Microarray-Based Analysis of the Complements in Kidney Reveals a Therapeutic Target in Lupus Nephritis

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Abstract

Background: To screen abnormal pathways and complement components in the kidney of lupus nephritis (LN), and determine a local C3 related therapeutic target in LN.

Methods: KEGG and GO enrichment assay were used to analyze the kidney microarray data of LN patients and NZB/W mice. Immunohistochemistry and immunofluorescence assays were used to measure renal expression of complement-related proteins and TGFβ1. Cytokines were measured using RT-qPCR and ELISA.

Results: We screened the local pathogenic pathways shared by LN patients and NZB/W mice in the kidney and selected the complement activation pathway for further study. We found greater expression of C1QA, C1QB, C3、C3AR1 and C5AR1 at the mRNA and protein levels. C3 is a key factor of the disease and the downstream of C1 is inhibited in the kidney. There were significant correlations between the expression of TGFβ1 and C3 in LN. In addition, our analysis of the primary cell-cultures indicated that TGFβ1 promoted the expression of C3 and that a TGFβ1 antagonist decreased the levels of C3 and C3AR in LN. TGFβ1 inhibition significantly inhibited the deposition of complement-related factors in the kidneys of NZB/W mice.

Conclusions: At the onset of LN, there was significant renal up-regulation of C3 and other complement pathway-related factors in the kidneys of human and NZB/W mice. C3 may lead to albuminuria and participate in the pathogenesis of LN. TGFβ1 promotes C3 synthesis, and TGFβ1 inhibition may block the progression of LN by inhibiting the synthesis of C3 and other complement components.

1. Background

Lupus nephritis (LN) is one of the most common and serious complications of systemic lupus erythematosus (SLE) [1], and a 2012 systematic review reported that 40 to 82% of Asian patients with SLE also had LN [2]. LN is a major cause of renal failure in SLE patients, and it directly increases their morbidity and mortality [3]. However, the mechanism of LN is not completely clear, in-depth investigation of LN's molecular pathogenesis will lay foundation for its precision therapy. Given the lack of sequential biopsies from patients, it remains essential to study informative mouse models of LN. Cross-species analyses can identify genes or pathways that are relevant to human disease and can be further studied in mouse models. Therefore, we screened the local pathogenic pathways in the kidneys of LN patients and NZB/W model mice, and selected the common complement activation pathway of LN patients and mice for further study.

The *in situ* deposition of immune complexes from the circulatory system or kidney may promote the accumulation of inflammatory cells, and then cause kidney damage [4, 5]. The liver produces most complement components (C1 to C9), but recent studies reported production by the kidneys, brain, blood vessels, lungs, and other organs [6, 7]. Renal tubules and renal podocytes directly produce most of the C3 [8, 9], which is then cleaved into multiple fragments (C3A, C3B, and C3C). C3 plays an important role in the classical, lectin-mediated, and alternative immune pathways [10]. C3A is an allergic toxin that stimulates mast cell degranulation after binding to its receptor C3AR; it also has chemotactic and antimicrobial activity and functions in inflammatory responses [11].

Immunofluorescence studies indicated abundant deposition of TGFβ and fibrin in the renal tissues of patients with LN [12]. Our previous research found excessive activation of the TLR9/TGFβ1/PDGF-B pathway in the peripheral blood of patients with SLE, and that TGFβ1 promoted the formation of platelet-derived growth factor subunit B (PDGF-B) [13]. In patients with glomerulonephritis, TGFβ1 and PDGF-B are important mediators of extracellular matrix (ECM) accumulation, glomerular fibrosis, and mesangial cell proliferation [14–18]. Other research reported that the interaction between TGFβ1 and complement exacerbates epithelial damage in pulmonary fibrosis [19]. However, the relationship of TGFβ1 with different complements during the pathogenesis of LN is unclear.

We selected patient samples combined with model mice and mainly studied the local changes in the kidney. Our general finding was that excessive activation of the complement pathway with up-regulation of specific complement components, including C3, occurs during the pathogenesis of LN. Moreover, TGFβ1 promotes the expression of C3 at the mRNA and protein levels, and a TGFβ1 inhibitor (SB431542) significantly inhibits the expression of C3 and renal complement deposition in a mouse model of SLE.

2. Methods

2.1 Patient data and samples

Complete clinical data were collected from newly diagnosed SLE patients from July 2018 to December 2019 at the first Hospital of Jilin University (Additional file 1: Supplementary Table S1). All patients met at least 4 of the 11 diagnostic criteria that were revised by the American College of Rheumatology (ACR) for SLE from the American Rheumatology Association [20]. LN was defined as 24-h urinary protein more than 0.5 g/day or 3 + on a dipstick test. There were 69 cases of SLE only and 76 cases of LN. Blood samples were collected before onset of

treatment. Blood samples from 47 healthy persons matched for age and sex (control group) were also collected. Peripheral blood mononuclear cells (PMBCs) were isolated from whole blood by density gradient centrifugation.

The renal tissues of 23 patients with LN were collected and classified according to the International Society of Nephrology/Renal Pathology (ISN/RPS) 2003 criteria [21]. Renal tissues were also collected from 13 controls. These tissues were from kidney donor before kidney transplantation (n = 6) or normal tissues distal to kidney tumors in patients (n = 7).

2.2 Ethics approval and consent to participate

Ethical approval for this study was received from the Institutional Medical Ethics Review Board of the First Hospital of Jilin University (reference number: 2017-087) and all procedures were in compliance with the Declaration of Helsinki.

2.3 Microarray analysis

Microarray analysis of gene expression profiles were based on data from the GEO database [22]. Kidney tissues were from the whole kidneys of NZB/W mice (accession number: GSE32583) or human glomerulus and renal tubule tissues (accession number: GSE32591). Mice were divided into three groups: control (16-week-old NZB/W mice without disease, n = 8); LN1 (23-week-old NZB/W mice with proteinuria and glomerulonephritis, n = 6); and LN2 (36-week-old NZB/W with proteinuria and glomerulonephritis, n = 10). Details are provided at the GEO, https://www.ncbi.nlm.nih.gov/geo/geo2r/?acc=GSE32583. The samples of human glomeruli and renal tubules were from renal biopsies. The glomerular samples were divided into two groups: Ctrl-GLO (glomeruli from control living donors, n = 14); and LN-GLO (glomeruli from LN patients, n = 32). Renal tubule samples were divided into two groups: Ctrl-Tub (tubulointerstitium from control living donors, n = 15); and LN-Tub (tubulointerstitium from LN patients, n = 32). Details are provided at the GEO, https://www.ncbi.nlm.nih.gov/geo/geo2r/?acc=GSE32591. The standard of a p-value below 0.05 for a fold-change of more than 1.2 was used for differential gene analysis.

2.4 Immunohistochemistry and Immunofluorescence staining

Immunohistochemistry assay: Kidney tissue was fixed in 10% neutral formalin solution, embedded in paraffin, and then dewaxed and sliced. After incubation at room temperature with an oxidase blocking solution, animal serum was added, and then the primary antibody (C3, C3AR, C5, C5AR1, C1Q, or TGFβ1; Bioss Biotechnology Co. LTD, Beijing, China) was added for overnight incubation. Then the secondary antibody and hematoxylin were added for visualization. A cell staining score was determined using Image J software [23].

Immunofluorescence assay: Kidney tissue was embedded in OCT (optimal cutting temperature compound), frozen and sectioned. After dried at room temperature, sections were stained with Complement C3 Antibody 11H9 (Cat. # NB200-540AF594, Novus Biologicals, shanghai, China, Alexa Fluor 594). Incubate the sections with Complement C3 Antibody (1.1mg/ml,1: 200) for 1h at 37 ℃ without light. After wash and dry, add 50 μl Hoechst 33342 (5mg/ml, Sigma-Aldrich, Germany) and incubate for 20min at 37 ℃ without light for nucleus staining. The section was finally sealed and observed under laser confocal microscope.

2.5 ELISA

A human complement protein C3 ELISA kit (#CSB-E08665h, Wuhan Huamei, China) was used according to the manufacturer's instructions. A human TGFβ1 ELISA kit was used following a previously described procedure [13]. A human TGFβ1 ELISA kit (#EK0513, Boster, China) was used to detect TGFβ1 in urine of patients following the manufacturer's instructions.

2.6 Real-time quantitative PCR

Cells were treated with the TRIzol reagent (Invitrogen, Carlsbad, California, USA), stored at − 80°C (5×10^6 cells/mL) for extraction of total RNA, and RT-qPCR was then performed as previously described [13].

2.7 Animal experiments

Female NZB/W mice were purchased from Jackson Laboratory (Bar Harbor, USA). Mice were randomly divided into two groups at the age of 16 weeks. One group received 4 cycles of 200 μL (1.5 mg/ml) intraperitoneal injections of SB431542 and the other group received the same routine with saline. Each treatment cycle consisted of an injection every other day for 1 week, followed by no injections for 3 weeks. The mice were sacrificed at 32 weeks of age, and kidneys were then harvested.

2.8 Cell culture

The mouse bone marrow was extracted under sterile condition, erythrocytes were lysed, washed and centrifuged. Mouse bone marrow cells were cultured using DMEM with penicillin (100 U/mL), streptomycin (100 μg/mL), and 10% FBS. These cells were divided into three groups: control; mouse recombinant TGFβ1 (2.5 ng/mL R&D Systems, Minneapolis, MN, USA); and SB431542 (5 μM; Sigma-Aldrich, St. Louis, MO, USA). Mouse fresh kidney was fully shredded to the size of 1 mm^3 with ophthalmic scissors. Add type I collagenase solution (0.1 U/ml) and shake for 60 min at 37 ℃. Single primary renal cells were obtained by filtration and cultured in RPMI1640 with medium penicillin (100 U/mL),

streptomycin (100 μg/mL), and 10% FBS. The cells were divided into three groups: control, mouse recombinant TGFβ1 (2.5 ng/ml) and SB431542 (5 μM). Human PBMCs were cultured in RPMI 1640 medium with penicillin (100 U/mL), streptomycin (100 μg/mL), and patient plasma (9:1 ratio of RPMI 1640: plasma). The cultured cells were divided into three groups: control; human recombinant TGFβ1 (7.5 ng/mL, R&D Systems, Minneapolis, MN, USA); and SB431542 (5 μM). The cells were collected after culturing in an incubator (37°C and 5% CO_2) for 5 h.

2.9 Statistical methods

GraphPad Prism 8.0 was used for statistical analysis and production of figures (GraphPad Software, San Diego CA, USA). To compare different matrices, all data between the maximum (red) and minimum (blue) values for each concentration were used to generate an unsupervised heat map. The Wilcoxon signed rank test was used to compare paired samples, the Mann-Whitney U test was used to compare unpaired samples, and the nonparametric Spearman rank correlation test was used to determine correlations. A p-value below 0.05 was considered significant.

3. Results

3.1 Activation of the complement pathway and up-regulation of local complement components in the kidneys of patients with LN

Our initial microarray analysis compared renal tissue samples from controls and patients with LN and identified 4754 differentially expressed genes. There were 2632 up-regulated genes and 2122 down-regulated genes in the glomeruli of LN patients. (Additional file 2: Supplementary Figure S1). A similar analysis indicated there were 3725 differentially expressed genes in the renal tubules, with 2359 up-regulated genes and 1366 down-regulated genes in the renal tubules of LN patients (Additional file 2: Supplementary Figure S1).

Using KEGG enrichment analysis, we found that there are 89 significantly up-regulated pathways in LN patients (Additional file 3: Supplementary Figure S2). We compared the differential pathways in patients and that in mice, selected some common differential pathways and showed them in Fig. 1A. This includes NF-kappa B signaling pathway, B cell receptor signaling pathway, Toll-like receptor signaling pathway, Complement and coagulation Cascades, T cell receptor signaling pathway, TGF-beta signaling pathway and so on.

Cluster analysis showed close similarities in the expression patterns of C1QA and C1QB and of C3AR1 and C5AR1, but the expression pattern of C3 was similar to C1QA and C1QB (Fig. 1A). Quantitation of these microarray data (Fig. 1B) indicated that LN patients had increased expression of C1QA, C1QB, C3, C3AR1, and C5AR1 in glomeruli, and increased expression of all these mRNAs except C5AR1 in renal tubules.

Next, we performed immunohistochemical analysis of the kidneys of LN patients and controls. The results indicated greater protein levels of C1Q, C3, C5, C3AR, C5AR1, and CR3 in the renal tubules of patients with LN (Fig. 2A). Quantitation of expression using cell staining scores from Image J software confirmed that these results were significantly different (Fig. 2B-G) in the renal tubules and glomeruli. These results indicate increased renal expression of complement proteins in patients with LN, possibly because of over-activation of the complement pathway.

3.2 Up-regulation of local complement components and activation of the complement pathway in the kidneys of NZB/W mice

We further analyzed the data of NZB/W mice. A comparison of whole kidney tissues before and at early-stage disease indicated there were 1725 differentially expressed genes, with 1375 up-regulated genes and 350 down-regulated genes in mice with early-stage disease (Additional file 2: Supplementary Figure S1). A comparison of whole kidney tissues before and at late-stage disease indicated 7966 differentially expressed genes, with 2863 up-regulated genes and 5103 down-regulated genes in the mice with late-stage disease (Additional file 2: Supplementary Figure S1).

Cluster analysis of complement-related factors in these mice indicated that the expression of C1QA, C1QB, C1QC, C3, C3AR1, C5AR1, CR3, CR4 in NZB/W mice gradually increased from before disease onset to early-stage disease, and to late-stage disease (Fig. 3A). The expression patterns of factors had some similarities to those in human glomeruli and renal tubules. In particular, the expression of C1QA and C1QB clustered together (as in humans), the expression of C1QC and C3AR1 clustered together. However, the expression of C3 clustered with C5AR1, and the expression pattern of C3AR1 was similar to C1QA, C1QB and C1QC.

Quantitation of these microarray data (Fig. 3B) indicated progressively increased expression of C1QA, C1QB, C1QC, C3, C3AR1, C5AR1, CR3, CR4 as disease progressed. In particular, C3 mRNA had significantly greater expression at late-stage disease than early-stage disease.

We also performed immunohistochemical analysis of C3 and C3AR on the kidneys of 5 NZB/W mice. The results indicated increased deposition of both proteins as disease progressed (Fig. 3C). These results confirmed that complement components may play an important role in the pathogenesis of LN in this mouse model.

To identify the upstream and downstream relationships of these significantly up-regulated genes (C1Q, C3, C3AR1, C5AR1, CR3, CR4), we performed KEGG pathway analysis based on the lupus mouse microarray database (Fig. 3D). The starting component C1qrs causes the activation of the downstream complement C3. Then C3 is cleaved into downstream factors (C3A, C3B, and C3C) which activate complement

C5. These different complements bind to receptors (C3AR1, C5AR1, CR3, etc.), inducing a series of responses, including phagocytosis, degranulation, and chemotaxis. These responses lead to the release of inflammatory mediators and kidney damage. Notably, we found significant up-regulation of C1Q, C3, CR3, CR4, C3AR1, and C5AR1. We also found up-regulation of C1INH (another name is SERPING1), which inhibits the down-stream signaling of classical pathway and lectin pathway. These results confirm that complement components function in the pathogenesis of LN.

3.3 Complement related up-regulated GO terms and elevated C3 levels in LN patients

GO enrichment analysis of biological process class help us to explore the biological functions of the differentially expressed genes. We chose the immune response related complement and coagulation cascades pathway for further study. The complement related up-regulated genes were depicted as directed acyclic graphs in Fig. 4A. The detailed information of the significantly increased complement related GO terms was listed in Table 1. The significantly up-regulated genes including serpin family G member 1 (SERPING1) and alpha-2-macroglobulin (A2M) were significantly enriched into the GO term of negative regulation of complement activation, lectin pathway (GO:0001869) in the kidney of both LN patients and NZB/W mice. This result suggests that the downstream signaling of C1 is inhibited in the kidney of LN patients. The significantly up-regulated genes including formyl peptide receptor 1 (FPR1), formyl peptide receptor 2 (FPR2), and complement C3A receptor 1 (C3AR1) were significantly enriched into the GO term of complement receptor mediated signaling pathway (GO:0002430) in the kidney of both LN patients and NZB/W mice. This result suggests that C3A is a key pathogenic factor in the kidneys of LN patients. The up-regulation of the term of regulation of complement activation (GO:0030449) was significant only in LN patients and the up-regulation of the term of complement activation, alternative pathway (GO:0006957) was significant only in NZB/W mice.

Table 1
Details of the four significantly different GO terms associated with complement

Species	GO_ID	GO_Name	Population_ mapped_id	Study_ mapped_id	Gene_symbol	Description	Enrichment	p_value	FDR
Human	GO:0001869	negative regulation of complement activation, lectin pathway	2	2	SERPING1, A2M	serpin family G member 1, alpha-2-macroglobulin	14.158	0.005	0.062
Mouse	GO:0001869	negative regulation of complement activation, lectin pathway	2	2	Serping1, A2m	serine (or cysteine) peptidase inhibitor, clade G, member 1, alpha-2-macroglobulin	21.701	0.002	0.019
Human	GO:0002430	complement receptor mediated signaling pathway	9	3	FPR1, FPR2, C3AR1	formyl peptide receptor 1, formyl peptide receptor 2, complement C3a receptor 1	4.719	0.021	0.153
Mouse	GO:0002430	complement receptor mediated signaling pathway	11	4	Fpr1, C3ar1, Fpr2, C5ar1	formyl peptide receptor 1, complement component 3a receptor 1, formyl peptide receptor 2, complement component 5a receptor 1	7.891	0.001	0.013
Human	GO:0030449	regulation of complement activation	29	7	C5AR1, C3, C3AR1, C1QBP, CFH, CFP, PROS1	complement C5a receptor 1, complement C3, complement C3a receptor 1, complement C1q binding protein, complement factor H, complement factor properdin, protein S (alpha)	3.417	0.003	0.052
Mouse	GO:0006957	complement activation, alternative pathway	10	3	C3, Cfh, Cfp	complement component 3, complement component factor h, complement factor properdin	6.510	0.009	0.055

In order to further explore the role of C3 in the pathogenesis of LN, we examined the expression of C3 in the urine, blood, and kidney tissues of LN patients. The results indicated that LN group had a significantly higher urinary level of C3 than the healthy control group and the SLE group (non-LN patients) ($p < 0.001$; Fig. 4B). However, the plasma of the LN group had a decreased level of C3 ($p < 0.001$; Fig. 4C) relative to the SLE group (non-LN patients). This presumably indicates that C3 was excreted from the kidneys of patients with LN. Analysis of the relationship between urinary C3 and 24-h urinary protein indicated a significantly positive correlation ($p < 0.0001$, $r = 0.6346$; Fig. 4D). The plasma of the LN group had a decreased level of C1Q compared to the SLE group (non-LN patients) ($p < 0.05$; Additional file 4: Supplementary Figure S3 A), but no significant change was observed on the level of C4 between the two groups ($p > 0.05$; Additional file 4: Supplementary Figure S3 B). Significant correlations were observed between the plasma levels of C3 and that of C1Q/C4 ($p < 0.0001$, $r = 0.6345$; $p < 0.0001$, $r = 0.5261$; Figure

S3 C/D). However, there is no significant correlation between C1Q/C4 and 24-h urine protein ($p > 0.05$; Additional file 4: Supplementary Figure S3 E/F). These results suggest that an elevated level of C3 may contribute to the pathogenesis of LN and proteinuria. Our immunohistochemistry analysis indicated that the expression of C3 was similar in LN patients with class Ⅱ, Ⅲ, Ⅳ+V disease, but expression was greater in those with class Ⅴ LN than healthy controls (Additional file 5: Supplementary Figure S4).

3.4 Up-regulation of TGFβ1 and C3 occurs during the pathogenesis of LN

Our research team previously found a correlation between the level of TGFβ1 and C3 in whole blood cells of SLE patients [24], and proposed that the levels of TGFβ1 and C3 may have similar changes in urine and blood of patients with LN. We found the plasma of the LN group had a decreased level of TGFβ1 relative to the SLE without LN group ($p < 0.05$; Fig. 5A). Our measurements of TGFβ1 and C3 in patients with LN indicated positive correlations in the levels of these proteins ($p = 0.0150$, $r = 0.3916$; Fig. 5B) and mRNAs ($p = 0.0014$, $r = 0.6949$; Fig. 5C) in blood. However, LN group had a significantly higher urinary level of TGFβ1 than the SLE without LN group ($p < 0.001$; Fig. 5D). Analysis of the relationship between urinary TGFβ1 and 24-h urinary protein indicated a significantly positive correlation ($p < 0.0001$, $r = 0.7120$; Fig. 5E). In addition, we also found that the levels of TGFβ1 significantly correlated with that of C3 in urine of the patients ($p = 0.0054$, $r = 0.5115$; Fig. 5F).Our renal immunohistochemistry results (Fig. 5G), also indicated a correlation of cell staining scores for TGFβ1 and C3 ($p = 0.0149$, $r = 0.7714$; Fig. 5H). These findings strongly suggest that TGFβ1 and C3 function in the pathogenesis of LN.

3.5 TGFβ1 upregulates C3

To confirm that TGFβ1 regulates the expression of C3, we cultured NZB/W mouse bone marrow cells, added TGFβ1 or SB431542 for 5 h, and then harvested cells for extraction of RNA and quantitation using RT-qPCR. The results indicated that TGFβ1 significantly increased the expression of C3 mRNA and that SB431542 significantly decreased the expression of this mRNA ($p < 0.001$; Fig. 6A). Interestingly, SB431542 also decreased the level of C3AR1 mRNA ($p < 0.001$; Fig. 6A), although TGFβ1 had no significant effect on this mRNA ($p > 0.05$; Fig. 6A). However, TGFβ1 and SB431542 had no significant effect on the level of C1Q mRNA ($p > 0.05$; Fig. 6A). At the same time, we used the same method to culture NZB/W mouse kidney primary cells. SB431542 also decreased the level of C3 mRNA ($p < 0.05$; Fig. 6B) and C3AR1 mRNA ($p < 0.001$; Fig. 6B),although TGFβ1 had no significant effect on these mRNA ($p > 0.05$; Fig. 6B). TGFβ1 and SB431542 have no significant effect on C1Q mRNA ($p > 0.05$; Fig. 6B).

We performed similar experiments using the PBMCs from SLE patients. TGFβ1 and SB431542 had identical effects on C3 mRNA ($p < 0.01$; Fig. 6C). However, analysis of C3AR1 indicated that TGFβ1 and SB431542 had no significant effect relative to the controls, although expression of C3AR1 mRNA was greater in cells given TGFβ1 than SB431542 ($p < 0.05$; Fig. 6C). TGFβ1 and SB431542 have no significant effect on C1Q mRNA ($p > 0.05$; Fig. 6C).

In addition, we administered SB431542 or saline to NZB/W mice for 3 months. The results indicated that SB431542 significantly reduced the levels of C1Q, C3, C5, C3AR, C5AR1, and CR3 in the kidneys of these mice (Fig. 6D). To confirm these results, we performed immunofluorescence staining on C3 using a different antibody. NZB/W mouse kidney immunofluorescence staining also indicated that SB431542 significantly reduced the levels of C3 (Fig. 6E).

4. Discussion

In the current study, by screening and analyzing the kidney genome data of LN patients and NZB/W mice, we found the pathogenesis related pathways and selected the complement activation pathway for further study. We observed the activation of various complement related factors and GO terms in patients and analyzed their roles in the pathogenesis of LN. We found that C3 is key factor of LN in the kidney. TGFβ1 can promote the synthesis of C3 in both NZB/W mice and LN patients, but has no effect on C1Q. TGFβ1 inhibitor SB431542 significantly inhibited C3 synthesis in primary kidney cells and peripheral blood cells, and improved the local complement deposition in the kidney of NZB/W mice.

In order to lay the foundation for the precise treatment of LN, we selected differential pathways and genes shared by patients and mice, so as to facilitate the in-depth study of drug administration in mice *in vivo*. We analyzed the mRNA expression of complement related factors in the kidney tissue of LN patients and NZB/W mice by bioinformatics. KEGG enrichment analysis showed that there was a common differential expression pathway of complex and coagulation cascades in LN patients and NZB/W mice. The present study is the first to use big data from microarray analysis to demonstrate that the mRNA levels of multiple complements (C1QA, C1QB, C3) and complement receptors (C3AR1 and C5AR1) were up-regulated in glomeruli and renal tubules of the kidneys of patients with LN. We found similar results in kidney tissue specimens of NZB/W mice. Moreover, as disease progressed in these mice, the levels of C3 and C1Q (C1QA, C1QB, C1QC), C3, C3AR, C5AR1, CR3 and CR4 continued to increase. The deposition of complement related factors including C1Q, C3, C3AR, C5, and CR3 was confirmed by immunohistochemistry in the kidneys of LN patients. Further analysis of these mice indicated significant up-regulation of C1Q mRNA (an upstream component) as well as C3 and its receptors C3AR1, CR3, CR4 (downstream components). Therefore, complement factors and pathways in the kidneys of LN patients are up-regulated.

Previous studies of mouse models of lupus (NZB/W and MRL/*lpr* mice) reported increased renal expression of complement mRNA and protein[25, 26]. Moreover, complement inhibitors reduce proteinuria and prevent the deterioration of renal function [25, 26]. Excessive activation of the renal complement system can cause renal tubular interstitial cytotoxicity, in which renal tubular epithelial cells are a key target of C3, resulting in proteinuria and promotion of renal fibrosis [27–29]. These previous interpretations are consistent with our results, and taken together confirm the presence of activation of the local complement pathway in the kidneys of patients with LN.

GO enrichment analysis showed activated immune response cascade in LN patients and NZB/W mice. The up-regulated FPR1, FPR2, and C3AR1 were significantly enriched into the GO term of complement receptor mediated signaling pathway in the kidney of both LN patients and NZB/W mice. FPR1 and FPR2 are Gi-protein-coupled receptors that are expressed mainly by mammalian phagocytic leukocytes. FPRs are involved in antibacterial host defense and inflammation. C3AR1 is a receptor for the chemotactic and inflammatory peptide anaphylatoxin C3A. This result suggests that C3 is a key pathogenic factor in the kidneys of LN patients. C3 functions as the core of the three pathways of complement system activation, and forms a C5b-9 membrane attack complex that attacks kidney tissue through a cascade reaction [30]. This leads to the release of proinflammatory mediators and other factors (e.g., reactive oxygen species and tissue degradation proteases) that damage endothelial cell surfaces and the glomerular basement membrane, destroy glomerular podocyte foot processes, and disrupt kidney filtration processes, ultimately leading to proteinuria, kidney tissue damage, and then LN [30].

We found that the reduced blood level of C3 in patients with LN was associated with an increased level of C3 in their urine. Liu et al. [31] reported that a lower serum level of C3 was associated with a higher risk of focal segmental glomerulosclerosis and end-stage renal disease. Our results also indicated that NZB/W mice had increasing renal expression of C3 and C3AR as disease progressed. This is consistent with previous reports that there is deposition of large amounts of C3 in the kidneys of patients with LN and in mouse models of this disease [32, 33]. Our finding of a significant decrease of plasma C3 in the presence of LN is consistent with previous clinical and animal experiments which reported that complement activation leads to secondary decreases in the blood levels of C4 and C3 during the active period of SLE [34]. We speculate that the loss of C3 through urine and deposition in the kidney are partly responsible for the decreased plasma level of C3 in LN.

Urinary protein is an important diagnostic indicator for LN. We found a correlation of urinary C3 level with 24-h urinary protein in patients with LN. Similar reports found that the increased level of complement in the urine of patients with LN was related to the overall level of urinary protein [35]. We also found that complement components downstream of the C3 pathway (C3AR and C5AR1) were significantly up-regulated in the kidneys of LN patients and NZB/W mice. In fact, there is evidence that C3A and C5A are powerful chemoattractants that guide neutrophils, monocytes, and macrophages to the site of complement activation, and thus promote inflammation [36]. Studies of the kidney tissues of patients with LN reported up-regulation of C3AR and C5AR at the mRNA and protein levels, and that C3AR and C5AR antagonists reduced the symptoms of LN [25, 26, 37].Through clinical data analysis on the patients' blood, we found that the levels of C1Q and C4 were positively correlated with that of C3. As an upstream factor, C1Q activates C3 with the help of C4 in the classical pathway. However, GO analysis showed that up-regulated SERPING1 and A2M were significantly enriched into the GO term of negative regulation of complement activation lectin pathway in the kidney of both LN patients and NZB/W mice. This result suggests that the downstream signaling of C1Q is significantly inhibited by SERPING1 and A2M in the kidney of patients. This may explain why C1Q and C4 have no significant correlation with 24-h urinary protein in LN patients. C1Q mediates a variety of immunoregulatory functions considered important in the prevention of autoimmunity such as the enhancement of phagocytosis and the regulation of cytokine production by antigen presenting cells [38]. The deficiency of C1Q strongly predisposes individuals to SLE development [39], which is thought to be related to the role of C1Q in the removal of apoptotic cells [40]. Lupus autoantigen on the surface of apoptotic cells is thought to stimulate an inappropriate immune response in SLE [41–43]. Decreased serum levels of C1Q protein and increased titres of C1Q antibodies may be involved in the pathogenesis of SLE, especially LN [44]. It was also found that the prognosis of the LN patients is poor when there is no C1Q deposition in the kidneys [45]. Taken together, these results support the conclusions that C3 is a key pathogenic factor in the kidneys of LN patients, and the downstream signaling of C1 is inhibited in the kidney of LN patients.

We looked for a factor other than C1, which can regulate the complement C3 in LN patients. We found that the levels of TGFβ1 decreased in the plasma and increased in the urine of LN patients. The levels of TGFβ1 in their urine were positively correlated with the levels of their urinary protein. Our analysis of the peripheral blood and kidney tissues of patients with LN indicated a correlation in the levels of TGFβ1 and C3 at the mRNA and protein levels. In addition, our analysis of primary cells-cultures of NZB/W mice indicated that TGFβ1 promoted the expression of C3 and that a TGFβ1 antagonist (SB431542) decreased the levels of C3 and C3AR.Similar effects on C3 were also observed in the PBMCs of patients. In agreement, People found that TGFβ1 modulates C3 in cultured human monocytes [46]. Another research group observed that the serum TGFβ1 level had a positive correlation with eGFR, C3, and C4 in SLE patients [47]. These reports are consistent with our findings. Therefore, these data support the effect of TGFβ1 on C3 and on the process of LN.

To examine the possible therapeutic effects of a TGFβ1 inhibitor, we treated NZB/W mice with a TGFβ1 antagonist (SB431542). This inhibitor markedly decreased the renal expression of C3 and C3AR and other complement components *in vivo*. Administration of a TGFβ1 inhibitor to patients with LN may reduce the deposition of immune complexes, the inflammatory response, and renal fibrosis. Although many previous

studies examined the effect of TGFβ1 inhibition as treatment for various diseases, use of this approach for treatment of LN requires further research.

Our research has several limitations. First, our studies of NZB/W mouse kidney tissues indicated increased C3 deposition as disease progressed. However, because renal puncture is an invasive operation, we cannot track the deposition of C3 in human kidneys during the progression of LN. Thus, it is uncertain whether C3 gradually increases in the kidneys of LN patients as disease progresses. Second, although we found a correlation of C3 and TGFβ1 expression, we did not demonstrate a causal relationship. However, previous studies found that the combination of C3A and C3AR upregulated the expression of TGFβ1, thereby promoting the progression of pulmonary fibrosis [48]. We need further research to confirm that C3 increases TGFβ1 expression in patients with LN.

To sum up, we found that the complement factors and pathways are up-regulated in the kidneys of LN patients. C3 is a key factor of the disease and the downstream of C1Q is inhibited in the classical complement pathway of the kidney. TGFβ1 up-regulates C3 to participate in the pathogenesis of LN. A suitable TGFβ1 inhibitor may help to inhibit the deposition of complement components in the kidney and provide effective treatment of LN.

5. Conclusions

There was significant renal up-regulation of C3 and other complement pathway-related factors in the kidneys of human and NZB/W mice. C3 may participate in the pathogenesis of LN and lead to albuminuria. TGFβ1 promotes C3 synthesis, and TGFβ1 inhibition may block the progression of LN by inhibiting the synthesis of C3 and other complement components.

Abbreviations

ACR, American College of Rheumatology;

C1, Complement C1;

C3, Complement C3;

C3AR1, Complement C3a Receptor 1;

C5AR1, Complement C5a Receptor 1;

CR3, Complement Component 3 Receptor 3;

CR4, Complement Component 3 Receptor 4;

ECM, Extracellular Matrix;

ELISA, Enzyme Linked Immunosorbent Assay;

FBS, Fetal Bovine Serum;

FPR, Formyl Peptide Receptor;

GO, Gene Ontology;

KEGG, Kyoto Encyclopedia of Genes and Genomes;

LN, Lupus Nephritis;

PBMC, Peripheral Blood Mononuclear Cell;

PDGF-B, Platelet-Derived Growth Factor B;

qPCR, Real-time quantitative PCR;

SLE, Systemic Lupus Erythematosus;

SLEDAI, SLE Disease Activity Index;

TGFβ, Transforming Growth Factor-β;

TLR9, Toll-like Receptor 9

Declarations

Ethics approval and consent to participate

Ethical approval for this study was received from the Institutional Medical Ethics Review Board of the First Hospital of Jilin University (reference number: 2017-087) and all procedures were in compliance with the Declaration of Helsinki.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

TL collected the clinical samples, contributed to experiments and wrote the manuscript; MY performed experiments, did the statistical analysis and created figures and tables; YX is involved in cell-culture; CJ performed mouse experiments; CL is involved in RT-qPCR experiments; ZJ provided clinical samples and analyzed clinical information; XW designed the study, analyzed data, completed and revised the manuscript; all of the authors read and approved the manuscript.

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Figures

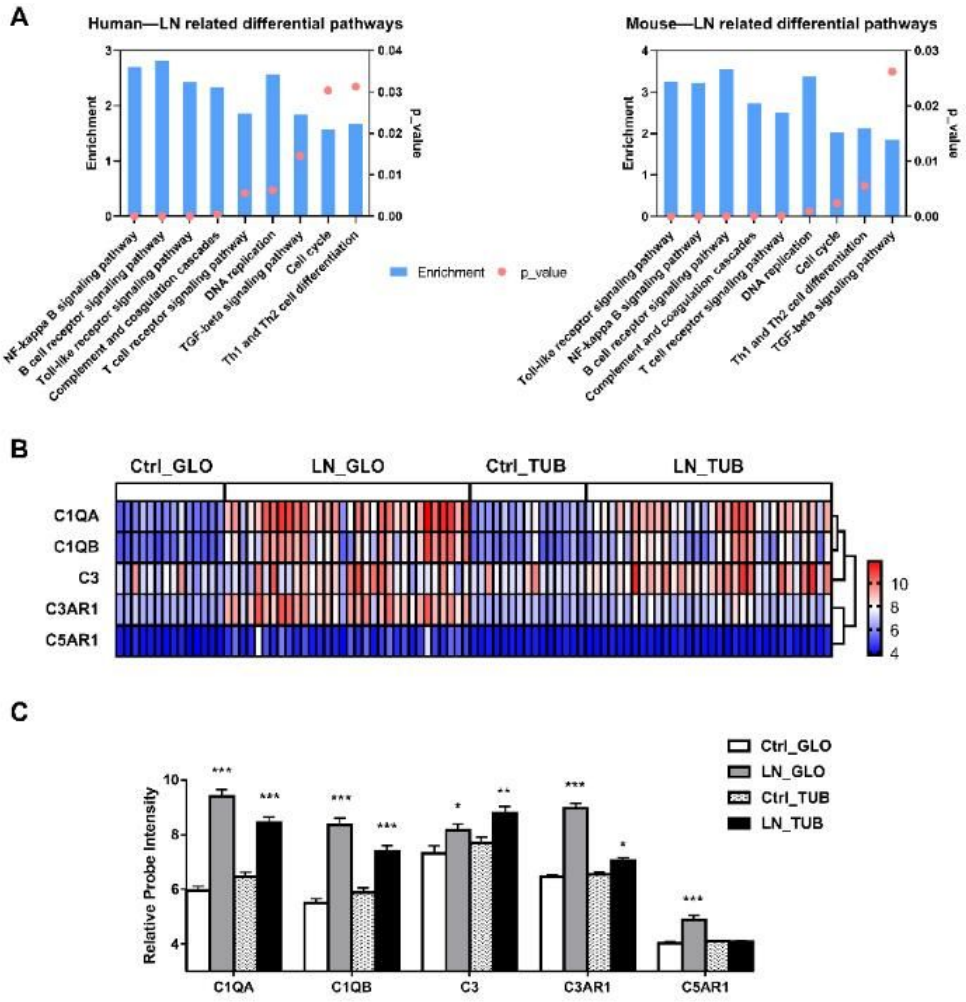

Figure 1

Renal pathways and complement genes in LN patients and healthy controls based on microarray results. (A) The statistical graph of enrichment degrees and p-values of some upregulated KEGG pathways on the kidneys of LN patients and NZB/W F1 mice. (B) Cluster analysis showing expression patterns of C1QA, C1QB, C3, C3AR1 and C5AR1 in glomerulus (GLO) or renal tubule (TUB) in each group. (C) mRNA levels of C1QA, C1QB, C3, C3AR1 and C5AR1 in renal tubules of patients with LN (N = 32) and healthy controls (N = 15). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

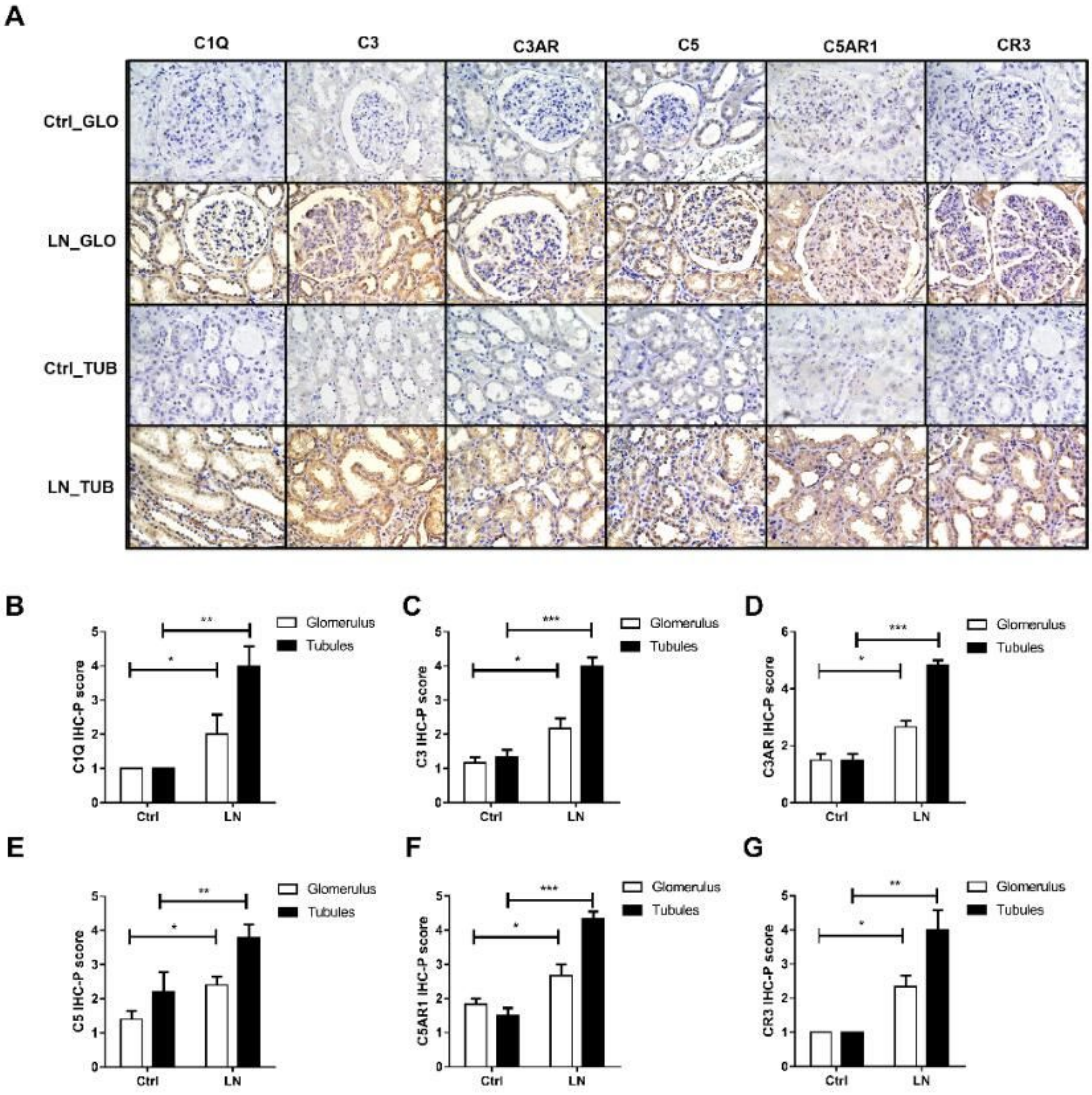

Figure 2

Distribution of the complement components in glomerulus and renal tubules of patients with LN or healthy controls based on immunohistochemical analysis. (A) Expression levels of C1Q, C3, C5, C3AR, C5AR1 and CR3 in glomeruli (GLO) and renal tubules (TUB) of patients with LN and healthy controls. (B) The IHC-P (Immunohistochemistry -Paraffin section) scores of C1Q (N = 3, $p < 0.01$). (C) The IHC-P scores of C3 (N = 6, $p < 0.001$). (D) The IHC-P scores of C3AR (N = 5, $p < 0.01$). (E) The IHC-P scores of C5 (N = 6, $p < 0.001$). (F) The IHC-P scores of C5AR1 (N = 6, $p < 0.001$). (F) The IHC-P scores of CR3 (N = 3, $p < 0.01$). The IHC-P scores was quantitatively analyzed using imageJ software.

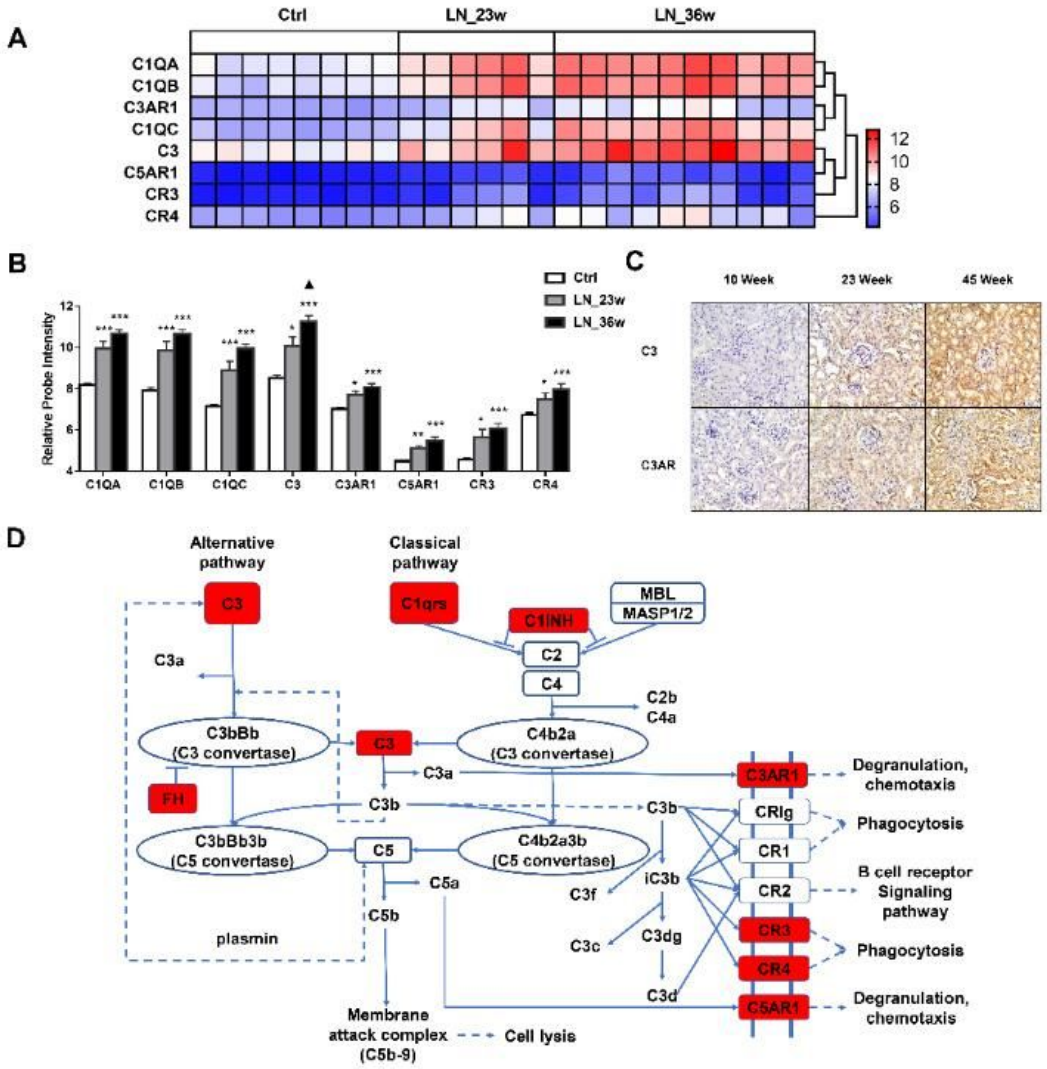

Figure 3

Expression levels of the complement components in NZB/W mice before and after the onset of LN. (A) Cluster analysis results showing expression patterns of C1QA, C1QB, C1QC, C3AR1, C3, C5AR1, CR3 and CR4 in control group (16w, N = 8), early stage group (23w, N = 6) and late stage group (36w, N = 10). (B) mRNA levels of the complement components in the kidney from each group (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ▲ represents the significant difference between late stage and early stage). (C) Immunohistochemical analysis of C3 and C3AR in the kidney of NZB/W mice. (D) Complement activation pathway analysis based on kidney microarray results of the LN patients and NZB/W mice, the red boxes show the significantly up-regulated genes.

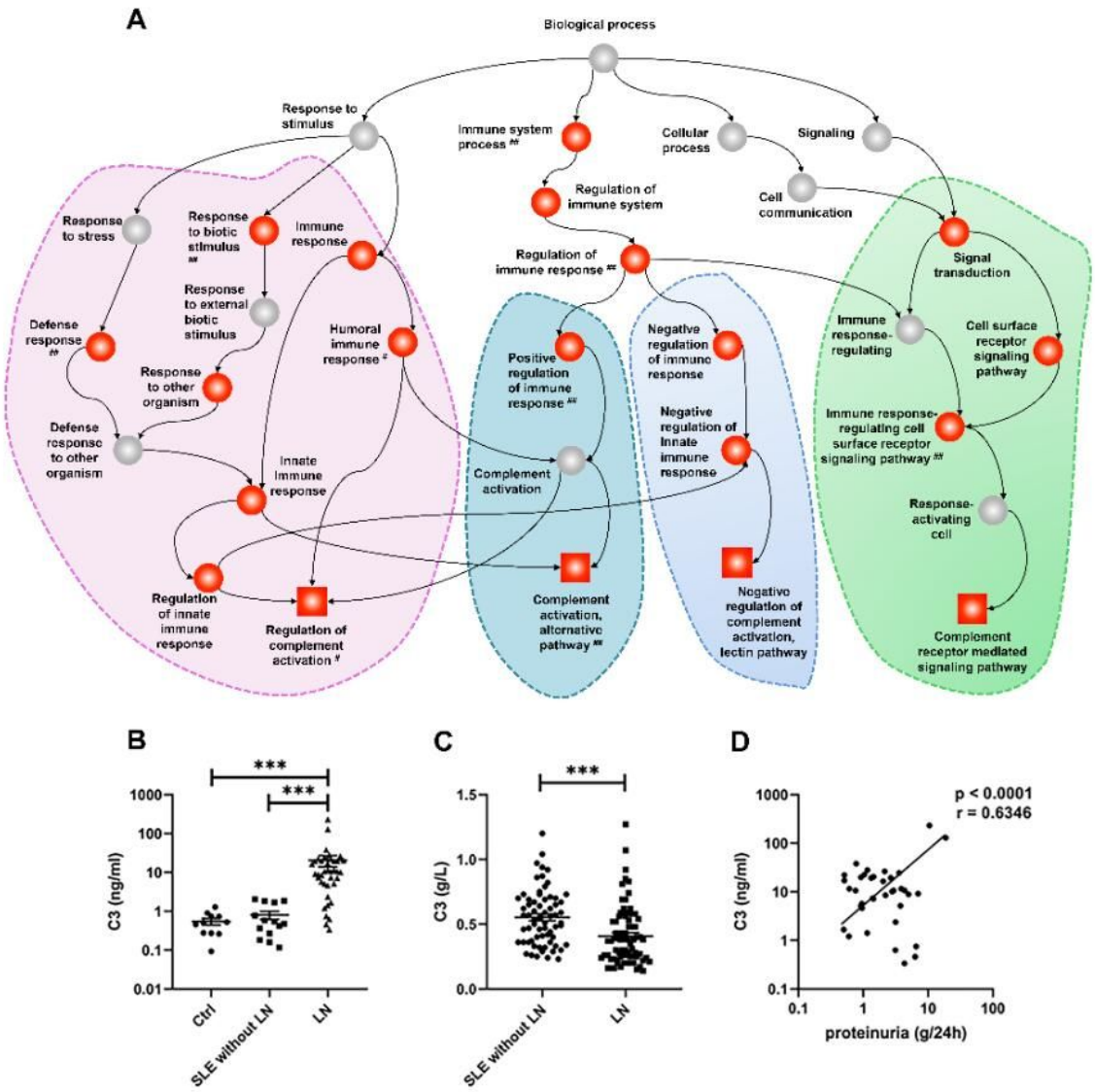

Figure 4

GO enrichment analysis and the levels of complement C3 in LN patients. (A) Directed acyclic graph of GO enrichment of up-regulated genes of LN patients and NZB/W mice in biological process, the squares represent the complement-related GO terms; red squares or red circles represent significant enrichment; # represents significant enrichment found only in LN patients but not in NZB/W mice; ## represents significant enrichment found only in NZB/W mice but not in LN patients; the unmarked terms have significant enrichment in both LN patients and NZB/W mice. (B) The levels of C3 in the urine of healthy control (N = 11), SLE patients without LN (N = 14) and patients with LN (N = 39). *** $p < 0.001$. (C) The levels of C3 in the plasma of SLE patients without LN (N = 66) and patients with LN (N = 76). *** $p < 0.001$. (D) Correlation between the urine levels of C3 and the levels of 24-h urine protein (N = 37).

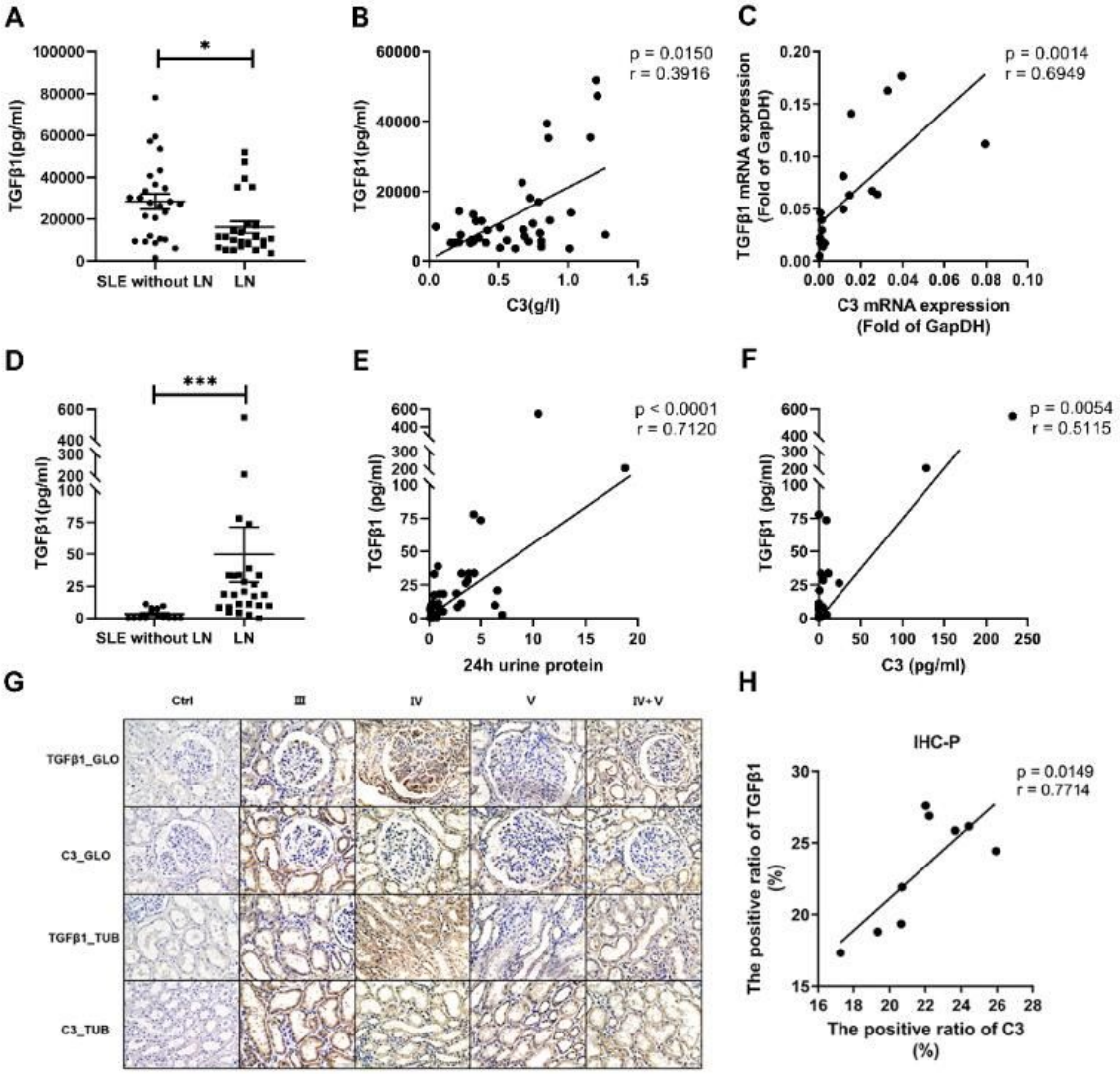

Figure 5

Relationships between the levels of TGFβ1 and C3 in LN patients. (A) The levels of TGFβ1 in the plasma of SLE patients without LN (N = 26) and patients with LN (N = 25). * $p < 0.05$. (B) Correlation analysis of the protein levels of TGFβ1 and C3 in plasma of LN patients (N = 38). (C) Correlation analysis of the mRNA levels of TGF β1 and C3 in PBMC of LN patients (N = 18). (D) The levels of TGFβ1 in the urine of SLE patients without LN (N = 14) and patients with LN (N = 26). *** $p < 0.001$. (E) Correlation between the levels of TGFβ1 and the levels of 24-h urine protein of patients with LN (N = 26). (F) Correlation analysis of the levels of TGFβ1 and C3 in urine of LN patients (N = 26). (G) Immunohistochemical analysis on the levels of TGFβ1 and C3 in glomeruli (GLO) or renal tubules (TUB) of patients with different classes of LN. (H) Correlation analysis on the levels of TGFβ1 and C3 in the kidney of patients with LN (N = 9).

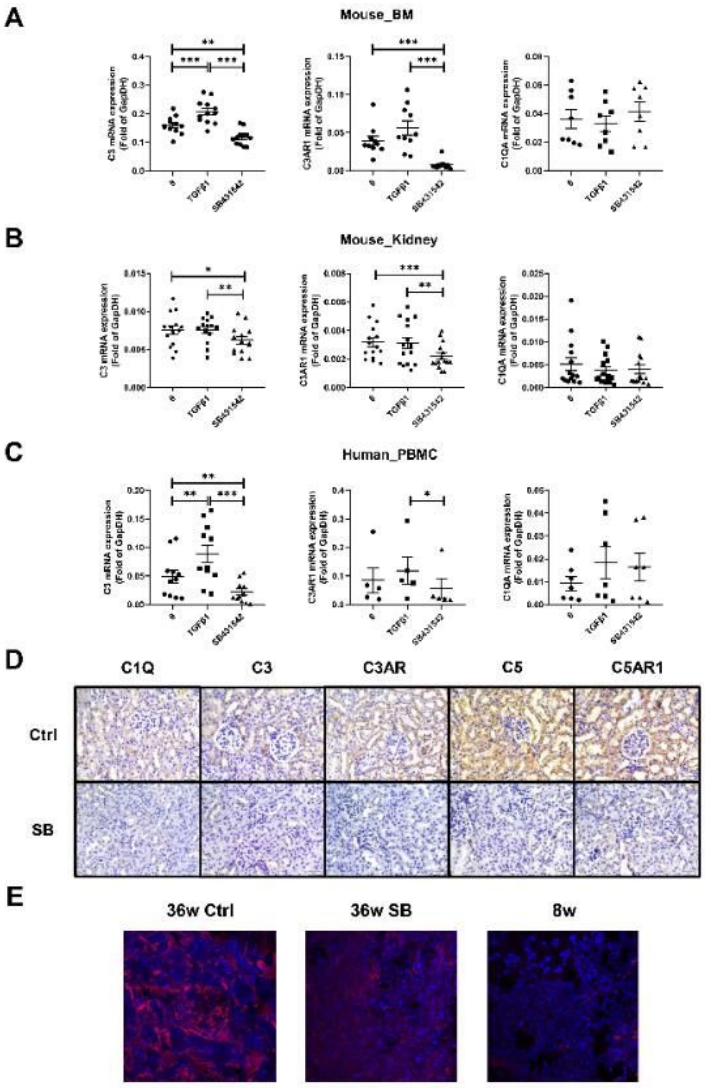

Figure 6

The effects of TGFβ1 on the production of C3 and other complement components. (A) mRNA levels of C3 (N = 12), C3AR1 (N = 10) and C1QA (N = 8) in the presence of TGFβ1 or SB431542 in bone marrow cell-cultures of NZB/W mice. (B) mRNA levels of C3 (N = 14), C3AR1 (N = 15) and C1QA (N = 14) in the presence of TGFβ1 or SB431542 in kidney primary cell-cultures of NZB/W mice. (C) mRNA levels of C3 (N = 11), C3AR1 (N = 5) and C1QA (N = 7) in the presence of TGFβ1 or SB431542 in PBMC-cultures of LN patients. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. (D) Immunohistochemical analysis of C1Q, C3, C5 and their receptors (C3AR, C5AR1) in the kidneys of NZB/W mice treated with SB431542 or saline as control. (E) Immunofluorescence analysis of C3 in kidney of NZB/W mice of 36 weeks old treated with SB431542, 36 weeks old treated with saline and 8 weeks old. The red fluorescence represents C3 and the blue fluorescence represents nucleus.

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