

Integrative bioinformatics and experimental analysis reveals FRA1 as a key mediator of tubulointerstitial inflammation in lupus nephritis

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Abstract. Tubulointerstitial injury is a key driver of lupus nephritis (LN) progression, and dysregulation of the immune microenvironment is a central feature of this process. The molecular mediators of this dysregulation remain incompletely defined. In the present study an integrated bioinformatics and experimental analysis was performed of the Activator Protein 1 (AP-1) family transcription factor Fos-related antigen 1 (FRA1) in LN tubulointerstitium. Analysis of gene expression omnibus datasets (GSE113342, GSE200306 and GSE127797) showed that FRA1 was markedly upregulated in the tubulointerstitium of LN samples and that its expression positively correlated with CD8⁺ T cells, regulatory T cells, monocytes, M1 macrophages and activated mast cells, but negatively correlated with plasma cells, resting CD4⁺ memory T cells, M0/M2 macrophages, resting dendritic cells and resting mast cells. *In vivo* experiments revealed that, FRA1 expression was also increased in kidneys from MRL/lpr mice. Furthermore, *in vitro*, lentiviral overexpression of FRA1 in HK-2 cells induced robust upregulation of IL-6, IL-1 β , IL-8, MCP-1 and RANTES, whereas FRA1 knockdown selectively decreased IL-6 and RANTES levels. Together, these results indicate

that FRA1 is significantly elevated in the LN tubulointerstitium and may foster a proinflammatory microenvironment by regulating key cytokines. The FRA1/AP-1 axis therefore represents a potential regulator of renal inflammation in LN and a candidate therapeutic target.

Introduction

Systemic lupus erythematosus (SLE) is a chronic, multisystem autoimmune disease, and renal involvement, which is referred to as lupus nephritis (LN), occurs in >60% of patients with SLE (1-3). LN is among the most severe complications of SLE; if uncontrolled, it can progress to renal failure and remains a leading cause of mortality in patients with SLE (4-6). Therefore, elucidating the pathogenic mechanisms of LN has important clinical significance.

Traditionally, research on LN has focused primarily on glomerular injury, but growing evidence indicates that tubulointerstitial pathology plays a key role in both the onset and progression of LN (7). Tubulointerstitial lesions are more notably associated with LN severity than glomerular fibrosis, and markers of tubular injury (such as tubular proteinuria) often precede microalbuminuria (8-12). In addition, severe tubulointerstitial damage has been identified as a major risk factor for LN progression (13,14). Mechanistically, epithelial-mesenchymal transition of renal tubular epithelial cells promotes extracellular matrix deposition and fibrosis, while infiltration of immune cells (such as macrophages and lymphocytes) and release of proinflammatory mediators exacerbate tubular and interstitial injury (14-16). Multiple signaling pathways, including NF- κ B, TGF- β and Wnt/ β -catenin, have been implicated in tubulointerstitial lesion regulation in LN, underscoring the complex and multifactorial immune-inflammatory mechanisms involved (17,18).

Fos-related antigen 1 (FRA1) is a member of the activator protein 1 (AP-1) transcription factor family, which comprises Fos-family proteins (c-Fos, FosB, FRA1 and FRA2) that dimerize with Jun-family proteins (19-21). AP-1 regulates key processes such as cell proliferation, differentiation and inflammatory responses, and controls cytokine expression in

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Abbreviations: LN, lupus nephritis; AP-1, Activator Protein 1; FRA1 Fos-related antigen 1; GEO Gene Expression Omnibus; Tregs, regulatory T cells; SLE, systemic lupus erythematosus; DEGs, differentially expressed genes; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; IHC, immunohistochemistry

Key words: lupus nephritis, FRA1, AP-1, transcription factor, tubulointerstitial inflammation, cytokines, immune infiltration

various immune and inflammatory disorders (22). FRA1 has been implicated not only in tumorigenesis but also in modulation of inflammatory and autoimmune diseases, including arthritis, pneumonia, psoriasis, myasthenia gravis and cardiovascular disorders (22). In immune cells, FRA1 influences B-cell fate; for example, FRA1 is upregulated in activated B cells and negatively regulates follicular B-cell differentiation into plasma cells by suppressing the expression of the key transcription factor Blimp-1, thereby limiting antibody production (23). In epithelial cells, Li *et al.* (24) reported that FRA1 disrupts inflammatory cytokine secretion by medullary thymic epithelial cells. Thus, the role of FRA1 in immune regulation has received increasing attention. Promoter regions of numerous inflammatory cytokines and chemokines, such as TNF- α , IL-1 β , IL-11, IL-8 and MCP-1, contain AP-1 binding sites, suggesting that FRA1 may directly regulate their expression (22).

In the context of renal injury, FRA1 also exerts notable functions; for example, in an acute kidney injury model, FRA1 expression is upregulated in proximal tubular cells and mitigates tubular cell damage and inflammation by maintaining expression of the anti-aging protein Klotho (25). Moreover, a gene-screening study in IgA nephropathy identified FRA1/2 as novel prognostic biomarkers, implicating TNF and MAPK signaling pathways in disease progression (26). Notably, METTL3 has been shown to exacerbate renal inflammation by enhancing m6A modification of FRA1 transcripts (27). Nevertheless, the role of FRA1 in LN and other autoimmune kidney diseases remains unexplored, and its functions and molecular mechanisms in LN-associated tubulointerstitial injury remain unclear.

Based on these observations, bioinformatic analysis of public gene expression datasets was performed to identify FRA1 as a potential key candidate in LN. The present study aimed to investigate the role of FRA1 in the pathogenesis of LN and to elucidate its underlying molecular mechanisms. The bioinformatics analyses were combined with validation in the MRL/lpr mouse model and *in vitro* experiments using HK-2 cells to systematically characterize FRA1 expression patterns and its regulatory effects on the inflammatory cytokine network in LN. By constructing FRA1 overexpression and knockdown cell models, the impact of FRA1 on tubular epithelial cell function and inflammatory responses, was further examined aiming to provide new insights into LN pathogenesis and to identify potential therapeutic targets.

Materials and methods

Data sources. Gene expression data for renal tubulointerstitial samples from patients with LN and healthy controls were obtained from the gene expression omnibus (GEO) database (<https://www.ncbi.nlm.nih.gov/geo/>). A total of three datasets were included: GSE113342 (47 LN and 10 control tubulointerstitial samples), GSE200306 (14 LN and 10 control samples) and GSE127797 (44 LN samples) (28–30). Probe identifiers were annotated to gene symbols using the platform annotation file; for genes represented by multiple probes, one probe was randomly selected to avoid redundancy. Background correction and normalization were performed with the ‘limma’ package (v3.6.2) (31) in R (v4.2.1; RStudio, Inc.) to ensure data quality

and comparability, yielding the final gene expression matrices. All analyses adhered to the principles of The Declaration of Helsinki (2013 revision) (32).

Identification of differentially expressed genes (DEGs). Differential expression analysis between LN and control tubulointerstitial samples was conducted separately for GSE113342 and GSE200306 using the ‘limma’ package (31). Genes with $\log_2$ fold change ($\log_2\text{FC}$) > 1 and adjusted $P < 0.05$ were defined as DEGs. The intersection of DEGs from both datasets was taken to identify consistently dysregulated genes, and results were visualized using ggplot2 (v3.3.6; <https://ggplot2.tidyverse.org/>) and VennDiagram (v1.7.3).

Functional enrichment analysis. To explore the potential biological roles of DEGs, gene identifiers were converted using the org.Hs.eg.db package (v3.22; <https://bioconductor.org/packages/org.Hs.eg.db>), and Gene Ontology (GO) (<https://geneontology.org/>) was used to determine relevant biological processes (BPs) and molecular functions (MFs) and Kyoto Encyclopedia of Genes and Genomes (KEGG) (www.genome.jp/kegg/) for pathway enrichment analyses were performed with clusterProfiler, with adjusted $P < 0.05$ considered significant (33). Enrichment results were visualized using ggplot2 (v3.3.6), igraph (v2.2.1; <https://r.igraph.org/>) and ggraph (v2.2.2; <https://ggraph.data-imaginat.com/>).

Relationship between FRA1 and immunity. To assess the association between FRA1 expression and immune cell infiltration in LN tubulointerstitial samples, the CIBERSORT algorithm was applied, which is a linear support vector regression-based deconvolution tool that estimates immune cell proportions from microarray or RNA-seq data (34). In GSE127797, LN samples were stratified into high- and low-FRA1 expression groups based on the median, and only samples with CIBERSORT $P < 0.05$ were retained for subsequent analyses. The correlations between FRA1 expression and infiltrating immune cell fractions and correlations between FRA1 and tubular epithelial cell-related cytokines were examined; all visualizations were generated with ggplot2 (v3.3.6).

Animal experiments in MRL/lpr mice. A total of three female MRL/lpr mice and three female MRL/MPJ control mice (age, 16 weeks; body weight, 38–46 g) were obtained from Cavens Bioglo (Suzhou) Model Animal Research Co. Ltd. Mice were maintained under standard housing conditions, which included a climate-controlled environment at $22 \pm 2^\circ\text{C}$ and $50 \pm 10\%$ relative humidity, a 12-h light/dark cycle and free access to food and water, for 4 weeks and then euthanized for kidney tissue collection to assess protein expression. All procedures complied with the AVMA Guidelines for the Euthanasia of Animals (2020) and approved institutional animal care protocols. Specific humane endpoints included: Weight loss $\geq 20\%$ of body weight; inability to eat or drink; severe dehydration; severe respiratory distress, paralysis or continuous convulsions; uncontrolled progressive infection; or unrelievable pain or clinical signs severely compromised quality of life. Health and behaviour were routinely monitored, with weekly weighing and observation after enrolment, and immediate escalation to daily or more frequent monitoring if abnormalities arose.

Mice were deeply anesthetized with pentobarbital sodium (150-200 mg/kg, intraperitoneally) until loss of the pedal withdrawal reflex, followed by cervical dislocation to ensure complete euthanasia. Death was confirmed by the absence of heartbeat and respiration, dilated pupils and lack of reflex response. No experimental intervention was performed; MRL/lpr mice, which spontaneously develop LN (35), were used as the disease model, whereas MRL/MPJ mice served as healthy controls and were used solely for terminal tissue collection. The power calculation for the mouse experiments is shown in Table SI, following the methodology described by Festing and Altman (36).

Immunohistochemistry (IHC). Kidney tissues were fixed in 10% neutral-buffered formalin for 24-48 h at room temperature and were then processed for paraffin embedding. Paraffin-embedded tissues were cut into 5- μ m sections. The sections were dewaxed twice in xylene (15 min each), rehydrated through a descending ethanol series (100% ethanol twice, then 95, 85 and 75% ethanol, 5 min each), and rinsed in phosphate-buffered saline (PBS, pH 7.4) three times for 5 min each. Antigen retrieval was performed in citrate buffer (pH 6.0) in a microwave: Medium power for 8 min, rest for 8 min, then low power for 7 min. Subsequently, the slides were allowed to cool to room temperature for 20-40 min and then rinsed in PBS (three times, 5 min each). Endogenous peroxidase activity was quenched by incubation in 3% hydrogen peroxide at room temperature in the dark for 25 min, followed by three PBS washes (5 min each). Blocking was performed using the normal goat serum blocking solution supplied with the Vectastain ABC Elite HRP Kit (cat. no. SAP-9100; Beijing Zhongshan Jinqiao Biotechnology Co., Ltd.) according to the manufacturer's instructions (incubation at room temperature for 10-15 min). For primary antibody incubation, the sections were incubated with rabbit polyclonal anti-FRA1 (cat. no. A5372; ABclonal Biotech Co., Ltd.) at a dilution of 1:100 (diluted in PBS) and incubated overnight at 4°C. After three PBS washes (5 min each), the sections were incubated with the biotinylated goat anti-rabbit IgG provided in the Vectastain ABC Elite HRP Kit at a working dilution of 1:500 for 10-15 min at 37°C, followed by PBS rinses (3x3 min). The HRP-labeled streptavidin working solution (provided in the Vectastain ABC Elite HRP Kit) was applied and incubated for 10-15 min at 37°C according to the manufacturer's instructions, followed by PBS washes (3x3 min). Chromogenic detection was performed with DAB substrate (cat. no. ZLI-9017; Beijing Zhongshan Jinqiao Biotechnology Co., Ltd.); color development was monitored under the microscope and stopped with distilled water once an optimal signal was achieved. The sections were then counterstained with hematoxylin at room temperature for 1-2 min, differentiated in 1% hydrochloric acid-ethanol (~1 sec), rinsed in running tap water, blued in ammonia water and rinsed again. Finally, the slides were dehydrated through an ascending ethanol series (75, 85, 95 and 100%), cleared in xylene and mounted with neutral resin. The percentage of FRA1-positive area/unit tissue area was quantified using ImageJ (v2.3.0; National Institutes of Health). All stained sections were examined and images were captured using an Olympus BX40 upright light microscope (Olympus Corporation).

Culture of HK-2 cells and retroviral infection. HK-2 cells (Pricella; Elabscience Bionovation Inc.) were maintained in DMEM/F12 (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% heat-inactivated fetal bovine serum (Beijing Baiao Leibo Technology Co., Ltd.). A total of four groups were established: FRA1 overexpression (FRA1-OE), FRA1 knockdown (FRA1-shRNA) and their respective empty-vector controls (control-OE and control-shRNA). The FRA1 overexpression vector (pCDH_CMV_MCS_EF1_copGFP-FRA1) and shRNA vector (pMAGic7.1-FRA1-shRNA) were constructed at the Clinical Laboratory, Boai Hospital of Zhongshan (Zhongshan, China); plasmid maps and sequences are provided in Fig. S1 and Table SII. For retroviral packaging, 293T cells (Pricella; Elabscience Bionovation Inc.) were co-transfected with 4 μ g total plasmids using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C for 48 h. Specifically, the packaging system consisted of the packaging plasmid (psPAX2), the envelope plasmid (pMD2.G) and one of the four aforementioned transfer plasmids (FRA1-OE, FRA1-shRNA, control-OE or control-shRNA) at a mass ratio of 4:3:2. For gene manipulation, 2×10^5 HK-2 cells were infected with retroviral particles [2×10^7 transducing units/ml; multiplicity of infection=30; supplemented with 6 μ g/ml polybrene (MedChemExpress); infection efficiency $\geq 85\%$]. The infection was performed at 37°C for 24 h, after which the medium was replaced with fresh complete medium. For the FRA1-shRNA and control-shRNA groups, stably transduced cells were selected using 3.0 μ g/ml puromycin (MedChemExpress) starting 48 h post-infection for 4 days. No additional selection was performed for the FRA1-OE and control-OE groups, as the high transduction efficiency was sufficient for subsequent experiments. All groups were harvested for further analysis at 144 h post-transduction.

Western blotting. Kidney tissues or HK-2 cells were homogenized in RIPA buffer (Biosharp Life Sciences), and protein concentrations were determined using a BCA Protein Assay Kit (Wuhan Servicebio Technology Co., Ltd.). For HK-2 experiments, cells were harvested at 144 h post-transduction to allow time for lentiviral reverse transcription, genomic integration and establishment of stable transgene expression and for accumulation of downstream protein and secreted cytokine changes. Lentiviral-mediated expression commonly requires on the order of 5-7 days to reach stable levels, and molecular-kinetic studies indicate that reverse transcription and integration occur during the first few days post-transduction (~3 days) (37,38). Prior studies have documented progressively increased reporter expression at 48-, 96- and 144-h post-transduction, and have selected 144 h post-transduction as the endpoint for subsequent mRNA and protein analyses of FRA1 effects (24,39,40); inflammatory mediators in renal cell models are also commonly measured at ~5 days or later following perturbation (41). Equal amounts of protein (40 μ g/lane) were separated by SDS-PAGE on 10% gels and were transferred to PVDF membranes (MilliporeSigma). Protein molecular weight markers (10-180 kDa; cat. no. 20350ES90; Shanghai Yeasen Biotechnology Co., Ltd.) were run alongside samples to confirm target sizes. Membranes were blocked in 5% skim milk at 37°C for 1 h, incubated with primary antibodies at 4°C overnight, then washed with TBS-0.1% Tween-20 (TBST).

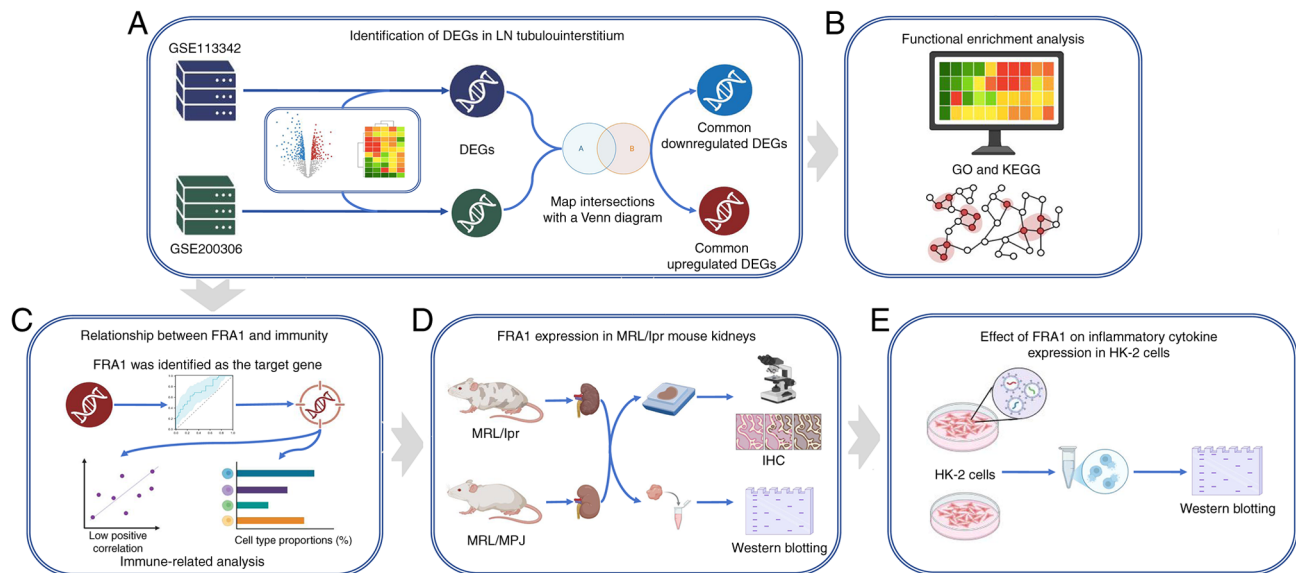


Figure 1. Workflow of the study. (A) Differential expression analysis was performed separately on two public tubulointerstitial datasets (GSE113342 and GSE200306) and the intersecting up and downregulated DEGs were identified. (B) Functional enrichment analysis was applied to the intersected DEGs to explore enriched biological processes and pathways. (C) Among upregulated DEGs, receiver operating characteristic curve analysis prioritized FRA1 as the top target gene, which was then subjected to downstream correlation and immune-infiltration analyses. CIBERSORT and correlation analyses were performed in GSE127797 to evaluate relationships between FRA1 expression, immune-cell fractions and tubular epithelial-related cytokines. (D) *In vivo* validation of FRA1 expression was performed by immunohistochemistry and western blot analysis in MRL/lpr mouse kidneys. (E) *In vitro* functional experiments were carried out in HK-2 cells with lentiviral FRA1 overexpression and knockdown to assess effects on cytokine expression. Together, these steps integrate bioinformatic discovery with *in vivo* and *in vitro* validation to evaluate FRA1 as a candidate mediator of tubulointerstitial inflammation in lupus nephritis. DEGs, differentially expressed genes; GO, gene ontology; KEGG, Kyoto encyclopedia of genes and genomes.

The primary antibodies used in the present study included: β -actin (1:5,000; cat. no. bs-0061R; BIOSS), FRA1 (1:1,000; cat. no. A5372; ABclonal Biotech Co., Ltd.), IL-1 β (1:1,000; cat. no. HA601002; clone no. A7F9; HUABIO), IL-8 (1:1,000; cat. no. ab289967; clone no. EPR26511-74; Abcam), RANTES (1:1,000; cat. no. 36467; CST Biological Reagents Co., Ltd.), IL-6 (1:2,000; cat. no. DF6087; Affinity Biosciences, Ltd.), MCP-1 (1:2,000; cat. no. A7277; ABclonal Biotech Co., Ltd.), TNF- α (1:2,000; cat. no. 17590-1-AP; Proteintech Group, Inc.), and TGF- β (1:2,000; cat. no. 81746-2-RR; clone no. 230544B7; Proteintech Group, Inc.). The membranes were then incubated with HRP-conjugated secondary antibodies at a 1:50,000 dilution for 30 min at room temperature. The secondary antibodies used included HRP-Goat anti Rabbit (cat. no. 5220-0336) and HRP-Goat anti Mouse (cat. no. 5220-0341; SeraCare; LGC Clinical Diagnostics). After further washing with TBST, bands were visualized using an ECL Chemiluminescence Detection Kit (Dalian Meilun Biology Technology Co., Ltd.) with the JS-1070P imaging system (Shanghai Peiqing Technology Co., Ltd.) and densitometric analysis was performed with IPWIN60 software (v6.0; Media Cybernetics, Inc.).

Statistical analysis. All analyses were performed in R (v4.0.3). For two-group comparisons of continuous variables the following tests were used: Unpaired Student's t-test for normally distributed data with equal variances; Welch's t-test for normal data with unequal variances; and the Wilcoxon rank-sum test for non-normal data. For multi-group comparisons, one-way ANOVA followed by Tukey's Honestly Significant Difference was applied to normal data with equal variances and the Kruskal-Wallis test followed by Dunn's post

hoc test was applied for non-normal data. Pearson's correlation was used for normally distributed variables, and Spearman's correlation otherwise. Receiver operating characteristic curve analysis was employed to evaluate the predictive performance of upregulated DEGs for the disease. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Identification of DEGs in LN tubulointerstitium. Design of the present study is illustrated in Fig. 1. To determine gene expression differences between LN and healthy controls tubulointerstitial samples, the datasets GSE113342 and GSE200306 from GEO were analyzed. Using $\log_2\text{FC} > 1$ and adjusted $P < 0.05$, 74 DEGs were identified in GSE113342 (Fig. 2A) and 16 DEGs were identified in GSE200306 (Fig. 2B). The intersection of these yielded 15 common DEGs (Fig. 2C), of which FRA1, C1R, CCL19, STAT1 and MX1 were upregulated in LN, whereas THY1, MME, ARG2, EGR1, KIT, IL6R, FKBP5, MRC1, ZBTB1 and RORC were downregulated. A heatmap illustrates their expression patterns across both datasets (Fig. 2D and E).

Functional enrichment of DEGs. GO and KEGG enrichment analyses of the 15 shared DEGs revealed significant associations with immune and developmental processes (Fig. 3A). In GO-BP, DEGs were enriched in 'T cell differentiation', 'kidney development', 'renal system development' and 'alpha-beta T cell activation' (Fig. 3B). In GO-MF, terms included 'histone acetyltransferase binding', 'transcription corepressor binding', 'CCR chemokine receptor binding', 'promoter-specific

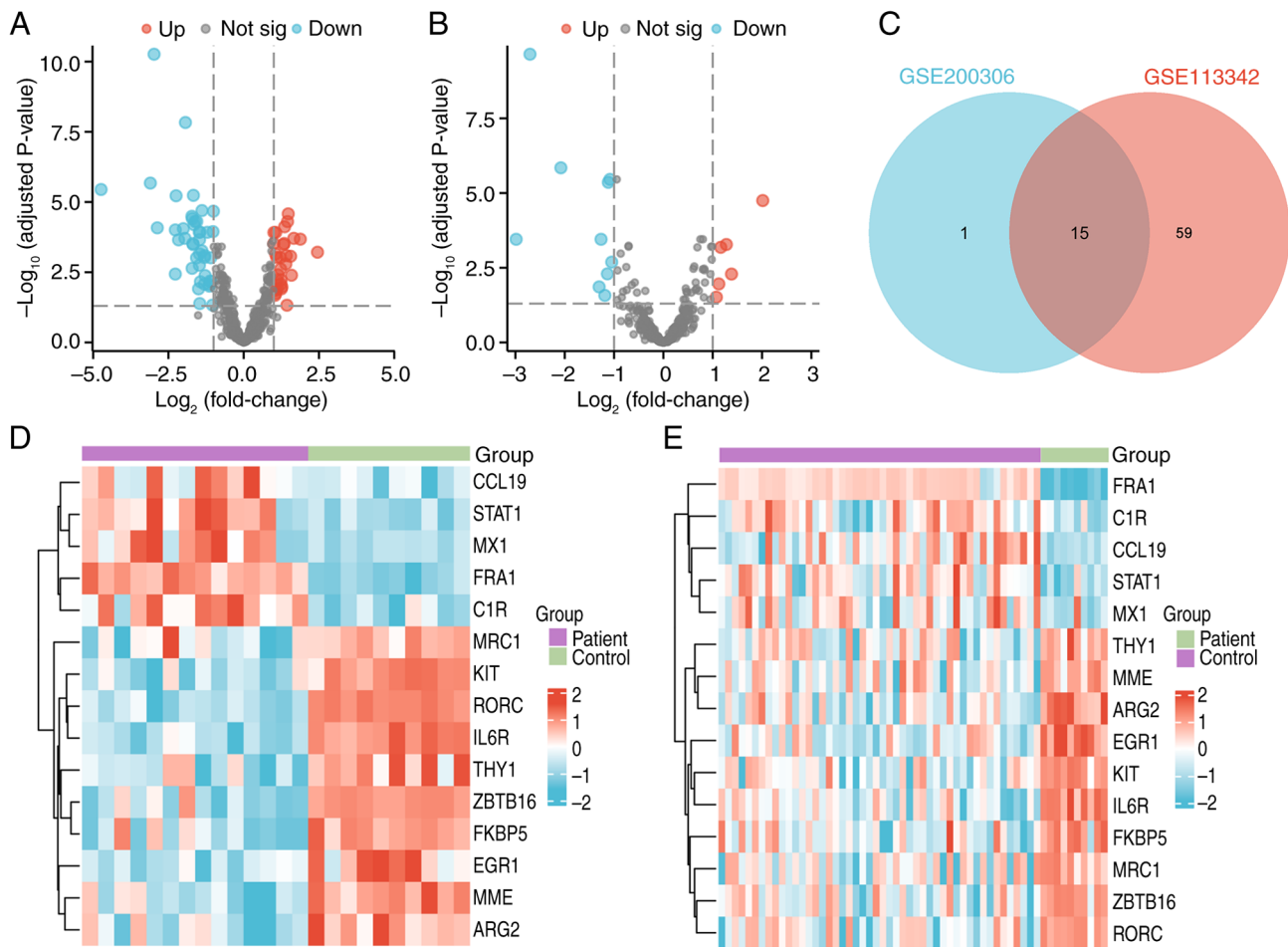


Figure 2. Identification of DEGs in LN tubulointerstitium. Volcano plots showing DEGs between patients with LN and healthy controls in (A) GSE113342 and (B) GSE200306. (C) Venn diagram of DEGs overlapping between GSE113342 and GSE200306, yielding 15 common genes. Heatmap of the 15 shared DEGs in (D) GSE113342 and (E) GSE200306. DEGs, differentially expressed genes; LN, lupus nephritis; Not sig, not significant; up, upregulated; down, downregulated; sig, significant.

chromatin binding' (Fig. 3B). KEGG pathways highlighted 'Coronavirus disease-COVID-19', 'Hematopoietic cell lineage', 'Th17 cell differentiation' and 'Inflammatory bowel disease' as pathways significantly associated with the DEGs (Fig. 3C).

Association between *FRA1* and immune cell infiltration. To prioritize candidates for functional follow-up, the diagnostic performance of five upregulated DEGs was assessed, and *FRA1* was identified as the top performer (AUC=0.977; Fig. 4); therefore, downstream analyses focused on *FRA1*. In addition, further analysis of the datasets showed that the trend of *FRA1* remained stable across different subgroups and after exclusion of outliers, indicating that the upregulation of *FRA1* in LN is robust and minimally affected by dataset heterogeneity (Fig. S2). Next, the relationship between *FRA1* expression and immune cell infiltration in LN tubulointerstitial samples was explored using CIBERSORT on dataset GSE127797.

The results showed statistically increased proportions of CD8⁺ T cells, regulatory T cells (Tregs), monocytes, M1 macrophages and activated mast cells in the high-*FRA1* group, whereas plasma cells, resting CD4⁺ memory T cells, M0/M2 macrophages, resting dendritic cells and resting mast cells were reduced (Fig. 5A-C). Correlation analyses further confirmed

these associations. Specifically, Fig. 5D illustrates that *FRA1* expression was significantly and positively correlated with several immune cell types, most notably naive CD4⁺ T cells, monocytes and Tregs. Conversely, *FRA1* showed strong negative correlations with M0/M2 macrophages, resting CD4⁺ memory T cells and plasma cells. Furthermore, *FRA1* expression was positively correlated with key tubular epithelial-related cytokines, including IL-6, IL-1 β , IL-8, MCP-1, RANTES, TGF- β and TNF (Fig. 5E-K). Collectively, these findings underscore the diagnostic potential of *FRA1* among upregulated DEGs and also suggest a key immunomodulatory role for *FRA1* in the tubulointerstitial microenvironment of LN.

***FRA1* expression in MRL/lpr mouse kidneys.** To verify the expression of *FRA1* in LN kidneys, kidneys from 20-week-old MRL/lpr mice were used. Compared with the control group, H&E staining of MRL/lpr mouse kidneys showed marked inflammatory infiltration (Fig. 6A and B), and tubulointerstitial injury was a distinctive and prominent feature of the diseased kidneys. Moreover, IHC revealed significantly increased *FRA1* expression in tubular epithelial cells of MRL/lpr kidneys compared with the control (Fig. 6C-I), which was corroborated by elevated protein levels in western blot analysis (Fig. 6J and K).

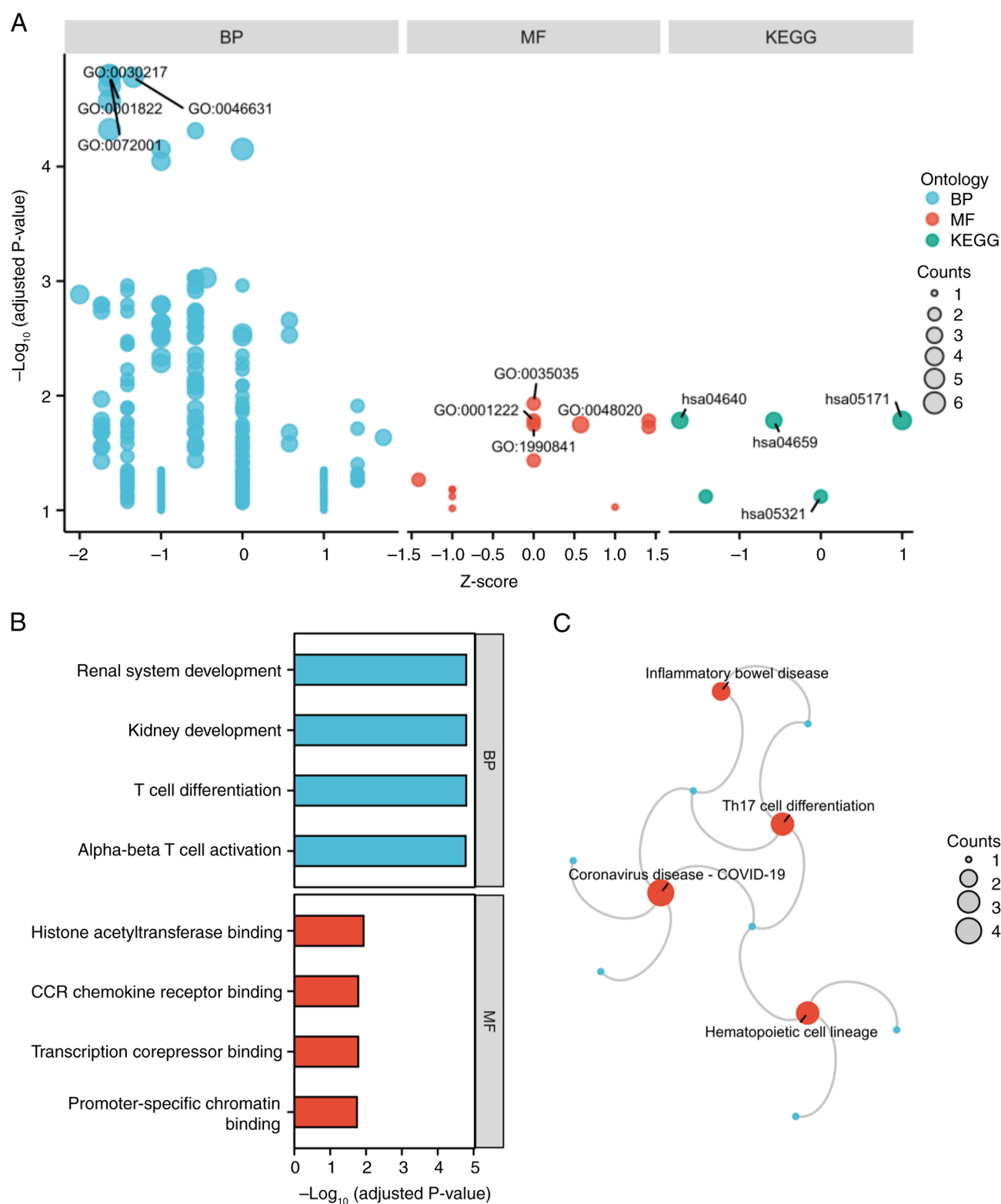


Figure 3. Functional enrichment analysis. (A) Bubble plot illustrating the GO and KEGG enrichment results for the 15 DEGs. (B) Bar plot of GO enrichment analysis (C) KEGG enrichment network of the 15 DEGs. DEGs, differentially expressed genes; GO, gene ontology; KEGG, Kyoto encyclopedia of genes and genomes; Bp, biological process; MF, molecular function.

Effect of FRA1 on inflammatory cytokine expression in HK-2 cells. To investigate the functional role of FRA1 in renal tubular epithelial cells, the expression of key cytokines were measured in HK-2 cells following lentivirus-mediated FRA1 overexpression (FRA1-OE) or knockdown (FRA1-shRNA).

Western blot analysis at 144 h post-infection showed that IL-6, IL-1 β , IL-8, MCP-1 and RANTES were significantly upregulated in the FRA1-OE group, whereas IL-6 and RANTES levels were decreased in the FRA1-shRNA group (Fig. 7). TNF- α and TGF- β expression remained unchanged

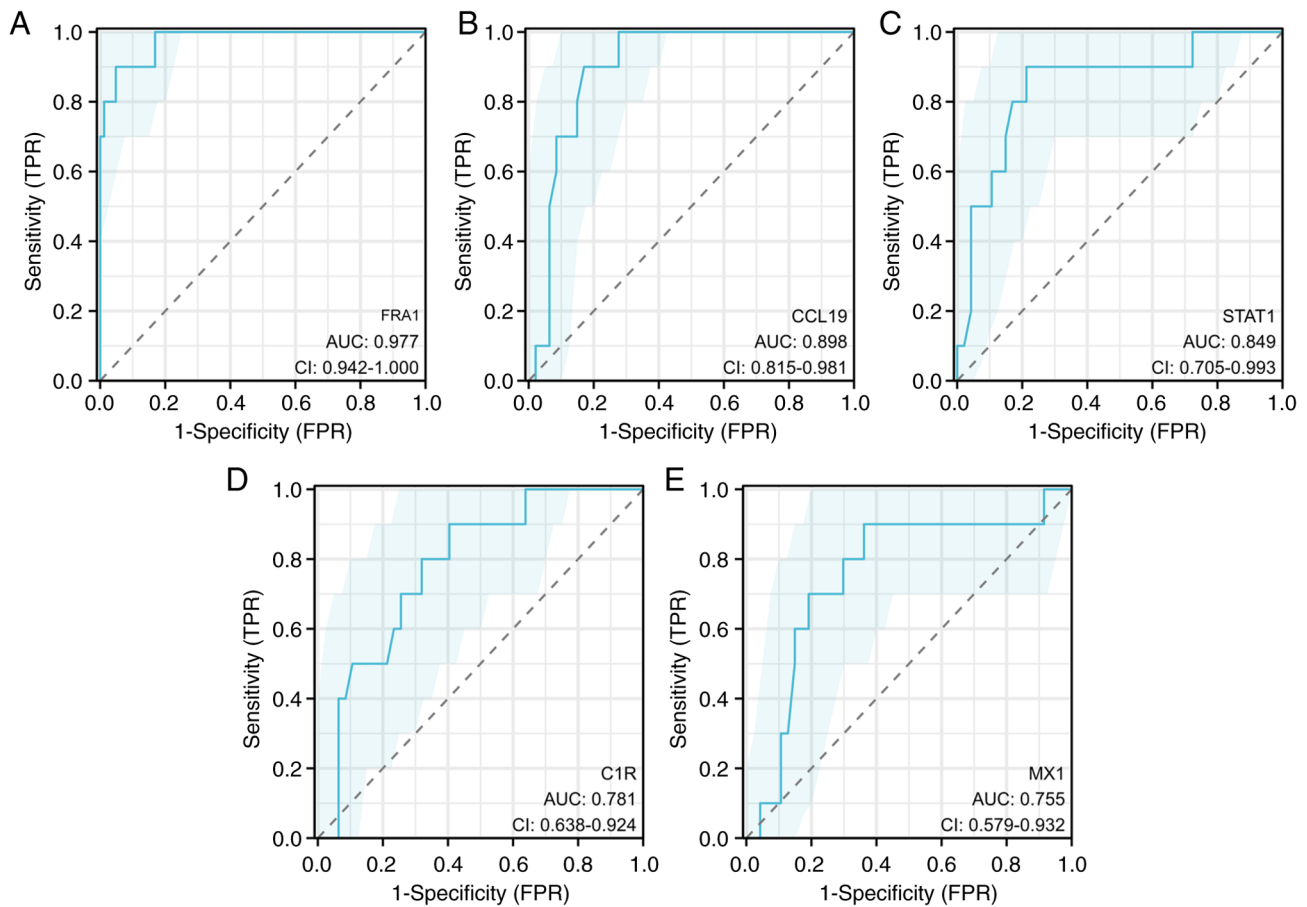


Figure 4. ROC curves for predictive performance of five DEGs upregulated in LN group. Panels (A-E) display the ROC curves for (A) FRA1 (AUC=0.977), (B) CCL19 (AUC=0.898), (C) STAT1 (AUC=0.849), (D) C1R (AUC=0.781) and (E) MX1 (AUC=0.755), respectively. ROC, receiver operating characteristic; AUC, area under the curve; TPR, true positive rate; FPR, false positive rate; CI, confidence interval.

in both FRA1-OE and FRA1-shRNA cells. These results demonstrate that FRA1 modulates multiple proinflammatory cytokines in human renal tubular epithelial cells, suggesting its potential role as a regulator of the tubulointerstitial immune microenvironment in LN.

Discussion

In the present study, an integrated analysis of FRA1 expression and its association with the immune microenvironment in the renal tubulointerstitium of patients with LN was performed. Bioinformatic analyses revealed significant upregulation of FRA1 in LN tubulointerstitial samples, and CIBERSORT deconvolution demonstrated that high FRA1 expression coincided with increased infiltration of CD8⁺ T cells, Tregs, monocytes, M1 macrophages and activated mast cells, alongside decreased proportions of plasma cells, resting CD4⁺ memory T cells, M0/M2 macrophages, resting dendritic cells and resting mast cells. These correlations suggest that FRA1 contributes to shaping a proinflammatory microenvironment in LN. Consistent with the bioinformatics analysis of GEO datasets, kidneys from MRL/lpr mice exhibited elevated tubular epithelial FRA1 expression. *In vitro*, FRA1 overexpression in HK-2 cells markedly increased levels of IL-6, IL-1 β , IL-8, MCP-1 and RANTES, whereas FRA1 knockdown resulted in reduced expression of IL-6 and RANTES. Collectively, these

data indicate that FRA1 may promote tubulointerstitial inflammation in LN by modulating key proinflammatory cytokines.

Prior research has indicated that LN renal tissue exhibits abundant immune cell infiltration and a pro-inflammatory cytokine environment, and that the renal microenvironment can bidirectionally regulate immune cell recruitment, survival and function (42). M1 macrophages in LN are considered to be associated with disease activity, whereas M2 macrophages are more commonly associated with the remission phase (43). In the present study, an increase in M1 macrophages and a decrease in M2 macrophages in the FRA1-high group was observed, consistent with the proinflammatory and alleviating roles attributed to these subsets in LN (43). Additionally, the increased monocyte infiltration may also promote macrophage proliferation and activation (44). Notably, Tregs were also increased in the FRA1-high group, despite their canonical role as suppressors of SLE/LN inflammation (43); this apparent paradox can be explained by several non-exclusive considerations: i) Increased Foxp3⁺Treg infiltration has been reported in active/proliferative LN and lupus-prone mouse models and may reflect compensatory recruitment or expansion in response to intense local inflammation rather than restored suppressive function (45,46); ii) inflammatory milieu, characterized by cytokines such as IL-6, can impair Treg stability and suppressive capacity and promote phenotypic plasticity toward exhausted or effector-like states (such

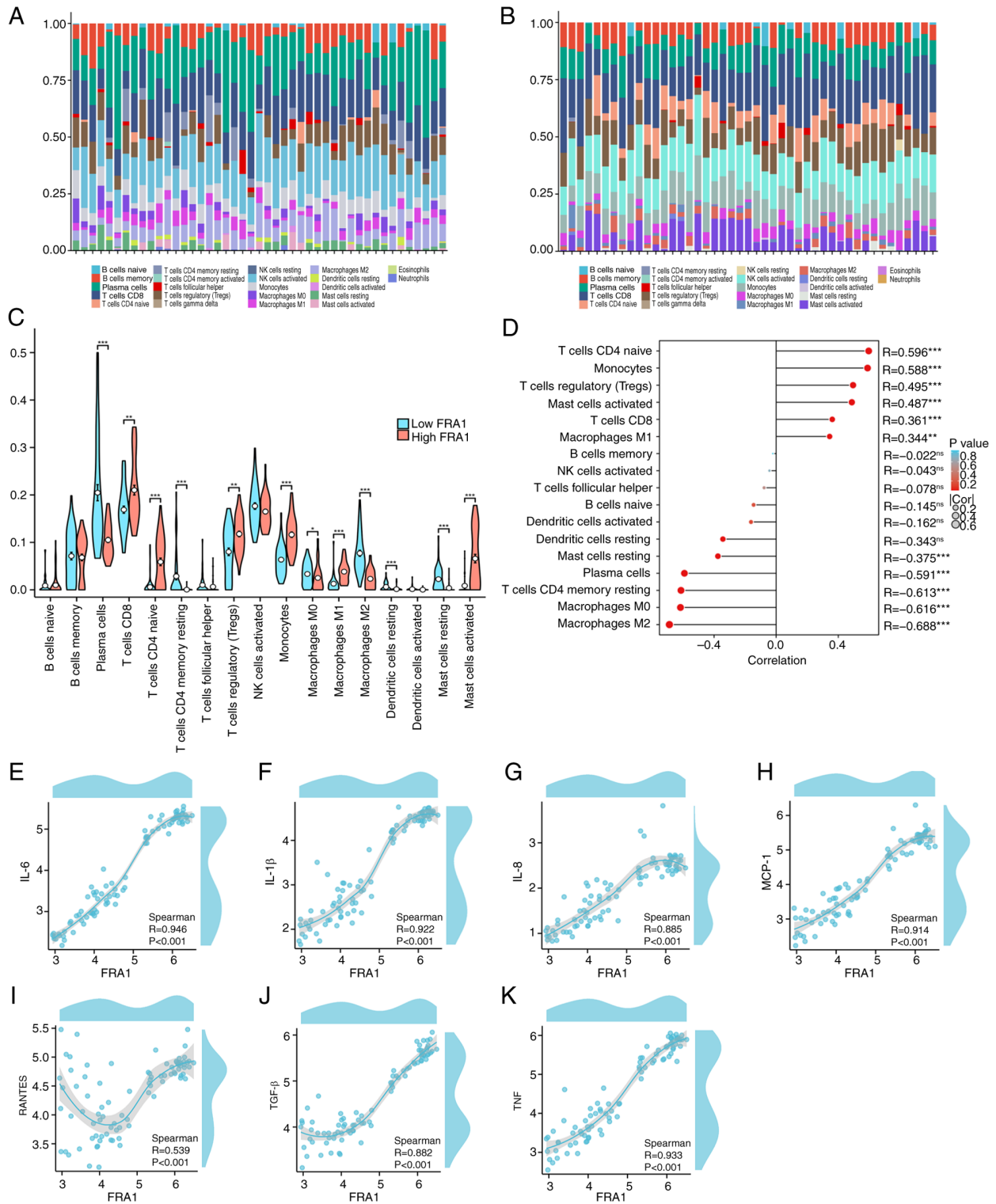


Figure 5. Association between FRA1 and immune cell infiltration. CIBERSORT-estimated immune cell proportions in each sample stratified by FRA1 expression (median split): (A) Low-FRA1 group; (B) high-FRA1 group. (C) Bar plots comparing the proportions of immune cell types between high- and low-FRA1 groups. Cells significantly increased in the high-FRA1 group included CD8⁺ T cells, T regulatory cells, monocytes, M1 macrophages and activated mast cells; cells reduced in the high-FRA1 group included plasma cells, resting CD4⁺ memory T cells, M0/M2 macrophages, resting dendritic cells and resting mast cells (mean $\pm$ SEM; n=44; group comparisons were performed using two-sided Wilcoxon rank-sum tests; *P<0.05; **P<0.01, ***P<0.001). (D) Spearman correlation analyses showing associations between FRA1 expression and immune cell fractions, as well as tubular epithelial-related cytokines, including (E) IL-6, (F) IL-1 β , (G) IL-8, (H) MCP-1 (CCL2), (I) RANTES (CCL5), (J) TGF- β and (K) TNF. All correlation tests are two-sided Spearman rank tests; *P<0.05, **P<0.01 and ***P<0.001.

as IL-17 production (46,47); and iii) CIBERSORT estimates only relative cell proportions from bulk transcriptomes and cannot determine Treg functional status. Thus, the increased

Treg frequency observed in the FRA1-high group may represent numerically expanded but functionally compromised Tregs that fail to restrain local LN inflammation (46,48). The

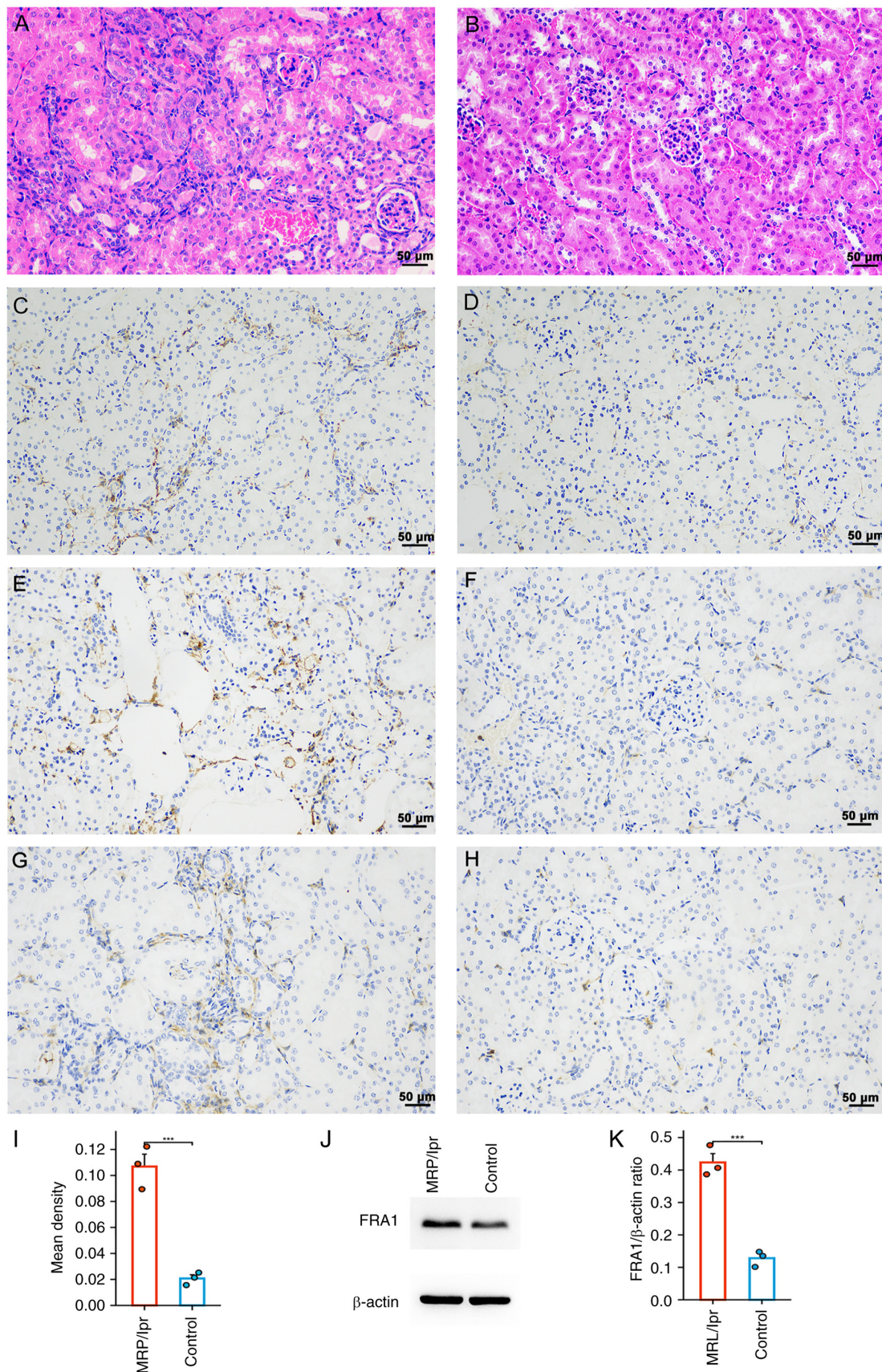


Figure 6. FRA1 expression in MRL/lpr mouse kidneys. Representative H&E staining of kidney sections from 20-week-old (A) MRL/lpr mice and (B) MRL/MPJ controls, showing tubulointerstitial inflammation and damage in MRL/lpr mice. MRL/lpr mice exhibit diffuse and extensive tubulointerstitial inflammation and structural damage. (C-H) FRA1 expression in mouse kidney sections. (C, E and G) Three independent mice from the MRL/lpr group, all demonstrating elevated FRA1 levels in the tubular epithelium. (D, F and H) Three independent mice from the control group, displaying baseline FRA1 immunoreactivity. (I) Quantification of FRA1-positive area from IHC images. (J) Western blot analysis of FRA1 protein levels from MRL/lpr and control mice. (K) Densitometric quantification of FRA1 bands normalized to β-actin. Scale bar, 50 μm; Data are plotted as the mean ± SEM; n=3 per group; group comparisons were performed using two-sided Welch's t-tests; ***P<0.001.

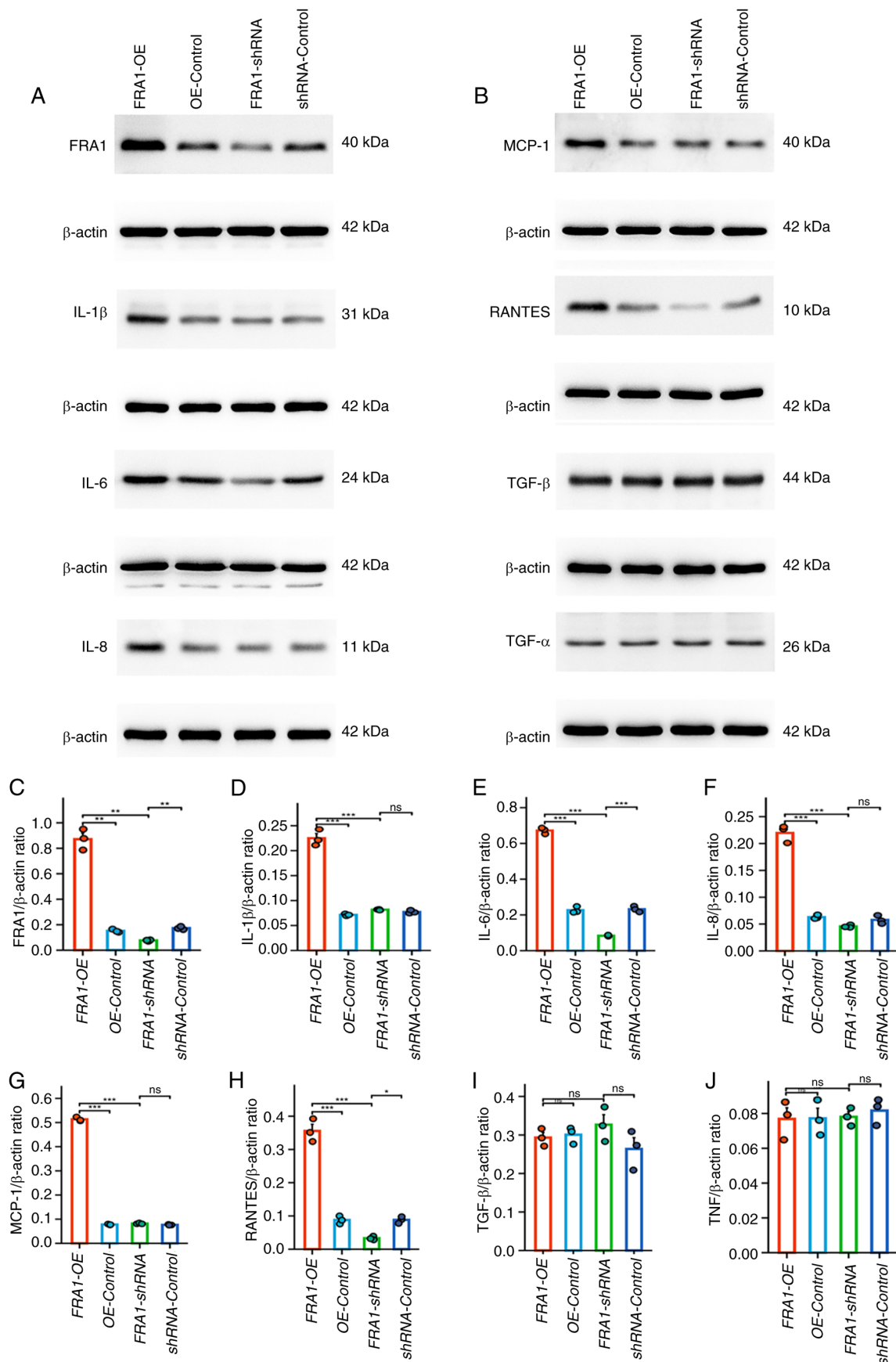


Figure 7. Effect of FRA1 on inflammatory cytokine expression in HK-2 cells. (A) Representative western blots of FRA1 and inflammatory cytokines (IL-1 β , IL-6, and IL-8) in HK-2 cells 144 h after transduction with FRA1-OE, FRA1-shRNA or their corresponding controls. (B) Representative western blots of MCP-1, RANTES, TGF- β and TNF- α in HK-2 cells following the same transduction conditions. Densitometric semi-quantification of protein bands normalized to β -actin for: (C) FRA1, (D) IL-1 β , (E) IL-6, (F) IL-8, (G) MCP-1, (H) RANTES, (I) TGF- β and (J) TNF- α . Data are presented as the mean $\pm$ SEM; n=3 per group; comparisons among subgroups were assessed using the two-sided Kruskal-Wallis test; *P<0.05, **P<0.01 and ***P<0.001. OE, over expression; ns, not significant; sh, short hairpin.

reduction in plasma cells in the FRA1 high-expression group may reflect the fact that plasma cell aggregation and antibody production mainly occur in the glomerular region (23,49), whereas the present analysis focused on interstitial infiltration. Overall, upregulation of FRA1 is consistent with increased proinflammatory cell infiltration and aligns with the inflammatory phenotype of LN renal tissue.

FRA1, as a member of the AP-1 transcription factor family, regulates the expression of various inflammation-related genes, including pro-inflammatory cytokines (such as IL-6 and TNF), chemokines (such as MCP-1 and CXCL1) and matrix metalloproteinases (22). Prior research has indicated that the promoter regions of several pro-inflammatory cytokines contain AP-1 binding sites, suggesting that AP-1 can directly participate in the transcriptional regulation of these genes (22). FRA1 can both form heterodimers with the Jun family to activate gene expression and may also suppress target genes under specific contexts (50-52). The present data demonstrated that FRA1 overexpression upregulates IL-6, a cytokine implicated in promoting antibody-mediated renal inflammation; IL-6 deficiency delays LN onset and reduces renal macrophage and T-cell infiltration in MRL/lpr mice (53). Therefore, FRA1-mediated upregulation of IL-6 may exacerbate LN pathogenesis. MCP-1 is a key chemokine in LN, produced by renal intrinsic cells and extensively attracting monocytes/macrophages to inflammatory sites (54); the present study revealed that FRA1 overexpression induced MCP-1 upregulation, which may explain the increased monocyte and M1 macrophage infiltration in the high-FRA1 group. Similarly, RANTES recruits immune cells such as T cells and natural killer cells, and research has reported elevated urinary RANTES levels in patients during active LN (55); FRA1-driven upregulation of RANTES may also promote T cell (including CD8⁺ T cells and Tregs) chemotaxis to the kidney (56-58). IL-8 is a known neutrophil chemokine that is upregulated in various nephritis conditions, and its pro-inflammatory effects may indirectly affect other cell types through interactions (59). FRA1 can exert context-dependent regulatory effects on IL-6 and other cytokines, influenced by cell type, microenvironmental stimuli and dimer composition (60-62). Future work should investigate whether the selective effects of FRA1 knockdown on IL-6 and RANTES involve distinct AP-1 dimer configurations or specific co-factor interactions.

FRA1 appears to carry out context-dependent roles across different forms of renal injury. In an acute kidney injury model, FRA1 was reported to be protective by preserving expression of the anti-aging protein Klotho, thereby mitigating tubular damage and inflammation (25). By contrast, a bioinformatic screen in IgA nephropathy identified FRA1/2 among prognostic candidates associated with TNF and MAPK signaling, consistent with an inflammatory/prognostic association (26); the present data in LN align more closely with a proinflammatory role. These apparently divergent roles may reflect several non-exclusive, context-dependent factors, for example, acute vs. chronic injury dynamics, differences in the principal affected cell types, variation in upstream signaling milieus that influence AP-1 dimerization and post-transcriptional or

epigenetic modulation (such as m6A) of FRA1 expression or activity (27). These factors can alter the partner selection, co-factor recruitment and promoter occupancy of FRA1, thereby producing disease-specific transcriptional outputs.

The present study has several limitations; first, the present data are primarily correlative and derived from *in vitro* and *ex vivo* analyses. Definitive causal roles for FRA1 in LN pathogenesis will require *in vivo* manipulation (such as tubular epithelial-specific FRA1 knockout or pharmacological inhibition) with larger sample sizes to assess its effects on renal inflammation and function. Although empty-vector controls were used to minimize non-specific viral effects, potential vector integration and shRNA off-target effects cannot be completely excluded. Second, although the present study demonstrated that FRA1-dependent changes in selected cytokines, the direct transcriptional targets of FRA1 in tubular cells remain to be identified; chromatin immunoprecipitation or promoter-reporter assays were not performed to confirm FRA1 binding at putative AP-1 sites, and these experiments constitute a clear next step. Third, the different AP-1 dimer combinations were not distinguished or the contributions of other Fos/Jun family members were not evaluated, which may modulate FRA1 activity and selectivity. Fourth, the present focus on the tubulointerstitium warrants complementary studies in glomerular compartments and in additional cell types; in particular, functional interrogation of FRA1 in LN-relevant immune cells (such as macrophages and T cells) and in glomerular cells would help determine cell-type-specific roles. Moreover, species-specific differences between rodent models and human LN necessitate analyses of human clinical samples to confirm the translational relevance of FRA1 as a potential biomarker or therapeutic target. Further investigation of classical profibrotic and epithelial-mesenchymal transition markers (such as fibronectin, collagen I and α -SMA) in FRA1 overexpression and knockdown models would be valuable. Finally, it is worth considering the translational potential of targeting FRA1/AP-1; for example, small-molecule AP-1 inhibitors have shown efficacy in preclinical kidney disease models: The selective c-Fos/AP-1 inhibitor T-5224 ameliorates renal inflammation and fibrosis in murine injury models, and other AP-1 pathway blockers (such as the JNK inhibitor SP600125) exhibit protective effects in inflammatory disease (63). Thus, FRA1-directed therapies or biomarker strategies, leveraging existing AP-1 modulators, could be explored in future studies of LN.

In conclusion, the present integrative analysis and experimental validation revealed that FRA1 was upregulated in LN tubulointerstitium and likely contributes to a proinflammatory immune microenvironment by driving expression of key cytokines. These findings position the FRA1/AP-1 axis as a novel regulator of renal inflammation in LN and highlight its potential as a target for therapeutic intervention. Further mechanistic and *in vivo* studies are warranted to exploit FRA1 modulation for LN treatment.

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Availability of data and materials

The gene expression datasets analyzed in the present study are publicly available from the Gene Expression Omnibus (<https://www.ncbi.nlm.nih.gov/geo/>) under accession numbers GSE113342, GSE200306 and GSE127797. All other data generated during the present study may be requested from the corresponding author upon reasonable request.

Authors' contributions

WN, JH, ZZ, JK, RL and GG contributed to the research conception and design. WN, JH, ZZ, JK and RL performed the experiments and drafted the manuscript. LW, YC, CZ, YL, JP and KD analyzed and interpreted the raw data. WN, JH, ZZ, JK, RL, GG, JP and KD confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

All animal studies were approved by the ethics committee of Boai Hospital of Zhongshan (approval no. WHMYS-20250096). All animal operations were performed in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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