Identification of functional heterogeneity of immune cells and tubular-immune cellular interplay action in diabetic kidney disease

Yunfeng Bai, Kun Chi, Delong Zhao, Wanjun Shen, Ran Liu, Jing Hao, Guangyan Cai, Xiangmei Chen, Quan Hong

Department of Nephrology, First Medical Center of Chinese PLA General Hospital, State Key Laboratory of Kidney Diseases, National Clinical Research Center for Kidney Diseases, Beijing Key Laboratory of Kidney Disease Research, Beijing 100853, China

ABSTRACT

Background: Renal inflammation plays key roles in the pathogenesis of diabetic kidney disease (DKD). Immune cell infiltration is the main pathological feature in the progression of DKD. Sodium glucose cotransporter 2 inhibitor (SGLT2i) were reported to have anti-inflammatory effects on DKD. While the heterogeneity and molecular basis of the pathogenesis and treatment with SGLT2i in DKD remains poorly understood. **Methods:** To address this question, we performed a single-cell transcriptomics data analysis and cell cross-talk analysis based on the database (GSE181382). The single-cell transcriptome analysis findings were validated using multiplex immunostaining. **Results:** A total of 58760 cells are categorized into 25 distinct cell types. A subset of macrophages with anti-inflammatory potential was identified. We found that Ccl3+ (S100a8/a9 high) macrophages with anti-inflammatory and antimicrobial in the pathogenesis of DKD decreased and reversed the dapagliflozin treatment. Besides, dapagliflozin treatment enhanced the accumulation of Pck1+ macrophage, characterized by gluconeogenesis signaling pathway. Cell-cross talk analysis showed the GRN/SORT1 pair and CD74 related signaling pathways were enriched in the interactions between tubular epithelial cells and immune cells. **Conclusions:** Our study depicts the heterogeneity of macrophages and clarifies a new possible explanation of dapagliflozin treatment, showing the metabolism shifts toward gluconeogenesis in macrophages, fueling the anti-inflammatory function of M2 macrophages, highlighting the new molecular features and signaling pathways and potential therapeutic targets, which has provided an important reference for the study of immune-related mechanisms in the progression of the disease.

Key words: diabetic kidney disease, inflammation, single-cell RNA-seq analysis, intercellular communication

Address for Correspondence:
Xiangmei Chen, Department of Nephrology, First Medical Center of Chinese PLA General Hospital, National Key Laboratory of Kidney Diseases, National Clinical Research Center for Kidney Diseases, Beijing Key Laboratory of Kidney Disease Research, 28# Fuxing Road, Haidian District, Beijing, China. E-mail: xmchen301@126.com. https://orcid.org/0000-0001-8774-6021

Quan Hong, Department of Nephrology, First Medical Center of Chinese PLA General Hospital, National Key Laboratory of Kidney Diseases, National Clinical Research Center for Kidney Diseases, Beijing Key Laboratory of Kidney Disease Research, 28# Fuxing Road, Haidian District, Beijing, China. E-mail: hongquan@301hospital.com.cn. https://orcid.org/0000-0002-6839-7695

Access this article online

Website: www.intern-med.com

DOI: 10.2478/jtim-2023-0130

INTRODUCTION

Chronic kidney disease (CKD) is a global public health problem.[1] Diabetic kidney disease is the leading cause of CKD and accounts for approximately half of the end-stage renal disease (ESRD) burden in the developed world.[2]

Diabetic substrates such as elevated glucose environment and advanced glycation end products induce renal cell death leading to the leakage of intracellular damage-associated molecular patterns into the extracellular, and then these danger signals elicit the activation of downstream immune signaling pathways.[3] Increasing evidence showed that renal inflammation caused by immune injury is the key driving factor to the progression of DKD, characterized by an increasing number of macrophages, and activated T cells, macrophage accumulation is directly proportional to the progression to ESRD in patients with diabetes,[4] and the aberrant intrarenal infiltration and activation of T cells lays the foundation of immunopathological mechanism of diabetic kidney injury.[5]

SGLT2i reduces the renal glucose excretion threshold, which results in a decrease in mean plasma glucose concentration and ameliorates glucotoxicity.[6-8] Dapagliflozin decreased macrophage infiltration, suppressed the gene expression of inflammatory cytokines, and attenuated proteinuria, mesangial expansion, interstitial fibrosis in a dose-dependent manner in diabetic mice.[9] Similarly, empagliflozin reduced the production of the inflammatory molecules NF-κB and toll-like receptor-4 induced by high glucose.[10]

Single-cell transcriptomic profiling is a powerful tool to observe the genetic changes in the tissue microenvironment and disease progression in a high-resolution and depth view and provides key insights into the mechanism of disease. Studies to dissect the landscape of DKD progression have emerged. In streptozotocin-induced diabetic endothelial nitric oxide synthase (eNOS)–deficient ($eNOS^{-/-}$) mice, single-cell RNA profiling shows that macrophages are the predominant immune cell type in diabetic glomeruli, contributing to diabetic kidney injury.[11] Single-nucleus RNA sequencing (snRNA-seq) analysis showed that in patients with early diabetic nephropathy samples, the gene expression that is essential for immune cell activation, ion transport and angiogenesis.[12] Wu *et al.* identified a new proximal tubular subcluster and revealed gene signatures in response to angiotensin receptor blockers (ARBs) and SGLT2i. Wu *et al.* elucidated that SGLT2i may act on proximal tubules through alternative splicing as a potential mechanism.[14] While what remains unknown is the heterogeneity of infiltrating immune cells in the progression of DKD and treatment with SGLT2i, and how immune cells intercross with tubular cells promotes the development of DKD.

Here, our findings from scRNA-seq analysis of 58760 cells from db/mc, db/db, and dapagliflozin-treated group showed that two new macrophage subtypes Ccl3+ macrophage and Pck1+ macrophage exhibited anti-inflammatory activity, delineating the heterogeneity of immune cells, underlining the importance of intracellular crosstalk between tubular epithelial cells and immune cells, revealing potential therapeutic targets for DKD.

MATERIALS AND METHODS

Single-cell transcriptomic data preparation

First, we downloaded scRNA-seq data from the Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo) dataset GSE181382, which contained the single-nucleus transcriptomic data of db/mc control group, db/db group and db/db SGLT2i groups produced by 10× genomics.

Determination of cell type

We followed the methods of Zhang *et al.* 2022. The clusters were annotated using cell type-specific signatures and marker genes, for example, macrophages of *C1qa*, *C1qb*, *Cd74*, *Cd79a* and *Cd79b*, and T cell of *CD4*, *CD8*, *Ltb*, *Nkg7 Gzma*.

Pathway analysis and intercellular crosstalk analysis

We followed the methods of Zhang *et al.* 2022.[15]

Single cell RNA sequence data preparation

The raw gene expression matrix was obtained and processed to align reads with the reference genome (Homo_sapiens_GRCh38_96) using Cell Ranger (version 5.0.0). Data filtration and normalization were performed using the R package Seurat (version 4.0.0) according to the manufacturer's manual. We used Seurat (4.0.0) to filter low quality cells, cells with the number of detected genes < 200 or > 5000, and cells with > 25% of the UMI counts belonging to mitochondrial genes were also omitted. Only scRNA seq data that met the quality control criteria were analyzed in this study.

Canonical correlation analysis, dimensionality reduction, and clustering

After quality control and filtering, the clustering and visualization was finished by Seurat (V4.0.0), with the following steps: First, library-size normalization to each cell was performed by NormalizeData. The variable genes were calculated by FindVariableGenes. Then, all libraries were combined using FindIntegrationAnchors and IntegrateData with default parameters, and using ScaleData to regress out the variability of the number of UMIs. Then the RunPCA and RunUMAP were used to reduce dimensions. FindClusters was used to cluster cells using 20 dims at a resolution of 0.8 (total 27 clusters). FindAllMarkers was used to compare each cluster to all others to identify cluster-specific marker genes. Each retained marker gene was expressed in a minimum of 25% of cells and at a minimum log-fold change threshold of 0.25. The clustering differential expressed genes were considered significant if the adjusted P-value was less than 0.05, and the avg_$\log_2$FC was ≥ 0.25.

Deferentially expressed genes in specified clusters

After cell annotation, differentially expressed genes in specified cell types were analyzed using the FindMarkers function based on the bimod algorithm of the R package Seurat (version 4.0.0). Fold changes 1.25 and $P < 0.05$ were considered significantly modulated.

Cell-cell interactions

CellPhoneDB is a public repository of curated receptors, ligands, and their interactions. In this study, cell crosstalk interaction was performed using CellPhoneDB (version

2.1.1) according to the manufacturer's manual (https://www.cellphonedb.org/). The mean value represents the average ligand and receptor expression in a specific cell type, which is calculated based on the percentage of cells expressing the specific gene and the gene expression mean. The *P*-value is calculated based on the proportion of means that are as high as or higher than the actualmean, which represents the likelihood of a specific cell type of a given receptor–ligand complex.

Spontaneous type 2 diabetic db/db mice model and measurements of urinary albumin excretionWe purchased the db/db and db/mc mice and measured the UAE based on our previous research.[16] The method of treatment to db/db and db/mc mice were followed by the methods of Wu *et al.* 2021.[13] The db/db mice were defined as DKD by albuminuria at 10 weeks of age and gavaged for 8 weeks, vehicle control (phosphate-buffered saline [PBS]) ($n = 4$), 3 mg/kg/d of dapagliflozin ($n = 4$), and sacrificed at the age of 18 weeks. All animal studies were performed according to the protocol approved by the Animal Care Committee at the First Medical Center of Chinese PLA General Hospital. The urine albumin concentration was measured using the ELISA kit (Bethyl Laboratories, Montgomery, TX, USA).

Antibodies and reagents

The antibodies and reagents used in this study included rabbit anti-CD206 (ab64693, 1:400, Abcam, Cambridge, UK), goat anti-S100a8 (AF3059, 1:100, R&D systems, MN, USA), goat anti-S100a9 (1:100, AF2065, R&D systems, MN, USA), DNA was stained with Hoechst 33342 (1:1000, H3570; Thermo Fisher Scientific, CA, USA), dapagliflozin (3 mg/kg/d, S1548, Selleck, TX, USA)

Immunofluorescence microscopy and kidney histology

For tissue immunofluorescence and histology analysis, we keep following the methods in our previous research.[16] Images were acquired at room temperature with a 60×/1.42 oil objective on an Olympus FV1000. All acquisition settings were kept constant for the experimental and control groups in the same experiment. All raw images were analyzed with Velocity 6.0 software (Perkin Elmer).

Statistical analysis

Statistical analysis was performed with GraphPad Prism. The statistically analyzed data are expressed as the mean ± SD. To compare the means between the three groups, one-way ANOVA followed by Dunnett's multiple comparison test was used, as noted in the figure legends. For all tests, differences were considered statistically significant if *P* values were < 0.05 (as indicated with $^{***}P < 0.001$). Investigators were blinded during the assessment of all staining assays.

RESULTS

Immune cells atlas at single-cell resolution in the pathogenesis and treatment with SGLT2i in DKD

To decipher the comprehensive landscape of potential dynamic features of macrophages in the DKD progression and treatment with SGLT2i, a total of 58760 single-cell transcriptomes from GSE138182 dataset of db/mc, db/db and SGLT2i-treated groups were analyzed. Using unsupervised clustering (uniform manifold approximation and projection [UMAP]), 27 separate cell clusters were identified after pooling all samples together (Figure 1A and 1B) and then categorized into ten distinct cell types, including proximal tubules (PT), neutrophil, distal convoluted tubule (DCT), loop of henle (LOH), T cells (T), macrophage, B cells (B), collecting duct (CD), endothelial cell (EC), proliferative PT (prolif-PT) (Supplementary Figure S1). Cluster 19 was annotated as macrophage since it is characterized by representative macrophage genes (*C1qa*, *C1qb* and *CD74*) (Figure 1C). Cluster 15 was annotated as T cell, characterized by its marker genes (*Ltb*, *Nkg7*, and *Gzma*) (Figure 1D).

Dapagliflozin enhanced the portion S100a8/a9 high macrophage, possibly by glucogenesis to fuel anti-inflammatory function. These 1306 macrophages were further divided into 5 clusters after passing mitochondrial quality control. Through unsupervised clustering, the representative genes for each cluster were C1qa, CCl_3, H2-DMb2, Spp1, Pck1(Figure 2A). After dapagliflozin treatment, compared with the db/db and db/mc groups, the portion of Ccl3+ group (S100a8/a9 high) and Pck1+ group significantly increased (Figure 2B)

We next focused on the potential function of Ccl3+ macrophages in the three groups, and found that it exhibited a high level of anti-inflammatory and antimicrobial signatures such as *Lcn2*, *Hp*, *S100a8*, *S100a9* (Figure 2C), while the portion of Ccl3+ macrophage in db/db group decreased and reversed in the db/db_SGLT2i treated groups (Figure 2C), suggesting the host susceptibility to severe bacterial infection in the progression of DKD.

S100a8/a9/a11, S100 calcium-binding protein a8/a9/a11 (S100a8/a9/a11) is a member of the S100 family, S100a8/a9 is related to inflammation, obesity and diabetes.[17-19] S100a8 attenuates the production of proinflammatory mediators[20] and decreases mast cell degranulation and cytokines secretion.[21] S100a8 forms a homodimer or a heterodimer with S100a9.

Pathway analysis of the unique gene sets in subcluster Ccl3 and Pck1 revealed that the pathways enriched in subcluster Ccl3 were absent in subcluster Pck1, while the pathways

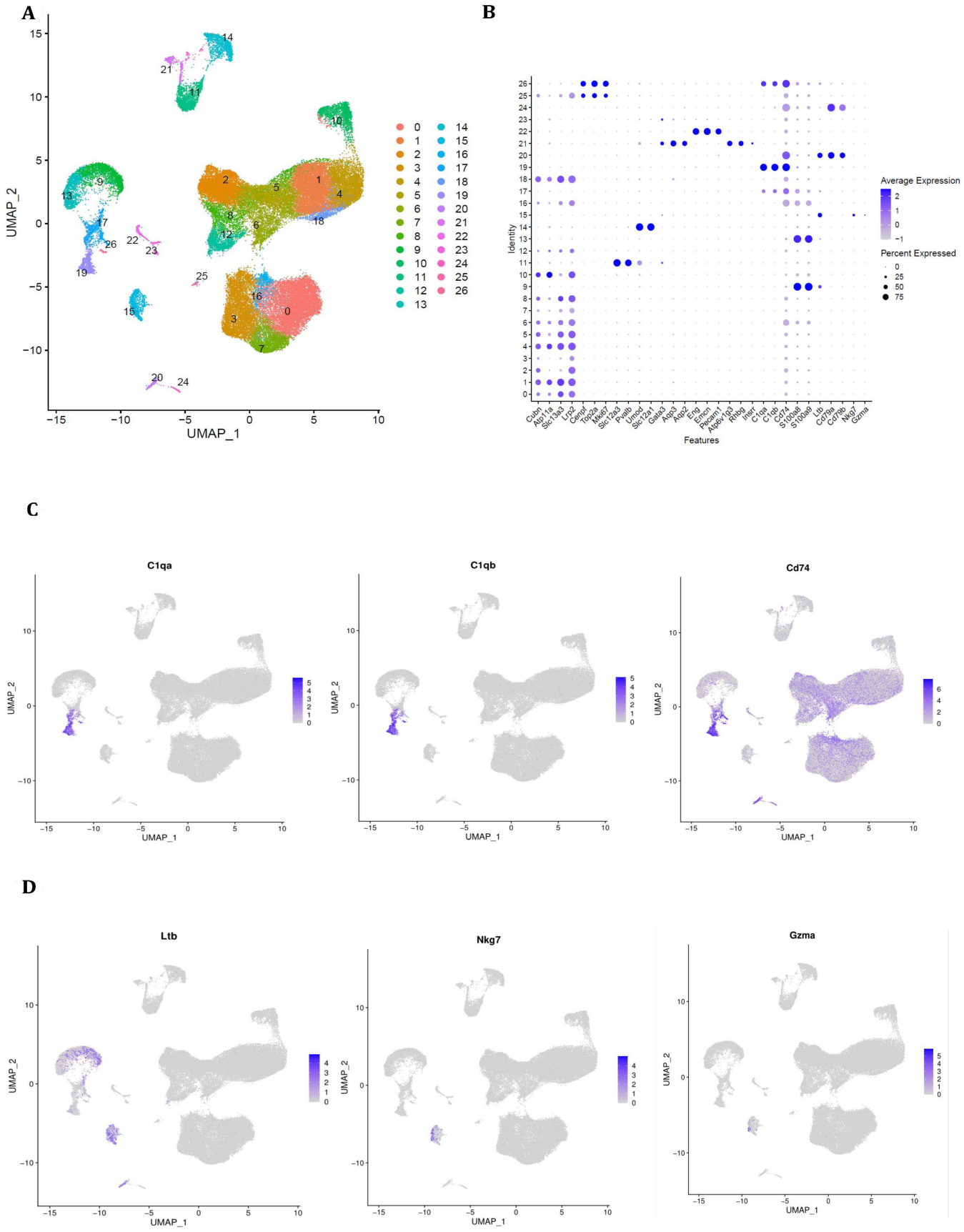

Figure 1: Integrated scRNA-seq of db/mc, db/db and SGLT2i-treated samples. We performed UMAP analysis (A), identified gene markers (B, C, D), of the 58760 cells.

Table 1: Summarization of key signaling pathways enriched in tubular-immune cell communication

Pathogenesis				Treatment			
db/db (+) db/mc (-)		db/db (-) db/mc (+)		db/db (+) SGLT2i (-)		db/db (-) SGLT2i (+)	
EGFR_GRN	PT/M	C5AR1_RPS19	M/LOH	EGFR_GRN	PT/M	C5AR1_RPS19	M/LOH
GRN_SORT1	M/LOH PT/LOH	CD74_COPA	M/T PT/LOH	GRN_SORT1	M/LOH PT/LOH	CD74_COPA	M/T PT/LOH
TNFRSF1A_GRN	PT/M	CD74_APP	PT/PT	TNFRSF1A_GRN	PT/M	CD74_APP	PT/PT
LAMP1_FAM3C	DCT/T			LAMP1_FAM3C	DCT/T		
	M/M				M/M		
	M/T				M/T		
	PT/M				PT/M		
	PT/T				PT/T		

+: significant enrichment; −: poor enrichment; DCT: distal convoluted tubule; LOH: loop of Henle; M: macrophage; PT: proximal tubules; T: T cells.

enriched in subcluster Pck1 were absent in subcluster Ccl3, indicating that these two subclusters likely have an exclusive function in DKD (Figure 2D). The GO term enrichment analysis of the marker revealed that subcluster Ccl3+ macrophage was enriched by response to a molecule of bacterial origin and lipopolysaccharide while the portion of Ccl3+ macrophage in total macrophage was decreased, suggesting that during the progression of diabetic kidney disease, the anti-inflammatory activity of subcluster Ccl3+ macrophage was inhibited and then reversed by SGLT2i treatment.

To further validate these results, type 2 diabetic db/db mice at 10 weeks of age were treated with vehicle, SGLT2i (dapagliflozin) for a total of 8 weeks (Figure 3A). Nondiabetic db/mc mice were used as their controls. Compared with db/m mice, db/db mice had increased 24-h urinary albumin excretion (UAE), a reduction in UAE was observed in SGLT2i-treated groups (Figure 3B). Histologically, both glomerular volume and mesangial matrix expansion were increased in db/db mice compared with db/mc mice, but these were reduced by dapagliflozin treatment (Figure 3C). The specific markers of Ccl3+ macrophage (S100a8/S100a9) were validated by immunostaining of mouse kidneys (Figure 3D) from db/mc, db/db and SGLT2i-treated mice.

SGLT2i treated group also showed the increased portion of Pck1+ macrophage, Pck1 is a main control point for the regulation of gluconeogenesis, showing the metabolism shifts toward gluconeogenesis, fueling the anti-inflammatory function of M2 macrophage.[22] So, we hypothesized that dapagliflozin can promote its anti-inflammatory effect mainly by changing the glucose metabolism pathway of macrophages.

Characteristics and dynamics of T cells in DKD

T cells were categorized into 7 subclusters based on available markers (*Ltb*, *Nkg7*, and *Gzma*) by UMAP analysis (Supplementary Figure S2A), and were performed to functionally annotate the seven T cell subsets (Supplementary Figure S2B). CD8a+ Tcm (central memory) subcluster decreased in the db/db group and reversed by the SGLT2i treatment (Supplementary Figure S2C). CD8a+ Tcm is characterized by its signature genes, *Ms4a4b*, *Ms4a6b*, *Nkg7*, and *Ccl5*. *Ms4a4b*, *Ms4a6b*, and *Nkg7*, preoccupied with immune defense, use MS4A family to enhance sensitivity to extrinsic antigen stimulation, modulates T cell activation and cytotoxic function.[23,24] *Ccl5* is a superfamily of secreted proteins involved in immunoregulatory and inflammatory processes, leading to an innate immune response.[25] The GO term enrichment analysis of the marker genes revealed that subcluster Cd8a+ Tcm was enriched by the phosphatidylinositol-3-kinase signaling pathway (Supplementary Figure S2D). It suggested that the central T cell memory activation and function was inhibited in the progression of diabetic kidney disease and reversed by dapagliflozin treatment.

Intracellular immune-tubular cell communication in DKD

Since cross-talk between cell types might underline renal inflammation, promoting the progression of diabetic kidney disease. We characterized the intercellular receptor-ligand pairs and molecular interactions of the two cell types by the CellPhoneDB algorithm. Interestingly, we found that the frequency of receptor-ligand interactions in Figure 4A and 4B varies markedly when comparing the dbdb group, dbmc group versus dbdb_SGLT2i (SGLT2i) group, and summarized the key interactions between ligand and receptor from tubular-immune cell communication (Table 1). Notably, the GRN plays central roles in the most active receptor-ligand interactions.These results indicate that the crosstalk between macrophages via diverse receptor-ligand signals may exert a profound effect on diabetic kidney disease

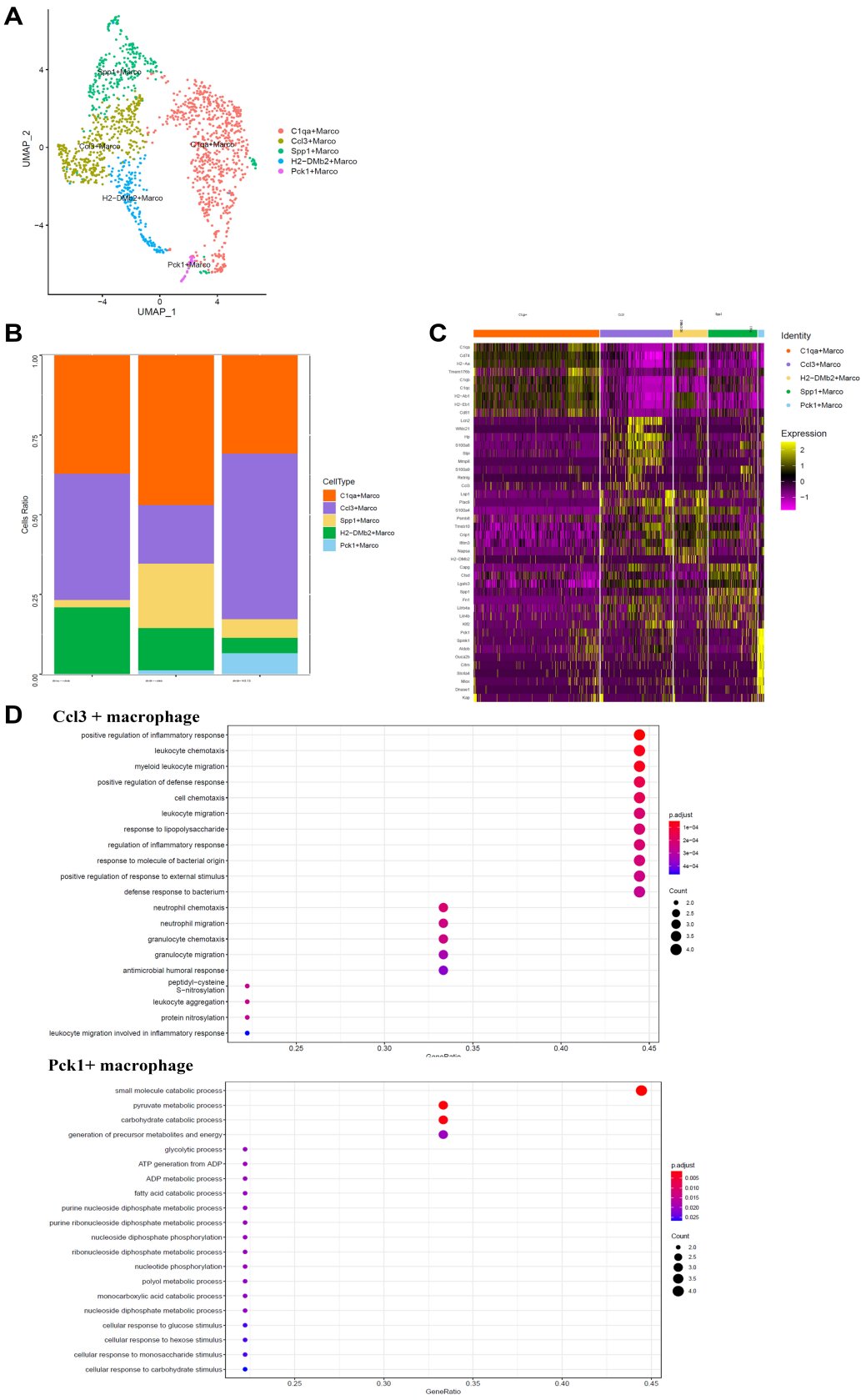

Figure 2: Functional heterogeneity of macrophage subcluster in the DKD. The distributions of the 5 subcluster macrophage in db/mc, db/db, and SGLT2i treated-db/db groups (A). The portion of subcluster in each group (B). The heatmap shows the top 9 significantly differentially expressed (SDE) genes in each subset of macrophages (C). GSVA analysis indicates enriched pathways of Ccl3+ macrophages (D).

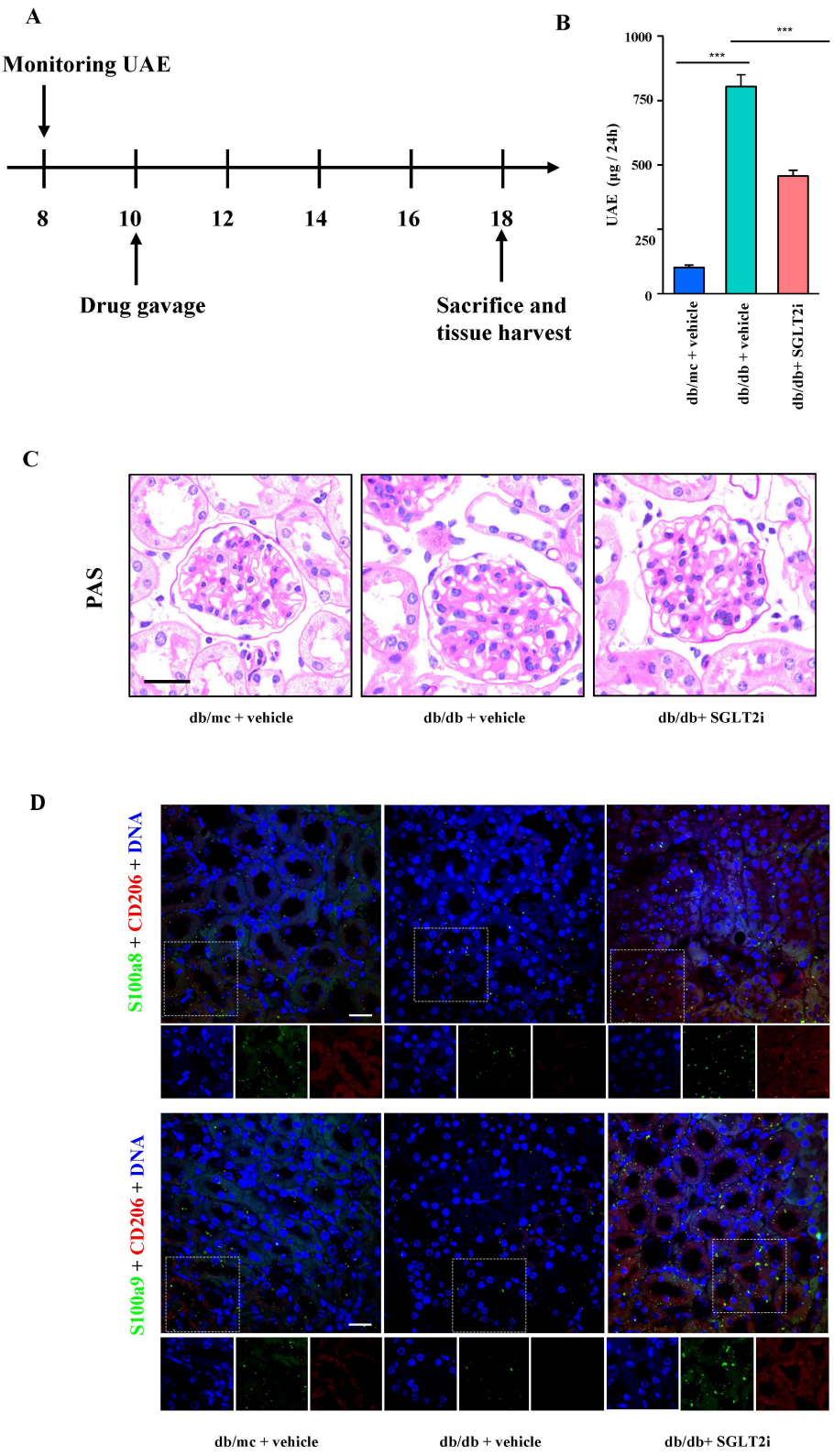

Figure 3: Characterization of mice and validation of macrophage after 8 weeks of treatments. (A) Schematics of the experimental design. UAE were monitored in 8-week-old db/db mice. Treatment (PBS, SGLT2i) was started after db/db mice developed DKD approximately 10 weeks of age. All mice were sacrificed 8 weeks after treatment, Four mice per group. (B) Urinary albumin excretion (UAE) in db/mc, db/db and db/db+SGLT2i group. (C) Kidney sections from each group were stained with PAS. Scale bars, 20 μm. (D) Kidney samples of each group were stained with the S100a8 (green), S100a9 (green) and M2 macrophage (CD206, red). Scale bars, 20 μm. The data are the means ± SD. One-way ANOVA was performed followed by Dunnett's multiple comparisons. $^{***}P < 0.001$.

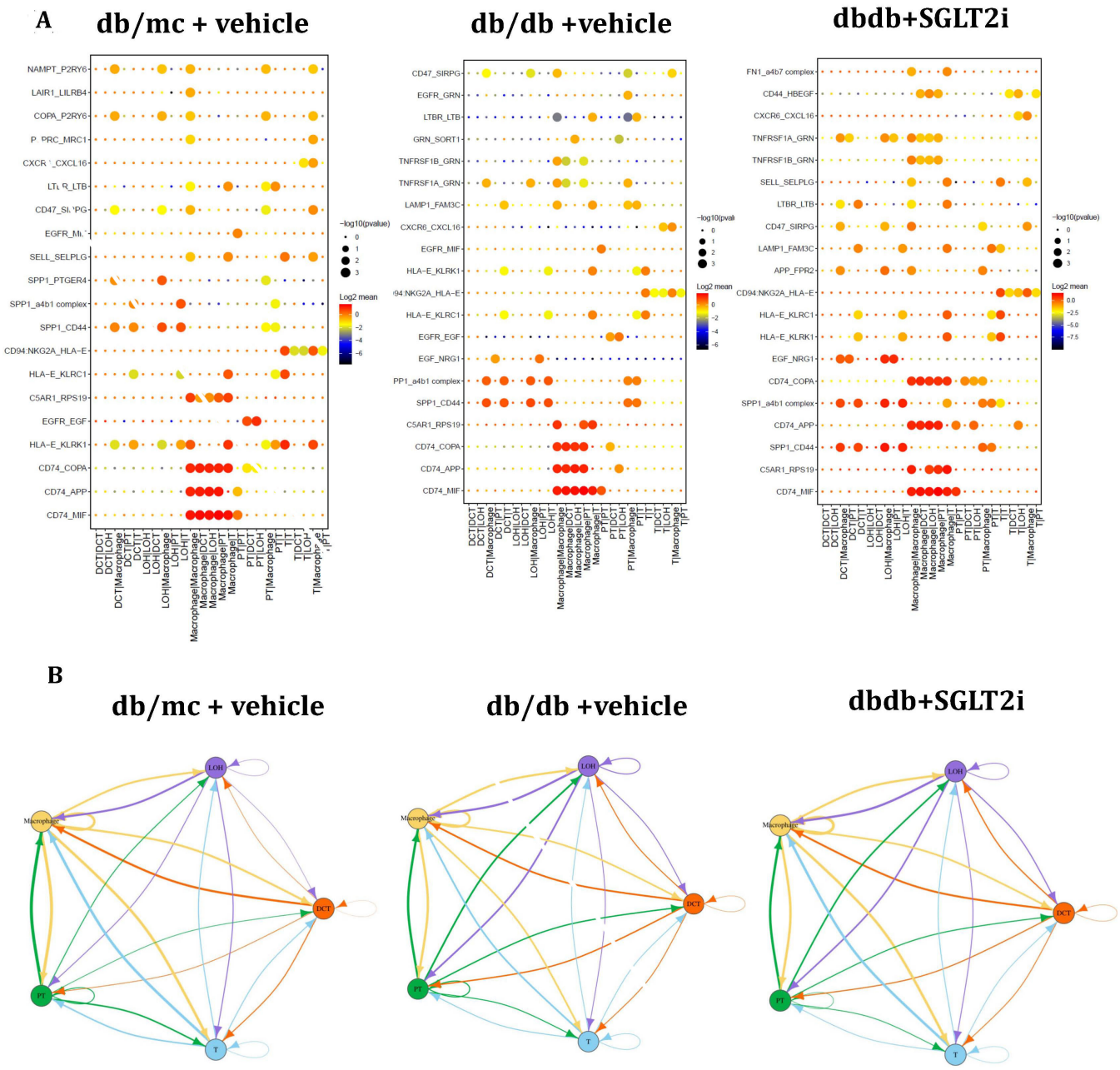

Figure 4: Intracellular immune-tubular cell communication in DKD. Overview of selected interactions of ligands and receptors (A). Detailed view of ligand-receptor connections between tubular cell and immune cell subclusters. Each color arrow line indicates the ligands to receptors from one cell population to another. The thickness of arrow line is proportional to the number of ligand-receptor pairs (B). The purple circle represents LOH, the yellow circle represents macrophage, the green circle represents PT, the blue circle represents T cell, The orange circle represents DCT.

progression and treatment.

Progranulin (GRN) is a secreted glycoprotein that is widely expressed in many cell types including leukocytes and neurons, and is involved in various physiological and pathological progressions, including embryogenesis, wound healing, hostdefense, and tumorigenesis.[26] GRN has been shown to inhibit the inflammatory response in chronic inflammatory conditions such as rheumatoid arthritis, osteoarthritis, and inflammatory bowel disease.[27] Macrophage-derived progranulin associated with M2 phenotypes[28-30] Besides, Zhou *et al.* reported that PGRN prevents podocyte injury by facilitating mitophagy and mitochondrial biogenesis in DKD. Sortilin 1 (SORT1) endocytoses and delivers PGRN to lysosomes, sortilin-mediated PGRN endocytosis is likely to play a central

role in DKD pathophysiology. Under diabetes conditions, insufficient PGRN accelerates DKD progression by disrupting mitochondrial homeostasis.[31]

Our data analysis showed that macrophage and PT clusters were revealed by enriched signaling pathways, including EGFR-GRN pair, suggesting that skewed macrophage plorization from M2 to M1, accelerating the immunologic injury in DKD. Macrophage-derived TNFRSF1A drives the progression to DKD.

In normal mouse and human kidneys, low CD74 expression limits inflammation to extracellular stimuli. The increased renal cell CD74 expression observed during kidney injury (*e.g.*, abnormally high concentrations of specific metabolites, glucose, and lyso-Gb3), including kidney cancer may contribute, together with increased ligand availability, to elicit biological responses during kidney disease. Thus, overexpression of CD74 led to upregulation of NF-κB-dependent genes encoding cytokines in macrophages and to NFκB activation and proliferation in human embryonic kidney cells. CD74, a chaperone molecule expressed in antigen-presenting cells, mediates the load of antigen peptides onto the MHC class-II molecule.[32,33]

Notably, EGFR_GRN_SORT1 and TNFRSF1A_GRN complexes were likely the most active receptor-ligand interactions between macrophage and epithelial cells in dbdb group. The CD74 related signaling pathways were enriched in the db/mc and SGLT2i group than those in db/db group, suggesting that these cell-to-cell connections might be important for delay the progression to DKD.

Together, our unbiased dissection of the key ligand-receptor interactions between tubular and immune cells highlights EGFR-GRN-SORT1 and CD74 signaling axes as essential to delay the progression of the to DKD.

DISCUSSION

Diabetic kidney disease is the most serious outcome of diabetic complications, which exacerbate its progression to CKD. Renal inflammation lays the foundation of DKD pathogenesis, which undergoes infiltration of immune cells, renal microenvironment alterations.[3] Dissecting the key immune cell subclusters and intercellular crosstalk associated with DKD pathology and treatment is critical for clarifying the mechanisms, precision diagnosis, and developing novel therapy strategies for DKD.

Several studies have elucidated diverse infiltrated immune cells, predominantly macrophages are presented along with the DKD progression. Fu *et al.* utilized scRNA-seq to uncover the increase of macrophages with M1 phenotype are the major inflammatory cells in early DKD.[11] Moon *et al.* reported that aberrant intrarenal infiltration and the activation of T cells in the interstitium are the underlying immunopathological mechanisms of diabetic kidney injury.[5] Wu *et al.* conducted scRNA seq to resolute the kidney cell transcriptome of DKD mice treated with ARBs, SGLT2i, or combination of ARBs and SGLT2i, and identified SGLT2i affected more mitochondrial function in PT, while ARBs had more anti-inflammatory and anti-fibrotic effects.[13] Wu *et al.* showed a 1-million-cell atlas showing heterogeneity in kidney cell responses to DKD and treatments.[14] These studies have broadened our knowledge of understanding kidney injury in diabetes progression. However, the molecular characteristics of immune cells and how infiltrating immune cells influence the process of DKD and treated with SGLT2i remains obscure.

In this study, we performed scRNA-seq analysis, using the immune cell atlas, and immune-tubular cell crosstalk of db/db mice treated with SGLT2i. We identified two macrophage subclusters respectively, and indicated that each subtype exhibits unique characteristics. Interestingly, the Ccl3+ macrophage exhibited anti-inflammatory and antimicrobial features and showed a decreased portion in the db/db group. The antimicrobial signature genes (*Lcn2*, *Hp*, *S100a8*, *S100a9*) were enriched in Ccl3+ macrophage. The antimicrobial peptide S100a8/a9 produced by macrophage may function as a potent and direct regulator of tubular function. *Pck1* is a key control gene for the regulation of gluconeogenesis, stimulating the anti-inflammatory function of activated macrophages, supplying a possible explanation for the mechanism of SGLT2i treatment.

The kidney microenvironment shows obvious regional heterogeneity that is highly dynamic and depends on local physiological and pathological conditions.[35] Define cell ontogeny and understand the relationship of tubular epithelial-immune cells is necessary to clarify the pathogenesis of diabetic kidney disease.

Taken together, our study provides a comprehensive immune cell atlas for depicting the pathogenesis and key molecular pathways that are disturbed in DKD. The results presented here highlighted EGFR-GRN-SORT1 and CD74 signaling pathways were potential targets of kidney injury, which may be a benefit for DKD therapeutic targets.

In conclusion, single-cell transcriptomics analysis of kidney samples in db/db and SLT2i-treated db/db mice reveals five distinct macrophage subclusters and seven T cell subclusters. Macrophage accumulation is the driving factor for the inflammation of diabetic kidney disease. The antimicrobial Ccl3+ macrophage suggests the potential

targets on SGLT2i treatment of its anti-inflammatory function, possibly by fueling glycogenesis signaling pathway in macrophage. Our work identifies essential factors underlying the pathophysiology of diabetic kidney disease progression and points to potential new therapeutic targets.

LIMITATIONS OF STUDY

Due to the limited numbers of other types of kidney cells in the reported studies, we mainly clarified the macrophage and T subclusters and cross-talk between tubular cells, macrophages, and T cells. Further studies are required to delineate the mechanism by which SGLT2i promotes anti-inflammatory activity by altering the metabolic pathway of macrophages in our future study. Also, our study did not verify the detailed mechanism by which the signaling axis between tubule epithelial cells and immune cells and molecules targeting the signaling axis may be applied to the db/db mice to validate its potential clinical application value. Besides, we need to demonstrate the complementary roles of kidney resident macrophages and monocyte-derived infiltrated macrophages in the pathogenesis of DKD.

Acknowledgements

None.

Author Contributions

QH, X-MC, and G-YC supervised the project; Y-FB and KC designed and carried out most of the experiments; JH carried out the statistics. W-JS and RL provided reagents and suggestions; J-NL; Y-FB and QH analyzed the data and wrote the paper. All authors discussed the results and commented on the manuscript. All authors contributed to the article and approved the submitted version.

Source of Funding

This work was supported by the National Natural Science Foundation of China (Grant No. 32141005, 82070741, 82270758, 82200797).

Informed Consent

Not applicable.

Ethical Approval

Not applicable.

Conflicts of Interest

There is no conflict of interest among the authors.

Data Availability Statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

REFERENCES

1. Levey AS, Coresh J. Chronic kidney disease. Lancet 2012;379:165-180.
2. Oshima M, Shimizu M, Yamanouchi M, Toyama T, Hara A, Furuichi K, *et al*. Trajectories of kidney function in diabetes: a clinicopathological update. Nat Rev Nephrol 2021;17:740–750.
3. Tang SCW, Yiu WH. Innate immunity in diabetic kidney disease. Nat Rev Nephrol 2020;16:206–222.
4. Nguyen D, Ping F, Mu W, Hill P, Atkins RC, Chadban SJ. Macrophage accumulation in human progressive diabetic nephropathy. Nephrology (Carlton) 2006;11:226–231.
5. Moon JY, Jeong KH, Lee TW, Ihm CG, Lim SJ, Lee SH. Aberrant recruitment and activation of T cells in diabetic nephropathy. Am J Nephrol 2012;35:164–174.
6. Merovci A, Solis-Herrera C, Daniele G, Eldor R, Fiorentino TV, Tripathy D, *et al*. Dapagliflozin improves muscle insulin sensitivity but enhances endogenous glucose production. J Clin Invest 2014;124:509–514.
7. DeFronzo RA, Hompesch M, Kasichayanula S, Liu X, Hong Y, Pfister M, *et al*. Characterization of renal glucose reabsorption in response to dapagliflozin in healthy subjects and subjects with type 2 diabetes. Diabetes Care 2013;36:3169–3176.
8. Merovci A, Mari A, Solis-Herrera C, Xiong J, Daniele G, Chavez-Velazquez A, *et al*. Dapagliflozin lowers plasma glucose concentration and improves β-cell function. J Clin Endocrinol Metab 2015;100:1927–1932.
9. Terami N, Ogawa D, Tachibana H, Hatanaka T, Wada J, Nakatsuka A, *et al*. Long-term treatment with the sodium glucose cotransporter 2 inhibitor, dapagliflozin, ameliorates glucose homeostasis and diabetic nephropathy in db/db mice. PLoS One 2014;9:e100777.
10. Panchapakesan U, Pegg K, Gross S, Komala MG, Mudaliar H, Forbes J, *et al*. Effects of SGLT2 inhibition in human kidney proximal tubular cells--renoprotection in diabetic nephropathy?PLoS One 2013;8:e54442.
11. Fu J, Akat KM, Sun Z, Zhang W, Schlondorff D, Liu Z, *et al*. Single-Cell RNA Profiling of Glomerular Cells Shows Dynamic Changes in Experimental Diabetic Kidney Disease. J Am Soc Nephrol 2019;30:533–545.
12. Wilson PC, Wu H, Kirita Y, Uchimura K, Ledru N, Rennke HG, *et al*. The single-cell transcriptomic landscape of early human diabetic nephropathy. Proc Natl Acad Sci U S A 2019;116:19619–19625.
13. Wu J, Sun Z, Yang S, Fu J, Fan Y, Wang N, *et al*. Kidney single-cell transcriptome profile reveals distinct response of proximal tubule cells to SGLT2i and ARB treatment in diabetic mice. Mol Ther 2022;30:1741–1753.
14. Wu H, Gonzalez Villalobos R, Yao X, Reilly D, Chen T, Rankin M, *et al*. Mapping the single-cell transcriptomic response of murine diabetic kidney disease to therapies. Cell Metab 2022;34:1064–1078.
15. Zhang M, Wu L, Deng Y, Peng F, Wang T, Zhao Y, *et al*. Single Cell Dissection of Epithelial-Immune Cellular Interplay in Acute Kidney Injury Microenvironment. Front Immunol 2022;13:857025.
16. Bai Y, Li P, Liu J, Zhang L, Cui S, Wei C, *et al*. Renal primary cilia lengthen in the progression of diabetic kidney disease. Front Endocrinol (Lausanne) 2022;13:984452.
17. Foell D, Roth J. Proinflammatory S100 proteins in arthritis and autoimmune disease. Arthritis Rheum 2004;50:3762–3771.
18. Goyette J, Geczy CL. Inflammation-associated S100 proteins: new mechanisms that regulate function. Amino Acids 2011;41:821–842.
19. Odink K, Cerletti N, Brüggen J, Clerc RG, Tarcsay L, Zwadlo G, *et al*. Two calcium-binding proteins in infiltrate macrophages of rheumatoid

arthritis. Nature 1987;330:80–82.
20. Wang S, Song R, Wang Z, Jing Z, Wang S, Ma J. S100A8/A9 in inflammation. Front Immunol 2018;9:1298.
21. Zhao J, Endoh I, Hsu K, Tedla N, Endoh Y, Geczy CL. S100A8 modulates mast cell function and suppresses eosinophil migration in acute asthma. Antioxid Redox Signal 2011;14:1589–1600.
22. Certo M, Tsai CH, Pucino V, Ho PC, Mauro C. Lactate modulation of immune responses in inflammatory versus tumour microenvironments. Nat Rev Immunol 2021;21:151–161.
23. Howie D, Nolan KF, Daley S, Butterfield E, Adams E, Garcia-Rueda H, *et al*. MS4A4B is a GITR-associated membrane adapter, expressed by regulatory T cells, which modulates T cell activation. J Immunol 2009;183:4197–4204.
24. Wen T, Barham W, Li Y, Zhang H, Gicobi JK, Hirdler JB, *et al*. NKG7 Is a T-cell-Intrinsic Therapeutic Target for Improving Antitumor Cytotoxicity and Cancer Immunotherapy. Cancer Immunol Res 2022;10:162–181.
25. Ohtani N, Ohtani H, Nakayama T, Naganuma H, Sato E, Imai T, *et al*. Infiltration of CD8+ T cells containing RANTES/CCL5+ cytoplasmic granules in actively inflammatory lesions of human chronic gastritis. Lab Invest 2004;84:368–375.
26. Nguyen AD, Nguyen TA, Martens LH, Mitic LL, Farese RV Jr. Progranulin: at the interface of neurodegenerative and metabolic diseases. Trends Endocrinol Metab 2013;24:597–606.
27. Tang W, Lu Y, Tian QY, Zhang Y, Guo FJ, Liu GY, *et al*. The growth factor progranulin binds to TNF receptors and is therapeutic against inflammatory arthritis in mice. Science 2011;332:478–484.
28. Quaranta V, Rainer C, Nielsen SR, Raymant ML, Ahmed MS, Engle DD, *et al*. Macrophage-Derived Granulin Drives Resistance to Immune Checkpoint Inhibition in Metastatic Pancreatic Cancer. Cancer Res 2018;78:4253–4269.
29. Nielsen SR, Quaranta V, Linford A, Emeagi P, Rainer C, Santos A, *et al*. Macrophage-secreted granulin supports pancreatic cancer metastasis by inducing liver fibrosis. Nat Cell Biol 2016;18:549–560.
30. Cheung PF, Yang J, Fang R, Borgers A, Krengel K, Stoffel A, *et al*. Progranulin mediates immune evasion of pancreatic ductal adenocarcinoma through regulation of MHCI expression. Nat Commun 2022;13:156.
31. Zhou D, Zhou M, Wang Z, Fu Y, Jia M, Wang X, *et al*. PGRN acts as a novel regulator of mitochondrial homeostasis by facilitating mitophagy and mitochondrial biogenesis to prevent podocyte injury in diabetic nephropathy. Cell Death Dis 2019;10:524.
32. Bruchez A, Sha K, Johnson J, Chen L, Stefani C, McConnell H, *et al*. MHC class II transactivator CIITA induces cell resistance to Ebola virus and SARS-like coronaviruses. Science 2020;370:241–247.
33. Strubin M, Berte C, Mach B. Alternative splicing and alternative initiation of translation explain the four forms of the Ia antigen-associated invariant chain. EMBO J 1986;5:3483–3488.
34. Shahbazian H, Rezaii I. Diabetic kidney disease; review of the current knowledge. J Renal Inj Prev 2013;2:73-80.
35. Sato Y, Yanagita M. Immunology of the ageing kidney. Nat Rev Nephrol 2019;15:625–640.

How to cite this article: Bai Y, Hong Q, *et al*. Identification of functional heterogeneity of immune cells and tubular-immune cellular interplay action in diabetic kidney disease. J Transl Intern Med 2024; 12: 395-405.