The American Journal of Pathology, Vol. 195, No. 1, January 2025

ELSEVIER

The American Journal of PATHOLOGY

ajp.amjpathol.org

Advances in Understanding Renal Diseases with Single-Cell Sequencing Theme Issue

REVIEW

Single-Cell Analysis Provides New Insights into the Roles of Tertiary Lymphoid Structures and Immune Cell Infiltration in Kidney Injury and Chronic Kidney Disease

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Accepted for publication July 2, 2024.

Chronic kidney disease (CKD) is a global health concern with high morbidity and mortality. Acute kidney injury (AKI) is a pivotal risk factor for the progression of CKD, and the rate of AKI-to-CKD progression increases with aging. Intrarenal inflammation is a fundamental mechanism underlying AKI-to-CKD progression. Tertiary lymphoid structures (TLSs), ectopic lymphoid aggregates formed in nonlymphoid organs, develop in aged injured kidneys, but not in young kidneys, with prolonged inflammation and maladaptive repair, which potentially exacerbates AKI-to-CKD progression in aged individuals. Dysregulated immune responses are involved in the pathogenesis of various kidney diseases, such as IgA nephropathy, lupus nephritis, and diabetic kidney diseases, thereby deteriorating kidney function. TLSs also develop in several kidney diseases, including transplanted kidneys and renal cell carcinoma. However, the precise immunologic mechanisms driving AKI-to-CKD progression and development of these kidney diseases remain unclear, which hinders the development of novel therapeutic approaches. This review aims to describe recent findings from single-cell analysis of cellular heterogeneity and complex interactions among immune and renal parenchymal cells, which potentially contribute to the pathogenesis of AKI-to-CKD progression and other kidney diseases, highlighting the mechanisms of formation and pathogenic roles of TLSs in aged injured kidneys. *(Am J Pathol 2025, 195: 40–54; https://doi.org/10.1016/j.ajpath.2024.07.008)*

Chronic kidney disease (CKD), defined as a reduction in kidney function or the presence of pathologic albuminuria,[1] is a global health concern, which has affected 9.1% of the global population and caused 1.2 million deaths in 2017.[2] CKD is a potent risk factor of cardiovascular diseases, with high mortality rates.[1,3] Acute kidney injury (AKI), a pathologic condition that causes a rapid decline in kidney function, is a pivotal risk factor for the development of CKD and escalates end-stage kidney disease, the most advanced stage of CKD.[3,4] Patients with end-stage kidney disease require kidney transplantation or dialysis for survival, which increases the socioeconomic burden; therefore, preventing AKI-to-CKD progression is crucial.[4] However, limited understanding of the underlying mechanisms of this progression hinders the development of effective therapeutic strategies.[5]

Intrarenal inflammation plays a crucial role in normal and maladaptive repair following AKI.[6–8] Complex interactions

Supported by the Japan Agency for Medical Research and Development grants 23ek0310020, 23gm1210009, 23zf0127003, and 21lm0203006; the Japan Society for the Promotion of Science KAKENHI Grant-in-Aid for Scientific Research B 20H03697; and the World Premier International Research Center Initiative, the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan (M.Y.); and by the Japan Society for the Promotion of Science KAKENHI Grant-in-Aid for Early-Career Scientists 24K19147 (T.Y.).

This article is part of a review series on recent advances in understanding renal diseases with single-cell sequencing.

https://doi.org/10.1016/j.ajpath.2024.07.008

among resident immune cells, infiltrating immune cells, and parenchymal cells within the renal microenvironment are fundamental to the AKI-to-CKD progression. Moreover, dysregulated immune responses are also implicated in the etiology of various kidney diseases, including IgA nephropathy (IgAN),[9,10] lupus nephritis (LN),[11] diabetic kidney disease (DKD),[12] and graft rejection.[13,14]

Tertiary lymphoid structures (TLSs), also known as tertiary lymphoid tissues, tertiary lymphoid organs, or ectopic lymphoid organs, are ectopic lymphoid aggregates that develop in nonlymphoid organs with chronic inflammation during autoimmune diseases,[15] infection,[16] age-dependent inflammation,[17,18] and cancer.[19–21] Notably, TLSs develop in the chronic phase after AKI in aged kidneys, but not in young kidneys, and potentially exacerbate tissue damage by promoting inflammation, leading to AKI-to-CKD progression.[17,21–23] Furthermore, TLSs are formed in various common kidney diseases, including IgAN,[24] LN,[25–27] transplanted kidneys,[13,28–32] and renal cell carcinoma (RCC).[33–35] Consequently, therapies targeting TLSs may offer a promising avenue for treating various kidney diseases.

Recent advancements in single-cell techniques have provided innovative insights into nephrology because of their comprehensive analytical capabilities.[36–38] Traditional investigations of immune cells in kidney diseases have been limited by the focus on specific cell types and methods, such as flow cytometry and immunostaining of specific cellular markers. Additionally, bulk RNA sequencing (RNA-seq) by averaging the expression profiles across diverse cell types inevitably dilutes the gene expression of immune cells that are often less numerous than renal parenchymal cells, such as tubular cells, resulting in difficulty in capturing rare cell types and their gene expression at low levels. In contrast, the advent of single-cell technologies has facilitated in-depth analysis of immunologic dynamics of various biological entities, including blood cells, secondary lymphoid organs, and nonlymphoid organs, including kidneys, by enabling precise phenotypic characterization of individual immune cells.[11,39,40]

This review focuses on the latest immunologic findings from single-cell technologies in healthy and diseased kidneys of mice and humans, describing the heterogeneous phenotypes of immune cells, renal parenchymal cells, and their interactions that play pivotal roles in AKI-to-CKD progression and several other kidney diseases. Additionally, recent discoveries regarding the cellular and molecular mechanisms of TLS formation and its pathologic roles in aged injured kidneys and other kidney diseases are highlighted.

Single-Cell and Single-Nucleus RNA-Seq for Investigating the Immune Cells

Single-cell RNA-seq (scRNA-seq) and single-nucleus RNA-seq (snRNA-seq) are popular methods for single-cell transcriptomics in nephrology, each exhibiting distinct advantages and limitations.[41,42] Dissociation of the kidney is challenging because of its complex microsegments and a large number of cell types. The extent of the adhesive interaction among cells and their microenvironment may lead to differential retention or loss of specific cell populations after tissue dissociation.[41] scRNA-seq is sensitive in detecting gene expression of immune cells.[41,43,44] Conversely, snRNA-seq is advantageous in capturing fragile cell types, such as podocytes and fibroblasts, and in attenuating the bias caused by up-regulation of stress-response genes, such as heat shock proteins and apoptosis-related genes,[41] because the nucleus can be mechanically dissociated on ice without the necessity of warm enzyme-mediated tissue dissociation used in scRNA-seq.[42] Despite these differences, scRNA-seq and snRNA-seq have similar detection capabilities of the majority of gene expression.[42] These technologies are complementary in several respects, underlining the importance of strategic applications based on the cell types of interest.[23,43]

Single-Cell Analysis of Resident Immune Cells in the Healthy Kidney

Resident immune cells exist in all organs and are crucial for maintaining tissue homeostasis by interacting with tissue parenchymal cells.[39] In healthy kidneys, these immune cells predominantly occupy the interstitial spaces between epithelial cells, endothelial cells, and fibroblasts, and respond to various insults, including kidney injury and infection, to protect kidney function and homeostasis.[40] Understanding the composition and function of resident immune cells in healthy kidneys is critical for elucidating the pathophysiology of immune-mediated kidney diseases. The first large-scale droplet-based scRNA-seq of murine kidneys classified 43,745 cells into 16 clusters, including immune cells, such as macrophages, neutrophils, B cells, T cells, and natural killer (NK) cells.[45] In another study, single-cell assay for transposase-accessible chromatin with high-throughput sequencing demonstrated the chromatin accessibility landscapes and heterogeneity of resident immune cells in murine kidneys.[46] However, investigations on the resident immune cells in the human kidney have been partially hindered by limited access to fresh and healthy samples and relatively low abundance of immune cells. Advances in single-cell analysis technologies have the potential to overcome these limitations. Mapping of the landscape of healthy human kidneys through scRNA-seq analysis of 19 living donor biopsy samples revealed that immune cells constitute $<1\%$ of all cells.[47] Subsequently, scRNA-seq of $CD45^+$ immune cells enriched by flow cytometry identified unexpectedly diverse subpopulations of myeloid cells and lymphocytes. Unlike $CD68^+$ cells in discarded deceased donor kidneys and specimens of tumor nephrectomy,[48–50] the enrichment of resident macrophages

expressing genes associated with alternatively activated macrophages was uniquely identified in the kidneys of healthy living donors, suggesting that altered kidney environments affect myeloid populations.[47] Additionally, by comparing with the publicly available data set on scRNA-seq of peripheral blood mononuclear cells, this study highlighted the elevated expression of chemokine receptors, such as *CXCR6* and very late antigen-4 (VLA-4) integrin components, in T cells within healthy kidneys, potentially underlining their tissue residency based on *CXCL16* expression by renal myeloid cells and the expression of fibronectin and vascular cell adhesion molecule 1 (VCAM1) in the kidney. Furthermore, NK cells expressing amphiregulin more abundantly than that by their circulating counterparts have been identified in healthy kidneys, suggesting their role in tissue repair.[51] Comparative analyses using single-cell technologies facilitate the identification of cellular heterogeneity and state changes attributable to environmental differences (Figure 1A). This large-scale immune cell atlas of steady-state and healthy kidneys will serve as a relevant reference for future studies on immune-mediated kidney diseases.[47]

Single-cell analysis demonstrates how immune cell populations change in developing kidneys.[48] scRNA-seq of human fetal kidney obtained at 7 to 16 post-conceptional weeks demonstrated that a macrophage subpopulation that dominated the resident immune cells at 7 to 10 post-conceptional weeks showed transcriptional profiles similar to that of murine kidney F4/80^{high} yolk sac–derived macrophages,[52] indicating that macrophage populations in the human kidney are also established from erythromyeloid precursors in the fetal yolk sac during early embryonic stages. From 9 weeks post-conception, a remarkable increase was noticed in different types of immune cells, such as dendritic, T, and NK cells, and B cells appear at late stages from 12 weeks after conception (Figure 1B), mirroring the development of the fetal thymus and spleen. Further investigations into the composition of immune cells of the adult human kidney coupled with ligand-receptor analysis indicated enrichment of neutrophils and antimicrobial mononuclear phagocytes in the renal pelvis, based on the elevated expression of various chemokines of pelvic epithelial cells, which was confirmed by histologic analyses. Such a highly organized pattern of immune cell localization was absent in fetal kidneys because of the minimal expression of neutrophil-recruiting chemokines in pelvic epithelial cells. These results suggest that immune zonation develops to combat bacterial infection in mature kidneys.[48]

Resident macrophages play a pivotal role in regulating homeostasis and disease progression in the kidney. Despite the identification of several well-defined markers in murine models,[53] the lack of conserved markers across different species has hindered their translatability. Zimmerman et al[49] revealed the gene expression profiles of innate immune cells in the kidney using scRNA-seq across several species, such as mice, rats, pigs, and humans, and identified two

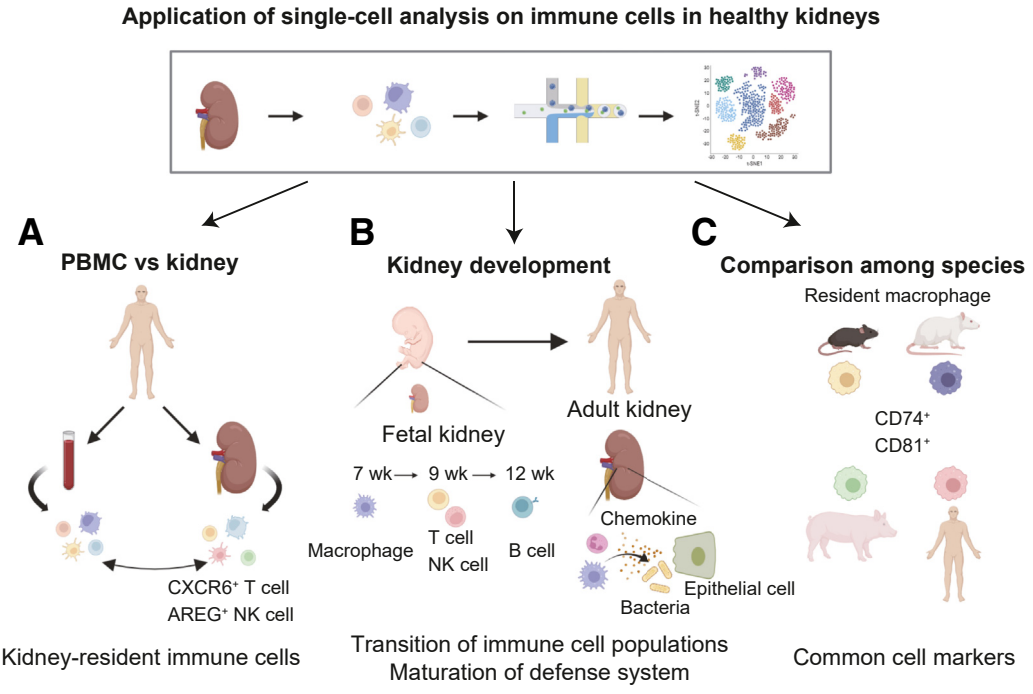

Figure 1 Application of single-cell analysis on immune cells in the healthy kidney. **A:** Comparison study of peripheral blood mononuclear cells (PBMCs) and renal immune cells identified kidney-resident immune cells with tissue-specific phenotypes. **B:** Temporal investigation on fetal kidneys demonstrated transition of resident immune cell populations during kidney development. Furthermore, comparison between fetal and adult kidneys revealed the mechanism for the maturation of defense systems against pathogens in the urinary tract. **C:** Comparison study of immune cells among different species identified common cell markers for resident macrophages in kidneys. Created with BioRender.com (Toronto, ON, Canada). AREG, amphiregulin; CXCR6, CXC motif chemokine receptor 6; NK, natural killer.

conserved markers of resident macrophages, CD74 and CD81, which were validated by comparative flow cytometry analysis. This study indicates that single-cell technologies can pave the way for cross-species translational research (Figure 1C).

Single-Cell Analysis of Immune Cells Involved in Kidney Injury and Regeneration

Intrarenal inflammatory responses are triggered by cellular injury and subsequent cell death, which is pivotal in the pathophysiological processes underlying AKI.[3,6,7] The inflammatory response involves infiltration and proliferation of various immune cell types and interactions between immune and parenchymal cells within injured kidneys. Immune cells contribute to the repair processes by eliminating dead cells and damage-associated molecular patterns.[3,7,8] Concurrently, they play a crucial role in exacerbating tubular damage and interstitial fibrosis by sustaining an inflammatory environment, thereby significantly affecting AKI-to-CKD progression. However, the heterogeneity of immune cells, their activation mechanisms, and cell-cell interactions involved in AKI-to-CKD progression remain incompletely understood. To address these unresolved issues, single-cell analyses of injured kidney tissues were performed.[54–57] Kirita et al[54] performed snRNA-seq on healthy and temporally sampled kidneys following ischemia-reperfusion injury (IRI), an established AKI model, of young adult mice, and constructed a single-cell atlas of AKI-to-CKD progression. In this data set, 26 distinct cell clusters were identified, including epithelial and endothelial cells, fibroblasts, and immune cells, such as macrophages, dendritic cells, and T and B cells. Notably, the population of VCAM1$^+$ failed-repair proximal tubular (PT) cells, which was characterized by profibrotic and proinflammatory signatures, increased 1 week after IRI and persisted thereafter. A ligand-receptor analysis to examine leukocyte chemotactic signaling across all cell types revealed strong signaling in fibroblasts and endothelial cells in the acute phase after IRI, which continuously increased over time until 6 weeks after IRI. In contrast, in the chronic phase after IRI, injured or failed-repair PT cells significantly increased leukocyte chemotactic signaling pronounced by the upregulation of *Ccl2-Ccr2* signaling, which contributes to the recruitment of monocytes and T cells, thereby highlighting the differences in cell types that promote intrarenal inflammation depending on the phase after kidney injury. In this study, the limited sensitivity of snRNA-seq in detecting gene expression in immune cells was an obstacle in elucidating the complexity of immune cell populations and phenotypes.[41,42] scRNA-seq combined with bulk RNA-seq and flow cytometry of kidneys 3 and 14 days after short-duration IRI (a model of adaptive response) and long-duration IRI (a model of maladaptive response leading to fibrosis) indicated that a diverse spectrum of immune cells, including T cells, macrophages, neutrophils, basophils, eosinophils, mast cells, and dendritic cells, increased at 14 days after IRI, particularly in maladaptive kidneys.[55] Furthermore, a maladaptive PT subcluster was identified in fibrotic kidneys after long-duration IRI, in addition to the previously reported *Vcam1*$^+$*Havcr1* [encoding kidney injury molecule-1 (KIM1), a well-known PT injury marker]$^+$ injured PT subclusters.[54,58] Maladaptive PT cells were characterized by the elevated expression of various cytokines and chemokines (*Il1b*, *Cxcl2*, and *Ccl3*) and complement 3. Furthermore, receptors for these proinflammatory mediators were expressed in both myeloid and maladaptive PT cells, suggesting a pivotal role for these cells in mediating inflammatory responses through interactions with myeloid cells and autocrine signaling. Novel insights into the role of basophils in renal fibrosis were provided using scRNA-seq of fibrotic kidneys after unilateral ureteral obstruction (a model of renal fibrosis) in mice.[56] Several profibrotic and proinflammatory PT cells expressing *Pdgfb*, *Cd74*, *Tnfrsf12a*, *Il34*, *Cxcl1*, *Cxcl10*, and *Cxcl16* were identified, which were predicted to recruit myeloid cells and lymphocytes by ligand-receptor analysis. Interestingly, unexpected interactions between profibrotic PT cells and basophils via *Cxcl1-Cxcr2* were predicted. Furthermore, basophils were demonstrated to express high levels of *Il6* in response to IL-18 and IL-33, which were mainly expressed in stromal cells, and type 17 helper T cells expressed genes encoding IL-6 receptors (*Il6st* and *Il6ra*), *Il17a*, and *Tgfb1*, suggesting strong interactions among stromal cells, basophils, and type 17 helper T cells. Notably, basophil depletion or blockade of IL-6 receptor attenuated renal fibrosis, suggesting their contribution to renal fibrosis in kidneys with unilateral ureteral obstruction. An increasing number of basophils and an obvious correlation between their number and renal fibrosis were also identified in kidney samples from patients with CKD, indicating a crucial contribution of basophils to renal fibrosis in humans.[56] Notably, the above-mentioned studies demonstrated that injured PT cells with various proinflammatory phenotypes serve as inflammatory hubs by orchestrating interactions between immune and parenchymal cells, thereby driving chronic inflammation and maladaptive repair (Figure 2A). Xu et al[57] conducted scRNA-seq on kidney samples after unilateral IRI (U-IRI; a model to induce renal atrophy) and kidney samples subjected to U-IRI with the contralateral nephrectomy (a model to induce adaptive repair) to understand the pathogenesis of tubular atrophy during AKI-to-CKD progression. Although macrophages and dendritic cells were the predominant immune cell types on day 7 after IRI in both models, kidneys after U-IRI exhibited a marked increase in macrophages, infiltrating neutrophils, and T cells between days 7 and 14 after injury, despite the plateauing number of these cells in the U-IRI with the contralateral nephrectomy model. Between 7 and 14 days after injury, renal macrophages were characterized by the upregulation of various chemokines, such as *Ccl2*, *Ccl7*, *Ccl8*, *Ccl12*, and *Cxcl16*. A ligand-receptor analysis predicted that *Ccl2*, *Ccl7*, and *Ccl12* may mediate chemotaxis of *Ccr2*$^+$ macrophages. Similarly, *Ccl8* and *Cxcl16* probably attracted

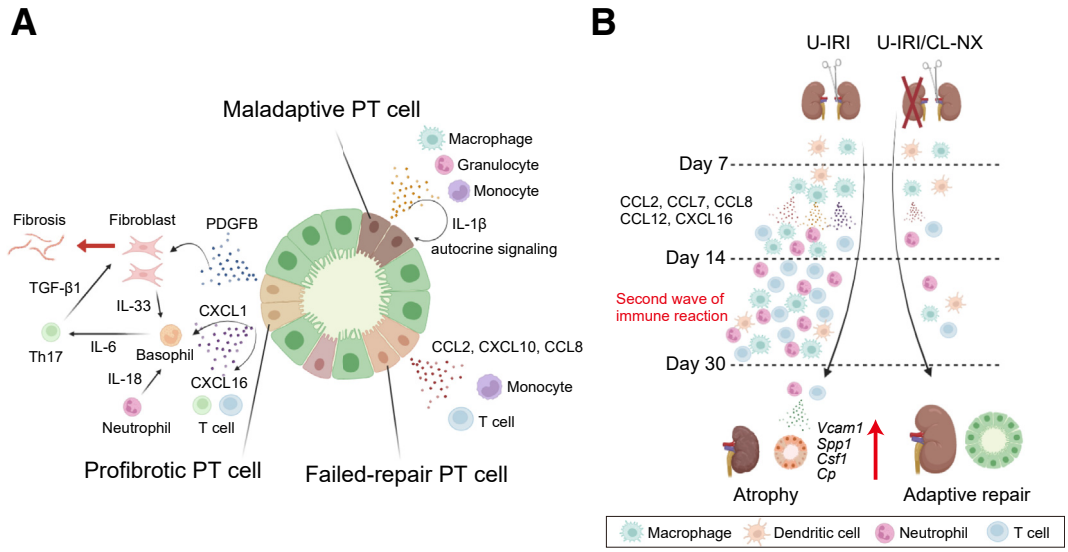

Figure 2 Immunologic mechanisms of acute kidney injury (AKI)—to—-chronic kidney disease (CKD) progression. **A:** Heterogeneous injured proximal tubular (PT) cells exacerbate inflammation and fibrosis in the kidney, potentially aggravating AKI-to-CKD progression. They recruit a wide range of immune cells and promote their cell-cell interactions by secreting various chemokines and cytokines. **B:** Macrophages producing excessive chemokines significantly increase in the kidney between 7 to 14 days after unilateral ischemia-reperfusion injury (U-IRI), compared with those after U-IRI with the contralateral nephrectomy (U-IRI/CL-NX). The increased macrophages recruit neutrophils and T cells after 14 days postinjury (second wave of immune reaction), which potentially damage tubular cells by cytokine productions, leading to renal atrophy. Created with BioRender.com (Toronto, ON, Canada). CCL, chemokine (C-C motif) ligand; PDGFB, platelet-derived growth factor B; TGF-β1, transforming growth factor-β1; Th17, type 17 helper T cell.

Ccr1^{+} neutrophils and *Cxcr6*$^{+}$ T cells, respectively. These results suggest that macrophages play a central role in recruiting more macrophages and the second wave of infiltrating neutrophils and T cells after 14 days post-U-IRI.[57] Neutrophils recruited to the kidney after U-IRI up-regulated the expression of numerous inflammatory genes, and T cells showed elevated expression of genes associated with their activation. Additionally, ligand-receptor-target analysis for mapping ligands secreted by effector cells to their receptors and corresponding targets suggested a potential mechanism wherein ligands released by neutrophils and T cells induce the expression of injury markers, such as *Vcam1*, *Spp1*, *Csf1*, and *Cp*, in PT cells. These data were experimentally validated by the finding that combined depletion of neutrophils and T cells at 5 days after IRI markedly ameliorated PT atrophy at 30 days after IRI, suggesting that a second wave of immune reaction from 14 days after IRI significantly promoted the loss of nephron mass (Figure 2B). Furthermore, analyses of 10 biopsy samples of kidneys from patients diagnosed with AKI demonstrated a negative correlation between the number of infiltrating T cells or neutrophils and recovery of estimated glomerular filtration rate after AKI, indicating the potential of these immune cells as therapeutic targets for AKI.[57] These studies, using scRNA-seq and snRNA-seq, revealed the unprecedented heterogeneous populations in injured PT cells and the phenotypes of various immune cells and predicted a range of cell-cell interactions between them that have not been revealed by conventional methods; however, information on their localization in injured kidneys could not be provided. Recently, Lake et al[59] combined large-scale scRNA-seq and snRNA-seq data with spatial transcriptomics to analyze healthy and injured kidneys of individuals with AKI or CKD and identified different types of cellular neighborhoods with cycling, transitioning, adaptive (successful or maladaptive repair), or degenerative states. Furthermore, multiplexed immunofluorescence imaging revealed distinct neighborhoods predominantly occupied by myeloid or T cells. Immune composition within these neighborhoods was associated with distinct epithelial responses to injury. Neighborhoods rich in myeloid cells were linked to adaptive epithelial states, and T-cell—dominant areas correlated with epithelial cells in degenerative states and fibrosis. This study indicates that spatial technologies can provide detailed and accurate insights into how immune cells influence tubular injury, repair, and fibrosis through interactions among parenchymal cells in AKI-to-CKD progression.

Single-Cell Analysis of TLSs

TLSs are ectopic lymphoid aggregates, which develop in nonlymphoid organs during chronic inflammatory conditions, such as infections,[16] autoimmune diseases,[15] age-associated inflammation,[17,18,21–23] and cancers.[19–21] TLSs are characterized by lymphoid aggregates predominantly composed of T and B cells along with fibroblast networks that serve specific functions by producing homeostatic chemokines recruiting B and T cells, such as CXCL13 and chemokine (C-C motif) ligand 19 (CCL19) (Figure 3).[17,21,60] TLSs are similar to secondary lymphoid organs in terms of components and functions. However, they are distinct in their capability of directly contacting adjacent microenvironments

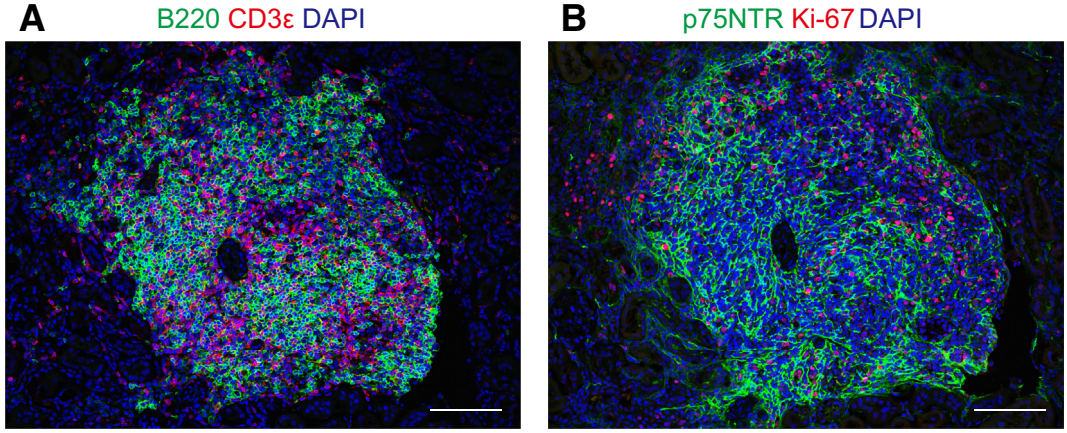

Figure 3 Tertiary lymphoid structures (TLSs) in injured kidneys in aged mice. **A:** An immunofluorescence image staining for CD3ε and B220 shows a TLS that is composed of T- and B-cell aggregates in the aged injured kidney 45 days after 30-minute ischemia-reperfusion injury. **B:** An immunofluorescence image staining for p75 neurotrophin receptor (p75NTR) and Ki-67 shows fibroblasts and proliferating immune cells within the TLS. Scale bar = 100 μm (**A** and **B**).

because of their nonencapsulated nature.[17,23,61] Their pathogenicity is context dependent. For instance, in infectious diseases and malignancies, TLSs exert beneficial effects by activating immune responses to eliminate pathogens and tumor cells.[19] Conversely, in autoimmune diseases, TLSs facilitate the activation of autoreactive immune cells, which is detrimental to the host.[15] TLSs were identified in several kidney diseases in humans, including IgAN,[24] LN,[25,26] anti-neutrophil cytoplasmic antibody–associated glomerulonephritis,[62,63] transplanted kidneys,[28,30,31] pyelonephritis,[16] and RCC,[19,32–35] which lead to CKD. Furthermore, TLSs were found to be associated with disease severity or poor renal prognosis in patients with IgAN,[24] lupus nephritis,[25] and transplanted kidneys.[28,30] This section describes recent cellular and molecular findings obtained by single-cell analysis of TLSs in aged injured kidneys, transplanted kidneys, and RCC. Additionally, a brief overview of single-cell studies related to TLSs in organs other than kidneys is provided. Other review articles can be referred to for additional studies on single-cell analyses of transplanted kidneys and RCC.[40,64]

TLS Formation in AKI-to-CKD Progression in Aged Mice and Humans

AKI is an important risk factor for the progression of CKD, particularly in aged individuals.[4,65] TLSs develop and expand from approximately 2 weeks after AKI in aged mice with chronic inflammation and maladaptive repair but not in young injured kidneys, suggesting that TLSs can potentially contribute to AKI-to-CKD progression.[17] Additionally, TLSs were exclusively identified in approximately 30% to 50% of nontumorous kidney tissues from patients aged >60 years with RCCs but not in those aged <40 years.[16,17] Age-dependent TLS formation extends to other organs, including the liver and bladder, and may contribute to age-related chronic inflammation.[18,66] Renal TLSs expand and mature over time, and their maturation steps are common between mice and humans, indicating that TLS formation is a conserved phenomenon across species.[16] To evaluate the clinical relevance of TLSs, staging of TLS maturation was conducted as follows: a stage I TLS is immature aggregates of proliferating T and B cells with chemokine-producing fibroblasts, a stage II TLS contains $CD21^+$ follicular dendritic cells, which promote B-cell maturation and proliferation, and a stage III TLS develops germinal center response at the site of follicular dendritic cells. On the basis of this staging strategy, elderly patients with CKD exhibit a higher prevalence of advanced-stage TLSs compared with those without CKD, indicating that a TLS potentially serves as a potential marker for the severity of kidney injury. The detrimental effects of such age-dependent TLSs on renal repair were supported by the fact that therapeutic interventions, such as administration of dexamethasone or anti-CD4 monoclonal antibodies (clone GK1.5), significantly reduced the size and prevalence of TLSs even after they have developed, thereby ameliorating tubular injury, interstitial fibrosis, and the decline of kidney function in animal models.[16,17] Although the specific mechanisms underlying the pathogenic effects of TLSs on the kidney have not been fully elucidated, previous reports have suggested that autoantibodies and proinflammatory cytokines produced within TLSs might aggravate inflammation and damage kidney tissues.[23,27,67] Further elucidation of the mechanisms is necessary for the development of new therapeutic approaches for TLSs in the kidney.

A comprehensive approach using single-cell analysis provided novel insights into the pathophysiological mechanisms of age-dependent TLSs in kidneys.[22,23] By applying scRNA-seq to $CD45^+$ immune cells derived from aged injured kidneys with TLSs, we identified unique lymphocyte populations and their interactions as pivotal players in TLS formation.[22] Specifically, programmed cell death-1 $(PD\text{-}1)^+CD153^+CD4^+$ memory T cells, referred to as

senescence-associated T cells, increased with age and were characterized by impaired proliferation and abundant production of proinflammatory chemokines and cytokines in mice.[68,69] B cells that increase with age are known as age-associated B cells (ABCs) and contribute to autoimmune diseases through autoantibody production and effective antigen presentation.[70–72] scRNA-seq detected the presence of senescence-associated T cells and ABCs in aged injured kidneys with TLSs, and histologic analysis demonstrated their synchronized accumulation within TLSs during their expansion and maturation.[22] Furthermore, a ligand-receptor analysis predicted their cell-cell interactions via the *Tnfsf8* (encoding CD153)—*Tnfrsf8* (encoding CD30) signaling pathway. Both CD153- and CD30-deficient mice showed a marked decrease in functional senescence-associated T cells and ABCs, with notable inhibition of renal TLS expansion and maturation, thereby underlining the essential role of CD153-CD30—mediated interactions between senescence-associated T cells and ABCs during TLS development. Moreover, attenuation of tubular injury, interstitial fibrosis, and kidney dysfunction in CD153-deficient mice indicated that the CD153-CD30 signaling pathway may be a promising therapeutic target for ameliorating kidney injury with TLS formation.[22] Despite the interactions between immune cells that contribute to TLS development, the contribution of renal parenchymal cells to TLS development and pathogenic effects of TLSs on surrounding tissues were not fully elucidated. Using snRNA-seq coupled with histologic analysis, we identified KIM1^{+}VCAM1^{+} PT cells that preferentially localized adjacent to TLSs in aged injured kidneys.[23] VCAM1^{+} PT cells had gene expression profiles similar to those of failed-repair or late injured PT cells, as previously reported,[54,58] which expressed proinflammatory chemokines (*Ccl2*, *Cxcl10*, and *Cxcl16*) and profibrotic ligands (*Pdgfb*, *Pdgfd*, and *Tgfb2*), thereby underlining their contribution to chronic inflammation and renal fibrosis in the neighborhood of TLSs. Furthermore, lymphocytes within TLSs were suggested to directly contribute to maladaptive repair and promotion of the proinflammatory phenotypes of VCAM1^{+} PT cells surrounding TLSs by the production of tumor necrosis factor-α and interferon-γ (IFN-γ) (Figure 4A). Subsequently, profibrotic and proinflammatory fibroblast subpopulations localized around and within the TLSs, respectively, were identified.[23] Proinflammatory fibroblasts potentially contributed to the recruitment of IFN-γ—producing CXCR3^{+} T cells and survival and proliferation of B cells by producing CXCL9, CXCL10, and B-cell—activating factor. Moreover, the up-regulation of several IFN-stimulated transcription factors and their downstream targets in proinflammatory fibroblasts and the STAT1-dependent up-regulation of *Cxcl9*, *Cxcl10*, and *Tnfsf13b* (encoding B-cell—activating factor) by IFN-γ in cultured fibroblasts suggested that bidirectional interactions between proinflammatory fibroblasts and IFN-γ—producing CXCR3^{+} T cells amplify inflammation and

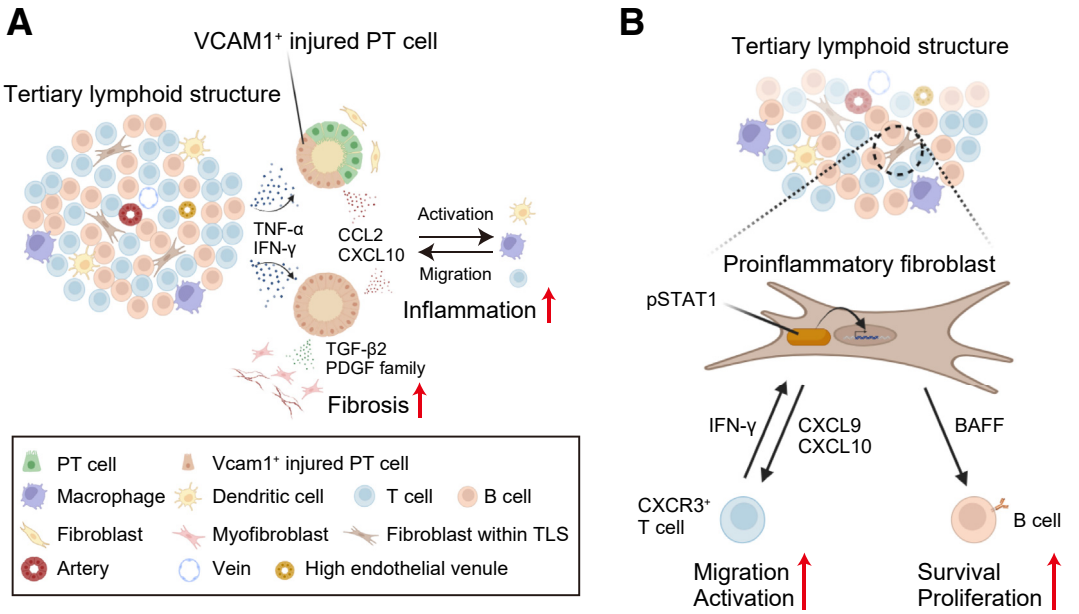

Figure 4 Cell-cell interactions between immune and proinflammatory parenchymal cells contribute to tertiary lymphoid structure (TLS) formation and pathogenicity in kidneys. **A:** Vascular cell adhesion molecule 1 (VCAM1)$^{+}$ injured proximal tubules (PTs) are preferentially localized adjacent to a TLS in aged injured kidneys and produce various chemokines and cytokines that recruit and activate immune cells and fibroblasts, promoting inflammation and fibrosis in the kidney tissue surrounding the TLS. Lymphocytes within the TLS highly express proinflammatory cytokines [tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ)], which have the potential to induce tubular maladaptive repair and promote the proinflammatory phenotypes in PT cells. **B:** Fibroblasts within TLSs produce CXCL9, CXCL10, and B-cell—activating factor (BAFF), which recruit and activate T and B cells, thereby contributing to TLS expansion. The proinflammatory fibroblasts enhance the production of these chemokines and cytokines in a STAT1-dependent manner by IFN-γ secreted by CXC motif chemokine receptor 3 (CXCR3)$^{+}$ T cells. Created with BioRender.com (Toronto, ON, Canada). CCL2, chemokine (C-C motif) ligand 2; PDGF, platelet-derived growth factor; pSTAT1, phosphorylated STAT1; TGF-β2, transforming growth factor- β2.

contribute to TLS expansion (Figure 4B). In summary, snRNA-seq reveals distinct proinflammatory profiles of parenchymal cells in aged kidneys with TLSs and their interactions with immune cells, thereby elucidating the mechanisms that promote inflammation and TLS expansion.[23]

TLSs in Transplanted Kidneys

B-cell infiltration, including TLSs, can develop in 10% to 59% in both rejected and nonrejected transplanted kidneys, sparking a debate regarding their influence on graft outcomes.[13,28] The presence of B-cell infiltrates, including TLSs, is associated with poor graft prognosis and resistance to glucocorticoids in cases of acute rejection.[73–75] Our study of protocol biopsy specimens without rejection revealed that TLS formation was identified in 46.9% (stage I, 43.2%; stage II, 3.65%) in a 1-month biopsy, and that stage II TLTs increased up to 18.9% in a 12-month biopsy. Moreover, the presence of stage II TLSs was associated with diminished graft function, independent of interstitial inflammation scores (Banff i-score).[28,29] In contrast, studies on a murine model of kidney transplantation identified the formation of regulatory T-cell–rich organized lymphoid structures, a variety of TLSs containing numerous $CD3^+$forkhead box P3 $(FoxP3)^+$ regulatory T cells, which is crucial for graft acceptance.[76,77] This notion was further supported by several human studies showing that graft acceptance was associated with the B-cell signature,[13] indicating a potential positive contribution of TLSs to transplanted kidneys. To explore the role of B cells in graft tolerance, scRNA-seq was performed on accepted renal grafts with T-cell–rich organized lymphoid structure development at serial time points for 6 months after transplantation and rejecting grafts a week after transplantation in a murine model.[78] They revealed a transition from T- to B-cell–rich immune microenvironments in accepted grafts from 1 week to 6 months after transplantation, highlighting distinct subpopulations of B cells. Crucially, markers indicative of regulatory B cells, such as *Cd5*, *Cd24a*, *Cd38*, *Cr2*, *Fcer2a*, *Il10*, and *Havcr1*, were up-regulated in B cells of accepted grafts unlike those in rejected grafts, thereby suggesting a pivotal role for regulatory B cells in fostering graft acceptance with T-cell–rich organized lymphoid structures in the chronic phase after transplantation.[78] However, the presence of T-cell–rich organized lymphoid structures in renal grafts of human has not yet been confirmed, and further studies are needed to determine the potential contributory role of B cells and TLSs in graft acceptance.

TLSs in Renal Cell Carcinomas

TLSs can develop in various cancers and are associated with improved prognosis and response to immunotherapy.[19,21] However, the drivers of TLS formation and contribution of TLSs to antitumor immunity have not been fully elucidated. Clear cell renal cell carcinoma (ccRCC) is the most common kidney cancer and a highly immune-infiltrated type of cancer.[79,80] Although immune checkpoint blockade improves the prognosis of patients with ccRCC, resistance to treatment remains common. Characterization of the microenvironment of immune cells is crucial for improving therapeutic approaches for ccRCC. Therefore, single-cell analyses were conducted focusing on immune cells in kidneys of patients with ccRCC to provide novel insights into their roles in tumor progression and antitumor immunity.[34,35,81–86] Tumor-infiltrating B cells, including TLSs, are often identified in the invasive margins of tumors and less often within tumors.[33,34] The cellular and molecular mechanisms underlying the antitumor function of TLSs in ccRCC were recently demonstrated using spatial transcriptomics and analyzing B-cell repertoire,[35] which showed that B-cell maturation and clonal expansion occurred within TLSs, and that the generated plasma cells disseminated from TLSs into the surrounding kidney tissues along CXCL12-producing fibroblastic tracks.[34] Additionally, tumors harboring mature TLSs with germinal centers were associated with the enrichment of multiple myeloma oncogene 1 $(MUM1)^+IgG^+$ plasma cell infiltrates in tumors and frequency of IgG-labeled tumor cells, suggesting the production of antitumor antibodies by plasma cells matured within TLSs. This study also demonstrated that cleaved caspase-3^+ apoptotic tumor cells proximal to macrophages existed more frequently in tumors with more intensive IgG^+ tumor cells than in tumors with less IgG^+ tumor cells, indicating that IgG may exert antitumor effects via promoting antibody-dependent cellular cytotoxicity by macrophages. Furthermore, immune checkpoint blockade–treated patients with ccRCC with more frequent IgG^+ tumor cells showed better therapeutic responses and survival outcomes than those with ccRCC with less IgG tumor cells, indicating that immune checkpoint blockade could facilitate antitumor immunity by promoting the production of antibody-producing cells within TLSs.[35] For more information on tumor-associated TLSs in the kidneys and other organs, other review articles may be referred.[19,20,87]

TLSs in Other Organs

Single-cell analysis has been used to characterize immune cells composed of TLSs in various organs other than kidneys in the context of autoimmune diseases, cancers, infection, and aging.[15,19,21,66] Age-dependent TLSs spontaneously develop in the murine liver and bladder, and contribute to inflammation-related conditions.[18,66] scRNA-seq on enriched $CD45^+$ immune cells from young and aged bladders identified *Cxcl13*-producing macrophages specifically in aged bladders, suggesting that this cell type was a potential source contributing to the recruitment of immune cells during aging in the bladder.[18] Additionally, the most striking difference was a drastic increase in B and plasma cells in the aged bladder; one of the B-cell subpopulations was ABCs that were also identified in age-

dependent TLSs in the kidney,[22] suggesting that ABCs contribute to age-dependent TLS formation in multiple organs. TLS-composing cells in thyroid tissues of patients with Hashimoto thyroiditis were characterized using scRNA-seq.[61] *ACTA2*$^+$ myofibroblasts and a subpopulation of fibroblasts shared common expression profiles of chemokines, such as *CCL2*, *CXCL12*, *CCL19*, and *CCL21*, suggesting that these stromal cells played key roles in promoting the infiltration of myeloid cells and lymphocytes and TLS formation. A fibroblast subpopulation highly expressed the IFN-γ–inducible T-cell homing chemokine, CXCL9, and B-cell activating cytokine, B-cell–activating factor, which was similar to that by the proinflammatory fibroblasts identified in renal TLSs,[23,88] thereby suggesting a pivotal role of interactions among IFN-γ–responding fibroblasts, T cells, and B cells in TLS formation across multiple organs. Furthermore, a comparative analysis of immune cells in thyroid tissues with peripheral blood mononuclear cells of patients with Hashimoto thyroiditis identified thyroid tissue–specific inflammatory macrophages and dendritic cells that highly expressed chemokines and cytokines, such as IL-1β, thereby potentially playing a crucial role in thyrocyte destruction by mediating the apoptosis of thyroid follicular cells. This study and our research on age-dependent TLSs in the kidney[23] suggest that TLSs can directly damage the surrounding tissue parenchymal cells by producing various proinflammatory cytokines and deteriorating their functions, resulting in organ failure.

Single-Cell Analysis of Immune Cells in Various Kidney Diseases

Immune cells play a crucial role in the pathogenesis of various kidney diseases.[10,11,89] Single-cell analyses of kidney biopsy samples from patients and murine models elucidated cellular heterogeneity and the role of immune cells in the pathogenesis of kidney diseases. Here, we describe novel findings from recent applications of single-cell technologies in relatively common kidney diseases, including LN, IgAN, and DKD.

LN

LN is the most common manifestation and a major risk factor for morbidity and mortality in systemic lupus erythematosus, an autoimmune disorder that affects any organ.[11,90] Approximately 50% of patients with systemic lupus erythematosus develop LN, and 10% of patients with LN develop end-stage kidney disease.[90] LN is generally classified on the basis of the 2003 International Society of Nephrology/Renal Pathology Society classification (revised in 2018),[91,92] which focuses on histologic findings in glomeruli. Nevertheless, tubulointerstitial findings, including immune cell infiltration, correlate with poor prognosis, suggesting a crucial role for immune cells in the pathophysiology of kidney injury in LN.[93,94] scRNA-seq of biopsy samples of kidneys and skin from patients with LN revealed that tubular cells and keratinocytes showed correlated elevations in type I IFN-response signatures, particularly in treatment responders. In this study, immune cell populations were classified into one small cluster with mixed expression of marker genes for myeloid, T, and B cells, thereby limiting the analysis of immune cell phenotypes.[95] In another study, scRNA-seq was performed for CD45$^+$ immune cells sorted from kidney biopsy samples from patients with LN to reveal detailed landscapes of immune cells.[96] The analysis identified 21 distinct immune cell clusters, including macrophages, dendritic, T, NK, and B cells, and plasma cells/plasmablasts, with a detailed subclassification of each cell cluster. IFN-stimulated genes were up-regulated in all immune cell clusters compared with those in living donor controls, demonstrating IFN-rich microenvironments in LN, which was consistent with a previous study.[95] Notably, naïve and activated B cells expressing signatures consistent with ABCs were identified, and the continuum state between them was determined by trajectory analysis, indicating that activation and differentiation of ABCs possibly occur in kidneys with LN. This analysis did not demonstrate the differentiation of ABCs into plasma cells; however, ABCs were previously reported to have the potential to differentiate into plasma cells that produce autoantibodies.[97] This inconsistency could possibly originate because of the sudden transcriptomic transition from ABCs to plasma cells, with minimal intermediate phases that were not detectable by trajectory analysis.[11] Comprehensive single-cell B-cell receptor sequencing is required to delineate the clonal relationships among these B-lineage cells. In contrast, scRNA-seq of CD8$^+$ T cells from spleens and kidneys of murine models of LN delineated the progression of CD8$^+$ T cells from naïve to several terminally differentiated phenotypes, including effector memory, exhausted, and resident memory phenotypes, via a transitional phenotype identified in kidney samples.[98] Additional single-cell T-cell receptor sequencing demonstrated that high-frequency clones were enriched in exhausted CD8$^+$ T cells with relatively high proliferation signatures. These results suggest that clonal expansion of CD8$^+$ T cells can occur in kidneys with LN, partly after their differentiation into exhausted states, thereby suggesting continuous T-cell receptor stimulation of exhausted T cells in kidneys and their contribution to the pathogenesis of LN.

Previous studies reported that TLSs and B-cell infiltrates were identified in 46% to 61.5% of patients with LN, and that they were associated with higher disease activity, the severity of histologic findings, and poor renal prognosis.[25,27] Considering the rarity of B-cell infiltrates outside TLSs in aged kidneys,[16] B-cell infiltrates in LN in the previous report might include TLSs, depending on their definition.[25] On the basis of the above-mentioned studies, differentiation and expansion of the pathologic lymphocytes

within TLSs are anticipated; however, further studies are needed to address this issue.

IgAN

IgAN is the most common primary glomerular disease worldwide and a leading cause of CKD.[99] IgAN is defined as the deposition of IgA derived from circulating IgA immune complexes composed of galactose-deficient IgA1 and IgG antibodies targeting galactose-deficient IgA1 in the mesangial region, which activates inflammatory responses that lead to glomerulosclerosis.[9] In addition, a recent study reported the potential involvement of IgA autoantibodies targeting to mesangial cell components in the pathogenesis of IgAN.[100] However, the precise immunologic mechanisms underlying mesangial IgA deposition remain unclear. scRNA-seq of glomerular, tubular epithelial, and immune cells separately isolated from renal biopsy samples of patients with IgAN and normal tissues of patients who had undergone nephrectomy demonstrated cell-type−specific up-regulated genes of IgAN.[101] Interestingly, mesangial cells in IgAN up-regulated the expression of joining chain of multimeric IgA and IgM (JCHAIN) that is necessary for dimerization of immunoglobulin and transepithelial transport of IgA, thereby suggesting the role of JCHAIN produced by mesangial cells in recognizing, transporting, and depositing IgA and demonstrating its potential as a therapeutic target. Mesangial cells in IgAN also up-regulated the expression of inflammatory mediators, such as *SPP1*, *KNG1*, *PLGRKT*, *CCL2*, and *AOC3*, indicating that mesangial cells can potentially amplify inflammation by trafficking and recruiting immune cells, such as monocytes and macrophages.[101] Additionally, $CD8^+$ T cells in IgAN exhibited elevated levels of exhaustion-related genes and decreased the expression of effector markers and T cell cytotoxicity-related genes, including *FCGR3A*, *GZMB*, *KLRD*, *FBFBP2*, and *GZMH*, suggesting the contribution of $CD8^+$ T-cell dysfunction in IgAN progression.[101] scRNA-seq of glomerular cells isolated from murine kidneys at the early stage of IgAN demonstrated that glomerular endothelial cells exhibited elevated expression of genes related to endothelial dysfunction (*Edn1*, *F8*, and *Nostrin*), adhesion molecules (*Sele*, *Icam1*, and *Vcam1*), and proinflammatory cytokines (*Cx3cl1*, *Cxcl1*, *Cxcl10*, *Cxcl11*, and *Cxcl16*).[102] Additionally, ligand-receptor analysis between glomerular endothelial cells and immune cells predicted several cell-cell interactions; however, mesangial cells and podocytes demonstrated molecular changes fewer than that reported by Zheng et al,[101] suggesting a crucial role of glomerular endothelial cells in the initial stage of IgAN pathogenesis.[102]

TLSs were identified in 37.5% of the patients with IgAN, and patients exhibiting renal TLSs showed worse clinicopathologic features than those without TLSs.[24] However, the specific pathologic roles of TLSs in IgAN remain unclear. Zheng et al[103] reported that toll-like receptor 7−expressing B cells that develop nodular lesions in the kidney potentially produce pathogenic galactose-deficient IgA1, suggesting pathogenicity of TLSs in IgAN. Single-cell analysis, including single-cell B-cell receptor sequencing, is expected to shed light on this challenging issue.

DKD

DKD is the most common cause of CKD and end-stage kidney disease worldwide, and is associated with high mortality. The number of patients with DKD is predicted to increase by approximately 50% from 2021 to 2045.[104] Inflammation is the key contributor to the onset and progression of DKD.[12,104,105] Single-cell analyses of human and murine kidney samples with DKD have indicated the occurrence of immune cell infiltration.[106−110] Wilson et al[106] performed snRNA-seq of kidney samples from patients with diabetes and demonstrated a sevenfold to eightfold increase in the number of immune cells, including monocytes, and T, B, and plasma cells compared with those in healthy controls, thereby indicating the progression of inflammation in diabetic kidneys. Additionally, multiple immune cell types showed increased expression of several genes included in the kidney risk inflammatory signature that is a panel of 17 circulating plasma proteins to predict the progression of diabetic nephropathy[111] scRNA-seq of $CD45^+$ cells isolated from kidneys of type I diabetic mice (OVE26 mice) and control kidneys at 3 and 7 months of age revealed 11 distinct immune cell clusters, including neutrophils, macrophages, and dendritic, innate lymphoid, T, NK, B, and plasma cells.[108] Additional analysis of mononuclear phagocytes identified heterogeneous cells, including infiltrating macrophages and various types of resident macrophages, in the diabetic and nondiabetic groups. A specific increase was noticed in the number of infiltrating macrophages with signatures of inflammatory gene expression and mannose receptor C type-1 $(MRC1)^+$ or triggering receptor expressed on myeloid cells 2 $(TREM2)^+$ resident macrophages that attenuate macrophage activation in diabetic kidneys,[112] indicating increases in both proinflammatory and anti-inflammatory macrophage subtypes. Moreover, studies with single-cell transcriptome-based annotation tools (MacSpectrum) to examine polarization and maturation of macrophages indicated that macrophages of 7-month−old diabetic mice showed more obvious shifts toward a proinflammatory M1-like phenotype and reduced reparative M2-like phenotype than those of 3-month−old diabetic mice.[113] These data were consistent with a continuum of macrophage activation characterized by an enhanced inflammatory status in DKD progression.[108] scRNA-seq of kidney samples from patients with DKD, nondiabetic kidneys, and healthy kidneys from patients with diabetes showed an increased number of venous endothelial cells with enrichment of pathways related to inflammation and immunity and fibroblast subsets with high expression of several types of chemokines, such as *CCL19*, *CCL21*, and *CCL2*, which were predicted to contribute to the recruitment of lymphocytes, mononuclear phagocytes, and plasmacytoid

dendritic cells in DKD kidneys.[109] Furthermore, spatial transcriptomics demonstrated that these inflammatory cells were preferentially localized in the area of renal fibrosis in DKD kidneys, indicating the contribution of their interactions to the progression of kidney fibrosis and their potential as promising therapeutic targets in DKD.[109]

No study focusing on TLSs in diabetic kidneys has been published. Considering the involvement of inflammation in the pathogenesis and progression of DKD,[12] TLSs may potentially develop in diabetic kidneys. The frequency and characteristics of TLSs and their clinical relevance in DKD needs to be investigated in the future.

Conclusions and Future Perspectives

Recent advancements in scRNA-seq and single-cell assay for transposase-accessible chromatin with high-throughput sequencing analyses have provided novel findings in the field of renal immunology. Although a droplet-based single-cell platform is widely adopted, its high costs for the reagent limit the access to the technology for analyzing a large number of samples. Single-cell analysis with split-pool barcoding methods has the potential to address the limitations, which can uniquely label individual cells and their transcriptomes through multiple rounds of combinational barcoding.[114] This method enables researchers to multiplex a large size of samples and profile transcriptomes of hundreds of thousands of cells in a single experiment, leading to relatively lower cost and fewer batch effects, and it has recently been applied to the nephrology field.[115,116] However, a notable limitation of these techniques is their inability to detect protein expression. The advent of single-cell proteomics can potentially overcome this limitation. Mass cytometry has emerged as a more powerful tool than traditional flow cytometry for understanding cellular heterogeneity and enables concurrent detection of >40 distinct proteins with minimum spectral overlap.[117–119] The combination of cellular indexing of transcriptomes and epitomes by sequencing with scRNA-seq enables simultaneous detection of protein and gene expression at the single-cell level through sequencing-based methods using antibodies conjugated with unique short DNA barcodes.[120] These technologies are expected to revolutionize the characterization of immune cells and offer unprecedented insights into renal immunology. Additionally, recent developments in spatial transcriptomics and proteomics have facilitated comprehensive detection of gene and protein expression while preserving the spatial information of each cell type,[121–124] which will be used in future research on kidneys.

Several questions regarding the roles of immune cells and TLSs in kidney injury and repair are still unanswered. For instance, it has not been fully revealed whether immune cell types and their functions that contribute to TLS formation and kidney injury are conserved across various kidney diseases or are disease specific. Single-cell analysis potentially answers this question by integrating immune cell profiles across various types of diseased kidneys, leading to elucidation of pathophysiology of TLSs and identification of comprehensive therapeutic targets. Moreover, the precise mechanisms initiating renal TLS formation, particularly the functions of immune cells as lymphoid tissue initiator cells, remain unknown. Temporal single-cell analyses from the early phase before complete TLS formation may provide crucial insights to answer this question. In conclusion, the application of comprehensive single-cell multiomics may help identify novel immune cell types and phenotypes, thereby deepening our understanding of the pathophysiology of immune-mediated kidney diseases and accelerating the development of novel therapeutic strategies.

Acknowledgments

We thank the Single-Cell Genome Information Analysis Core at the Institute for Advanced Study of Human Biology (Kyoto University) for support; Prof. Seishi Ogawa (Kyoto University) for allowing us to use machines for single-nucleus RNA sequencing (snRNA-seq); and Dr. Yuhei Kirita and Prof. Benjamin D. Humphreys (Washington University School of Medicine) for valuable advice on snRNA-seq analysis.

Author Contributions

T.Y. wrote the article and M.Y. revised the article and approved the final version.

Disclosure Statement

M.Y. received research grants from Mitsubishi Tanabe Pharma and Boehringer Ingelheim.

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