Kidney intercalated cells are phagocytic and acidify internalized uropathogenic *Escherichia coli*

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Kidney intercalated cells are involved in acid-base homeostasis via vacuolar ATPase expression. Here we report six human intercalated cell subtypes, including hybrid principal-intercalated cells identified from single cell transcriptomics. Phagosome maturation is a biological process that increases in biological pathway analysis rank following exposure to uropathogenic *Escherichia coli* in two of the intercalated cell subtypes. Real time confocal microscopy visualization of murine renal tubules perfused with green fluorescent protein expressing *Escherichia coli* or pHrodo Green *E. coli* BioParticles demonstrates that intercalated cells actively phagocytose bacteria then acidify phagolysosomes. Additionally, intercalated cells have increased vacuolar ATPase expression following in vivo experimental UTI. Taken together, intercalated cells exhibit a transcriptional response conducive to the kidney’s defense, engulf bacteria and acidify the internalized bacteria. Intercalated cells represent an epithelial cell with characteristics of professional phagocytes like macrophages.
The kidney-collecting tubule is the terminal location for acid–base regulation. The collecting tubule consists of principal cells and intercalated cells (ICs). Principal cells (PCs) mediate salt and water resorption and are characterized by claudin-1 (CLDN1) and α-enolase (ENOA). PCs help maintain acid–base homeostasis and have three described subtypes, type A (A-IC), type B (B-IC), and nonA–nonB-ICs. A-ICs secrete protons via apical vacuolar H-ATPase (V-ATPase) and regenerate bicarbonate via the basolateral chloride/bicarbonate (Cl–/HCO₃⁻) transporter, anion exchange 1 (AE1) and band 3 (SLC4A1)3,4. B-ICs secrete HCO₃⁻ via an apical Cl–/HCO₃⁻ transporter, pendrin (SLC26A4), and express basolateral V-ATPase5,6. Rodent nonA–nonB ICs have apical pendrin and V-ATPase, but no AE1, and have been identified in the collecting duct and connecting tubule (CNT)3. Increasing evidence supports an innate immune role for ICs along with their traditional function of pH regulation. First, the majority of ten unrelated patients with distal renal tubular acidosis, a condition characterized by IC dysfunction, were reported to have a UTI history. Second, uropathogenic Escherichia coli (UPEC), the organism isolated in 70% of acute pyelonephritis in males and 80% in females, selectively localizes to the cytoplasm of ICs6,7. Third, we identified that ICs express antimicrobial peptides (AMPs) such as ribonuclease 7 (RNAse7)8–11. These AMPs have direct activity against a range of pathogens, including bacteria, at baseline and/or in response to infection8–11. Fourth, we and others have independently reported that mice with abnormal IC development have increased susceptibility to urinary tract infections (UTIs) and/or pyelonephritis12,13. Fifth, ICs both sense and mediate inflammation14. Last, we have demonstrated innate immune gene expression in isolated murine ICs15,16.

Understanding how ICs defend the urinary tract from infection is important because UTIs are frequently encountered. Over 50% of women experience a UTI during their lifetime, and UTIs account for 1–6% of all medical visits15. When bacteria ascend to the kidneys resulting in pyelonephritis, potential complications include hypertension, chronic kidney disease, and urosepsis18,19. An estimated 250,000 cases of pyelonephritis occur annually in the United States17. Multidrug resistance is emerging as a critical challenge regarding treatment of UTIs and other infections20. Therefore, the mechanisms ICs use to defend against ascending uropathogens might be therapeutic targets that could be exploited to develop kidney-specific pyelonephritis therapy. Because most IC studies involve animal models, a better understanding of human ICs will be needed to guide basic science studies into improved clinical care.

Because the kidney is a complex organ, mRNA profiles can be difficult to interpret. For example, the nephron has regional specialization with distinct tubular segments and cell subtypes. The kidney contains endothelial, epithelial, interstitial, and recruited inflammatory cells, all with distinct biological functions and gene expression patterns. Single-cell RNA sequencing (scRNA-seq) represents a rapidly advancing methodology to identify and profile distinct cell types at baseline or in response to stressors21. Here, we show using scRNA-seq technology that human kidney ICs consist of six different subtypes that include three A-ICs, one hybrid IC–PC, one nonA–nonB IC, and one B-IC. RNA velocity analysis revealed transcriptional shift from PCs toward A-IC subtypes upon UPEC exposure. Ingenuity Pathway Analysis revealed phagosome maturation as the key biological pathway in an A-IC subset upon UPEC exposure. Within this pathway, V-ATPases are the key involved components. We used intravital microscopy to visualize in vivo UPEC uptake by ICs and confirmed acidification of UPEC internalized in these ICs. Finally, a murine UTI model demonstrated increase in V-ATPase (Atp6v1b1) mRNA expression. This study will provide the foundation to explore and therapeutically manipulate V-ATPase expressing epithelial cells as bacterial phagocytosis.

Results

Human ICs can be enriched via magnetic-activated cell sorting (MACS) with mast/stem cell growth factor receptor (CD117/c-KIT) coated magnetic beads. Human kidney tissue, primarily from normal margins of kidney mass resections, was dissected into 2–4 mm pieces then overnighted to our lab in Dulbecco’s Modified Eagle Medium (DMEM) on ice by the Cooperative Human Tissue Network [https://www.chn.org]. We evaluated c-KIT as cell surface marker to sort human ICs because c-KIT has been successfully used for this purpose in mice22. c-KIT expression localized to human ICs based on the finding that c-KIT and the human IC marker V-ATPase, B1 subunit (gene ATP6V1B1), co-labeled the same cells in the human kidney section (Supplementary Fig. 1)22,23. Following magnetic sorting which included removal of CD45+ immune cells and dead cells; the enrichment of c-KIT-positive presumed ICs was demonstrated by ATP6V1B1 mRNA expression that increased by 8–12-fold in c-KIT-positive compared to the c-KIT-negative cells. Both SLCA4A1 and SLCA26A4 were variably enriched in ICs demonstrating that both A-IC and/or B-IC enrichment is possible using this methodology (Supplementary Fig. 2). The background of the kidney tissue used in this study is presented in Supplementary Table 1.

Integrated cluster analysis identified six IC subtypes. ScRNA-seq was completed on 1861 dissociated kidney cells that were enriched for ICs as described above. The enriched ICs were obtained from the normal margins of a single kidney mass resection. Quality control (QC) filtering was passed by 859 cells exposed to UPEC in vitro for 1 h and 1002 cells exposed to saline in vitro for 1 h. Seurat function analysis identified 12 clusters in the sorted cell preparation (Fig. 1a). The nine most conserved markers within each cluster are presented in Supplementary Figs. 3–14. Overall, IC subtypes accounted for 1066 (57%) of the 1861 cells. Six of the clusters represented IC subtypes. Specifically, we found three A-IC, one B-IC, one nonA–nonB-IC, and one hybrid PC–IC subtype(s). Kidney epithelial cell nomenclature recommended by Chen and colleagues, conserved makers that have been previously reported to be cell-type markers or conserved markers that differentiated one of the 12 clusters from others were used to assign a cell type to each cluster (Supplementary Table 2, Fig. 1b, c, Fig. 2)24–28. Expression levels of all genes and cluster-specific marker genes can be found at https://hpcwebapps.cit.nih.gov/ESBL/Database/IU-Data/Human-c-KIT-Sorted-Single-Cell-RNASeq.htm.

A-IC subtype and hybrid PC–IC protein expression correlates with scRNA-seq results. We validated key scRNA-seq findings, notably that SLCA8A1 (also referred to as Na+/Ca2+ exchanger 1 (NCX1)) is a marker for hybrid PC–ICs and that heat shock protein family A (Hsp70) member 1A (HSPA1A) along with early growth response 1 (EGR1) are markers that can differentiate A-IC subtypes (Fig. 3). Because we used the normal margins of kidney cancer resections, we validated that our SLCA8A1, HSPA1A, and EGR1 kidney expression patterns were consistent to that seen in normal kidney tissue from the Human Protein Atlas available from http://www.proteinatlas.org (Supplementary Fig. 15)29.

Collecting duct cell subtype marker profiles previously demonstrated in murine kidney cells remain largely intact in human kidney cells. Because it will be important to contextualize future murine model findings to human pathophysiology, we evaluated human IC expression of murine-collecting duct cell
markers identified previously in scRNA-seq studies performed by Chen et al.\textsuperscript{22} and Ransick et al.\textsuperscript{30}. A dot plot of the results is presented in Fig. 4. Murine-collecting duct cell-type markers are mostly conserved in human collecting duct cells.

A-IC subtype A increases in relative percentage with 1 h of UPEC exposure. The percentages of cells within clusters were consistent between exposure type with the exception of A-IC subtype A/cluster 0, which increased from 16.2 to 20.1%, and A-IC subtype C/cluster 5, which decreased from 9.4 to 6.6%, with saline vs. UPEC exposures, \( P = 0.03 \) (Table 1).

Hybrid PC–IC cells change their RNA velocity away from PCs and toward A-ICS in response to 1 h of UPEC exposure. The relative abundance of recently transcribed unspliced pre-mRNAs vs. mature spliced mRNA can be used to calculate the change in mRNA abundance, termed mRNA velocity.\textsuperscript{31} A positive mRNA velocity in single-cell studies indicates that genes are being upregulated where a negative mRNA velocity means that genes are being downregulated.\textsuperscript{31} The mRNA velocity direction infers that a cell has a mRNA expression trajectory toward another cell type.\textsuperscript{31,32} The mRNA velocity in response to UPEC exposure is presented in Fig. 5. Of importance, hybrid PC–ICs change their RNA velocity away from PCs and toward ICs in response to UPEC. In addition, ICs maintain their transcription activity, whereas transcriptional activity becomes minimal in other kidney cell types.

The kidney innate immune profile demonstrates some early single-cell expression changes following 1 h of UPEC exposure. scRNA-seq expression patterns of select innate immune genes following UPEC vs. saline exposure for 1 h are presented in Supplementary Figs. 16 and 17. Key findings include high expression of the AMP adrenomedullin (ADM) in A-IC subtypes A and B, hybrid PC–ICs, and B-ICs. PCs do not express adrenomedullin at baseline but have significantly increased expression in response to UPEC exposure. Cytokine-inducible SH2-containing protein (CISH) and barrier to autointegration factor 1 (BANF1) expression is significantly induced in non-A–non-B ICs following UPEC exposure. Interleukin 18 (IL18), galectin 3 (LGALS3), beta defensin 1 (DEFB1), and signal transducer CD24 lipocalin 2/neutrophil gelatinase-associated lipocalin (LCN2/NGAL) was only identified...
in hybrid PC–ICs and only minimal toll-like receptor 4 mRNA expression was present. We did not identify expression of ribonuclease 7 (RNASE7) on scRNA-seq. We evaluated RNASE7 mRNA expression in magnetically sorted pooled IC and non-IC kidney cells. RNASE7 mRNA expression was identified in ICs from one out of four kidneys (Supplementary Fig. 18).

The biological process of phagosome maturation is associated with ICs exposed to UPEC for 1 h. The ten leading biological pathways associated with each cluster were ranked by p value as determined by Ingenuity Pathway Analysis. Three collecting duct-assigned clusters A-IC subtype A/cluster 0, A-IC subtype B/cluster 3, and A-IC subtype C/cluster 5 had differential biological pathway processes following UPEC exposure compared to saline (Fig. 6a–c). Phagosome maturation was the top ranked function in cells exposed to UPEC and saline in A-IC subtype C/cluster 5, moved from second to first position in A-IC subtype A/cluster 0 and from fourth to third highest position in A-IC subtype B/cluster 3. None of the remaining collecting duct cell subtypes (Supplementary Fig. 19) had top ten associated pathways that were different following UPEC exposure compared to saline. The gene expression profile that resulted in the phagosome maturation ranking for A-IC subtype A/cluster 0 is presented in Supplementary Table 3.

ICs phagocytize UPEC in vivo. We developed methodology to perfuse a single kidney tubule with UPEC in a live mouse with an intact kidney. Green fluorescent protein (GFP)-expressing UPEC aggregates microperfused into a single kidney tubule lumen of “IC reporter” mice were visualized flowing through tubules, selectively adhering to the luminal surface and being internalized by ICs (Fig. 7) during intravital microscopy.

ICs acidify UPEC containing phagolysosomes in vivo and in vitro. We also perfused tubules with pHrodo Green E. coli BioParticles that only fluoresce when acidified. Uptake and fluorescence of these bioparticles was only visualized during time following tubular perfusion with pHrodo Green E. coli BioParticles (Fig. 9b). The linear regression results from each cell are presented in Supplementary Table 4. The live in vivo intravital imaging findings of phagocytosis and acidification of pHrodo Green E. coli BioParticles by ICs were validated using an in vitro flow cytometry assay. Murine kidney cell suspension from “IC reporter” mice in which ICs (CD45+ICs) endogenously express tdtT was exposed to pHrodo Green E. coli BioParticles versus cells with media alone. CD45+ resident immune cells in cell suspension were gated as a positive control and CD45−tdtT− (non-ICs) cells were gated as a negative control. Comparing media alone to
bioparticle exposure, green fluorescence indicative of bioparticle acquisition and acidification increased from 0.7 to 62.8% of CD45+ cells. This finding demonstrates the expected bacterial uptake by professional phagocytes such as neutrophils. Interestingly, 22.2% of the ICs had uptake of green bioparticles compared to 5.6% of non-ICs (Supplementary Fig. 20), media control had uptake in 0.7% of ICs and 0% of non-ICs.

**Discussion**

ICs are difficult to study, particularly in humans, because they comprise only 7% of kidney cells, consist of a range of subtypes, and do not retain their phenotype in culture. Chen et al. reported single-cell analysis on murine kidney cells and Lake et al. reported a single-nucleus RNA sequencing pipeline on human kidney cells. These studies identified two subtypes of A-ICs. Here, we have developed methodology to perform single-cell sequencing on viable enriched human ICs. We enriched for viable ICs rather than sequencing all kidney cells to allow increased focus on this cell type. Our primary findings were (a) identification that hybrid PCs–ICs can switch their RNA velocity direction away from PCs and toward A-ICs in response to UPEC exposure, (b) at least three subtypes of A-ICs exist and their relative frequencies can shift in response to UPEC exposure, (c) phagosome maturation is a leading biological pathway in multiple A-IC subtypes and can increase in relative significance with UPEC exposure, and (d) ICs phagocytose and acidify UPEC in vivo during imaging of live mice.

ICs differentiate in response to acidosis by reversing their polarity. ICs have been reported to arise from AQP2-expressing cells. In 2017, Chen and colleagues identified double-positive cells that expressed Aqp2 along with Slc4a1 or Slc26a4 by murine single-cell profiling. These findings were validated by Park and colleagues the following year. In 2019, we demonstrated by protein and mRNA immunolabeling that hybrid PC–ICs exist. Specifically, 9% of murine ICs defined by V-ATPase B1 fluorescent immunolabeling also expressed Aqp2 mRNA defined by fluorescent in situ hybridization. The present study defined a population of human kidney-collecting duct cells that express AQP2, ATP6V1G3, and SLC4A1 consistent with hybrid PC–ICs. On tSNE and UMAP plots, these hybrid cells clustered closest to PCs, but were distinct from traditional PCs based on IC marker expression. SLC8A1 expression was distinct to this cell type. Interestingly, SLC8A1 is highly involved with epithelial-to-mesenchymal transition. Specifically, the absence of SLC8A1 transforms epithelial cell phenotypes via β-catenin-mediated destabilization of E-cadherin. Here, we demonstrate that ICs differentiate in response to uropathogen exposure. Hybrid PC–IC cells change their RNA velocity, the time derivative of mRNA expression, away from PCs and toward A-ICs in response to UPEC. In mice, Ransick et al. identified Slc8a1 expression primarily in the distal convoluted tubule and cells resembling PCs in the connecting tubule. On our confocal microscopy images evaluating kidney SLC8A1 expression, we found isolated cells that expressed SLC8A1 in AQP2-positive tubules (e.g., collecting ducts or connecting tubules). We also

![Fig. 3 Hybrid PCs–ICs and IC subtypes are seen on fluorescent immunolabeling.](image-url)
identified AQP2-negative tubules in which most cells expressed SLC8A1, potentially consistent with the aforementioned distal convoluted tubule Slc8a1 expression in mice. Some A-IC subtypes have differential leading biological pathways following UPEC exposure and A-IC subtype A increased in relative frequency in response to UPEC. These findings indicate that A-IC subtype A may represent an innate immune variant. The role of SLC8A1 in IC differentiation warrants investigation in future studies.

We performed scRNA-seq on 1861 human kidney cells of which 1066 were ICs. To contextualize murine experimental models to human pathophysiology, it will be important to compare human and murine ICs. Chen and colleagues enriched for murine ICs with c-KIT and performed scRNA-seq. They classified 74 cells as PCs, 87 as A-ICs, and 23 cells as B-ICs. Ransick et al. performed scRNA-seq on 688 ICs. They did not enrich for ICs but rather divided the kidney into the cortex, inner medulla, and outer medulla to evaluate zonal differences. We demonstrated that murine-collecting duct cell-type marker expression appeared to be largely conserved in human collecting duct cells. Whether mice have comparable A-IC subtypes to humans will likely require a targeted single-cell evaluation of a larger number of murine ICs.

| Cluster | Assigned cell type | Saline (n = 1002 cells) | UPEC (n = 859 cells) | P value |
|---------|--------------------|-------------------------|----------------------|---------|
| 0       | A-IC, subtype A    | 162 (16.2%)             | 173 (20.1%)          | 0.03*   |
| 1       | NonA nonB-IC       | 152 (15.2%)             | 119 (13.9%)          | 0.43    |
| 2       | Proximal tubule S2a| 133 (13.3%)             | 121 (14.1%)          | 0.64    |
| 3       | A-IC, subtype B    | 94 (9.4%)               | 84 (9.8%)            | 0.81    |
| 4       | Lymphatic endothelium | 95 (9.5%)            | 72 (8.4%)            | 0.42    |
| 5       | A-IC, subtype C    | 94 (9.4%)               | 57 (6.6%)            | 0.03*   |
| 6       | Proximal tubule S2b| 86 (8.6%)               | 64 (7.5%)            | 0.39    |
| 7       | Hybrid PC-IC       | 54 (5.4%)               | 57 (6.6%)            | 0.28    |
| 8       | Thick ascending LOH| 57 (5.7%)               | 44 (6.1%)            | 0.61    |
| 9       | Glomerular endothelium | 52 (5.2%)           | 42 (4.9%)            | 0.83    |
| 10      | PC                 | 14 (1.4%)               | 15 (1.7%)            | 0.58    |
| 11      | B-IC               | 9 (0.9%)                | 11 (1.3%)            | 0.50    |

The Fisher exact test (two-tailed) was used to compare groups. *indicates statistically significant differences.
ICs is consistent to what has been reported in murine ICs by our research group along with Ransick et al.\textsuperscript{30} [https://cello.shinyapps.io/kidneycellexplorer/\textsuperscript{15}]. Chen et al.\textsuperscript{22} did report some TLR4 and NGL1 mRNA expression in ICs, but several fold less than in PCs. We did evaluate pooled human ICs for RNASE7 expression and identified it in ICs from 1 out of 4 kidneys (Supplementary Fig. 18), indicating that its expression may be intermittent depending on region, time point, and physiological conditions. CISH expression, which is induced in nonA nonB ICs in response to UPEC, has been demonstrated to regulate the innate immune response to \textit{Mycobacterium tuberculosis} in the lung and spleen.\textsuperscript{43} ICs phagocytosed bacteria over several minutes, and our cells for scRNA-seq were exposed to UPEC for a relatively short 1 hour time point. Future studies are needed to determine if other IC pathways, such as AMP expression or endocytosis capabilities,\textsuperscript{8,16,49,57,58} with macrophages, including V-ATPase expression, redox potential, AMP secretion, and endocytosis capabilities.\textsuperscript{8,16,49,57,58} Whether ICs can phagocytose cellular debris and bacteria other than UPEC in a similar manner to professional phagocytes remains to be determined.

Confocal imaging of a single-kidney tubule perfused in vivo with bacteria expands on prior in vitro techniques like cell culture and perfusion of dissected tubules. Here, we developed methodology to directly test in live mice whether phagocytosis is an IC function. Intravital microscopy allows for intricate study of dynamic cellular processes within functioning murine kidneys.\textsuperscript{59} Past strategies to utilize intravital microscopy have involved systemic injection of intravital dyes or markers into mice to study proximal tubule endocytosis, kidney blood flow dynamics, and vascular or glomerular permeability.\textsuperscript{60} When intravital microscopy is combined with sophisticated surgical techniques, difficult-to-evaluate early time points of infective processes can be studied.\textsuperscript{61} Previously, bacteria were microperfused in proximal tubules of rat to evaluate blood flow, neutrophil recruitment, and urinary obstruction.\textsuperscript{62,63} The vast majority of UTIs are due to ascending pathogens and would initially encounter the collecting duct.\textsuperscript{64,65} Thus, we propose that the collecting duct is a critical tubular region to evaluate host-pathogen interactions. Our intravital model system effectively recreates a classical

**Fig. 5** UPEC exposure results in human hybrid PC–IC RNA velocity toward A-ICs. At baseline (a) represented by 1h of control saline exposure, human hybrid PC–ICs have an RNA velocity that moves counterclockwise toward PCs. Following 1h of UPEC exposure, hybrid PC–IC’s RNA velocity changes directions toward A-ICs. Also, non-IC cell types, including proximal tubule, PCs, endothelial, and thick ascending loop of Henle (LOH) have much smaller arrows with UPEC exposure (b) compared to saline (a) indicating that these cells have decreased transcriptional activity in response to infection. In contrast, ICs had similar sized arrows with saline and UPEC exposure, indicating that they maintain their transcriptional activity when infected. The color shade of the collecting duct cell clusters matches the cluster color in Fig. 1, while the non-collecting duct kidney cell clusters are shaded gray.
single collecting tubule perfusion study, but in a live mouse with an intact kidney, blood, lymphatic, and neuronal supply along with retained ability to interact with immune cells.

Pyelonephritis pathophysiology has male versus female distinctions. For example, in humans, females are likely to develop acute pyelonephritis but male mice have androgen-mediated increased severity of pyelonephritis. Ransick and colleagues demonstrated differential male and female differences in proximal tubule cells, particularly in organic anion transporters. Male ICs were evaluated in both our single-cell experiments. Monitoring of a microinjection procedure under a fluorescence confocal/two-photon microscope brings advantages, but also challenges. First, a unique organ positioning may have regionalized diversity as demonstrated by Ransick and colleagues. Male ICs were evaluated in both our single-cell sequencing and intravital studies. Because of the role of ICs in the bacterial defense of the kidney and electrolyte balance, sex-related diversity in IC functions, such as phagocytosis and single-cell gene expression, will be key areas for future studies.

This study does have limitations. The UPEC exposure to human ICs in our scRNA-seq was in vitro to a single kidney and may have distinctions compared to in vivo UPEC exposure. In addition, our findings represented the anatomical region of the human kidney sampled and future studies with different locations may have regionalized diversity as demonstrated by Ransick et al. ICs comprised 57% of the cells in our scRNA-seq, enriched from the ~7% IC composition in kidneys at baseline. Our sample did contain some other epithelial and endothelial cell types, but was negative for CD45+ immune cells that were sorted off prior to enrichment of ICs (Fig. 1, Supplementary Fig. 1). With scRNA-seq, the target ICs could be identified and evaluated separately. Hematopoietic stem cells express c-KIT, but they do not likely represent the kidney resident myeloid cell populations. We did not identify other c-KIT-expressing cells and evaluated hematopoietic stem cells expressing c-KIT, but they do not likely represent the kidney resident myeloid cell populations. We did not identify other c-KIT-expressing cells.

Murine intravital imaging limitations were also present due to the challenging nature of the single-tubule microperfusion experiments. Monitoring of a microinjection procedure under a fluorescence confocal/two-photon microscope brings advantages, but also challenges. First, a unique “angled” organ positioning required for a successful injection eliminated usage of a preferred two-photon scanning mode. In most cases, only a narrow band of a focal volume could be visualized, as dictated by the depth discrimination nature of two-photon microscopy. Switching to a confocal mode with a slightly open pinhole eliminated this problem. The second limitation we encountered was dictated by the depth discrimination nature of two-photon microscopy. Switching to a confocal mode with a slightly open pinhole eliminated this problem. The second limitation we encountered was dictated by the depth discrimination nature of two-photon microscopy. Switching to a confocal mode with a slightly open pinhole eliminated this problem.

The ten leading biological pathways ranked by p-value are presented and the colored box beside the cell type corresponds to the cluster color in Fig. 6. a With saline exposure, cluster 0/A-IC subtype A, nuclear factor, erythroid 2-like 2 (NRF2)-mediated oxidative stress response, phagosome maturation, and antigen presentation were the 3 leading biological pathways. With UPEC exposure, cluster 0/A-IC subtype A, phagosome maturation surpassed NRF-mediated oxidative stress response to become the leading pathway. TNF receptor superfamily member 4 (OX40) signaling and cell division cycle 42 (Cdc42) signaling appeared in the top ten pathways. b Cluster 3/A-IC subtype B also had differential rankings of the ten most involved pathways. Phagosome maturation moved from the fourth to third most involved pathway and Cdc42 signaling, OX40 signaling, and ketogenesis replaced xenobiotic metabolism, T-cell exhaustion signaling, and aldosterone signaling in epithelial cells as the eighth, ninth, and tenth most involved pathways with UPEC compared to saline exposure. c Cluster 5/A-IC subtype C was the remaining cell type that had differential top ten involved pathways between saline and UPEC exposure. Ketolysis moved from fourth to second, aryl hydrocarbon response went from glutathione-mediated detoxification appearing with UPEC-replacing sirtuin signaling pathway that moved off of the list. d “IC reporter” mice were challenged with UPEC strain CFT073 or saline transurethrally for 1 h. ICs were flow sorted and Atp6v1b1 mRNA expression was analyzed. Atp6v1b1 mRNA expression increased by a median average of ~2-fold (35.23 (20.40–67.17) saline vs. 73.40 (51.97–115.00)) (UPEC) in ICs following transurethral inoculation of UPEC strain CFT073 versus saline; n = 11 mice in saline and n = 12 mice in UPEC group. Statistical significance (asterisk) between saline and UPEC exposure was determined by the Mann-Whitney test because the data were not parametric. The results are presented as the median and interquartile range. Source data are provided as a Source Data File.
Intravital confocal microscopy demonstrates murine IC phagocytosis of UPEC in real time. A single renal connecting and/or collecting tubule of a "IC reporter" mouse was perfused with GFP expressing UPEC and imaged during confocal intravital microscopy. An example of the imaging with tdT expressing ICs (red arrows), the needle tip (asterisk) and GFP expressing UPEC (green arrows) flowing through a tubular lumen are presented. Approximately, 1 min after the UPEC injection into the tubule, target tdT+ ICs were identified and imaging was started with the 0 min time points presented. UPEC (arrows) can be seen adhering to the lumen of ICs (arrowheads) at early time points (d). At the 6.5 min time point, ICs have luminal UPEC aggregates (arrow) and punctate areas of yellow (arrowheads) consistent with internalization of the green UPEC into the red ICs. At the 8.4 min time point, more UPEC uptake takes place in some ICs (star) compared to other ICs (arrowhead), while tubule areas with no tdT expression (pound sign) have little-to-no green UPEC uptake. UPEC aggregates remain around the luminal IC surface (arrow).

Methods

Regulatory approval. Murine studies were approved by the Institutional Animal Care and Use Committee (IACUC) at the Indiana University School of Medicine, protocol number 11333, and adhered to the "NIH Guide for the Care and Use of Laboratory Animals". Human tissue was obtained from the Cooperative Human Tissue Network (CHTN) Midwest Division (Columbus, OH) and details regarding the policies and procedures that govern collection of specimens and distribution to investigators are found at their website: [https://www.chtn.org/policies/index.html]. The key policies include protection of subjects with all policies being consistent with repository guidance and regulations from the Office of Human Research Protections in the Department of Health and Human Services (OHRP, DHHS), review/approval of the local Institutional Review Boards (IRB), specimen derivation from residual sample material removed as a component of routine medical care in accordance with state and local laws, each CHTN institution has received human subject assurance from the OHRP that the institutions comply with the "Common Rule" (45 CFR part 46), each CHTN division has local IRB approval to collect and distribute biospecimens that are reviewed yearly, donor identities and identifying information is not provided to investigators, and participating investigators must document IRB review (and approval if deemed necessary) by the IRB where the proposed research will be taking place. The use of CHTN samples for this study was reviewed by the Indiana University Institutional Review Board and was exempt (protocol 1802253259) because patient samples did not include identifying information and permissions and consents for human sample collection were from the CHTN and its member institutions. The research complied with all relevant ethical regulations for work with human participants.

Human kidney tissue. Fresh human kidney biopsy samples were obtained from the CHTN. The kidney tissue consisted of normal kidney margins from kidney resection surgeries. The tissue was cut into small, ~2-mm pieces, placed in sterile DMEM, and shipped overnight in cold packs. Supplementary Table 1 outlines the clinical characteristics from the patients that the kidney margins were obtained from. For immunofluorescence, microscopy samples were obtained from the CHTN.

Mice. Strains and generation of "IC reporter" mice. V-ATPaseB1-cre transgenic mice (kindly provided by Dr. Raoul Nelson, University of Utah, UT) in which 7-kb B1 promoter drives the cre expression in ICs were used. The V-ATPaseB1-cre mice were crossed with tdTomato-loxP homzygous mice (B6.Cg-Gt(ROSA)26Sortm9(CAG-tdTomato) (Stock no. 007909, Jackson lab, Maine). The progeny of the V-ATPase-B1 and tdTomato-loxp mice were genotyped for the cre gene (see Supplementary Table 1). Mice positive for the cre gene were used and termed "IC reporter" mice. All mice were on a C57BL/6 background.
Housing and husbandry. All mice used for this experiment were bred and kept in a pathogen-free housing facility at the Indiana University School of Medicine, Indianapolis, IN, which is Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) accredited. The day light cycle of the colony is 7 AM–7 PM and mice are kept at an ambient temperature of 65–75 °F with 40–60% humidity. Mice for experiments were all male, except those used for in vivo UTI experiments, and kept separated by sex, with no more than five male mice being in a single cage at one time. Bedding material was used for wood shaving for the main base with tissue paper also being provided to mice for bedding. Mice for these breeding strains were bred with 1 male with 2 females under normal, nonaltered conditions with water, light, or food. Animals were weaned from breeding pairs at 28 days and separated into separate cages depending on date of birth as well as sex. Animals were cared for by vivarium staff before being selected for the experimental procedures.

Bacteria. GFP expressing uropathogenic E. coli (UPEC) strain CFT073 (originally a gift from Matthew A. Mulvey, University of Utah) was used in the study. It was stored at −80 °C in 50% glycerol until use. For murine experimental UTI induction and intravitral tubular perfusion, the following bacterial growth protocol was used: (a) a culture tube was prepared with 4 ml of Luria broth and labeled time and date, (b) CFT073 GFP expressing UPEC was retrieved from −80 °C, (c) the pipette tip was used to scrape some frozen bacterial stock and the tip was placed in the tube to transfer over frozen bacteria, (d) the tube with Luria broth and bacteria was placed into a 37°C incubator overnight for 16–18 h, and (e) the next day, the bacterial stock was spun down and resuspended in sterile PBS at a concentration of 1 × 10^8 CFT073 in 50 µl of sterile PBS for use in experimental UTI.

Enrichment of human kidney ICs with MACS. Upon arrival, kidney biopsy tissues were further dissected into small pieces and transferred to a C tube (Miltenyi Biotec, Bergisch Gladbach, Germany) containing 5 ml Liberase TL (500 µg/ml) (Sigma, St Louis, MO) with DNase I (100 µg/ml) (Sigma, St Louis, MO) containing DMEM media. Single-cell suspension was prepared with GentleMACS (Miltenyi Biotec) using lung 02_01 program. Cells were incubated at 37 °C for 15 min and then program spleen 04_01 was run, cells were again incubated for 15 min at 37 °C. Cell suspension was filtered with 70-µm basket filter (Fisher Scientific, Waltham, MA). Cells were centrifuged for 10 min at 1000 × g, RBCs were lysed with 1X RBC lysing buffer (Biolegend, San Diego, CA) for 5 min on ice with agitation, cells were then washed with DMEM and resuspended in 5 ml fresh DMEM and filtered with 70-µm basket filter. Dead cells were removed from cell suspension using dead cell removal microbeads (150 µl beads/10 × 10^6 cells, Cat. No. 130-090-101, Miltenyi Biotec) on LS column. Live cells were counted and CD45⁺ immune cells were removed using anti-human CD45 microbeads (20 µl beads/10 × 10^6 cells, Cat. No. 130-045-801, Miltenyi Biotec) before the ICs were enriched. CD45⁺-depleted cells were centrifuged at 300 × g for 10 min and counted and then incubated with 100 µl FcR blocking reagent and 100 µl anti-human CD117 (c-Kit) microbeads (Cat. No. 130-091-332, Miltenyi Biotec) at 4 °C. Cells were applied over MS column and c-Kit⁺ (CD117⁺) cells were flushed out of the column with 1 ml MACS buffer (PBS containing 0.5% bovine serum albumin (BSA) and 2 mM EDTA). RNA quality was tested on Agilent Bioanalyzer (Agilent, Santa Clara, CA). Enriched cells were filtered for mRNA expression of ATP6V1B1, SLC4A1, and SLC26A4 gene by RT-PCR to confirm the relative IC enrichment (Supplementary Fig. 2).

Fig. 8 Murine ICs internalize and acidify E. coli. An “IC reporter” kidney tube with tdT-expressing ICs (red) was perfused with pHrodo Green E. coli-coated BioParticles for phagocytosis that fluoresce green when acidified. a At the 0 min time point red ICs are seen (arrow). Because the 0 min time point started ~1 min after injection when we identified our target tubule and narrowed the focus, some cells may be already starting to uptake bioparticles (arrowhead). b Increasing green fluorescence occurs in patchy areas in the cytoplasm in an isolated IC (arrow) as the experiment progresses to the 2.8 min time point but not some remain red (arrow). c At the 7.1 min time point, most ICs are increasingly green (arrowheads), but an isolated IC remains red (arrow). d At the 13.5 min time point, variable uptake/acidification can be seen with relatively high (arrowhead), medium (star), or low (arrow) green fluorescence visualized. No appreciable green fluorescence is present in red fluorescence-negative cells (presumed PCs) (pound symbol) within the tubule. a–d Magnification 20×, with digital zoom. Images representative of imaging from three tubules from two mice. Each image is from the same tubule and imaging plane, but at a different time point. Because of breathing-induced cell movement, cellular morphology and position will vary from image to image. Images are representative of intravitral experiments on two individual “IC reporter” mice. Source data are provided as a Source Data File.
The slopes of BT1 and BT2 were both significantly higher than the slope of CT ($P < 10^{-15}$), but not different from each other ($P = 0.221$). Within the bioparticle-perfused tubules, 12 of 16 tubules had a slope that increased significantly compared to a zero-slope line. Thus, there is some heterogeneity in IC phagocytic capabilities. During linear regression, Graphpad Prism compares slopes by calculating a two-tailed p value to test the null hypothesis that the slopes are identical. For the tubule analysis (b), slopes were compared to one other tubule (BT1 vs. BT2, BT1 vs. CT, and BT2 vs. CT). For the cell analysis (c), the slope of each cell was compared individually to a zero-slope line. Multiple comparisons were not used. Analysis included 2 bioparticle tubules (one with 11 ICs and the other with 5 ICs) and 1 control tubule (with 5 ICs) from one mouse each. Source data are provided as a Source Data File.

**Single-cell RNA sequencing (scRNA-seq).** The single-cell 3′ RNA-seq experiment was conducted using the Chromium single-cell system (10× Genomics, Pleasanton, CA). The reads from the NovaSeq 6000 sequence (Illumina, CA). Each single-cell suspension was first counted on the counter II FL (Thermo Fisher Scientific) as well as under light microscope for cell number, cell viability, and cell size. Single-cell preparation included resuspension, centrifugation, and filtration to remove cell debris, dead cells, and cell aggregates. The resulting single-cell suspensions had viabilities of 75% and 83% with concentrations of 200 and 310 live cells per μl of sample for saline- and UPEC-exposed cells, respectively. Five- to ten-thousand cells per sample were added to a single-cell master mix, following the Chromium Single Cell 3′ Reagent Kits v2 (User Guide, CG00052 Ver B) along with the single-cell gel beads and partitioning oil in separate wells of a Single Cell A Chip, the single-cell master mixture containing an aliquot of single-cell suspension was loaded to the Chromium controller for gel bead-in-emulsions (GEM) generation and barcoding, followed by cDNA synthesis and library preparation. At each step, the quality of cDNA and library was examined by Bioanalyzer. The resulting libraries were sequenced using a custom program for generating 26 and 98-bp paired-end reads on an Illumina NovaSeq 6000 (Illumina NovaSeq 6000 control software version 1.6.0 and the Real time analysis version 3.4.4).

**ScRNA-seq data analysis.** Data processing and filtering. CellRanger version 3.0.2 (10x Genomics) was utilized to process the raw sequence data generated. Briefly, CellRanger uses bc2fastq to demultiplex raw base sequence calls generated from the sequencer into sample-specific FASTQ files. The FASTQ files were then aligned to the reference genome with RNA-seq aligner STAR. The aligned reads were traced back to the individual cells and the gene expression level of individual genes was quantified based on the number of UMIs (unique molecular indices) detected in each cell. The filtered gene-cell barcode matrices generated with CellRanger were used for further analysis with the R package Seurat development version 3.0.9.0000. QC of the data was implemented as the first step in our analysis. We first filtered out genes that detected in less than five cells and cells with less than 200 genes. To further exclude low-quality cells in downstream analysis, we used the function Outlier from R package scatter together with visual inspection of the distributions of the number of genes, UMIs, and mitochondrial gene content. Cells with high or low number of detected genes/UMIs were excluded. In addition, cells with greater than 25% mitochondrial reads were also filtered out. After removing likely multiplets and low-quality cells, the gene expression levels for each cell were normalized with the NormalizeData function in Seurat. To reduce variations sourced from different number of UMIs and mitochondrial gene expression, we used the ScaleData function to linearly repress out these variations. Highly variable genes were identified with the FindVariableFeatures function (selection.method = "var").

Cluster analysis. To integrate the single-cell data from the treated and untreated samples, functions “FindIntegrationAnchors” and “IntegrateData” from Seurat v3 were implemented with a dimensionality of 30. The integrated data were then scaled and PCA performed. Clusters were identified with the Seurat functions “FindNeighbors” and “FindClusters” using a resolution of 0.8 and the first 12 PCs. The “FindConservedMarkers” function was used to identify cell cluster marker genes. To compare average gene expression within the same cluster between cells of different samples, AverageExpression function was applied. “FindMarkers” function was also used to investigate the differences of gene expression induced by treatment within the same cell cluster. The cell clusters were visualized using the t-Distributed Stochastic Neighbor Embedding (t-SNE) plots and Uniform Manifold Approximation and Projection (UMAP) plots. R packages ggplot2 ([https://ggplot2.tidyverse.org](https://ggplot2.tidyverse.org)) and ggrepel ([https://github.com/sliverow/ggrepel](https://github.com/sliverow/ggrepel)) were used to plot the average gene expression. Violin plots (ViolinPlot) and feature plots (FeaturePlot) were used to visualize specific gene expressions across clusters and different sample conditions. Figures of canonical pathways were made with the Ingenuity Pathway Analysis Software. The raw datasets for scRNA-seq have been deposited in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) repository under accession number GSE159085.

**Pathway analysis.** Canonical pathway analysis was performed using Ingenuity Pathway Analysis (QIAGENhttps://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis) (Qiagen, Hilden, Germany) to identify biological pathways associated with single-cell clusters (April 2019 release, analysis performed in August, 2019). Entrez gene ID, log2 fold change, and adjusted p-values from Seurat scRNA-seq are the IPA inputs. The log2 fold change is the expression relative to other clusters. Seurat uses the Wilcoxon rank sum test to generate a p-value for gene expression and a Bonferroni correction to account for a false discovery rate and to calculate an adjusted p-value. Ingenuity reports a p-value regarding pathway enrichment versus a random list of genes within a cluster using a right-tailed Fisher’s exact test to calculate the probability that assigned biological functions are not due to chance alone.

**RNA velocity analysis.** Mapping position sorted.bam files generated by cellRanger were converted to cellsorted.bam files through the velocyto pipeline v0.17 (velocyto.org) to obtain loom files for each experimental condition. These loom files...
along with their associated UMAP positions and principal component tables extracted from the merged Seurat file were then fed individually into the RNA Velocity pipeline as described in the Velocyto.R Dentate Gyrus/loom tutorial.

The default settings described in the tutorial were used, except for ISNE positions that were overwritten with the associated UMAP positions from the merged Seurat object, as well as the principal component table. This generated an RNA velocity figure mapped using the merged Seurat object cell positions for both conditions. The code used for the RNA velocity is presented in Supplementary Software 1.

Intravital imaging of kidney. Mouse conditions. Eight experimental intravital sessions and 1 control session (bleaching control) were undertaken. Animal groups were subdivided into 5 groups, with 3 female mice in each group. The age of mice ranged from 55 to 103 days, and the mouse weight ranged from 19.3 to 22.4 g, median weight: 20.3 g, mean weight: 20.6 g.

Sample size. With prior experiments evaluating the murine transcriptional response to experimental UTI, a broad standard deviation occurred. Therefore, we have developed the strategy of initially experimenting with 4–6 mice, depending on availability, per group (saline vs. E. coli inoculation). If significance is achieved or if the trend toward a difference the experiment is complete. If a trend toward significance is obtained, the experimental number is expanded to another 4–6 group.

Immunofluorescence and confocal microscopy. Seven-µm thick paraffin-embedded human kidney sections were stained with polyclonal rabbit anti-human c-KIT antibody (Cat no. A540229-2, Agilent Dago, Santa Clara, CA) to localize collecting duct ICs at 1:100 dilution followed by anti-rabbit Cy2 (1:200 dilution) secondary antibody was used (Jackson Immunoresearch, West Grove, PA). For SLCA1 immunolabeling, a 1:1 dilution of polyclonal rabbit anti-human SLCA1 (Sigma Aldrich, St. Louis, MO) was used along with an anti-rabbit Cy3 (1:500 dilution) secondary conjugate (Jackson Immunoresearch, West Grove, PA). To visualize V-ATPase E1 subunit (1:200 dilution) followed by anti-chicken Cy3 (1:600 final dilution) secondary antibody was used (Jackson Immunoresearch, West Grove, PA). V-ATPase E1 staining was performed using a polyclonal chicken anti-human ATP6V1E1 antibody (Sigma Aldrich, St. Louis, MO) and visualized with an anti-chicken DyLight405 secondary antibody at 1:200 final concentration of 1:2000. Last, monoclonal Rabbit Anti-human EGR1 was stained at 1:100 dilution followed by a secondary antibody with a 1:800 final concentration of anti-rabbit-AF488 (Jackson Immunoresearch, West Grove, PA).

RT-PCR of IC marker genes in enriched human cells. Human kidney biopsy samples from four individual patients were processed. Single-cell suspension was prepared and fixed cells were removed using cell removal microbeads, and c-KIT + and c-KIT− cells were magnetically enriched after depletion of CD45 immune cells as described earlier. The cells were pelleted, and RNA was prepared using the RNAeasy mini kit (Qiagen). CDNA was prepared using high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). In all, 1 µl cDNA was amplified with ATP6V1B1, SLCA4A1, SLCA6A4, and RNASE7 primers (Kicqstart Primers, Sigma) in a 20 µl reaction on a CFX connect real-time system (Bio-Rad Laboratories, Hercules, CA). GAPDH was used as an internal control. Relative mRNA expression comparison to GAPDH was calculated using ddCT method. Primer sequences for all PCR experiments are reported in Supplementary Table 5.
In vitro E. coli phagocytosis assay. To further confirm the live animal intratubal imaging bacterial uptake, pHrodo E. coli BioParticle uptake was measured in vitro by flow cytometry. This method uses a unique pHrodo dye that fluoresces in response to an acidic environment. Kidney cell suspension was prepared from “IC reporter” mice using Accumax solution (Innate Cell Technology, San Diego, CA) in combination with GentLeMacs (Miltenyi Biotec). In this mouse strain, ICs are endogenously marked by tdiTomato (tdt) red fluorescence. In total, 250,000 cells were surface labeled with Alexa-488-conjugated with APC (1:250 dilution, clone 30-F11, Cat no. 17-0451, eBioscience, San Diego, CA) for 30 min at 4 °C and then incubated with pHrodo Green-E. coli BioParticles or media alone and incubated for 15 min at 37 °C as recommended (Invitrogen, CA). After that cells were kept on ice to stop the reaction. Cells were immediately acquired on Attune Flow cytometer (Invitrogen, Carlsbad, CA) to measure green fluorescence on CD45-tdt+ (intercalated cell) and CD45+ immune cells. Data were analyzed using FlowJo version 10 software (Becton Dickinson, Franklin Lakes, NJ). The gating strategies for flow cytometry experiments are presented in Supplementary Fig. 21.

Statistical analysis. Unless stated otherwise, statistical evaluation was performed using GraphPad Prism. The Fisher exact test was used to compare percentages or proportions. Continuous variables were tested for normalcy with the Shapiro–Wilks test. If the data were parametric, a two-tailed t test was used, while the Mann–Whitney, also two-tailed, was used for nonparametric data. Linear regression was used to compare slopes.

Figure generation. Intratubal figures were generated using Microsoft PowerPoint, GraphPad Prism, Adobe Photoshop, Leica Application Suite X, and/or Imaris software. Background fluorescence was only removed from nontargeted fluorescent cells/tubules on images with numbered cells presented as segmentation cartoons. Any brightness and contrast adjustments were performed uniformly across all images in a time series. Immunofluorescence microscopy and confocal microscopy figures were generated using Microsoft PowerPoint, Leica Application Suite X, and/or Keyence BZ Analyzer software. Image adjustments included black balance along with brightness and contrast adjustments. Canonical pathway figures were generated using IPA software.

Key resources. Key resources for the experimental methodology are presented in Supplementary Table 6.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. Contacting the originating investigators or source companies is needed to obtain key biological material. The scRNA-seq processed data can be found at https://hpcwebapps.cit.nih.gov/ESBL/Dataset/IA-DataHuman-e-KitSorted-Single-Cell-RNASeq.htm. The scRNA-seq raw data are available at NCBI gene expression omnibus (GEO). Its GEO accession number is GSE159006. Kidney Cell Explorer (https://cellsofthinys.cells.org/kidneycellexplorer/) was used to compare our scRNA-seq findings to comparable past murine studies including GSE129798. For references not found to the aforementioned links, we have submitted source data with this paper. Source images from the Human Protein Atlas version 20.0 may be obtained at https://www.proteinatlas.org/ENSG00000120738-EGR1/tissue/kidney#img, https://www.proteinatlas.org/ENSG00000243899-HSPA1A/tissue/kidney#img, and https://www.proteinatlas.org/ENSG00000183023-SLC8A1/tissue/kidney#img. Additionally, other data supporting this publication can be obtained by contacting the lead author. Source data are provided with this paper.

Code availability. Code used for the RNA velocity analysis presented in Fig. 5 is available as Supplementary Software 1.
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Author contributions
A.L.S., D.S.H., V.S., and T.H. supervised the study. A.L.S., D.S.H., V.S., T.H., M.H.Z., and M.M.K. developed the experimental protocol. V.S., S.A., M.M.K., A.Z., P.M., X.X., and H.G. performed the experiments. H.G., D.S.H., A.L.S., H.G., and M.M.K. performed the data analysis. A.L.S., V.S., D.S.H., S.A., T.H., XX., H.G., M.H.Z., and M.M.K. performed the critical edits.

Competing interests
The authors declare no competing interests.
