

Immune gene expression in kidney biopsies of lupus nephritis patients at diagnosis and at renal flare

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ABSTRACT

Background. Up to 50% of lupus nephritis (LN) patients experience renal flares after their initial episode of LN. These flares contribute to poor renal outcomes. We postulated that intrarenal immune gene expression is different in flares compared with *de novo* LN, and conducted these studies to test this hypothesis.

Methods. Glomerular and tubulointerstitial immune gene expression was evaluated in 14 patients who had a kidney biopsy to diagnose LN and another biopsy at their first LN flare. Ten healthy living kidney donors were included as controls. RNA was extracted from laser microdissected formalin-fixed paraffin-embedded kidney biopsies. Gene expression was analyzed using the Nanostring nCounter[®] platform and validated by quantitative real-time polymerase chain reaction. Differentially expressed genes were analyzed by the Ingenuity Pathway Analysis and Panther Gene Ontology tools.

Results. Over 110 genes were differentially expressed between LN and healthy control kidney biopsies. Although there was considerable molecular heterogeneity between LN biopsies at diagnosis and flare, for about half the LN patients gene expression from the first LN biopsy clustered with the repeated LN biopsy. However, in all patients, a set of eight interferon alpha-controlled genes had a significantly higher expression in the diagnostic biopsy compared with the flare biopsy. In contrast, nine tumor necrosis factor alpha-controlled genes had higher expression in flare biopsies.

Conclusions. There is significant heterogeneity in immune-gene expression of kidney tissue from LN patients. There are limited but important differences in gene expression between LN flares, which may influence treatment decisions.

Keywords: gene expression, interferon signature, lupus nephritis, transcriptomics, tumor necrosis factor

INTRODUCTION

Lupus nephritis (LN) affects 40–60% of systemic lupus erythematosus (SLE) patients during the course of their disease [1, 2]. The development of kidney disease increases morbidity and mortality rates up to 26-fold compared with age-matched individuals from the general population [3]. With current therapies remission is achieved in only 50–70% of patients and ~10–20% still progress to end-stage kidney disease over 5–10 years [4].

A contributing factor to poor kidney outcomes in LN is the high frequency of disease flare [5–7]. Up to 50% of LN patients experience renal flares after achieving complete or partial remission [8]. Based on current guidelines [9–11], these patients are usually treated with the same drug regimen that was successful in initially controlling disease activity. While this makes clinical sense, LN flares are often more resistant to treatment [12]. It is conceivable that the pathogenic mechanisms responsible for LN flare are different from those that were operating during the initial episode of disease activity, due in part to changes in the nature of LN with time, chronic changes in the kidney and/or alterations in patients' immune systems by prior and ongoing immunosuppressive therapy. This study was undertaken to examine differences in intrarenal gene expression between the diagnosis and flare of LN.

MATERIALS AND METHODS

Experimental design

This investigation focused on patients who had a kidney biopsy to diagnose LN, and another biopsy when LN flared after treatment-induced remission. The transcriptomes of these paired biopsies were interrogated and compared to identify differences in intrarenal transcript expression between LN episodes. Such differences were assumed to reflect, in part, changes

in disease pathogenesis over time and after exposure to immunosuppression.

Patients and kidney biopsy specimens

Fourteen Hispanic patients who had a kidney biopsy at the initial diagnosis of LN (biopsy A) and a second biopsy at LN flare (biopsy B) were included. All patients had a pathologic diagnosis of active proliferative or mixed LN (Classes III/IV \pm V) on first and repeat biopsies. Several patients were immunosuppressive treatment-naïve at LN diagnosis (biopsy A) and most were receiving maintenance immunosuppression at LN flare (biopsy B).

Kidney biopsy tissue was available from archived paraffin blocks and clinical information was obtained from the prospective local LN registry. Normal control renal tissue was obtained from archived kidney biopsies of living-kidney donors ($n = 10$) obtained at implantation of the allograft from the same institution. The protocol was approved by the Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán Research and Ethics board and The Ohio State University Institutional Review Board.

RNA extraction and analysis

Formalin-fixed and paraffin-embedded (FFPE) kidney biopsy tissue was cut into 10 μm sections. After deparaffinization, all available glomeruli and tubulointerstitium were separated by laser microdissection (PALM MicroBeam, Zeiss Labs, Munchen, Germany), captured and digested with proteinase K. From each biopsy, a median glomerular cross-sectional area of 1 970 000 μm^2 , corresponding to ≈ 50 glomerular cross-sections, and a tubulointerstitial area of 3 940 000 μm^2 was collected. DNA was removed with DNase. RNA was precipitated and extracted with RNeasy MinElute spin columns (Qiagen, Redwood City, CA, USA) as previously described [13]. Gene transcript expression was analyzed using the Nanostring nCounter[®] platform and the nCounter Immunology Panel (Nanostring Technologies, Seattle, WA, USA) [14]. The panel consists of 579 immune response genes, 6 positive controls, 6 negative controls and 15 housekeeping genes [15].

Quantitative real-time polymerase chain reaction

cDNA was obtained from renal biopsy RNA of three randomly selected patients using a high-capacity RNA-to-cDNA kit (ThermoFisher Scientific Cat # 4387406), followed by Taqman gene expression assay (ThermoFisher Scientific, Waltham, MA, USA) with the following primers to validate differentially expressed genes: Promyelocytic Leukemia (*PML*, Hs00971694), Myxovirus Resistance 1 (*MX1*, Hs00895608), Signal Transducer And Activator Of Transcription 1 (*STAT1*, Hs01013996), Interferon Alpha 2 (*IFNA2*, Hs00265051), Fibronectin 1 (*FNI*, Hs01549976), Vascular Cell Adhesion Molecule 1 (*VCAM1*, Hs01003372), C-C Motif Chemokine Ligand 19 (*CCL19*, Hs00171149), Colony Stimulating Factor 1 Receptor (*CSF1R*, Hs00911250), C-C Motif Chemokine Ligand 2 (*CCL2*, Hs00234140). Glyceraldehyde-3-Phosphate Dehydrogenase (*GADPH*, Hs02758991), Ornithine Decarboxylase Antizyme 1 (*OAZ1*, Hs01548012) and Ribosomal Protein L19 (*RPL19*, Hs01577060) were included as housekeeping genes.

Statistical analysis

Descriptive statistics are given as median and interquartile range or as relative frequencies. For baseline comparisons, Fisher's exact test, Chi-square or the Kruskal–Wallis test was applied as appropriate. For gene expression data, raw counts were normalized to the positive spiked-in controls and then \log_2 transformed. Genes with an expression level below the mean plus two standard deviations of the negative controls were filtered out. Quantile normalization was applied to the remaining transcripts (≈ 500 for glomeruli and tubulointerstitium). Comparisons between biopsies A and B, and between biopsies A and B and healthy controls were done using linear mixed effect models. For any specific gene to be considered differentially expressed, at least a 1.5-fold change in transcript expression and a P-value of 0.01 were required (false detection rate of 5 out of 500 genes). Differentially expressed genes were analyzed by the Ingenuity Pathway Analysis tool (Qiagen Inc., Germantown, MD, USA, <https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis>) and by Panther Gene Ontology pathway tools [16, 17]. Ingenuity pathway analysis clusters differentially expressed genes into formal molecular pathways based on levels of transcript expression and the (currently) known informatics of gene–gene interactions. This is a tool to identify dysregulated pathways in a disease process.

RESULTS

Characteristics of the LN cohort

The demographic, clinical and histopathological characteristics of the patients and healthy controls are shown in Table 1. At initial diagnosis, patients had relatively normal serum creatinine concentrations, heavy proteinuria, high histologic activity and low chronicity on biopsy. Patients were generally treated with corticosteroids plus cyclophosphamide, and most achieved a complete renal response within a year. All patients had responded at least partially by 18 months. Between biopsies A and B, most patients (85.7%) were maintained with azathioprine and low-dose prednisone. At LN flare, patients had significantly impaired kidney function, greater complement consumption, and more histologic evidence of chronicity on biopsy, although the degree of proteinuria and histologic activity were similar to the initial episode of LN. At flare, patients were generally re-induced with corticosteroids plus mycophenolate mofetil, reflecting the changing global trends in the treatment of LN between initial diagnosis and flare. Complete renal response rates were diminished in the patients who flared compared with their initial episode.

Molecular heterogeneity of LN

A principal component analysis of transcript expression for the glomerular and tubulointerstitial compartments from biopsies A and B is shown in Figure 1. Glomerular and tubulointerstitial transcripts from healthy kidneys clustered and separated from LN groups in both compartments. In contrast, transcript expression from LN biopsies (A and B) did not cluster well, revealing the molecular heterogeneity of LN. However, in about half the LN cases glomerular and tubulointerstitial transcript

Table 1. Demographic and clinical data

	Biopsy A ^a n = 14	Biopsy B ^b n = 14	P	Normal controls
Demographic				
Age at biopsy (years)	26 ± 5 ^c	30 ± 7	<0.001	32 ± 9
Female (%)	14 (100)	14 (100)	1.000	8 (80)
Hispanic (%)	14 (100)	14 (100)	1.000	10 (100)
Delay from symptoms to biopsy (months)	3 (1–5) ^d	1 (0–2)	<0.001	–
Time between biopsies A and B (years)	3.5 (1.5–6.5) ^d	–	–	–
Laboratory^d				
Serum creatinine (µmol/L)	80 (71–133)	168 (97–256)	0.011	62 (53–80)
24 h-urine protein/creatinine (mg/mmol)	475 (260–746)	520 (260–836)	0.667	0 (0–2.3)
ds-DNA antibodies (u/ul) ^e	118 (34–602)	123 (47–539)	1.000	–
C3 ratio ^f	0.7 (0.6–1.3)	0.4 (0.2–0.8)	0.004	–
C4 ratio ^f	0.5 (0.3–1.2)	0.5 (0.4–1.0)	0.280	–
Histopathology				
Class III (%)	2 (14.5)	0 (0)	0.481	–
Class IV (%)	4 (28.5)	3 (21.4)	1.000	–
Class III+V (%)	4 (28.5)	2 (14.3)	0.648	–
Class IV+V (%)	4 (28.5)	9 (64.3)	0.128	–
Activity score ^d	7 (4–9)	8 (3–11)	0.734	–
Chronicity score ^d	2 (2–4)	5 (3–7)	0.016	–
Treatment history before biopsy A				
No immunosuppression	6 (42.9)	–	–	–
Corticosteroids <10 mg	6 (42.9)	–	–	–
Antimalarial	4 (28.6)	–	–	–
Induction treatment^g				
Corticosteroids (%)	14 (100)	14 (100)	1.000	–
Mycophenolate mofetil (%)	3 (21.4)	10 (71.4)	0.021	–
Cyclophosphamide (%)	8 (57.2)	4 (28.6)	0.252	–
Azathioprine (%)	3 (21.4)	0 (0)	0.222	–
Maintenance treatment between biopsies A and B				
Azathioprine (%)	12 (85.7)	–	–	–
Mycophenolate mofetil (%)	2 (14.3)	–	–	–
Corticosteroids (%)	12 (85.7)	–	–	–
Antimalarial (%)	6 (42.9)	–	–	–
12-month response^h				
Complete response (%)	9 (64)	3 (21)	0.054	–
Partial response (%)	2 (14)	4 (29)	0.648	–
No response (%) ⁱ	3 (21)	7 (50)	0.236	–

^aBiopsy A was done at initial LN diagnosis.

^bBiopsy B was done at LN flare.

^cMean ± standard deviation.

^dData presented as median and interquartile range.

^eNormal ds-DNA is <9.6 UI/mL.

^fC3 and C4 ratios were calculated as the reported value divided by the lower limit of the reference level.

^gAll patients were also given high-dose corticosteroids.

^hComplete response is defined as stable renal function (within 15% of baseline) plus 24 h-uPCR <0.5 g/g; partial response is defined as stable renal function plus 50% reduction of baseline 24 h-uPCR; no response indicates a patient did not achieve complete or partial response by 12 months, but responded later.

ⁱThese patients achieved response beyond 12 months.

expression from biopsy A clustered with biopsy B. Many of the same patients whose A and B biopsies clustered from the glomerular compartment also clustered from the tubulointerstitial compartment. There were no differences in clinical or histological parameters between patients with concordant gene expression between biopsies A and B when compared with patients with discordant gene expression (data not shown).

Immune gene expression patterns in LN and healthy controls

Compared with healthy tissue, biopsies A and B showed 139 and 119 differentially regulated glomerular transcripts, respectively. Most of these transcripts clustered into similar pathways in both biopsies. A list of the top canonical pathways

differentially expressed in LN compared with healthy controls is shown in [Supplementary data](#), Table S1. Based on the enrichment P-value, the enriched pathways included interleukin-6 (IL-6) and lipopolysaccharide (LPS)-stimulated pathways, nuclear factor (NF)-κB activation pathway, leukocyte recruitment and extravasation, T-cell signaling [Th1 pathway, Protein Kinase C-γ, Tec kinase and inducible T cell costimulator- inducible T cell costimulator ligand (iCOS-iCOSL) signaling] and B-cell signaling [CD28 co-stimulation pathway and Phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) signaling pathway].

In the tubulointerstitial compartment there were 140 and 135 differentially expressed transcripts between healthy tissue and biopsies A and B, respectively. As for glomeruli, these

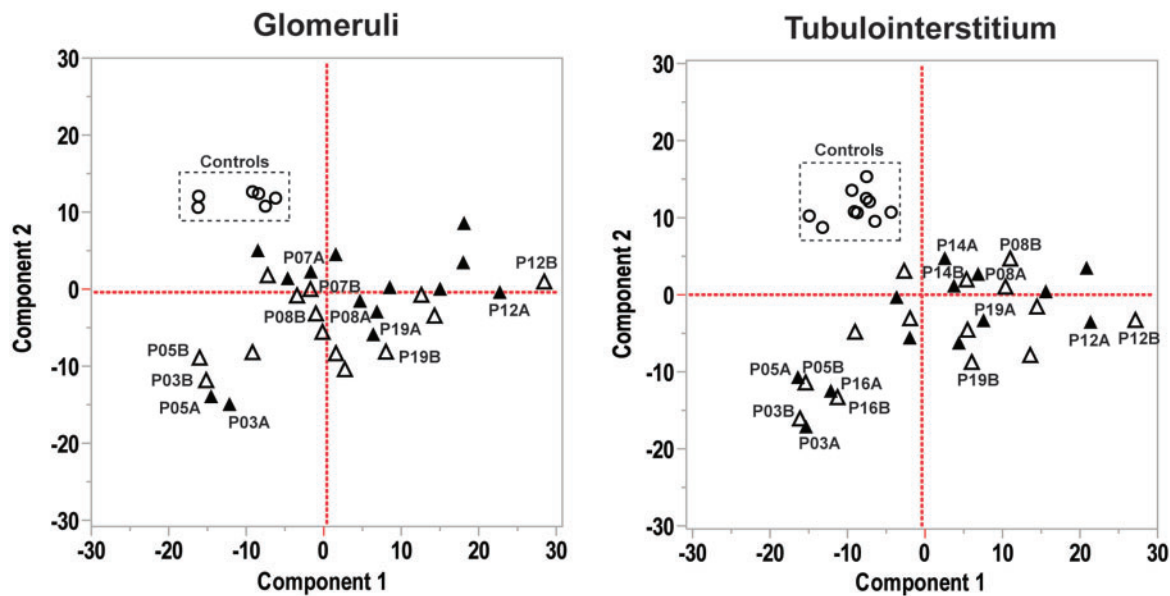


FIGURE 1: Principal component analysis of immune gene expression in glomeruli and tubulointerstitium. In both compartments healthy controls clustered separately from LN biopsies. In contrast, transcript expression in LN did not cluster, but for some individual patients biopsy A (open triangles) and biopsy B (filled triangles) immune gene expression clustered closely.

transcripts clustered into similar pathways in both biopsies. A list of the top canonical pathways that differed between LN and control is provided in [Supplementary data](#), Table S2. Based on the enrichment P-value, pathways enriched in the tubulointerstitial compartment of LN kidneys included the complement system, Nuclear Factor kappa B (NF- κ B) activation pathway, B-cell activation pathways (B-cell receptor signaling, PI3K signaling, OX40 signaling and B-cell activating factor (BAFF) signaling), acute phase response (IL-6 signaling), apoptosis signaling, co-stimulation (CD40 signaling) and T-cell activation (CD28 signaling), among others.

Many toll-like receptor (TLR) pathway transcripts were upregulated in the glomeruli [Toll-like Receptor 7 (*TLR7*), Fc Fragment of IgG Receptor IIa (*FCGR2A*), Myeloid Differentiation Primary Response 88 (*MYD88*), Interleukin 1 Receptor Associated Kinase 1 (*IRAK1*), Interferon Regulatory Factor 7 (*IRF7*) and Secreted Phosphoprotein 1 (*SPPI* or osteopontin)] and tubulointerstitium (*TLR7*, *FCGR2A* and *MYD88*) of LN biopsies compared with healthy controls ([Supplementary data](#), Figure S1). There were no differences in *TLR9* expression between LN and controls in either compartment, whereas *TLR3*, *TLR4* and *TLR5* were significantly downregulated in glomeruli from LN patients compared with controls.

Differences in immune gene expression between episodes of active LN

In the glomerular compartment, 23 genes were differentially expressed between biopsies A and B. Eight genes were higher in biopsy A, including five transcripts regulated by interferon alpha (IFN α): *MX1*, *STAT1*, *IRF7*, *PML*, major histocompatibility complex, Class I, A (HLA-A), and *CCL19*, a chemokine induced by IFN α . With the exception of *HLA-A*, these genes were also upregulated in biopsy A when compared with controls. In contrast, none of these genes was differentially expressed in biopsy B

compared with controls ([Figures 2 and 3](#)). All these transcripts clustered into the type I IFN signaling pathway [gene ontology (GO) fold-enrichment 70.2, $P = 7.80 \times 10^{-05}$] ([Table 2](#)).

Among the nine differentially expressed transcripts between biopsies A and B in the tubulointerstitial compartment, four were regulated by IFN α and had higher levels of expression in biopsy A: *MX1*, *PML*, *STAT4* and *IRF7* ([Figures 2 and 4](#)). Only *MX1* and *PML* were differentially expressed between biopsy A and normal controls. All of these transcripts clustered into the type I IFN signaling pathway (GO-fold enrichment >100, $P = 1.97 \times 10^{-07}$) ([Table 3](#)).

In the glomerular compartment, 15 genes had higher expression in biopsy B than biopsy A. These included nine genes regulated by the tumor necrosis factor (TNF): Integrin Subunit Alpha 5 (*ITGA5*), NF- κ B Inhibitor Zeta (*NFKBIZ*), *CSF1R*, *RELA* Proto-Oncogene (*RELA*, NF- κ B subunit), Protein Kinase C Delta (*PRKCD*), Lypopolysaccharide Induced TNF Factor (*LITAF*), Colony Stimulating Factor 1 (*CSF1*) and S100 Calcium Binding Protein A8 (*S100A8*) and *CD40*. Most of these genes (*ITGA5*, *NFKBIZ*, *RELA*, *PRKCD*, *CSF1* and *S100A8*) were also downregulated compared with healthy controls in biopsy A and were not different than healthy controls in biopsy B ([Figure 2](#)). These transcripts clustered into the TNF receptor binding pathway by GO analysis (GO-fold enrichment 24.4, $P = 7.42 \times 10^{-03}$). However, TNF gene expression itself was not different between biopsies A and B (GO-fold enrichment 1.07, $P = 0.554$). Other genes with higher expression in the glomeruli and tubulointerstitium of biopsy B than A included those for fibronectin (*FN1*) and vascular cell adhesion molecule (*VCAM1*).

With respect to TLR genes, only *TLR7* (fold-change 1.48, $P = 0.008$) and *IRF7* (GO-fold enrichment 1.65, $P \leq 0.001$) were differentially expressed between biopsies A and B in glomeruli, and only *IRF7* (fold-change 1.52, $P = 0.003$) in

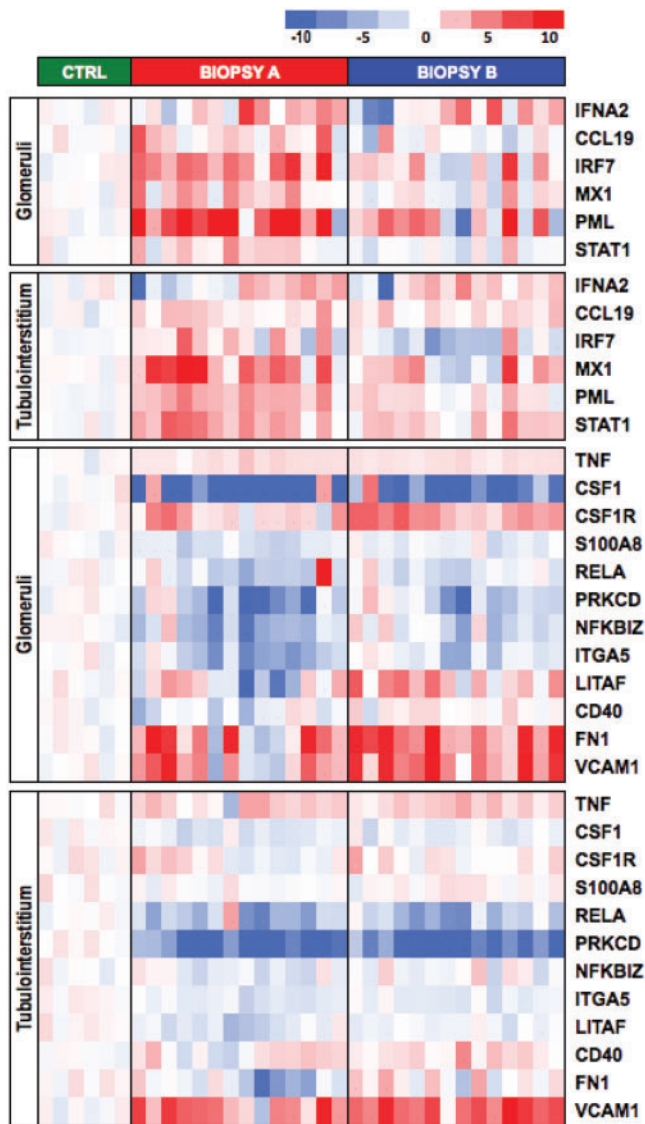


FIGURE 2: IFN α - and TNF α -regulated genes differentially expressed between biopsy A or B and normal controls. Genes are listed in the heatmap using z-score of normalized gene expression level. Each column is an individual patient or healthy control. Column 1 in biopsy A and column 1 in biopsy B correspond to the same patient.

tubulointerstitium (Supplementary data, Figure S1). Transcript expression changes were similar when patients with Class III/IV LN were examined separately from patients with Class III/IV + V LN, suggesting that the presence of Class V did not affect the results (data not shown).

Quantitative real-time polymerase chain reaction validation

To validate the Nanostring findings quantitative real-time polymerase chain reaction (qPCR) was performed for selected genes using glomerular and tubulointerstitial RNA. *IFNA2*, *PML*, *CCL19*, *CSF1R*, *VCAM1* and *MX1* were evaluated in glomerular samples and *IFNA2*, *PML*, *CCL19*, *MX1* and *STAT1* evaluated in tubulointerstitial samples. Transcript expression

by qPCR followed the same trends as Nanostring (examples shown in Figures 3 and 4).

DISCUSSION

When LN flares, the usual clinical approach to therapy has been to repeat the treatment regimen that had been successful in the past [9–11]. This approach may be suboptimal given the increased resistance to therapy observed in LN flares [12]. Mechanistically, it is conceivable that prior immunosuppression, along with chronic damage to the kidney and duration of LN alter the pathogenesis of disease flares compared with the initial episode of LN.

Using molecular interrogation of paired kidney biopsies from patients who flared after successful treatment of LN, we have shown that global intrarenal transcript expression from the flare biopsy is very similar to the transcriptome of the initial biopsy. We did, however, observe a few key differences. The IFN α signature was significantly attenuated in flare biopsies compared with initial biopsies. While this may not have much impact on non-specific immunosuppression with cyclophosphamide or mycophenolate mofetil, it may affect the response to targeted therapies like antibodies to type 1 IFN or the IFN receptor (e.g. Safety Efficacy of Two Doses of Anifrolumab Compared to Placebo in Adult Subjects With Active Proliferative Lupus Nephritis [TULIP-LN1] trial NCT02547922). Furthermore, a strong TNF α signature appeared in the flare biopsy that was not present in the first biopsy, supporting previous concepts that TNF α counterbalances IFN α in some autoimmune diseases [19–21]. These findings may be especially important for clinical trial design of novel anti-IFN therapeutics, as most trials enroll patients with active disease, regardless of whether the disease is *de novo* or established.

Type I IFNs (mainly IFN α and IFN β) are central players to both innate and adaptive immunity [22]. An intense upregulation of IFN-controlled genes has been described in active SLE patients [23–26], which is globally known as the ‘interferon signature’. The interferon signature locally occurs in the kidneys [27] and has been demonstrated to occur predominantly in mesangial [28, 29] and endothelial cells [30], where it has a pathogenic effect in animal models [31]. We found that *IFNA2* expression and the interferon signature differ between LN flares, with a more intense expression in an initial episode compared with repeated ones. This response occurs in both glomerular and tubulointerstitial compartments. The patients included in this study were under minimal or no immunosuppression at LN diagnosis, whereas at flare they had received intense induction immunosuppression and were receiving maintenance therapy, including antimalarial agents that have been shown to decrease IFN response [32, 33]. These findings are supported by a recent study that showed lower MX1 protein staining in the kidneys of LN patients after immunosuppression compared with immunosuppression-naïve patients [34]. All this suggests that immunosuppression modifies the interferon signature and limits its expression at LN flare. Therefore, response to IFN-targeted therapies may be lower in patients with established LN who flare.

Contrasting with the interferon signature, we found that TNF α -controlled gene expression (‘TNF α signature’) was

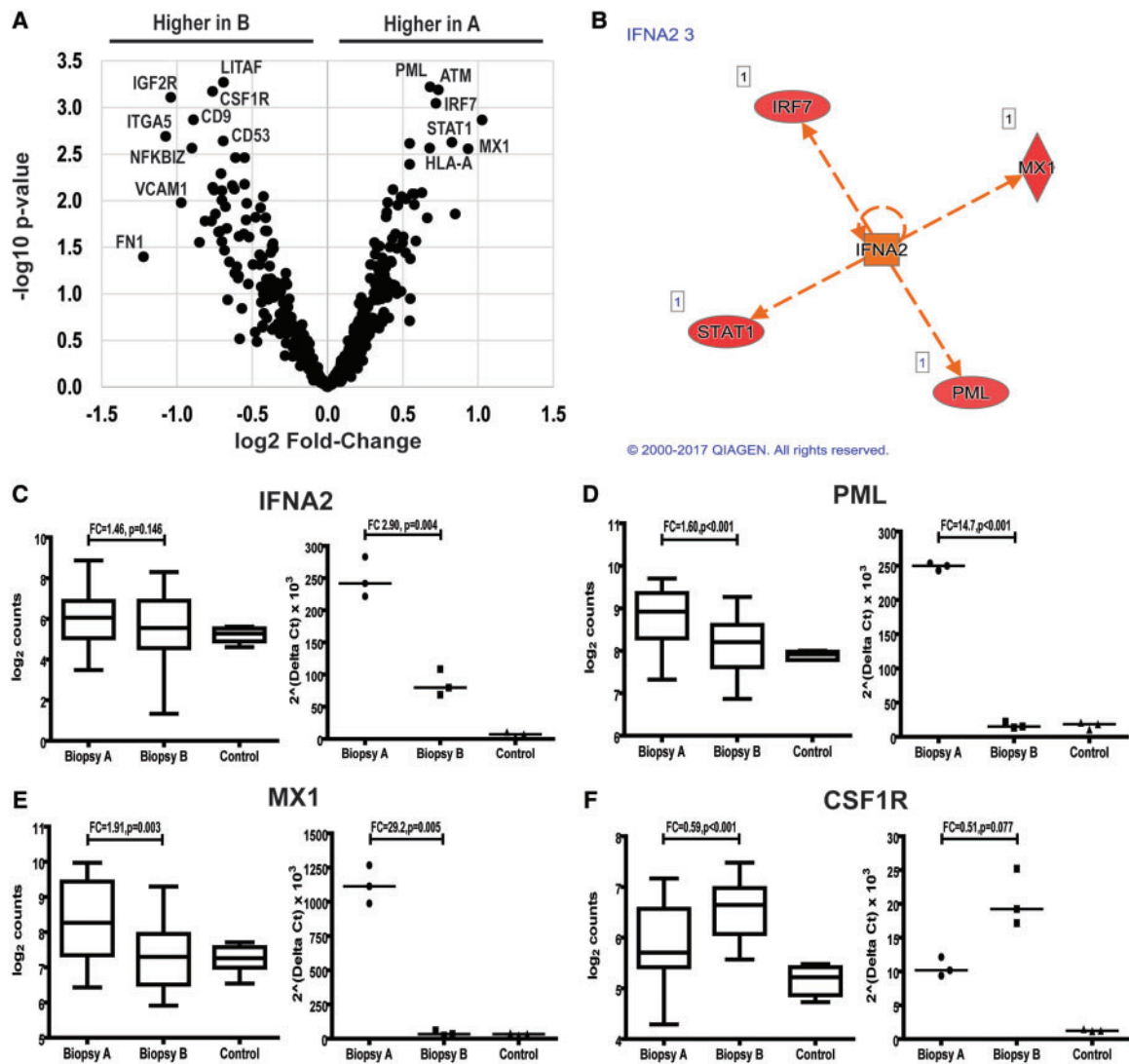


FIGURE 3: Glomerular immune gene expression from LN patients. (A) Volcano plot showing gene expression in biopsies A and B. A value of 2.0 on the ordinate corresponds to a P-value of 0.01 and a value of ± 1.0 on the abscissa corresponds to a 1.5 fold-change in expression. (B) Ingenuity Pathway Analysis showing IFNA2 as a central upstream regulator of differentially expressed genes between biopsies A and B. Expression of IFNA2 (C), PML (D), MX1 (E) and CSF1R (F) measured by Nanostring (left graph) and by qPCR (right graph).

Table 2. GO biological processes from differentially expressed genes between biopsies A and B in the glomerular compartment

GO biological process	Ref. list	Hits	Fold enrichment	Biopsy A versus B	P
Complement activation, alternative pathway (C3, C7, CFH)	13	3	>100	>B	1.97×10^{-02}
IFN γ -mediated signaling pathway (HLA-A, HLA-DRB1, PML, VCAM1, PRKCD, STAT1, IRF3, IRF7, HLA-DRB3)	71	9	66.6	>A	1.23×10^{-10}
Type I IFN signaling pathway (HLA-A, STAT1, IRF3, PML, MX1, IRF7)	65	6	48.5	>A	1.42×10^{-02}
Negative regulation of leukocyte apoptotic process (CCL19, LILRB1, BCL6, IRF7)	44	4	47.7	>B	1.42×10^{-03}
Regulation of immunoglobulin-mediated immune response (C3, LTA, CD40, BCL6)	48	4	43.8	>B	2.00×10^{-02}
Regulation of IL12 production (RELA, CCL19, LILRB1, CD40)	51	4	41.2	>B	2.54×10^{-02}
T cell co-stimulation (CCL19, HLA-DRB1, DPP4, ICOS, HLA-DRB3)	82	5	32.0	>A	4.56×10^{-03}
PRR signaling pathway (RELA, MAPKAPK2, IRF3, IRF7, S100A8)	122	5	21.5	>A	3.14×10^{-02}
Regulation of humoral immune response (C3, CFH, LTA, CIQB, C7)	128	5	20.5	>B	3.96×10^{-02}
Positive regulation of I κ B kinase/NF- κ B signaling (RELA, CCL19, LITAF, CD40, HLA-DRB1, TRAF5, IRF3)	183	7	20.1	>B	4.73×10^{-04}

Ref. list (reference list) indicates the number of genes in a particular biological pathway. Hits, indicates the number of differentially expressed genes included in the pathway. Fold enrichment represents the enrichment score that reflects the degree to which a set of differentially expressed genes is overrepresented in the biopsies A or B [18]. The P-value is the estimation of significance level of the enrichment score.

PRR, pattern recognition receptor.

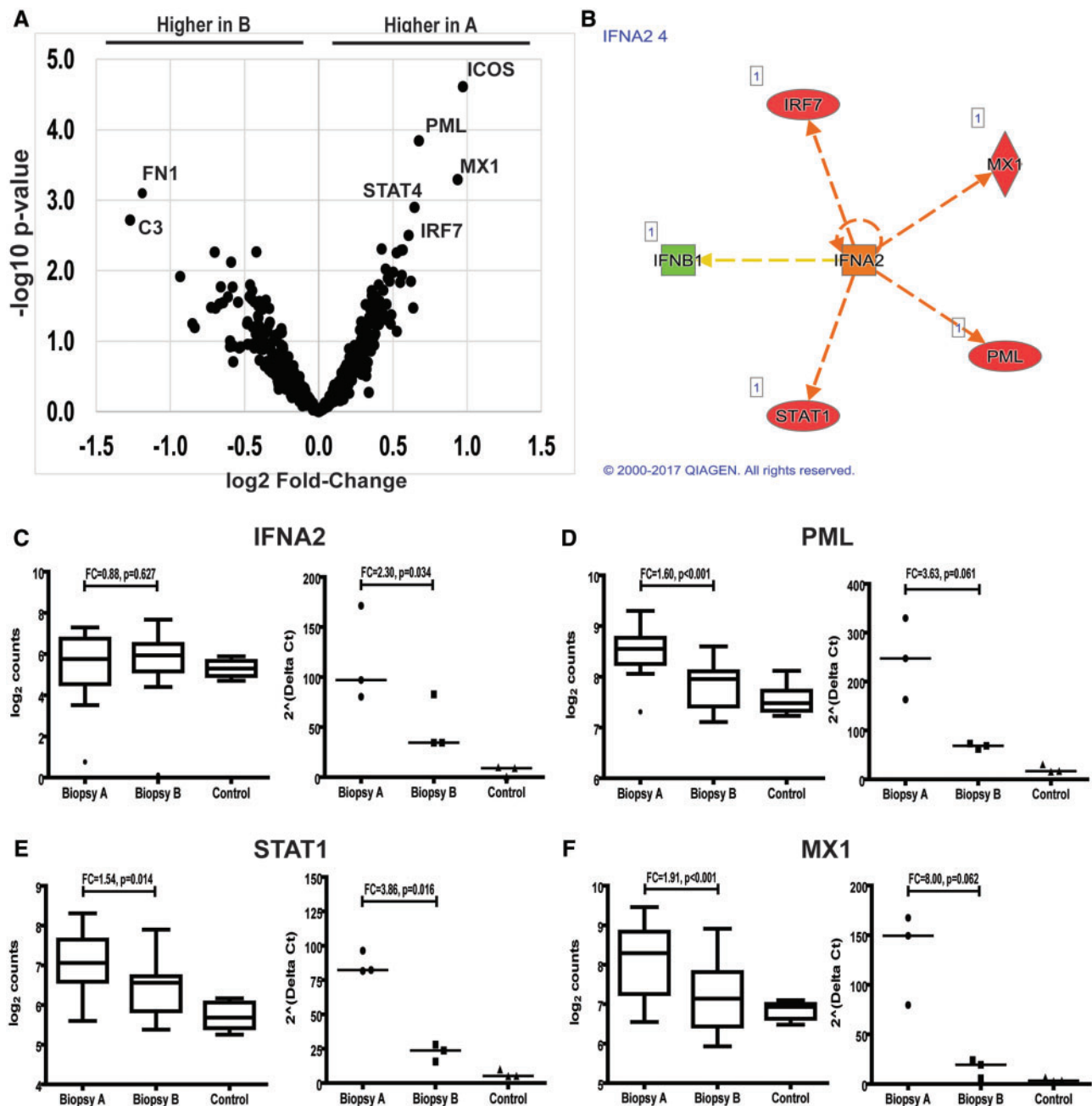


FIGURE 4: Tubulointerstitial immune gene expression from LN patients. (A) Volcano plot showing immune gene expression in biopsies A and B. A value of 2.0 on the ordinate corresponds to a P-value of 0.01 and a value of ± 1.0 on the abscissa corresponds to a 1.5 fold-change in expression. (B) Ingenuity Pathway Analysis showing IFNA2 as a central upstream regulator of differentially expressed genes between biopsies A and B. Expression of *IFNA2* (C), *PML* (D), *STAT1* (E) and *MX1* (F) measured by Nanostring (left graph) and by qPCR (right graph).

higher at flare than at diagnosis. The existence of a cross-regulation between $TNF\alpha$ and $IFN\alpha$ has been previously suggested to be a determinant in autoimmune diseases [19–21]. For example, an intense expression of $TNF\alpha$ is associated with the development of rheumatoid arthritis while an intense $IFN\alpha$ expression is associated with active SLE. The bridge between these two cytokines is supported by multiple reports of ‘lupus-like’ disease secondary either to IFN agents [35, 36] or TNF blockers [37, 38]. However, others have proposed using TNF blockers to treat lupus [39–41]. This ambiguous role of $TNF\alpha$

has been described in animal models of SLE where $TNF\alpha$ displays an initial immunoregulatory role by inhibiting the emergence of early autoreactivity [42–45], and a late pro-inflammatory role leading to end-organ damage [46–48]. Our data suggest TNF blockers may not be successful in *de novo* LN but may have a place in the treatment of LN flares.

Previous studies have emphasized the role of TLR signaling pathways in the pathogenesis of LN [49–51]. Nucleic acid immune complexes activate TLR in plasmacytoid dendritic cells, macrophages and B-cells mainly through TLR4, TLR7 and

Table 3. GO biological functions from differentially expressed genes between biopsies A and B in the tubulointerstitium

GO biological process	Ref. list	Hits	Fold enrichment	Biopsy A versus B	P
Regulation of type I IFN-mediated signaling pathway (<i>IFNA1, IFNB1, STAT1, IRF7</i>)	42	4	>100	>A	5.18×10^{-04}
Type I IFN signaling pathway (<i>IFNA1, IFNB1, STAT1, EGR1, MX1, IRF7</i>)	65	6	>100	>A	1.97×10^{-07}
Negative regulation of adaptive immune response based on somatic recombination of immune receptors built from immunoglobulin superfamily domains (<i>IL7R, IFNB1, IL4R</i>)	36	3	92.1	>B	4.09×10^{-02}
Negative regulation of viral life cycle (<i>PML, IFNB1, LTF, MX1</i>)	75	4	59.0	>A	5.17×10^{-03}
Leukocyte proliferation (<i>IL7R, KIT, IFNA1, IFNB1</i>)	88	4	50.2	>B	9.72×10^{-03}
Lymphocyte differentiation (<i>IL7R, KIT, IFNA1, IFNB1, EGR1</i>)	221	5	25.0	>B	1.13×10^{-02}
T cell activation (<i>IL7R, KIT, IFNA1, IFNB1, EGR1</i>)	226	5	24.5	>B	1.27×10^{-02}
Regulation of vasculature development(<i>C3, KIT, PML, STAT1, EGR1</i>)	248	5	22.3	>B	1.99×10^{-02}

Ref. list (reference list) indicates the number of genes in a particular biological pathway. Hits indicates the number of differentially expressed genes included in the pathway. Fold enrichment represents the enrichment score that reflects the degree to which a set of differentially expressed genes is overrepresented in the biopsies A or B [18]. The P-value is the estimation of significance level of the enrichment score.

TLR9, which in turn activate the MyDosome that mediates the production of IFN α and other cytokines. We did not find differences in *TLR4* and *TLR9* gene expression in kidney tissue from LN patients compared with normal controls, whereas *TLR7* and several transcripts involved in its activation pathway were highly upregulated. This suggests that *TLR7* has the predominant TLR role in human LN pathogenesis both in the glomerular and tubulointerstitial compartments. In LN animal models, *TLR7* is an essential mediator of kidney disease [52–54] and its overexpression accelerates renal damage [55], while its blockade [54] or knockout [56] diminishes damage. In contrast, dual inhibition of *TLR9* and *TLR7* has no additional effects beyond single *TLR7* blockade [52], and *TLR9* inhibition alone even increased damage in animal models [57]. Therefore, the *TLR7* pathway may be more important in human proliferative LN.

VCAM1 has been studied as a potential LN biomarker in serum [58–60] and urine [61, 62]. We found that kidney *VCAM1* expression increased between the initial and flare biopsy. This highlights the importance of determining the potential differences between LN flares when trying to identify disease biomarkers.

There are important limitations to this study. All patients were Hispanic and from a single center in Mexico, which may limit generalizability of the results to other races and ethnicities. Although the number of patients assessed was small, it must be emphasized that this is a rare disease and few SLE patients have had a kidney biopsy at diagnosis, responded to therapy and then had a repeat kidney biopsy at their first LN flare. While more patients would be desirable, our cohort provides an initial look into an important question that could affect therapy and has identified several candidate pathways for later directed investigation. The nCounter platform does not cover all transcripts as microarray or RNAseq do, but the advantage of this platform is the ability to work with FFPE tissues [63, 64]. Finally, due to the limited amount of tissue that can be extracted from samples, only a small number of key transcripts could be validated by qPCR.

In summary, we showed: (i) significant intrarenal immune gene expression heterogeneity among patients with proliferative LN; (ii) within a single patient, immune gene expression may be similar or different during subsequent disease flares; (iii) IFN

expression and the interferon signature in glomeruli and the tubulointerstitium is higher during the initial episode of LN compared with disease flares, whereas the opposite occurs for TNF α -regulated genes; and (iv) these gene expression changes between LN flares can be used to inform the treatment of a flaring patient and suggest a way to enrich clinical trials of specific targeted therapies for patients likely to respond to the experimental therapeutic.

SUPPLEMENTARY DATA

Supplementary data are available at ndt online. The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus and is accessible through GEO Series accession number GSE113342 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE113342>).

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AUTHORS' CONTRIBUTIONS

J.M.M.-V., S.V.P. and B.H.R. contributed to conception and study design. N.U.-U. and J.M.M.-V. contributed to biopsy handling and histopathological analysis. H.S., P.F., J.P.S., I.A. and J.M.M.-V. contributed to laser microdissection, sample management, nCounter and PCR analyses. Analysis and interpretation of data were done by L.Y., J.Z., I.A., S.V.P.,

J.M.M.-V. and B.H.R. J.M.M.-V., S.V.P., I.A. and B.H.R. contributed to drafting the manuscript and revising it.

CONFLICT OF INTEREST STATEMENT

None declared.

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