

Single cell transcriptomics of human nephritis reveal the cellular origin of kidney disease associated genes

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Research

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Single cell transcriptomics of human nephritis reveal the cellular origin of kidney disease associated genes

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Abstract

Background Single cell RNA sequencing (scRNA-seq) have

become a powerful tool in discovering a novel cell type and pinpointing cellular specific gene within tissues. However, in diseased kidneys, especially caused by glomerulonephritis, there have few study focusing on revealing gene expression changes at the cellular level.

Methods To reveal cellular gene expression profiles of glomerulonephritis, we performed scRNA-seq of 2 human kidney transplantation donor samples, 4 human glomerulonephritis samples, 1 human malignant hypertension sample and 1 human chronic interstitial nephritis sample, all tissues were taken from biopsy.

Results Upon disease occur, immune cells infiltrate which can be proved by our dataset. In the cluster of podocyte, two glomerulonephritis related genes named FXYD5 and CD74 were found, and interferon alpha/beta signalling pathway and antigen processing and presentation was enriched in podocyte of LN and IgAN comparing with other samples, thus inhibiting these signalling pathway may alleviate the symptom of these disease.

Conclusions Cellular origin of kidney disease associated genes and the activated signaling pathways the genes involved in can be found by scRNA-seq.

Keywords single cell RNA-seq, kidney disease, nephritis, transcriptomics

Background

The kidney is a highly complex tissue with a broad range of specialized cell types organized into functionally distinct compartments. Previous bulk RNA-seq cannot detect heterogeneity of renal cells, thus specific renal cells functioning abnormal may be covered up by the majority of cell types(1).

Glomerulonephritis is a major reason leading to renal failure. The clinical manifestation of glomerulonephritis is a heterogeneous group of diseases, which were diagnosed by biopsy. However specific pathogenesis underlying histologic findings seems to be covered(2).

As one of most common renal diseases, IgA nephropathy is characterized by deposition of IgA immune complexes in glomerulus. The mesangial IgA is exclusively of the IgA1 subclass, which is mostly in a dimeric form that is composed of two IgA1 monomers and J chain(3).

Genome-wide association studies(GWAS) have found IgAN contributing genes including HLA-DRB1, -DQA1 and DQB1, which mediate the MHC class II response. In addition, TAP1, TAP2, PSM8 and PSM9 involved in

antigen generation are also IgAN associated genes(4). CFH and the related genes have a protective role via inhibiting the C3 and C5 convertases, while a common deletion of CFHR1 and CFHR3 found in the GWAS can help CFH-mediated inhibition of the complement cascade. Chinese GWAS found a new loci centered on gene TNFSF13 encoding a proliferation-inducing ligand (APRIL) influences IgA-producing cells(5). However, whether these IgAN associated genes expressed abnormal comparing with healthy kidney at cellular level is still unknown.

A major cause of nephrotic syndrome of non-diabetic origin in adults is membranous nephropathy(MN), which is the second or third leading cause of end-stage renal disease in patients with primary glomerulonephritis. The disease is characterized by the formation of immune deposits on the outer aspect of the glomerular basement membrane resulting in complement activation. Podocyte phospholipase A2 receptor (PLA2R) was found as an antigenic target in autoimmune adult MN. Measurement of anti-PLA2R antibodies in serum and detection of PLA2R antigen in glomerular deposits can be a very

good indicator for diagnosis and treatment of MN(6). However, in addition to primary MN (caused by auto antibody), there have other forms of MN in which anti-PLA2R antibodies can not be detected(7). The mechanisms underlying the common clinical manifestations also need to be clarified.

Lupus nephritis(LN) is a common and serious complication of systemic lupus erythematosus (SLE).To date, renal biopsy remains to be the gold standard for the severity of LN as current laboratory markers for LN lack sensitivity and specificity for renal disease activity and damage in lupus nephritis(8). Thus, novel biomarkers should be found to act as surrogate indicators instead of invasive method. However whether these functionally disease-related genes originating from specific cell type remain unknown. So this study wants to find whether the disease associated genes originate from specific cell type .

Methods

Clinical Sample

The patient described in this study consented under

Ethics committee review of renji hospital affiliated to Shanghai Jiao Tong University School of Medicine.

Tissue Processing and Single-Cell Dissociation

The renal biopsy was preserved in cold PBS, then minced into small pieces with a razor blade and incubated at 37°C in freshly prepared dissociation buffer containing enzymes from Multi Tissue Dissociation Kit (Miltenyi Biotec) with rotation speed at 200rpm.

Dissociated cells were harvested every 10minutes by filtering the cell suspension through a 70-mm cell strainer (FALCON) into 10% FBS buffer on ice. The residual biopsy tissue was dissociated once again with 1 ml dissociation buffer for 10 minutes and passed through the cell strainer into the same FBS buffer from the first collection. We repeated this dissociation procedure three times until most of the tissue had been dissociated into single cells (total dissociation time was 30 minutes).

Finally, cells were collected by centrifugation at 1500rpm for 5 minutes, resuspended in PBS containing 5% FBS, and strained through a 40-mm cell strainer (FALCON) to further remove cell clumps and large fragments. Cell viability was approximately 85% for the

biopsy used in this study as assessed by Trypan Blue staining.

Single-cell RNA sequencing and library construction

According to manufacturer's (10X Genomics) protocol, single cell suspensions were used to generate single-cell libraries with the Chromium Single Cell Gene Expression system using 3' Library & Gel Bead Kit v2 (10X Genomics) and paired-end sequencing was performed on a HiSeq.

Bioinformatic Analysis

According to the instructions of SATIJA LAB (<https://satijalab.org/seurat/>), integrated sample was performed by using R toolkit, then calculation of high-variance genes, dimensional reduction, graph-based clustering, and the identification of cluster markers was done.

Gene enrichment analysis

This analysis was done by metascape (<http://metascape.org/gp/index.html#/main/step1>)(9).

Results

Overall cell clusters and marker genes of 21 cell clusters from integrated 8 samples.

After filtering the cells with <200 genes and >10% MT genes, the resulting 14932 cells, as we can see in figure 1A, can be divided into 20 cell clusters including 5 types of proximal tubules (PT), loop of henle (LOH)+distal tubule (DCT), collecting duct-intercalated cell(CD-IC), collecting duct-principal cell(CD-PC), endothelial cell(EC), monocyte, myofibroblast, B cell, podocyte, mesangial cell, neutrophil, LTB high, NK, CD4 T, NKT, mast cell and plasma cell . Next we want to seek some novel marker genes in the already known cell clusters. By using violin plot, we found SPINK1 was expressed high and exclusively in CD-IC(Figure1C and Figure1E). Furthermore, dotplot was used to find the expression of SPINK1 in CD-IC across different kidney samples(Figure1F). Meanwhile, comparing with ATP6V1G3, SPINK1 seems to be a marker of CD-A-IC as there exists low level of SPINK1 in CD-B-IC subtype(Figure1F). AND there exists two subtypes of CD-A-IC which has not been reported to data. One of CD-A-IC seems to express genes of DCT(Figure 1G). By

calculating the cell proportion across 8 samples , we found decreased PT cell clusters and increased immune cell clusters in diseased samples comparing with two donors(Figure 1H).

Sub cell clusters of NK, NKT, LTB high and CD4 T and characteristic of PT

As increased immune cells were observed in kidney biopsy of diseased patients comparing with that of donor ones and a special NK subtype expressing LTB exists, next we want to subdivide NK cell clusters to find whether there have some diseased associated subtypes of NK. As figure 2A and 2B showed, there exists a subtype adjacent to NK/NKT cell cluster expressing IL7R, CD3D and CD3E instead of NKG7. In our data, besides LTB high cluster, the level of LTB was also higher in CD4 T of LN and CIN than that of other samples(Figure 2D).

Next we subdivide the PT cells. Interestingly, an inflammatory subtype named PT6 was found in our dataset(Figure 2H).Figure 2F show the top genes, defined as high fold change, of PT6.And we can find wnt- β catenin target genes(MMP7 AND MYC) was also

activated in PT6(Figure 2H). Comparing with diseased samples, the number of PT1, PT4 and PT7 cell clusters was increased, while the number of other PT cell clusters was decreased in donor samples(Figure2G).Figure 2I show the cell cycle status of each cell clusters. B and NK cell seems to be at status of cell division comparing with other cell types.

GWAS genes and target genes of promising biologics in the specific cell clusters

The dotplot in Figure 3A shows that GWAS genes focus on clusters of glomerulus and immune cells. GWAS genes expressed across different samples were showed in Figure 3A. The target genes of current and evolving biologics expression profile across different cell clusters was showed in Figure 3B. As we know, IgAN, LN and MN are three types of glomerulonephritis which affect podocyte most. Thus we use plots to show specific differential expressed genes(DEGs), which was enriched in top signalling pathway, in podocyte of MN(Figure 3D), LN(Figure 3E), IgAN1(Figure 3F) and IgAN2(Figure3G) comparing with that of donor. IHC

staining of two genes(FXYD5 and CD74) which is enriched in podocyte of LN, MN, IgAN1 and IgAN2 can be detected in glomerulus(Figure 3C).

The activated signaling pathway at cellular level

As immune complex was deposited in mesangial cell of IgAN, firstly the genes, which was up-regulated in mesangial cell of IgAN comparing with that of donor, were enriched in peptide chain elongation, respond to growth factor and ossification, while the down-regulated genes were enriched in negative regulation of hydrolase activity, respond to decreased oxygen level and protein processing in ER, which indicated hyperactivity of hydrolase, unable to cope with decreased oxygen level and ER stress were existed in mesangial cell of IgAN(Figure4A).Figure4B shows the interaction of down regulated genes was enriched in oxidation respiratory chain(showed in green) and muscle contraction(showed in purple).As humoral immunity was activated in MN, the up regulated genes in plasma of MN was enriched in B cell activation signaling compared with donor (Figure4C). While in LN sample, interferon

signaling was activated in monocyte (Figure4D), B(Figure4F), plasma(Figure4H), neutrophil(Figure4G) and NKT(Figure4I). TNFSF13B was gathered in monocyte of LN (Figure4E). Some chemokines such as CXCL1, CXCL2 and CXCL3 can interact with C5AR1, CCR1 and FPR3 to recruit more monocyte to LN kidney (Figure4D). AND in neutrophil of LN, there also have CXCL8 and CXCL16 interacting with FPR3 to recruit more neutrophil to LN kidney (Figure4G).

Table1. Clinical Demographics of biopsy samples.

Sample ID	age	sex	Serum creatinine(uM)	laboratory examination	complications
Donor1(IgA)	46	male	50	Glu 19.77 mmol/L RBC by urine sediment:211/ul	NA
Donor2	N	male	88	NA	NA
IgAN1	61	male	107.9	RBC:17/HP, 24h proteinuria:1.19g	NA
LN	27	male	145	eGFR-EPI Cr 56	NA
MN	60	male	62	24h proteinuria:16g eGFR-EPI Cr 103	NA
CIN	62	male	203	HbA1c:10.0 eGFR-EPI Cr:29	renal arteriolar sclerosis

IgAN2	38	female	117	RBC:22.1/HP
				24h proteinuria:2.075g
				eGFR-EPI Cr:72
				HBV-DNA:1.14E+05
MH	38	male	184	eGFR-EPI Cr:39

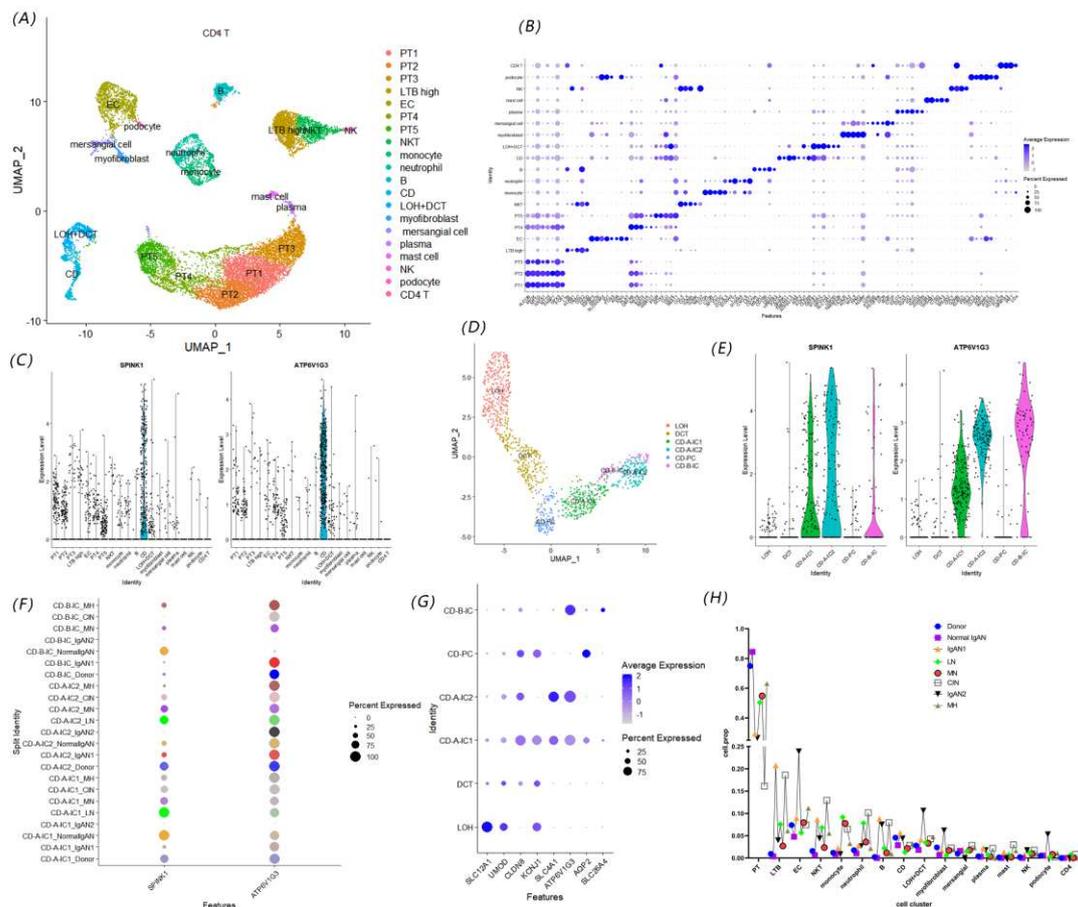


Figure 1. Biopsy of two kidney transplantation donors, two IgAN, one LN, one MN, one MH and one CIN were merged. (A) One of the donors was confirmed as IgA nephropathy by histopathology. And 20 cell clusters including 5 types of proximal tubules (PT), loop of henle (LOH)+distal tubule (DCT), collecting duct-intercalated cell (CD-IC), collecting duct-principal cell (CD-PC), endothelial cell (EC), monocyte, myofibroblast, B cell, podocyte, NKT, NK, LTB high, CD4, mast cell and plasma cell were found. (B) The marker genes of each cell clusters and (C) The gene SPINK1 was expressed high and exclusively in CD, while ATP6V1G3 was a known accepted marker of CD-IC. (D) Sub clusters of CD AND LOH-DCT. (E) The violinplot shows SPINK1 was expressed higher in CD-A-IC comparing with its level in CD-B-IC. (F) The expression profile of SPINK1 across different samples. (G) The marker of two CD-A-IC subtypes. (H) The figure shows cell proportion of different kidney samples across 20 cell clusters.

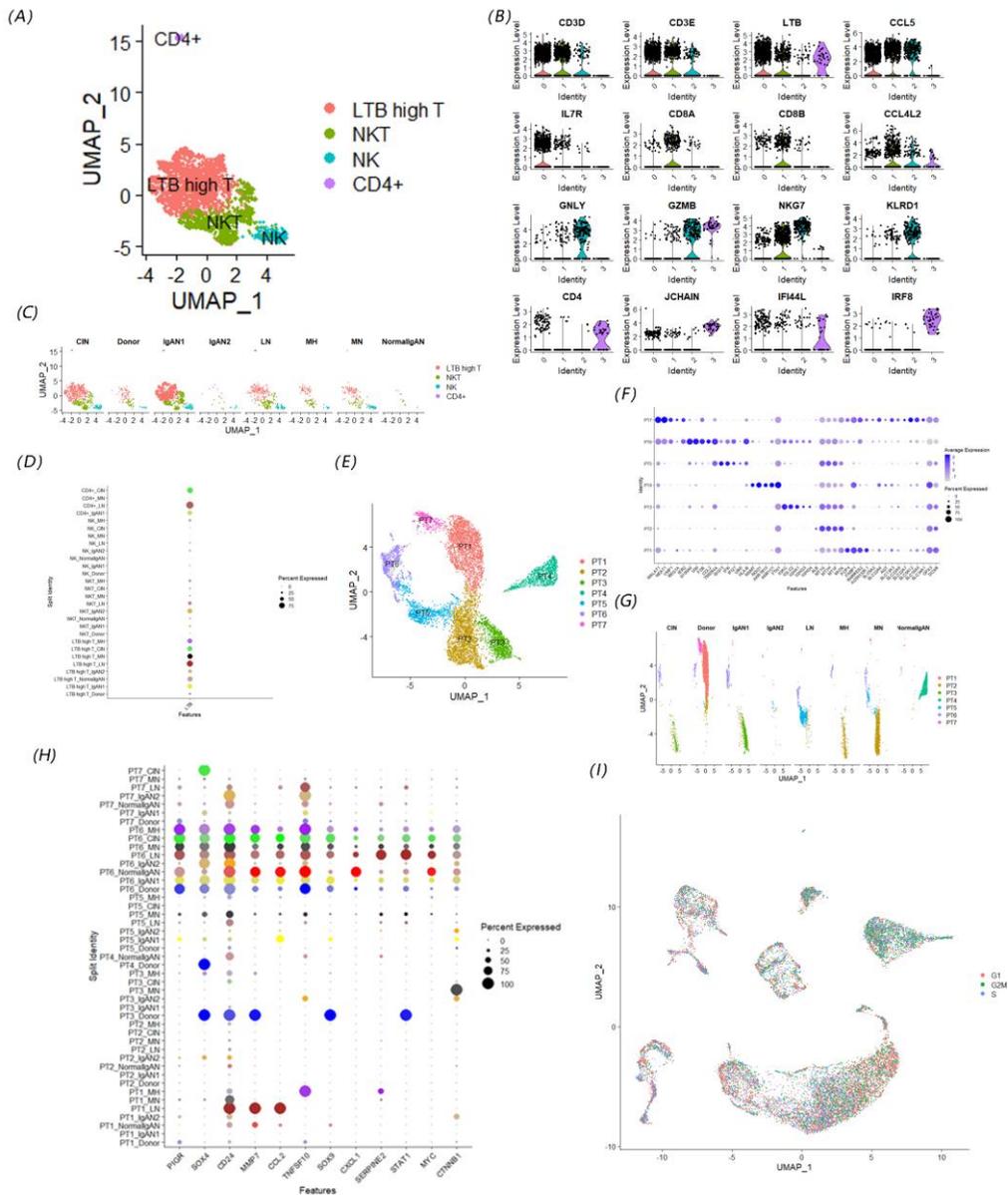


Figure 2. Sub cell clusters of NK clusters and characteristic of PT. (A) Umap show subclusters of NK cell. (B) The violinplot show the marker gene of NK subclusters. (C) The split view of NK subclusters from different samples. (D) Our data show LTB seems to express in T cell instead of NK cell types. (E) Subclusters of PT. (F) The marker genes of PT subclusters. (G) The split view of PT subclusters from different samples. (H) The top genes, defined as high fold change, of PT6 was showed in dotplot across different samples. (I) The umap show the cell cycle status of each cell clusters.

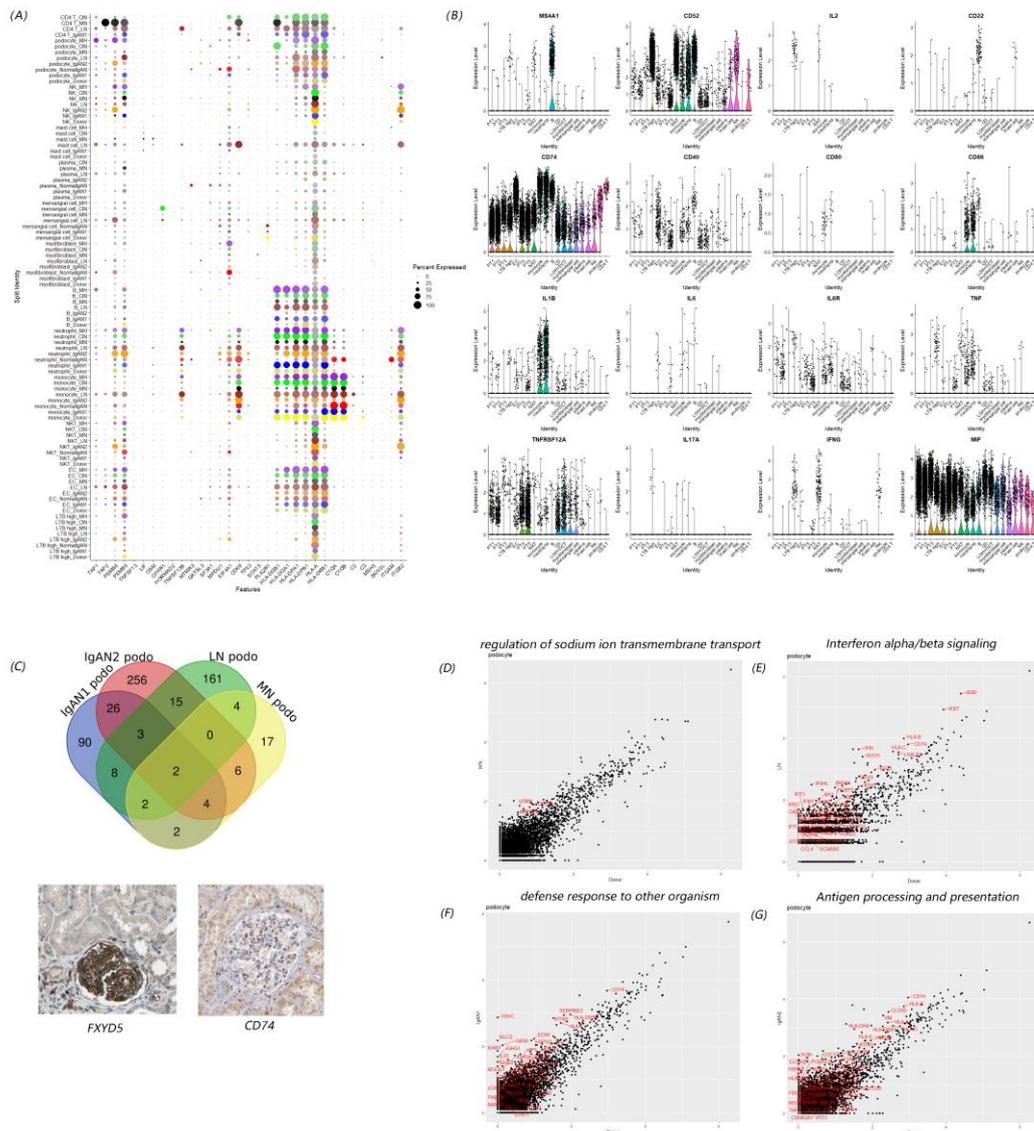


Figure3 GWAS genes and target genes of promising biologics in the specific cell clusters.(A)Dotplot shows cellular specific expression of GWAS genes(10, 11) (B)Vlnplot shows the target genes of current and evolving biologics expressed in specific cell types(12) (C)The higher expressed gene in podocyte of MN, LN, IgAN comparing with that in donor was merged in Venn diagram, while IHC staining of all highly expressed two gene CD74 and FXYD5 ,which is from the Human Protein Atlas (<https://www.proteinatlas.org/>), was showed. Disease specific differential expressed genes(DEGs) which was enriched in top signaling pathway was found between MN(D),LN(E),IgAN1(F),IgAN2(G) and Donor.

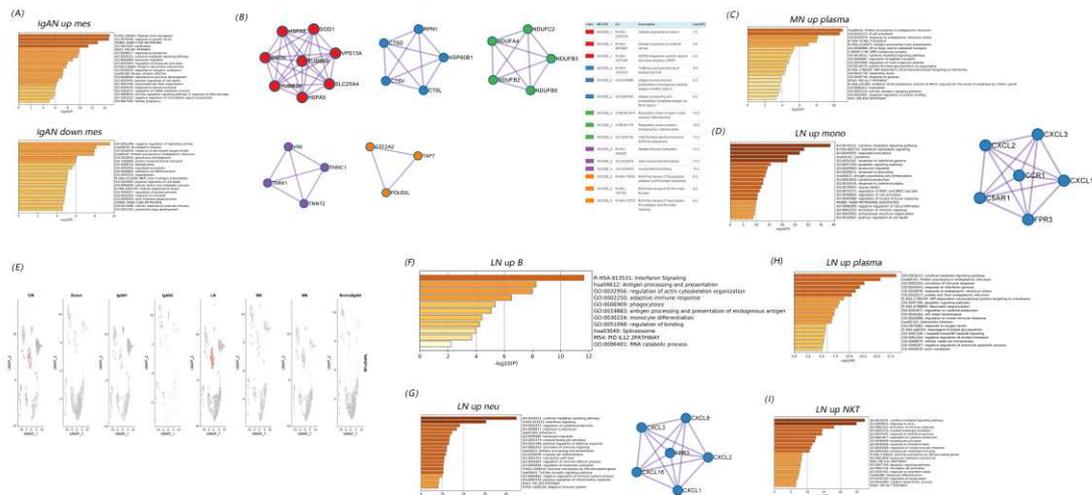


Figure 4 The activated signaling pathway at cellular level. (A) As immune complex was deposited in mesangial cell of IgAN, the up-regulated genes in mesangial cell of IgAN comparing with that of donor were enriched in peptide chain elongation, respond to growth factor and ossification, while the down-regulated genes were enriched in negative regulation of hydrolase activity, respond to decreased oxygen level and protein processing in ER. (B) The interaction of down-regulated genes was enriched in oxidation respiratory chain (showed in green) and muscle contraction (showed in purple). (C) As humoral immunity was activated in MN, we want to see the up-regulated genes of plasma, and B cell was indeed activated in MN. While in LN sample, interferon signaling was activated in monocyte (D), B (F), plasma (H), neutrophil (G) and NKT (I) (13). TNFSF13B was gathered in monocyte of LN (E).

Discussion

We adopt two transplantation donor biopsy samples from transplantation center as normal controls, while other samples acquired from nephrology department were used as group of kidney disease whose tissues were taken from biopsy to confirm their cause of proteinuria or rise in the serum creatinine. As the pathological results show, one donor sample and two patients were confirmed as IgA nephropathy with other four patients diagnosed as

lupus nephritis, membranous nephropathy, malignant hypertension and chronic interstitial nephropathy respectively. And another donor sample was relatively normal besides reperfusion injury. This study wants to bring kidney biopsy interpretation into single cell transcriptomics of human nephritis(14). Firstly, As we know, PT cells was mainly divided into three subtypes: S1, S2 and S3. While in this data, *SLC5A2*(the marker of S1), *SLC22A6*(the marker of S2) and S3 marker gene *AGT(15)* didn't have obviously differential expression across PT cell clusters(Extended figure2). Thus subclusters of PT cells need to be done. However, after subdividing the PT cells, we can find *SLC5A2*(the marker of S1), *SLC22A6*(the marker of S2) and S3 marker gene *AGT* were relatively expressed in PT1, PT4 and PT7, the number of which was more in donors than that in diseased patients(Figure2F). This somehow indicates that normal PT cell numbers was decreased in diseased patients. Indeed, PTs are susceptible to the derangements of some nephritis such as diabetic kidney disease(16) and are vulnerable to various injury. And PTs are the primary sensor and effector in the

progression of CKD(17). Given that SPINK1 was high and exclusively presented in CD-A-IC, further study should be performed to investigate why the gene SPINK1 was activated in CD-A-IC of LN and MN. As more immune cells infiltrate the diseased sample. Next, we want to find diseased associated NKT, and LTB high T cell cluster was found in our data, this cell cluster seems to enrich in IgAN, the up regulated genes in this cluster of IgAN comparing with that of donor was enriched in B cell activation, this suggests LTB high cell may have a role in B cell activation. While the data from humphreyslab show LTB level was higher in lymphocyte of diabetes than that of control. Their data also show LTB was expressed only in T and B cell cluster(18-20). As an inducer of the inflammatory response system, LTB was also involved in normal development of lymphoid tissue(21). Mast cell, which has a profibrogenic role (22, 23), expressing TPSB2 and TPSAB1 was easy to find in IgAN(24) and CIN comparing with LN and MN(Figure1H). This is why tubular interstitial fibrosis can be easily found in IgAN and CIN. Monocyte was enriched in LN, and its high expressed genes was

enriched in interferon alpha/beta signaling pathway (9, 25) which was activated in LN.

To date, there exist lots of GWAS study exploring the relationship between gene SNP or mutation and occurring and development of disease. Somehow, the GWAS studies only display the relationship but cannot explain how the SNP lead to occurring and development of disease or some SNPs may be the result of the disease. So we want to find whether GWAS genes have some association with diseases. And we find GWAS genes focus on clusters of glomerulus and immune cells. From this we can know nephritis was most relevant with malfunction of glomerulus and immune cell clusters. From the Figure 3B, we can find big side effect of some biologics was not only due to deleting the normal function of target genes but also affecting target genes of various cell types as CD52, CD74, MIF and TNFRSF12A was displayed across various cell types. So biologics targeting these genes may have some side effects. CD74 is a protein trafficking regulator and a cell membrane receptor for MIF (26). In our dataset, four glomerulonephritis samples including two IgAN, one LN

and one MN express higher CD74 mRNA level comparing with donor sample within podocyte, which indicates activation of MIF/CD74 between immune cells and podocyte exists in glomerulonephritis sample, providing a potential marker for glomerulonephritis status. While FXYD proteins belong to a family of small-membrane proteins. Among them, FXYD4 (also called CHIF) are one subunits of Na-K-ATPase(27). In our data, we found FXYD4 was enriched in podocyte of glomerulonephritis, this situation needs to be further investigated for the role of FXYD4 within podocyte in the progression of glomerulonephritis. By doing gene enrichment analysis, B cell activation signaling was activated in plasma of MN. While in LN sample, interferon signaling was activated in immune cells including monocyte, B, plasma, neutrophil and NKT. Hyper-activation of type I interferon (IFN-I) signaling pathway is associated with progression and prognosis of LN(28). Many molecules(29-31), such as JAK inhibitor (tofacitinib), monoclonal antibodies targeting IFN alpha (sifalimumab) and IFN-I receptor (anifrolumab), by blocking IFN-I signaling pathway have been developed

to ameliorate the symptoms of SLE. As this data shows, interferon signaling was activated in immune cells of LN comparing with that of donor, so targeted therapy should be applied to over-activation of interferon signaling within immune cells. Interestingly, it was found that TNFSF13B(also called BAFF) was exclusively expressed in monocyte. Previous study found the levels of BAFF were significantly higher in active compared to inactive LN(32), and from this data the BAFF level within monocytes may be relatively higher in LN comparing with other kidney disease and donors. So the high level of BAFF in monocyte may be a good biomarker for the activity of LN.

A limitation of this study is the relatively small number of patients, and the sample size should be increased in future studies to better delineate the occurrence, development and outcome of kidney diseases. Our results lay the foundation for such efforts.

Conclusion

Single cell RNA-seq is a powerful tool for mining kidney disease associated genes and the activated signaling pathways the genes involved in at cellular level, and such

datasets of nephritis were provided by this paper to some extent.

Competing interests :

The authors declare that they have no competing interests.

Availability of data and material

The datasets generated during the current study are available for uploading if required.

Consent for publication

Not applicable

Ethics approval and consent to participate

The patient described in this study consented under Ethics committee review of renji hospital affiliated to Shanghai Jiao Tong University School of Medicine

Authors' contributions :

ZJ Chen analyzed the data and was a major contributor in writing the manuscript. T Zhang helped process the single cell RNA-seq. Q Wang, ZY Li, YY Xie, XD

Yuan, L Ying, M Zhang helped provide the tissue biopsy and performed the histological examination of the kidney, S Mou directed the research design. All authors read and approved the final manuscript.

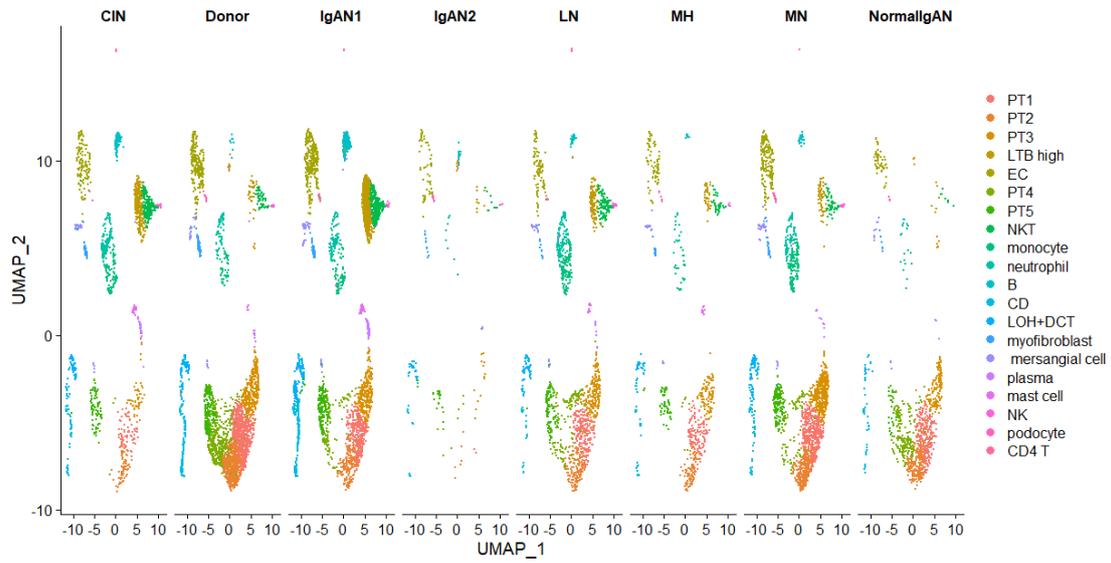
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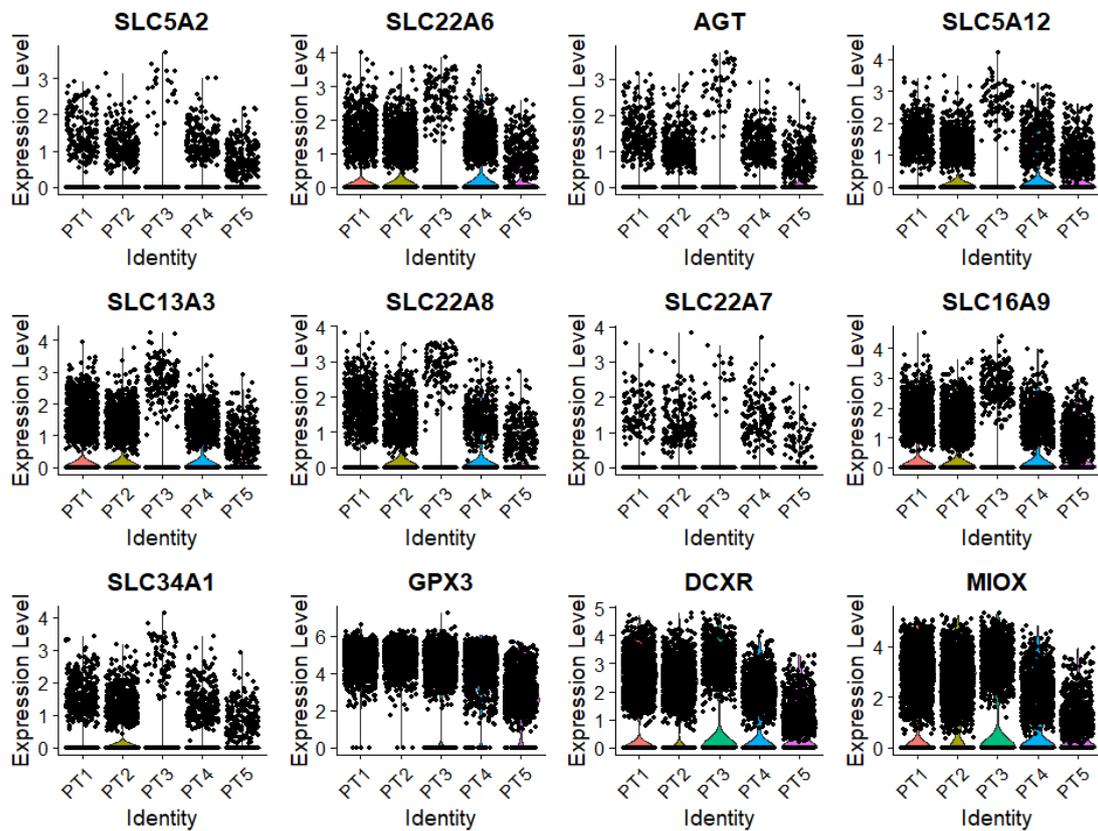
Acknowledgement

Not applicable

Extended figure1 The split view of cell clusters at the level of different samples



Extended figure2 The expression of known PT markers across different PT types before subclustering (33-35)



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Figures

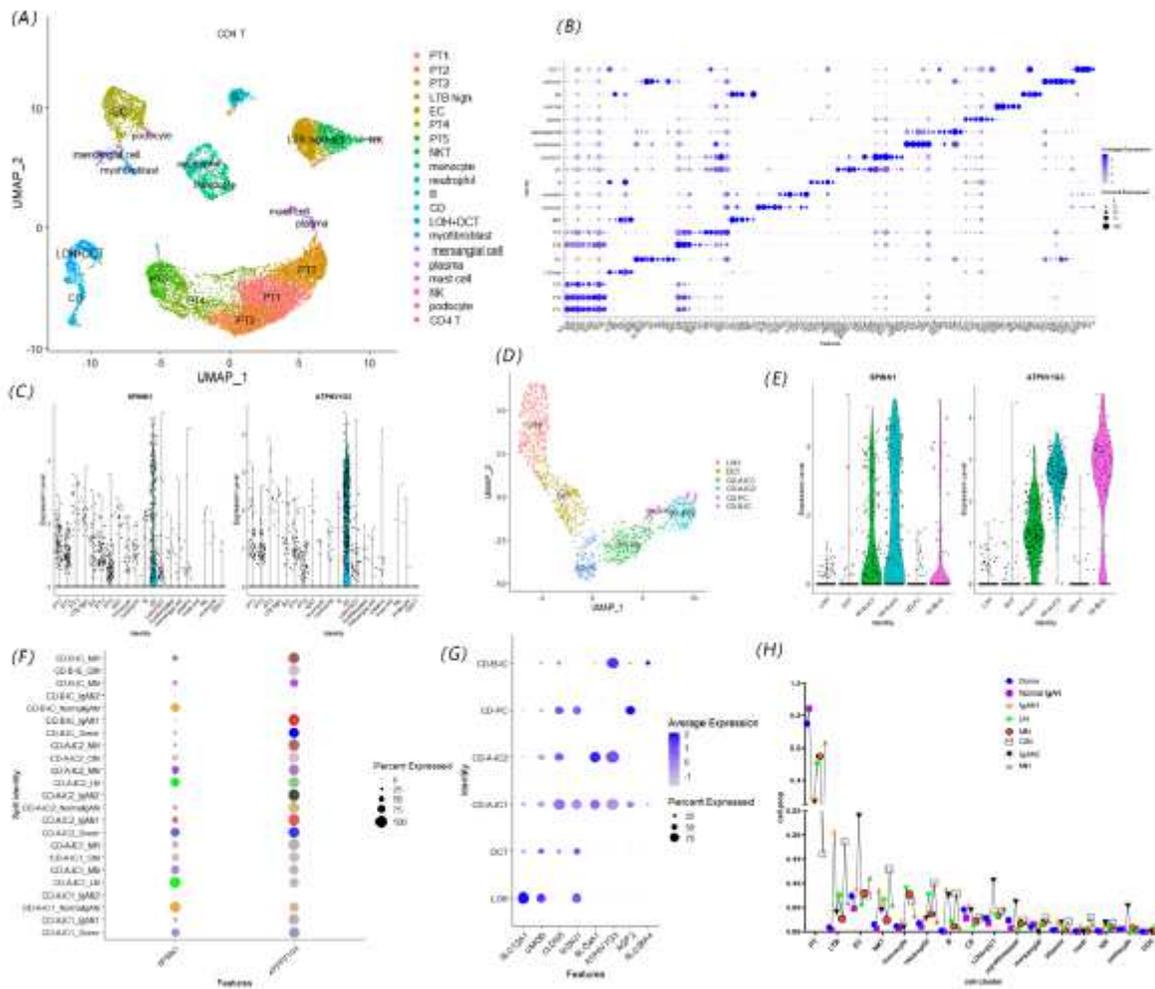


Figure 1

Biopsy of two kidney transplantation donors, two IgAN, one LN, one MN, one MH and one CIN were merged. (A) One of the donors was confirmed as IgA nephropathy by histopathology. And 20 cell clusters including 5 types of proximal tubules (PT), loop of henle (LOH)+distal tubule (DCT), collecting duct-intercalated cell (CD-IC), collecting duct-principal cell (CD-PC), endothelial cell (EC), monocyte, myofibroblast, B cell, podocyte, NKT, NK, LTB high, CD4, mast cell and plasma cell were found. (B) The marker genes of each cell clusters and (C) The gene SPINK1 was expressed high and exclusively in CD, while ATP6V1G3 was a known accepted marker of CD-IC. (D) Sub clusters of CD AND LOH-DCT. (E) The violinplot shows SPINK1 was expressed higher in CD-A-IC comparing with its level in CD-B-IC. (F) The expression profile of SPINK1 across different samples. (G) The marker of two CD-A-IC subtypes. (H) The figure shows cell proportion of different kidney samples across 20 cell clusters.

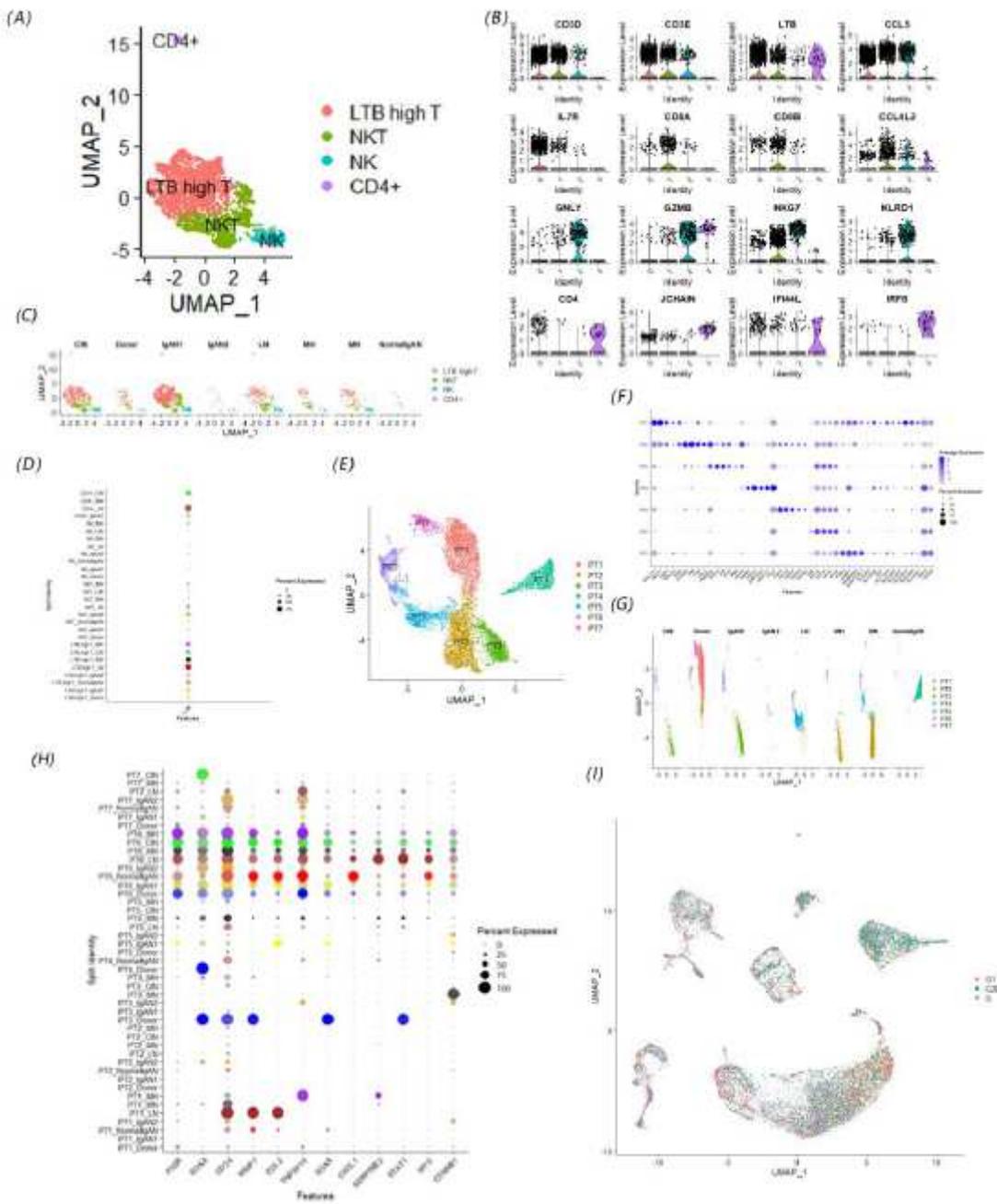


Figure 2

Sub cell clusters of NK clusters and characteristic of PT.(A)Umap show subclusters of NK cell.(B)The violinplot show the marker gene of NK subclusters.(C)The split view of NK subclusters from different samples. (D) Our data show LTB seems to express in T cell instead of NK cell types. (E)Subclusters of PT. (F)The marker genes of PT subclusters. (G)The split view of PT subclusters from different samples. (H)The top genes, defined as high fold change, of PT6 was showed in dotplot across different samples. (I)The umap show the cell cycle status of each cell clusters.

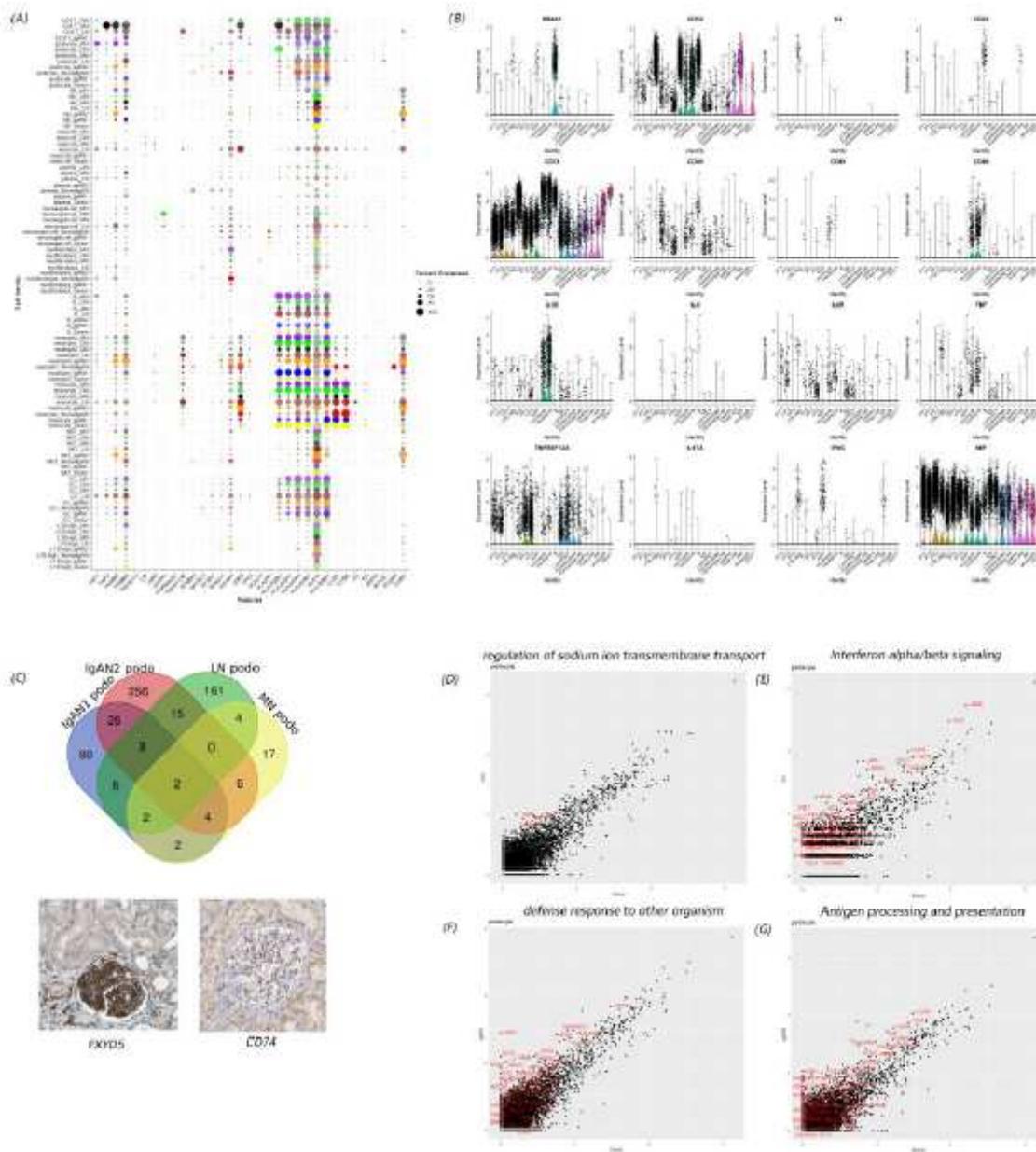


Figure 3

GWAS genes and target genes of promising biologics in the specific cell clusters. (A) Dotplot shows cellular specific expression of GWAS genes(10, 11) (B) Vlnplot shows the target genes of current and evolving biologics expressed in specific cell types(12) (C) The higher expressed gene in podocyte of MN, LN, IgAN comparing with that in donor was merged in Venn diagram, while IHC staining of all highly expressed two gene CD74 and FXD5, which is from the Human Protein Atlas (<https://www.proteinatlas.org/>), was showed. Disease specific differential expressed genes(DEGs) which was enriched in top signaling pathway was found between MN(D), LN(E), IgAN1(F), IgAN2(G) and Donor.

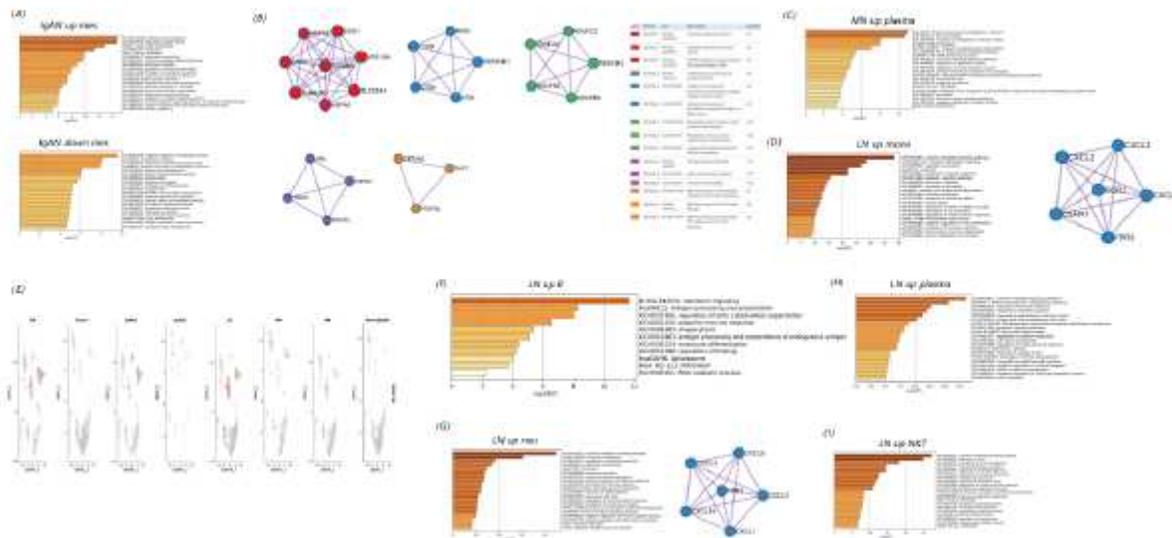


Figure 4

The activated signaling pathway at cellular level. (A) As immune complex was deposited in mesangial cell of IgAN, the up-regulated genes in mesangial cell of IgAN comparing with that of donor were enriched in peptide chain elongation, respond to growth factor and ossification, while the down-regulated genes were enriched in negative regulation of hydrolase activity, respond to decreased oxygen level and protein processing in ER. (B) The interaction of down regulated genes was enriched in oxidation respiratory chain (showed in green) and muscle contraction (showed in purple). (C) As humoral immunity was activated in MN, we want to see the up regulated genes of plasma, and B cell was indeed activated in MN. While in LN sample, interferon signaling was activated in monocyte (D), B (F), plasma (H), neutrophil (G) and NKT (I) (13). TNFSF13B was gathered in monocyte of LN (E).

Supplementary Files

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