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RENAL C3 COMPLEMENT COMPONENT: FEED FORWARD TO DIABETIC KIDNEY DISEASE

Katherine. J. Kelly, MD, MSc[1], **Yunlong Liu, PhD**[2], **Jizhong Zhang, PhD**[1], and **Jesus H. Dominguez, MD**[1,3]

[1]Department of Medicine, Indiana University School of Medicine, Indianapolis, IN

[2]Department of Medical and Molecular Genetics, Indiana University School of Medicine, Indianapolis, IN

[3]Roudebush Veterans' Affairs Medical Center, Indianapolis, IN

Abstract

Background—Diabetic nephropathy is the main cause of end-stage renal disease and has reached epidemic proportions.

Methods—Comprehensive genomic profiling (RNA Seq) was employed in the ZS (F_1 hybrids of Zucker and spontaneously hypertensive heart failure) model of diabetic nephropathy. Controls were lean littermates.

Results—Diabetic nephropathy in obese, diabetic ZS was accelerated by a single episode of renal ischemia (DI). This rapid renal decline was accompanied by activation of the renal complement system in DI, and to a lesser extent in sham operated diabetic rats (DS). In DI there were significant increases in renal mRNA encoding C3, C4, C5, C6, C8 and C9 over sham operated lean normal controls (LS). Moreover, mRNAs encoding the receptors for the anaphylatoxins C3a and C5a were also significantly increased in DI compared to LS. The classic complement pathway was activated in diabetic kidneys with significant increases of C1qa, C1qb, and C1qc mRNAs in DI over LS. In addition, critical regulators of complement activation were significantly attenuated in DI and DS. These included mRNAs encoding CD55, decay accelerating factor, and CD59, which inhibits the membrane attack complex. C3, C4 and C9 proteins were demonstrated in renal tubules and glomeruli. The complement RNAseq data were incorporated into a gene network showing interactions among C3 generating renal tubular cells and other immune competent migratory cells.

Conclusions—We conclude that local activation of the complement system mediates renal injury in diabetic nephropathy.

Keywords

complement; diabetic nephropathies; ischemia; kidney failure; chronic

Address Correspondence to: K.J. Kelly, MD, MSc, Indiana University School of Medicine-Nephrology, 950 West Walnut Street, RII-202, Indianapolis, IN USA 46202, Telephone: 01-317-274-7453 FAX: 01-317-274-8575, kajkelly@iu.edu.

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INTRODUCTION

Diabetic nephropathy is the main reason for the vast numbers of patients entering end-stage renal disease (ESRD) programs [1]. Unfortunately, current therapies of diabetic nephropathy fail to stop progression to ESRD [2]. To gain insight into its progressive pathophysiology, we have studied diabetic nephropathy in the ZS rat, a model of obesity and diabetes that progresses to ESRD [3], with features similar to human diabetic nephropathy [4, 5]. ZS rats suffer an accelerated decline following renal ischemia with associated inflammation. These renal responses include leukocyte infiltration and broad activation of pro-inflammatory genes [6]. We hypothesized that this complexity can be dissected by systems biology, and used deep sequencing with advanced bioinformatics tools to study disease mechanisms of diabetic nephropathy [7]. We found that obese, diabetic rats with renal ischemia exhibited general and prominent activation of the renal complement system along with interacting pro-inflammatory gene networks. The complement system is a mainstay of systemic innate immunity comprising several interacting components [8]. The master element is complement component 3 (C3), which is in a perpetual state of contained activity [9], restrained by specific proteins [8, 9]. Failure of the regulatory proteins leads to uncontrolled activation and injury [8].

The presence of renal C3 in human diabetic nephropathy was reported from the beginning of immunofluorescence, but was thought secondary to non-specific trapping of plasma C3 [10]. Experiments on diabetic animals also found renal C3; and again, it was thought to be blood-derived [11]. In our studies of renal transcriptomes in diabetes (7) we found major activation of the renal complement system in rats with diabetic nephropathy.

SUBJECTS and METHODS

Animals

The three groups of rats included here, and their core renal transcript networks, including inflammation, have been reported elsewhere [7]. Lean and obese, diabetic male ZS rats (Charles River, Wilmington, MA) were acquired at 8 weeks of age and fed Purina diet #5008. Their body weights were measured and sera plus urine samples analyzed at biweekly intervals. One group of obese/diabetic rats was subjected to bilateral renal ischemia at 10 weeks of age as described (DI, n=11) [7]. The lean rats (LS, n=6) and a second obese/diabetic group (DS, n = 7) were subjected to sham surgery. These rats were terminated at 28 weeks of age, their kidneys removed, immediately frozen in liquid nitrogen, and RNA extracted (below). In addition, renal tubular cells from 12 week-old normal Sprague Dawley male rats (n=4) were isolated as previously described [12].

Histology and immunohistochemistry

Kidney sections were stained for histology and changes quantified in blinded sections as described [7, 10]. Immunostaining was performed as described [10] using goat anti-complement component 3 (CAT #c312-A, Alpha Diagnostics, San Antonio, TX), rabbit anti-complement component 4 (CAT #hp8023, Hycult Biotech, Plymouth Meeting, PA) and mouse anti-complement component 9 (CAT #ab17931, Abcam, Cambridge, MA) and then

Texas Red-conjugated donkey anti-rabbit (CAT# 111-075-045, Jackson ImmunoResearch, West Grove, PA) secondary antibody.

Hypoxia chamber

Kidney tubular cells, derived from normal Sprague Dawley rats [10] were grown in $38\%O_2/5\%CO_2$ until confluent and then subjected to anoxia ($1\%O_2/5\%CO_2$ in a hypoxia chamber [Hypoxia workstation, Sci-Tive-Dual, Ruskinn Technology, Bridgend, UK]) for periods of 8–48 hours ("ischemia"). The cells were then switched back to $38\%O_2/5\%CO_2$ for 16–24 more hours ("reperfusion"). Control cells were always maintained in $38\%O_2/5\%CO_2$. Total RNA were isolated from these cells and mRNAs encoding C3, C4, C5, C8, C9 were measured by RT^2PCR.

RNA-seq and RT^2PCR

Total kidney RNA isolation was performed as described [7].. RNA, 3 ug, was fragmented with RNAase III, cDNA libraries constructed with SOLiD adaptors by reverse transcription (RT), and then sequenced by strand specific RNA-seq of short 50 bp reads using the SOLiD 4 platform (Center for Medical Genomics at Indiana University School of Medicine) as described [7]. Sequence alignment to the UCSC rat genome database was performed using BFAST [13]. Gene expression was calculated in the form of Reads per Kilobase Exon Model per million mapped reads (RPKM) [14]. To identify differentially expressed genes, we conducted student *t*-test on the logarithmically transformed RPKM values, comparing DI vs. LS and DS vs. LS. Network analysis was performed using Metacore Software (GeneGo, Carlsbad, CA).

RT^2PCR was performed [7] using primers from Qiagen, Valencia, CA. The samples were run in quadruplicate, normalized to β-actin mRNA and are reported as amount of mRNA compared to control samples [15].

Animal Use Statement

The experiments were conducted in conformity with the "Guiding Principles for Research Involving Animals and Human Beings." The investigations were approved by the Institutional Animal Care and Use Committee of Indiana University School of Medicine.

Statistics

Results are expressed as means ± 1 standard error. Differences in renal and metabolic parameters were determined by 1 way ANOVA with subsequent *t*-tests (when ANOVA indicated statistical significance, GraphPad Prism, LaJolla, CA). Bonferroni correction was used for multiple comparisons. The null hypothesis was rejected at $p<0.05$.

RESULTS and DISCUSSION

The abnormal metabolic and renal phenotypes in the ZS rats included in this report were described in more detail in an earlier article [7]. There were 3 groups of ZS rats: lean sham-operated (LS), obese/diabetic sham-operated (DS), and obese/diabetic subjected to a short period of bilateral renal ischemia (DI). At termination, DS and DI rats were heavier, 539±28

and 526±26 than the LS controls, 466±67 (weight in gm $p < 0.05$ for both). The blood glucose levels were also higher in DS and DI, 29.4±16 and 30.5±1.7, respectively, when compared to LS controls, 7.8±0.6 (glucose in mM, significantly higher for both, $p < 0.05$). Creatinine clearance rates were also depressed in DS and DI rats when compared to LS controls (figure 1). Proteinuria, normal in LS, was increased 27 and 30 fold in DS and DI, respectively [7]. Renal histology in ZS rats included the following stains: Leder's stain to identify leukocytes, PAS to outline cell structure and extracellular matrix, and Mason's trichrome to label fibrosis, figure 1. Leder's stain in DS and DI rats revealed clusters of densely packed cellular infiltrates that contained neutrophils. PAS demonstrated increases in tubular damage plus interstitial and glomerular extracellular matrix, particularly in the DI rats (vs LS). Trichrome stain showed early stages of peritubular fibrosis in DS rats, and more advanced in DI rats (vs LS), figure 1.

Renal histology revealed the stark complexity of diabetic nephropathy; migrating inflammatory cells could be found in the vicinity of stressed and damaged renal epithelial and endothelial cells. These intricate relations were approached by systems biology, and we analyzed the interactions among expressed renal transcriptomes in the three conditions: LS, DS, and DI. Among the many up-regulated renal transcripts in diabetic nephropathy [7], a subset of stimulated mRNAs stood out because it contained most elements of the complement system. The up-regulation of these transcripts is shown on the top in figure 2a. We verified these RNAseq results with RT^2PCR as an independent measurement. The correlations of mRNA results obtained between the two methods, RNAseq and RTPCR, were excellent and are shown in figure 2b.

The renal protein expression of the complement components C3, C4, and C9 was then examined by immunofluorescence. There was very faint expression of these proteins in LS rats. However in DS, and particularly in DI, C3, C4, and C9 were broadly expressed in renal tubules and glomeruli. C3, nearly absent in lean group glomeruli (LS), was robustly expressed in diabetes/sham (DS) and more so in diabetes/ischemia (DI) kidneys, figure 3.

The kidney has been viewed as a passive target of circulating complement components of hepatic origin which result in immune complex-mediated glomerular injury. It is now recognized that vast local expansion of complement can occur in injured kidneys [16] and, in animal models, can affect progression of renal failure and survival [17]. Our data showing increased expression of complement components in the renal transcriptome are consistent with local complement activation in diabetic nephropathy. To test the hypothesis that C3 is derived from the tubular epithelium and that ischemia is a critical mediator of the C3 production, primary cultures of normal Sprague Dawley rat renal tubular cells were exposed to acute anoxic conditions, followed by re-oxygenation, i.e., in vitro "ischemia/reperfusion." Following anoxia, mRNA encoding C3 was markedly elevated, 2.34 ± 0.05 fold over normoxic cells ($p<0.05$; $n=4$), while mRNAs encoding C1, C3, C5, C8, and C9 were slightly depressed (not shown). C3 protein was also increased by anoxia (figure 3). In addition to ischemia, inflammatory cytokines and loss or decrease in regulatory proteins have been shown important in local complement expression [18, 19].

Transcripts of the complement system were assembled in a gene network (figure 4). These transcripts were differentially expressed in both conditions, DS and DI, when compared to controls (LS). The center piece of the network is complement component C3 (C3); activated 5.2 fold in chronic diabetic nephropathy with ischemia over lean rat controls, and 2.3 fold after 24 hours of acute anoxia/reoxygenation in isolated renal tubular cells. These results confirm intrinsic renal expression of C3, as previously suggested [20, 21]. C3b, derived from up-regulated renal C3, generates the classical pathway C5 convertase (C4bC2aC3b) that cleaves C5. Renal C5 and C5aR1 mRNAs were not among the 13,453 detected and measured renal transcripts reported by RNAseq, and we resorted to RT^2-PCR to measure these transcripts in the three groups of rats: C5 mRNA in DS and DI was 58% and 35% higher than in LS ($p<0.03$). C5aR1 mRNA in DS and DI were 2.2 fold ($p<0.07$) and 3.4 fold higher than LS ($p <0.04$). The C1 complex was well represented in the RNAseq analysis, with up-regulation of C1q, C1s, and C1r, and C2. In the network, C1q is acting on C2 and C4, forming the C3 convertase (C4bC2a), leading to the assembly of the classical pathway C5 convertase, C4bC2aC3b [9]. C1q also stimulates the release of interleukin-12 (IL-12) [22], also activated by other components (below), and the pro-inflammatory homing receptor CCR7, which enhances the effects of its up-regulated ligand, CCL19 via PI3K [23].

CD55, or decay accelerating factor (DAF), is restricted to cell membranes [24] where it antagonizes both C3 and C5 convertases. This transcript encodes a critical controller of activation, and it is suppressed in kidneys from DI rats 35% ($p<0.004$). The attenuation of renal DAF may cede the modulatory role to up-regulated complement factor I (CFI) [25] (increased 10.5 fold, $p=1.25\times10^5$) and complement factor H (CFH) [26] (increased 1.8 fold, $p<0.002$). However, the up-regulation of CFI and CFH failed to prevent complement activation. It is likely that complement was activated by up-regulation of complement factor D (CFD) and unchanged factor B (CFB) that cause the formation of the C3 amplification convertase C3bBb [27]. These events lead to the formation of the membrane attack complex (MAC). The cytotoxic activity of MAC in diabetic nephropathy was further enhanced by the attenuation of CD59, which promotes the clearance of MAC [28]. Renal C3 activation generates the anaphylatoxins C3a and C5a, and the portal for C3a, the receptor C3ar1, is also up-regulated in diabetic nephropathy. Activated CD80 (or B7-1), the receptor for cytotoxic T lymphocyte antigen 4 (CTLA4), is a downstream target for C3a [29]. C3a and C5a both act on unchanged renal extracellular signal-regulated kinases 1/2 (ERK), in accord with a role for ERK signaling in complement activation [30–32]. IL-12 is another downstream target for activated C3a [33][36] and C5a [34]. The network also shows C3a acting on ICAM-1, a relationship previously reported [35]. C3a also modulates T cell maturation [37]. The importance of the renal IL-12 gene in diabetic nephropathy is further emphasized by additional IL-12 activation from up-regulated C1q [38] and C5a [39]. C3a also acts on TGFb [40] a fibrogenic interaction exemplified by up-regulation of Collagen 4.

C5a works through its receptor C5ar1, found in most renal cells [41]. Renal C5a acts on up-regulated p-selectin (Selp) [42], which also amplifies complement activation [43] and may account for grievous clinical states [44]. Selp is also activated by C5b when integrated as the MAC [45]. Renal ICAM-1 was up-regulated in DI/DS rats, and it is shown as a downstream target for activation not only by C3ar1 [35] but also by C5ar1 [46]. In addition, C3a and C5a activate PI3K via independent pathways [47]. The gene encoding the co-stimulatory

receptor CD40 is also up-regulated in diabetic nephropathy, and it is shown interacting with both up-regulated C3a and C5a, suggesting that adaptive immunity, or a form of autoimmunity, might occur in diabetic nephropathy [31]. Some of these pro-inflammatory actions are mediated by up-regulated NFkB, which is a downstream target of both C3a and C5a [31], and may include up-regulated CD40 [48]. NFkB activates up-regulated CXCR4, suggesting a role for the CXCR4/CXCL12 signaling axis in diabetic nephropathy [49]. The network also shows NF-kB acting on up-regulated CCR2, a receptor involved in monocyte activation [50], on up-regulated CXCL1, the prototypical neutrophil chemokine [51], on up-regulated CXCL10, a leukocyte chemoattractant [52], and on up-regulated CCL19, a chemokine for dendritic cells and T lymphocytes [53]. NF-kB, activated by IL1R, the receptor for IL-1 [54], acts on up-regulated collagen I gene, a redundant effect mediated by C3a, C5a, and IL-1 [55]. C5a also acts on the fibronectin gene [56], and presumably its cognate protein, which can then activate up-regulated BMP1 and promote the formation of collagens I and III [57]. C5a also acts on up-regulated TLR4 gene [58], potentially acting CASP1, which is required to cleave Pro-IL-1b and generate renal IL-1b, the master cytokine [59].

In conclusion, our data point to renal C3 activation in diabetic nephropathy of rats. C3 reactivity in tubules leads to broader renal activation of the complement system, which is sustained by suppression of complement regulators, and contributes to renal inflammation, impaired function and fibrosis.

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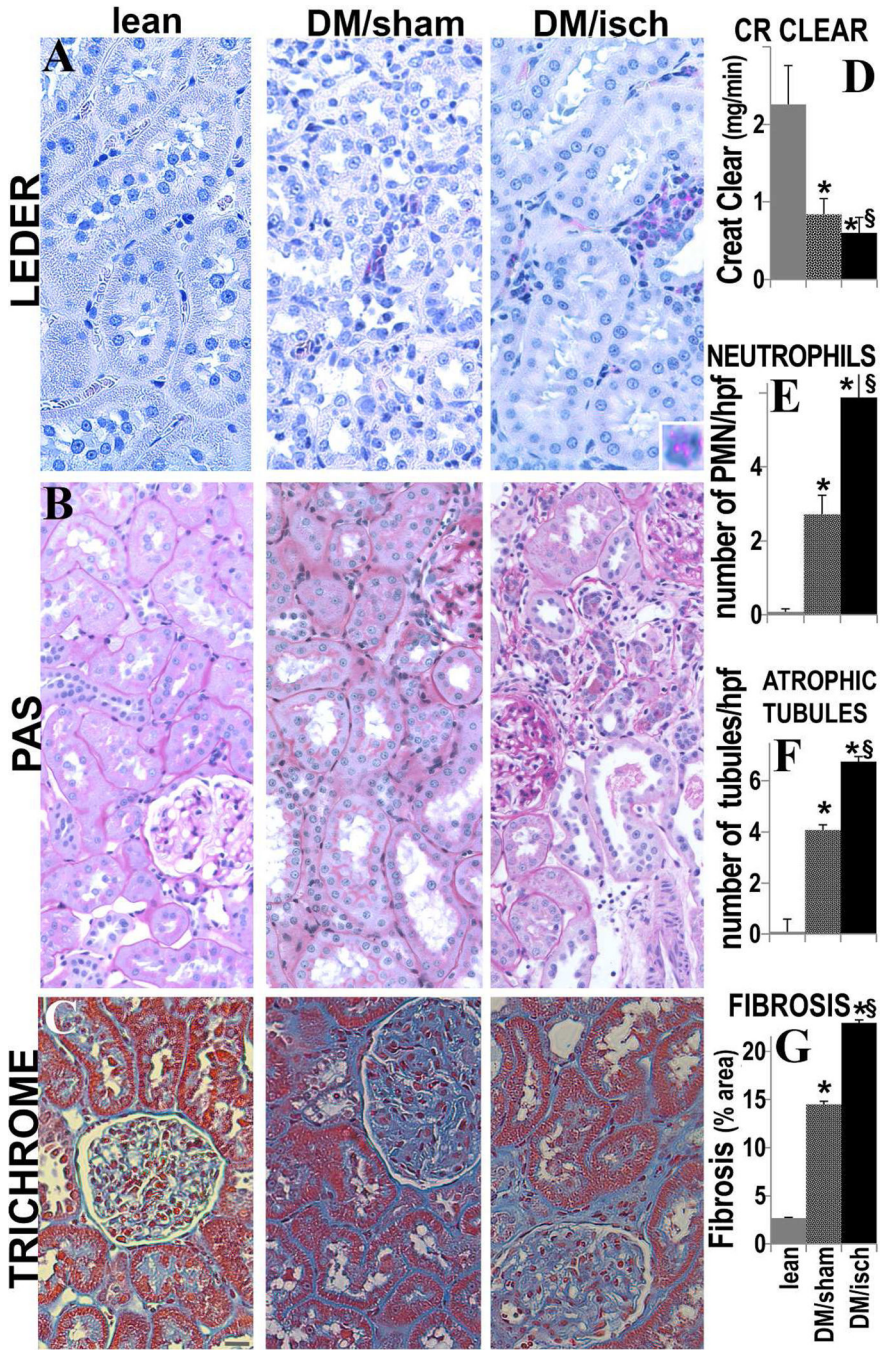

Figure 1. ZS rat diabetic nephropathy

Representative renal sections (from top to bottom: Leder's stain, Periodic acid Schiff (PAS) stain, and Masson's trichrome stain) in the three groups of rats (from left to right, lean controls [lean], diabetic sham operated [DM/sham], and diabetic postischemic rats [Diabetes/Ischemia]) are presented. Leder's stain (A) labeled clusters of leukocytes (pink), nearly absent in the Lean (LS) group, clearly present in Diabetes/Sham group (DS) and very prominent in Diabetes/Ischemia rats group (DI). The inset shows a neutrophil with typical nuclear morphology. PAS stain (B) demonstrated normal glomerular and tubular structures

in LS. The Diabetes/Sham group exhibited mild increases of interstitial and glomerular extracellular matrix, which were far more pronounced in the Diabetes/Ischemia group. Masson's trichrome (C) stained connective tissue (blue) was normal in LS. Increases in connective tissue were evident in the glomeruli and interstitium of Diabetes/Sham group, and more severe in Diabetes/Ischemia group. Mean creatinine clearance and quantification of histological parameters are shown in the graphs (D-G). *$p<0.05$ vs LS; §$p<0.05$ vs DS, Scale bar=50 microns.

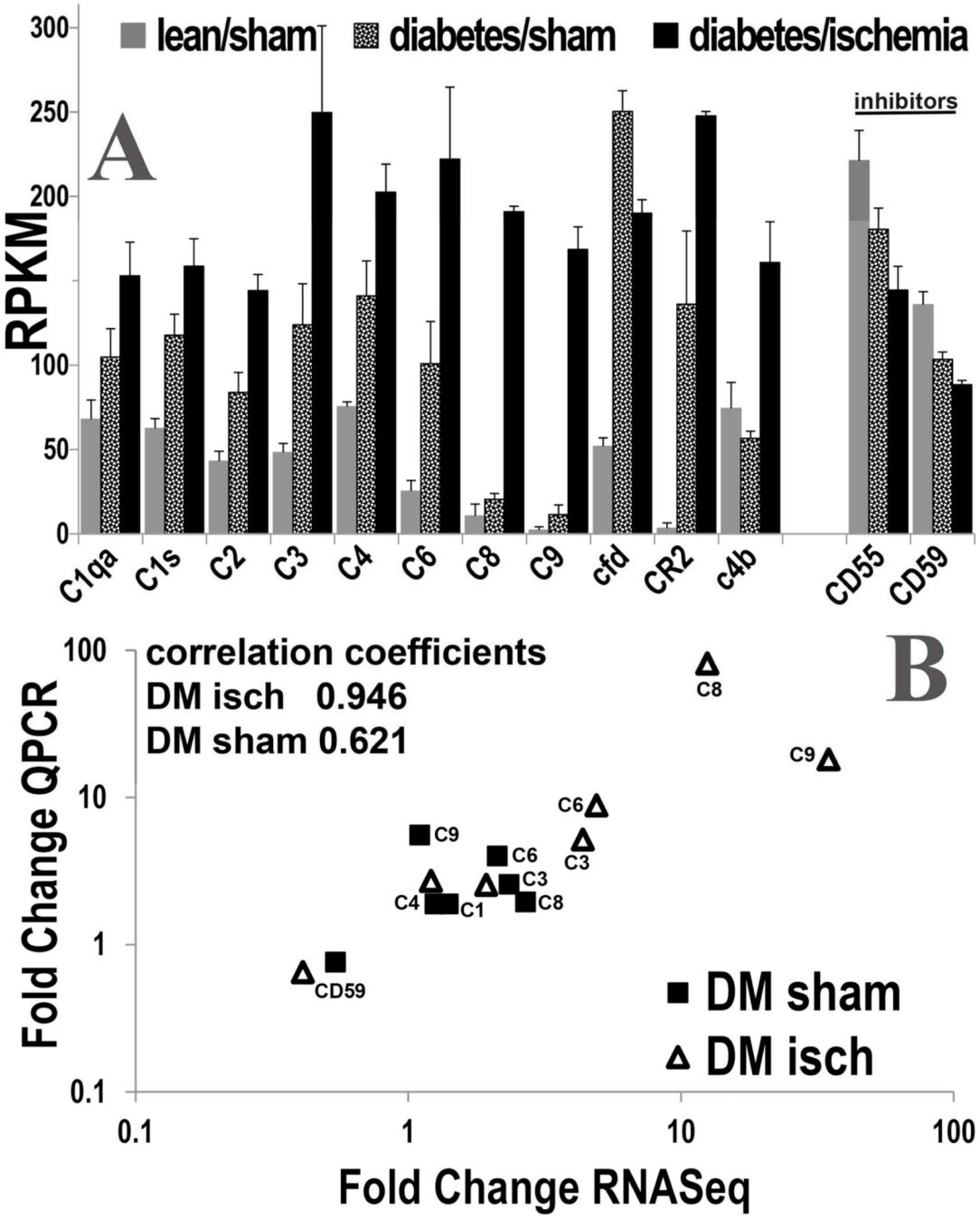

Figure 2. Complement system renal transcripts detected by RNAseq and RT^{2}PCR

Transcript levels by RNAseq (A) are expressed as Reads per Kilobase Exon Model per Million mapped reads (RPKM, see text) and represent mRNAs encoding complement components (C1qa, C1s, C2–4, C6–9), as well as complement factor D (cfd), complement receptor type 2 (CR2) and c4 binding protein (C4b). These renal transcripts are significantly elevated in Diabetes/Sham and Diabetes/Ischemia groups. CD55, decay accelerating factor for complement, and CD59, encode complement regulatory proteins that limit the complement cascade. The two regulatory renal genes are suppressed in both Diabetes/Sham

and Diabetes/Ischemia groups. Significant correlations between transcript levels measured by RNAseq (horizontal axis) and quantitative PCR (QPCR) vertical axis are shown in B. These comparisons were made to verify the accuracy of the RNAseq determinations. To facilitate visualization on one axis, RNA Seq data are presented as follows: C1s/2, C2*2, C4/3, C6*10, C8*50, C9*10, cfd*100, CD55*3, CD59/10, CR2*50, C4b*10.

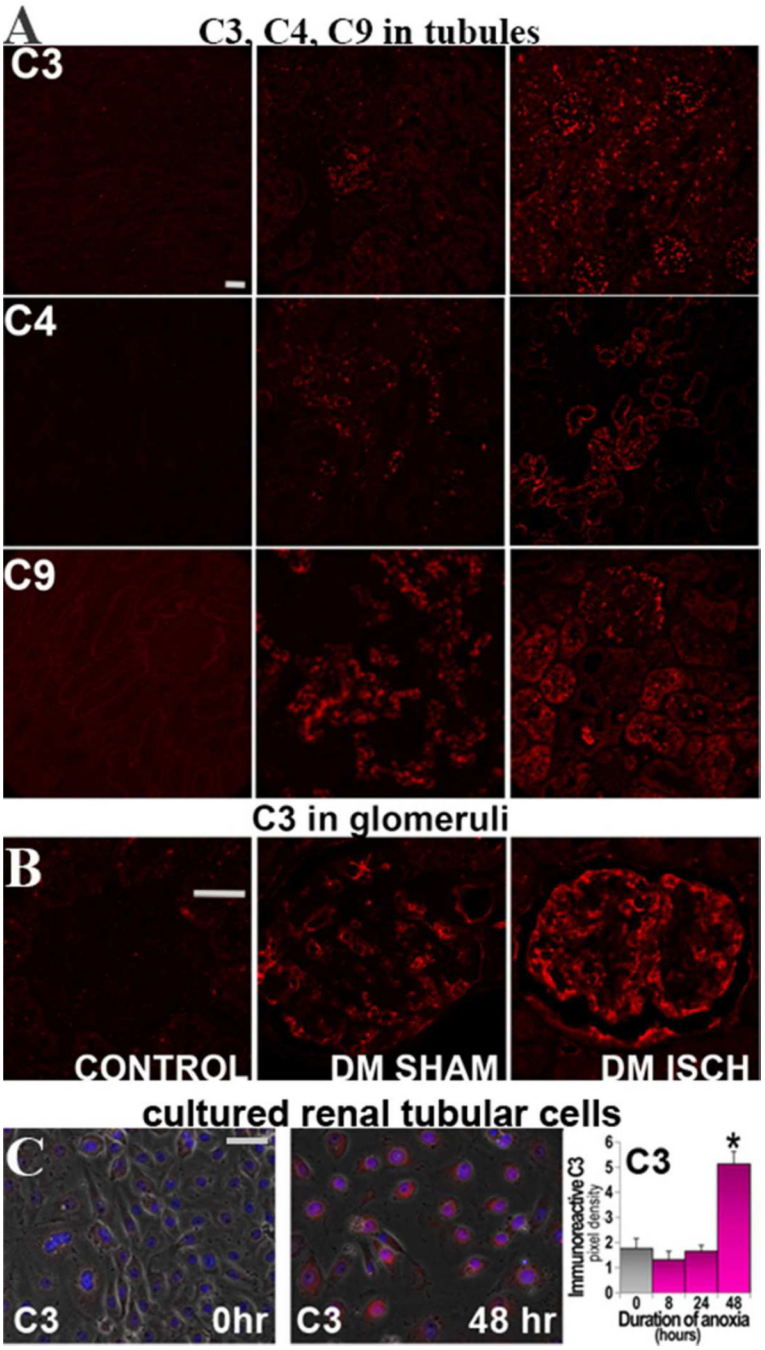

Figure 3. Renal complement components in ZS rat diabetic nephropathy

Complement components 3, 4, and 9 are shown (A) in (Left to Right) Lean control rats (Control), Diabetic/Sham group (DM Sham) and Diabetes/Ischemia group (DM Isch). Complement (3,4,9) protein expression was barely detectable in the Lean control group, but, in contrast, was robustly expressed in the two diabetic groups. Similarly, glomerular C3 levels (B) were nearly undetectable in the Lean group (Left), and rose prominently in the two diabetic groups (right). To examine complement generation in tubule cells, primary renal cells (C) were cultured in 38%O_2 and 5% CO_2 and transferred to 1%O_2 and 5% CO_2

for the designated times. The cells were then returned to 38%O_2 and 5%CO_2 for 24 hours. C3 mRNA was increased at 24 hours, whereas levels of immunoreactive C3 increased at 48 hours of anoxia. Scale bar=50 microns; *$p<0.05$ vs time 0

	Fold change	
	DS	DI
C1r	1.5	1.7
C1s	1.9	2.5
C1qa	1.5	2.3
C1qb	1.7	2.7
C1qc	1.4	2.4
C2	2.0	3.4
C3	2.6	5.2
C4	2.1	3.0
C6	4.0	8.8
C8	1.9	18.1
C9	5.5	81.2
cfd	4.8	3.7
CD55	0.82	0.65
CD59	0.66	0.64

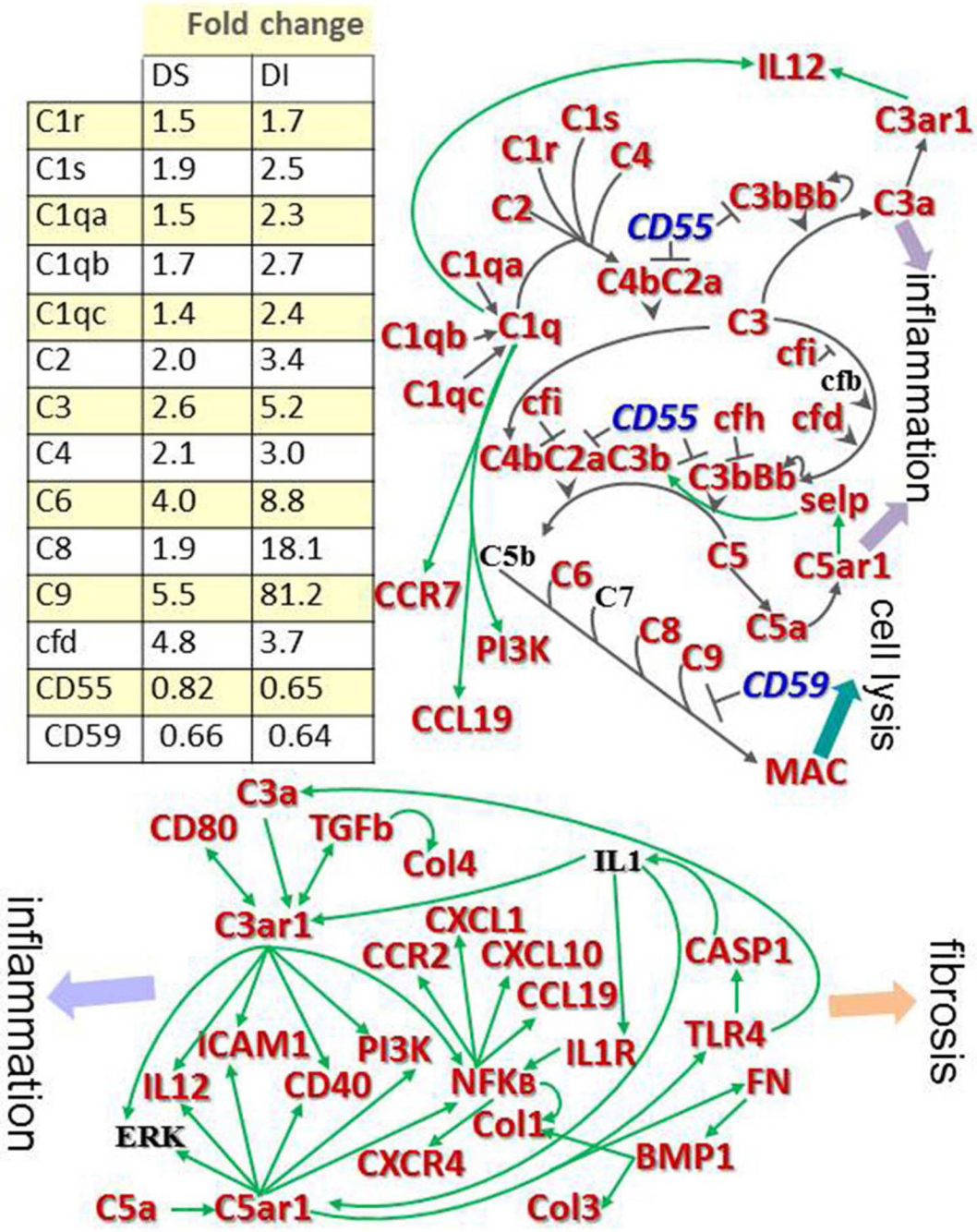

Figure 4. Complement system gene pathways in diabetic nephropathy

The numerical table on the upper left corner shows the fold changes in the different transcripts of the complement system. These values were all statistically significant ($p<0.05$). The network on the right upper corner contains up-regulated (red), inhibited (blue, italics) or unchanged transcripts (black). Green arrows show positive interactions. The main elements of this network were organized to show activation of the local classic and alternative pathways. The end result was the production of the anaphylatoxins C3a and C5a and the formation of the membrane attack complex (MAC). The bottom network delineates

critical interactions among the anaphylatoxins C3a and C5a with pro-inflammatory and pro-fibrotic transcripts. These morbid interactions might be responsible, at least in part, for the development and eventual progression of diabetic nephropathy.