Single-Cell RNA Sequencing Identifies Candidate Renal Resident Macrophage Gene Expression Signatures across Species

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ABSTRACT

Background Resident macrophages regulate homeostatic and disease processes in multiple tissues, including the kidney. Despite having well defined markers to identify these cells in mice, technical limitations have prevented identification of a similar cell type across species. The inability to identify resident macrophage populations across species hinders the translation of data obtained from animal model to human patients.

Methods As an entry point to determine novel markers that could identify resident macrophages across species, we performed single-cell RNA sequencing (scRNAseq) analysis of all T and B cell–negative CD45+ innate immune cells in mouse, rat, pig, and human kidney tissue.

Results We identified genes with enriched expression in mouse renal resident macrophages that were also present in candidate resident macrophage populations across species. Using the scRNAseq data, we defined a novel set of possible cell surface markers (Cd74 and Cd81) for these candidate kidney resident macrophages. We confirmed, using parabiosis and flow cytometry, that these proteins are indeed enriched in mouse resident macrophages. Flow cytometry data also indicated the existence of a defined population of innate immune cells in rat and human kidney tissue that coexpress CD74 and CD81, suggesting the presence of renal resident macrophages in multiple species.

Conclusions Based on transcriptional signatures, our data indicate that there is a conserved population of innate immune cells across multiple species that have been defined as resident macrophages in the mouse. Further, we identified potential cell surface markers to allow for future identification and characterization of this candidate resident macrophage population in mouse, rat, and pig translational studies.


Following the discovery of mononuclear phagocytes by Eli Metchnikoff,1 the innate immune system has received notoriety as the first line of defense against foreign pathogens. The innate immune system consists of a complex array of cells, including neutrophils, natural killer cells (NK cells), innate lymphoid cells (ILCs), monocyte-derived macrophages, tissue resident macrophages, and dendritic cells.

Two major populations of macrophages are present within most solid tissues of mice and can be
classified on the basis of ontological origin and the cell surface markers F4/80 and CD11b. Although multiple reports have identified well-defined markers of resident macrophages across tissues in the mouse, whether these cells are evolutionarily conserved in higher mammalian species, including humans, remains uncertain because of a lack of conservation of markers between these species. For example, one of the markers used to define tissue resident macrophages in mice (adgre1, identified by the F4/80 antibody) is not expressed by macrophage populations in human tissue. Further, recent literature highlights the fundamental importance of resident macrophages in maintaining homeostasis and controlling disease progression. Therefore, the inability to identify resident macrophage populations across species is a major hurdle in translating data obtained in animal model systems to human patients. In these studies, we address this issue by using single-cell RNA sequencing (scRNAseq) to identify a set of genes whose expression is enriched in renal resident macrophages of mice, and show that these genes are also expressed in a distinct cluster of innate immune cells in multiple species. On the basis of the scRNAseq data, we identified two candidate cell surface markers that may define resident macrophage populations in mouse, rat, pig, and human kidney tissue.

METHODS

Animals

We purchased 8-week-old C57BL/6 male mice from the Jackson Laboratory (Bar Harbor, ME). We bred 3-month-old male Sprague–Dawley rats in house at University of Alabama at Birmingham (UAB). A 6-month-old male, uncastrated pig (Sus scrofa) was obtained from Synder Farms at 8–10 weeks of age and maintained at UAB. Animals were maintained in Association for Assessment and Accreditation of Laboratory Animal Care International-accredited facilities in accordance with Institutional Animal Care and Use Committee (IACUC) regulations at UAB (approval numbers 10130 and 21072).

Mouse Single-Cell Isolation

Eight-week-old male C57BL/6 wild-type mice were anesthetized with avertin (2,2,2-tribromoethanol; catalog no. T48402; Sigma-Aldrich), opened, and perfused with 20 ml 1× Dulbecco’s-PBS (catalog no. 21–030-CV; Corning). The kidney capsule was removed, kidneys (approximately 0.15 g) were minced with a sterile razor blade, and digested in 1 ml of RPMI 1640 containing 1 mg/ml collagenase type I (Sigma-Aldrich) and 100 U/ml DNase I (Sigma-Aldrich) for 30 minutes at 37°C, with agitation. For the 10× Genomics studies, three wild-type male kidneys were combined for sequencing.

Rat Single-Cell Isolation

A 3-month-old male Sprague–Dawley rat was anesthetized with carbon dioxide, opened, and perfused with 50 ml 1× Dulbecco’s-PBS. The kidney capsule was removed and approximately 2.5 g of kidney tissue containing both the cortex and medulla was weighed. The kidney was minced with a sterile razor blade and digested in 10 ml of RPMI 1640 containing 1 mg/ml collagenase type I and 100 U/ml DNase I for 30 minutes at 37°C, with agitation.

Pig Single-Cell Isolation

A 6-month-old male pig (S. scrofa) was anesthetized with combination of tiletamine and xylazine and the kidney was removed without perfusion. The kidney capsule was removed and approximately 2.5 g of kidney tissue containing cortex and medulla was weighed. The kidney was minced with a sterile razor blade and digested in 10 ml of RPMI 1640 containing 1 mg/ml collagenase type I and 100 U/ml DNase I for 30 minutes at 37°C, with agitation.

Human Single-Cell Isolation

Remnant human kidney tissues were collected from surgical nephrectomies within 4 hours of resection, deidentiﬁed, and analyzed according to a protocol approved by the Institutional Review Board of UAB, as described previously. For scRNAseq, normal kidney tissue was obtained from a 60-year-old white male with normal serum creatinine (0.8–1.2 mg/dl) and an exophytic 3 cm lesion in the upper pole of the kidney. The validation of markers identiﬁed by scRNAseq (Figure 7) was performed on kidney tissues from three adult patients (average age 63 ± 8 years, two men and one woman, two white and one black). In two cases, normal-appearing tissue was collected from a kidney pole opposite to a well demarcated, relatively small, peripherally located mass; in one case, relatively unaffected tissue was collected from a patient with end-stage autosomal dominant polycystic kidney disease. Remnant tissue was collected and processed within 4 hours of resection according to a protocol approved by the Institutional Review Board of UAB. For isolation of single cells, 2.5 g of tissue containing both the cortex and medulla were minced with a sterile razor blade and digested in 10 ml of RPMI 1640 containing 1 mg/ml collagenase type I and 100 U/ml DNase I for 30 minutes at 37°C, with agitation.

Significance Statement

Despite abundant research focused on understanding the importance of mouse renal resident macrophages in homeostatic and disease settings, these findings have unknown relevance to higher-order species, including humans, because markers to identify a similar population of cells across species are lacking. This hinders translating data obtained in animal model systems to human patients. In this study, the authors used a single-cell RNA sequencing approach, followed by validation using flow cytometry, to identify novel markers of mouse resident macrophages and show that these markers also identify a population of macrophages in rat, pig, and human kidney tissue. Over all, their findings serve as an entry point to study candidate kidney resident macrophages across species.

Single-Cell Isolation and Flow Cytometry

After digestion, kidney fragments were passed through a 70-μm mesh filter (Falcon; BD Biosciences), yielding single-cell
suspensions. Cells were centrifuged at 1200 rpm (220 × g) for 5 minutes at 4°C, resuspended in 5 ml red blood cell lysis buffer and incubated at 37°C for 5 minutes. Cells were spun at 1200 rpm (220 × g) for 5 minutes, resuspended in 1 ml Dulbecco’s-PBS containing 0.04% BSA (catalog no. BP1600–100; Fisher) and Fc blocking solution (dilution 1:200; catalog no. CUS-HB-197; BioXcell), and incubated for 30 minutes on ice. Cells were spun (220 × g), washed with 0.04% BSA, and stained for 30 minutes at room temperature with the following antibodies, diluted according to manufacturers’ recommendations: mouse: PE rat anti-mouse CD45 (catalog no. 12–0451, 30–F11; ebioscience), BV786 hamster anti-mouse CD3e (catalog no. 564379, 145–2C11; BD Horizon), and PerCP-Cy5.5 rat anti-mouse B220 (catalog no.561101, RA3–6B2; BD Bioscience); rat: PE anti-rat CD45 (catalog no. 202207, OX-1; Biolegend), APC anti-rat CD3 (catalog no. 201413, 1F4; Biolegend), and PE/Cy7 anti-rat CD45RA (catalog no. 202315, OX-33; Biolegend); pig: mouse anti-pig CD45 (catalog no. MCA1222GA; Bio-Rad), and Ftc mouse anti-human CD3e (catalog no. 556611; BD Biosciences), secondary antibodies included Cy5 donkey anti-mouse IgG (catalog no. 715–175–151; Jackson ImmunoResearch); and human: Pacific Blue anti-human CD45 (catalog no. 304029, HI30; Biolegend), BV605 anti-human CD3 (catalog no. 317322, OKT3; Biolegend), and Alexa Fluor 647 anti-human CD19 (catalog no. 302220, HB19; Biolegend). All species were stained with a Fixable Aqua Dead Cell Stain (catalog no. L34957; Invitrogen). After addition of primary antibody, cells were spun (220 × g), washed in 0.04% BSA, and resuspended in 1 ml of 0.04% BSA. Samples were taken to the flow cytometry core and sorted using a Becton–Dickenson FACSAriaII.

**Flow Cytometry Validation of Novel Resident Macrophage Markers**

After digestion, a single-cell suspension was generated as described above, blocked, washed, and stained with the following antibodies according to manufacturers’ recommendations: mouse: PE anti-mouse/rat CD81 (catalog no. 559519; BD Biosciences), Ftc anti-mouse/rat/human C1Q (catalog no. MA1–40313; Invitrogen), Ftc anti-mouse CD74 (catalog no. 561941; BD Bioscience), and Alexa-647 conjugated anti-mouse Apoe (catalog no. NB110–6053155; Novus Biologicals); rat: PE anti-mouse/rat CD81 (catalog no. 559519), Ftc anti-mouse/rat/human C1Q (catalog no. MA1–40313), Ftc anti-rat CD74 (catalog no. 21419; Abcam); and human: Ftc anti-mouse/rat/human C1Q (catalog no. MA1–40313), Ftc anti-human CD81 (catalog no. 551108; BD Pharmingen), Ftc anti-human CD74 (catalog no. 555540; BD Pharmingen), PE anti-human CD81 (catalog no. 561957; BD Bioscience), and PE anti-human CD74 (catalog no. 326807; Biolegend). After addition of primary antibody, cells were spun (220 × g), washed in 0.04% BSA, and fixed for 30 minutes in 2% PFA at room temperature. After 30 minutes, cells were spun (220 × g), washed in Dulbecco’s-PBS, and resuspended in Dulbecco’s-PBS. Samples were run on a Becton–Dickenson LSRII and analyzed using FlowJo software.

**Fluidigm C1 Sequencing**

Fluidigm single-cell cDNA libraries were prepared according to standard procedures outlined in Using C1 to Generate Single-Cell cDNA Libraries for mRNA Sequencing (https://www.fluidigm.com/binaries/content/documents/fluidigm/resources/c1-mrna%E2%80%90seq-pr-100%E2%80%9097168/c1-mrna%E2%80%90seq-pr-100%E2%80%9097168/fluidigm%3Afile). Briefly, sorted macrophages were resuspended into a final concentration of 300–350 cells/µl in Dulbecco’s-PBS and mixed with Fluidigm suspension reagent to reach 5% final buoyancy, as determined by pretesting. Six microliter of mixed cells were loaded into the Fluidigm C1 IFC plate for mRNA seq (10–17 µm capture site) (PN 100–5760; Fluidigm). Captured single cells were confirmed via brightfield microscopy. Next, lysis mix, transverse transversion mix, and preamplification mix were loaded into the C1 plate as instructed and single-cell preamplified cDNA libraries were generated by the C1 machine using the program “mRNA Seq: RT and Amp (1771 × 1772/1773 × script.” Illumina sequencing libraries were prepared from preamplified single-cell cDNA with Nextera XT DNA Sample Preparation Kit (PN FC–131–1096; Illumina), following the sequential steps of tagmentation, PCR amplification and pooling, and cleaning of the libraries. The final constructed libraries were sequenced by an Illumina Nextseq machine with the minimum reads per cell set at 250,000.

**10× Genomics**

10× Chromium single-cell libraries were prepared according to the standard protocol outlined in the manual. Briefly, sorted single-cell suspension, 10× barcoded gel beads, and oil were loaded into Chromium Single Cell A Chip to capture single cells in nanoliter-scale oil droplets by Chromium Controller and to generate Gel Bead-In-Emulsions (GEMs). Full-length cDNA libraries were prepared by incubation of GEMs in a thermocycler machine. GEMs containing cDNAs were broken and all single-cell cDNA libraries were pooled together, cleaned using DynaBeads MyOne Silane beads (PN 37002D; Fisher), and preamplified by PCR to generate sufficient mass for sequencing library construction. Sequencing libraries were constructed by following the steps cDNA fragmentation, end repair and A-tailing, size selection by SPRIselect beads (PN B23318; Beckman Coulter), adaptor ligation, sample index PCR amplification, and repeat SPRIselect beads size selection. The final constructed single-cell libraries were sequenced by Illumina Nextseq machine with total reads per cell targeted, for a minimum of 50,000.

**Single-Cell Sequencing Data Processing**

For single cell RNA sequences generated from the Fluidigm platform, RSEM (version 1.2.31) was used to align the raw sequence reads obtained using STAR (version 2.5.3a) to the reference genome from Gencode.12,13 Transcript abundances represented as tags per million were then loaded into Fluidigm’s R package, SINGuLAR (version 3.6.1), for differential gene expression analysis. In brief, the function “identifyOutliers” in SINGuLAR was used to identify outliers and remove them from
downstream analysis. After the removal of outliers, the function “autoAnalysis” was used to create the principal component analysis (PCA), t-distributed stochastic neighbor embedding (tSNE), hierarchical cluster, and correlation analysis of the gene clusters.

For 10× Genomics single cells, the 10× Genomics Cellranger software (version 2.1.1), “mkfastq,” was used to create the fastq files from the sequencer. After fastq file generation, Cellranger “count” was used to align the raw sequence reads to the reference genome using STAR. The “count” software created three data files (barcodes.tsv, genes.tsv, matrix.mtx) from the “filtered_gene_bc_matrices” folder that were loaded into the R package Seurat version 2.3.4,14 which allows for selection and filtration of cells on the basis of quality control metrics, data normalization and scaling, and detection of highly variable genes. We followed the Seurat vignette (https://satijalab.org/seurat/pbmc3k_tutorial.html) to create the Seurat data matrix object. In brief, we kept all genes expressed in more than three cells and cells with at least 200 detected cells. Cells with mitochondrial gene percentages >5% and unique gene counts >2500 or <200 were discarded. The data were normalized using Seurat’s “NormalizeData” function, which uses a global-scaling normalization method, LogNormalize, to normalize the gene expression measurements for each cell to the total gene expression. The result is multiplied by a scale factor of 1e4 and the result is log-transformed. Highly variable genes were then identified using the function “FindVariableGenes” in Seurat. Genes were placed into 20 bins on the basis of their average expression and removed using 0.0125 low cut-off, 3 high cut-off, and a z-score cut-off of 0.5. We also regressed out the variation arising from library size and percentage of mitochondrial genes using the function “ScaleData” in Seurat. We performed PCA of the variable genes as input and determined significant principal components on the basis of the “JackStraw” function in Seurat. The first 20 principal components were selected as input for tSNE using the functions “FindClusters” and “RunTSNE” in Seurat. To identify differentially expressed genes (DEGs) in each cell cluster, we used the function “FindAllMarkers” in Seurat on the normalized gene expression data.

All relevant data have been deposited in the Gene Expression Omnibus under accession number GSE128993.

Parabiosis

The parabiosis protocol was modified from the procedure published by Kamran et al.15 Mice were anesthetized using 1.5%–2.0% vol/vol isoflurane for induction and 1.0%–1.5% vol/vol for maintenance. The surgical site, which was the entire flank region from rostral of the cervical spine to rostral of the hip joint, as well as the involved extremities distally to the knee and elbow joints, was shaved. The mice were laid supine and the site was disinfected with betadine followed by 70% ethanol. Incisions were made through the skin and muscular layer, starting from the elbow joint and extending down the flank to the knee joint. Nonabsorbable 3–0 interrupted sutures were placed around the knee and elbow joints to prevent strain along the suture lines, taking care not to obstruct blood flow to the distal extremities. Starting rostrally, interrupted sutures were used to attach the skin and muscular layers of the two mice. Working on the ventral side, continuous 3–0 absorbable Vycril sutures through the skin and muscular layers were used, followed by the same technique through the dorsal skin and muscular layer. Analgesia was maintained on all mice according to IACUC guidelines. Topical antibiotics were applied to the surgical site upon completion of the procedure. Animals were provided with water in a plastic dish on the floor of the cage as well as moistened chow and a gel food diet supplement daily for the duration of the experiment. Mouse weights were monitored throughout the experiment.

Statistical Analyses

t test and one-way ANOVA were used to analyze data. A value was considered statistically significant if P<0.05.

RESULTS

scRNAseq Reveals Distinct Clusters of Innate Immune Cells in the Mouse Kidney

We sorted populations of immune cells (CD45+ from the kidney, excluded lymphoid cells, and subjected the remaining cells to scRNAseq using the 10× Genomics platform (Figure 1A). For this analysis, we excluded cells from both the T cell (CD3e in mouse and pig, CD3 in rat and human) and B cell lineage (B220 in mouse, CD45RA in rat, CD19 in human) using fluorescence-labeled antibodies that were commercially available. Using this approach, we analyzed 3013, 3935, 4671, and 2868 single cells from mouse, rat, pig, and human kidney tissue, respectively (Supplemental Table 1). The mean reads per cell were 80,508, 54,456, 61,638, and 112,080 for mouse, rat, pig, and human cells, respectively. Unbiased hierarchical clustering and heatmap analysis using Seurat shows a unique innate immune cell landscape in each species with each color representing a different cell population (Figure 1, B and C).

To understand the conservation of innate immune cells and to identify markers for these cells across species, we began by defining cell populations from mouse scRNAseq data using canonical markers of innate immune cell populations derived from published literature and online databases.16 Lcn2 encodes a 25 kD secreted protein that is produced by activated neutrophils.17 Gama is an effector molecule that is preferentially expressed by NK cells.18 Il7r is expressed by ILCs.19 Infiltrating (Cd11bhi, F4/80lo) and resident (Cd11blo, F4/80hi) macrophages were originally identified on the basis of differential expression of Cd11b (Ilgam) and F4/80 (Adgre1).2 Follow-up studies indicate that Cd64 (Fcgfr1)20 and Ccr2 (Ccr2)21 are additional markers of resident and infiltrating macrophages, respectively. S100a9 and Batf are recently discovered genes that can successfully distinguish dendritic cells from resident macrophages in the kidney.22 Using the indicated genes, we used tSNE projections and violin plots to identify the following types of...
Figure 1. scRNAseq identifies clusters of cells with unique gene expression patterns in the renal innate immune compartment. (A) Schematic of experimental design. (B) tSNE plot and (C) heatmap of innate immune cells in mouse, rat, pig, and human kidneys. The heatmap depicts the top five DEGs in each cluster of innate immune cells.
innate immune cells in the mouse kidney: neutrophils (Lcn2 +, Cluster 9, Figure 2A), NK cells (Gzmα +, Cluster 2, Figure 2B), ILCs (Il7r +, Cluster 6, Figure 2C), infiltrating macrophages (Ccr2 +, Itgam +, Figure 2D), resident macrophages (Adgre1 +, Fcgr1 +, Cluster 0, Figure 2E), dendritic cells (Sna22 +, Batf3 +, Clusters 1 and 7, Figure 2F), and unknown (Cluster 5). Additional markers including Plac8, Lyz2, and Cebpb were used to identify infiltrating macrophages as clusters 3 and 4 (Figure 1). We also identified a small population of B cells (Cd79a +, Cluster 8, Supplemental Figure 1) in our lineage-negative scRNAseq data, despite the use of a pan B cell marker (B220).24,25

Comparative Analysis Identifies Novel Genes Whose Expression Is Enriched in Infiltrating or Resident Macrophages

Using the markers described above, we manually annotated clusters of cells in the tSNE plots to reflect known innate immune cell populations that are present in the mouse kidney (Figure 3A). Our results highlight the presence of distinct clusters of neutrophils, NK cells, ILCs, infiltrating macrophages, resident macrophages, and dendritic cells in the mouse kidney. In this analysis, infiltrating macrophages were further subdivided into Ly6c hi (Ly6c1, Ly6c2) and Ly6c lo (Cebpb, Nr4a1) subpopulations. Similarly, dendritic cells were subdivided into cDC1 (Batf3, Ifr7) and cDC2 (Sirpa) compartments. These data support recent findings showing that resident macrophages and dendritic cells are unique populations in the kidney as they cluster to distinct regions of the tSNE plot.22

To identify novel markers of innate immune cells in the mouse kidney, we used Seurat to identify the top differentially expressed genes (DEGs) that are present in each cluster of cells in Figure 3A. This approach identified a list of candidate genes whose expression was enriched in each innate immune compartment in mice and comprised both known and unknown markers (Figure 3B, Supplemental Table 2). Using this unbiased approach, we were able to identify transcripts uniquely expressed in Ly6c hi (Chil3, Plac8) and Ly6c lo (Fapb4, Ear2) infiltrating macrophages as well as embryonic-derived resident (C1qc, Cd81) macrophages (Figure 3C).

Fluidigm C1 scRNAseq of Infiltrating and Resident Macrophages Using Conventional Flow Sorting Approaches (CD11b and F4/80) Confirms 10× Genomics Data

To further validate the novel infiltrating and resident macrophage markers identified by 10× Genomics, we used FACS to sort infiltrating (CD11b hi F4/80 lo) and resident (CD11b lo F4/80 hi) macrophage populations according to conventional markers (Supplemental Figure 2) and performed scRNAseq using Fluidigm C1 technology. PCA plots, violin plots, and heatmap analysis of the C1 data show that infiltrating and resident macrophages express a unique gene signature and that several of the top DEGs between infiltrating and resident (C1qa, Cd81, Apoe) macrophages match the 10× Genomics data (Figure 4, A–C). Our data indicate that 13 out of the top 20 resident macrophage markers identified using 10× Genomics were also observed in resident macrophages using the Fluidigm C1 platform. The number of reads per cell from Fluidigm C1 scRNAseq is shown in Supplemental Table 3.

scRNAseq Reveals the Conservation of the Resident Macrophage Gene Expression Patterns across Species

Next, we assessed whether similar gene expression patterns could be found in lineage negative CD45+ innate immune cells isolated from rat, pig, and human kidneys. We began by choosing the top DEG used to unambiguously identify resident macrophages in the mouse (C1qc) and looked for the presence of this gene in scRNAseq data from each species. Remarkably, tSNE projections and violin plots show the presence of a unique cluster of cells with enriched expression of C1qc in single-cell data from mouse, rat, pig, and human tissue (Figure 5A). To test whether the candidate resident macrophage gene expression pattern exists across species, we searched for the presence of three other top DEGs that were enriched in mouse resident macrophages (Cd81, Cd74, Apoe; Supplemental Table 2) in single-cell data from rat, pig, and human kidney tissue. tSNE projections and violin plots show that each gene, including C1qc, is found in a common cell cluster in each respective species (Figure 5, B–D). These data indicate that the candidate resident macrophage gene expression pattern may be conserved in resident macrophages across species.

To determine if gene expression within other innate immune cell populations are conserved across species, we repeated this analysis using a top DEG found in each innate immune cell population in the mouse. The gene that we selected to identify each population of innate immune cells (Supplemental Table 2; red highlight) was chosen according to the calculated P value and the presence of an orthologous gene in higher-order species. tSNE projections show the presence of a unique cluster of cells expressing the neutrophil (S100a8) and NK cell (Nkg7) specific marker in single-cell data from rat (Supplemental Figure 3, A and B), pig (Supplemental Figure 4, A and B), and human (Supplemental Figure 5, A and B) kidney tissue. In contrast, the DEG used to identify Ly6c hi (Ifr7) and Ly6c lo (Cebpb) infiltrating macrophages and cDC1 (Naaa) and cDC2 (Clec10a) dendritic cells in mice could not distinguish between these subtypes in rat (Supplemental Figure 3, C–E), pig (Supplemental Figure 4, C–E), or human kidney tissue (Supplemental Figure 5, C–E).

On the basis of gene expression profiles, additional DEGs to identify each cell type (Supplemental Table 2), and other common lineage defining markers reported in the literature (CD14 for infiltrating macrophages in humans), we manually annotated clusters of cells into neutrophils, NK cells, infiltrating macrophages, resident macrophages, and dendritic cells in each species (Figure 6A). In the rat, pig, and human, we defined infiltrating macrophages as the cluster of cells that was negative for the neutrophil marker S100a8 and had enriched
Figure 2. Canonical markers can be used to identify distinct clusters of innate immune cells in the mouse kidney. tSNE projections and accompanying violin plots depicting genes used to identify (A) neutrophils (Lcn2), (B) NK cells (Gzma), (C) ILCs (Il7r), (D) infiltrating macrophages (Itgam, Ccr2), (E) resident macrophages (Adgre1, Cd64), and (F) dendritic cells (Snx22, Batf3).
Figure 3. scRNA-seq can be used to identify novel markers of innate immune cells in the mouse kidney. (A) Single cells from the mouse kidney were manually annotated according to expression of the canonical genes identified in Figure 2. (B) Heatmap identifying novel gene expression signatures in each innate immune compartment. A more detailed list of genes is included in Supplemental Table 2. (C) tSNE and violin plots depicting novel genes used to identify Ly6c⁺ infiltrating macrophages (Chil3, Plac8), Ly6c⁻ infiltrating macrophages (Fabp4, Ear2), and resident macrophages (C1qc, Cd81).
expression of both the Ly6c\(^{hi}\) (Irf7) and Ly6c\(^{lo}\) (Cebpb) mouse infiltrating macrophage markers. Dendritic cells in the rat and human were identified using Clec10a; however, the ortholog of this gene is not present in pigs, thereby precluding us from identifying dendritic cells in this species. Further, we were also unable to identify ILCs (Cxcr6) in rat, pig, or human kidney tissue using this approach, despite recently published literature identifying ILC2s in human kidney tissue.\(^{26}\) Therefore, we grouped clusters of cells into broad immune cell subtypes (i.e., infiltrating macrophage, dendritic cells) rather than subtypes, in rat, pig, and human tissue. Of note, many of the canonical markers used to identify macrophages in human samples (Cd68, Axl, Tmcc3, Slamf9, Plaur, Notch2, Marcks, Cx3cr1, Dusp3, Nupr1, Sub1, Nr4a1, Gpr65, Dnajc8, Mrc1, mt-Rnr1, Lgmn, Cdkn1b, Rab7b, Sik1, Kdm6b, Creb5, Tgfbr1, Siglece, Ctsc, Gm26473, Ppp1r15a, Btg1, Hpgds, S100a10, Gpx1, Selenop, Zfhx3, Pdcd4, BCO35044, Cttnbp2nl, Cd63, Rps10, Srgn, Arrb2, Scamp2, Hnmpa1, Ear2, Fh1, Oas2, Rgs10, Slco2b1, Adgre4, C5ar2, Btg2, Ccnd3, Son, Lgals3bp, Gusb, Slc9a9, MUSG0000011, Ube2n-ps1, Gm20659, Ccl12, Scimp, Smpdl3a, Pim1, S100a4, St3gal6, Adap2os, Lcp1, Lsp1, Prdx5, Stab1, Fyb, Adap2, Gatm, Cxcl16, Aft3, Napsa, Ahnak, Tmsb10, Casm1, Sdc4).
Figure 5. scRNAseq data identifies a unique cluster of cells in multiple species that share the same gene expression pattern as mouse resident macrophages. (A–D) tSNE and violin plots showing the top DEGs (C1qc, Cd81, Cd74, Apoe) that were used to identify candidate resident macrophages in scRNAseq data from mouse, rat, pig, and human kidneys.
Novel Resident Macrophage Markers Identified by 10X Genomics Are Present at the Protein Level in a Population of Cells in Mouse, Rat, and Human Kidney Tissue

We used our scRNAseq data to identify novel markers of mouse resident macrophages that may be present on candidate

Figure 6. scRNAseq identifies distinct renal innate immune cells. (A) tSNE plot of manually annotated mouse, rat, pig, and human innate immune compartments. (B) tSNE and violin plots showing expression of commonly used human macrophage markers (CD68, CD163, CD14, FCGR3A) in scRNAseq data from human kidney tissue.

Cd163, Cd14, Fcgr3a) were not specific to human renal macrophage populations and could not separate human macrophages into infiltrating or resident macrophages (Figure 6B). Gene expression patterns from 10X Genomics observed in human tissue were further confirmed using Fluidigm C1 technology (Supplemental Figure 6).

We used our scRNAseq data to identify novel markers of mouse resident macrophages that may be present on candidate
To establish that the candidate resident macrophages identified were enriched in mouse renal resident macrophages, we sorted single cells obtained from wild-type mouse kidneys and looked for the presence of our novel resident macrophage markers (C1q, CD81, CD74, Apoe) in resident macrophages that were gated based on the canonical approach (CD11b, F4/80). As a control, we also analyzed expression of these markers in infiltrating macrophages. Analysis of flow cytometry data shows that the number and mean fluorescence intensity of cells expressing C1q, CD81, and CD74 is enhanced in resident macrophages (CD11b<sup>lo</sup> F4/80<sup>hi</sup>) compared with infiltrating macrophages (CD11b<sup>hi</sup> F4/80<sup>lo</sup>) (Figure 7, A and B). Although the percentage of resident macrophages expressing Apoe was slightly higher than the percentage of infiltrating macrophages expressing Apoe, the mean fluorescence intensity (x-axis) was similar between the two groups, suggesting that it is not a good marker to distinguish the two populations. Therefore, this marker was excluded in analysis of macrophage populations in higher-order species.

On the basis of these data, we created a novel gating strategy to test whether CD74 and CD81 were able to identify resident macrophages in a mouse kidney (Supplemental Figure 7). These markers were chosen on the basis of antibody availability and potential cell surface localization. In mice, the resident macrophages identified using the novel approach are almost exclusively found in the resident macrophage gate using CD11b and F4/80 (Supplemental Figure 7). We also identified a population of CD74/CD81 double positive cells in kidney tissue from rat and human samples (Figure 7C, red circles). In addition, we compared protein expression of C1q, CD81, and CD74 in candidate resident macrophages (red) or nonresident (blue) macrophages identified using the CD81 antibody. Our data indicate that C1q, CD81, and CD74 protein expression is enriched in CD81<sup>+</sup> cells from mouse and rat tissue (Figure 7, D and E). Of interest, although CD74 and CD81 protein expression was enriched in human CD81<sup>+</sup> cells, we did not observe a similar enrichment C1q at the protein level in these cells (Figure 7, D and E), which may reflect the limited number of patients analyzed or their disease state. Collectively, these data indicate that CD74 and CD81 can be used to identify a population of candidate resident macrophages across multiple species, including mouse, rat, and human tissue.

Resident Macrophages Identified Using the Novel Markers Have Minimal Exchange with the Peripheral Blood

To establish that the candidate resident macrophages identified using our novel approach demonstrate a lack of exchange with the peripheral blood, a defining feature of tissue resident macrophages, we applied the parabiosis model in CD45 congenic mice (CD45.2 and CD45.1). Our data indicate that the percent chimerism of candidate resident macrophages identified using our novel markers is 2%–6%, similar to the percent chimerism observed using canonical markers and in agreement with previous reports (Figure 8). In contrast, we observe 40%, 25%, 15%, and 25% chimerism in T/B cells, infiltrating macrophages, neutrophils, and NK cells, respectively. The level of chimerism is significantly higher in these populations compared with resident macrophages.

The low level of chimerism that we observe in neutrophils is likely due to the difficulty that these cells have in migrating across the anastomotic vessels and their short lifespan. Analysis of percentage of chimerism of neutrophils in the blood confirms this idea and suggests that neutrophils in the kidney are fully exchanged with the peripheral blood (i.e., neutrophil chimerism is approximately 15% in both the blood and kidney; Supplemental Figure 8). The level of chimerism observed in renal immune cell populations 8 weeks after parabiosis surgery in our study is similar to previously published studies in the kidney. Overall, our data validate that the population of cells we identify as candidate resident macrophages using our novel markers are minimally replaced by cells from the peripheral blood in the mouse.

**DISCUSSION**

Our findings shed light on the composition of the innate immune system in the kidney across multiple mammalian species using an unbiased lineage negative scRNAseq approach. The goal of this scRNAseq study was to determine a list of novel candidate genes that were uniquely expressed in mouse resident macrophages. Using this list of candidate genes, we identified a unique cluster of cells in rat, pig, and human kidney tissue that had enrichment of several of the same genes that were identified in mouse resident macrophages. Further, we were able to use this list of genes to identify potential cell surface markers that could identify mouse resident macrophages and may be used to identify and characterize resident macrophages in other species.

One of the key markers resulting from this study was C1q, whose expression was enriched at the RNA level in the candidate resident macrophage populations across species. Intriguingly, scRNAseq data from other groups demonstrate the presence of a unique cluster of cells that express C1q in mouse and human kidney tissue. Park et al. performed scRNAseq of the mouse kidney and identified a distinct cluster of cells, which they referred to as macrophages, that had enriched expression of C1qa and C1qb and comprised approximately 0.5% of total cells. Further, studies by Wu et al. identified a distinct cluster of cells, which the authors referred to as monocytes, that were isolated from human kidney allograft biopsy samples that had enriched expression of C1qa, C1qb, and C1qc. On the basis of our data, we propose that the population of cells with enriched expression of C1q genes identified in each study is likely resident macrophages. Although C1q expression was enriched in a cluster of cells across species at the RNA level, we did not...
Figure 7. Genes identified by scRNAseq are enriched in mouse resident macrophages at the protein level. (A) Representative histograms depicting C1q, CD81, CD74, and Apoe expression in mouse infiltrating and resident macrophages that were gated on CD11b and F/480 (Supplemental Figure 2). FMO, fluorescence minus one antibody of interest; Inf., infiltrating macrophage; Res., resident macrophage. (B) Quantification of the percentage of resident and infiltrating macrophages that express each marker. Data are shown as mean±SEM; ****P<0.001. (C) Representative flow cytometry plots showing expression of CD81 and CD74 in innate immune cells from mouse, rat, and human kidney tissue. (D) Representative histograms depicting protein expression of C1q, CD81, and CD74 in candidate resident (red) and nonresident macrophages (blue) identified using the CD81 antibody. (E) Quantification of the percentage of CD81+ candidate resident macrophages and CD81− nonresident macrophages that express C1q and CD74 in mouse, rat, and human kidney tissue. Each point represents an individual kidney sample that was harvested from mice, rats, or humans. Data are shown as mean±SEM. *P<0.01; ****P<0.001. mouse n=5, rat n=3 or 6, and human n=3.
observe a corresponding enrichment of C1q at the protein level in candidate resident macrophages isolated from human kidney tissue. This discrepancy may be because of a divergence in C1q protein expression between species, the limited number of kidneys that were analyzed, the disease state of the kidney tissue, or a lack of a reliable antibody to detect human C1q.

Although these studies suggest that resident macrophages exist in the kidney of multiple species, whether CD81/CD74 double positive cells share a gene expression signature across species is unknown, nor is it known whether candidate resident macrophages maintain a functional equivalency across species during homeostatic and disease processes. Overall, this study provides an optimistic outlook for the potential translatability of kidney resident macrophage studies in mouse and provides a template for future comparative immunologic studies in resident macrophages across tissues and species.

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DISCLOSURES

M. Mrug has received research/clinical trial support from and has consulted for Otsuka and Sanofi-Genzyme not related to this project.

SUPPLEMENTAL MATERIAL

This article contains the following supplemental material online at http://jasn.asnjournals.org/lookup/suppl/doi:10.1681/ASN.2018090931/-/DCSupplemental.

Supplemental Table 1. Number of cells, genes, and reads in single-cell data from mouse, rat, pig, and human kidneys.

Supplemental Table 2. Top DEGs in mouse innate immune cell populations.

Supplemental Table 3. Number of reads in individual cells from mouse Fluidigm C1 scRNAseq data.

Supplemental Figure 1. scRNAseq reveals the presence of CD79a+ B cells in mouse kidney tissue.

Supplemental Figure 2. Gating strategy used in Fluidigm C1 studies to identify infiltrating and resident macrophages on the basis of canonical markers.

Supplemental Figure 3. scRNAseq reveals the presence of distinct clusters of innate immune cells in rat kidney tissue.

Supplemental Figure 4. scRNAseq reveals the presence of distinct clusters of innate immune cells in pig kidney tissue.

Supplemental Figure 5. scRNAseq reveals the presence of distinct clusters of innate immune cells in human kidney tissue.

Supplemental Figure 6. Fluidigm C1 scRNAseq of single cells from human kidney tissue.

Supplemental Figure 7. Gating strategy used to identify candidate resident macrophages on the basis of novel markers.

Supplemental Figure 8. Chimerism of blood-derived cells in mouse parabiosis studies.
REFERENCES


Supplemental Material

Table S1-S3

Figure S1-8

Supplementary Table 1

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**Figure S1.** tSNE projection and violin plot showing expression of *Cd79a* in mouse renal innate immune cells.

**Figure S2** Gating strategy used to isolate infiltrating and resident macrophages based on the canonical Cd11b and F/480 approach. Infiltrating and resident macrophages were collected and subjected to C1 Fluidigm single cell RNA sequencing.

**Figure S3** tSNE projections and violin plots showing a top DEG that was used to identify (A) neutrophils (*S100a8*), (B) NK cells (*Nkg7*), (C) innate lymphoid cells (ILCs, *Cxcr6*), (D) infiltrating macrophages (*Irf7, Cebpb*), (E) and dendritic cells (*Naaa, Clec10a*) in single cell data from a rat kidney.

**Figure S4** tSNE projections and violin plots showing a top DEG that was used to identify (A) neutrophils (*S100A8*), (B) NK cells (*NKG7*), (C) innate lymphoid cells (ILCs, *CXCR6*), (D) infiltrating macrophages (*IRF7, CEBPB*), and (E) dendritic cells (*NAAA*) in single cell data from a pig kidney. Unfortunately, the *Clec10a* orthologue has not been identified in the pig.

**Figure S5** tSNE projections and violin plots showing a top DEG that was used to identify (A) neutrophils (*S100A8*), (B) NK cells (*NKG7*), (C) innate lymphoid cells (ILCs, *CXCR6*), (D) infiltrating macrophages (*IRF7, CEBPB*), (E) and dendritic cells (*NAAA, CLEC10A*) in single cell data from a human kidney.

**Figure S6** Heat map of top 100 DEGs in lineage negative CD45+ innate immune cells from human kidney tissue identified using Fluidigm C1 single cell RNA sequencing.

**Figure S7** Gating strategy used to identify resident macrophages in multiple species using CD74 and CD81 antibodies.
**Figure S8** Quantification of the percent chimerism of B/T cells, infiltrating monocytes/macrophages, and neutrophils in the blood of mice undergoing parabiosis. ****

P<0.0001.
Figure S3

A) S100a8 (Neutrophils)  
B) Nkg7 (NK Cells)  
C) Cxcr6 (ILCs)  
D) Irf7 (Infiltrating MΦ)  
E) Cebpb (Infiltrating MΦ)  
F) Naaa (Dendritic Cells)  
G) Clec10a (Dendritic Cells)
Figure S4

Pig

A B C

D E F

S100A8
(Neutrophils)

NKG7
(NK Cells)

CXCR6
(ILCs)

IRF7
(Infiltrating MΦ)

CEBPB
(Infiltrating MΦ)

NAAA
(Dendritic Cells)

Normalized Expression

Normalized Expression

Normalized Expression
Figure S5

Human

A  S100A8  (Neutrophils)

B  NKG7  (NK Cells)

C  CXCR6  (ILCs)

D  IRF7  (Infiltrating MΦ)

E  CEBPB  (Infiltrating MΦ)

F  NAAA  (Dendritic Cells)

G  CIEC10A  (Dendritic Cells)
Figure S6

ZFAND2A
EIF5
FPR1
TCP1
OLIG1
ATP10D
LST1
RPL26
DNAJB1
HSPH1
ENSG00000278217
IFI30
S100A9
S100A8
S100A6
FTL
NFKBIZ
HLA-DRA
LYZ
FTH1
RGS2
CD74
CST7
BRD2
DBI
RPS11
GZMB
PRF1
CCL5
CCL4
FGFBP2
CX3CR1
PLAC8
GNLY
CD247
NKG7
FCER1G
YWHAZ
ARPC2
UBC
RPS10
TYROBP
DUSP1
HSP90AB1
ARHGDIB
RPS27A
RPS29
RPS18
RPS3A
IFITM2
RPL10
EEF1A1
RPL27
HSPE1
RPL11
RPS23
H3F3A
RPL19
SERF2
RPS16
RPL27A
HSPA8
S100A11
RPL9P9
HNRNPA2B1
UBB
RPL37A
RPL21P16
RPL21
CASP1
EEF1B2
ALDOA
CD52
PABPC1
FOS
SAT1
RPL13A
RPL23
RPL17
DDX5
RPL18A
RPS27
EIF1
TMSB4X
B2M
RPL38
RPL35
RPL8
RPL31
RPL32
RPL23A
TMSB10
PFDN5
ENSG00000278483
RPL41
RPS15
RPL30
RPS19
RPL3
RPS14
NKG7+ NK Cells
S100A8+ Neutrophils
Neutrophils
S100A11+ Inf. Mac.

Global Z Score
Figure S8

Blood

** ****

% Chimerism

B/T Cells
Infiltrating
Neutrophil