

Fava Andrea (Orcid ID: 0000-0001-6738-4836)
Rao Deepak A (Orcid ID: 0000-0001-9672-7746)
Zhang Ting (Orcid ID: 0000-0002-8616-9733)
Izmirly Peter (Orcid ID: 0000-0001-5445-2182)
James Judith A. (Orcid ID: 0000-0002-9574-7355)
Buyon Jill (Orcid ID: 0000-0002-3852-7129)
Petri Michelle A (Orcid ID: 0000-0003-1441-5373)

Urine Proteomics and Renal Single Cell Transcriptomics Implicate IL-16 in Lupus Nephritis.

Authors: Andrea Fava MD*¹, Deepak A. Rao MD PhD², Chandra Mohan MD PhD³, Ting Zhang PhD³, Avi Rosenberg MD PhD⁴, Paride Fenaroli MD^{4,5}, H. Michael Belmont MD⁶, Peter Izmirly MD⁶, Robert Clancy MD⁶, Jose Monroy Trujillo MD⁷, Derek Fine MD⁷, Arnon Arazi PhD⁸, Celine C. Berthier PhD⁹, Anne Davidson MBBS¹⁰, Judith A. James MD PhD¹¹, Betty Diamond MD⁸, Nir Hacoheh PhD¹², David Wofsy MD¹³, Soumya Raychaudhuri MD PhD^{2,14-16}, William Apruzzese MPH², the Accelerating Medicines Partnership in RA/SLE network[‡], Jill Buyon MD⁶ and Michelle Petri MD MPH¹

*Corresponding author:

Andrea Fava, MD
1830 East Monument Street, Suite 7500
Baltimore, MD 21205
Telephone: 410 955 9114 – Fax 410 614 0498
Email: afaval@jh.edu

Affiliations:

1. Division of Rheumatology, Johns Hopkins University, Baltimore, MD, USA
2. Division of Rheumatology, Immunology, Allergy, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA.
3. University of Houston, Houston, USA
4. Division of Renal Pathology, Johns Hopkins University, Baltimore, MD, USA
5. Nephrology Unit, Parma University Hospital, Department of Medicine and Surgery, Parma, Italy
6. New York University School of Medicine, New York, New York, USA
7. Division of Nephrology, Johns Hopkins University, Baltimore, MD, USA
8. Feinstein Institutes for Medical Research, Manhasset, NY, USA
9. University of Michigan, Ann Arbor, MI, USA
10. Northwell Health, New York, NY, USA
11. Oklahoma Medical Research Foundation, Oklahoma City, OK, USA
12. Broad Institute, Boston, MA, USA
13. University of California San Francisco, San Francisco, CA, USA
14. Center for Data Sciences, Brigham and Women's Hospital, Boston, MA 02115, USA.
15. Program in Medical and Population Genetics, Broad Institute of MIT and Harvard, Cambridge, MA, USA
16. Centre for Genetics and Genomics Versus Arthritis, Centre for Musculoskeletal Research, Manchester Academic Health Science Centre, The University of Manchester, Oxford Road, Manchester, UK.

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‡The Accelerating Medicines Partnership in RA/SLE network include the following authors
Andrea Fava, Deepak A. Rao, Chandra Mohan, Ting Zhang, H. Michael Belmont, Peter Izmirly,
Robert Clancy, Jose Monroy Trujillo, Derek Fine, Arnon Arazi, Celine C. Berthier, Anne
Davidson, Judith A. James, Betty Diamond, Nir Hacohen, David Wofsy, Soumya Raychaudhuri,
William Apruzzese, Jill Buyon, Michelle Petri, Yanyan Liu, Paul J. Hoover, Adam Chicoine,
Thomas M. Eisenhaure, A. Helena Jonsson, Shuqiang Li, David J. Lieb, Fan Zhang, Ilya
Korsunski, Joseph Mears, Kamil Slowikowski, Edward P. Browne, Akiko Noma, Danielle
Sutherby, Scott Steelman, Dawn E. Smilek, Patti Tosta, Elena Massarotti, Maria Dall’Era,
Meyeon Park, Diane L. Kamen, Richard A. Furie, Fernanda Payan-Schober, William F.
Pendergraft III, Elizabeth A. McInnis, Chaim Putterman, Kenneth C. Kalunian, E. Steve
Woodle, James A. Lederer, David A. Hildeman, Chad Nusbaum, Matthias Kretzler, Jennifer H.
Anolik, Michael B. Brenner, Dia Waguespack, Sean M. Connery, Maureen A. McMahon,
William J. McCune, Ruba B. Kado, Raymond Hsu, Melissa A. Cunningham, Paul J. Utz, Mina
Pichavant, Holden T. Maecker, Rohit Gupta, Joel M. Guthridge, Chamith Fonseka, Evan Der,
Thomas Tuschl, Hemant Suryawanshi, Daniel Goldman.

Abstract

Objectives. Current treatments are effective only in 30% of lupus nephritis patients emphasizing the need for novel therapeutic strategies. To develop mechanistic hypotheses and explore novel biomarkers, we analyzed the longitudinal urinary proteomic profiles in patients with lupus nephritis undergoing treatment.

Methods. We quantified 1,000 urinary proteins in 30 patients with lupus nephritis at the time of the diagnostic renal biopsy and after 3, 6, and 12 months. The proteins and molecular pathways detected in the urine proteome were then analyzed with respect to baseline clinical features and longitudinal trajectories. The intrarenal expression of candidate biomarkers was evaluated using single cell transcriptomics of renal biopsies from lupus nephritis patients.

Results. Our analysis revealed multiple biological pathways including chemotaxis, neutrophil activation, platelet degranulation, and extracellular matrix organization that could be noninvasively quantified and monitored in the urine. We identified 237 urinary biomarkers associated with lupus nephritis as compared to controls without SLE. IL-16, CD163, and TGF- β mirrored intrarenal nephritis activity. Response to treatment was paralleled by a reduction of urinary IL-16, a CD4 ligand with proinflammatory and chemotactic properties. Single cell RNA sequencing independently demonstrated that IL16 is the second most expressed cytokine by most infiltrating immune cells in lupus nephritis kidneys. IL-16 producing cells were found at key sites of kidney injury.

Conclusion. Urine proteomics may profoundly change the diagnosis and management of lupus nephritis by noninvasively monitor active intrarenal biological pathways. These findings implicate IL-16 in lupus nephritis pathogenesis designating it as a potentially treatable target and biomarker.

Introduction

Lupus nephritis (LN) is a severe manifestation of systemic lupus erythematosus (SLE) that frequently leads to end-stage kidney disease despite treatment¹. Diagnosis and treatment of LN rely on histopathological features of kidney biopsies in patients with proteinuria. Kidney biopsies have an indispensable role in that they can distinguish active nephritis from chronic damage, both of which manifest with proteinuria. However, kidney biopsies have limitations. Most notably, histology does not capture patient-specific active biological pathways. Further, the histological class frequently changes on repeat kidney biopsies, suggesting that the histological classification may artificially divide patients based on one point in time^{2,3}. Procedure-related complications may occur⁴, and up to 35% of kidney biopsies may fail to obtain an adequate sample⁵. Access to kidney biopsies may delay diagnosis and treatment, and can be limited by antithrombotic and anticoagulation treatments, severe thrombocytopenia, and in resource poor settings. Finally, because the presence of proteinuria implies that underlying kidney damage has already happened, kidney biopsies are a lagging indicator. Thus, there is a pressing need for a noninvasive biomarker to probe in “real-time” the active molecular pathological processes in the kidney and to monitor them over time in response to treatment.

Several available biomarkers correlate with histological features, but none are currently used in clinical practice^{6,7}. These lack the sensitivity and specificity to detect active renal inflammation, predict flares, and reliably inform prognosis, and do not add actionable information in addition proteinuria or renal function^{6,7}. Unbiased proteomic screenings carry a high potential for discovery, but these have been limited to the evaluation of proteins or peptides sufficiently abundant to be detectable by mass spectrometry^{8,9}. More sensitive aptamer-based arrays identified

candidate urinary biomarkers associated with proteinuria, but their ability to predict nephritis activity and clinical outcomes is still to be determined ¹⁰.

Management of LN could be greatly enhanced by a resource which can identify candidate biomarkers that predict histological features and clinical outcomes, as well as infer the renally active biological pathways. Here, we used a glass slide based protein microarray to screen and quantify 1000 proteins covering a wide range of biological processes in longitudinal urine samples of patients with LN (starting at the time of biopsy) to develop mechanistic hypotheses and explore novel biomarkers. This array allowed the unbiased, precise and sensitive quantification of the concentration of each of the 1000 proteins as validated in previous studies¹¹⁻¹³. We found that protein expression patterns define distinct molecular pathways that are differentially expressed among LN patients. We also discovered that IL-16, a proinflammatory chemokine, is tightly associated with LN activity and may have role in LN pathogenesis thus nominating IL-16 as a potentially treatable target.

Methods

Patients and sample collection.

This study enrolled SLE patients with urine protein/creatinine ratio greater than 0.5 undergoing clinically indicated renal biopsy. Only patients with a pathology report confirming LN were included in the study. Renal biopsies were scored by one renal pathologist at each site of the two sites according to the International Society of Nephrology/Renal Pathology Society (ISN/RPS) guidelines and NIH activity and chronicity indices¹⁴. Clinical information, including serologies, were collected at the most recent visit before the biopsy. Response status at week 52 was defined as follows. Complete: pr/cr \leq 0.5, normal serum creatinine (sCr) or $<$ 25% increase from baseline if abnormal, and prednisone \leq 10mg daily; partial: pr/cr $>$ 0.5 but \leq 50% of the baseline value and identical sCr and prednisone rules as complete response; no response: pr/cr $>$ 50% of baseline value or new abnormal elevation of sCr or \geq 25% from baseline or prednisone \geq 10mg daily. Urine samples from healthy volunteers (all females, median age 42 years [32-54], 3 identifying as Caucasian and 4 as African American) were included. Urine specimens were acquired on the day of the biopsy (before the procedure) at 2 clinical sites in the United (Johns Hopkins University, JHU, and New York University, NYU). For the validation cohort (n=101), urine samples were collected on the day of (73%) or within 3 weeks (27%) of kidney biopsy. Serological features and complement levels were determined at the clinical visit preceding the biopsy. Proteinuria was measured on or near the day of the biopsy.

Study approval.

Human study protocols were approved by the institutional review boards at JHU and NYU, and written informed consent was received from all participants.

For healthy controls, IRB approval was obtained from the Oklahoma Medical Research Foundation. After informed consent, controls were recruited through the Oklahoma Rheumatic Disease Research Cores Center, cohort matching for gender, race, ethnicity and age. Subjects were screened by a questionnaire and tested negative for ANA, dsDNA, Chromatin, RiboP, Ro, La, Sm, SmRNP, RNP, Centromere B, Scl-70, and Jo-1 antibodies. Samples were processed, stored, and shipped using the AMP RA/SLE protocols to align with the patient samples.

Urine Quantibody assay

The Kiloplex Quantibody protein array platform (Raybiotech) was used for screening urine samples as previously described¹². Validation was performed using an immunoquantitative (PCR-Based) IL-16 ELISA (Raybiotech) to match and improve the sensitivity and dynamic range provided by the kiloplex array. These were summarized in the Supplementary Methods.

Renal tissue single cell RNA sequencing

Renal tissue was collected, stored and processed as previously described¹⁵. Briefly, research biopsy cores were collected from consented subjects as an additional biopsy pass or tissue from routine clinical passes. Only biopsies with confirmed LN were included. Kidney tissue was frozen on site and shipped to a central processing location where it was thawed and disaggregated. Individual cells were retrieved and sorted by flow cytometry. For each sample, 10% of the sample was allocated to sort CD10+CD45- epithelial cells as single cells, and the remaining

90% of the sample was used to sort CD45+ leukocytes as single cells. For each single cell, the whole gene expression profile was sequenced using the CEL-Seq2 method.

Statistical analyses.

The differential protein abundance was calculated using a moderated t statistic. To achieve normal distribution, the protein abundances were log-transformed after adding 10% (arbitrary constant empirically shown not to significantly alter distributions) of the lowest measured abundance to remove zeros. With 30 LN and 7 HD samples, using a two-sided .05-level test adjusting for 1000 comparisons (Bonferroni), there was 80% power to detect a difference in mean peptide magnitude of 1.2 standard deviations (i.e., an effect size of 1.2). Concentrations of all urinary proteins for all urine samples were available without missing data. Clustering was performed using the Ward's minimum variance method. ROC curves and areas under the curve (AUCs) were calculated using the function `roc` within the `pROC` R package. The impact of confounders on the association between the ISN Activity Index and the urinary abundance of a biomarker was tested using one confounder at the time (given limited sample size) using a linear regression model as follows: $activity \sim biomarker_abundance + confounder$. The models were fitted using the `lm` function within the `stats` R package. See Supplementary Methods for pathway enrichment analysis. Pearson correlation coefficients were used throughout the manuscript. All analyses were performed in R.

Prevalence of cytokine positive cells.

Analysis of cytokine-positive cells was based on a compendium of 237 cytokines obtained from Gene Ontology ¹⁶ and manually extended using the Cytokine Registry

(<https://www.immport.org/resources/cytokineRegistry>), the iTalk database ¹⁷, and the International Union of Basic and Clinical Pharmacology (IUPHAR) and British Pharmacological Society (BPS) database. For each cytokine, we calculated the prevalence of the cells with at least one transcript over the total number of cells.

Immunohistochemistry.

See Supplementary Methods.

Results

Urine proteomics identifies biologically relevant active pathways in LN

Urine samples from 30 subjects with active LN were collected near or at the time of a renal biopsy. Clinical and demographic characteristics are summarized in **Supplementary Table 1**. Compared to healthy donors, there were 237 proteins significantly elevated in the urine of patients with LN (FDR < 10%) as displayed in **Figure 1A**. This list included both novel and previously described urinary biomarkers (**Supplementary File 1**). Pathway enrichment analysis of the proteins the proteins significantly elevated in LN identified 12 enriched non-overlapping pathways, including relevant biological processes such as chemotaxis, neutrophil activation, platelet degranulation, and extracellular matrix organization (**Figure S1**). Hierarchical clustering using enriched pathways segregated LN patients into 2 groups, with 80% of those who later achieved a complete renal response being in the same group with overall less inflammatory pathways (OR 12.6, p=0.03) (**Figure 1B**). Baseline parameters such as proteinuria, creatinine, histologic activity or chronicity scores, or class were present in similar frequencies in both clusters suggesting that urine proteomics may provide unique informative features (**Figure 1B**).

Identification of urinary biomarkers of renal histology.

We sought to identify urinary proteins that could identify renal histology. LN can be classified in two broad categories based on the presence of a glomerular endocapillary immune infiltrate or “proliferation”. Proliferative LN (ISN class III or IV) is a more aggressive phenotype associated with glomerular endocapillary hypercellularity, abundant immune cell infiltration and higher risk of permanent renal damage. Compared to pure membranous LN (n=9), patients with proliferative LN (n=14) showed higher concentration of several urine cytokines and molecules

involved in immune activation and chemotaxis (**Figure 2A-B**). IL-16 was the most significantly enriched urinary protein in proliferative LN (**Figure 2A**). Pathway enrichment analysis revealed that the pattern of chemokines matched the chemokine released in response to interferon-gamma (IFN- γ), IL-1 β , and TNF (**Figure 2B**).

Many of the urinary proteins that were differentially abundant when comparing proliferative and membranous were not significantly more abundant when comparing all LN patients to healthy controls. In fact, although most of the proteins enriched in proliferative LN were generally more abundant in LN vs healthy controls, these were not among the most abundant (> 2 SD) (**Figure S2 A-B**). This is because the first comparison (LN vs healthy) is aimed to identify proteins that are generally more abundant in all LN patients, regardless of class. Not surprisingly, the most abundant protein in all LN was RBP4, a general marker of tubular impairment¹⁸. These findings indicate that contrasting well defined subgroups allowed to identify relevant biomarkers that could have been missed by analyzing all LN patients together. Different pathogenic processes may underlie each histological subgroup and thus these biomarkers may provide insight into the relative active pathways.

Urinary IL-16 reflects histological activity.

The degree of histological activity is often used to inform clinical decisions, so we sought to identify noninvasive urinary biomarkers that reflect histological activity. We studied the correlation of the urinary abundances of all 1000 biomarkers in urine samples collected at the time of biopsy with the histological NIH activity index. We found that IL-16 was the urinary protein most strongly positively correlated with the NIH activity index (Pearson's r 0.73, $p = 1.2 \times 10^{-5}$, FDR $< 10\%$, $n=28$) followed by CD163, and TGF- β (FDR $< 10\%$) (**Figure 3A-D**). We validated

the significant concurrent correlation between urinary IL-16 abundance and NIH activity index in an independent cohort of 101 patients ($r = 0.59$, $p = 9.3 \times 10^{-11}$, **Figure S3** and **Supplementary Table 2**) and with a PCR-based ELISA (**Figure S4**). Notably, IL-16 was the only one not associated with proteinuria (**Figure 3H**), suggesting the potential to provide actionable information in addition to classic biomarkers such as proteinuria. In multivariate models, IL-16, CD163, and TGF- β retained their association with histological activity after adjustment for multiple confounders, including proteinuria (**Supplementary Table 3**). The pathways associated with histological activity are displayed in **Figure S4**.

In addition to having the strongest correlation with histological activity, IL-16 was the urinary protein most associated with proliferative LN (**Figure 2A**). The receiver operating characteristic curve revealed that IL-16 was a promising urinary biomarker to identify patients with proliferative LN with an area under the curve (AUC) of 0.85 ($p = 0.016$) and 0.89 ($p = 0.037$) in association with CD163 and TGF- β (**Figure S5**).

Urinary biomarkers correlating with activity decrease with clinical response in longitudinal samples.

A goal of immunosuppression in LN is to eradicate pathological renal inflammation to ultimately prevent irreversible renal damage and preserve function. The NIH activity index captures many renal inflammatory features and, as a consequence, it improves with treatment in patients achieving renal remission^{2,19}. However, it is impractical to monitor in clinical practice as it requires frequent repeat renal biopsies. Thus, we hypothesized that the 3 urinary biomarkers associated with histological activity would decline over time in patients who are responding to treatment and might serve as noninvasive biomarkers of response. The urinary concentration of

all 3 candidate biomarkers declined in complete and partial responders but not in non-responders (**Figure 4A-C**). The average decline was most striking in IL-16 with a decrease in partial and complete responders by week 12. CD163 concentration improved by week 12 in complete responders but not in partial responders. TGF- β showed a more modest decline.

Since response status is defined by reduction of proteinuria, we wanted to ensure that the observed biomarker trajectories were not simply a reflection of a decline in all urinary protein in responders. The trajectories of 3 urinary proteins that were selected among those that did not correlate with histological activity demonstrated that there was no non-specific decline (**Figure 4D-F**). These findings indicate that IL-16, CD163, and TGF- β trajectories represent a specific decrease in the production and excretion of these molecules and, as they correlated with activity at baseline, likely reflect a corresponding improvement of intrarenal LN activity supporting their value as biomarkers.

IL16 is one the most expressed cytokines in kidney infiltrating immune cells in LN.

To determine whether the urinary concentration of the 3 candidate biomarkers reflects an active intrarenal process rather than passive filtration through a damaged glomerular membrane, we evaluated the intrarenal relative gene expression using single cell RNA sequencing of LN renal biopsies. IL16 was abundantly expressed by most immune infiltrating cells, CD163 by a subset of myeloid cells, and TGF β 1 mostly by NK cells (**Figure 5A-D**).

In LN, most of IL-16 expression was in immune infiltrating cells, especially the lymphoid lineage (**Figure 5C-D**). In renal allograft rejection, single cell RNA-seq showed that IL16 is expressed by endothelial, epithelial, and immune cells, but immune cells were the main source (**Figure S6A**)²⁰. Conversely, in the healthy kidney, single nuclear RNA sequencing and ATAC-

seq (Assay for Transposase-Accessible Chromatin using sequencing) revealed substantial IL16 expression by podocytes, fibroblasts, endothelial, mesangial, and proximal tubular cells (**Figure S5B-C**)^{21,22}. These findings suggest that while immune cells are likely the major intrarenal source of IL-16 in LN, IL-16 secretion by endothelial and tubular cells may precede immune infiltration. Speculating, this initial event can then be amplified by infiltrating immune cells as seen in LN and allograft rejection.

Finally, we explored whether IL16 was disproportionately more expressed as compared to other cytokines in LN. Out of a compendium of 237 cytokines, IL16 was the second most commonly expressed cytokine (49% of all infiltrating immune cells) (**Figure 5E**). These findings independently nominate IL-16 as a major cytokine involved in LN.

Tissue expression of IL-16 correlate with LN activity and urinary IL-16 abundance.

To establish the location of IL-16 secreting cells in renal tissue, we performed immunohistochemical staining of human IL-16 in 7 LN kidney biopsies with matching urine IL-16 collected at or near the time of biopsy. We observed abundant interstitial and glomerular IL-16 expression in proliferative LN (**Figure 6 and S7A-C**), with the exception of one case (**Figure S7D**) in which the activity index was uncharacteristically low (2) and IL-16 was not detectable in the urine. In contrast, there was very scant IL-16 positivity in membranous LN (**Figure 6 and S7E-G**) and marginal in a class I LN biopsy used as negative control (**Figure S7H**). These findings matched the urinary IL-16 profile. Furthermore, there was a qualitative correlation between the number of IL-16 positive cells and urinary IL-16 abundance as well as the NIH activity index (**Figure 6**). This was particularly evident for glomerular IL-16 positive cells. These findings

indicate that IL-16 is intrarenally produced in proliferative LN and urinary IL-16 reflects the abundance of intrarenal IL-16 positive cells and LN activity.

Discussion

Leveraging urine proteomics in patients with LN and healthy controls, the results of this study confirmed that the pathological processes in LN can be noninvasively captured and monitored over time. We found: (1) 237 urinary proteins associated with LN that represented at least 12 distinct molecular pathways; (2) a strong chemokine signature characterizing the urine of patients with proliferative LN; and (3) several candidate biomarkers to detect active nephritis that can be monitored over time to assess response to treatment. Overall IL-16 emerged as the most robust correlate of histological activity implying a role in LN pathogenesis and thus subsequent translation to clinical application both as a biomarker and treatable target.

Proteomic analysis revealed that the intrarenal activation of several pathogenic mechanisms contributing to LN can be quantified in the urine. These biological processes were previously implicated in LN including neutrophil immunity ^{23,24}, platelet degranulation ²⁵, extracellular matrix organization ²⁶, and chemotaxis ²⁷. Patients did not cluster based on the abundance of a single or a group of signatures. Rather, we observed two clusters characterized by high and intermediate abundance of all signatures, respectively. This is consistent with previous findings from an agnostic approach to urine proteomics in LN that showed that patients stratify on a gradient ²⁷. Importantly, 80% of complete responders clustered in the intermediate abundance group. The predictive value of this approach needs to be validated in a larger cohort given the small number of responders.

In this study, urinary abundance of proteomic signatures was independent from proteinuria, suggesting that these signatures specifically reflect active biological processes rather than a non-specific increase or decrease of all urine proteins. In particular, pathway enrichment analysis revealed a strong chemokine signature in proliferative LN suggesting active recruiting of immune cells in the kidney in these patients. This is biologically consistent with the abundant immune cell infiltration and more aggressive phenotype observed in class III and IV LN, further supporting the ability of urine proteomics to infer intrarenal biological processes.

Ideal biomarkers in LN should noninvasively infer nephritis activity, longitudinally track response to treatment, and ideally capture the intrarenal biology. Based on feasibility, the current management of LN hinges on monitoring proteinuria to establish renal activity rather than frequent biopsies. However, proteinuria is a poor marker of nephritis activity. Six-month repeat biopsies after induction therapy revealed that about half of the patients in complete clinical remission (proteinuria <0.5 g/24h and no increase in sCr) had persistent histologically active proliferative nephritis ²⁸. Conversely, $> 50\%$ of patients who achieved complete histological remission had persistent proteinuria >0.5 g/24h. Moreover, patients in clinical remission 3 years after induction treatment may show persistent nephritis activity on per protocol biopsies which is associated with flares of nephritis as immunosuppression is tapered ². Using an unbiased approach, we discovered a previously unrecognized biomarker of intrarenal activity, IL-16, in addition to two previously recognized LN biomarkers, CD163 ²⁹ and TGF- β ³⁰. IL-16 showed the strongest and most significant association with the renal activity index of any marker measured, and urinary abundance of IL-16 decreased over time in patients who ultimately responded to treatment after 1 year. IL-16, CD163, and TGF-beta were selected based on their correlation with histological activity; thus, it is conceivable that their decreasing urinary abundance mirrored an improvement

of intrarenal histological activity. In fact, urinary proteins that did not correlate with activity did not decrease over time in responders.

Renal single cell RNA sequencing revealed that IL16, CD163, and TGFB1 are actively expressed by immune infiltrating cells in LN kidney biopsies, suggesting that their detection in the urine reflects intrarenal immune activity. Because their expression was in distinct immune cell types, their urinary abundance could identify the activity of distinct immune processes. We discovered that IL16 was the second most expressed cytokine in LN kidneys (49% of all infiltrating immune cells). This striking concordant result was independent of the urine proteomics dataset, thus demonstrating the relevance of IL-16 in LN in an orthogonal approach. Furthermore, we demonstrated prominent intraglomerular and interstitial renal production of IL-16 in proliferative LN by immunohistochemistry. Although we did not evaluate circulating cells or serum, IL-16 urinary abundance correlated with intrarenal IL-16 positive cells implicating that urinary IL-16 is the direct consequence of intrarenal IL-16 secretion. Because urinary IL-16, intrarenal IL-16 positive cells, and histological activity are positively co-correlated and IL16 is one the most expressed cytokines in LN, our findings suggest that IL-16 may be implicated in LN pathogenesis and this process can be non-invasively measured in urine.

IL-16 is a proinflammatory chemokine secreted by immune cells and non-immune cells (endothelial, epithelial cells, fibroblasts, and neurons) in response to several stimuli such as complement activation, antigen stimulation, interferon, hypoxia, and cell injury³¹⁻³⁴. Because the release of bioactive IL-16 depends on caspase 3 activation³³, apoptosis and pro-apoptotic stimuli including sublethal doses of granzymes may also lead to its release. IL-16 can also be released upon cleavage by proteinase 3³⁵ suggesting that urinary IL-16 may indicate neutrophil

degranulation. IL-16 is the natural ligand for CD4 and CD9, and is a strong chemoattractant for CD4⁺ T cells (especially Th1 cells) as well as CD8 T, NK, B cells monocytes, neutrophils, dendritic cells, and mast cells³¹. IL-16 can activate CD4 T cells independently of T-cell receptor (TCR) activation³⁶ and may lead to the release of proinflammatory cytokines such as TNF, IL-1 β , IL-6, IL-15, and IL-12³¹. IL16 polymorphisms were associated with increased risk of SLE (OR 3.3-10.4) suggesting a potential causal role³⁷. Plasma IL-16 levels were associated with SLE severity including renal involvement³⁸. Finally, IL-16 was mechanistically linked to lung disease in the pristane model of SLE³⁹. The role of IL-16 in LN is yet to be fully understood, but it has been implicated in several other immune mediated diseases such as multiple sclerosis, scleroderma, rheumatoid arthritis, and allograft rejection^{31,40,41}. Further studies are needed to address the efficacy of IL-16 blockade in LN.

Our study demonstrated the power of integrating urinary proteomic screening platforms with matching clinical and pathological information and with tissue single cell transcriptomics⁴². In fact, in addition to a newly discovered biomarker, our approach detected CD163 and TGF- β that are proven biomarkers in LN. Similar to our findings, soluble CD163 was shown to correlate with LN nephritis activity and improve with treatment²⁹. CD163 is a scavenger receptor expressed on phagocytic monocytes, especially in M2c polarized macrophages that infiltrate tissues during the healing phase of inflammation and are implicated in fibrosis resolution⁴³. Notably, M2c macrophage are inducible by TGF- β ⁴⁴. CD163⁺ cells are a dominant macrophage subtype in LN⁴⁴, thus again supporting the capability of urinary proteomic to infer intrarenal biology. CD163⁺ cells have been detected in proliferative glomerular lesions and in tubulointerstitial inflammation⁴⁵ and they constitute ~80% of the urinary cells in LN⁴⁶. Similarly consistent with our results, urinary TGF- β correlated with nephritis activity and response in previous studies^{30,47,48}, but

sensitive immunoassays (such as the one used here) are required to reliably detect urinary TGF- β ⁴⁸. TGF- β regulates inflammation and progression of renal fibrosis. Notably, TGF- β increased IL-16 release in synovial fibroblasts suggesting a possible similar interplay between these two cytokines in LN⁴⁹. Here, we have shown that NK cells are the major immune cell type expressing TGFB1 in LN, whether NK or tubular cells⁵⁰ are responsible for urinary TGF- β in LN is to be determined.

We acknowledge the limitations of our study. Since we did not analyze serum or plasma, we could not establish with definitive certainty whether the concentration of specific proteins in the urine was the consequence of extrarenal leakage from the circulation through a damaged glomerular basement membrane or of intrarenal production. For example, plasma IL-16 levels were associated with disease severity including renal involvement in a group of SLE patients³⁸, but whether the source IL-16 was intra or extrarenal was not established. We have unequivocally demonstrated that there is high intrarenal production of IL-16 in LN indicating that urinary IL-16 derives, at least in part, from active intrarenal secretion. Importantly, the association between urinary IL-16 and proliferative LN activity was independent of proteinuria suggesting that a change in urinary IL-16 abundance is an independent process rather than nonspecific leakage from plasma. Future studies will be needed to address the power of urinary IL-16 to discriminate “active” from “non active” proliferative LN. In addition, as there was a limited number of complete responders, we could not study biomarkers to predict future response with statistically robust confidence nor confidently test if the longitudinal trajectories were statistically significant. Ongoing studies as part of the AMP RA/SLE consortium will allow us to address these questions.

In summary, this study linked IL-16 release with lupus nephritis activity suggesting a possible role as a biomarker and in LN pathogenesis thus nominating IL-16 as a potentially

treatable target. Further, our study demonstrated the feasibility to detect novel and biologically relevant biomarkers in LN using a urine proteomic platform in a well characterized longitudinal cohort. Further ongoing studies are required to confirm the clinical applicability of these findings, this unprecedented dataset may further discovery by allowing investigators to research and validate new biomarkers, test new hypotheses, and complement mechanistic studies in LN.

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Data and materials availability: The data reported in this publication, including the clinical and serological data of the study participants, are deposited in the ImmPort repository (accession code SDY997). The raw single-cell RNA-seq data are also deposited in dbGAP (accession code phs001457.v1.p1).

Figure Legends:

Figure 1. Identification of pathogenic pathways by urine proteomics. (A) Volcano plot illustrating the differential abundance of 1000 urinary proteins in lupus nephritis (LN, n=30) and healthy controls (HC, n=7). There were 237 proteins significantly more abundant in LN (>2 fold, false discovery rate (FDR) <10%, moderated t test). Thresholds for an FDR of 1% and nominal p=0.05 are shown by dotted lines. (B) Heatmap of the abundance of the 12 non-overlapping pathways enriched in LN urine samples by pathway enrichment analysis (GO Biological Process). Twenty of the thirty patients displayed a LN cluster with higher abundance of all pathways, whereas the patients in the other cluster exhibited an intermediate abundance as compared to healthy controls. Clustering was otherwise not explained by other clinical variables such as proteinuria, renal function, nephritis activity, chronic damage, or class. Values were scaled by rows. Clustering was performed using the Ward's minimum variance method.

Figure 2. Proteomic profile of proliferative lupus nephritis. (A) Volcano plot illustrating the differential abundance of 1000 urinary proteins in proliferative LN (n=14) and pure membranous LN (n=9). (B) Pathway enrichment analysis (GO Biological Process) of the urinary proteomic profile revealed that chemotaxis was the process most enriched in proliferative LN. In particular, these were chemokines secreted in response to TNF, IL-1, and IFN- γ . The enrichment FDR (GSEA rank permutation) was <5% for all pathways except for "Natural killer activation" (16%).

Figure 3. Urinary biomarkers of histological nephritis activity. (A) Pearson's correlations of the urinary abundance of 1000 proteins and the histological NIH Activity Index in near or same day renal biopsies. Each dot represents a protein within the array. The dashed line marks the

significance threshold after correcting for multiple comparisons (FDR 10%). The area of the dot is proportional to the absolute of the correlation coefficient. Three proteins showed an FDR <10%. The FDR of IL-16 was 1.2%. Scatter plots displaying the Pearson's r and p values of correlations of the urinary abundance of IL-16, CD163, and TGF- β 1 with the NIH Activity Score (B-D), NIH Chronicity Score (E-G), and proteinuria (H-J).

Figure 4. Biomarkers associated with nephritis activity decrease in responders. Urinary concentration of all biomarkers was measured at time of biopsy (or “week 0”, W0) and after 12, 24, and 52 weeks. Thin lines depict the trajectories of each patient categorized based on the response status determined at week 52. Thick lines represent the average for each group. The urinary concentration of the 3 biomarkers significantly correlated with histological activity declined in complete and partial responders but not in non-responders (A-C). In contrast, 3 biomarkers that did not correlate with histological activity (Pearson's r range -0.0018 - 0.0015, p=ns) did not show a decline over time.

Figure 5. High expression of IL16 in lupus nephritis kidney. (A) UMAP plot of scRNA-seq of renal biopsies (3131 cell) by lineage. (B) Feature plot displaying IL16 expression at single cell level. Violin (C) and bar (D) plots summarizing the expression of the genes coding for the urinary proteins associated with nephritis activity. IL16 was abundantly expressed by most kidney infiltrating immune cells, CD163 mostly by macrophages, and TGFB1 by NK cells. (E) Prevalence (%) of cytokine positive cells out of a compendium of 237 cytokines ranked decreasingly (top 20 are shown). IL16 (in red) was the second most expressed cytokine in LN kidneys.

Figure 6. IL-16 positive cells are abundant in proliferative LN and qualitatively correlate with urinary IL-16 and LN activity. Immunohistochemical (IHC) staining of human IL-16 were performed in 7 LN kidney biopsies with matching urine IL-16 collected at or near the time of biopsy. The corresponding urinary abundance of IL-16 (A) and NIH Activity Index (B) of the patients whose biopsy depicted in C are plotted according to ISN class. Lower case letters (“a” to “g”) in A, B, and C identify information from the same patients. (C) IHC of IL-16 in 4 proliferative LN biopsy sections (a-d) and 3 pure membranous LN (e-g). Abundance of IL-16+ cells was noted in proliferative LN (C, a-d) with qualitatively more prominent intraglomerular IL-16 positivity in patients with higher urinary IL-16 and NIH Activity Index. Images are magnified 33.6x. Lower magnification images with larger representation of the interstitium are displayed in Supplementary Figure 6.