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**Shuangyu Tangke Decoction ameliorates diabetic nephropathy by
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Insights from multi-omics analysis**

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Abstract

Background: Diabetic nephropathy (DN) is a major microvascular complication of diabetes and a leading cause of end-stage renal disease, yet effective therapies remain limited. Shuangyu Tangke Decoction (SKD), a traditional Chinese herbal formula, has shown promising clinical benefits in treating diabetes and DN. Nevertheless, the specific bioactive constituents and underlying molecular mechanisms of SKD remain incompletely understood.

Purpose: This study aimed to systematically evaluate the renoprotective effects of SKD and elucidate its mechanism of action through integrated multi-omics profiling, computational modeling, and experimental validation.

Methods: The bioactive serum-absorbed constituents of SKD were characterized using ultra-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF/MS). The therapeutic efficacy was evaluated in db/db mice through biochemical measurements and renal histopathology. Integrated transcriptomic, proteomic, and metabolomic analyses were utilized to identify the central dysregulated pathway and key molecular targets. Molecular docking, molecular dynamics simulations, microscale thermophoresis (MST), and cellular thermal shift assays (CETSA) were used to validate the interaction between Robinin (a major active constituent) and Corol1a. Functional rescue experiments involving Corol1a overexpression and knockdown in macrophages, along with site-directed mutagenesis, were conducted to identify the critical binding residue.

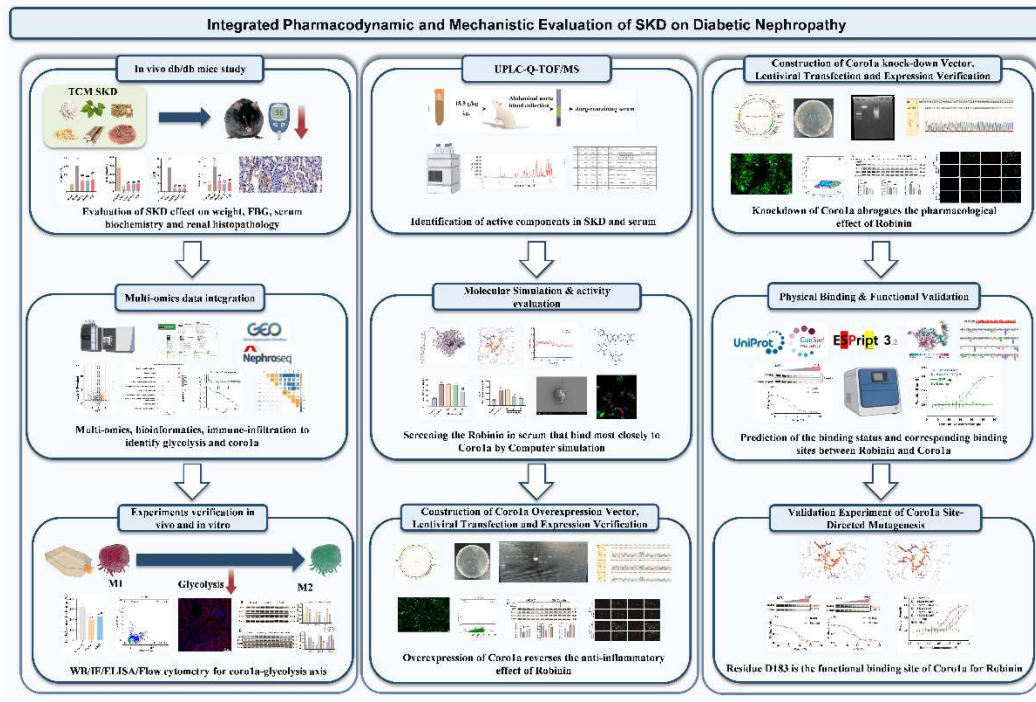
Results: SKD reduced blood glucose levels, improved renal function index, and attenuated renal pathological injury in db/db mice. Multi-omics integration identified glycolysis as the central dysregulated pathway, with Corol1a serving as a key molecular node. SKD suppressed macrophage glycolysis, inhibited M1 polarization, and reduced the production of pro-inflammatory cytokines. UPLC-Q-TOF/MS identified 63 prototype compounds and nine serum-absorbed constituents of SKD. Robinin binds to Corol1a at D183, disrupts glycolytic flux, and alleviates inflammation.

Conclusion: These findings demonstrate that SKD exerts renoprotective effects in DN

by targeting macrophage metabolism through inhibition of glycolysis via the Robinin–Coro1a interaction. This study uncovers a mechanism linking herbal medicine to immune-metabolic crosstalk and identifies Coro1a as a potential therapeutic target for metabolic kidney diseases. Our work provides a mechanistic foundation for the clinical application of SKD and advances the evidence-based development of plant-derived therapeutics for DN.

Key words: Shuangyu Tangke decoction; Diabetic nephropathy; Multi-Omics analysis; Coro1a/glycolysis axis; Robinin

65 **Graphic abstract:**



66

67 **Abbreviations:** TCM, Traditional Chinese Medicine; SKD, Shuangyu Tangke
68 Decoction; DM, diabetes mellitus; DN, diabetic nephropathy; ESRD, end-stage renal
69 disease; ALB, albumin; BUN, blood urea nitrogen; Scr, serum creatinine; GO, gene
70 ontology; BP, biological process; MF, molecular function; CC, cellular component;
71 KEGG, Kyoto Encyclopedia of Genes and Genomes; LPS, lipopolysaccharides; H&E,
72 hematoxylin and eosin; PAS, periodic acid-schiff stain; UPLC-Q-TOF-MS, ultra-
73 performance liquid chromatography/quadrupole time-of-flight-tandem mass
74 spectrometry; GSEA, gene set enrichment analysis; OPLS-DA, orthogonal partial least
75 squares discriminant analysis; BPI, base peak ion; CCK8, cell counting kit-8; FBS, fetal
76 bovine serum; FC, fold change; DGEs, differential expression genes; DEPs, differential
77 expression proteins; FBG, fasting blood glucose; IF, immunofluorescence; FC, flow
78 cytometry; ELISA, enzyme linked immunosorbent assay; SD, Sprague-Dawley; NS,
79 normal saline; OCR, oxygen consumption rate; ECAR, extracellular acidification rate;
80 MST, microscale thermophoresis; CETSA, cell thermal shift assay; MD, molecular
81 dynamics; RMSD, root mean square deviation; RMSE, root mean square fluctuation;
82 SASA, solvent accessible surface area; SEM, scanning electron microscope

83

Introduction

Diabetic nephropathy (DN), the most prevalent microvascular complication of diabetes mellitus (DM), remains a leading cause of end-stage renal disease (ESRD) worldwide [1]. As a chronic kidney disease now regarded as an epidemic, DN imposes an increasing burden on global healthcare economies [2, 3]. The hallmarks of DN include progressive renal function decline, sustained proteinuria, and a diminished glomerular filtration rate, primarily driven by chronic hyperglycemia and metabolic disorder [4]. Despite the widespread use of renin–angiotensin system (RAS) inhibitors and sodium-glucose cotransporter-2 inhibitors (SGLT2) as standard-of-care therapies, a significant proportion of patients continue to experience progressive renal function decline, underscoring the urgent need for novel therapeutic strategies that target the underlying pathogenic mechanisms of DN [5, 6].

Increasing evidence indicates that DN involves not only hemodynamic and metabolic abnormalities, but also persistent renal inflammation and immune dysregulation [7-9]. Among the various immune cell types involved, macrophages play a pivotal role in orchestrating renal inflammation and fibrosis [10]. Within the DN microenvironment, macrophages undergo profound phenotypic and metabolic shifts, marked by excessive polarization toward the pro-inflammatory M1 subtype. This shift drives the release of inflammatory cytokines, including tumor necrosis factor- α (TNF- α) and interleukin-6 (IL-6), which in turn exacerbate tubular damage and interstitial fibrosis [11, 12]. Accompanying this phenotypic switch is a metabolic reprogramming characterized by suppressed oxidative phosphorylation and upregulated aerobic glycolysis [13]. These alterations in macrophage glucose metabolism present a compelling therapeutic target for mitigating chronic renal inflammation and potentially slowing the progression of DN [14].

Coronin-1A, encoded by the *Coro1a* gene, is an actin-binding protein involved in regulating cytoskeletal dynamics, immune cell migration, and phagocytosis. [15, 16]. Studies have found that alterations in *Coro1a* expression are closely associated with the severity of inflammatory responses, highlighting its potential as a diagnostic biomarker

or therapeutic target [17]. For instance, CREBH-mediated regulation of Coro1a enhances autophagy in hepatocytes and attenuates oxidative stress and inflammation in non-alcoholic fatty liver disease [18]. However, whether Coro1a regulates macrophage glycolysis and contributes to DN pathogenesis remains unknown.

Traditional Chinese Medicine (TCM) has a long history of clinical application in treating complex chronic diseases such as obesity, metabolic dysfunction-associated fatty liver disease (MAFLD), and diabetic complications [19-21]. is a modified herbal formulation developed from the classical Chinese medicinal prescriptions Yuye Decoction and Xiaoke Decoction, both of which have long been documented and used in clinical practice for managing diabetes and its complications [22, 23]. A patent application for this formulation is currently underway (Application No.: 202511056471.9). However, its active constituents, circulating metabolites, and mechanisms of renal protection remain insufficiently defined. This limitation is particularly relevant for complex herbal formulas, whose numerous chemical constituents undergo extensive biotransformation after administration. Characterization of serum-exposed constituents is therefore essential for identifying pharmacologically relevant compounds [24, 25].

Multi-omics approaches, encompassing transcriptomics, proteomics, and metabolomics, offer complementary insights into the complex mechanisms underlying disease pathogenesis and the therapeutic actions of herbal medicines [26]. The integration of these multi-layered datasets enables a holistic and unbiased view of biological systems, thereby deepening our understanding of pharmacological effects at the systems level [27]. In the present study, we evaluated the renoprotective effects of SKD in db/db mice. By integrating multi-omics analysis with rigorous biochemical validation, we demonstrated that SKD exerts its therapeutic effects by targeting the Coro1a/glycolysis axis. Moreover, we identified robinin as the principal bioactive component underlying the anti-inflammatory actions of SKD. Robinin exerts its effects by directly binding to Asp183 of Coro1a, thereby suppressing glycolytic reprogramming and pro-inflammatory activation.

Materials and methods

Preparation of aqueous extract and pharmacologically active serum of SKD

The following herbs were obtained from Zhejiang Chinese Medical University of Traditional Pharmaceutical Slices Co., Ltd. (Production License: (Zhejiang) 2000-0072): *Fragrant Solomonseal Rhizome* (Yuzhu), *Corn Silk* (Yumixu), *Common Yam Rhizome* (Shanyao), *Mulberry Leaf* (Sangye), *Lobed Kudzuvin Root* (Gegen), and *Glutinous Rice Root* (Nuodaogen). The combined aqueous extracts were concentrated using a multifunctional traditional Chinese medicine (TCM) extraction apparatus (70 °C, 0.8 Pa) and further reduced via rotary evaporation to a final concentration of 18.9 g/kg.

For the preparation of medicated serum, Sprague-Dawley (SD) rats (n = 6, 6 weeks old, male) were obtained from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China) and acclimatized under standard laboratory conditions for 7 days. Rats were randomly assigned to two groups: control (n = 3) and SKD-treated (n = 3). Animals in the SKD group received oral gavage of SKD aqueous extract at a dose of 18.9 g/kg. twice daily for six consecutive days. The control group received an equal volume of normal saline via the same route.

On the seventh day, after a 12-hour fast, rats were anesthetized with isoflurane (induction: 3%, maintenance: 1.5–2%). Blood samples were collected from the abdominal aorta, immediately centrifuged at 4 °C and 4000 rpm for 10 min to separate serum. Serum was heat-inactivated at 56 °C for 30 min to deactivate complement proteins, followed by sterile filtration through a 0.22 µm membrane filter (Biosharp, China). All animal procedures were conducted in accordance with the guidelines of the Ethics and Welfare Committee of ZClab (ZCMUJHYJY-202508-02).

Chemical component profile analysis of SKD and drug-serum

The components of SKD and drug-serum were analyzed using UPLC-Q-TOF/MS on a Waters SYNAPT G2-Si system (Shanghai, China). Chromatographic separation was performed under the following conditions: the mobile phase consisted of (A) acetonitrile and (B) water containing 0.1% formic acid, with a gradient elution program

as follows: 0-2 min, 5% A; 2-30 min, 5-50% A; 30-40 min, 50-100% A; 40-42 min, 100% A; 42-42.5 min, 100-5% A; 42.5-48 min, 5% A. The flow rate was set at 0.3 mL/min, the injection volume was 2 μ L, and the column temperature was maintained at 35 °C.

Mass spectrometric detection was conducted using an electrospray ionization (ESI) source. Data were acquired in both positive and negative ion modes with MSE continuous full scan acquisition. The scan time was 0.2 s, and the mass range was set from 50 to 1200 m/z. In MSE mode, a low collision energy of 6 V and a high collision energy ramp from 15 to 45 V were applied. Mass accuracy was calibrated using sodium formate, and real-time mass correction was performed with leucine enkephalin as the reference lock mass (m/z 556.2771 for positive mode; m/z 554.2615 for negative mode).

Animals and experiment design

Eight-week-old male C57BLKs/J db/m and db/db mice were purchased from Saiye (Suzhou) Biological Technology Co., Ltd. The feeding and rearing conditions for animals were identical to those stated in the previous section. After one-week adaptation, Mice were randomly divided into 5 groups (n = 6 for each group): (1) db/m group, (2) db/db group, (3) db/db + SKD-L group (10.8 g/kg), (4) db/db + SKD-H group (21.6 g/kg), and (5) db/db + Dapagliflozin group (1.3 mg/kg). The dosage of SKD was calculated based on the conversion of animal body surface area to the human clinical dosage [28].

Drug intervention was carried out once daily for 10 weeks, while the db/m (Control group) and db/db (Model group) received 0.9 % (W/V) normal saline (NS). Animal experiments followed standards set by Animal Ethical and Welfare Committee of ZCMU (Approve number: IACUC-202501-14).

Serum and urine samples analysis and Histological examination

Urine levels of glucose, albumin (ALB), urinary creatinine (UCR), and 24-hour urinary protein quantity (24h-UTP) as well as serum concentrations of blood urea nitrogen (BUN), creatinine (SCR) were measured using an automatic biochemical analyzer (Toshiba, Japan). Serum insulin, C-peptide, and inflammatory cytokines

including IL-6, IL-1 β , TNF- α , and IL-1RA were quantified using commercial ELISA kits (CUSABIO, Wuhan, China; MULTI SCIENCES, Hangzhou, China). Renal tissues were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned at a thickness of 4 μ m. The sections were stained with hematoxylin-eosin (HE) and periodic acid–Schiff (PAS) for histological evaluation. Pathological changes were examined under a light microscope (Leica, Germany).

Transcriptomic, proteomic, and metabolomic analysis

Total RNA was extracted from renal tissues using TRIzol reagent. mRNA enrichment was performed using oligo(dT) magnetic beads, fragmented into ~200–300 bp fragments, and reverse-transcribed into cDNA using random hexamers. cDNA was end-repaired, adenylated, and ligated to Y-shaped adapters. Libraries were quantified using the TBS380 system. Differential expression analysis was conducted based on Fragments Per Kilobase of transcript per Million fragments mapped (FPKM).

Proteomic profiling was performed using data-independent acquisition (DIA) on an Orbitrap Astral mass spectrometer (Thermo Fisher Scientific). Chromatographic separation was carried out using a Vanquish Neo system (Thermo Fisher Scientific). Positive ion mode was used with precursor scan range of 380–980 m/z and a primary resolution of 240,000 at 200 m/z.

Untargeted metabolomics was performed on renal tissue homogenates extracted with 80% methanol. Supernatants were lyophilized, reconstituted in 10% methanol/90% water, and subjected to LC-MS analysis. Quality control (QC) samples were prepared by pooling aliquots from all samples.

Multi-omics integration and bioinformatics analysis

Immune cell infiltration was estimated using the CIBERSORT algorithm (v1.0) in R (v4.3.1), employing the murine LM25 signature matrix as the reference. Immune cell composition was visualized using box plots, and statistical differences were assessed by Wilcoxon rank-sum test ($P < 0.05$).

Over-representation analysis (ORA) of differentially expressed genes (DEGs) and proteins (DEPs) was performed using the KEGG pathway database via the

clusterProfiler R package [29]. Pathways with adjusted $P < 0.05$ were considered significantly enriched. Spearman's rank correlation was used to assess relationships among DEGs, immune cell proportions, and clinical phenotypes.

Clinical relevance of Coro1a expression

GSE30528 and GSE30529 datasets were retrieved from the Gene Expression Omnibus (GEO) database (<https://www.ncbi.nlm.nih.gov/geo/>). Differential expression analysis was conducted using the Limma package. Box plots were generated using ggplot2 to visualize Coro1a expression. Correlation analysis between Coro1a mRNA levels and renal function markers (eGFR, SCR, Proteinuria) was performed using the Nephroseq v5 database (<https://nephroseq.org>) via Spearman's rank correlation.

Flow cytometry

Kidney tissues were mechanically dissociated, filtered through a 70- μ m strainer, and red blood cells lysed. Single-cell suspensions were incubated with fluorochrome-conjugated antibodies against CD45, F4/80, and CD86 (BioLegend, USA) for 30 min at 4 °C. Data were acquired on a CytoFLEX S flow cytometer (Beckman Coulter, USA) and analyzed using FlowJo software (v10.4.0).

Cell culture and stimulation

RAW264.7 and HEK 293T cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin at 37 °C, 5% CO₂. For M1 polarization, RAW264.7 cells were stimulated with LPS (100 ng/mL, Solarbio, China) and IFN- γ (50 ng/mL, DAKWE, China) for 6 hours. SKD serum and Robinin (Ambeed, USA) were pre-treated 1 hour.

Molecular docking and molecular dynamics simulation

The 3D structure of Coro1a was obtained using the AlphaFold 3 (<https://alphafoldserver.com/>) (pLDDT > 90) and prepared for docking with the AutoDock Tools (ADT). Binding pockets were predicted using ProteinsPlus. Ligand structures were sourced from PubChem. Molecular docking was performed using AutoDock Vina (v1.2.0), with the top-scoring pose selected based on binding energy.

Visualization was performed using Chimera X (v1.5).

MD simulations (100 ns) were conducted using GROMACS 2023.4. The complex was solvated in a TIP3P water box (10 Å margin), neutralized, and equilibrated under NVT (100 ps) and NPT (100 ps) conditions at 310 K and 1 bar. Production runs used a 2 fs time step, PME for electrostatics, and cutoffs of 1.0 nm. Trajectory analysis included root mean square deviation curve (RMSD), root mean square fluctuation (RMSF), Solvent accessible surface area (SASA), and Radius of gyration (Rg). Binding free energy was calculated via MM/PBSA.

Scanning Electron Microscope (SEM) observation of macrophage morphological changes

For scanning electron microscopy, treated macrophages were fixed with 2.5% glutaraldehyde, post-fixed with 1% osmium tetroxide, and dehydrated through graded ethanol. After critical-point drying and gold-palladium sputter-coating, cells were visualized under a scanning electron microscope (Quanta 450, FEI) at 5 kV to evaluate surface morphology and membrane architecture.

Immunohistochemistry and Immunofluorescence analysis

Kidney sections underwent antigen retrieval (65 °C, overnight), quenching of endogenous peroxidase with 3% H₂O₂, and blocking with 3% FBS. Sections were incubated with anti-Nephrin (PSH07-70, HUABIO, 1:100) at 4 °C overnight, followed by secondary antibody and DAB staining. For immunofluorescence, RAW264.7 cells were fixed, blocked, and incubated with anti-CD206 (DF4149, Affinity, 1:500) and anti-CD86 (HUABIO, 1:500), followed by fluorescent secondary antibodies and DAPI nuclear staining. Images were acquired using the Leica confocal microscope.

Western blot analysis

Cells and tissues were lysed in RIPA buffer (Cat# R0020, Solarbio) on ice for 30 min. Protein concentration was determined using the BCA assay (Beyotime, # p0012). Proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, blocked with rapid blocking buffer, and probed with primary antibodies: anti-Corola

(EPR19467-36, 1:2000, abcam), anti-Nephrin (PSH07-70, HUABIO, 1:1000), anti-CD86 (251860A8, 1:2000, Proteintech), anti-Arg-1 (16001-1-AP, 1:2000, Proteintech), anti-G6PI (ab66340, 1:1000, abcam), anti-PFK-1 (55028-1-AP, 1:2000, Proteintech), and anti-PKM2 (84281-5-RR, 1:2000, Proteintech). Detection was performed using HRP-conjugated secondary antibodies and ECL substrate.

Knockdown and Overexpression assays in Macrophages

The plasmid template for Corol1a was purchased from MiaoLing Plasmid Biotech (Wuhan, China). The core open reading frame (ORF) region of the vector was replaced with the full-length coding sequence (CDS) of Corol1a using standard molecular cloning techniques. The resulting recombinant plasmids were designated as: pLVX-Corol1a (mouse)-P2A-mEGFP-IRES-Puro for overexpression of Corol1a and pSIH1-H1-Corol1a(mouse)-shRNA-CopGFP-PuroR for knockdown of Corol1a expression. The primer sequence was shown in Table S1.

After successful plasmid construction, the recombinant plasmids were co-transfected into HEK 293T cells together with helper plasmids via the PEI transfection reagent. The harvested lentiviral supernatant was then used to infect RAW264.7 cells. Following antibiotic selection with puromycin, the stable transfected cell lines were subjected to subsequent experimental verification.

Construction of the Corol1a-mut expression plasmid

The overexpression plasmid was used as the template to construct two mutant plasmids, D183A and D183G. The sequence was shown in Table S2. After PCR amplification and digestion with Dpn I enzyme, the products were transformed into DH5 α competent cells, followed by plasmid extraction. The subsequent procedures were consistent with those described above. Mutations were confirmed by Sanger sequencing.

Cellular thermal shift assay (CETSA) and microscale thermophoresis (MST)

For CETSA, RAW264.7 lysates were treated with Robinin (10 μ M) or DMSO, heated at 42–77 °C for 5 min, cooled at 4 °C for 3 min, and analyzed by Western blot.

For MST, interaction between Robinin and Coro1a was measured using the NanoTemper Monolith NT.115. Samples were diluted 5-fold in TNT buffer. Fluorescence was monitored at 25 °C, 40% excitation, and 40% MST power. Apparent K_D values were calculated using MO Affinity Analysis v2.3.

Lactic acid and metabolic assays

Lactic acid levels were measured using a commercial kit (Nanjing Jiancheng, China). Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were assessed using Elabscience OCR and Glycolysis Stress Assay Kits, respectively, according to manufacturer instructions.

Statistical analysis

Data are expressed as the mean $\pm$ standard error of the mean (SEM). Statistical significance among groups was assessed by one-way analysis of variance (ANOVA), followed by appropriate post hoc tests, and P value < 0.05 was considered statistically significant.

Results

UPLC-Q-TOF/MS-based identification of prototype compounds and serum-absorbed constituents

UPLC-Q-TOF/MS identified 63 prototype compounds in SKD (Fig. S1A). To identify serum-absorbed bioactive constituents, we compared the base peak intensity (BPI) chromatograms of serum samples from SKD-treated mice with those from blank serum under both positive and negative ionization modes. Potential constituents were defined as ion peaks detected exclusively in SKD serum, absent from blank serum, and displaying fragment-ion patterns consistent with the corresponding prototype compounds. Based on these criteria, 9 potential active ingredients were identified as serum-absorbed components of SKD (Fig. S1B, C, Table S3).

SKD ameliorates hyperglycemia and metabolic abnormalities in DN mice

To evaluate the therapeutic effects of SKD on metabolic abnormalities, db/db mice received SKD at 10.8 or 21.6 g/kg for 10 weeks (Fig. 1A). Throughout the treatment period, fasting blood glucose (FBG) and body weight were significantly elevated in the db/db model group compared with the control group. Both SKD and Dap treatment effectively reduced FBG levels, although no marked changes in body weight were observed (Fig. 1B, C). Metabolic-cage assessments conducted prior to sacrifice revealed increased food intake, water consumption, and urine output in db/db mice relative to controls. Notably, treatment with SKD or Dap significantly alleviated these abnormalities (Fig. 1D-F).

Serum biochemical analyses further demonstrated elevated blood urea nitrogen (BUN) and serum creatinine (SCR) levels, along with reduced C-peptide levels, in the DN model group. SKD and Dap treatment significantly reversed these changes, restoring BUN, SCR, and C-peptide levels toward normal (Fig. 1G-I).

SKD alleviates renal pathological damage and protects renal function in DN mice

To investigate the renal protective effects of SKD, urine samples were collected for the assessment of kidney injury biomarkers. Urinalysis revealed elevated levels of ALB, 24h-UTP, and ACR, along with reduced UCR levels in db/db mice. SKD and Dap

administration reduced ALB, 24h-UTP, and ACR levels and improved UCR excretion (Fig. 2A-D). HE and PAS staining showed glomerular hypertrophy, mesangial matrix expansion, vascular wall thickening, and evident inflammatory cell infiltration in db/db mice. SKD treatment attenuated these pathological alterations. IHC and WB analyses further showed that Nephrin was markedly downregulated in DN mice, and SKD administration effectively restored its expression (Fig. 2E-G).

Multi-omics analysis reveals that the therapeutic efficacy of SKD is closely linked to the Coro1a/glycolysis axis

Untargeted metabolomic profiling of kidney tissue was performed to characterize metabolic alterations after SKD treatment. OPLS-DA score plots and volcano plots showed clear separation between the db/db model group and SKD-treated mice, indicating distinct metabolic profiles in the two groups (Fig. 3A). Volcano and cluster analyses showed that differentially expressed metabolites (DEMs) were mainly associated with amino acid, carbohydrate, and lipid metabolism (Fig. 3B, C). KEGG pathway enrichment analysis showed that SKD-modulated metabolites were enriched in energy metabolism pathways, including the citrate cycle (TCA cycle), glyoxylate and dicarboxylate metabolism, and several amino acid catabolic pathways (Fig. 3D). Metabolite set enrichment analysis (MSEA) showed significant downregulation of the glycolysis pathway after SKD intervention (Fig. 3E), suggesting suppression of aerobic glycolysis, a hallmark of pro-inflammatory macrophage activation.

To investigate molecular changes associated with the renoprotective effects of SKD, integrated transcriptomic and proteomic analyses were performed. A total of 122 differentially expressed genes (DEGs) and 84 differentially expressed proteins (DEPs) were significantly reversed by SKD treatment relative to the db/db model group (Fig. S2A–C, Fig. S3A–C). Functional enrichment analysis indicated that these molecules were mainly involved in inflammation-associated signaling pathways, including Wnt, Hippo, Relaxin, and PI3K-Akt signaling (Fig. S2D, E, Fig. S3D, E). Gene Set Enrichment Analysis (GSEA) further showed downregulation of ECM-receptor interaction after SKD treatment (Fig. S2F, Fig. S3F), consistent with reduced renal

inflammation and ECM accumulation in pathological staining.

CIBERSORT analysis revealed notable shifts in immune-cell infiltration during the progression of DN. The abundance of M1 macrophages was significantly elevated in the db/db group compared to controls, and treatment with SKD effectively counteracted this polarization toward the M1 phenotype. (Fig. 4A, B). Integration of DEGs and DEPs from the multi-omics datasets, followed by KEGG pathway analysis, again identified the glycolytic pathway as a central node modulated by SKD (Fig. 4C). We then focused on mRNAs and proteins that were consistently downregulated in the SKD-treated model and performed correlation analyses. Corol1a showed a highly significant positive correlation with M1-polarized macrophages, as well as with multiple renal injury markers and inflammatory cytokines (Fig. 4D-H). Collectively, these omics-based findings provide robust evidence that the therapeutic effects of SKD in DN are mechanistically associated with the inhibition of the macrophage Corol1a/glycolysis axis.

Bioinformatics analysis of the clinical relevance of Corol1a

In both GSE30528 and GSE30529 datasets, Corol1a expression was markedly upregulated in DN samples relative to control groups (Fig. 5A, B). In the Nephroseq V5 database, Corol1a correlated positively with urinary protein and urinary creatinine levels, and inversely with glomerular filtration rate (GFR), suggesting that Corol1a is associated with the progression of DN (Fig. 5C-E).

SKD attenuates macrophage M1 polarization and glycolytic activity in vivo

Flow cytometric analysis of kidney and spleen tissues revealed that SKD treatment significantly reduced the proportion of M1-polarized macrophages (CD45⁺F4/80⁺CD86⁺) in both organs compared with the model group (Fig. 6A, B). Western blotting further demonstrated that SKD upregulated the M2-associated marker Arg-1 while downregulating the M1-specific marker CD86, accompanied by decreased expression of Corol1a and key glycolytic enzymes (G6PI, PFK1, and PKM2), suggesting inhibition of aerobic glycolysis in macrophages (Fig. 6C). Immunofluorescence staining corroborated these results, showing fewer M1

macrophages and more M2 macrophages in the renal interstitium following SKD administration (Fig. 6D, E), indicating that SKD modulates macrophage polarization within the renal microenvironment. ELISA assays revealed that SKD treatment significantly reduced pro-inflammatory cytokines (IL-1 β , IL-6, TNF- α) while increasing anti-inflammatory mediators (IL-1RA), consistent with a shift toward immune homeostasis. Moreover, the elevated lactic acid levels observed in model kidneys were markedly reduced after SKD intervention, providing functional evidence of suppressed glycolytic flux (Fig. S4A–E). These experimental results preliminarily indicate that SKD-mediated improvement of renal function in DN may be related to the regulation of the Coro1a/glycolysis axis in macrophages.

Effects of SKD serum on macrophage glycolysis in vitro

Given the protective effects of SKD observed in vivo, we next aimed to uncover the underlying molecular mechanisms using in vitro approaches. CCK-8 assays revealed that 2% SKD serum was the optimal concentration for subsequent experiments, as it exhibited no cytotoxicity while preserving strong biological activity (Fig. 7A). Metabolic profiling showed increased oxygen consumption rate (OCR), decreased extracellular acidification rate (ECAR), and lower lactic acid production after SKD serum treatment (Fig. 7B–D). In line with our in vivo observations, WB and IF assays confirmed that SKD serum effectively suppressed LPS+IFN- γ -induced M1 polarization, as evidenced by reduced CD86 expression and increased CD206 levels, along with downregulation of Coro1a and key glycolytic enzymes (G6PI, PFK1, PKM2) (Fig. 7E–G). ELISA showed lower levels of IL-1 β , IL-6, and TNF- α and a higher level of IL-1RA after SKD serum treatment (Fig. S4F–I). Collectively, these in vitro findings further provide robust functional evidence that SKD serum inhibits inflammation and M1 polarization via modulation of the Coro1a/glycolysis axis.

Computational docking and MD nominate Robinin and propose a Coro1a binding site

To prioritize the serum-absorbed constituents for experimental validation, we docked the candidate compounds against Coro1a. Robinin ranked highest among the tested

ligands, with a docking score of -10.9 kcal/mol, and was predicted to form hydrogen bonds with D183, W184, W139, G38, and V42 within the Corol1a binding pocket (Fig. 8A-G). This score serves as a relative ranking of the candidates and as a proposal of the binding site. It is not a quantitative binding free energy and was not used to infer affinity.

We next asked whether the predicted Robinin-Corol1a complex remains stable over a 100-ns MD trajectory. The ligand RMSD converged to approximately 0.12 nm, indicating that Robinin retained its docked pose, and hydrogen bonds between Robinin and Corol1a (1 to 11 contacts) persisted throughout the simulation (Fig. 9A, C). The radius of gyration ($R_g=2.23$ nm) and solvent-accessible surface area ($SASA=215$ nm²) remained essentially constant (Fig. 9D, E), indicating that the complex did not undergo large-scale unfolding. MM/PBSA estimated a favorable interaction, with Pro323, Asp36, and Asp183 contributing most (Fig. 9F, G). Consistent with the docking score, this value is interpreted as a relative, rather than quantitative, estimate of binding. Because docking scores and end-point free-energy methods overestimate affinities for charged, highly solvated sites, the computationally favorable ranking of Robinin was therefore validated experimentally, and the experimentally measured affinity, rather than the computational scores, is adopted throughout this study.

Robinin suppresses macrophage glycolysis and M1 polarization in vitro

The anti-inflammatory effectiveness of Robinin was investigated in vitro using RAW264.7 macrophages. Robinin exhibited no cytotoxicity at the tested concentrations (Fig. S5A), and dose-dependently suppressed the elevated glycolytic flux associated with M1 macrophage differentiation, accompanied by reduced pseudopodia formation and cytoskeletal remodeling. WB and IF results revealed a coordinated regulation of Corol1a expression with glycolytic activity (Figure 10A-C). In addition, the release of key pro-inflammatory cytokines (IL-6, IL-1 β , and TNF- α) and NO into the culture supernatant was markedly attenuated following Robinin treatment, as determined by ELISA and Griess assays (Fig. S5B-E). To better ascertain the effects of Robinin on macrophage metabolic phenotypes, we subsequently measured the ECAR, OCR, and lactic acid production in Robinin-treated macrophages. M1 polarization exhibited

enhanced glycolytic capacity and elevated lactic acid levels, whereas Robinin treatment reversed this metabolic shift in a dose-dependent manner (Fig. S5F-H).

Robinin inhibited LPS+IFN- γ -induced macrophage glycolytic reprogramming by targeting Coro1a

Raw264.7 cells with a plasmid containing Coro1a overexpression were transfected to clarify the relationship of Robinin and glycolysis. (Fig. S6A-C). In Coro1a-overexpressing cells, Robinin no longer reduced the expression of glycolytic rate-limiting enzymes (G6PI, PKM2, PFK1) or the levels of inflammatory cytokines in the supernatant upon LPS + IFN- γ stimulation. SEM and IF results demonstrated the Robinin failed to suppress pseudopodia formation and CD86 expression in macrophages. Collectively, these findings indicated that Coro1a overexpression reversed the anti-inflammatory effect of Robinin (Figure 11A-D).

In the meantime, we knocked down Coro1a using three specific shRNAs, among which Coro1a-sh1 exhibited the highest silencing efficiency (Figure S6D, E). In Coro1a-knockdown macrophages, LPS + IFN- γ stimulation failed to induce M1 polarization, as evidenced by blunted upregulation of G6PI, PKM2, and PFK1, reduced pseudopodia formation, and diminished inflammatory cytokine release. Under these conditions, the anti-inflammatory effect of Robinin was also largely abolished (Figure S7A-D). These gain- and loss-of-function experiments collectively demonstrate that Robinin exerts its anti-inflammatory activity through a Coro1a/glycolysis axis.

Coro1a D183 is the key amino acid that mediates the binding of Robinin

Prior to site-directed mutagenesis, CETSA was performed to evaluate the effect of Robinin on the thermal stability of Coro1a. The results demonstrated that Robinin significantly enhanced the thermal stability of Coro1a in a temperature-dependent manner. The MST fitting curve is "S" shaped, with obvious upper and lower plateaus and strong concentration dependence, indicating specific binding, with a K_D of 62.3 μ M (Figure 12A, B).

To identify the binding site of Robinin on Coro1a, and based on the molecular docking and dynamics simulation results, we hypothesized that D183 might be involved

in binding. Conservation analysis revealed that D183 is conserved across human, mouse, and rat Corol1a (Figure 12C, D). In silico mutagenesis of D183 to A183 and G183 using ChimeraX abolished the predicted interaction between Robinin and this residue (Figure 12E). The PoPMuSiC database suggested that mutation at the D183 site may markedly reduce the stability of the Corol1a protein (Figure 12F). Database analyses with DUET and DDMut revealed that substituting D183 with Ala (alanine) or Gly (glycine) leads to protein structural instability, which informs the selection of potential mutation sites (Table S4). On this basis, we mutated D183 into Ala (A) and Gly (G). Corresponding to a marked reduction in binding affinity relative to the wild-type protein, MST revealed that the D183A and D183G mutants increased the K_d to 382 μ M and 1125 μ M, respectively. CETSA further confirmed that Robinin failed to enhance the thermal stability of either mutant. Together, these results indicate that Asp183 contributes to the interaction between Robinin and Corol1a (Figure 12G, H).

Discussion

DN affects a substantial global population, yet effective treatment options remain limited [30]. Renal inflammation is not merely a consequence of DN but plays a pivotal role in driving disease progression [31, 32]. Cellular metabolic reprogramming and M1 macrophage polarization have emerged as key features underlying this inflammatory process [33]. Thus, targeting macrophage glycolytic reprogramming may therefore represent a promising therapeutic strategy against DN. In summary, the present study demonstrates that SKD exerts a protective effect against DN by suppressing the Corol1a/glycolysis axis, and identifies Robinin as a key bioactive constituent of SKD that binds to Corol1a at Asp183, thereby inhibiting macrophage glycolytic reprogramming and pro-inflammatory activation.

In db/db mice, SKD alleviated systemic metabolic disturbances, as evidenced by significant reductions in FBG, energy intake, and urine output. Furthermore, SKD improved key renal functional parameters, attenuated renal inflammatory cell infiltration, and restored the integrity of the glomerular filtration barrier. These findings indicate that SKD not only ameliorates systemic metabolic derangements but also preserves renal function in the setting of DN.

Multi-omics analysis revealed that DEMs mapped mainly to amino acid, carbohydrate, and lipid metabolism. MSEA further indicated suppression of glycolytic pathway activity following SKD treatment. Notably, glycolytic enrichment was consistently observed across the metabolomic, transcriptomic, and proteomic layers, while GSEA additionally demonstrated downregulation of ECM–receptor interaction signaling. ECM accumulation is a hallmark of renal fibrosis, a process driven largely by chronic inflammation [34]. Pro-inflammatory M1 macrophages exacerbate early renal injury by promoting ECM deposition and myofibroblast activation through the secretion of pro-inflammatory cytokines [35, 36]. Corroborating these findings, our immune infiltration analysis demonstrated that SKD significantly reduced M1 macrophage accumulation in the kidneys of db/db mice, providing a mechanistic basis for its anti-inflammatory properties. Furthermore, Corol1a expression was positively

correlated with M1 macrophage infiltration as well as with renal injury markers and pro-inflammatory cytokine levels. By integrating data from the GEO and Nephroseq database, we further confirmed that Corol1a is significantly upregulated in DN and is closely associated with declining renal function, suggesting its potential as a therapeutic target. Consistent with this notion, Mendelian randomization analysis has identified Corol1a as a druggable target in DN [37]. However, the causal relationship between Corol1a and aerobic glycolysis in macrophages has not been established.

Given that macrophages preferentially rely on glycolysis as their predominant energy metabolic pathway during M1 polarization [38], our data support a model in which SKD attenuates M1 macrophage polarization by targeting the Corol1a/glycolysis axis. In vivo, SKD suppressed M1 polarization of renal macrophages while promoting their shift toward the M2 phenotype. This phenotypic switch was accompanied by reduced levels of multiple pro-inflammatory cytokines in kidney tissue. Moreover, SKD decreased Corol1a expression and the activity of key glycolytic enzymes, ultimately leading to lower lactic acid production. In vitro, SKD-containing serum recapitulated these effects and effectively inhibited LPS + IFN- γ -induced M1 polarization. The above experimental results indicate that Corol1a may be a potential target for SKD to exert anti-inflammatory and renal protective effects.

Corol1a, a highly conserved actin-binding protein, plays a key role in regulating F-actin depolymerization, thereby influencing cell morphogenesis, migration, and intracellular transport. During macrophage polarization, cytoskeletal remodeling constitutes a critical step that drives phenotypic transition between distinct functional states. On the basis of these established roles, we hypothesize that Corol1a may modulate the inflammatory milieu in DN by regulating macrophage polarization and effector functions [39].

To further explore the function of Corol1a in macrophage glycolysis and elucidate the material basis responsible for the anti-inflammatory effect of SKD, we identified Robinin as an active component of SKD by molecular docking and MD. Robinin reduced Corol1a expression and glycolytic levels in macrophages, and Corol1a

knockdown or overexpression showed that Corol1a is required for M1 polarization and glycolysis. Furthermore, the anti-inflammatory activity of Robinin was shown to depend on its binding to Corol1a, with D183 identified as the critical binding residue. Firstly, our study provides the first evidence establishing the essential role of Corol1a in macrophage energy metabolism. Secondly, we identify D183 of Corol1a as a crucial functional residue for Robinin binding, laying an experimental foundation for the future development of Corol1a-targeted inhibitors. Finally, we propose that Robinin may serve as a promising lead compound, offering new therapeutic avenues for the development of anti-inflammatory agents.

Despite the systematic and comprehensive experiments performed in this study, some limitations remain to be addressed. The efficacy of the active Robinin should be further validated in vivo to provide additional evidence for the development of therapeutic agents against DN. The spatial expression pattern of Corol1a in clinical samples needs to be confirmed and the signal transduction linking Corol1a to glycolysis warrants further investigation.

Conclusions

In summary, our study demonstrates that SKD exerts renoprotective effects in DN through the inhibition of macrophage glycolysis. More importantly, we provide the first evidence that Corol1a regulates macrophage glycolytic metabolism, and identify Robinin as a bioactive natural compound capable of counteracting inflammation in a Corol1a-dependent manner. These findings offer a promising lead compound and a foundation for the development of natural product-based therapeutics against metabolic inflammatory diseases.

CRedit author statement

Lichao Wu: investigation, data curation, visualization, formal analysis, methodology, project administration, writing-original draft. Jiaqian Yao, Ai Mi & Renjie Li: investigation, data curation, formal analysis, visualization. Xueqian Peng, Lijian Wang & Yaping Fu: investigation, formal analysis, funding acquisition, supervision. Letian Shan, Christina Avanti, & Nanang Fakhruddin: supervision, writing-review & editing. Yanfang Yang, Wenhong Liu & Zhiwei Xu: funding acquisition, supervision, writing-review & editing. Hui Wang: conceptualization, funding acquisition, supervision and writing-review & editing. All authors agree to be accountable for all aspects of the work.

Declaration of Competing Interest

The authors declared there are no conflicts of interest for this study.

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Data Availability

Data will be made available on request.

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Figure legends

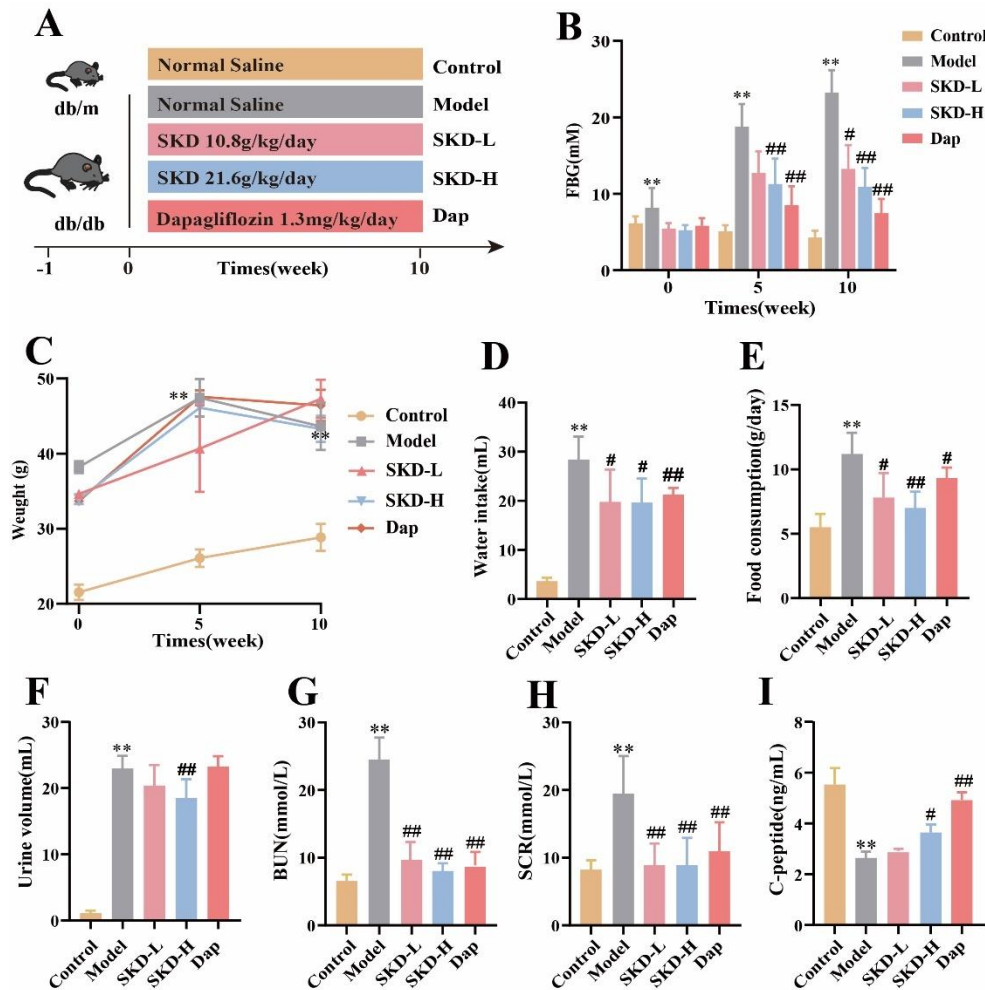


Fig.1. The effects of SKD on blood glucose, body weight, metabolism, and biochemical indicators in db/db mice. (A) Schematic diagram of the experimental design in mice. (B) The change of FBG in 10 weeks. (C) The change of body weight in 10 weeks. (D-F) Metabolic cage analyses about food intake, water consumption, and urine output. (G-I) BUN, SCR, C-peptide level in serum. Data were shown as the mean $\pm$ SEM. $N = 6$, * $p < 0.05$, ** $p < 0.01$ vs control group; # $p < 0.05$, ## $p < 0.01$ vs model group.

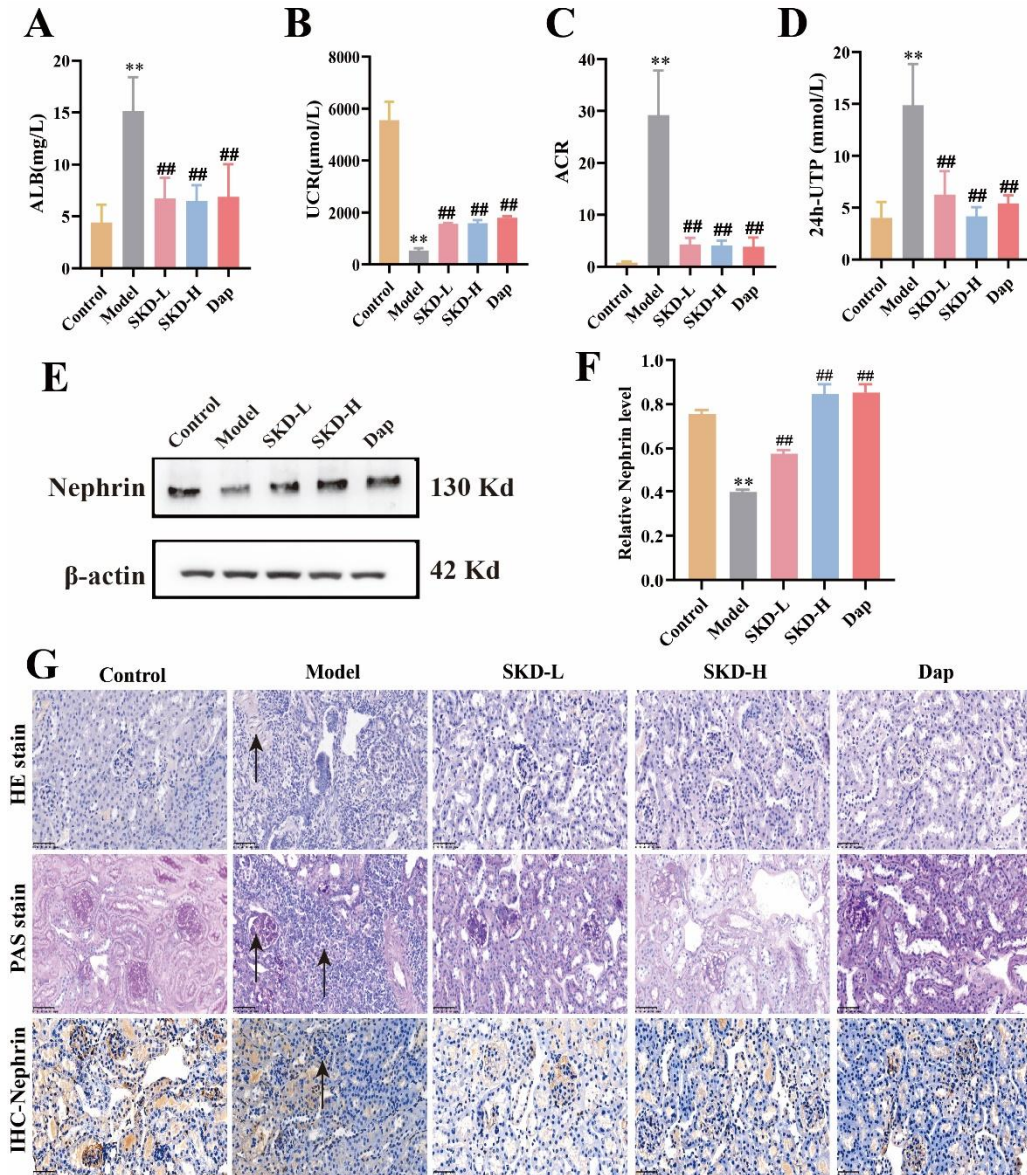


Fig.2. SKD intervention alleviated renal injury in db/db mice. (A-D) ALB, UCR, ACR and 24h-UTP in urine. (E) The relative protein level and quantitative analysis of Nephrin. (F) Representative kidney sections of HE, PAS staining; IHC of nephrin protein in renal tissue. Data were shown as the mean $\pm$ SEM. $N = 6$, * $p < 0.05$, ** $p < 0.01$ vs control group; # $p < 0.05$, ## $p < 0.01$ vs model group. Black arrow indicated the lesion site.

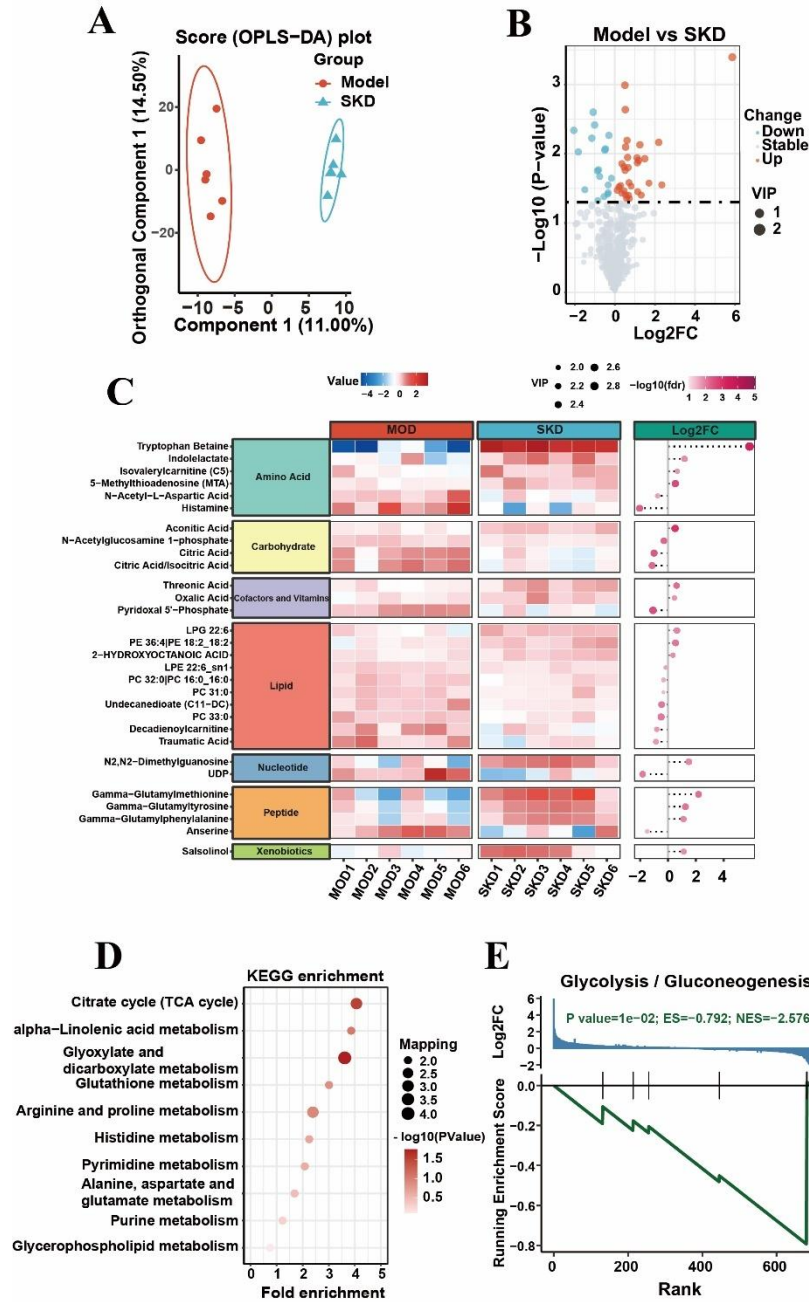


Fig.3. Effects of SKD treatment on the kidney metabolome. (A) The Score (OPLS-DA) plot of metabolomics between SKD and model groups. (B) Volcano plot of DEMs between SKD and model group. (C) The heatmap plot cluster of DEMs between SKD and model groups. (D) KEGG enrichment of DEMs between SKD and model. (E) MSEA analysis results of Glycolysis/Gluconeogenesis.

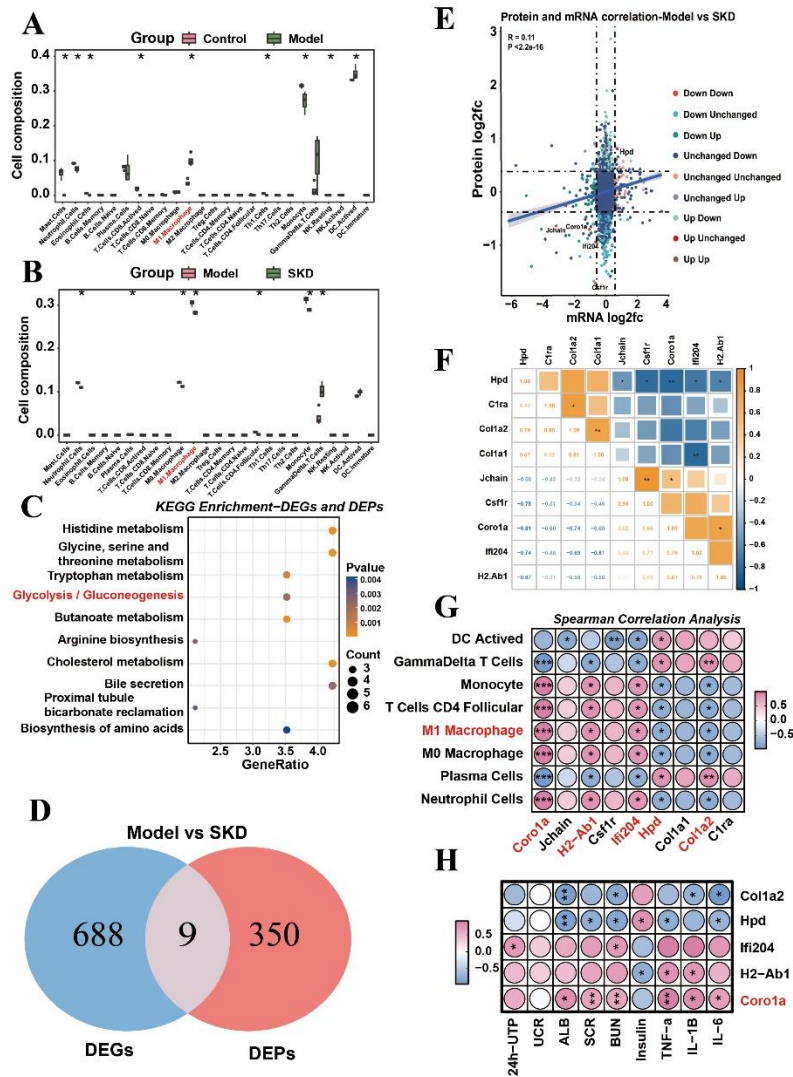


Fig.4. Immune cell infiltration and integrative analysis of transcriptomics, proteomics and metabolomics. (A) Boxplot of the proportion 25 types of immune cells in control and model groups. (B) Boxplot of the proportion 25 types of immune cells in SKD and model groups. (C) KEGG pathways in DEGs and DEPs. (D) Overlap mRNAs and Proteins between DEGs and DEPs. (E) The trend of changes in transcriptional and protein-level expression of homologous genes between the two groups of samples. (F) Correlation heatmap of nine genes. (G) Spearman's rank correlation analysis about immune cells and candidate genes. (H) Spearman's rank correlation analysis about Corro1a and renal injury indicators. Red shows a positive correlation and blue shows a negative correlation. The intensity of the color is directly proportional to the correlation coefficient.

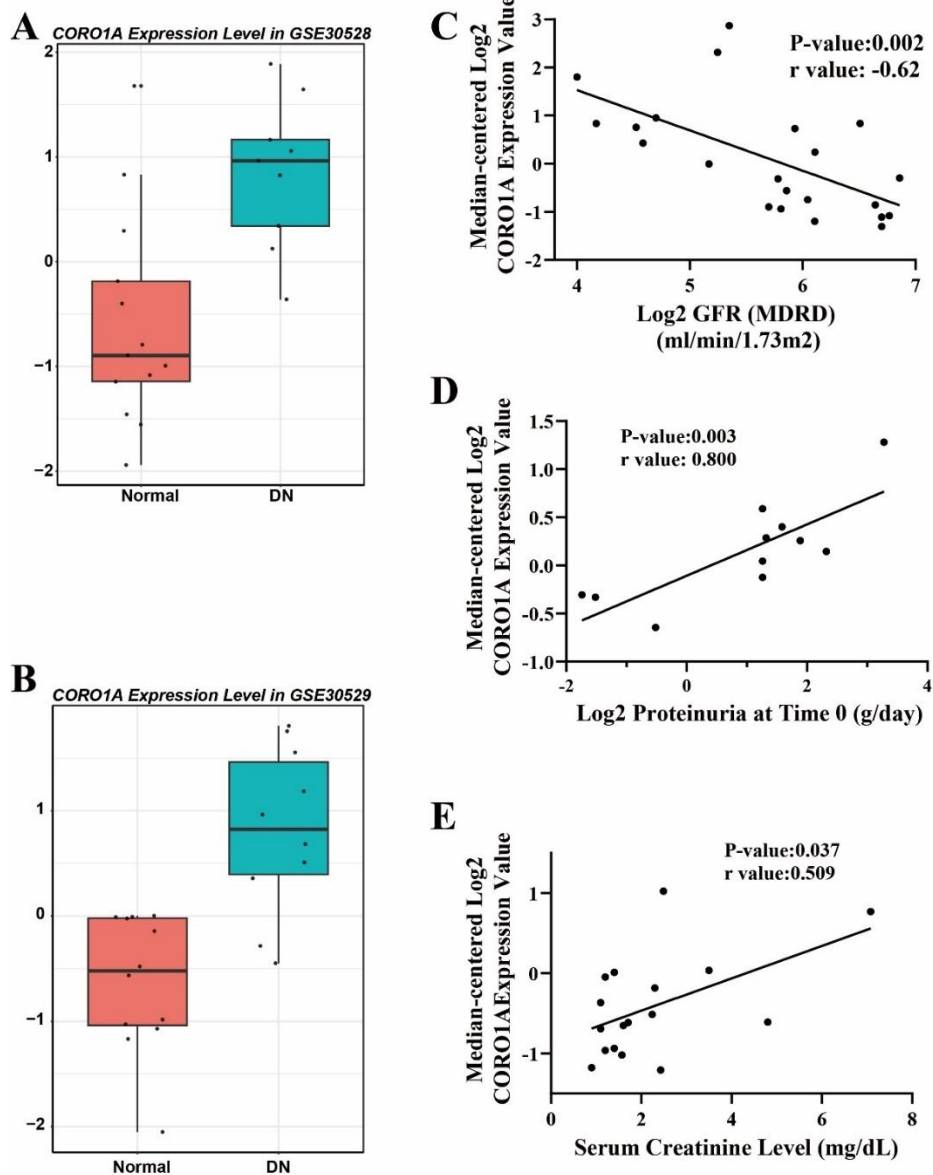
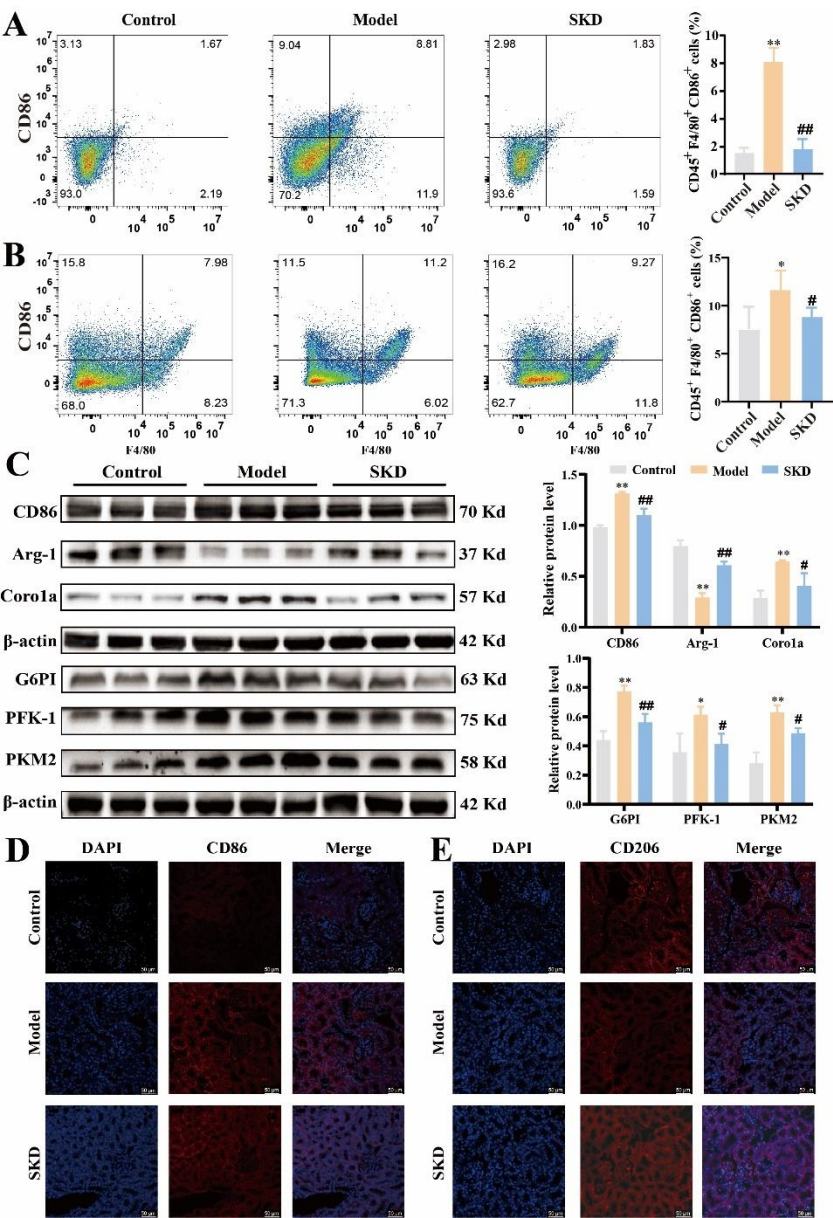


Fig.5. Bioinformatics analysis of the clinical relevance of Corol1a. (A) Boxplot of Corol1a expression in GSE30528. (B) Boxplot of Corol1a expression in GSE30529. (C-E) Correlation analysis of Corol1a with GFR, Proteinuria, and serum creatinine.



774 **Fig.6.** Effects of SKD treatment on the macrophage polarization, multiple cytokines,
775 and lactic acid in db/db mice. (A, B) Representative flow cytometry plots of the kidney
776 and spleen M1 macrophage populations, which were measured as CD86⁺ F4/80⁺ CD45⁺
777 live cell percentages. (C) Protein expression levels of CD86, Arg-1, Coro1a, G6PI,
778 PFK-1, and PKM2 in renal tissue. (D, E) Immunofluorescence staining of CD86 and
779 CD206 of kidney. Data were shown as the mean ± SEM. *N* = 3, **p*<0.05, ***p*<0.01 vs
780 control group; #*p*<0.05, ##*p*<0.01 vs model group.

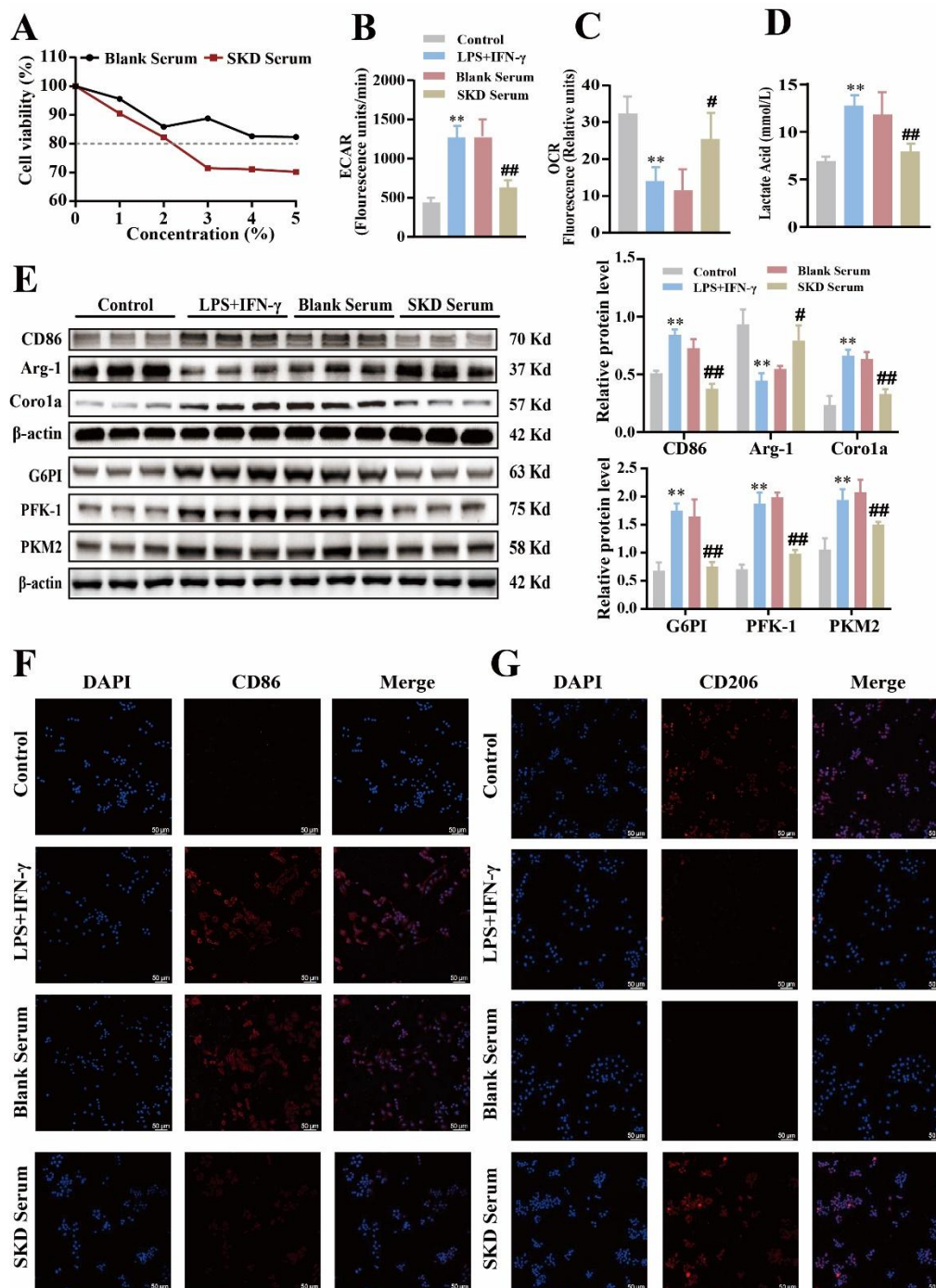


Fig.7. Anti-inflammation effects of SKD serum in RAW264.7 cell. (A) CCK-8 analysis results. (B-D) The OCR, ECAR, and lactic acid level in RAW264.7 cells. (E) Protein expression levels of CD86, Arg-1, Coro1a, G6PI, PFK-1, and PKM2 in cells. (F, G) Immunofluorescence staining of CD86 and CD206 in RAW264.7 cells. Data were shown as the mean $\pm$ SEM. $N=3$, * $p<0.05$, ** $p<0.01$ vs control group; # $p<0.05$, ## $p<0.01$ vs LPS+IFN- γ group.

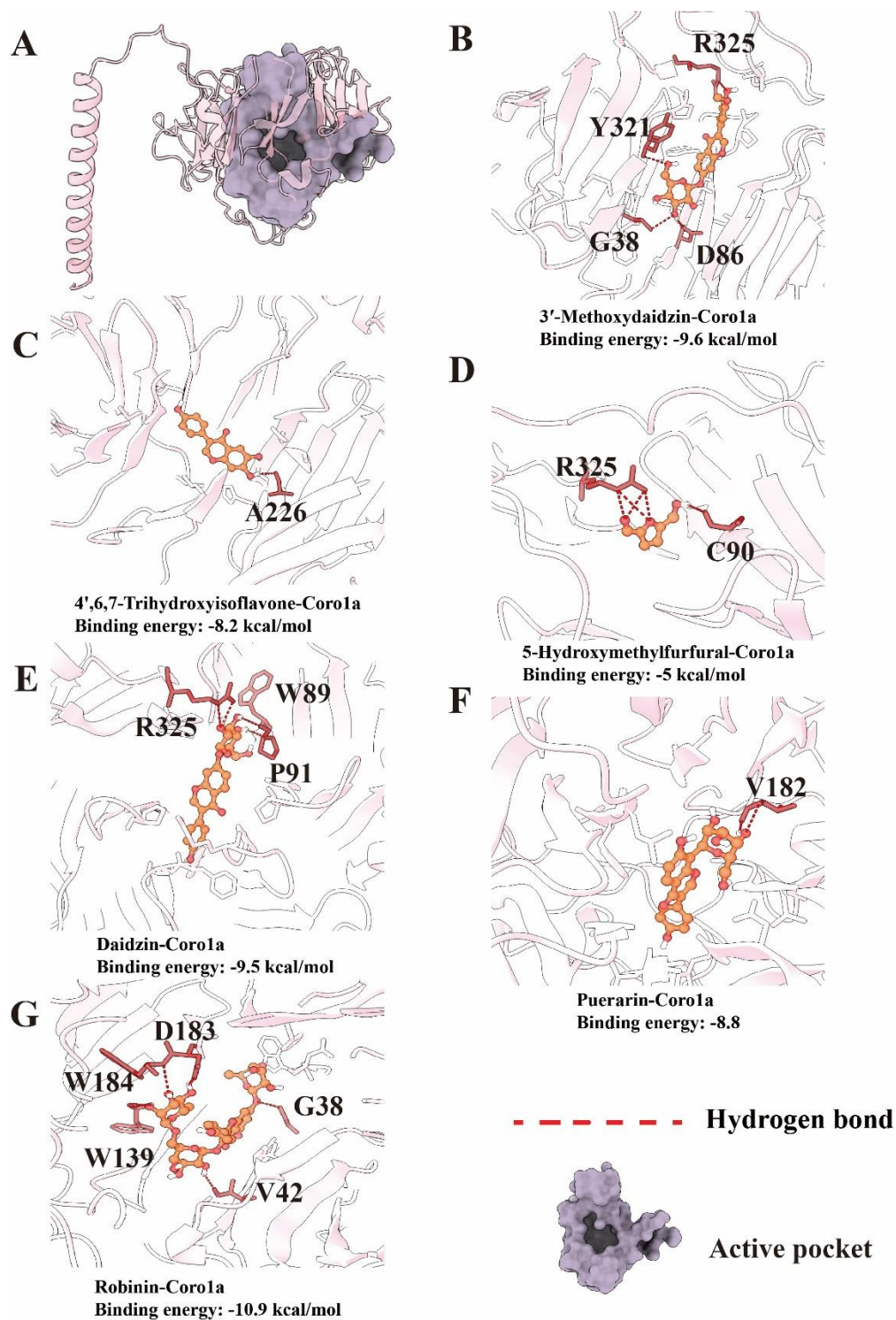


Fig.8. Molecular docking of six bioactive compounds with Corol1a. (A) Active pocket of Corol1a predicted by ProteinsPlus. (B-G) Binding Site maps with the lowest binding energies.

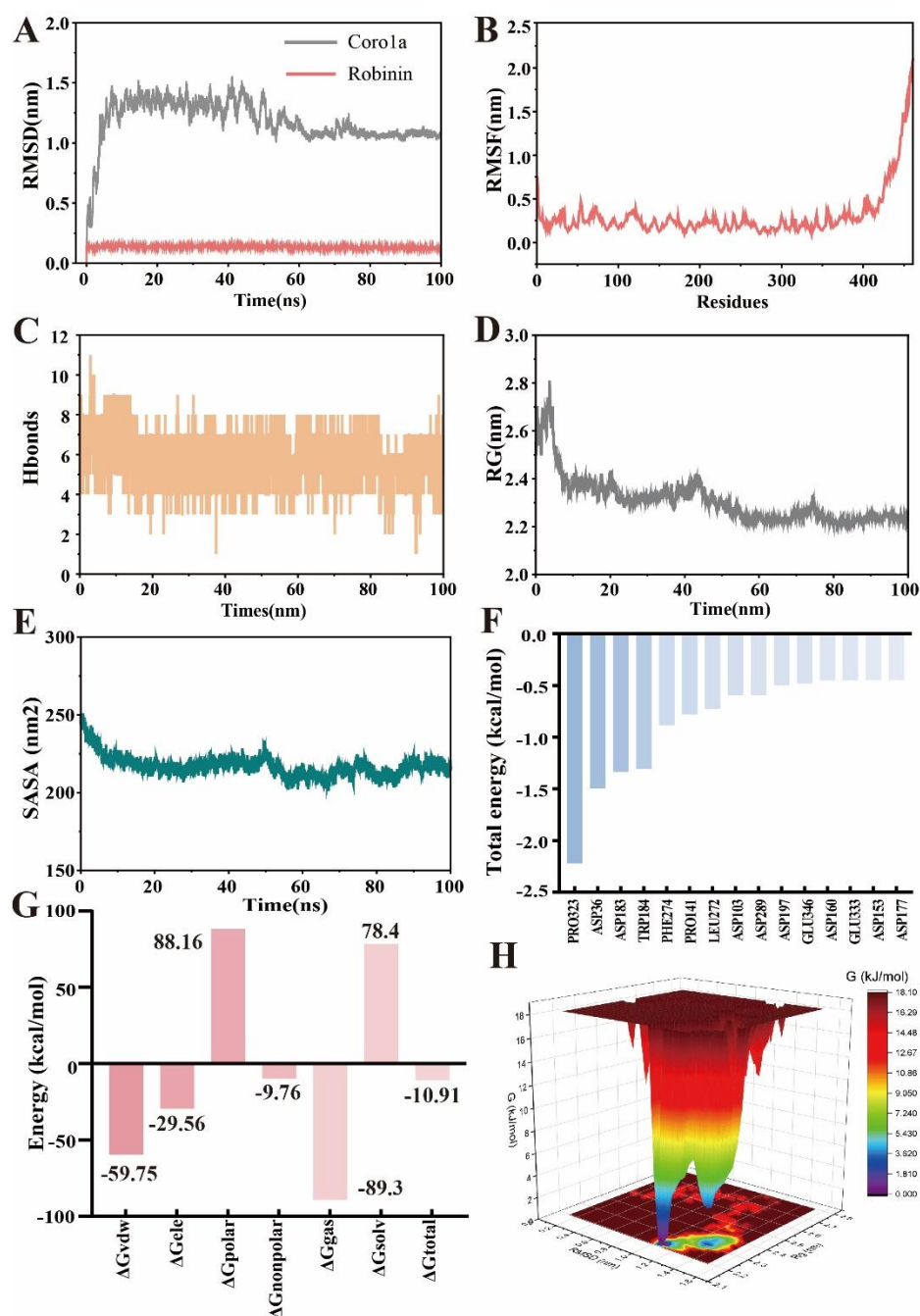


Fig.9. Molecular dynamics simulation of Robinin-Corola complex. (A, B) RMSD and RMSF value of Robinin-Corola complex. (C-E) Hbonds, RG, and SASA of Robinin-Corola complex. (F-H) Total energy and FEL of Robinin-Corola complex.

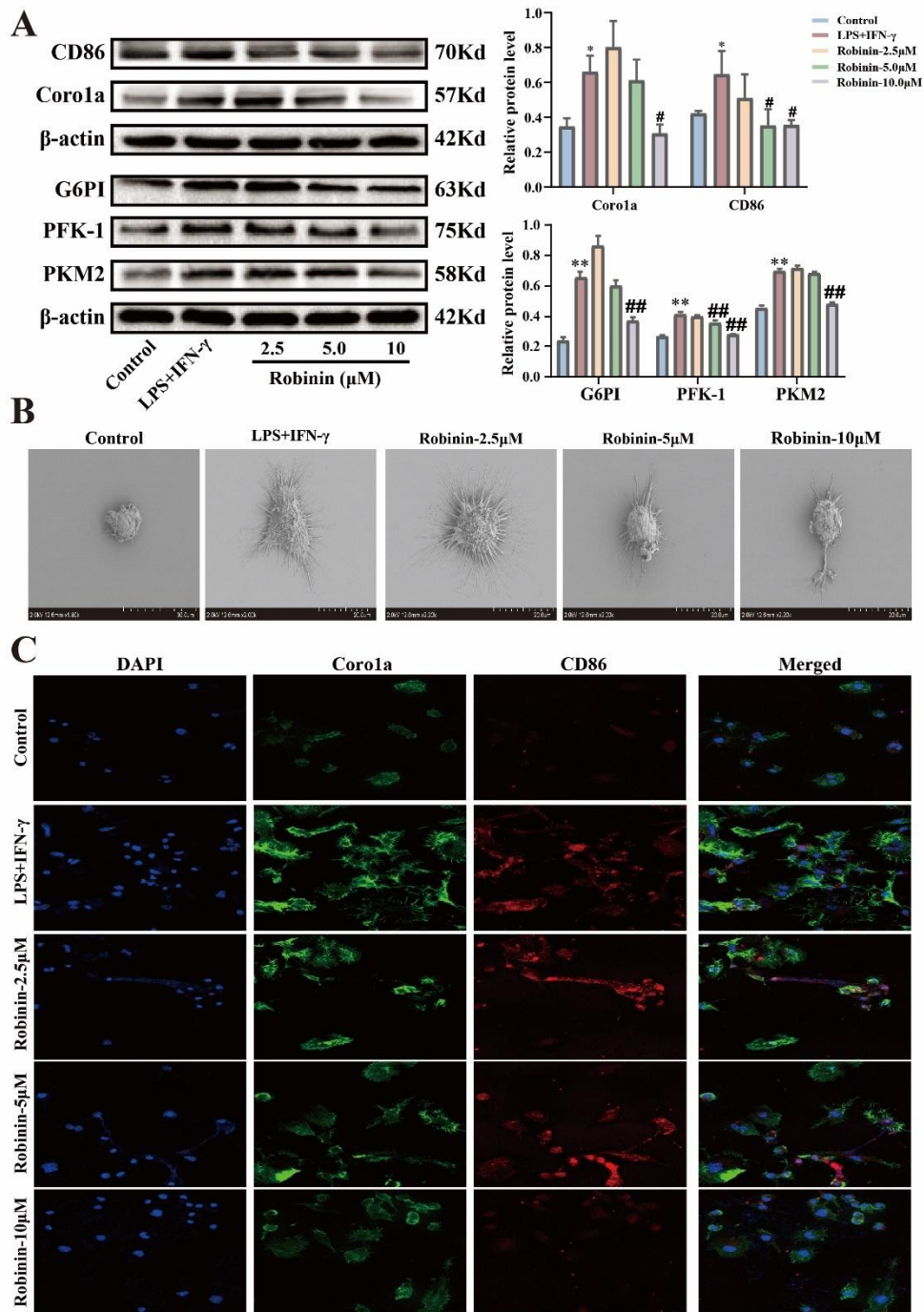


Fig.10. Anti-inflammation effects of Robinin in RAW264.7 cell. (A) Protein expression levels of CD86, Coro1a, G6PI, PFK-1, and PKM2 in cells. (B) SEM results of morphological changes in macrophages. (C) Immunofluorescence staining of CD86 and Coro1a in RAW264.7 cells. Data were shown as the mean $\pm$ SEM. $N = 3$, $^*p < 0.05$, $^{**}p < 0.01$ vs control group; $^{\#}p < 0.05$, $^{##}p < 0.01$ vs LPS+IFN- γ group.

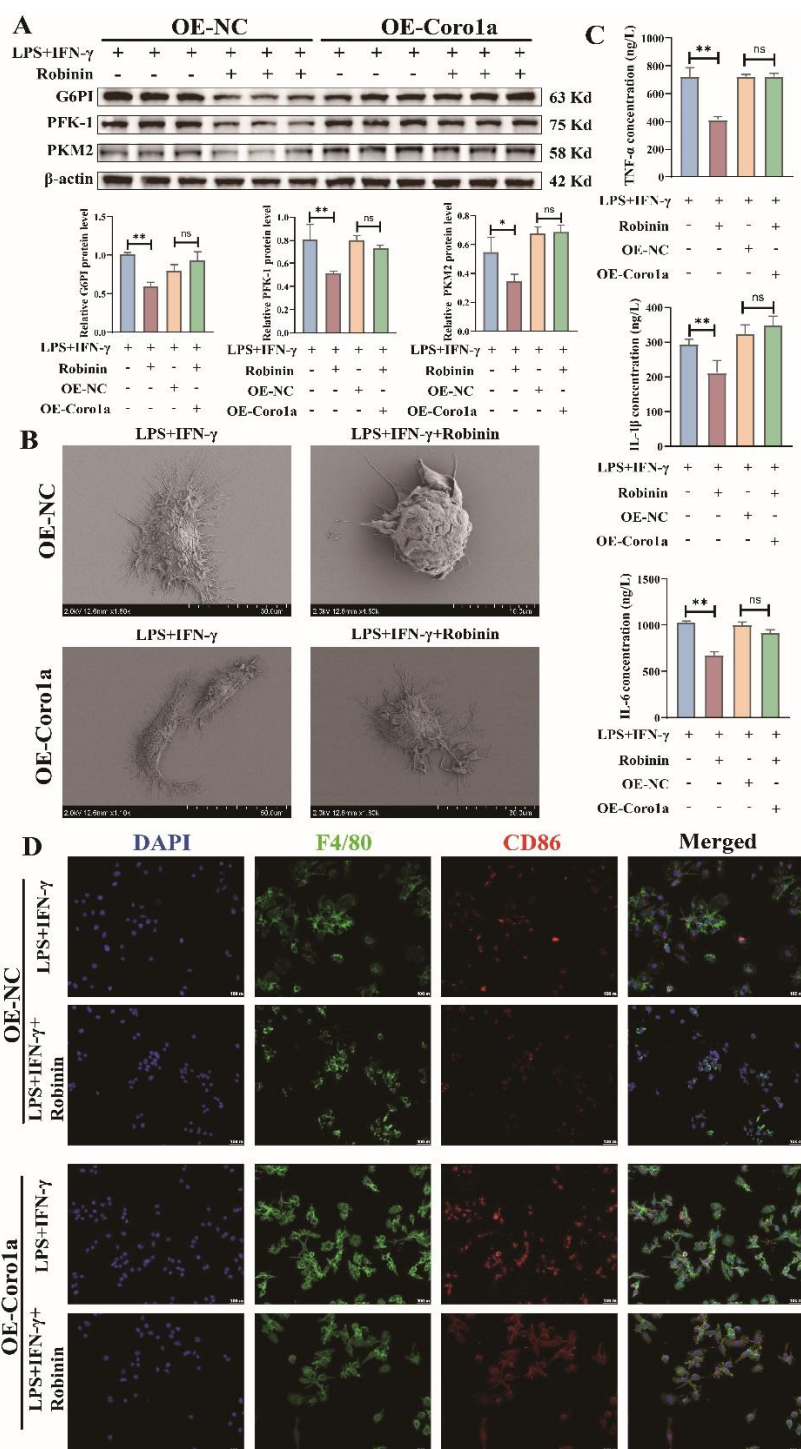
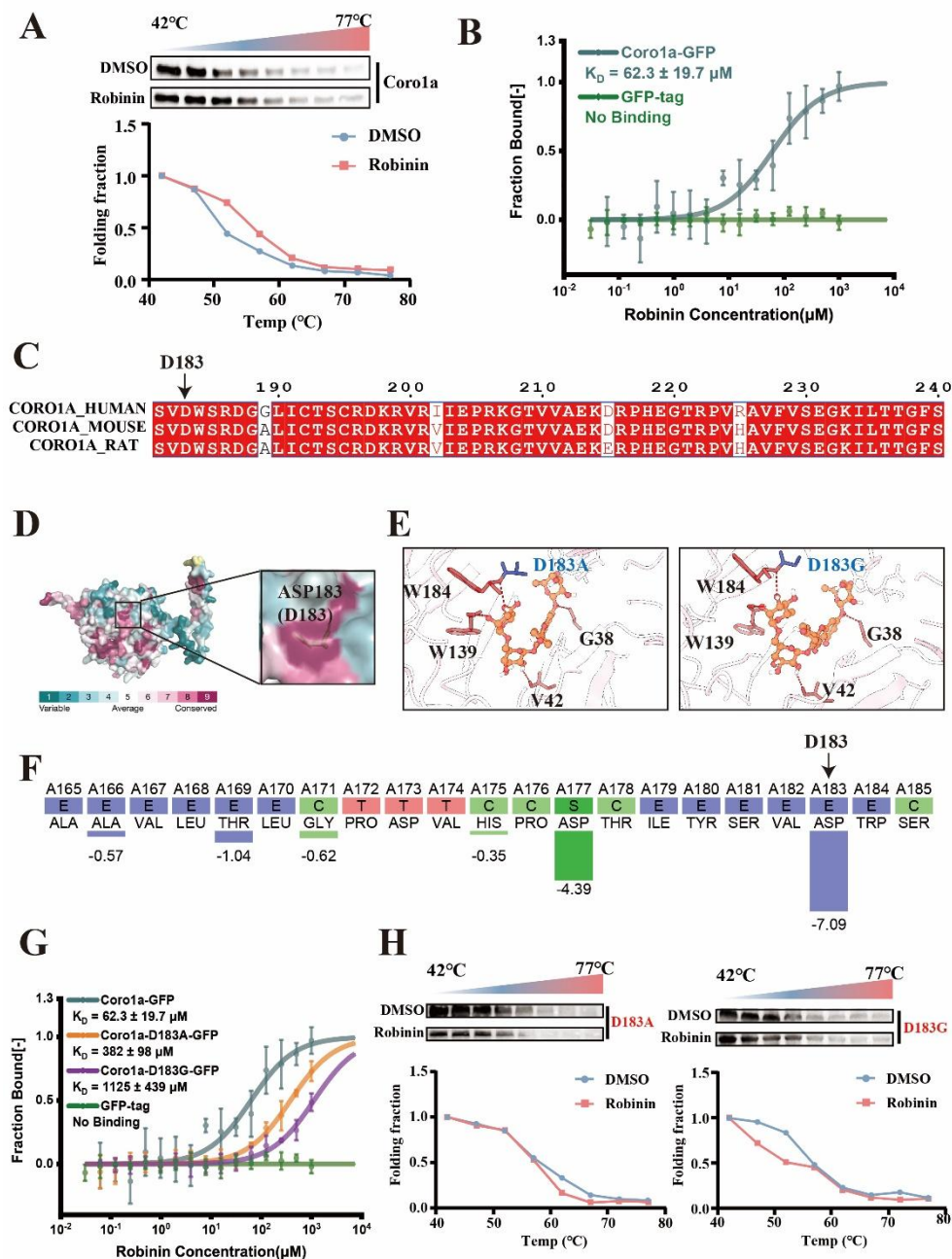


Fig.11. Robinin inhibited LPS+IFN- γ -induced macrophage glycolytic reprogramming by targeting Coro1a. (A) Protein expression levels of G6PI, PFK-1, and PKM2 in cells. (B) SEM results of morphological changes in macrophages. (C) The concentrations of IL-1 β , TNF- α , IL-6 in cell supernatant. (D) Immunofluorescence staining of F4/80 and CD86 in RAW264.7 cells. Data were shown as the mean $\pm$ SEM. $N = 3$, * $p < 0.05$, ** $p < 0.01$ vs LPS+IFN- γ group.



813

814 **Fig.12.** Robinin binds to D183 of Corol1a. (A, B) The CETSA and MST results of
815 Robinin and Corol1a. (C) Sequence conservation analysis of Corol1a protein using
816 ESPript 3.2. (D) Consurf analysis of Corol1a protein sequence conservation. (E)
817 Computer simulation results of point mutation. (F) Prediction results of mutation
818 stability using PoPMuSiC. (G, H) The CETSA and MST results of Robinin and Corol1a-
819 D183A, D183G.

Supplementary Files

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