripts may be involved in homeostatic function or may indicate priming towards the immediate early-response by this cluster, allowing for Cluster 1 to preferentially upregulate the immediate early-response during the dissociation protocol. Cluster 2 exhibited increased transcription of *Hmox1* (heme oxygenase-1), which is the rate-limiting step in the degradation of heme into iron, biliverdin, and carbon monoxide. Notably, we also observed increased expression of *Slc40a1* (ferroportin), *Prdx1* (peroxiredoxin), *Fth* (ferritin heavy chain), and *Ftl* (ferritin light chain), which supports the supposition that heme/iron handling is an important function of cluster 2 (Figure 2C, Figure S3A). Cluster 3 is enriched for genes associated with antimicrobial processes and inflammation such as *Cxcl2*, *Ccl3* (macrophage inflammatory protein 1- $\alpha$ ), and *Ccl4* (macrophage inflammatory protein 1- $\beta$ ), which are involved in the recruitment of inflammatory cells. *CD14*, which is engaged in toll-like receptor signaling by LPS, is expressed by these cells at high levels (Figure 2C, Figure S3B). Cluster 4 expresses transcripts involved in fibrosis, including *Pf4*, *CD206*, and *Stab1* (Fig S3C)(14–18). Cluster 5 can be distinguished by the expression of *Vim*, *Ccl2*, *Tnip3*, and *Ccr2* transcripts (Figure 2C, Figure S3D). Cluster 6 is enriched in the expression of several genes associated with type I interferon responses such as *Isg15*, *Ifit3*, and *Ifit2*, indicating a possible role in anti-viral responses (Figure 2C, Figure S3E). A full listing of the DEGs is provided in the supplementary data.

Gene ontology enrichment analysis (GO: biologic process) determines the extent to which genes associated with specific functions are represented in each cluster. As seen in

Table 1, enrichment of specific gene sets could be assigned with varying degrees of confidence to each cluster, except cluster 5, which contained too few cells in quiescence for a reliable estimation. The most significant terms in Clusters 1, 3, and 6 are involved in anti-bacterial, anti-viral, and anti-fungal responses. Cluster 2 contained terms related to responses to iron, phagocytosis, and wound healing, suggesting involvement in homeostatic functions. Clusters 0 and 4 mapped to few terms but the analysis contained references to tumor necrosis factor and apoptosis. These disparate gene ontology mappings suggest that each cluster executes a distinct transcriptional program which could be a function of the location in which each cluster resides.

### **Kidney resident macrophage subpopulations are localized to zones associated with specific nephron structures**

We identified KRM locations by integrating the scRNAseq data (Figure 2A) into spatial transcriptomic datasets generated from kidney sections of three individual mice. The spatial matrix was integrated with the scRNAseq dataset using the anchor-based workflow built into the Seurat package, which allows the probabilistic mapping of scRNAseq/snRNAseq-defined clusters onto a histological kidney image (Figure S4) (19–22). We validated this approach using a single-nuclear dataset from whole kidneys, in which the cell types identified by snRNAseq mapped to expected histologic locations in the kidney (Figure S5). The seven transcriptionally defined KRM subpopulations were localized to distinct locations in the kidney (Figure 3, right panel), corresponding to zones enriched for specific nephron structures (Figure 3, left panel), which were designated zones I - V and confirmed using zone-specific transcripts (23) (Figure 3, middle panel). The localization patterns suggest a zonal organization of KRMs from the cortex to the papilla. For example, Cluster 0, appearing in zone II, is largely concentrated in the cortico-medullary region and appears to be associated with the S3-segment of the proximal tubule, as determined by marker transcripts for the S3 proximal tubule (*Slc7a13*, *Napsa*, *Nudt19*). Cluster

4 and cluster 6 appear largely in zone III and their spatial distributions are strikingly similar to the distributions of genes associated with the ascending loop of Henle. Within zones III and IV, we observed the localization of more than one KRM cluster. In both zones, each cluster was mapped similarly, but with slightly different distributions. The spatial organization of each KRM cluster is unique and associated with specific zones within the kidney (Figure 3).

### **Validation of select KRM markers**

To validate the general location of specific clusters shown by spatial transcriptomics, we used flow cytometry to visualize the expression of select proteins which were transcriptionally distinct within clusters. Because cluster 3 resides primarily in the medulla (Figure 4A), we performed flow cytometry to compare expression levels of CD14 protein among cells isolated separately from the medulla and the cortex. Analysis of the most significant DEGs for each KRM cluster by adjusted p-value indicated that transcripts for CD14, a pathogen-associated molecular pattern receptor involved in the recognition of lipopolysaccharide, were expressed in all KRMs, but appeared to substantially increase in cluster 3 (dot plot, Figure 4A). Flow cytometry of kidneys manually dissected into cortex and medulla allowed for the identification of increased CD14<sup>++</sup> cells among CD45<sup>+</sup>TCRb<sup>-</sup>CD19<sup>-</sup>NK1.1<sup>-</sup>Gr1<sup>-</sup>CD11b<sup>INT</sup>F4/80<sup>HI</sup> KRMs in the medulla, but not the cortex of the kidney (Figure 4B). This aligned with the plotting of Cluster 3 using spatial transcriptomics, which appeared primarily in the medulla and papilla. In comparison, Mannose receptor C-type 1 (*Mrc1/CD206*) expression is increased in Cluster 4. This cluster is spatially distributed in the inner medulla and, to a lesser extent, the outer cortex (Figure 4C). Immunofluorescence imaging reveals CD206/*Mrc1*<sup>+</sup> KRMs in the outer cortex and inner medulla, however, they are largely absent from the inner cortex, which is consistent with the spatial transcriptomics data (Figs. 4D-E).

## **Ischemic injury alters the spatial and transcriptomic profiles of KRM subpopulations and they remain altered four weeks post-injury**

We next determined the effect of ischemic injury on transcriptomic programming and spatial distribution of KRMs as a function of time. Figure 5A shows UMAP plots for KRMs from quiescent kidneys, and also 12 hours, days one, six, and 28 post-injury. Relative to quiescent kidneys, proportional changes in the representation of each KRM cluster was observed within 12 h following the ischemic insult. The proportions at day 28 appear similar to those at quiescence (Figure 5B). Most notable among these acute changes are increases in the proportions of cells in clusters 2 and 5.

Following injury, there is a change in cluster spatial locations (Figure 5C). This was observed in the early inflammatory phase (12 hours and day one post-injury) as well as in the reparative phase (day six post-injury). For reference, kidney cell markers at each time point are provided and appear similar to the quiescent profile (Figure S6-9). At 12 hours, we identified clusters that appeared to be in intermediate locations between their initial sites and their positions on day one and day six post-injury. This is exemplified by the medullary subpopulations, Clusters 1 and 3. During quiescence, these populations are predominantly present in zones V (papilla) and IV (inner medulla), respectively. At 12 hours post-injury, Cluster 1 and Cluster 3 map to the cortical/medullary region. By day one post insult, Cluster 1 is concentrated at the corticomedullary junction but also appears to be distributed throughout the cortex. Cluster 3, initially found in the papilla, is also found in the cortex (Figure 5C).

Spatial transcriptomics from day 28 shows some KRM subpopulations do not map to their initial locations (5C). A comparison of KRM subpopulations at day 28 to their quiescent profile showed that while clusters 0, 1, and 2 mostly map to their quiescent location, clusters 3, 4, 5, and 6 appear scattered throughout the tissue (Figure 5C). Notably, the day 28 kidneys appear to lose spatially distinct medullary KRM subpopulations.

The GO terms for Clusters 1, 3, and 6 at day 28 remain anti-viral and anti-bacterial (Table S3). Cluster 2 has reestablished terms involved in iron handling. While GO terms appear for cluster 0, they have FDRs above the significance level. Clusters 4 and 5 continued to be involved in chemotaxis.

### **Discussion:**

Using scRNAseq in conjunction with spatial transcriptomics, we identified, characterized, and localized seven major subpopulations of KRMs in the mouse kidney before and after ischemic injury. We revealed that KRM subpopulations exist within microenvironmental niches associated with specific zones, roughly approximating layers from the cortex to the papilla. After the injury, the original compartmentalization of KRM subpopulations is lost and is still not fully regained 28 days post-injury.

Recent work in the area of the kidney myeloid immune system has focused on increasing the understanding of the various subpopulations in quiescence and following injury (15, 24). Our work expands on these evolving definitions and characterizes protein expression markers for multiple transcriptionally distinct subpopulations. Current studies analyze the macrophage compartment as a whole, which provides important information on infiltrating monocytes, but can cloud the understanding of the KRM population. By focusing solely on the KRMs, we have identified at least seven transcriptionally distinct major subpopulations. Some of these, such as the type I IFN-responding Cluster 6, share similarities with resident macrophage populations in other tissues (25, 26). Others, like Cluster 5 which expands after ischemic injury, may be unique to the kidney.

In an attempt to generate a comprehensive understanding of kidney macrophages, we identified quiescent protein expression markers for subpopulations that had been identified across injury models previously by other groups. We identified cluster 3 as CD14<sup>++</sup> and cluster 4 cells as CD206<sup>+</sup>. In human kidneys, CD14<sup>++</sup> macrophages have been identified as a

subpopulation primed against bacterial infection and responsive to salt gradients in models of urinary tract infections (27). Additionally, medullary CD14<sup>++</sup> cluster 3 KRMs express transcripts involved in neutrophil chemotaxis and activation. Previous studies by Berry et al. demonstrated that medullary CD14<sup>++</sup> macrophages isolated from human medulla activate neutrophils (27). CD206<sup>+</sup> macrophages have been identified by other groups in models of unilateral ureteral obstruction and polycystic kidney disease (15, 28). Like these studies, our CD206<sup>+</sup> cluster 4 KRMs express transcripts for *Stab1*, *Fcrls*, and *Igf1*, which are associated with tissue regeneration and fibrosis. In the liver, resident macrophages that express similar transcripts to cluster 4 are thought to be involved in the prevention of fibrosis following insult(29). Zimmerman et al. identified CD206<sup>+</sup> KRMs surrounding cysts in polycystic kidney disease development and inhibition of KRM proliferation decreased CD206<sup>+</sup> KRM numbers and lessened cyst growth and numbers, suggesting that cluster 4 cells could be involved in cyst formation or fibrosis progression.

Stratification of KRMs into specific zones within the kidney was previously unknown. The spatial location of macrophages impacts their function in other tissues, such as the lung, spleen, and liver, and response to an immunological challenge, in the case of tuberculosis or tumors (8–12). Although many disease states have known connections with KRMs (30–32) and targeting populations holds great therapeutic promise, successful design and implementation of such strategies are limited by our current understanding of KRM regulation and response to injury. As it relates to location, Cluster 0 is initially at the site of the greatest insult during an ischemic event. The lack of oxygen in the tissue damages the mitochondria-heavy S3 proximal tubule cells in the cortico-medullary junction (33, 34). Cluster 1 is the second-largest KRM population and is concentrated in the papilla. Its presence in zone V, which has aquaporin 2 gene expression, suggests that cluster 1 cells are found near the collecting ducts. Prior studies have indicated a relationship between macrophages and the collecting duct in the context of IgA nephropathy, with one study suggesting a role in driving fibrosis in that region (35, 36). Cluster

2, which is distinguished by its iron handling transcripts, is located in the cortex around proximal tubules, where iron is reabsorbed in healthy conditions and accumulates during iron disorders (37). Additionally, the subpopulation locations suggest a coordinated positioning to protect the kidney from infection. The transcriptome and location of clusters 1, 3, and 6 depict a strategic immune barrier from the ureter, the most common origin of kidney infections (38). Macrophage subpopulations in other tissues, such as the lung, gut, and skin, are known to perform this function (39–42). Our work highlights the diversity amongst the KRMs and the corresponding need to selectively target specific subpopulations with respect to the originating mechanism of insult.

It is similarly important to consider timing, as the KRMs alter their spatial location post-injury. This apparent movement can be ascribed either 1) to a physical translocation of the KRMs following injury and/or 2) to KRM subpopulations in specific locations undergoing a transition from transcriptional profiles associated with one cluster to another. The former hypothesis is supported by data showing that 12 hours post-injury, clusters 1 and 3 appear at intermediate locations between their quiescent and 24-hour locations. In addition, GO analysis from timepoints following injury identifies many terms involved in cell migration and motility. This further supports that the KRM subpopulations have location-specific responses to areas of damage within the kidney and may both relocate and alter their transcriptional profile in response to injury. While our data support the possibility that KRMs are migrating, future experiments will need to confirm KRM locomotion.

Nevertheless, the transcriptomic atlas, with many KRM subpopulations no longer expressing their original profiles and existing within new locations, is persistently altered and future therapeutics will need to consider the location at the time of treatment to accurately gain the desired effect. During the course of our studies, the transcriptomic profile failed to return to quiescence, even after 28 days. Given the continued disruption in transcriptional and spatial distribution beyond acute injury, KRMs may influence the AKI to chronic kidney disease (CKD)

transition. A single AKI event drastically increases the risk for the development of CKD (43–46), although the mechanisms that underlie the AKI to CKD transition remain unclear. Multiple cell types have been implicated, including damaged proximal tubules and vascular cells in addition to leukocytes (47). KRMs are of particular interest given their capacity for inflammation and fibrosis and continued presence in the tissue beyond an acute injury (48–52). Future studies should consider the effect of the altered KRM subpopulations on long-term kidney function both from native kidneys and the following transplant.

This work has characterized the subpopulations of KRMs and determined their location within healthy kidneys. Following insult, we tracked the subpopulations as they appear to relocate throughout the tissue, suggesting locomotion by these cells in response to injury, as a result of tubule cell death and/or transient ischemia to the various subpopulations.. Finally, our data confirm a long-hypothesized dysregulation of the immune system following AKI and provide a foundational framework for the increased risk for CKD following an AKI event. The result is a KRM atlas of the murine kidney that can provide a point of reference for future studies into the role of the resident macrophage system in the normal and injured kidney.

## **Methods:**

### Animals and Study Approval

Male C57BL/6J mice, 12-16 weeks of age, were obtained from The Jackson Laboratory. Mice were housed at the University of Alabama at Birmingham (UAB) animal facilities in compliance with the National Institutes of Health Guidelines regarding the care and use of live animals.

### Bilateral ischemia-reperfusion injury (BIRI)

Mice were subjected to BIRI as previously described (3, 4). All surgeries were performed in the morning. Mice were anesthetized using ketamine and xylazine (IP). Under aseptic precautions, both kidneys were clamped at the renal pedicle using a micro-serrefine vascular clamp (Fine Science Tools, 18055-05). After 19 minutes, the clamps were removed to allow reperfusion. Reperfusion was visually confirmed within one minute. Careful maintenance of body temperature at 37° C +/- 1° C was measured by a rectal thermometer.

### Flow Cytometry/Fluorescence-Activated Cell Sorting (FACS)

KRMs represent approximately 1-2% of total viable cells in a quiescent kidney. In order to obtain a minimum number of cells to draw meaningful conclusions about KRM subpopulations as determined by scRNAseq, we isolated KRMs using flow cytometric sorting. Leukocytes were isolated as previously described (3, 4). Mice were anesthetized under isoflurane and perfused through the left ventricle with 10 mL of cold PBS. Kidneys were removed, stripped of the capsule, minced with a razor blade on a glass slide, and placed into Liberase (Sigma) at 37 degrees Celsius for 30 minutes. The digestion was stopped by adding cold PBS containing 1% BSA and tissue was further disaggregated through an 18-gauge syringe. Red blood cells were lysed using ACK lysis buffer for 2 minutes at room temperature and the remaining leukocytes were then washed with ice-cold PBS. Cells were then stained with Aqua fixable viability dye, and treated with unlabeled anti-CD16/32 antibody to block Fcγ3 receptors. Cells were

subsequently stained using anti-Gr-1 Alexa-fluor(AF)-700 (Ly6G, clone 1A8, BioLegend), anti-CD11b super bright (SB)-600 (M1/70, Invitrogen), anti-F4/80 APC-eFluor-780 (BM8, Invitrogen), anti-NK1.1 PE-C7 (PK136, Invitrogen), anti-CD45.2 BV-650 (104, BioLegend), anti-MHC II (I-A/I-E) PerCP (M5/114.15.2, BD Biosciences), anti-CD19 SB-702 (6D5, BioLegend), and anti-TCR $\beta$  Pe-Cy5 (H57-597, BD Biosciences), anti-CD14 APC (Sa2-8, Invitrogen) (Fig S1).

#### Nuclear isolation from whole kidney

Nuclei were isolated using Nuclei Lysis Buffer containing Nuclei Isolation Kit: Nuclei EZ Prep Buffer (Sigma) supplemented with cOmplete ULTRA Tablets (Sigma) and SUPERase IN (Thermo) and Promega RNasin Plus nuclease inhibitors. Kidneys were minced into <1 mm pieces in 2 mL of Nuclei Lysis Buffer. Samples were transferred to a dounce homogenizer (Kimble) and homogenized. An additional 2 mL of Nuclei Lysis Buffer were added to the sample and incubated for 5 minutes on ice. Samples were passed through a 40  $\mu$ m filter into a 50 mL conical tube. Samples were centrifuged at 500 x g for 5 minutes at 4° C. The supernatant was removed and the pellet was washed with 4 mL of Nuclei Lysis Buffer containing 1% Bovine Serum Albumin for 5 minutes on ice. Samples were centrifuged at 500 x g for 5 minutes at 4° C. Samples were passed through a 5  $\mu$ m filter into a 50 mL conical tube and then centrifuged again. Nuclei were resuspended in a solution containing phosphate buffered saline (PBS), 1% BSA, and 0.1% RNase inhibitor.

#### Single-cell/Single-nuclear RNA sequencing

Purified cells or nuclei were transferred on ice to the UAB Flow Cytometry and Single Cell Core and immediately processed using the Chromium 3' Single Cell RNA sequencing kit (10X Genomics) according to the manufacturer's instructions. The cell suspension was counted and combined with a 10X Chromium reagent mixture and loaded into a microfluidic single-cell partitioning device in which lysis and reverse transcriptions occur in microdroplets. The resulting

cDNA was amplified by a polymerase chain reaction and subsequently processed to yield bar-coded sequencing libraries. Paired-end sequencing was carried out on an Illumina NovaSeq6000 sequencing platform (Illumina). Reads were processed using the 10x Genomics Cell Ranger Single-Cell Software Suite version 6.0 on the UAB Cheaha High-Performance Computing Cluster. BCL files were converted to FASTQ files using the CellRanger mkfastq function. CellRanger count was used to align the FASTQ files to the mouse genome (mm10). The genes table, barcodes table, and transcriptional expression matrices were created for the analysis indicated below.

### Spatial Transcriptomics

The Visium system relies on a 2-dimensional matrix of 5000 spots distributed on a microscope slide in a 6.5 by 6.5 mm square. Each spot, which contains a poly-dT oligonucleotide with a unique sequence (bar code), is 50um in diameter at a distance from the other spots of 100um from center to center. Kidneys were embedded in the Optimal Cutting Temperature matrix and stored at -80.C Before sectioning, blocks were equilibrated to -10C for 30 minutes. A 10 um section was placed onto specialized Spatial Gene Expression slides (10X Genomics) and processed according to the manufacturer's protocols. Briefly, slides were stained with hematoxylin and eosin and brightfield images were acquired using a Keyence BZ-X700 microscope. Tissues were permeabilized for 18 minutes, cDNA was generated, and used to create second strand DNA. The resulting cDNA was subject to downstream amplification and library processing for single-cell RNA sequencing. Reads were processed using the 10x Genomics Cell Ranger Single-Cell Software Suite version 6.0 on the UAB Cheaha High-Performance Computing Cluster. BCL files were converted to FASTQ files using the SpaceRanger mkfastq function. SpaceRanger count was used to align the FASTQ files to the mouse genome (mm10).

### Sequencing Analysis

Both scRNAseq and spatial transcriptomics analyses were carried out using packages created for the R statistical analysis environment (version 4.06). Data were primarily analyzed using Seurat version 3.2.3 and its associated dependencies (19, 53) as previously described (54). Data from each mouse were imported using the Read10X function and then structured into a Seurat object using CreateSeuratObject. For quality control, cells with unique feature counts over 2,500 or under 200. Data were normalized and scaled using SCTransform(55). Objects from each time point were labeled with unique group IDs and then merged into a single object using the Seurat merge function. Data objects were integrated using the Harmony R package (56). Principal component analysis was performed based on 30 principal components and then cells were clustered using FindAllMarkers set to a resolution of 0.4. The dimensional reduction was done using uniform manifold approximation and projection (UMAP). WebGestaltR was used for gene ontology analysis to identify pathways using the Biologic Process and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases (57).

### Integration of scRNAseq and spatial transcriptomics to resolve cell location

The spatial matrix was integrated with the scRNAseq dataset using the anchor-based workflow built into the Seurat package. FindTransferAnchors was used on the data object containing all seven KRM clusters. Using the generated anchor set, the TransferData function created predictions from the KRM reference clusters and applied that to the spatial dataset. Using a predictions assay, each subpopulation could be visualized using SpatialFeaturePlots.

### Immunofluorescence staining and Imaging

Kidneys from Cx3Cr1+/GFP mice (a gift from Dr. Bradley Yoder's laboratory) were cryosectioned onto glass slides and then fixed in 4% paraformaldehyde for 10 minutes. Tissues were permeabilized using 0.2% Triton X-100 in PBS for 8 minutes, washed three times with

PBS, and then blocked in a solution containing PBS, 0.1% Triton X-100, 1% bovine serum albumin, and 1% donkey serum for 30 minutes at room temperature. Primary antibodies including anti-CD206 (abcam, #ab64693, 1:250) were diluted in a blocking solution and stained overnight at 4C. Tissues were washed with PBS, stained with secondary antibodies including anti-rabbit-Alexa Fluor 595 (Invitrogen, #A21207, 1:1000) and anti-rat-Alexa Fluor 647 (Invitrogen, #A21247, 1:1000) for 30 minutes at room temperature, and then washed again with PBS. A 1:1000 DAPI solution was added for 5 minutes at room temperature and then washed with PBS. Slides were mounted with IMMU-MOUNT (Thermo Fisher). All images were captured on Nikon Spinning-disk confocal microscope with Yokogawa X1 disk, using a Hamamatsu flash4 sCMOS camera using the 40X Oil immersion objective. Images were processed and analyzed in NIS Elements software (Nikon) version 5.0 and ImageJ. A blinded observer quantified the number of CD206+Cx3cr1+ cells out of the total Cx3Cr1+ cells per high power field.

#### Data Sharing Statement

The scRNAseq and spatial transcriptomics data generated in this paper are available in GEO under accession number GSE200115.

#### Statistics

GraphPad Prism 8.0 was used for the statistical analysis. The mean  $\pm$  SEM was determined for each treatment group in the experiment. A one-way ANOVA followed by post-hoc analysis, was used to determine the statistical significance between groups. *P* values of less than 0.05 were considered significant.

#### Study Approval

All animal work performed was reviewed and approved by the Institutional Animal Care and Use Committee at UAB.

**Author Contributions:**

MDC, ENE, JML, AA, and JFG designed the experiments, MDC, EE, JML, JRL, SL, ZY, and ZL performed the experiments, MDC, EE, KHM, GG, AA, and JFG performed data analysis and interpretation, MDC, EE, KHM, AA, and JFG wrote the manuscript, all authors reviewed and approved the manuscript prior to submission. The authorship order among co-first authors was agreed upon after discussion by the authors.

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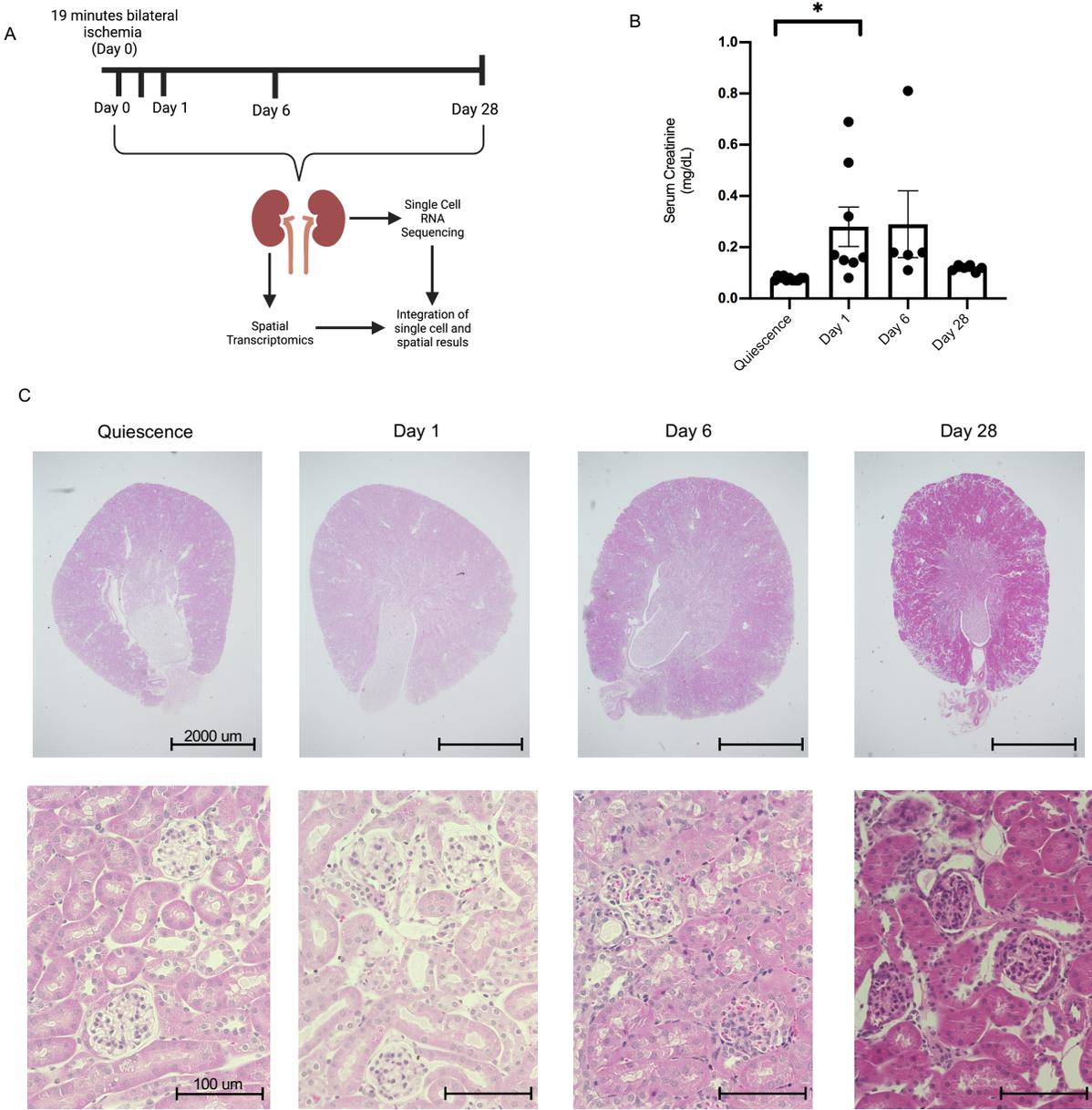
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**Figures:**

**Figure 1**

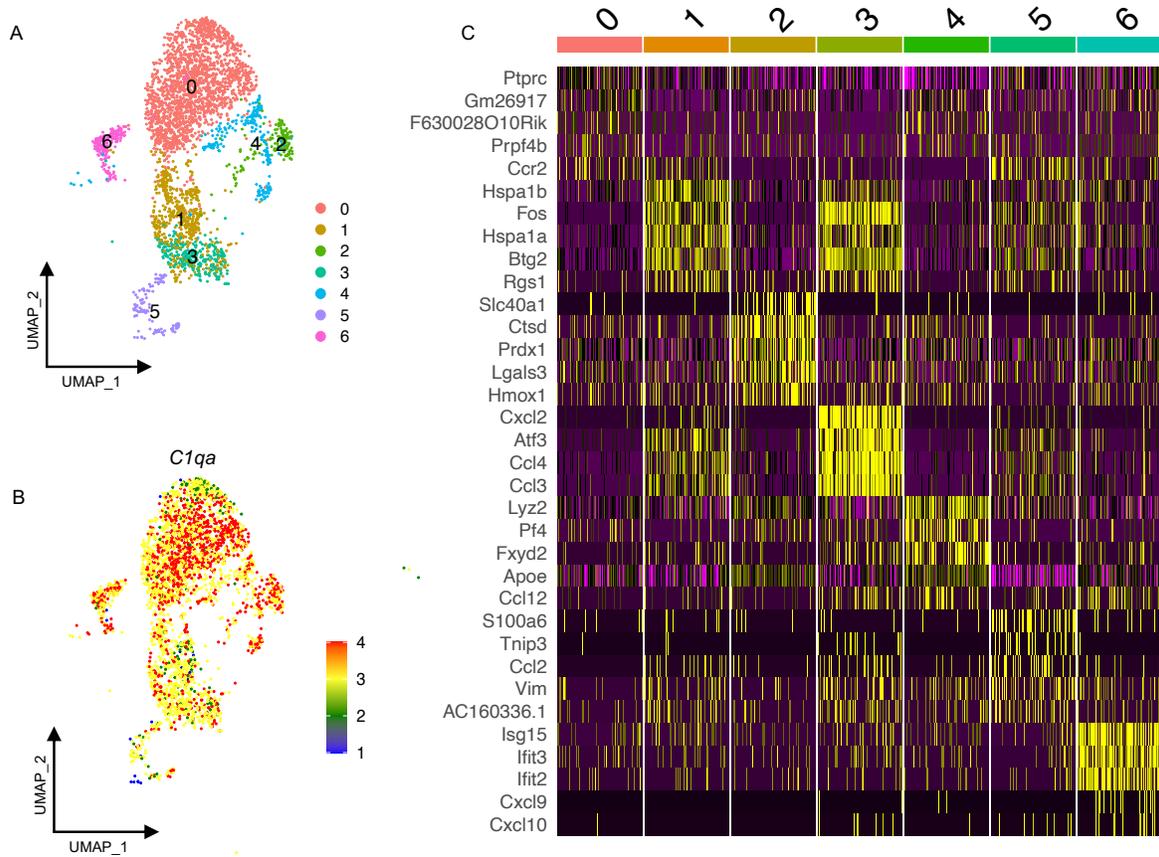


**Figure 1. Model of acute kidney injury**

A) Schematic depicting work flow for scRNAseq and spatial transcriptomics. Mice were subjected to bilateral ischemia-reperfusion injury for 19 minutes. Kidneys were harvested at day 0 and 12 hours, days 1, 6, and 28 after injury. Kidneys were either utilized for spatial

transcriptomics or digested and flow sorted for KRMs and subjected to scRNAseq. There were three biological replicates per timepoint. B) Serum creatinine levels (mg/dL) at quiescence (day 0) and days 1, 6, and 28 post-injury from at least two independent experiments. Data are reported as mean  $\pm$  SEM. A one way ANOVA was used to determine the statistical significance between groups.  $*P < .05$  C) Hematoxylin & Eosin-stained kidney sections at quiescence and days 1, 6, and 28 post-injury. Scale bar is 2,000  $\mu\text{m}$  for 2X objective images and 100  $\mu\text{m}$  for 40X objective images.

Figure 2

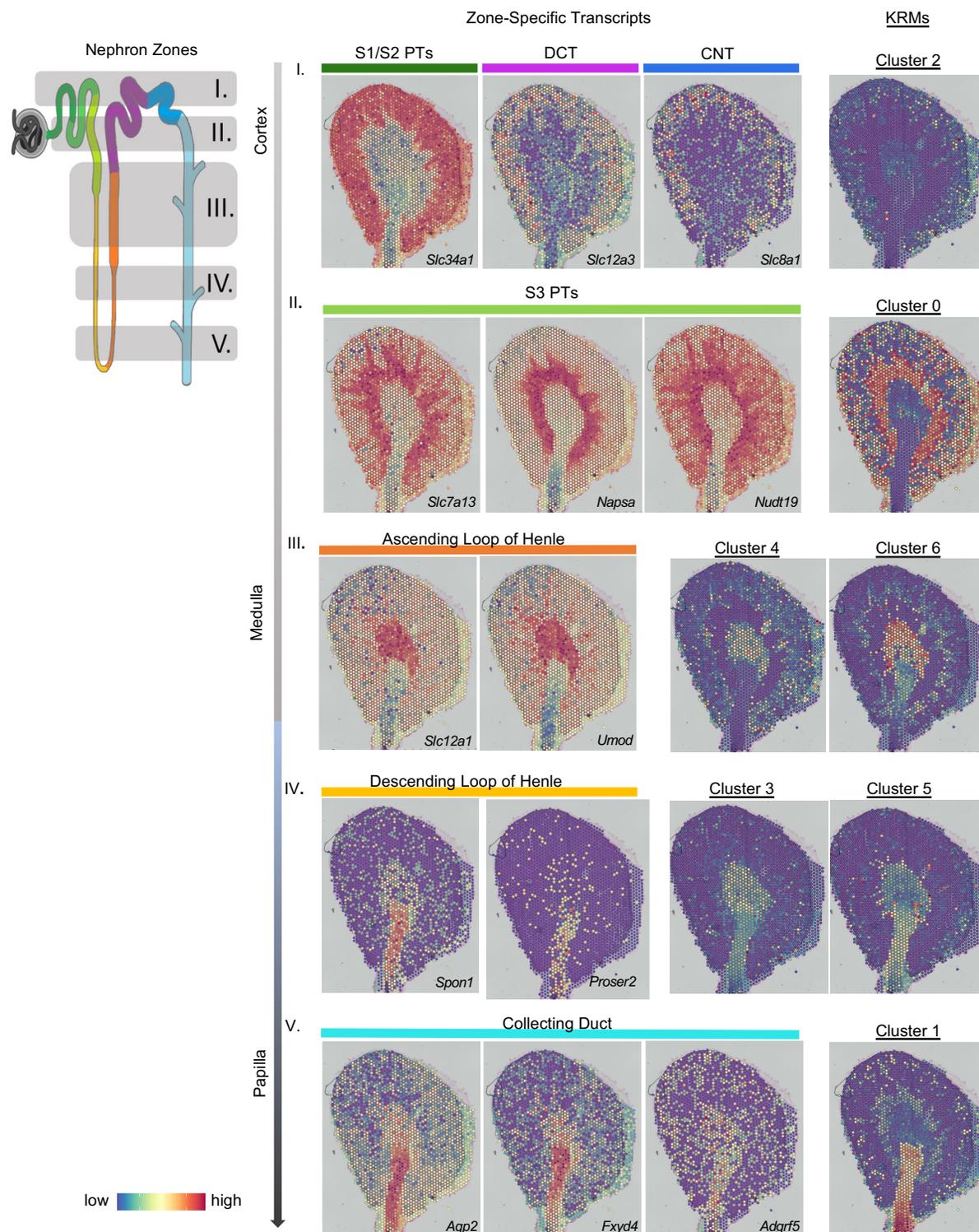


**Figure 2. Single-cell RNA sequencing (scRNAseq) and spatial transcriptomics reveals distinct subpopulations of kidney resident macrophages (KRM).**

A) Uniform manifold approximation and projection (UMAP) plot of sequenced kidney resident macrophages (KRM) demonstrates thirteen clusters. Contaminating kidney cells and clusters representing less than one percent were removed to leave seven unique clusters in quiescence.

B) *C1qa* expression in all KRM clusters during quiescence. C) Heatmap of top five differentially expressed genes amongst each subpopulation in quiescence ordered by adjusted p-value.

Figure 3

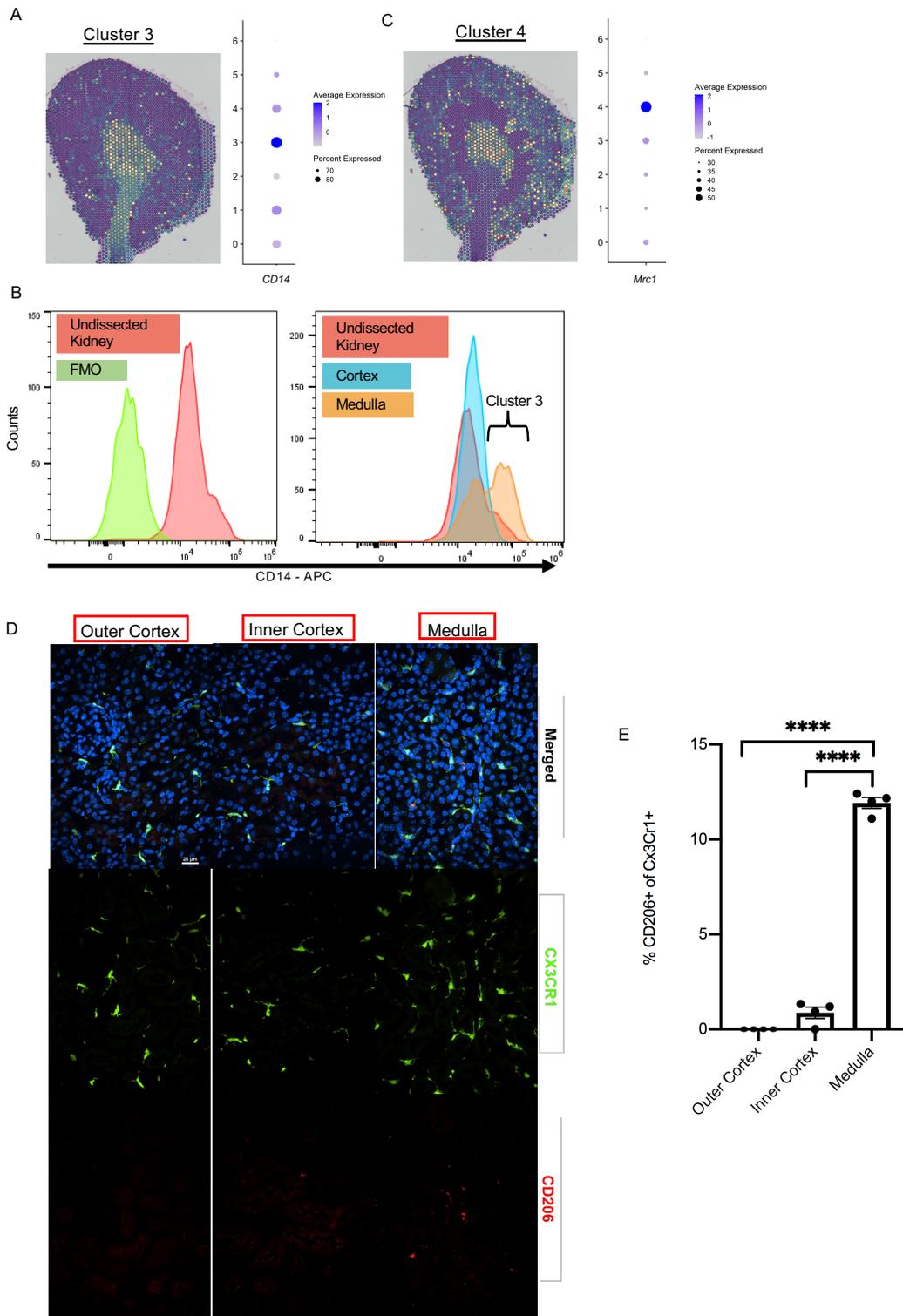


### **Figure 3. Kidney resident macrophages are found in distinct regions**

An integrated analysis of the scRNAseq and spatial transcriptomics data were performed to localize the KRM clusters on a kidney section. Nephron Zones (Left Panel): A diagram of a nephron is color coded to delineate different nephron segments. Gray shading and numbering (I-V) describes nephron zones that would be enriched in areas of a kidney cross-section. Zone-Specific Transcripts (Middle Panel): The spatial location of the nephron segments are shown by mapping segment-specific transcripts onto the histological image. Transcript markers are listed in the bottom right-hand corner of each section. Specific nephron segments are listed above each image. Colored bars correspond to the location of the segments from the nephron in the left panel. Row number (I-V) indicates the nephron zone. KRMs (Right Panel): The integration of the KRM scRNAseq data onto the spatial section plots the location of KRM subpopulations within the quiescent kidney. The clusters are aligned with the kidney nephron segments that are found in the same zone to highlight the colocalization between KRMs and kidney cells.

Abbreviations: CNT; connecting tubule, DCT; distal convoluted tubule, KRM; kidney resident macrophage, PT; proximal tubule

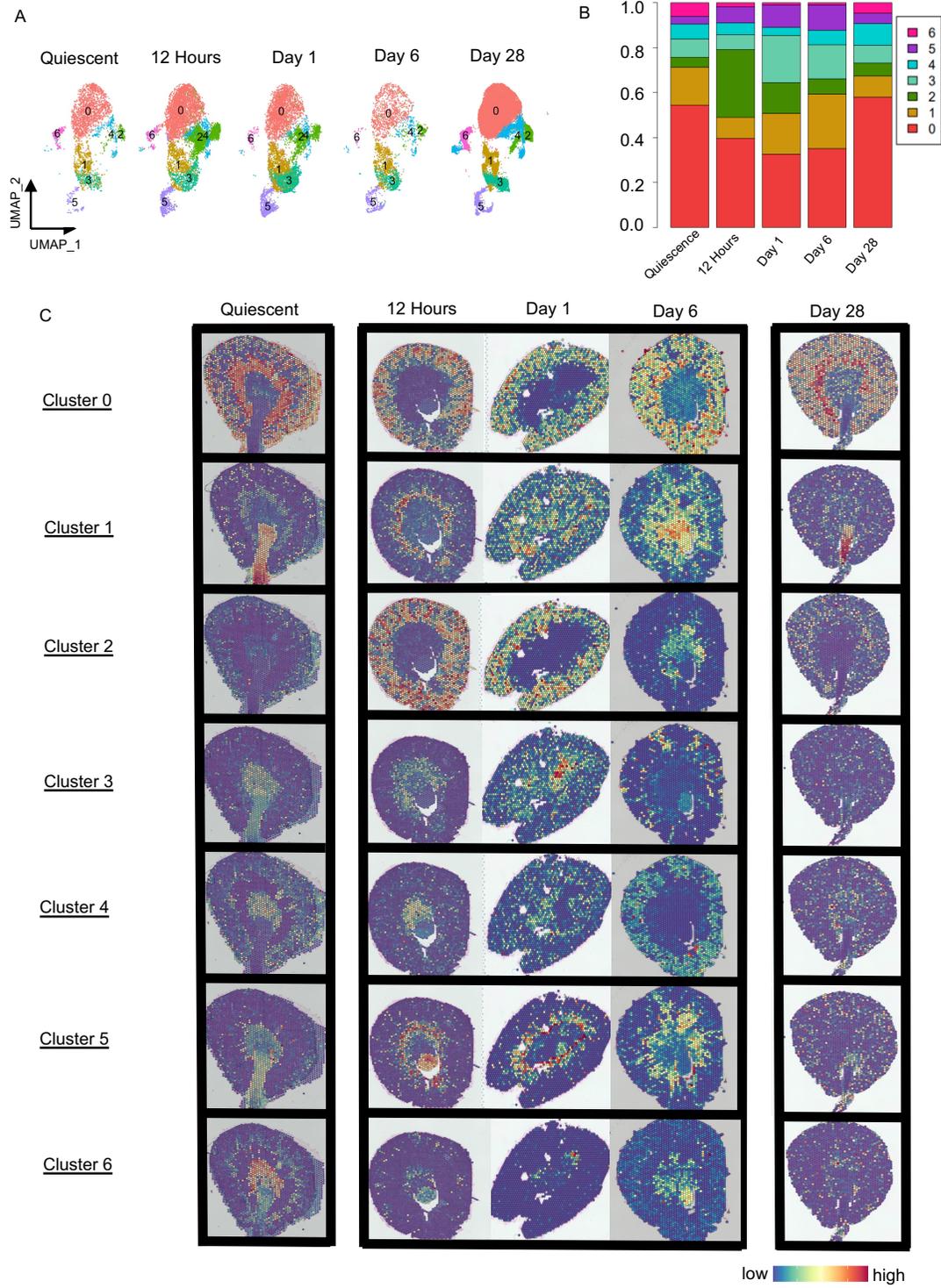
Figure 4



#### Figure 4: Spatial validation of protein markers

A) Spatial location of cluster 3 overlaid on the histological image and a dot plot of *CD14* transcript expression between each cluster. B) Kidneys were harvested and dissected to separate the cortex from the medulla to confirm the location of Cluster 3 CD14<sup>++</sup> cells in the medulla. Flow cytometry analysis of CD14 expression in KRM of the whole kidney (left), and dissected cortex compared to medulla (right) along with the fluorescence minus one (FMO) control. C) Spatial location of cluster 4 overlaid on histological image and a dot plot of *Mrc1* transcript expression show Cluster 4 is localized in the outer cortex and inner medulla, but not the inner cortex. D) Representative images from immunofluorescence of kidney sections of Cx3Cr1 GFP<sup>+/-</sup> mice stained with CD206, and the nuclear stain DAPI (4'6-diamidino-2-phenylidole) to validate cluster 4 KRM by confocal microscopy with a 40X objective. Results were averaged from four separate fields within each area with four mice in total over two independent experiments. Scale bar is 20  $\mu$ m. E) Quantitation from a blinded observer of CD206<sup>+</sup> KRM in the outer cortex, inner cortex, and medulla, expressed as a proportion of Cx3Cr1<sup>+</sup> cells. All p-values less than 0.0001 by one-way ANOVA followed by Tukey's Test. Error bars are SEM.

Figure 5



**Figure 5: Spatial and proportional changes to KRM subpopulations following injury**

A) Uniform manifold approximation and projection (UMAP) plot of KRM clusters at quiescence, 12 hours, day 1, day 6, and day 28 post-injury to assess changes after injury. B) Bar graphs depicting changes in proportions of each cluster over time. C) scRNAseq data from each time point integrated with their respective spatial transcriptomic kidney sections to resolve cluster locations. Each row represents a KRM cluster whereas each column depicts a timepoint from quiescence to day 28, left to right.

**Table 1: Gene ontology analysis of KRM clusters in quiescence**

Gene ontology analysis of terms with an FDR < 0.05.

**Table 1**  
**Gene Ontology Analysis<sup>1</sup>**  
**Quiescent**

Cluster	Gene Set	Description	Enrichment Ratio	p-Value	FDR
0	GO:0046631	alpha-beta T cell activation	15.9	1.13E-04	0.0470
0	GO:0032640	tumor necrosis factor production	15.6	1.19E-04	0.0470
0	GO:1903555	regulation of tumor necrosis factor superfamily cytokine production	15.6	1.19E-04	0.0470
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1	GO:0072593	reactive oxygen species metabolic process	9.6	1.70E-06	0.0026
1	GO:0034405	response to fluid shear stress	33.3	9.85E-05	0.0120
1	GO:2001057	reactive nitrogen species metabolic process	16.5	1.01E-04	0.0120
1	GO:0072538	T-helper 17 type immune response	30.1	1.33E-04	0.0140
1	GO:0042110	T cell activation	4.9	5.26E-04	0.0298
1	GO:0009611	response to wounding	4.6	7.66E-04	0.0361
1	GO:0002294	CD4-positive, alpha-beta T cell differentiation	15.3	9.93E-04	0.0424
-----					
2	GO:0042060	wound healing	7.2	3.96E-06	0.0012
2	GO:0006909	phagocytosis	10.7	2.09E-05	0.0038
2	GO:0010039	response to iron ion	26.2	2.03E-04	0.0178
2	GO:0006956	complement activation	19.8	4.69E-04	0.0328
2	GO:0002448	mast cell mediated immunity	18.9	5.34E-04	0.0342

2	GO:0031663	lipopolysaccharide-mediated signaling pathway	17.8	6.41E-04	0.0387
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3	GO:0071222	cellular response to lipopolysaccharide	8.8	6.14E-13	0.0000
3	GO:0030099	myeloid cell differentiation	6.3	2.30E-12	0.0000
3	GO:0032640	tumor necrosis factor production	8.8	2.91E-09	0.0000
3	GO:0035710	CD4-positive, alpha-beta T cell activation	11.0	2.55E-08	0.0000
3	GO:0032611	interleukin-1 beta production	13.0	2.83E-08	0.0000
-----					
4	GO:0034349	glial cell apoptotic process	102.0	3.17E-06	0.0099
4	GO:0071825	protein-lipid complex subunit organization	49.6	2.95E-05	0.0460
-----					
6	GO:0051607	defense response to virus	16.4	0	0.0000
6	GO:0034341	response to interferon-gamma	15.3	0	0.0000
6	GO:0034340	response to type I interferon	15.3	3.06E-07	0.0000
6	GO:0032606	type I interferon production	9.7	3.54E-07	0.0000
6	GO:0032496	response to lipopolysaccharide	4.3	5.44E-07	0.0000

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<sup>1</sup>No statistically significant results were found for cluster 5.