Arthritis & Rheumatology
Vol. 74, No. 5, May 2022, pp 829–839
DOI 10.1002/art.42023

American College of Rheumatology
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Urine Proteomics and Renal Single-Cell Transcriptomics Implicate Interleukin-16 in Lupus Nephritis

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Objective. Current lupus nephritis (LN) treatments are effective in only 30% of patients, emphasizing the need for novel therapeutic strategies. We undertook this study to develop mechanistic hypotheses and explore novel biomarkers by analyzing the longitudinal urinary proteomic profiles in LN patients undergoing treatment.

Methods. We quantified 1,000 urinary proteins in 30 patients with LN 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 biopsy sections from LN patients.

Results. Our analysis revealed multiple biologic pathways, including chemotaxis, neutrophil activation, platelet degranulation, and extracellular matrix organization, which could be noninvasively quantified and monitored in the urine. We identified 237 urinary biomarkers associated with LN, as compared to controls without systemic lupus erythematosus. Interleukin-16 (IL-16), CD163, and transforming growth factor β mirrored intrarenal nephritis activity. Response to treatment was paralleled by a reduction in 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 LN kidneys. IL-16–producing cells were found at key sites of kidney injury.

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

Supported by the Accelerating Medicines Partnership (AMP) in Rheumatoid Arthritis and Systemic Lupus Erythematosus Network. AMP is a public-private partnership (AbbVie, the Arthritis Foundation, Bristol Myers Squibb, the Foundation for the National Institutes of Health, the Lupus Foundation of America, the Lupus Research Alliance, Merck Sharp & Dohme, the National Institute of Allergy and Infectious Diseases, the National Institute of Arthritis and Musculoskeletal and Skin Diseases, Pfizer, the Rheumatology Research Foundation, Sanofi, and Takeda Pharmaceuticals International) created to develop new ways of identifying and validating promising biological targets for diagnostics and drug development. Funding was provided by the NIH (grants UH2-AR-067676, UH2-AR-067677, UH2-AR-067679, UH2-AR-067681, UH2-AR-067685, UH2-AR-067688, UH2-AR-067689, UH2-AR-067690, UH2-AR-067691, UH2-AR-067694, UM2-AR-067678, and AR-074096). The Oklahoma Rheumatic Disease Research Cores Center is supported by NIH grant P30-AR-073750. The Hopkins Lupus Cohort is funded by NIH grant AR-69572. Dr Fava's work was supported by the Jerome L. Greene Foundation and the Cupid Foundation.

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Author disclosures are available at https://onlinelibrary.wiley.com/action/downloadSupplement?doi=10.1002%2Fart.42023&file=art42023-sup-0001-Disclosureform.pdf.

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Submitted for publication February 18, 2021; accepted in revised form November 9, 2021.

INTRODUCTION

Lupus nephritis (LN) is a severe manifestation of systemic lupus erythematosus (SLE) that frequently leads to end-stage kidney disease despite treatment (1). Diagnosis and treatment of LN rely on histopathologic features of kidney biopsy samples from 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 biologic pathways. Further, the histologic class frequently changes on repeat kidney biopsies, suggesting that the histologic classification may artificially divide patients based on results from one point in time (2,3). Procedure-related complications may occur (4), and up to 35% of kidney biopsies may fail to obtain an adequate sample (5). Access to kidney biopsies may delay diagnosis and treatment, and can be limited by antithrombotic and anticoagulation treatments, severe thrombocytopenia, and resource-poor settings. Finally, because the presence of proteinuria implies that underlying kidney damage has already happened, kidney biopsy results are a lagging indicator. Thus, there is a pressing need for a noninvasive biomarker to probe in "real-time" the active molecular pathologic processes in the kidney and to monitor them over time in response to treatment.

Several available biomarkers correlate with histologic 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 to 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 have 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 that can identify candidate biomarkers that predict histologic features and clinical outcomes, as well as infer the renally active biologic pathways. Here, we used a glass slide–based protein microarray to screen and quantify 1,000 proteins covering a wide range of biologic processes in longitudinal urine samples from 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 1,000 proteins, as validated in previous studies (11–13). We found that protein expression patterns define distinct molecular pathways that are differentially expressed among LN patients. We also discovered that interleukin-16 (IL-16), a proinflammatory chemokine, is strongly associated with LN activity and may have role in LN pathogenesis, thus nominating IL-16 as a potentially treatable target.

PATIENTS AND METHODS

Patients and sample collection. This study enrolled SLE patients with a urine protein–to-creatinine ratio (UPr:Cr) of >0.5 who were undergoing clinically indicated renal biopsy. Only patients with a pathology report confirming LN were included in the study. Renal biopsy sections were scored by 1 renal pathologist at each of the 2 sites according to the International Society of Nephrology (ISN)/Renal Pathology Society guidelines and the National Institutes of Health (NIH) activity and chronicity indices (14). Clinical information, including serologies, were collected at the most recent visit before the biopsy. Response status at week 52 was defined as follows: complete response (UPr:Cr ≤0.5, normal serum creatinine or <25% increase from baseline if abnormal, and prednisone ≤10 mg daily), partial response (UPr:Cr >0.5 but ≤50% of baseline value, and identical serum creatinine and prednisone rules as complete response), or no response (UPr:Cr >50% of baseline value, new abnormal elevation of serum creatinine or ≥25% from baseline, or prednisone ≥10 mg daily). Urine samples from healthy volunteers (all women, median age 42 years [interquartile range 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 US (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 the kidney biopsy. Serologic features and complement levels were assessed 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 (IRBs) at JHU and NYU, and written informed consent was obtained 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 and were matched for sex, race, ethnicity, and age. Subjects were screened using a questionnaire and tested negative for the following antibodies: antinuclear, double-stranded DNA, chromatin, ribosomal P, Ro, La, Smith (Sm), SmRNP, RNP, centromere B, Scl-70, and Jo-1. Samples were processed, stored, and shipped using protocols from the Accelerating Medicines Partnership in Rheumatoid Arthritis and Systemic Lupus Erythematosus (AMP RA/SLE) Network to align with the patient samples. See Appendix A for a list of members of the AMP RA/SLE Network, and see Supplementary Acknowledgments (https://onlinelibrary.wiley.com/doi/10.1002/art.42023) for additional details.

Urine Quantibody assay. The Kiloplex Quantibody protein array platform (RayBiotech) was used to screen urine samples as previously described (12). Validation was performed

using an immunoquantitative (polymerase chain reaction [PCR]–based) IL-16 enzyme-linked immunosorbent assay (ELISA) (RayBiotech) to match and improve the sensitivity and dynamic range provided by the Kiloplex array. These are summarized in Supplementary Methods (https://onlinelibrary.wiley.com/doi/10.1002/art.42023).

Renal tissue single-cell RNA sequencing. Renal tissue was collected, stored, and processed as previously described (15). Briefly, research biopsy cores were collected from consenting subjects as an additional biopsy pass or tissue from routine clinical passes. Only biopsy samples 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% 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.

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 and British Pharmacological Society database. For each cytokine, we calculated the prevalence of the cells with ≥1 transcript over the total number of cells. For details on immunohistochemistry, see Supplementary Methods (https://onlinelibrary.wiley.com/doi/10.1002/art.42023).

Statistical analysis. 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 healthy donor samples, using a 2-sided test with a significance level of 0.05, adjusting for 1,000 comparisons (Bonferroni), there was 80% power to detect a difference in mean peptide magnitude of 1.2 SDs (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. Receiver operating characteristic (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 NIH activity index score and the urinary abundance of a biomarker was tested using 1 confounder at the time (given limited sample size), using a linear regression model as follows: *activity ~ biomarker_abundance + confounder*. The models were fitted

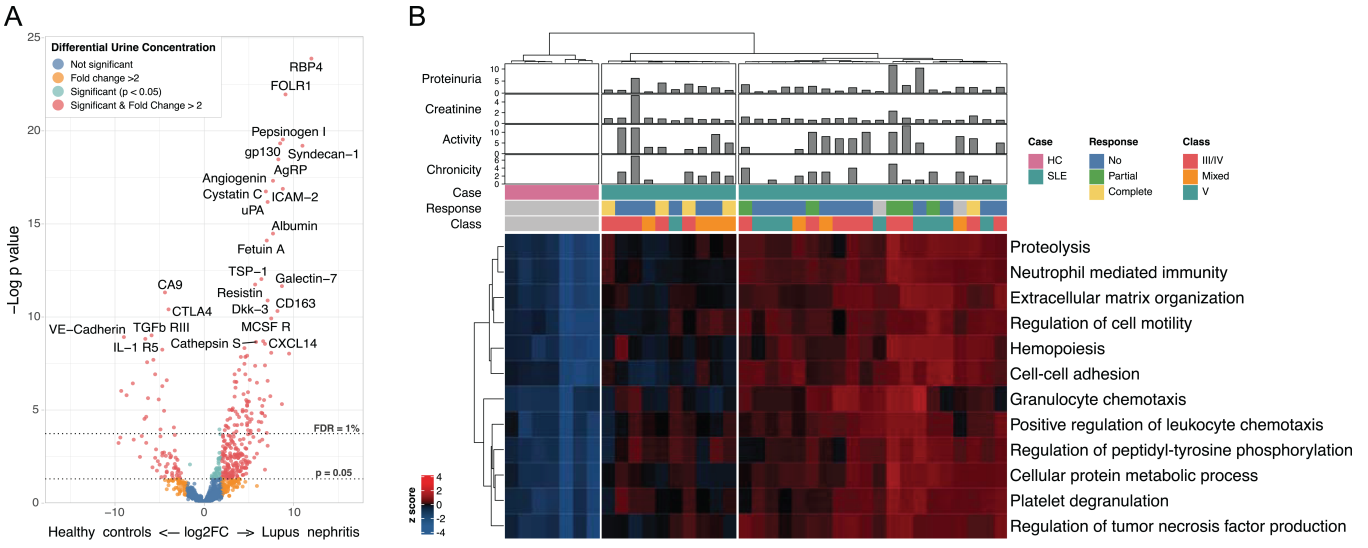

Figure 1. Identification of pathogenic pathways by urine proteomics. **A**, Volcano plot illustrating the differential abundance of 1,000 urinary proteins in patients with lupus nephritis (LN; n = 30) and healthy controls (HC; n = 7). There were 237 proteins that were significantly more abundant in LN (>2-fold change, false discovery rate [FDR] <10%, moderated *t*-test). **B**, Heatmap of the abundance of the 12 nonoverlapping pathways enriched in LN urine samples by pathway enrichment analysis (Gene Ontology biological process). Among the 30 patients, 20 displayed an 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 Ward's minimum variance method. TGFβRIII = transforming growth factor β receptor III; IL-1R5 = interleukin-1 receptor 5; TSP-1 = thrombospondin 1; RBP-4 = retinol binding protein 4; FOLR-1 = folate receptor 1; ICAM-2 = intercellular adhesion molecule 2; uPA = urokinase plasminogen activator; $\log_2$ FC = $\log_2$ fold change; SLE = systemic lupus erythematosus. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.42023/abstract.

using the *lm* function within the stats R package. See Supplementary Methods (https://onlinelibrary.wiley.com/doi/10.1002/art.42023) for pathway enrichment analysis. Pearson's correlation coefficients are used throughout the manuscript. All analyses were performed in R.

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

RESULTS

Urine proteomics identifies biologically relevant active pathways in LN. Urine samples from 30 patients with active LN were collected near or at the time of renal biopsy. Clinical and demographic characteristics are summarized in Supplementary Table 1 (https://onlinelibrary.wiley.com/doi/10.1002/art.42023). Compared to healthy donors, there were 237 proteins significantly elevated in the urine of patients with LN (false discovery rate [FDR] <10%), as shown in Figure 1A. This list includes both novel and previously described urinary biomarkers (Supplementary Data File 1, https://onlinelibrary.wiley.com/doi/10.1002/art.42023). Pathway enrichment analysis of the proteins that were significantly elevated in LN identified 12 enriched nonoverlapping pathways, including relevant biologic processes such as chemotaxis, neutrophil activation, platelet degranulation, and extracellular matrix organization (Supplementary Figure 1, https://onlinelibrary.wiley.com/doi/10.1002/art.42023). 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 (odds ratio 12.6, $P = 0.03$) (Figure 1B). Baseline parameters such as proteinuria, creatinine level, histologic activity or chronicity scores, and ISN 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 2 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 a higher concentration of several urine cytokines and molecules involved in immune activation and chemotaxis (Figures 2A and 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-γ (IFNγ), IL-1β, and tumor necrosis factor (TNF) (Figure 2B).

Many of the urinary proteins that were differentially abundant when comparing proliferative and membranous LN 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 compared to healthy controls, these were not among the most abundant (>2 SDs) (Supplementary Figures 2A and B, https://onlinelibrary.wiley.com/doi/10.1002/art.42023). This is because the first comparison (LN patients versus healthy controls) is aimed to identify proteins that are generally more abundant in all LN patients, regardless of ISN class. Not surprisingly, the most abundant protein in all LN patients was retinol binding protein 4, a general marker of tubular impairment (18). These findings indicate that contrasting well-defined subgroups allowed for identification of relevant biomarkers that could have been missed by analyzing all LN patients together. Different pathogenic processes may underlie each histologic subgroup, and thus, these biomarkers may provide insight into the relative active pathways.

Urinary IL-16 reflects histologic activity. The degree of histologic activity is often used to inform clinical decisions, so we sought to identify noninvasive urinary biomarkers that reflect histologic activity. We studied the correlation of the urinary abundances of all 1,000 biomarkers in urine samples collected at the time of biopsy with the histologic NIH activity index score. We found that IL-16 was the urinary protein most strongly positively correlated with the NIH activity index ($r = 0.73$, $P = 1.2 \times 10^{-5}$, FDR <10%) (n = 28), followed by CD163 and transforming growth factor β (TGFβ) (FDR <10%) (Figures 3A–D). We validated the significant concurrent correlation between urinary IL-16 abundance and NIH activity index score in an independent cohort of 101 patients ($r = 0.59$, $P = 9.3 \times 10^{-11}$) (Supplementary Figure 3 and Supplementary Table 2, https://onlinelibrary.wiley.com/doi/10.1002/art.42023) and with a PCR-based ELISA (Supplementary Figure 4, https://onlinelibrary.wiley.com/doi/10.1002/art.42023). Notably, IL-16 was the only protein 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 TGFβ retained their association with histologic activity after adjustment for multiple confounders, including proteinuria (Supplementary Table 3, https://onlinelibrary.wiley.com/doi/10.1002/art.42023). The pathways associated with histologic activity are displayed in Supplementary Figure 4 (https://onlinelibrary.wiley.com/doi/10.1002/art.42023).

In addition to having the strongest correlation with histologic activity, IL-16 was the urinary protein most strongly associated with proliferative LN (Figure 2A). The ROC curve revealed that IL-16 was a promising urinary biomarker to identify patients with

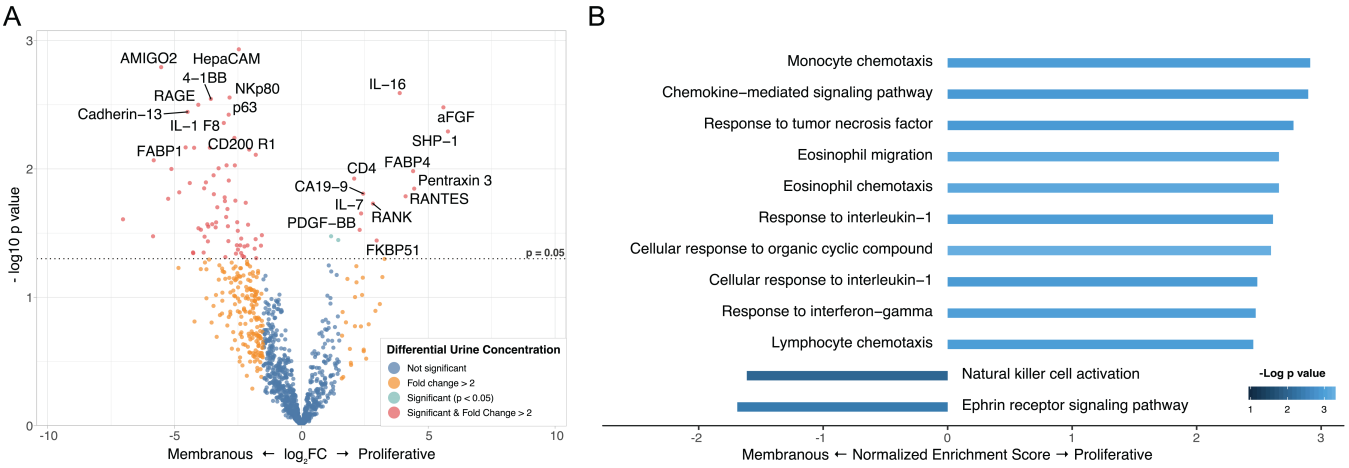

Figure 2. Proteomic profile of proliferative lupus nephritis. **A**, Volcano plot shows the differential abundance of 1,000 urinary proteins in proliferative LN (n = 14) and pure membranous LN (n = 9). **B**, Pathway enrichment analysis (Gene Ontology biological process) of the urinary proteomic profile revealed that chemotaxis was the process most enriched in proliferative LN. In particular, these were chemokines secreted in response to tumor necrosis factor, IL-1, and interferon-γ. The enrichment FDR (gene set enrichment analysis rank permutation) was <5% for all pathways except for "Natural killer cell activation" (16%). FABP-1 = fatty acid binding protein 1; PDGF-BB = platelet-derived growth factor BB; aFGF = acidic fibroblast growth factor; IGFBP-1 = insulin-like growth factor binding protein 1; FKBP51 = FK-506 binding protein 51; SHP-1 = SH2 domain–containing phosphatase 1 (see Figure 1 for other definitions). Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.42023/abstract.

proliferative LN, with AUCs of 0.85 ($P = 0.016$) and 0.89 ($P = 0.037$) in association with CD163 and TGFβ, respectively (Supplementary Figure 5, https://onlinelibrary.wiley.com/doi/10.1002/art.42023).

Correlation of urinary biomarkers with activity decrease, according to clinical response in longitudinal samples. A goal of immunosuppression in LN is to eradicate pathologic renal inflammation to ultimately prevent irreversible

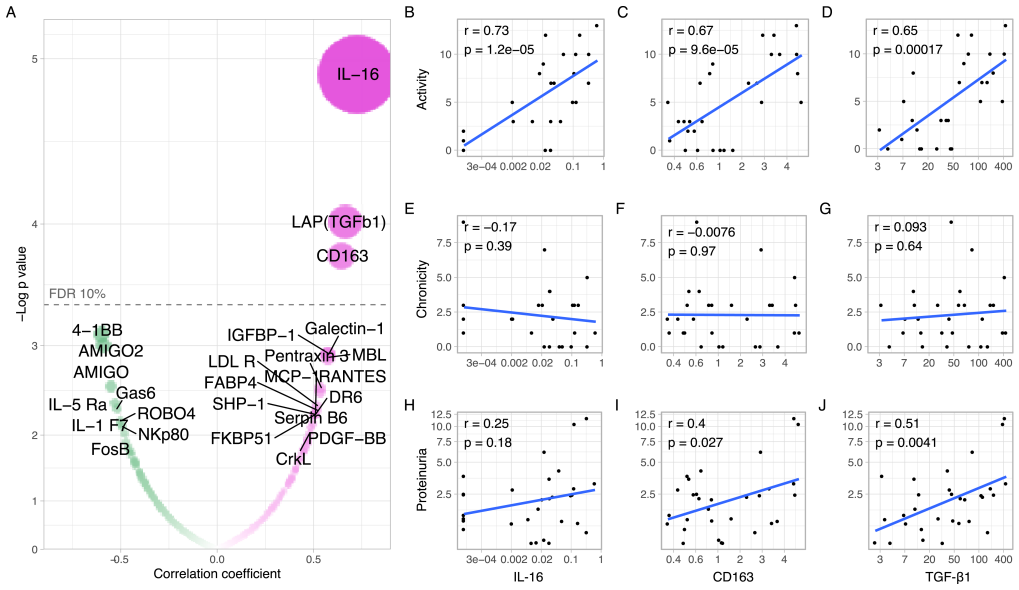

Figure 3. Urinary biomarkers of histologic nephritis activity. **A**, Pearson's correlation coefficients for the urinary abundance of 1,000 proteins and the histologic National Institutes of Health (NIH) activity index score in near or same-day renal biopsy samples. Each dot represents a protein within 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 of <10%. The FDR of IL-16 was 1.2%. **B–J**, Scatterplots displaying the Pearson's correlation coefficient and *P* value for correlations of the urinary abundance of IL-16, CD163, and TGFβ1 with the NIH activity score (**B–D**), NIH chronicity score (**E–G**), and proteinuria (**H–J**). GAS-6 = growth arrest–specific protein 6; SHP-1 = SH2 domain–containing phosphatase 1; PDGF-BB platelet-derived growth factor BB (see Figure 1 for other definitions). Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.42023/abstract.

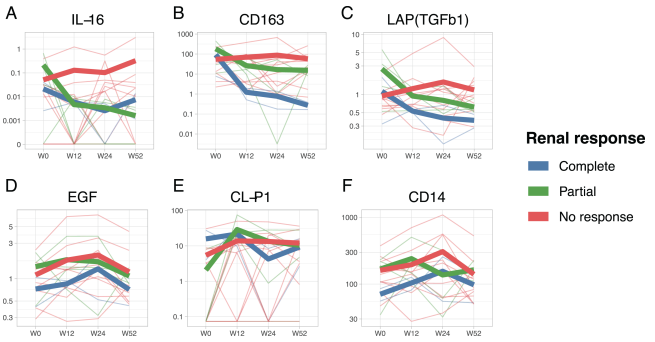

Figure 4. Biomarkers associated with nephritis activity decrease in responders. **A–F**, Urinary concentration of all biomarkers was measured at the time of biopsy (e.g., week 0 [W0]) and after 12, 24, and 52 weeks. Thin lines depict the trajectories of each patient categorized according to the response status determined at week 52. Thick lines represent the average for each group. The urinary concentration of the 3 biomarkers that significantly correlated with histologic activity declined in complete and partial responders but not in nonresponders (**A–C**). In contrast, 3 biomarkers that did not correlate with histologic activity (r values ranged from –0.0018 to 0.0015, *P* not significant) did not show a decline over time (**D–F**). IL-16 = interleukin-16; LAP = latency-associated peptide; TGFβ1 = transforming growth factor β type 1; EGF = endothelial growth factor; CL-P1 = collectin placenta 1. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.42023/abstract.

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 histologic activity would decline over time in patients 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 nonresponders (Figures 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. TGFβ showed a more modest decline.

Since response status is defined by reduction in 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 histologic activity demonstrated that there was not a nonspecific decline (Figures 4D–F). These findings indicate that IL-16, CD163, and TGFβ 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 of the most expressed cytokines in infiltrating immune cells in LN kidneys.** To determine whether the urinary concentration of the 3 candidate biomarkers reflects an active intrarenal process rather than passive filtration through

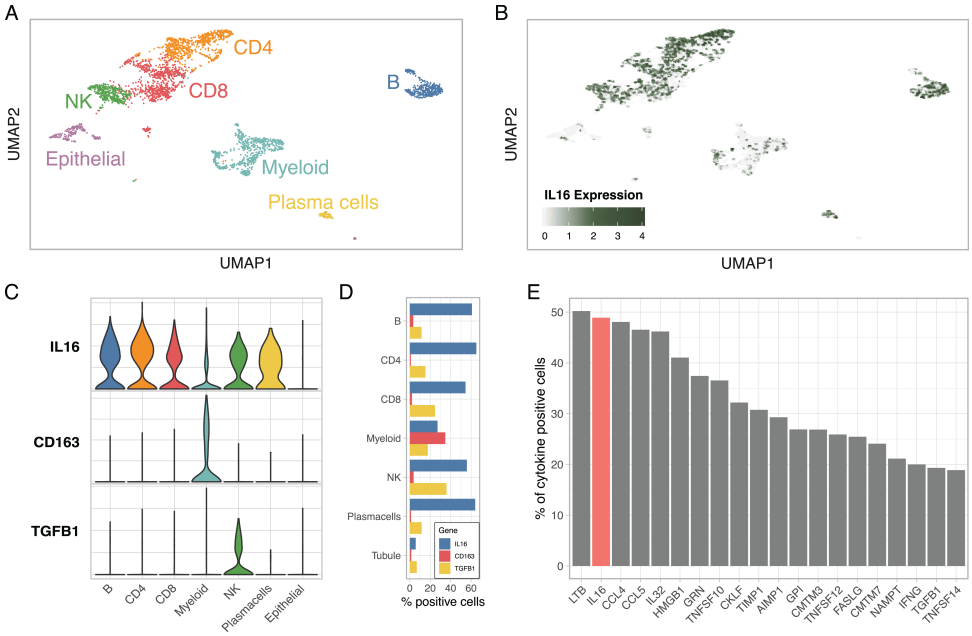

Figure 5. High expression of *IL16* in lupus nephritis (LN) kidneys. **A**, UMAP plot of single-cell RNA sequencing of renal biopsies (3131 cell) by lineage. **B**, Feature plot displaying *IL16* expression at the single-cell level. **C** and **D**, Violin plots (**C**) and bar plots (**D**) summarizing the expression of the genes coding for the urinary proteins associated with nephritis activity. *IL16* was abundantly expressed by most infiltrating immune cells in kidneys, *CD163* mostly by macrophages, and *TGFB1* by natural killer cells. **E**, Prevalence of cytokine positive cells out of a compendium of 237 cytokines ranked decreasingly (top 20 are shown). *IL16* (red) was the second most expressed cytokine in LN kidneys. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.42023/abstract.

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 *TGFB1* mostly by natural killer (NK) cells (Figures 5A–D).

In LN, most of *IL16* expression was in immune infiltrating cells, especially the lymphoid lineage (Figures 5C and D). In renal allograft rejection, single-cell RNA sequencing showed that *IL16* was expressed by endothelial, epithelial, and immune cells, but immune cells were the main source (20) (Supplementary Figure 6A, https://onlinelibrary.wiley.com/doi/10.1002/art.42023). Conversely, in healthy kidneys, single nuclear RNA sequencing and ATAC-seq (Assay for Transposase-Accessible Chromatin using sequencing) revealed substantial *IL16* expression by podocytes, fibroblasts, endothelial cells, mesangial cells, and proximal tubular cells (21,22) (Supplementary Figures 5B and C, https://onlinelibrary.wiley.com/doi/10.1002/art.42023). 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. It can be speculated that this initial event can then be amplified by infiltrating immune cells, as seen in LN and allograft rejection.

Finally, we explored whether *IL16* was disproportionally more expressed 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 suggest IL-16 as a major cytokine involved in LN.

Correlation of tissue expression of IL-16 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 biopsy samples, 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 Supplementary Figures 7A–C, https://onlinelibrary.wiley.com/doi/10.1002/art.42023), with the exception of 1 case (Supplementary Figure 7D), in which the activity index score was uncharacteristically low (score of 2) and IL-16 was not detectable in the urine. In contrast,

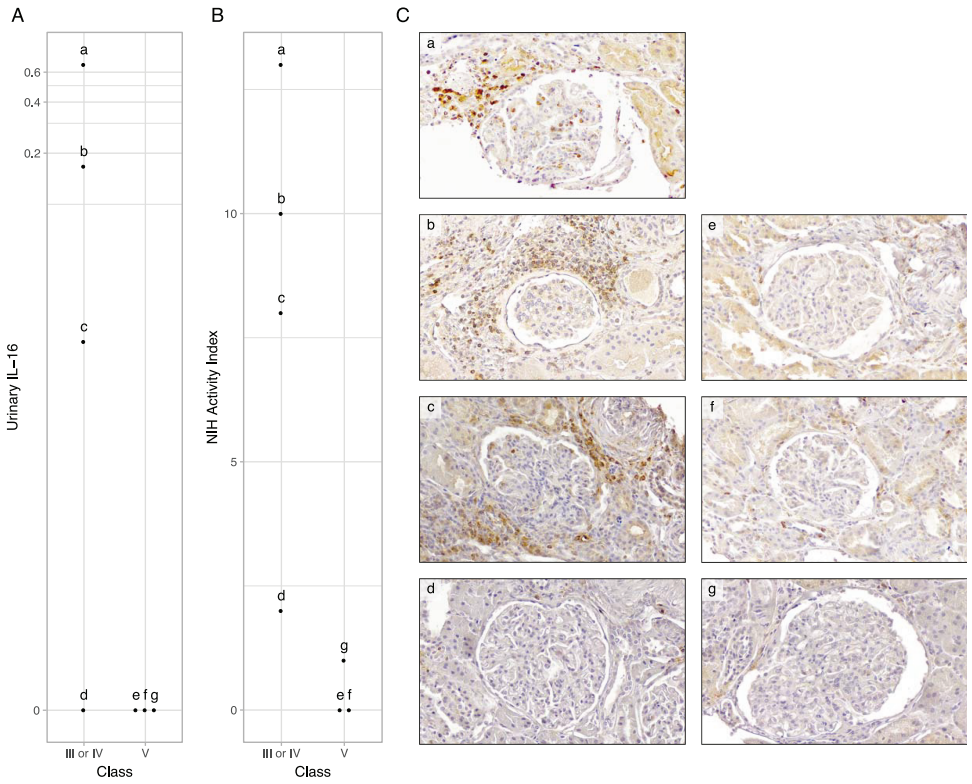

Figure 6. Interleukin-16 (IL-16)–positive cells are abundant in proliferative lupus nephritis (LN) and qualitatively correlate with urinary IL-16 and LN activity. Immunohistochemical staining for human IL-16 was performed in 7 LN kidney biopsy samples with matching urine IL-16 collected at or near the time of biopsy. **A** and **B**, The corresponding urinary abundance of IL-16 (**A**) and National Institutes of Health (NIH) activity index (**B**) of the patients whose biopsy results depicted in **C** are plotted according to the International Society of Nephrology class. Lower-case letters in **A–C** identify information from the same patients. **C**, Immunohistochemical staining of IL-16 in 4 proliferative LN biopsy sections (a–d) and 3 pure membranous LN biopsy sections (e–g). An abundance of IL-16–positive cells was noted in proliferative LN (**C**; a–d), with qualitatively more prominent intraglomerular IL-16 positivity in patients with higher urinary IL-16 levels and NIH activity index scores. Original magnification × 33.6. Lower-magnification images with larger representation of the interstitium are displayed in Supplementary Figure 6 (https://onlinelibrary.wiley.com/doi/10.1002/art.42023).

there was very scant IL-16 positivity in membranous LN (Figure 6 and Supplementary Figures 7E–G), and there was marginal IL-16 in a class I LN biopsy sample used as negative control (Supplementary Figure 7H). These findings were consistent with 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 with the NIH activity index score (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 LN patients and healthy controls, the findings of this study confirmed that the pathologic processes in LN can be noninvasively captured and monitored over time. In the present study, we found the following: 1) 237 urinary proteins associated with LN that represented ≥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 histologic activity, suggesting 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 biologic 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 signature or a group of signatures. Rather, we observed 2 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 are stratified on a gradient (27). Importantly, 80% of complete responders were 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, indicating that these signatures specifically reflect active biologic processes rather than a nonspecific 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 class IV LN, further supporting the ability of urine proteomics to infer intrarenal biologic processes.

Ideal biomarkers in LN should noninvasively infer nephritis activity, longitudinally track response to treatment, and 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 repeated biopsies after induction therapy revealed that ~50% of the patients with disease in complete clinical remission (proteinuria <0.5 gm/24 hours and no increase in serum creatinine) had persistent histologically active proliferative nephritis (28). Conversely, >50% of patients who achieved complete histologic remission had persistent proteinuria >0.5 gm/24 hours. 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 2 previously recognized LN biomarkers, CD163 (29) and TGFβ (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 TGFβ were selected based on their correlation with histologic activity; therefore, it is conceivable that their decreasing urinary abundance mirrored an improvement of intrarenal histologic 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 biopsy samples, suggesting that their detection in the urine reflects intrarenal immune activity. Because their expression was observed 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 data set, 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, indicating that urinary IL-16 is the direct consequence of intrarenal IL-16 secretion. Because urinary IL-16, intrarenal IL-16–positive cells, and histologic 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 noninvasively measured in urine.

IL-16 is a proinflammatory chemokine secreted by immune cells and nonimmune cells (endothelial cells, epithelial cells, fibroblasts, and neurons) in response to several stimuli, such as complement activation, antigen stimulation, IFN, hypoxia, and

cell injury (31–34). Because the release of bioactive IL-16 depends on caspase 3 activation (33), apoptosis and proapoptotic stimuli, including sublethal doses of granzymes, may also lead to its release. IL-16 can also be released upon cleavage by proteinase 3 (35), which suggests 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 cells, NK cells, B cells, monocytes, neutrophils, dendritic cells, and mast cells (31). IL-16 can activate CD4 T cells independently of T cell receptor activation (36) and may lead to the release of proinflammatory cytokines such as TNF, IL-1β, IL-6, IL-15, and IL-12 (31). *IL16* polymorphisms were associated with an increased risk of SLE (odds ratio 3.3–10.4), suggesting a potential causal role (37). Plasma IL-16 levels were associated with SLE severity, including renal involvement (38). Finally, IL-16 was mechanistically linked to lung disease in the pristane model of SLE (39). 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 pathologic information and with tissue single-cell transcriptomics (42). In fact, in addition to a newly discovered biomarker, our approach detected that CD163 and TGFβ are proven biomarkers in LN. Similar to our findings, soluble CD163 was shown to correlate with LN nephritis activity and to improve with treatment (29). CD163 is a scavenger receptor expressed on phagocytic monocytes, especially in M2c-polarized macrophages that infiltrate tissue during the healing phase of inflammation and are implicated in fibrosis resolution (43). Notably, M2c macrophages are inducible by TGFβ (44). CD163+ cells are a dominant macrophage subtype in LN (44), once again supporting the notion of capability of urinary proteomic to infer intrarenal biology. CD163+ cells have been detected in proliferative glomerular lesions and in tubulointerstitial inflammation (45), and they constitute ~80% of the urinary cells in LN (46). Similarly consistent with our results, urinary TGFβ 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 TGFβ (48). TGFβ regulates inflammation and progression of renal fibrosis. Notably, TGFβ increased IL-16 release in synovial fibroblasts, suggesting a possible similar interplay between these 2 cytokines in LN (49). Here, we have shown that NK cells are the major immune cell type expressing *TGFB1* in LN; whether NK cells or tubular cells (50) are responsible for urinary TGFβ in LN is yet 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 patients (38), 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 and suggests 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 "nonactive" 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 evaluate whether 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 LN 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 data set may further discovery by allowing investigators to research and validate new biomarkers, test new hypotheses, and complement mechanistic studies in LN.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Fava had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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APPENDIX A: THE ACCELERATING MEDICINES PARTNERSHIP IN RHEUMATOID ARTHRITIS AND SYSTEMIC LUPUS ERYTHEMATOSUS NETWORK

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