

FAP α targeted liposomal delivery of Toosendanin enables G2/M phase arrest for effective rheumatoid arthritis therapy

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ABSTRACT: Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by persistent synovial inflammation and hyperplasia, which is predominantly driven by abnormal activation of fibroblast-like synoviocytes (RA-FLS). Current therapeutic strategies are frequently plagued by major limitations such as suboptimal efficacy and adverse effects. In the present study, a novel nanotherapeutic system (TSN-FLP) was developed by encapsulating Toosendanin (TSN) into liposomes capable of recognizing fibroblast activation protein- α (FAP α), enabling targeted delivery to RA-FLS. The therapeutic potential and its underlying mechanism of TSN-FLP was systematically investigated. The findings indicated that TSN-FLP notably inhibited the proliferation of RA-FLS by inducing cell cycle arrest at the G2/M phase. This was accompanied by downregulating cyclin-dependent kinase 1 (CDK1), which is involved in the crucial regulatory pathway of cell cycle progression. Furthermore, *in vivo* studies revealed that TSN-FLP administration effectively alleviated joint swelling, attenuated cartilage and bone erosion, and reduced the secretion of pro-inflammatory cytokines, as evidenced by histopathological analysis, micro-computed tomography (CT) imaging, and enzyme-linked immunosorbent assay (ELISA). Collectively, these preclinical findings provide compelling evidence that TSN-FLP exerts a potent therapeutic effect on RA while maintaining a favorable safety profile. Thus, TSN-FLP represents a promising candidate for the development of novel therapeutic interventions against rheumatoid arthritis.

KEYWORDS: rheumatoid arthritis, fibroblast-like synoviocytes, targeting delivery, Toosendanin

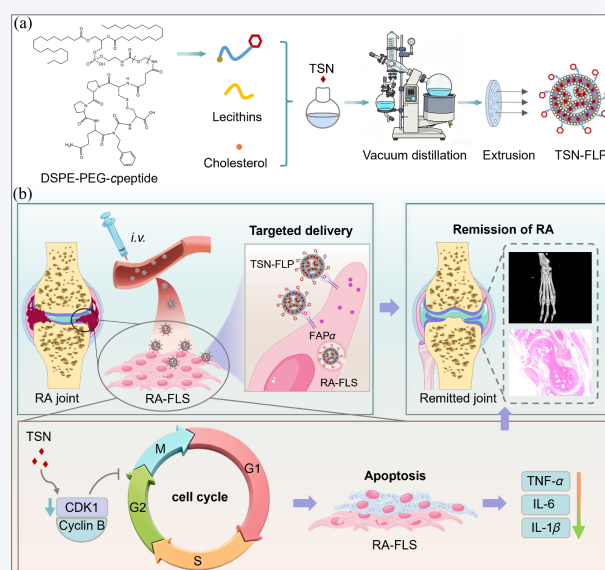
1 Introduction

Rheumatoid arthritis (RA) is a chronic, systemic autoimmune disease characterized by persistent synovial inflammation, which involves multiple immune cells, cytokines, and signaling pathways

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[1–3]. The complex pathological process of RA makes it challenging to eradicate, exerting a profound global burden [4]. Biologic disease-modifying antirheumatic drugs (DMARDs) represent the first-line therapeutic agents for RA management; however, their clinical efficacy is limited by suboptimal response rates and adverse events, necessitating the development of novel therapeutic approaches [5, 6]. Rheumatoid arthritis fibroblast-like synoviocytes (RA-FLS) are regarded as pivotal in the initiation and persistence of synovial inflammation and joint damage. These cells exhibit an aggressive, tumor-like phenotype that contributes significantly to pannus formation, ultimately leading to joint deformity and functional impairment [1, 7–10]. Consequently,

targeting RA-FLS and modulating their pathogenic manifestations have emerged as a prospective therapeutic approach for the management of RA [11, 12].

In recent years, numerous traditional natural compounds have exhibited remarkable anti-inflammatory, immunomodulatory, and anti-proliferative potential, attracting considerable attention in RA research [13–15]. A growing body of evidence indicates that certain natural compounds can directly modulate the aberrant activation and function of RA-FLS, thereby attenuating destructive processes within the joint microenvironment [16–19]. For instance, studies have shown that compounds such as curcumin, resveratrol, and epigallocatechin gallate (EGCG) can inhibit the proliferation, migration, and invasion of RA-FLS, as well as suppress the production of pro-inflammatory cytokines and matrix metalloproteinases (MMPs), which are critical for pannus formation and cartilage degradation [20, 21]. Toosendanin (TSN), the major active triterpenoid isolated from *Toosendan Fructus*, has been reported to exert potent anti-tumor and anti-inflammatory activities [22, 23]. However, its potential role in RA and the underlying mechanisms, particularly in regulating RA-FLS functions, remain largely unexplored. Given the similarities in the pathological processes of abnormal cell proliferation, migration, and invasion between tumor cells and RA-FLS, it is hypothesized that TSN may exert therapeutic effects on RA by modulating RA-FLS. In line with this notion, another unpublished study from our group demonstrated that TSN can effectively inhibit RA-FLS proliferation and ameliorate collagen-induced arthritis in mice. TSN is readily obtainable from natural sources, rendering it a promising candidate for clinical application. However, its clinical translation is largely hampered by several drawbacks, including poor water solubility, low bioavailability, and potential adverse effects [24, 25].

Functionalized liposomes (LPs), with their unique physicochemical properties, high surface-to-volume ratio, and flexibly tailorable surface modifications, exhibit tremendous potential in multiple biomedical application fields [26–30]. LPs not only possess excellent drug-loading capacity but also can achieve high-precision recognition and binding to specific cells or tissues through surface functionalization [31, 32]. Therefore, functionalized LPs have become important tools in fields such as drug delivery, molecular imaging, and vaccine delivery, with broad prospects for clinical translation [33–35]. Moreover, nanoscale therapeutics exhibit enhanced performance in drug delivery and intracellular redox catalytic activity, as validated by existing research [36, 37]. Based on these advantages, to overcome the limitations of TSN, a novel TSN-loaded functional liposome nano-system (TSN-FLP) has been designed to improve therapeutic efficacy and reduce the toxicity of TSN. A FAP α -targeting cyclic oligopeptide (cpeptide) was functionalized on the surface of TSN-loaded liposomes to mediate targeted delivery [38]. FAP α is a type II transmembrane serine protease that exhibits negligible expression in normal tissues but is significantly up-regulated in various pathological disorders, including fibrosis, arthritis, and cancer [8, 39]. In recent years, promising translational applications of FAP-targeted strategies have been explored in the field of diagnostic imaging [40–42]. In this study, the FAP α -targeting oligopeptide was innovatively conjugated to the terminal end of the 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-polyethylene glycol 2000 (DSPE-PEG2000) molecule in a circular form. The structure of the amphiphilic macromolecule thereby enables the cyclic oligopeptide display on the surface of FLP

through anchoring into the lipid membrane. Surface-modified PEG2000 is conducive to extending the blood circulation half-life of FLP. Notably, to the best of our knowledge, there have been no prior attempts to integrate this cyclic oligopeptide into liposomal drug delivery nano-system. By tracking the specific molecule expressed on the surface of RA-FLS, TSN-FLP is endowed with the capacity for precise targeted delivery of TSN (Scheme 1). The inhibitory effect and mechanism of TSN-FLP on RA-FLS were systematically investigated by *ex vivo* experiments, complemented by transcriptomic profiling and public database analyses. Furthermore, the biodistribution and therapeutic potential of TSN-FLP against RA were evaluated using synovial organoids and collagen-induced arthritis (CIA) mouse models. The present study aims to establish TSN-FLP as a promising nanopharmaceutical system for the treatment of RA, and to demonstrate that the modulation of RA-FLS serves as a pivotal therapeutic strategy for this disease.

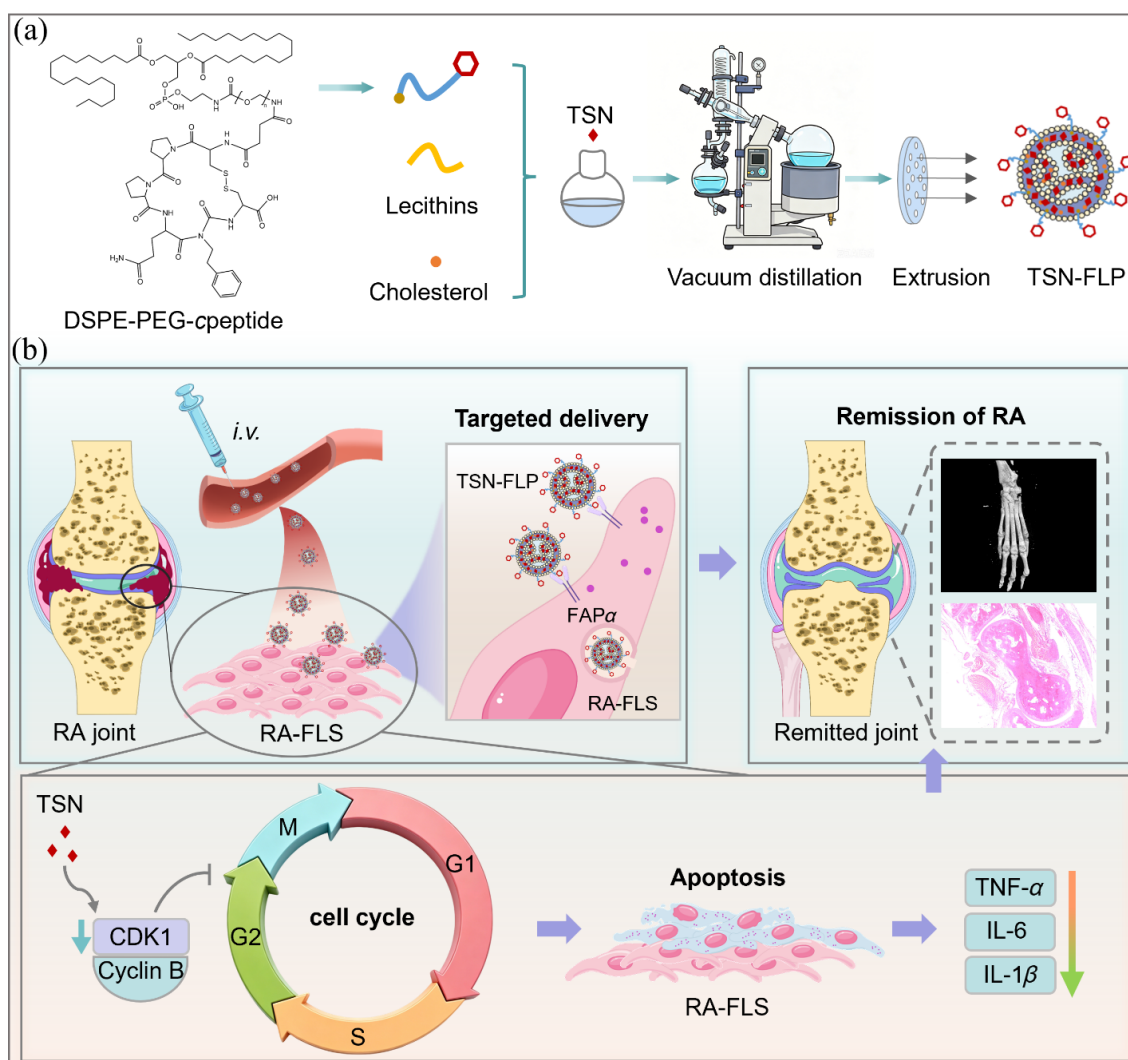
2 Experimental

2.1 Materials and reagents

DSPE-PEG2000-*N*-hydroxysuccinimide (NHS)-cpeptide (cysteine-proline-proline-glutamine-phenylalanine-cysteine, cCPPQFC) was customized from HongTide Biotechnology Co., Ltd. (Shanghai, China). Cholesterol and lecithin were procured from MedChemExpress (MCE, USA). Tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6), and interleukin-1 β (IL-1 β) enzyme-linked immunosorbent assay (ELISA) Kits were purchased from Multi Sciences Co., Ltd. (China). Toosendanin was acquired from Chengdu Push Bio-Technology Co., Ltd. (China). Dulbecco's modified Eagle medium (DMEM) and Roswell Park Memorial Institute 1640 (RPMI 1640) medium were obtained from Gibco (Thermo Fisher Scientific Inc., USA). Fetal bovine serum was purchased from ExCell Biotechnology Co., Ltd. (Suzhou, China). Transwell permeable supports (6.5 mm insert with 8.0 μ m polycarbonate membrane, 24-well plate) were purchased from Corning Incorporated (USA). Bovine type II collagen and complete Freund's adjuvant were acquired from Chondrex Inc. (USA). 3,3'-Diiodo-4,4'-dimethyldiphenylmethane perchlorate (DiO) and 1,1'-diiodo-4,4'-dimethyldiphenylmethane perchlorate (DiD) were purchased from MedChemExpress Inc. (USA). The Matrigel matrix was acquired from Corning Inc. (USA).

2.2 Preparation of TSN-FLP

Before use, the cCPPQFC-conjugated DSPE-PEG2000-NHS was characterized using high-performance liquid chromatography (HPLC) and mass spectrometry (MS) to confirm its purity and molecular weight. For the preparation of liposomes encapsulating TSN, the thin-film hydration technique was adopted. Specifically, cholesterol, lecithin, TSN, and the DSPE-PEG2000-NHS-cCPPQFC conjugate were dissolved in ethanol at a mass ratio of 5:1:1:0.1. Thereafter, the ethanol was evaporated at 35 °C under vacuum to form a thin film on the inner walls of a round-bottom flask. The film was subsequently hydrated with phosphate-buffered saline (PBS, pH 7.4) by gentle agitation of the container. The resultant mixture was extruded through a liposome extruder fitted with a polycarbonate membrane with a pore size of 200 nm. Finally, the LPs were purified via dialysis against PBS to remove any unincorporated substances. The blank liposome was prepared using



Scheme 1 Construction of TSN-FLP and inhibition mechanisms of synovial inflammation in rheumatoid arthritis. (a) Schematic diagram of the preparation of TSN-FLP. (b) Schematic diagram of TSN-FLP-mediated FLS targeting and therapy for RA. *i.v.*: intravenous injection.

the same process without the addition of TSN and DSPE-PEG2000-NHS-cCPPQFC.

2.3 Characterization of the properties of TSN-FLP

The size distribution and zeta potential of TSN-FLP were evaluated using dynamic light scattering (DLS). The drug encapsulation efficiency was determined by ultraviolet spectrophotometry. The morphological characteristics of TSN-FLP were further visualized by transmission electron microscopy (TEM). The stability of TSN-FLP was evaluated by incubating it in serum and PBS at 37 °C for 5 days, after which it was analyzed using DLS. The long-term stability for storage and transportation was examined by storing the sample at 4 °C for 20 days, followed by DLS analysis.

2.4 Cell culture for *ex vivo* experiments

For cell culture experiments, all reagents were of analytical grade and were used as received without further purification. RA-FLS were isolated from synovial tissues of RA patients following institutional ethical approval (No. 2024-176) granted by the Research Ethics Committee of Peking University Shenzhen Hospital, with written informed consent obtained in compliance with the Helsinki Declaration protocols. The cells were identified by

Vimentin staining and maintained in DMEM medium supplemented with 10% fetal bovine serum, 100 units·mL⁻¹ Penicillin, and 100 μ g·mL⁻¹ Streptomycin at 37 °C in a humidified atmosphere containing 5% CO₂. The HepG2 cells and THP-1 cells were purchased from the Shanghai Cell Bank of the Chinese Academy of Sciences and maintained in DMEM and RPMI 1640 medium under the same conditions as described above.

2.5 Cell uptake of TSN-FLP

RA-FLS were inoculated in 12-well plates and incubated in complete medium for at least 12 h. Subsequently, DiO-labeled TSN-FLP (equivalent TSN concentration of 100 ng·mL⁻¹) was introduced into the medium. The cells were cultured for 3 h, then washed with PBS and stained with DiD. Finally, the samples were observed under confocal laser scanning microscopy (CLSM).

2.6 Investigation of the *ex vivo* targeted delivery of TSN-FLP

RA-FLS and HepG2 cells were seeded in 6-well plates and allowed to adhere overnight. Subsequently, the culture medium was replaced with fresh medium containing DiO-labeled TSN-FLP

(equivalent TSN concentration of 100 ng·mL⁻¹) or non-targeting liposome formulations (equal amount to TSN-FLP), respectively. After incubation for 3 h, the cells were harvested, washed with PBS, and then analyzed by flow cytometry.

2.7 Detection of the tissue penetration capacity of TSN-FLP using an *ex vivo* three-dimensional synovial-like tissue (synovial organoid)

Synovial organoid was established by culturing RA-FLS in a three-dimensional Matrigel matrix. This model mimics the *in vivo* synovial environment, allowing for a more accurate assessment of tissue penetration. After the synovial organoid was formed, DiO-labeled TSN-FLP (equivalent TSN concentration of 100 ng·mL⁻¹) was introduced into the medium. The penetration depth and distribution of the formulations within the tissue were then evaluated by CLSM after a 6 h-incubation, providing insights into the tissue-penetrating ability of TSN-FLP.

2.8 Cell migration and proliferation assay

For the wound healing assay, RA-FLS were seeded in 12-well plates at a density of 5×10^4 cells per well and allowed to adhere overnight. A cross-shaped scratch was made in the confluent cell monolayer using a sterile pipette tip. After washing away the detached cells, fresh medium containing TSN-FLP (equivalent TSN concentration of 100 ng·mL⁻¹), FLP (equal volume to TSN-FLP stock solution), TSN (100 ng·mL⁻¹), or saline (equal volume to TSN-FLP stock solution) was added to each well for different experimental groups. The migration of RA-FLS towards the scratch area was monitored and photographed at specific time points (0, 24, and 48 h) under an inverted microscope.

For the transwell migration assay, RA-FLS were seeded in transwell supports at a density of 8×10^3 cells per well and allowed to adhere overnight. Fresh medium containing TSN-FLP (equivalent TSN concentration of 100 ng·mL⁻¹), FLP (equal volume to TSN-FLP stock solution), TSN (100 ng·mL⁻¹), or saline (equal volume to TSN-FLP stock solution) was added to the 24-well plate for different experimental groups before the transwell supports were transferred into the plate. After incubation for 48 h, the transwell supports were washed and stained with crystal violet before being observed under an inverted microscope.

For the EdU proliferation assay, RA-FLS were seeded in a 96-well plate at a density of 5×10^3 cells per well. Cells were divided into 4 groups (TSN-FLP, FLP, TSN, and saline) and cultured with medium containing TSN-FLP (equivalent TSN concentration of 100 ng·mL⁻¹), FLP (equal volume to TSN-FLP stock solution), TSN (100 ng·mL⁻¹), or saline (equal volume to TSN-FLP stock solution). After incubation for 48 h, the medium was removed, and EdU solution was added to each well for another 2 h to label the proliferating cells. Subsequently, the cells were fixed and stained according to the manufacturer's instructions of the EdU assay kit. The cells were observed under a fluorescence microscope.

For cell apoptosis analysis, RA-FLS were seeded in 6-well plates (3×10^5 cells per well) and cultured overnight. The cells were treated with medium containing TSN-FLP (equivalent TSN concentration of 100 ng·mL⁻¹), FLP (equal volume to TSN-FLP stock solution), TSN (100 ng·mL⁻¹), or saline (equal volume to TSN-FLP stock solution) for 24 h. Then, cell apoptosis was detected by flow cytometry using an Annexin V-FITC/PI apoptosis detection kit according to the manufacturer's protocol.

For live/dead staining, RA-FLS were incubated in 12-well plates

with 1×10^5 cells per well. After culture in media containing TSN-FLP (equivalent TSN concentration of 100 ng·mL⁻¹), FLP (equal volume to TSN-FLP stock solution), TSN (100 ng·mL⁻¹), or saline (equal volume to TSN-FLP stock solution), the cells were then treated with a staining solution containing Calcein-AM and propidium iodide (PI) for 30 min at 37 °C in the dark. After incubation, the cells were washed with PBS to remove excess stain. The stained cells were observed under a fluorescence microscope.

For the cell counting kit-8 (CCK-8) assay, RA-FLS were seeded in 96-well plates at a density of 5×10^3 cells per well. After group-based treatment with medium containing TSN-FLP (equivalent TSN concentration of 100 ng·mL⁻¹), FLP (equal volume to TSN-FLP stock solution), TSN (100 ng·mL⁻¹), or saline (equal volume to TSN-FLP stock solution) for 48 h, cell viability was assessed using CCK-8 assay according to the manufacturer's instructions.

2.9 Macrophage chemotaxis assay

THP-1 cells were seeded in transwell supports at a density of 1×10^4 cells per well and allowed to induce differentiation by medium containing phorbol myristate acetate (PMA, 100 ng·mL⁻¹) for 24 h. RA-FLS were incubated in 24-well plates with 2×10^4 cells per well with medium containing TSN-FLP (equivalent TSN concentration of 100 ng·mL⁻¹), FLP (equal volume to TSN-FLP stock solution), TSN (100 ng·mL⁻¹), or saline (equal volume to TSN-FLP stock solution) for different experimental groups for 24 h before the transwell supports were transferred into the plate. After incubation for 24 h, the transwells were washed and stained with crystal violet before being observed under an inverted microscope.

2.10 Transcriptome analyses

For transcriptome analyses, RA-FLS were seeded in 6-well plates and treated with TSN-FLP (equivalent TSN concentration of 100 ng·mL⁻¹) for 48 h. After the treatment, total RNA was extracted from the cells using an RNA extraction kit according to the manufacturer's instructions. The quantity of the extracted RNA was assessed by spectrophotometry. Then, the RNA samples were subjected to transcriptome sequencing. The sequencing data were analyzed using Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) pathway enrichment analyses to identify differentially expressed genes between the TSN-FLP-treated group and the control group.

2.11 Western blotting

Western blotting was conducted to ascertain whether the key molecules in the pathways responsible for cell cycle arrest were affected by TSN-FLP. The cells treated with TSN-FLP (equivalent TSN concentration of 100 ng·mL⁻¹) for 48 h were washed, lysed, and collected by centrifugation at 10,000g for 10 min. The total protein content of each group was determined using the bicinchoninic acid (BCA) assay. Subsequently, the proteins were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred onto a polyvinylidene difluoride (PVDF) membrane. After blocking in a Tris-buffered saline with Tween 20 (TBST) buffer containing 5% bovine serum albumin (BSA), the membrane was incubated overnight at 4 °C with primary antibodies against cyclin-dependent kinase 1 (CDK1), CDK2, CDK4, extracellular signal-regulated kinase (ERK), pERK, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), followed by incubation with the

secondary antibody for 2 h at room temperature. Finally, the membrane was visualized using the Clarity Western enhanced chemiluminescence (ECL) substrate and imaged using a gel documentation system (ChemiDoc MP, Bio-Rad, USA).

2.12 Animals

Male 8- to 10-week-old DBA/1J mice were purchased from Shanghai Slaccas Laboratory Animal Technology Co., Ltd. Experiments were approved by the Experimental Animal Ethics Committee of Peking University Shenzhen Hospital (No. 2024-461) and were conducted in accordance with the Guide for the Care and Use of Laboratory Animals.

2.13 Establishment of the CIA mouse model

The CIA model was established by two immunizations with bovine type II collagen emulsified in complete Freund's adjuvant. On day 0, mice were injected intradermally at the base of the tail with 100 µg of type II collagen dissolved in 0.1 mL of complete Freund's adjuvant. On day 21, a booster immunization was given with the same amount of mixture. The mice were monitored daily for signs of arthritis, such as joint swelling and redness, and the arthritis index was scored according to a standard scoring system.

2.14 Biodistribution of TSN-FLP

TSN-FLP and non-targeting liposomes were pre-labeled with DiO before intravenous injection into CIA mice. Fluorescence imaging was performed using a PerkinElmer IVIS Spectrum system at various time points from 0 to 24 h.

2.15 Evaluation of therapeutic effects

Mouse models were randomly divided into five groups: TSN-FLP, FLP, TSN, MTX (methotrexate), and CIA ($n = 5$). TSN-FLP was administered via intravenous injection at a TSN dose of 2 mg·kg⁻¹. For the FLP group, FLP was intravenously administered at an equivalent amount with TSN-FLP. TSN and MTX were administered via intraperitoneal injection at the doses of 2 and 23 mg·kg⁻¹, respectively. All drugs were administered three times per week. The treatment was continued for 21 days, and the arthritis index in each group was continuously recorded. A group of healthy mice (named Healthy) was kept during the period without administration and was used as a healthy control group. After the observation period, micro-computed tomography (CT), histological analysis, and immunohistochemical analysis were performed on the joint tissues. The blood was collected to analyze cytokines (tumor necrosis factor- α (TNF- α), IL-6, and IL-1 β) and blood biochemistry parameters, including alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transferase (γ -GT), blood urea nitrogen (BUN), and creatinine (Cr). Sections of heart, liver, spleen, lung, and kidney were then stained with hematoxylin and eosin (H&E) to observe pathological changes.

2.16 Data analysis

Quantitative data presented in this study are expressed as the mean $\pm$ standard error of mean (s.e.m.). Statistical analysis between two groups was performed using two-sided unpaired t-tests. For multiple comparisons, one-way ANOVA with Tukey's multiple comparisons test was applied. A probability value of less than 0.05 ($P < 0.05$) was regarded as statistically significant. Graphical analyses were conducted using GraphPad Prism 9 software. Data in

the graphs generated by GraphPad software are presented as mean $\pm$ S.E.M.

3 Results

3.1 Preparation and characterization of TSN-FLP

In this study, the compound DSPE-PEG2000-cpeptide was designed to equip the targeting cyclic oligopeptide onto the surface of nanoliposomes. The DSPE-PEG2000-cpeptide was custom-synthesized and characterized by MS. The molecular weight of the peptide determined to be 691.83 (Fig. S1 in the Electronic Supplementary Material (ESM)). The TSN-FLP was prepared via the film dispersion method, and an overview of the preparation process was presented in Fig. 1(a). The structure of TSN-FLP was observed under TEM, and the image in Fig. 1(b) showed the morphology of the nanoparticles. The TSN-FLP exhibited the characteristics of multichambered liposomes, which facilitate carrying more hydrophobic drug according to their larger specific surface area of membrane. The effective drug loading was determined to be $12.2\% \pm 0.6\%$ while the encapsulation efficiency was $84.5\% \pm 4.5\%$. The particle size of TSN-FLP was homogenized to 197.8 ± 2.8 nm (polydispersity index (PDI) = 0.099) after passing through the polycarbonate film with 200 nm-pores (Fig. 1(c) and Fig. S2 in the ESM). The zeta potential had been slightly altered (from -3.1 to -2.8 mV) after TSN was added to the membrane (Fig. 1(d)). The ultraviolet (UV)-absorption spectrum of TSN-FLP is shown in Fig. 1(e). The stability of TSN-FLP was detected by simulating the characteristics of both *in vivo* and *in vitro* environments. As shown in Figs. 1(f) and 1(g), TSN-FLP could stably keep in size of ~ 200 nm under 37 °C in PBS (pH 7.4) and serum, indicating that the prepared TSN-FLP can maintain its structural integrity during systemic circulation until it reaches the synovium. Furthermore, the TSN-FLP remained stable for up to 20 days when stored in PBS (pH 7.4) at 4 °C, suggesting that the formulation possesses favorable long-term storage and transportation stability.

3.2 The cell-interaction and biodistribution of TSN-FLP

The cyclic oligopeptide (cCPPQFC) on the particle surface was designed to direct nanoliposomes to the RA-FLS located in RA synovium via recognition of FAP α (Fig. 2(a)). In the *ex vivo* study, compared with non-targeting nanoliposomes, a greater quantity of TSN-FLP ($6.7\% \pm 0.5\%$ vs. $0.3\% \pm 0.1\%$, $P < 0.0001$) was internalized by RA-FLS after incubation for 3 h (Fig. 2(b)). The internalization of TSN-FLP exhibited a time-dependent pattern within 3 h (from $0.7\% \pm 0.04\%$ to $6.7\% \pm 0.5\%$) (Fig. 2(c) and Fig. S3 in the ESM). Furthermore, CLSM images demonstrated that FLS derived from RA patients could quickly internalize FLP within 3 h. Additionally, FLP was mainly localized in the cytoplasm of RA-FLS after internalization, which provided a favorable intracellular environment for subsequent drug release and biological effects (Fig. 2(d)). To further explore the selectivity of this enhanced internalization, competitive assays were performed by comparing RA-FLS with HepG2 cells that lack FAP expression. The results in Figs. 2(e) and 2(f) revealed that the uptake of FLP by RA-FLS was significantly larger than that by HepG2 cells ($15.4\% \pm 0.6\%$ vs. $0.4\% \pm 0.1\%$, $P = 0.0021$) within 3 h, suggesting that the targeting effect is mediated by specific interactions between the cyclic peptide and FAP on the surface of RA-FLS. To detect whether TSN-FLP could

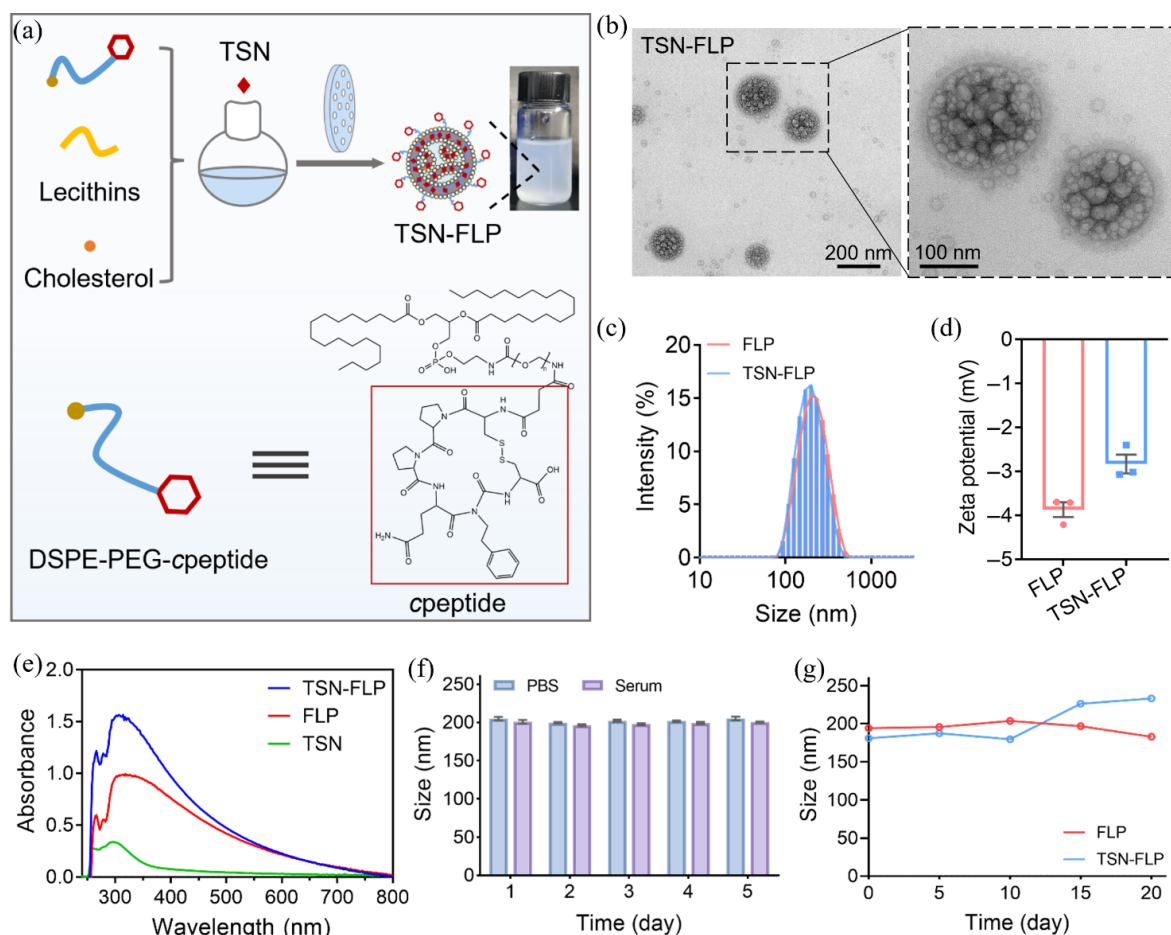


Figure 1 Synthesis and characterization of TSN-FLP. (a) Schematic diagram of the preparation of TSN-FLP. (b) TEM images of TSN-FLP. (c) The hydrated particle size of TSN-FLP was measured by DLS. (d) Zeta potential of TSN-FLP. (e) Absorption spectrum of TSN-FLP in 50% ethanol. (f) Hydrated particle size of TSN-FLP in PBS and fetal bovine serum (FBS) at 37 °C for 5 days ($n = 3$). (g) Hydrated particle size of TSN-FLP in PBS at 4 °C for 20 days ($n = 3$). Data are presented as mean $\pm$ s.e.m. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparisons test.

penetrate the abnormally hyperplastic synovial tissue, a 3D *in vitro* synovial tissue model was employed. As shown in Fig. 2(g), the CLSM image illustrated that DiO-labeled TSN-FLP was distributed throughout the 3D tissue (the maximum diameter was approximately 200 μm), indicating that TSN-FLP can penetrate multiple layers of RA-FLS for drug delivery. To evaluate the *in vivo* targeting property of TSN-FLP, biodistribution in the CIA mouse model was investigated after confirming the interaction between TSN-FLP and RA-FLS. Figure 2(h) shows that TSN-FLP significantly accumulated in RA joints and remained for more than 24 h. Furthermore, the *ex vivo* fluorescence images of organs and tissues presented in Fig. 2(i) indicated that TSN-FLP was concentrated in RA joints 24 h post-injection rather than the organs. Overall, these findings collectively indicate that the cpeptide-modified nanoliposomes (TSN-FLP) possessed excellent targeting ability and efficient cellular internalization toward RA-FLS, laying a solid foundation for their potential application in the targeted therapy of rheumatoid arthritis.

3.3 Suppression of *ex vivo* RA-FLS by TSN-FLP

To evaluate whether TSN-FLP could prevent the migration of RA-FLS, a wound healing assay was conducted by incubating RA-FLS in medium containing TSN-FLP for 48 h. The images in Fig. 3(a) illustrate that the migration distance of RA-FLS treated with TSN-

FLP was significantly shorter than that of the Saline group and the FLP group. Further quantitative analysis of the migration rate also confirmed that TSN-FLP treatment led to a significant decrease in the migratory activity of RA-FLS compared to the Saline group ($18.1\% \pm 2.0\%$ vs. $86.0\% \pm 6.4\%$, $P < 0.0001$) after 48 h (Fig. S4 in the ESM), indicating that the liposomal TSN presents an ideal inhibitory effect on RA-FLS migration. Moreover, transwell migration assays using RA-FLS showed that TSN-FLP suppressed cell migration more effectively than the other treatments, as it more strongly prevented the cells from penetrating the polycarbonate membrane (Figs. 3(b) and 3(c)). These results clearly demonstrated that TSN-FLP could effectively inhibit the migration ability of RA-FLS, which is crucial for reducing the invasive growth and joint destruction caused by activated RA-FLS in rheumatoid arthritis. To further explore whether the proliferative activity of RA-FLS was suppressed, EdU staining was performed after RA-FLS had been incubated in TSN-FLP for 48 h. The results in Figs. 3(d) and 3(e) showed that the number of EdU-positive cells in the TSN-FLP group was significantly lower than that in the Saline group ($0.3\% \pm 0.5\%$ vs. $28.1\% \pm 3.7\%$, $P < 0.0001$), suggesting that TSN-FLP could effectively inhibit the proliferation of RA-FLS. Cell apoptosis assay was subsequently applied to evaluate the impact of TSN-FLP on RA-FLS survival. The results in Figs. 3(f) and 3(g), and Fig. S5 in the ESM showed that the apoptotic rate in the TSN-FLP group was

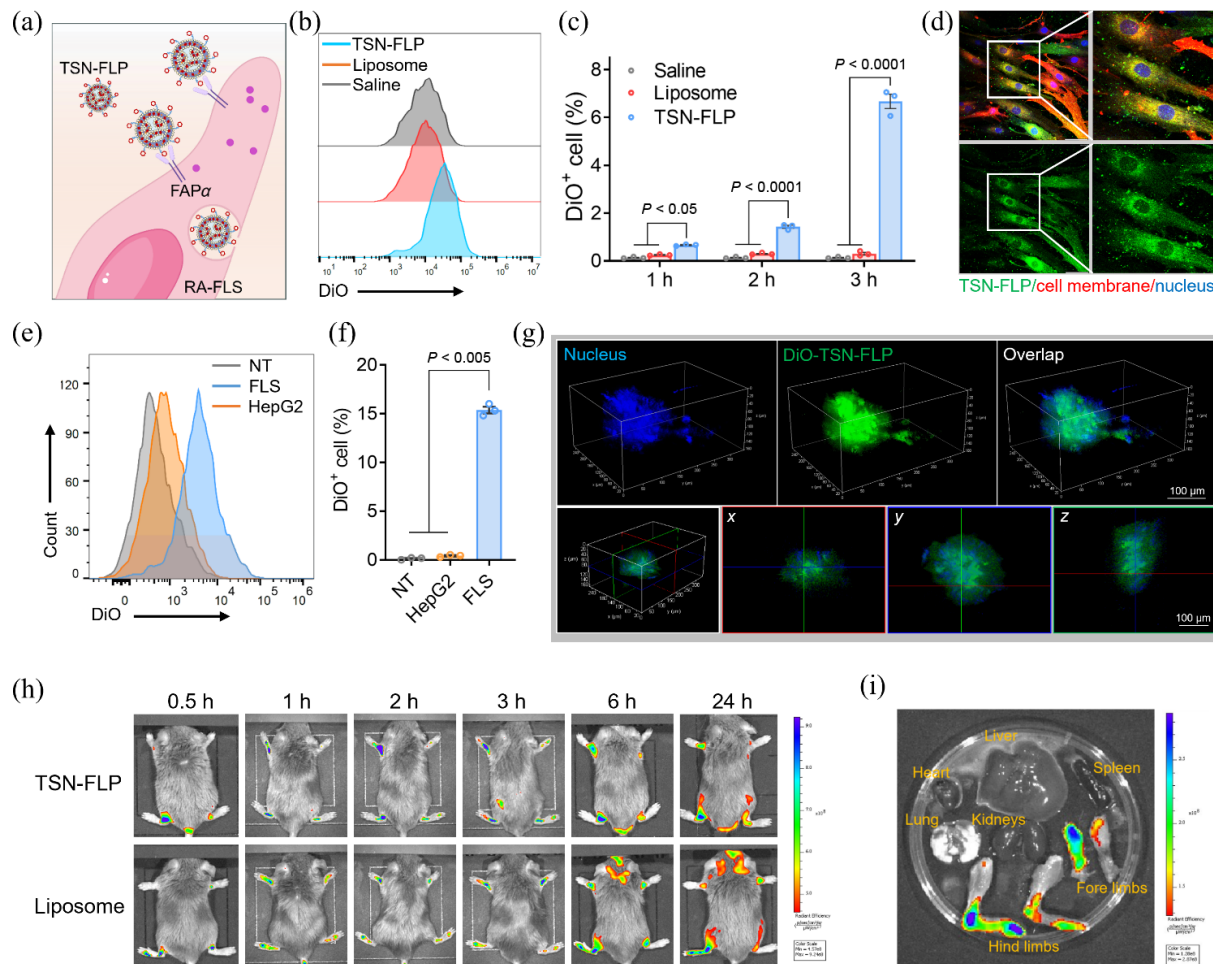


Figure 2 *In vitro* targeting and *in vivo* biodistribution of TSN-FLP. (a) Schematic diagram of the targeted delivery of TSN-FLP. (b) Flow cytometry analysis and (c) quantitative analysis of the cellular uptake of DiO-TSN-FLP and DiO-liposome in RA-FLS after incubation for 3 h. (d) Representative confocal images of DiO-TSN-FLP (green) colocalized with cell membrane (red) after 1 h of co-incubation at 37 °C (scale bars: 20 μm). (e) Flow cytometry analysis and (f) quantitative analysis of the cellular uptake of DiO-TSN-FLP by RA-FLS and HepG2 cells after incubation for 3 h. (g) Representative confocal images of DiO-TSN-FLP (green) infiltrating into 3D synovial organoids (scale bars: 100 μm). (h) *In vivo* biodistribution of DiO-TSN-FLP and DiO-liposome in CIA mice imaged under the IVIS system during 24 h. (i) IVIS images of the organs and limbs derived from CIA mice after administration of DiO-TSN-FLP and DiO-liposome for 24 h. Data are presented as mean ± s.e.m. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparisons test.

markedly higher compared to the Saline group ($40.3\% \pm 4.9\%$ vs. $2.4\% \pm 0.1\%$, $P < 0.0001$), as evidenced by increased Annexin V-FITC/PI double-positive staining. These findings indicated that TSN-FLP not only inhibited cell proliferation but also promoted the apoptosis of RA-FLS, further contributing to reduction in living cell numbers. This effect was further visualized by Calcein/PI staining (Fig. 3(h)), which showed that a large number of RA-FLS were dead, as evidenced by PI staining of leaked nuclear DNA. Moreover, the cell viability measured by CCK-8 assay showed a significant decrease in the TSN-FLP-treated group compared to the Saline group and TSN group (Fig. 3(i)). Specifically, at the same drug concentration of the TSN group and the TSN-FLP group, the cell viability dropped from $55.7\% \pm 9.1\%$ to $37.4\% \pm 5.3\%$ ($P = 0.0013$), indicating that TSN-FLP can effectively deliver TSN into RA-FLS, which is important for suppressing the cell growth and survival of RA-FLS. To further explore whether suppression of RA-FLS by TSN-FLP reduces inflammatory cell recruitment, a macrophage chemotaxis assay was performed. As shown in Figs. 3(j) and 3(k), RA-FLS pre-incubated with TSN-FLP displayed the least chemotactic attraction toward THP-1 cells compared to the other groups. These data collectively demonstrate that targeted

delivery of TSN effectively suppresses RA-FLS migration and proliferation, promotes apoptosis, and reduces their chemotactic attraction toward macrophages. These findings provide a solid basis for subsequent in-depth investigations.

3.4 TSN-FLP arrested the cell cycle by down-regulating CDK1

To further investigate the possible mechanism by which TSN-FLP influences RA-FLS activity, transcriptome sequencing was performed after the cells were treated with TSN-FLP for 48 h. Compared with the Saline group, 1157 upregulated genes and 1077 down-regulated genes were observed in the TSN-FLP-treated group (Fig. 4(a)). KEGG pathway enrichment analysis of the down-regulated genes showed that TSN-FLP-treated RA-FLS were enriched in pathways associated with cell cycle regulation and DNA replication (Fig. 4(b)). Similarly, GO analysis demonstrated that TSN-FLP primarily affects cell cycle arrest (Fig. 4(c)). To further examine the influence on the cell cycle, the RA-FLS were subjected to flow cytometry analysis after a 48-h incubation with TSN-FLP. As shown in Fig. 4(d), compared with the Saline group, the

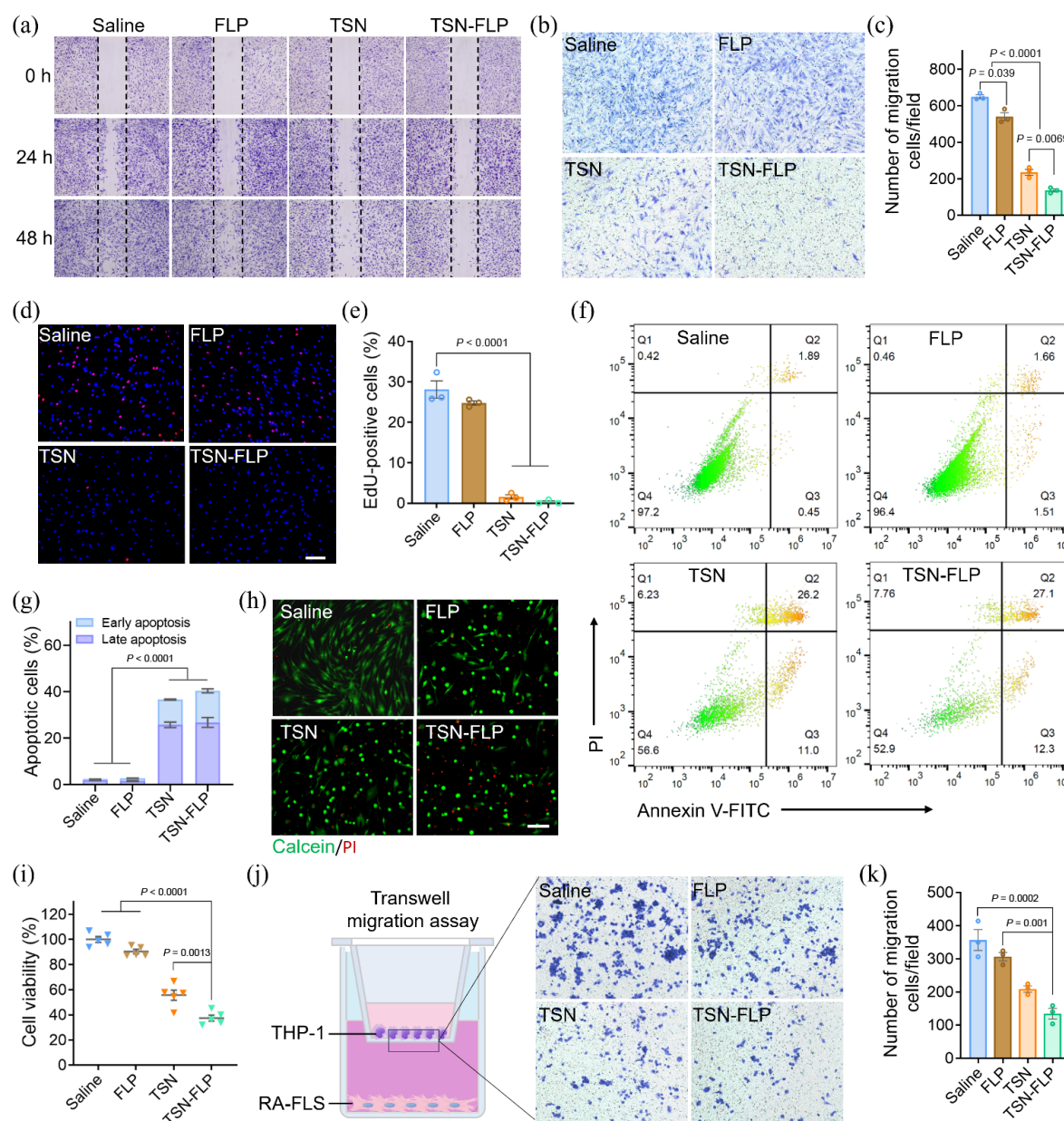


Figure 3 The inhibitory effects of TSN-FLP on *ex vivo* RA-FLS. (a) Representative images of RA-FLS migration evaluated by wound healing assay at 0, 24, and 48 h after incubation with TSN-FLP, TSN, FLP, or saline. (b) Representative images of RA-FLS migration evaluated by transwell assay at 0, 24, and 48 h after incubation with TSN-FLP, TSN, FLP, or saline. (c) Quantification of migrating RA-FLS from three fields of view per group. (d) Representative images of EdU assays assessing DNA replication in each group of RA-FLS (scale bar: 100 μ m). (e) Semi-quantitative analysis of EdU-positive cells. (f) Analysis of RA-FLS apoptosis by flow cytometry after treatment with TSN-FLP, TSN, FLP, or saline for 24 h. (g) Quantification of apoptotic cells identified by flow cytometry analysis. (h) Fluorescent images of RA-FLS stained with a live/dead Kit after being treated with TSN-FLP, TSN, FLP, or saline for 48 h (scale bar: 100 μ m). (i) Cell viability of RA-FLS after incubation with TSN-FLP, TSN, FLP, or saline for 48 h. (j) The scheme of the transwell migration assay of THP-1 cells and the representative images. (k) Quantification of migrating THP-1 cells from three fields of view per group. The concentrations used were as follows: TSN-FLP: 100 ng·mL⁻¹ of TSN; TSN: 100 ng·mL⁻¹; FLP: an equivalent amount to TSN-FLP; saline: an equivalent volume to TSN-FLP stock solution. Data are presented as mean $\pm$ s.e.m. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparisons test.

percentage of cells in G2 phase was significantly increased after treatment with TSN-FLP (11.9% $\pm$ 0.1% vs. 17.8% $\pm$ 0.1%). This finding suggests that TSN-FLP treatment induces G2 cell cycle arrest in RA-FLS, thereby inhibiting their abnormal proliferation.

Such cell cycle modulation could be one of the key mechanisms underlying the therapeutic effect of TSN-FLP in RA, as the arrest at the G2 phase prevents the cells from entering mitosis and subsequently induces cell apoptosis. Further validation using Western blot confirmed that TSN-FLP treatment led to a marked

decrease of CDK1 (Fig. 4(e)), which mediates the transition of cells from G2 phase to M phase, thereby inducing G2 phase cell cycle arrest in RA-FLS. Quantitative real-time PCR (qRT-PCR) analysis demonstrated that TSN-FLP significantly suppressed CDK1 gene transcription, confirming that the reduction of CDK1 occurs through downregulated expression (Fig. S6 in the ESM). In contrast, the expression of CDK2 and CDK4, which drive cells from G1 phase to S phase, remained constant after being treated with TSN-FLP (Fig. 4(f)). This trend is consistent with the cell cycle

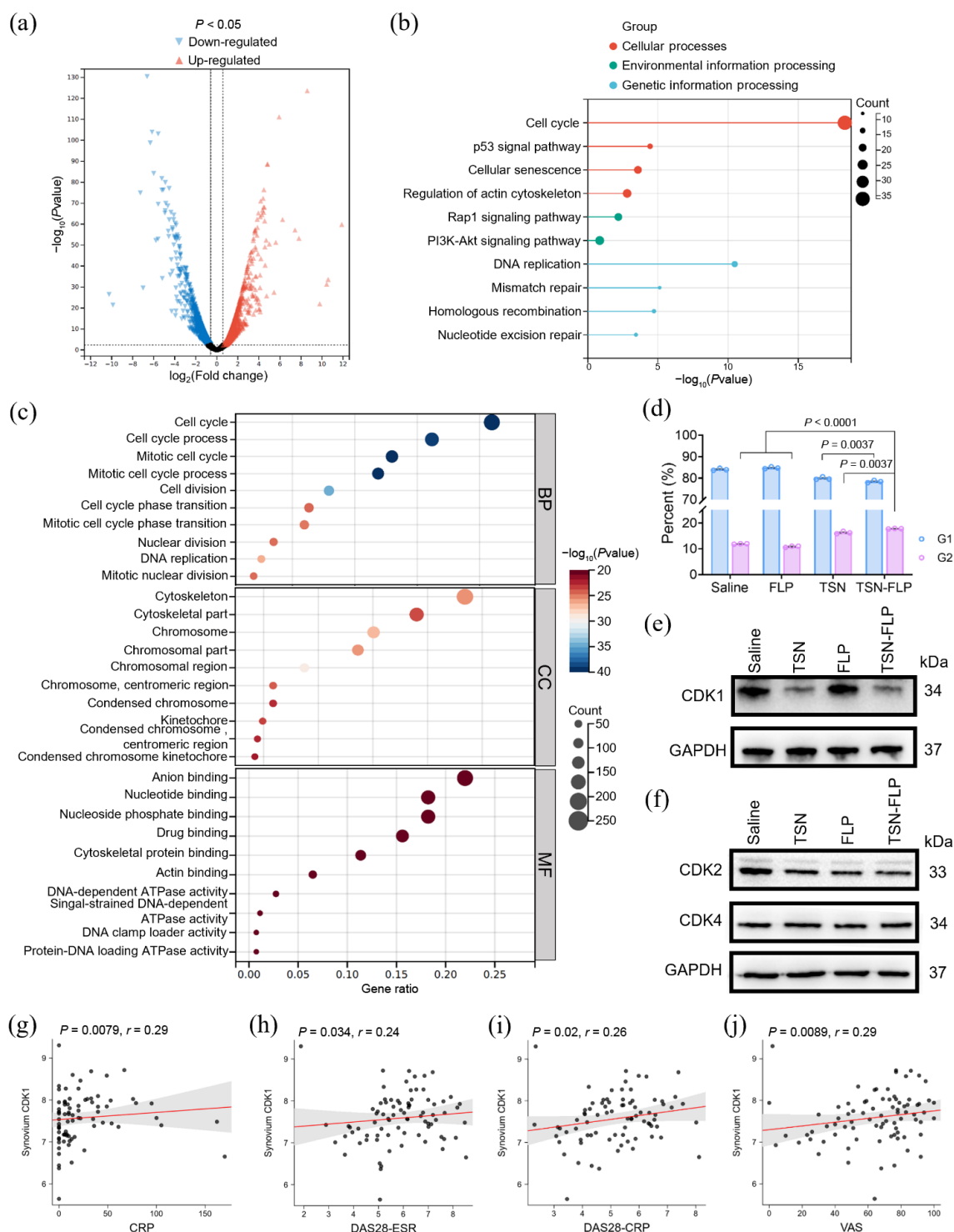


Figure 4 TSN-FLP arrests the cell cycle by downregulating CDK1. (a) Transcriptomic profiling revealing differentially expressed genes in TSN-FLP-treated RA-FLS versus controls ($n = 3$ for each group). (b) KEGG enrichment analyses of the top ten signaling pathways down-regulated by TSN-FLP in RA-FLS. (c) GO enrichment analyses of the genes down-regulated by TSN-FLP in RA-FLS (BP: biological process, CC: cellular component, MF: molecular function). (d) Cell cycle phase analysis of TSN-FLP-treated RA-FLS performed by flow cytometry. Western blot analysis of the levels of (e) CDK1, (f) CDK2, and CDK4. Correlations between CDK1 gene expression and RA disease severity: (g) CRP, (h) DAS28-ESR, (i) DAS28-CRP, and (j) VAS from the PEAC RNA-seq database. Data are presented as mean $\pm$ s.e.m. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparisons test.

analysis performed by flow cytometry. Further investigation found that the phosphorylation status of ERK in RA-FLS was down-regulated after incubation with TSN-FLP for 48 h (Fig. S7 in the ESM). It was hypothesized that TSN-FLP-induced cell cycle arrest

might be caused by downregulation of CDK1 through reduced phosphorylation of ERK in the mitogen-activated protein kinase (MAPK) pathway, and further by disruption of the interaction between these two proteins. To evaluate whether the expression of

the CDK1 gene affects clinical RA indicators, the expression level of the CDK1 gene in synovium was compared with clinical indicators through the Pathobiology of Early Arthritis Cohort (PEAC) database [43]. The results shown in Figs. 4(g)–4(j) and Fig. S8 in the ESM indicate that the CDK1 gene level was positively correlated with the clinical disease severity score (C-reactive protein (CRP), disease activity score 28-erythrocyte sedimentation rate (DAS28-ESR), disease activity score 28-c-reactive protein (DAS28-CRP), and visual analogue scale (VAS)) ($P < 0.05$) and negatively associated with therapeutic response score (delta ESR, delta DAS28-ESR, and delta CRP) ($P < 0.05$) as per the PEAC RNA-seq database. Based on these clinical clues, it was speculated that the down-regulation of CDK1 expression is meaningful for the alleviation of RA. The above results illustrate that the down-regulation of the CDK1 is a crucial molecular mechanism underlying the inhibitory effect of TSN-FLP on RA-FLS viability.

3.5 TSN-FLP significantly inhibited the progress of the CIA mouse model

To identify the *in vivo* therapeutic effect of TSN-FLP on RA, a further investigation was conducted using the CIA mouse model, a well-established animal model for RA research. The treatment schedule ($n = 5$) is shown in Fig. 5(a). As shown in Figs. 5(b) and 5(c), after administration of TSN-FLP, the severity of arthritis in the mice was significantly reduced compared to the CIA, TSN, and FLP group, as the mean score of TSN-FLP group was developed slowly during the period and was close to the MTX group at the endpoint (TSN-FLP: 3.2, CIA: 10.4, MTX: 3.4, FLP: 7.4, and TSN: 6.6). A more direct observation is shown in Fig. 5(d): the paw swelling, which is a typical clinical manifestation of rheumatoid arthritis, was markedly alleviated after TSN-FLP treatment. Moreover, micro-CT analysis showed that the CIA, FLP, and TSN groups developed bone erosion to varying degrees (Fig. 5(d) and Fig. S9 in the ESM). Among all CIA model groups except the MTX group, the TSN-FLP group had the least severe bone erosion and joint space changes, in line with the severity scores.

Histological analysis of the joint tissues after treatment with TSN-FLP revealed reduced inflammatory cell infiltration, reduced synovial hyperplasia, as well as cartilage and bone destruction (Fig. 6(a)). Moreover, immunohistochemical analysis (Fig. 6(b)) showed that the TSN-FLP group exhibited less invasive synovium expressing CDK-1 compared to the CIA, FLP, and TSN groups. The expression and distribution of IL-6, TNF- α , and IL-1 β in joints were further studied using immunohistochemical analysis. As shown in Fig. 6(c), compared with the Saline group and the FLP group, the expression and distribution of the cytokines were obviously reduced in the TSN-FLP-treated group. The results illustrate that TSN-FLP suppresses aberrant FLS activation, effectively reducing inflammatory cytokine levels in RA joints and thereby contributing to arthritis recovery. Additionally, as presented in Figs. 6(d)–6(f), serum levels of pro-inflammatory cytokines, including TNF- α , IL-6, and IL-1 β , were significantly lower in TSN-FLP-treated mice than in untreated CIA mice, indicating that suppression of RA-FLS effectively attenuates the systemic inflammatory response in rheumatoid arthritis. These *in vivo* findings are consistent with the *in vitro* results, further confirming the therapeutic potential of TSN-FLP for the treatment of rheumatoid arthritis.

3.6 The biosafety evaluation of TSN-FLP

Finally, the *in vivo* biosafety of TSN-FLP was evaluated. The body

weights of the mice in different groups were recorded and are presented in Fig. S10 in the ESM. In comparison with healthy mice, all the mice with arthritis exhibited a reduction in body weight (not exceeding 20%). Except for the CIA group, the mice in the other groups did not show a sudden or substantial decrease in body weight. Moreover, as demonstrated in Fig. 7(a), the H&E staining of heart, liver, spleen, lung, and kidney revealed that the administration of TSN-FLP did not lead to tissue damage, necrosis, or inflammatory infiltration in these organs. Furthermore, no obvious abnormalities were observed in plasma biochemical markers (ALT, AST, γ -GT, BUN, and Cr) after treatment with TSN-FLP (Figs. 7(b)–7(f)), suggesting that the administration caused neither acute nor chronic damage to liver and kidney function. These data collectively demonstrate that the TSN-FLP does not induce significant systemic toxicity or organ damage in CIA mice. This favorable biosafety profile is crucial for the potential clinical translation of TSN-FLP as a therapeutic agent for RA, as it ensures that the treatment could exert its anti-arthritis effects without inducing unacceptable adverse reactions in major organs or the overall physiological functions.

4 Discussion

The research findings demonstrate that TSN-FLP effectively inhibits the proliferation of RA-FLS, a key pathological feature of RA that contributes to joint destruction. The observations from *ex vivo* and *in vivo* experiments are consistent with previous studies indicating that cell cycle regulation is a valid target for inducing apoptosis, and they further support the notion that TSN-FLP modulates RA-FLS behavior by interfering with cell cycle progression. Furthermore, the biosafety evaluation of TSN-FLP revealed a favorable safety profile. *In vivo* assessments in animal models showed no significant adverse effects on organ function or overall health at the therapeutic doses tested. This is of paramount importance for the translation of TSN-FLP into clinical applications, as therapeutic agents with good efficacy but poor safety profiles often have limited clinical utility. The RA-FLS-targeted liposome effectively addressed the challenge of delivering hydrophobic drugs. However, several limitations of the current study should be acknowledged. First, the *in vivo* experiments were conducted over a relatively short period, and long-term safety and efficacy require further observation. Second, the specific molecular targets of TSN-FLP within the cell cycle machinery remain to be fully elucidated. According to recent studies, TSN exerts activity by targeting various proteins and regulating signaling pathways including JAK/STAT3, Wnt/ β -catenin, PI3K/AKT/mTOR, Hh, and ERK/Snai1 [24, 44]. The regulatory effect of TSN on cells represents a complex process involving the coordinated action of multiple signaling pathways. Moreover, there exists a cascade reaction of CDK and MAPK phosphorylation that has not been fully elucidated, which is interconnected with the cell cycle (G2/M phase) and can modulate its progression [45, 46]. Future studies should aim to identify the precise pathway involved in TSN-FLP-induced cell cycle arrest, including the analysis of specific regulatory patterns and upstream regulatory mechanisms involving CDK1. Such investigations would provide a deeper understanding of its mechanism of action and potentially lead to the development of more potent and specific derivatives. Additional preclinical and clinical investigations are necessary to verify these findings and facilitate the development of TSN-FLP as a valuable supplement to the existing therapeutic strategies for the management of rheumatoid arthritis.

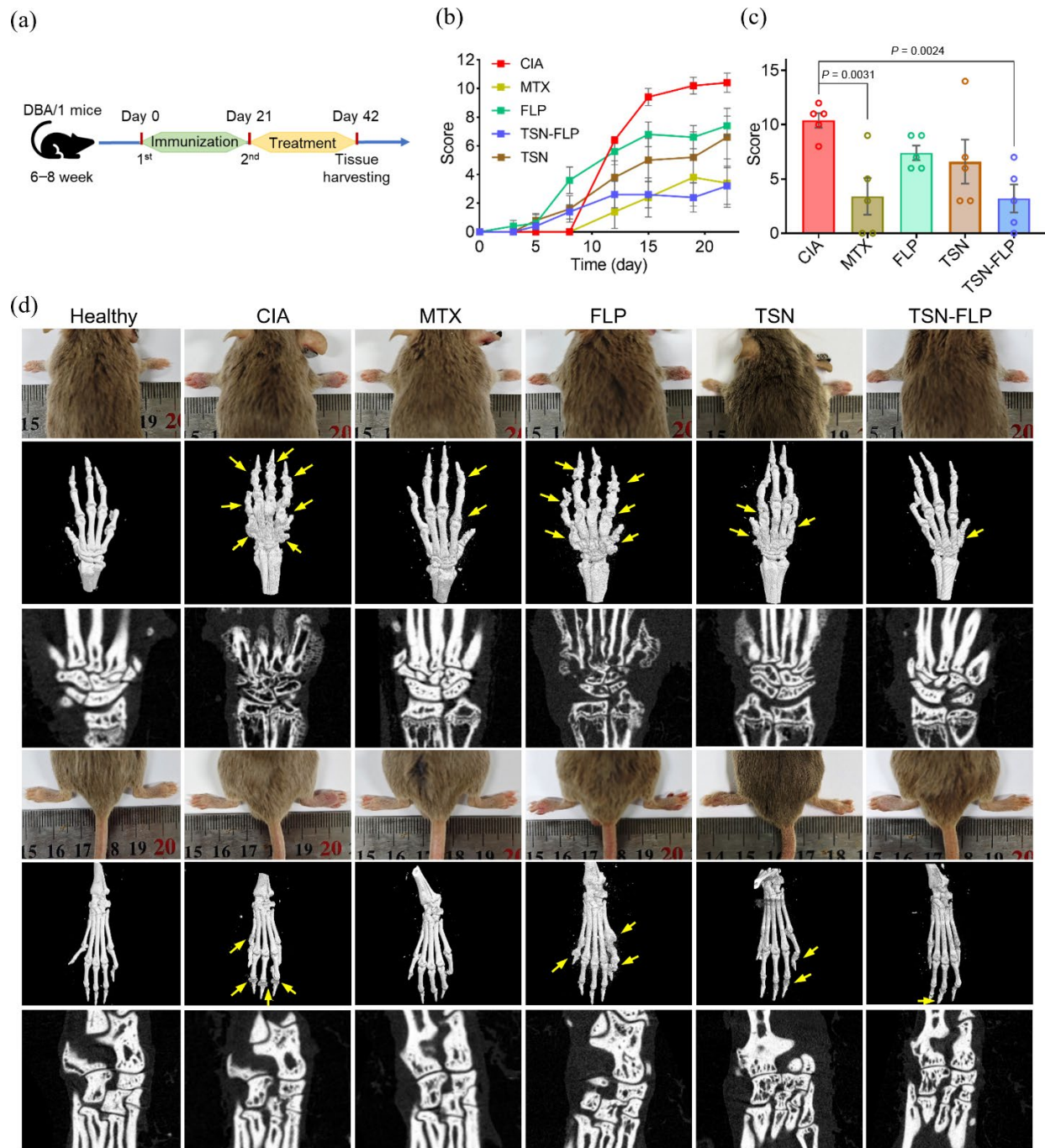


Figure 5 TSN-FLP attenuates RA progression in the CIA mouse models. (a) Schematic diagram of the animal experiment procedure. (b) Curves of the clinical severity scores of mice in various groups throughout the observation period. (c) Clinical severity scores of mice in various groups at the endpoint. (d) Representative photographs and micro-CT images of the limbs of the mice in various groups. Data are presented as mean $\pm$ s.e.m. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparisons test.

5 Conclusions

The present study systematically investigated the therapeutic effects and underlying mechanisms of TSN-FLP in RA. The *ex vivo* results demonstrated that TSN-FLP could significantly inhibit the viability and induce G2 phase cell cycle arrest in RA-FLS. Mechanistically, this was attributed to the downregulation of the CDK1, which plays a pivotal role in cell cycle progression from the G2 to M phase. Furthermore, in the CIA mouse model, TSN-FLP effectively alleviated arthritis severity, reduced paw swelling, mitigated bone

and cartilage destruction, and suppressed the secretion of pro-inflammatory cytokines, thereby confirming the *in vivo* anti-arthritic efficacy. Importantly, the biosafety evaluation revealed that TSN-FLP did not cause significant systemic toxicity or organ damage in CIA mice, as evidenced by stable body weight, normal histopathological features of major organs, and unaltered plasma biochemical markers. Collectively, these findings highlight that TSN-FLP exerts its therapeutic effects on RA by inhibiting RA-FLS proliferation through cell cycle arrest, while maintaining a favorable biosafety profile. Therefore, TSN-FLP demonstrates significant

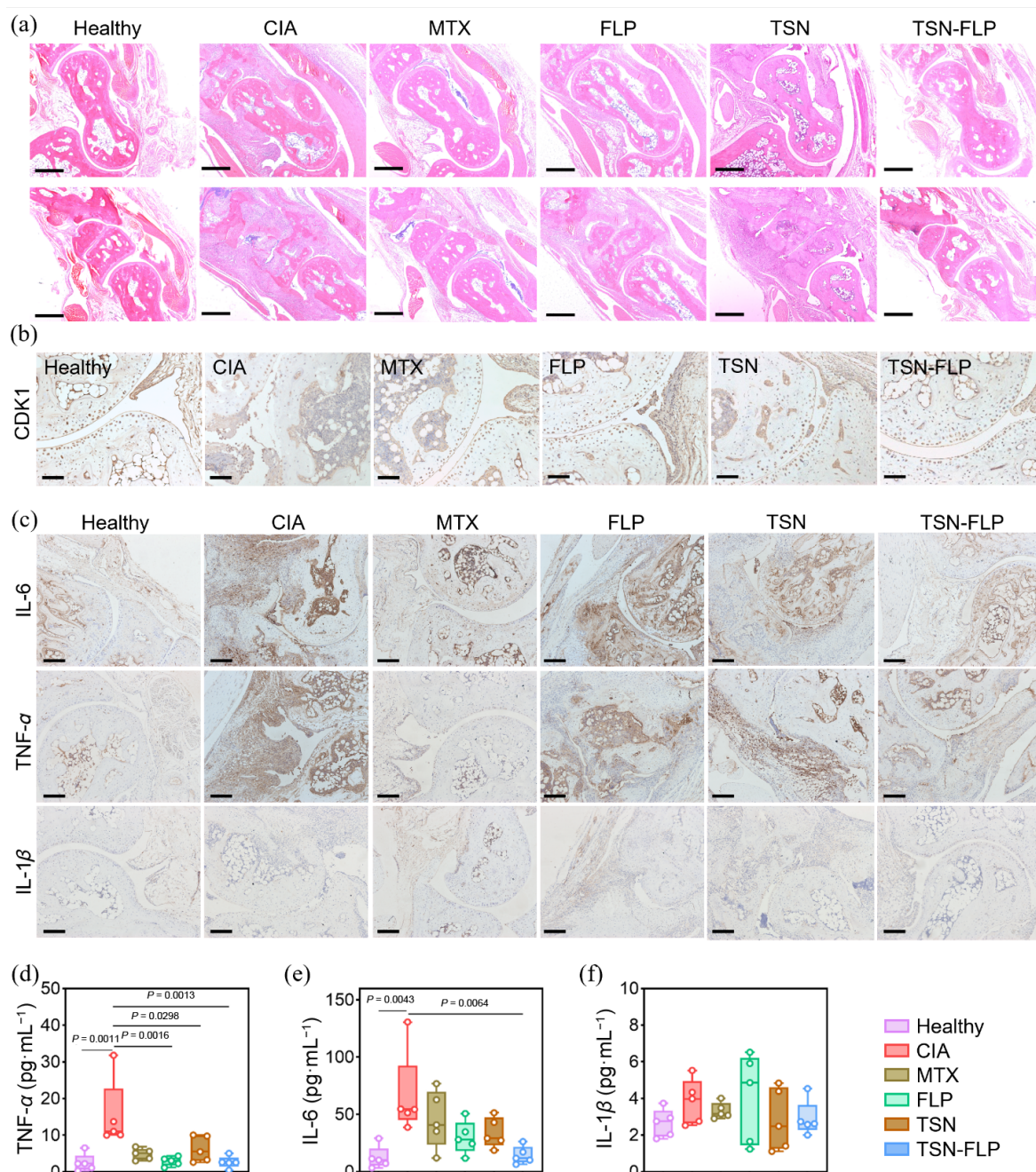


Figure 6 Histological analysis and serum analysis of the *in vivo* RA therapy. (a) Images of H&E staining of the joints of mice in different groups. Scale bars: 500 μ m. (b) Representative images of immunohistochemical staining for CDK1 in the joints of mice from different groups. Scale bars: 100 μ m. (c) Representative images of immunohistochemical staining for IL-6, TNF- α , and IL-1 β in the joints of mice from different groups. Scale bars: 200 μ m. Serum levels of (d) TNF- α , (e) IL-6, and (f) IL-1 β at the endpoint determined by ELISA kits. Data are presented as mean $\pm$ s.e.m. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparisons test.

potential and offers a new perspective for the therapeutic management of rheumatoid arthritis.

Electronic Supplementary Material: Supplementary material (Figs. S1–S10): MS graph of DSPE-PEG2000-NHS-cPPQFC, hydrated particle size of TSN-FLP before and after extruded, graphs of the cell uptake of TSN-FLP and non-targeting liposome by RA-FLS conducted via flow cytometry, migration rate calculated from microscope images, graphs of RA-FLS apoptosis analysis conducted by flow cytometry, result of RNA-seq, photograph of western blot

analysis of the expression levels of ERK1/2 and pERK1/2, graphs of correlations between CDK1 gene expression and RA therapeutic response score, micro-CT images of the ankle joints, and curves of body weight of mice) is available in the online version of this article at <https://doi.org/10.26599/NR.2026.94909013>.

Data availability

All data needed to support the conclusions in the paper are presented in the manuscript and/or the Electronic Supplementary Material. Additional data related to this paper may be requested

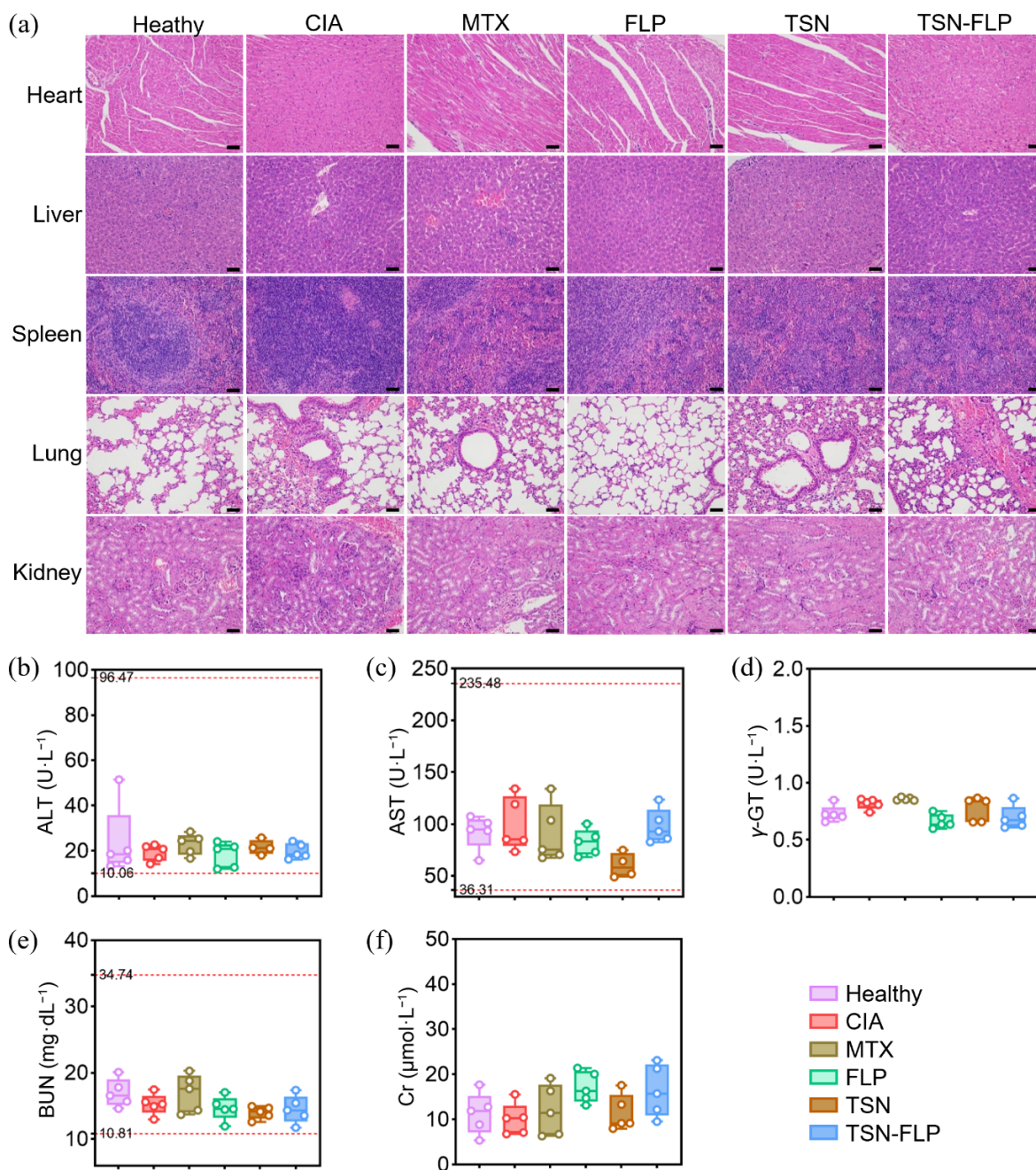


Figure 7 Biosafety analyses after TSN-FLP administration. (a) Images of H&E staining of the sections of heart, liver, spleen, lung, and kidney derived from mice in various groups at the end point. Scale bars: 50 μm. Analysis of blood biochemical indicators including: (b) ALT, (c) AST, (d) γ-GT, (e) BUN, and (f) Cr at the end point.

from the corresponding author upon request.

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Declaration of competing interest

All the contributing authors report no conflict of interests in this work.

Author contribution statement

Q. Z. and W. Y. P.: Data curation, project administration, validation, writing manuscript, and experimental design. X. G., X. C. W., and J. X. H.: investigation, software, and methodology. J. C. and X. L.: supervision and conceptualization. S. B. X.: investigation. F. P. and Q. W. W.: funding acquisition, project administration,

resources, and supervision. All the authors have approved the final manuscript.

Informed consent

Not applicable.

Ethics statement

The animal experiments were carried out following the guidelines of the Experimental Animal Ethics Committee of Peking University Shenzhen Hospital for the Care and Use of Laboratory Animals and were approved by the Committee on Ethical Use of Animals of Peking University Shenzhen Hospital (Ethical code: 2024-461).

Use of AI statement

None.

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