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Immunomodulatory effect and safety of TNF-α RNAi mediated by oral yeast microcapsules in rheumatoid arthritis therapy

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ABSTRACT

Rheumatoid arthritis (RA) is a chronic autoimmune disease that requires long-term treatment and monitoring. Inhibition of inflammatory gene expression by gene therapy is a significant breakthrough in RA treatment, but the lack of a safe and effective gene delivery system hinders its application. Since oral administration can significantly reduce wound infection caused by parenteral administration, it also has the advantages of high patient compliance and convenience. Therefore, oral administration may be the best option for the treatment of this chronic disease. In this study, we developed a novel oral drug system by delivering tumor necrosis factor-α (TNF-α) short hairpin RNA (shRNA) mediated by non-pathogenic yeast to evaluate its regulation of systemic immune inflammation and safety in RA. Non-pathogenic yeast can resist the destruction of the gastrointestinal acid-base environment and can be recognized by the intestinal macrophages and act on systemic inflammatory lesions. Oral administration of yeast-mediated TNF-α shRNA significantly reduced the expression of TNF-α predominant pro-inflammatory factors in intestinal macrophages and joint synovium, and up-regulated the expression of anti-inflammatory cytokine IL-10 and M2 macrophages, systematically regulating the inflammatory response. This yeast-mediated oral gene delivery system can not only significantly inhibit knee joint synovial inflammation, but also has no toxic effects on peripheral blood and major organs. Therefore, yeast-mediated oral delivery of TNF-α shRNA may be used as a novel gene therapy strategy to treat RA through immunomodulating the mononuclear phagocyte system from the intestine to the joint synovium, and ultimately regulating systemic and local immune inflammation, providing new ideas for the clinical treatment of RA.

1. Introduction

Rheumatoid arthritis (RA) is a chronic systemic autoimmune disease characterized by inflammation and subsequent destruction of multiple joints and other organs [1]. The disease affects approximately 0.2–1.0% of the population worldwide and 0.28–0.45% of Chinese [2,3]. Beginning with synovitis, joints destruction in RA may gradually involves cartilage and bone, causing pain and disability, which affects the overall health and quality of life [1,4].

With advances generated in understanding the RA pathogenesis, the treatments have focused on intercellular messengers or cytokines that play a pivotal role in RA, such as tumor necrosis factor (TNF)-α, the first target in RA immunotherapy [5–7]. TNF-α is very important in driving synovial inflammation and bone erosion, thereby promoting the development of RA [8]. TNF-α inhibitors have been considered revolutionary advances in RA treatment, followed by the continuous introduction of new biologic disease-modifying anti-rheumatic drugs (DMARDs) [9]. At present, the five available TNF-α blocking agents (etanercept, infliximab, adalimumab, certolizumab and golimumab) have markedly improved remission rate/treatment outcomes of RA [10,11]. However, the disadvantages of their invasive administration (intravenous or subcutaneous injection with short intervals) appear to be apparent and predominant in such chronic disorders. It is painful, expensive and time-consuming, which results in decreased patient compliance [12]. Especially in certain circumstances, such as the pandemic of Covid-19 in 2020, therapy with TNF-α blocking agents were discontinued in many RA patients in China, for they have to undergo home isolation. Therefore, a non-invasive, safe and effective

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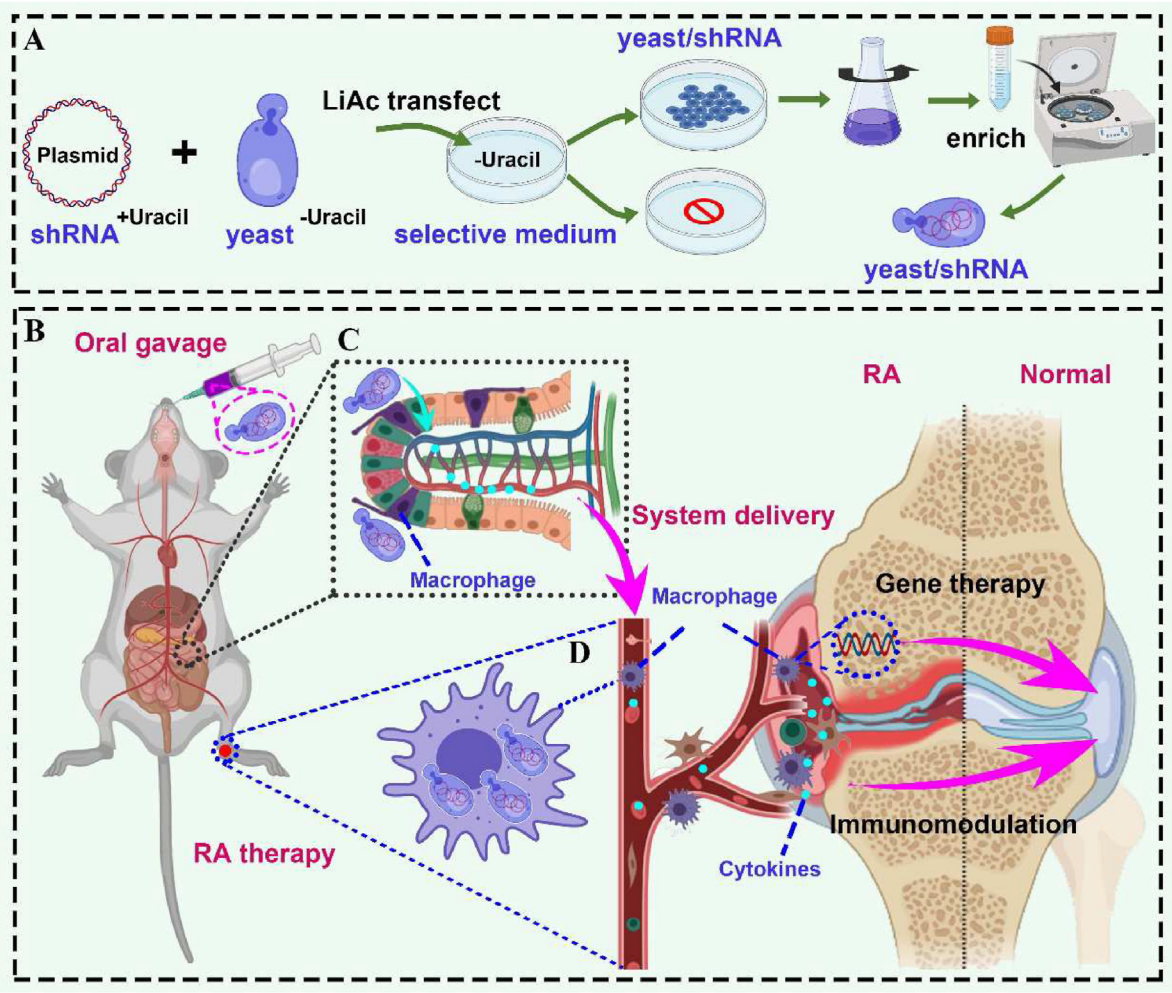
drug-delivery system for TNF-α blocking agents are urgently needed.

Appropriate delivery systems that match the drug properties and absorption mechanisms contribute to its highest bioavailability and effectivity [13]. The oral drug delivery system is the most attractive route due to its unique advantages, including ease of administration, non-invasiveness and feasibility for solid formulation [14,15]. It is suitable for long-term continuous therapy of chronic diseases and thus is preferred clinicians and RA patients. Despite so many advantages in drug delivery, the oral route still faces the harsh conditions along with the gastrointestinal (GI) tract, which can degrade or denature active bio-therapeutics, including the average length of the segment, pH, thickness of the mucus, the residence time of the drug and the bacterial diversity/population in different segments [16–19]. In recent years, the increasing demand for biopharmaceutical oral products in RA therapy impel the development of oral delivery systems.

Yeast microcapsules are potential carriers in oral drug delivery systems and have received increasing attention because of their specific recognition of immune cells [20]. This is due to three principal reasons. First, TNF-α exerts potent pro-inflammatory functions *via* activation of various immune cells, such as synoviocytes, macrophages, chondrocytes and osteoclasts. The non-starch polysaccharides on the yeast cell wall, called β-D-glucans, could be recognized by glucan receptors on the surface of immune cells. After being taken in the gastrointestinal tract, β-D-glucans are selectively recognized by pattern recognition receptors mainly expressed on macrophages and dendritic cells, such as Dectin-1 [21]. Then they would be transported with the circulation of macrophages to exert immunomodulatory activity [22–24]. Additionally, they can encapsulate drugs to protect them not only against harsh acidic/alkaline environment but against enzyme digestion in gastrointestinal tract. Lastly, yeast microcapsules have low-grade toxicity on tissues with a maximum tolerated dose proved in several clinical trials [25,26]. These characteristics make yeast microcapsules a preferred carrier for targeting the delivery of biological information or pharmaceutical compounds, thereby achieving target immunotherapy of RA.

In this study, we hypothesized that oral yeast microcapsule-mediated TNF-α shRNA can treat RA by antagonizing TNF-α. The present study aimed to develop a safe and efficient micro-drug-delivery system for chronic inflammatory diseases treatment not limited to RA therapy (see Scheme 1). Furthermore, the biosafety issues brought by the gene delivery system for oral administration of RA were evaluated.



Scheme 1. Schematic diagram of oral gene therapy for RA with recombinant yeast/TNF-α shRNA. (A) By transfecting shRNA vectors expressing uracil into yeast lacking uracil and culturing in selective medium (uracil deficient), the yeast clones grown out indicate successful yeast/shRNA construction. Cultures were then expanded and enriched to collect yeast/shRNA. (B) Oral gavage yeast/shRNA to RA rats. (C) The yeast/shRNA is recognized and phagocytosed by intestinal macrophages in the small intestine and be involved in regulating immune responses. (D) Macrophage@yeast/shRNA and the anti-inflammatory cytokines secreted by macrophages are transferred to distal inflammatory joints through humoral circulation and improve RA symptoms by gene therapy and immune regulation at synovial tissue sites.

2. Materials and methods

2.1. Construction of TNF-α shRNA expression vectors

Three different shRNA sequences that could target *TNF-α* mRNA were designed following the website (https://portals.broadinstitute.org/gpp/public/seq/search). The information of the three such short hairpin sequences of *TNF-α* and target sites were shown in Table 1. And the shRNA gene sequences containing *Sal*I and *Eco*RI digestion sites at the 5′ and 3’ ends were synthesized by GeneCreate (Wuhan, China). Subsequently, four TNF-α shRNAs (control shRNA, shRNA-1, shRNA-2 and shRNA-3) were cloned into pIN27-hU6 vector to get the pIN27-hU6-TNFα-shRNA vector. The yeast cloning vector pIN27-hU6-TNFα-shRNA was constructed as we described previously [27].

2.2. Construction of recombinant yeast/TNF-α shRNA

The yeast strain of Saccharomyces cerevisiae Scy27 (MATα, his3-Δ1 trp1-289 rad1-Δ ura3-52) was used in this study. Plasmids pIN27-hU6-TNFα-shRNA or pIN27-hU6-control-shRNA were respectively transformed into Scy27 by the LiAc method to construct yeast/TNF-α shRNA and yeast/shRNA NC [28]. Then the recombinant yeast/shRNA was cultured in selective medium lacking Urcial to reach a density of OD600 = 1. The harvested recombinant yeast/shRNA was then dissolved in PBS and stored at −20 °C before use.

2.3. Engulfment of yeast microcapsule by macrophages

The 5-(4,6-dichlorotriazinyl) aminofluoresce (Thermo; 1 mg/mL in DMSO) was used to prepare green fluorescently labelled recombinant yeast as we described before [29]. Then the green-labelled recombinant yeast/shRNA was to verify whether yeast could be recognized and engulfed by macrophage. To detect the uptake of yeast by macrophages, macrophages NR8383 were cultured at 37 °C under 5% CO_2 for 24 h in Ham's F–12K (Procell Life Science, PM150910) containing 20% fetal bovine serum (FBS, Gibco) supplemented with 1% penicillin streptomycin. About 10^5/12-well green labelled yeast microcapsule was added into the cell culture medium, and after culturing for 4 h at 37 °C under 5% CO_2, the phagocytosis of yeast microcapsule by macrophages was detected by fluorescence microscopy.

2.4. Functional detection of recombinant yeast/TNF-α shRNA in macrophages in vitro

To verify whether yeast/TNF-α shRNA can modulate the immune response of macrophage. We cultured bone marrow-derived macrophages in DMEM that containing 10% fetal bovine serum (FBS) supplemented with 100 ng/mL LPS (Sigma, L2880) for 24 h. Then recombinant yeast/TNF-α shRNA (10^5/6-well) were added into the LPS-induced macrophages culture medium for recombinant yeast/TNF-α shRNA functional detection. After incubation at 37 °C under 5% CO_2 for 24 h, cells were harvested by washing with phosphate buffered saline (PBS) before cell lysis for total RNA isolation. Finally, gene expression of TNF-α, IL-10 and IL-6 were detected by real-time PCR method. In addition, we also used NR8383 cells to examine the effect of yeast/TNF-α shRNA on cytokines secretion and TNF-a protein expression in macrophages. After 24 h of treatment with LPS, macrophage NR8383 was seeded into 12-well plates. Then, an equal amount of yeast/TNF-α shRNAs mixture (3 kinds of yeast/TNF-α shRNA were mixed according to 1:1:1) was added to the medium. After 36 h of co-culture, the cell suspension was collected, and the expression of cytokines TNF-α, IL-1β, IL-10 and IL-12 was measured by ELISA. Cells were harvested and lysed for TNF-α protein expression detection according to the protein extraction manual.

2.5. Animals

Female Dark Agoutid (DA) rats were generously offered by Professor Liesu Meng from Xi'an Jiaotong University, PR China. The experiment had been approved by the Institutional Animal Ethics Committee of Xi'an Jiaotong University (No:2021–710). Animals were bred specific pathogen–free (SPF) and were subjected to 12 h light/dark cycles. During the experiment, rats were housed with standard rodent chow and water. Forty rats at the age of 8 weeks were subcutaneously injected with 150 μL pristane (Acros Organics, 138460050, Belgium) at the base of the tail as described previously [30,31]. Nineteen days after injection, rheumatoid arthritis (RA) rat were randomly allocated to 3 groups (n = 12). Group RA-PBS was RA rats oral administration with PBS. Group RA-shR NC was oral administration of RA rats with negative control yeast/shRNA NC. Group RA-shR TNF was oral administration of RA rats with yeast/TNF-α shRNA. While the control group, also called group NC-PBS, was normal rats oral administration with PBS. Rats in RA-shR NC group and RA-shR TNF group were given 60 mg/kg yeast every other day. And rats in NC-PBS and RA-PBS groups were given the same volume of PBS as the experimental group. After 12 days of oral administration, whole blood samples were collected from the tail veins for blood routine tests. Samples of the small intestine and knee joints were collected and analyzed histologically.

2.6. Distribution of yeast in joint injury after oral administration

We used fluorescence imaging to detect whether yeast/TNF-α shRNA could transfer to the inflammatory tissue site after oral administration. First, near-infrared fluorescent dye Dir (AAT Bioquest, 22070) was used to label yeast into DirYeast, which emits red light at near-infrared wavelengths. For the preparation of DirYeast preparation, about 10^8/yeast/TNF-α shRNA was dissolved in 50 mL PBS. Then 20 μL of near-infrared fluorescent dye Dir (AAT Bioquest, 22070) was added into the yeast suspension and treated at 25 °C for 16 h in the dark. Discard the supernatant and wash the yeast with sterilized deionized water until the supernatant is colourless (about 4 times) to obtain the DirYeast. Subsequently, the RA rats that were induced by pristane were given 60 mg/kg DirYeast or yeast. After 10 h administration, we collected the knee joints

Table 1
Information of shRNA and primer sequence.

Name	Full sequence (5′-3′)
shR-1	CCGGAGGAGGAGAAGTTCCCAAATGCTCGAGCATTTGGGAACTTCTCCTCCTTTTTTG
shR-2	CCGGCAACTCCGGGCTCAGAATTTCCTCGAGGAAATTCTGAGCCCGGAGTTGTTTTTG
shR-3	CCGGATTGCACCTGTGACTATTTATCTCGAGATAAATAGTCACAGGTGCAATTTTTTG
IL-10 forward primer	AGAAGAGGGAGGAGCCTTTG
IL-10 reverse primer	GCCTTTGCTGGTCTTCACTC
TNF-α forward primer	AGGACACCATGAGCACGGAA
TNF-α reverse primer	GGGCCATGGAACTGATGAGA
IL-6 forward primer	AGACTTCCAGCCAGTTGCCT
IL-6 reverse primer	CTGACAGTGCATCATCGCTG
GAPDH forward primer	TCTCTGCTCCTCCCTGTT
GAPDH reverse primer	CTTGCCGTGGGTAGAGTC

of rats and used the Odyssey CLX imaging system for fluorescence imaging detection.

2.7. Immunofluorescence

Rats in RA-shR NC group and RA-shR TNF group were all given 60 mg/kg of yeast every other day. And rats in NC-PBS and RA-PBS groups were given the same volume of PBS as the experimental group. After 12 days of oral administration, rat knee joints and small intestine were collected for immunofluorescence analysis. Samples were fixed overnight in 4% paraformaldehyde, dehydrated and then paraffin-embedded. Immunofluorescence staining was performed with CD206 (Proteintech, 18704-1AP), TNF-α (Immunoway, YT4689) or IL-10 (Servicebio, GB11534) and FITC-TSA (Servicebio, G1222) or CY3-TSA (Servicebio, G1223).

2.8. Toxicity detection of yeast/shRNA drug delivery system

Hematoxylin-eosin (H&E) staining and blood routine tests were used to evaluate the *in vivo* toxicity of recombinant yeast/TNF-α shRNA. Rats were orally administrated with recombinant yeast/TNF-α shRNA or negative control shRNA every two days for 12 days. The whole blood collected from the tail vein was used for blood routine test (Mindray veterinary automatic blood cell analyzer, BC-2800vet). Lung, liver, spleen, kidney and intestine of rats were collected for histological analysis using hematoxylin-eosin (H&E) staining.

2.9. Statistical analysis

All statistical analyses were performed using Prism version 7 (GraphPad Software) for Windows. Data were presented as means ± standard deviations (SD). Comparisons were performed using an unpaired two-tailed Student's *t*-test between two groups. Differences among groups were analyzed by using analysis of variance (ANOVA) test. A p-value <0.05 was considered as statistically significant.

3. Results

3.1. Generation of recombinant yeast/TNF-α shRNA

Recombinant yeast/shRNA strains that contain these three TNF-α shRNA $^{+\text{Uracil}}$ or negative control shRNA $^{+\text{Uracil}}$ vectors were generated by the LiAc method and cultured in a selective medium lacking Uracil. The results showed that monoclonal colonies could only grow in the selective culture plates by transforming shRNA vectors expressing Uracil into yeast. Control without transformed shRNA vector, on the other hand, had no colony generation (Fig. 1A). A single yeast clone was subjected to bacterial solution PCR, indicating that the shRNA vector was transformed into yeast (Fig. 1B).

3.2. Recombinant yeast/TNF-α shRNA participates in immune regulation in macrophages in vitro

To demonstrate whether recombinant yeast can be specifically recognized and phagocytosed by macrophages, and then in turn, participate in the immune regulation of macrophages. We constructed gYeast by fluorescently labelling yeast with green amino fluorescent dye (Fig. 2A). Five hours after adding gYeast to the LPS-induced macrophages, the result showed that gYeast was phagocytosed by macrophages (Fig. 2B), indicating that yeast was able to be phagocytosed by macrophage-specific recognition.

To further detect the function of recombinant yeast/shRNA on macrophages, yeast/shRNA was added into macrophages medium induced by LPS. After 24 h co-culture, cells were collected to detect *Interleukin-6*

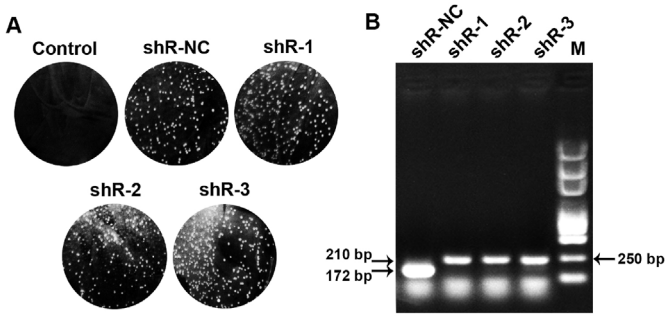


Fig. 1. Preparation of yeast/shRNA. (A) Construction of yeast/shRNA by LiAc method. The negative control shRNA plasmid shR-NC and TNF-a shRNA (TNF-a shR-1, -2, -3) that could express uracil were transfected into yeast, and separately cultured in selective medium $SD^{-\text{uracil}}$. The $\text{yeast}^{-\text{uracil}}$ alone was the control group. (B) Monoclonal bacterial solution PCR. Monoclonal colonies in Petri dishes were subjected to bacterial solution PCR to verify that the shRNA vector was in the yeast (n = 3).

(IL-6), *TNF-α* and *IL-10* gene expression by RT-qPCR. The results showed that three different yeast/TNF-α shRNA could inhibit inflammatory gene expression to varying degrees and promote *IL-10* expression (Fig. 2C). In addition, we also used NR8383 cells to examine the effect of yeast/TNF-α shRNA on cytokine secretion and TNF-a protein expression in macrophages. After 24 h of treatment with LPS, macrophage NR8383 was seeded into 12-well plates. Then, an equal amount of yeast/TNF-α shRNAs mixture (3 kinds of yeast/TNF-α shRNA were mixed 1:1:1) was added to the medium. After 36 h of co-culture, the cell suspension was collected. The expression of cytokines TNF-α, IL-1β, IL-10 and IL-12 in cell culture medium was measured by ELISA (Fig. 2D). The results showed that in LPS-treated macrophages, yeast/TNF-α shRNAs could effectively inhibits the expression of cytokine TNF-α, while increasing the expression of anti-inflammatory factors IL-10 and IL-12. But the use of recombinant yeast does not appear to have an effect on IL-1β. In macrophages without the LPS treatment group, although recombinant yeast/shRNA NC could promote the expression of TNF-α to a certain extent, the expression of anti-inflammatory factor IL-12 was also increased (Fig. 2D). This appears to have important implications for maintaining cellular immune balance. Moreover, the expression of TNF-α in western blot was similar to that in ELISA results (Fig. 2E and F).

3.3. Yeast was enriched in the joint inflammatory site after oral administration

Previous studies have demonstrated that oral yeast-mediated shRNA can effectively protect shRNA successfully passing through the gastrointestinal tract and reaching the inflammatory tissue [32]. In this study, we also demonstrated that yeast could successfully access the inflammatory site of RA joints after oral administration. Ten hours after intragastric administration of near-infrared fluorescent DirYeast, the fluorescence signal was detected by an oddessy fluorescence imaging system. Compared with the control group (yeast without fluorescent labelling), the hind limbs of rats in the DirYeast group had strong fluorescence signals (Fig. 3A and B), which indicated that after oral administration, yeast could successfully pass through the gastrointestinal tract and be delivered to inflammatory tissue. The mode of entero-articular delivery of this yeast may be based on yeast being phagocytosed by intestinal macrophages at the site of the small intestine and transferred to the joint toward distal inflammatory tissue [29,32]. By detecting liver, lung and spleen, the results showed that the fluorescence signal was stronger in the experimental group than the control group (Fig. 3C). This suggested that yeast can enter the circulation of body fluids to reach the lesion tissue by oral administration.

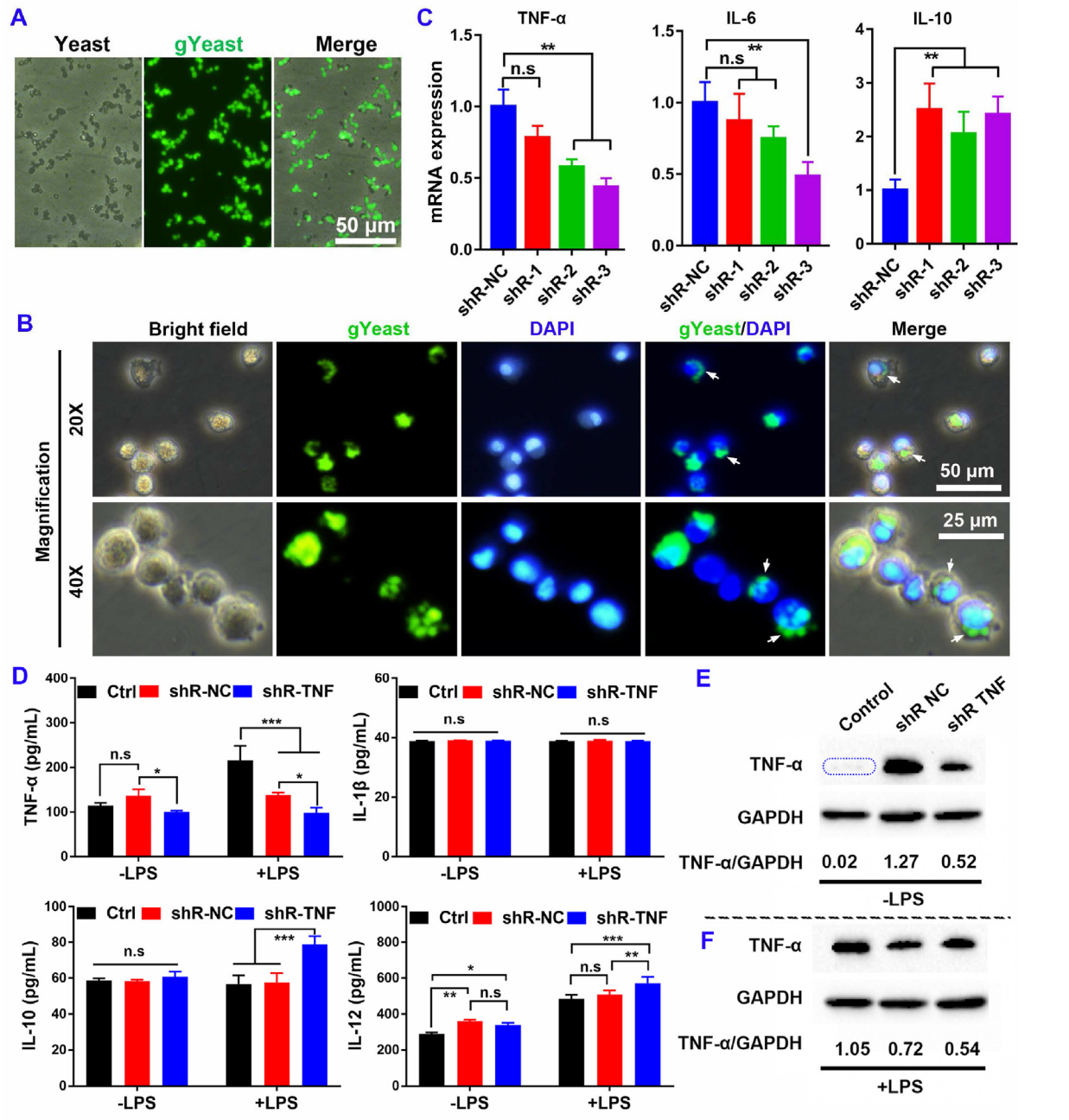


Fig. 2. ***In vitro*** **functional assays of recombinant yeast/shRNA.** (A) Yeast fluorescent staining to get green-labelled gYeast. (B) Green fluorescently labelled gYeast was co-cultured with macrophage. After 5 h co-culture, the effect of macrophages on recombinant yeast was determined by fluorescence imaging. (C) LPS-induced macrophages were treated with yeast/shRNA to examine the immune regulation of yeast/TNF-α shRNA by RT-qPCR method (shR-NC refers to yeast/shRNA negative control; shR-1, -2, -3 respectively refer to yeast/TNF-α shRNA-1, -2, -3). (D) Cytokines expression in macrophages cell culture medium after yeast/TNF-α shRNA treatment (+LPS or -LPS, respectively refers to macrophages with or without LPS treatment; Ctrl refers to macrophages without any treatment; shR-NC refers to yeast/shRNA negative control; shR-TNF refers to yeast/TNF-α shRNA). (E) TNF-α expression in macrophages without LPS treatment was detected *via* western blot after yeast/TNF-α shRNA treatment. (F) TNF-α expression in macrophages with LPS treatment was detected *via* western blot after yeast/TNF-α shRNA treatment. $*P<0.05$, $**P<0.01$, $***P<0.001$ and n.s. means no significance (n = 3). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.4. Recombinant yeast/TNF-α shRNA modulated the articular inflammation

Group RA-PBS was RA rats oral administration with PBS. Group RA-shR NC was oral administration of RA rats with negative control yeast/shRNA NC. Group RA-shR TNF was oral administration of RA rats with yeast/TNF-α shRNA. While the control group, also called group NC-PBS, was normal rats oral administration with PBS. Rats in RA-shR NC group and RA-shR TNF group were all given 60 mg/kg yeast every other day. And rats in NC-PBS and RA-PBS groups were given the same volume of PBS as the experimental group. After 12 days of oral administration, rats hind limbs and forepaws were collected. By anatomical analysis, the hind limbs and forepaws in RA-PBS and RA-shR NC groups had significant RA symptoms (joint swelling). While after yeast/TNF-α shRNA treatment, the joint swelling was significantly improved (Fig. 4A and B). Although some joints of the forepaw remained swollen in the TNF-treated group,

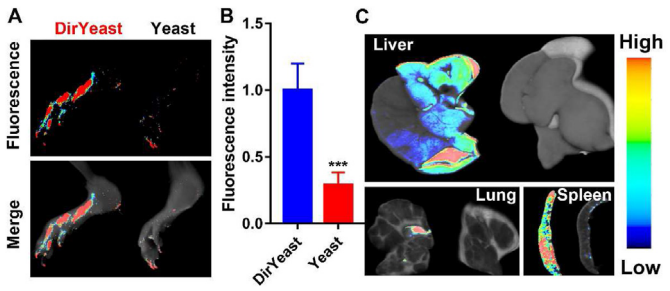


Fig. 3. Yeast phagocytized by macrophages was enriched in inflammatory joints. After administration of near-infrared fluorescently labelled DirYeast to RA rats, whether yeast could be delivered to inflamed tissues (joints) was detected by the oddessy fluorescence imaging system. (A) Fluorescence imaging of hind limbs of RA rats. (B) Hind limb fluorescence quantification. (C) Fluorescence imaging of liver, lung and spleen (left is the fluorescent labelling DirYeast group and right is the control group) (n = 3).

they were still significantly improved compared with the control group (Fig. 4B). And the RA scoring results also proved that yeast/TNF-α shRNA was found to be effective in improving RA symptoms. (Fig. 4C).

When recombinant yeast/shRNA was orally administered by rats, it was first phagocytosed by small intestinal macrophages and then carried to distant inflammation sites of joint [29]. RA-related inflammatory protein TNF-α and anti-inflammatory protein CD206 and IL-10 that related to M2 macrophages were used to test the function of yeast/TNF-α shRNA on the RA small intestine and articular synovium macrophages. The small intestine immunofluorescence results showed that compared with the control group, the expression of TNF-α (red) was significantly inhibited in the RA-shR TNF group, while the expression of CD206 (green) (Fig. 5A) and IL-10 were significantly increased (Fig. 5B). These results indicated that yeast/TNF-α shRNA could inhibit the inflammatory response of RA rats by oral administration. And the immunofluorescence results of TNF-α, CD206 and IL-10 in the synovial tissue also confirmed this conclusion (Fig. 5C and D).

3.5. *In vivo* biological safety of recombinant yeast/TNF-α shRNA in RA therapy

To verify the safety of recombinant yeast/TNF-α shRNA in RA therapy, 12 days after oral administration of recombinant yeast by RA and control rats, whole blood samples from their tail veins were collected for blood routing tests. And the samples of lung, liver, spleen, kidney and intestine were collected for hematoxylin-eosin (H&E) staining to evaluate the *in vivo* tissue toxicity of recombinant yeast/TNF-α shRNA. The results showed that the main cellular components and associated parameters in peripheral blood, such as red blood cells (RBC) counts, white blood cells (WBC) counts, lymphocyte counts (Lymph), monocyte counts (Mon), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), hemoglobin (HGB), mean platelet volume (MPV), and platelet distribution width (PDW) had no significant changes in the four groups, suggesting that oral yeast therapy had no significant effect on blood cells in rats (Fig. 6ABDE and Fig. 7).

We also found that two biomarkers commonly used in clinical practice to assess RA disease activity, platelet (PLT) (Fig. 6C) and neutrophil counts (Gran) (Fig. 6F) in RA rats (group RA-PBS) were higher than that in group NC-PBS. After treatment with recombinant yeast/shRNA, these two cell counts decreased significantly in group RA-shR TNF. Recently, the neutrophil-lymphocyte ratio (NLR) and the platelet-lymphocyte ratio (PLR) have been suggested as potential diagnostic biomarkers in RA. There was a significant association between NLR, PLR and RA [33–35]. In this study, NLR and PLR showed a similar trend in PLT and neutrophil

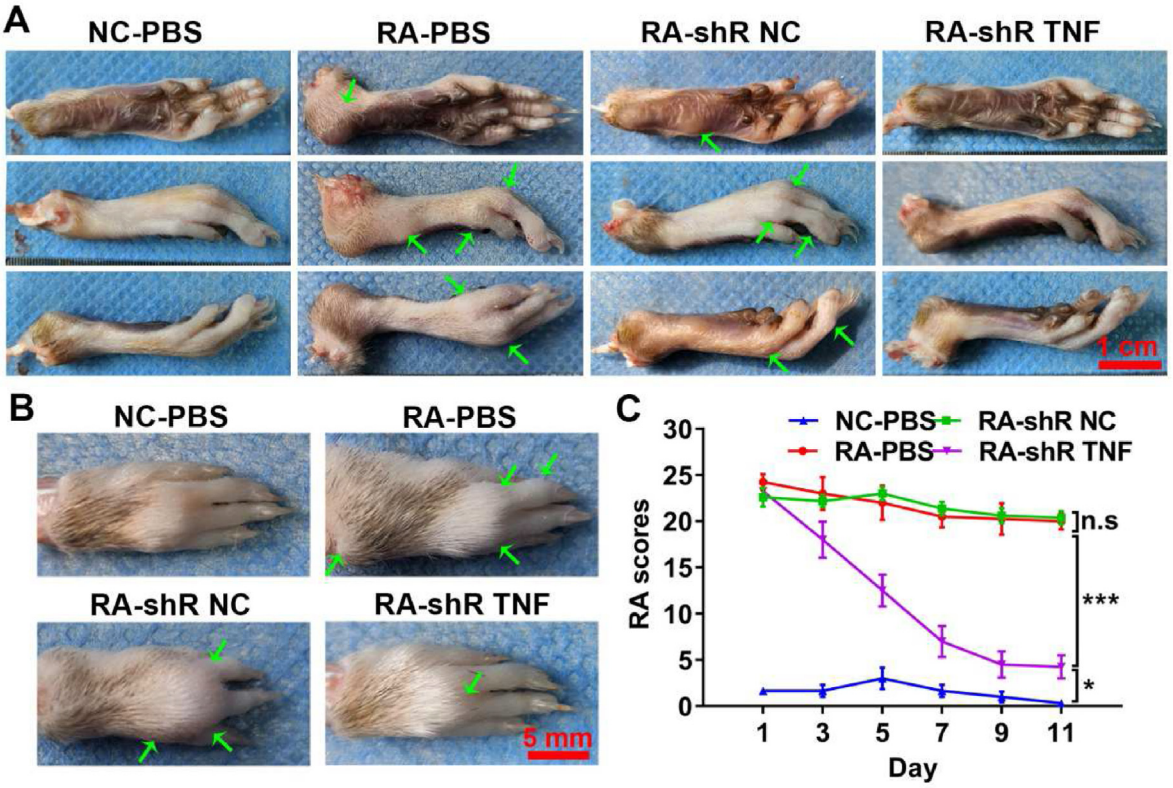


Fig. 4. RA symptoms in rat knuckles. After 12 days of oral administration of recombinant yeast, rats hind limbs and forepaws were collected for anatomical analysis. (A) Forelimb anatomical analysis. (B) Hind limb anatomical analysis. (C) Total RA score of fore and hind limbs in different groups. n.s (no significance). *$P < 0.05$, ***$P < 0.001$ (n = 6).

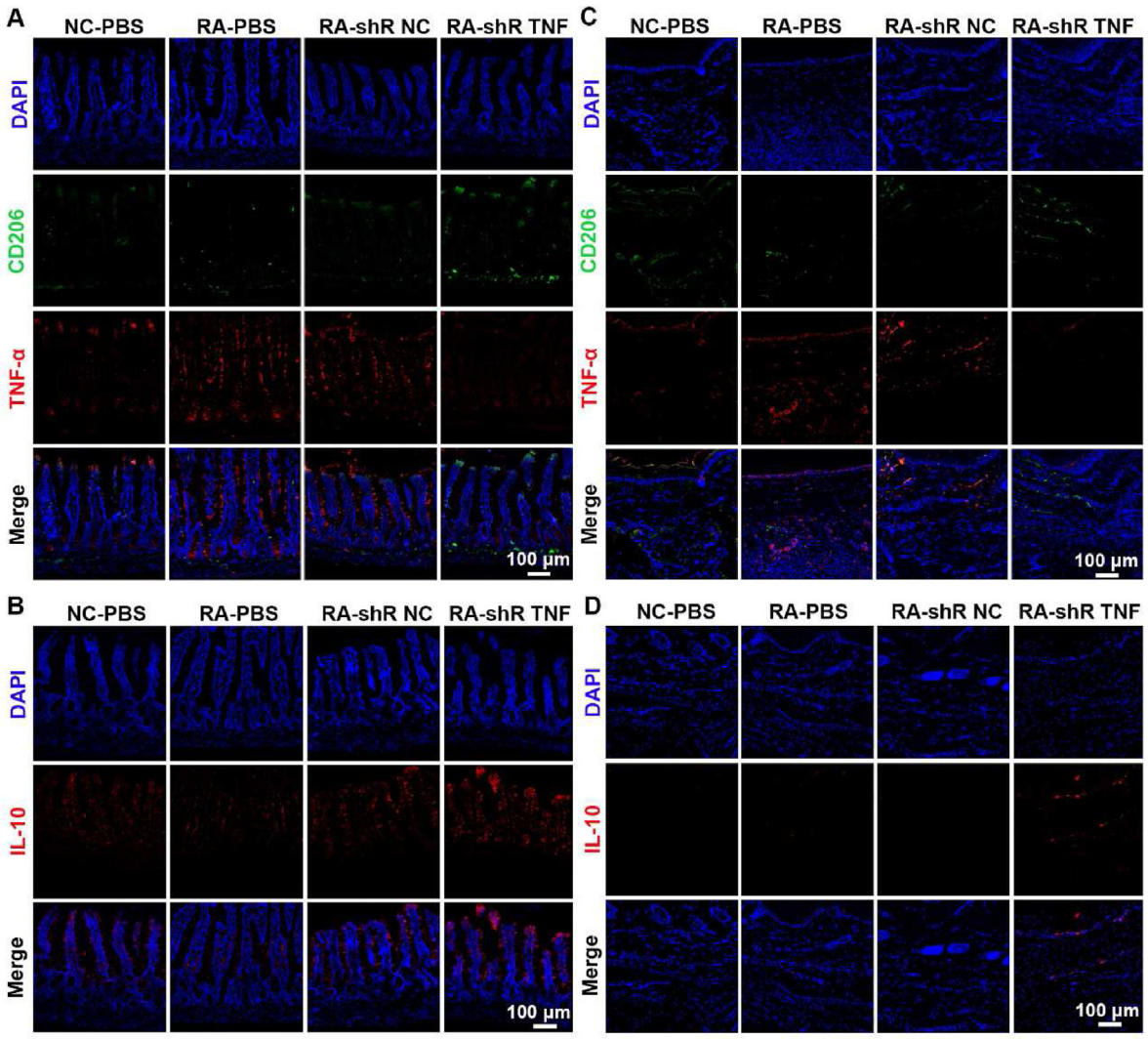


Fig. 5. Immunofluorescence staining of the small intestine and articular synovium. (A–B) The immunofluorescence staining of TNF-α, CD206 and IL-10 in the small intestine. (C–D) The immunofluorescence staining of TNF-α, CD206 and IL-10 in the articular synovium (n = 6).

counts among four groups (Fig. 6G and H). Compared with the control groups (NC-PBS and RA-PBS), no significant difference in tissue integrity, cell structure and morphology have been found in RA-shR NC and RA-shR TNF group (Fig. 8). Through the analysis of the main indicators related to peripheral blood and organs, it suggested recombinant yeast/TNF-α shRNA has no toxic effect on tissues *in vivo*.

4. Discussion

RA is a chronic systemic autoimmune disease involving not only joints but also multiple organs. Its etiology has not been clarified, requiring long-term treatment and monitoring. Cellular immune responses, as well as inflammatory bone erosion are its key pathogenic features [36]. Various immune cells, cytokines and signaling pathways, such as T/B lymphocytes, macrophages, TNF-α, interferon-γ, interleukin family and sphingosine-1-phosphate (S1P)–S1P receptor-1 (S1PR1) signaling are involved in the same microenvironment and processes during RA pathogenesis [37,38]. Based on this, a variety of intercellular messengers and cytokines have emerged as pivotal targets for RA treatment, such as TNF-α, Interleukin-6 (IL-6), cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), and Janus kinase (JAK) et al. TNF-α is one of the most important cytokines and the earliest target for biological therapy of RA.

As a representative autoimmune disease, the pathogenesis of RA involves complex immunological mechanisms. In addition to the cytokine network, various cell types of the innate and adaptive immune system, such as macrophages, also play an important role in RA synovitis and have become an important entry point for exploring the inflammatory joint erosion in RA. Inflammatory macrophages, which can differentiate directly into osteoclasts, are the main producers of pathogenic TNF [39,40], and can also induce disease initiation by affecting other cells involved in synovitis progression and bone erosion [41]. Therefore, targeting macrophages and macrophage-associated inflammatory pathways is an emerging strategy for RA immunotherapy in the future.

The biologics and small-molecule target drugs that are currently used in clinical practice are characterized by rapid onset, single target, and few side effects, but they still require long-term use. Therefore, improving patient compliance while meeting efficacy and safety becomes a new direction for global drug research and development. Compared to parenteral administration, the oral route is safe, convenient, and low cost of care, which is the most preferred treatment mode for this chronic condition of RA.

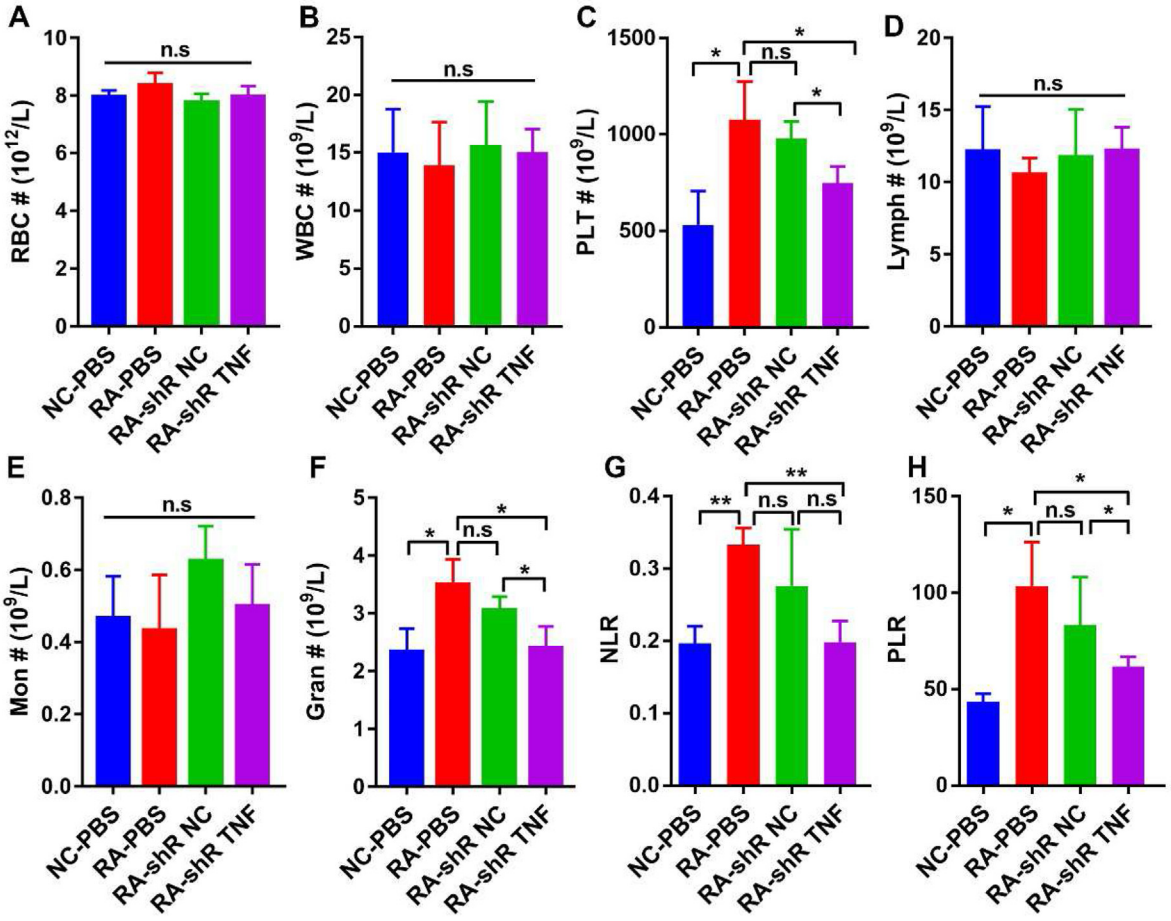


Fig. 6. Blood cell values were measured by blood routine tests. After 12 days of oral administration of recombinant yeast, whole blood samples from their tail veins were collected for blood routine tests. (A) RBC: red blood cell count; (B) WBC: white blood cell count; (C) PLT: platelet count; (D) Lymph: lymphocyte count; (E) Mon: monocyte count; (F) Gran: neutrophil count. (G) NLR: peripheral blood neutrophil-to-lymphocyte ratio; (H) PLR: platelet-to-lymphocyte ratio. n.s (no significance). $*P < 0.05$, $**p < 0.01$. (n = 6). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

With more and more generic drugs approved for marketing [42], gene therapy has become a powerful method to restore tissue function and disease treatment [43], such as the application study of siRNA in Zika virus (ZIKV) and severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) [44,45]; the development of DNA/RNA vaccine in the treatment of diseases such as new-coronary pneumonia COVID-19 and Ebola hemorrhagic fever. Our group has been working on the development of oral drug delivery systems and RA therapy for a long time. We plan to target the delivery of gene drugs in a simple and easily controlled oral manner starting from the level of gene regulation to provide new ideas for the treatment of RA. In this study, we constructed a novel strategy for RA therapy via yeast-mediated orally targeted delivery of TNF-α shRNA, which could regulate small intestine and joint synovial tissue inflammatory with high safety for RA treatment.

The probiotic Saccharomyces cerevisiae has been widely used in food fields such as brewing and baking, which is beneficial to the human body [46] and is approved as a safe product by the FDA [47]. Its main component, β-D-glucan, is a naturally occurring non-digestible [28] polysaccharide with immunomodulatory activities, attracting increasing attention to serve as therapeutic agents or immune-adjuvants. When administered orally, β-D-glucan is specifically recognized by glucan receptor dectin-1 on the surface of intestinal macrophages [48], then transported and enriched to distal inflammatory sites [49], exerting its immunomodulatory effects [50]. Due to these properties, including stability, biocompatibility and specificity, β-D-glucans can be used as promising carriers for targeting delivery for systemic immunotherapy of RA.

In our study, a recombinant yeast/TNF-α shRNA drug delivery system could inhibit pro-inflammatory genes TNF-α and IL-6 expression in macrophages to varying degrees *in vitro* and promoting anti-inflammatory gene/protein IL-10 expression (Fig. 2C and D), which was involved in immunoregulatory effects [51]. Similar results were observed in the small intestine and joint synovium of RA rats after oral yeast/TNF-α shRNA treatment (Fig. 5). Development of new technologies helps us to learn more about macrophage phenotypes reside in the synovium of RA. When stimulated by the different local microenvironments, macrophages display a spectrum of phenotypes, including M1 (pro-inflammatory) and M2 (anti-inflammatory) [52]. The immunofluorescence staining data of the small intestine and knee joint synovium showed that in the RA-shR TNF group, more M2 macrophages were detected (Fig. 5). These results illustrated that oral administration of yeast/TNF-α shRNA can target macrophages and their associated inflammatory cytokines, ultimately reducing the joint synovial inflammatory response and activating the anti-inflammatory phenotype.

Currently, there is already a small-molecule targeted drug, tofacitinib, that can be administered orally to treat RA. Unlike other biological disease-modifying antirheumatic drugs (bDMARDs), which target only one extracellular cytokine pathway in the inflammatory network,

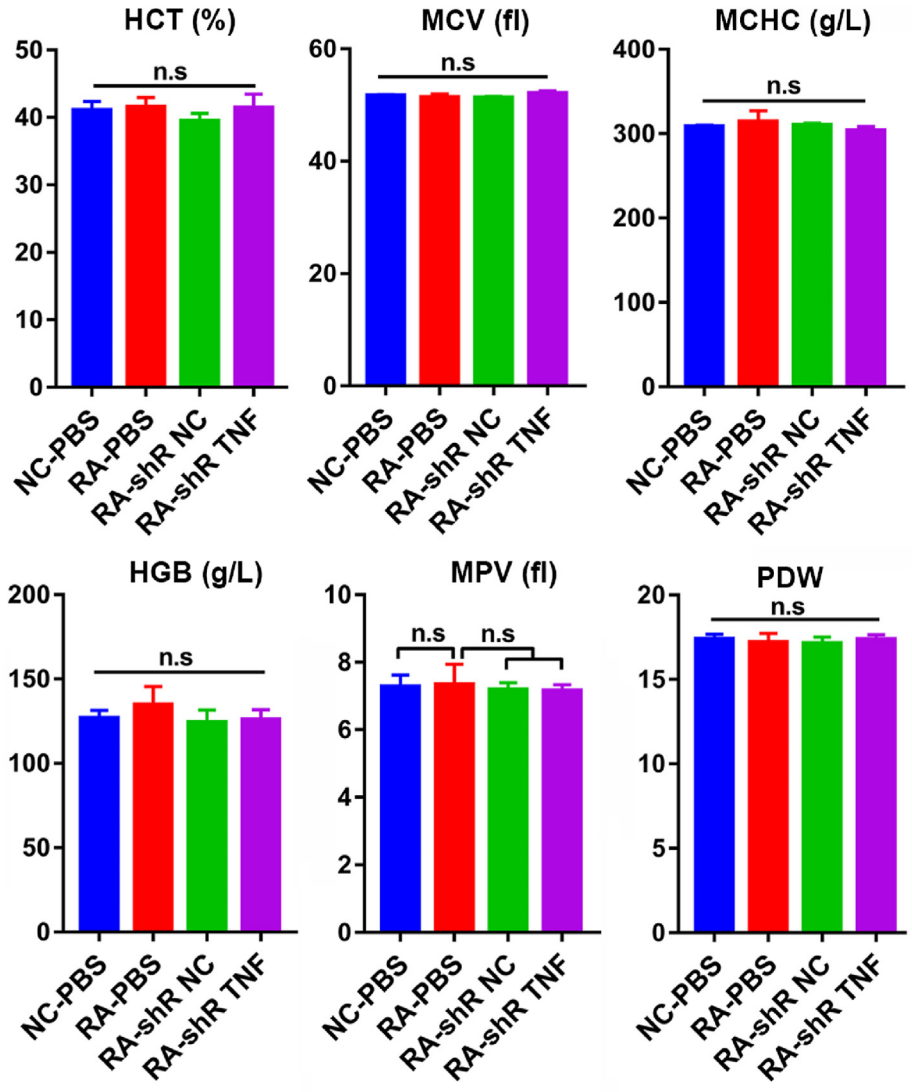


Fig. 7. Biosafety assessment of yeast/shRNA drug delivery systems on blood cell component analysis level. After 12 days of oral administration of recombinant yeast, whole blood samples from their tail veins. Blood cell components were used to assess the safety of this drug delivery system. HCT: hematocrit. MCV: mean corpuscular volume. MCHC: mean corpuscular hemoglobin concentration. HGB: hemoglobin. MPV: mean platelet volume. PDW: platelet distribution width. n.s (no significance) (n = 6).

tofacitinib exerts disease-modifying effects by partially inhibiting several intracellular inflammatory cytokines and modulating the overall immune and inflammatory response [53]. As a consequence of these mechanisms, tofacitinib, while achieving similar therapeutic effects with other bDMARDs [54,55], also has more significant risk of adverse events, particularly impact on the immune system. In a phase III clinical trial (The ORAL Standard trial), tofacitinib was compared with both placebo and an anti–TNF biologic agent (adalimumab) for efficacy and safety. The results showed that some adverse events including cytopenia, infections, and gastrointestinal side effects during months 0–3 occurred in 46.8% of the patients in 10 mg tofacitinib group. At month 3, they had a greater percentage of patients with aspartate aminotransferase (AST) levels one or more times the upper limit of the normal range than in the adalimumab or placebo group, which was dose-dependent. In months 0–3, the rates of serious adverse events and serious infectious events occurred more frequently in the tofacitinib group than in the placebo or adalimumab groups. In our study, main cell subsets and parameters in peripheral blood and histological staining of main organs (Figs. 6–8) were not affected by treatment, suggesting that oral administration of yeast/TNF-α shRNA for RA therapy is safe during the observation period. Notably, a dose-dependent mean decrease in absolute neutrophil counts was observed in both tofacitinib and adalimumab group along with corresponding reductions in acute phase reactants, consistent with our data (Fig. 6F). This result indicated that changes in neutrophils might be primarily related to the control of inflammation, rather than specific to the mechanism of drugs themselves.

However, this study also has some limitations. The intestine is full of a large population of innate and adaptive immune cells, and thus is often considered as the body's largest immunological organ [56]. Numerous studies in recent years have shown that gut-joint interactions constitute an important aspect of the pathogenesis of RA. Intestinal mucosal dysbiosis plays an essential role in the development and maintenance of systemic chronic inflammation in RA and may be a target of future preventive interventions in individuals at high risk of RA [57–60]. Probiotic therapies have received much attention for slowing inflammatory responses, regulating systemic immunity and promoting tissue repair by modulating intestinal microbiota and/or intestinal barrier function [61,62]. Probiotic yeast-mediated oral small molecule drug delivery systems can not only enhance the resistance of small molecule drugs to gastrointestinal environmental damage and improve the oral bioavailability of small molecule drugs, but also act by delivering drugs to distant lesion tissues through intestinal macrophages. However, it is unknown whether

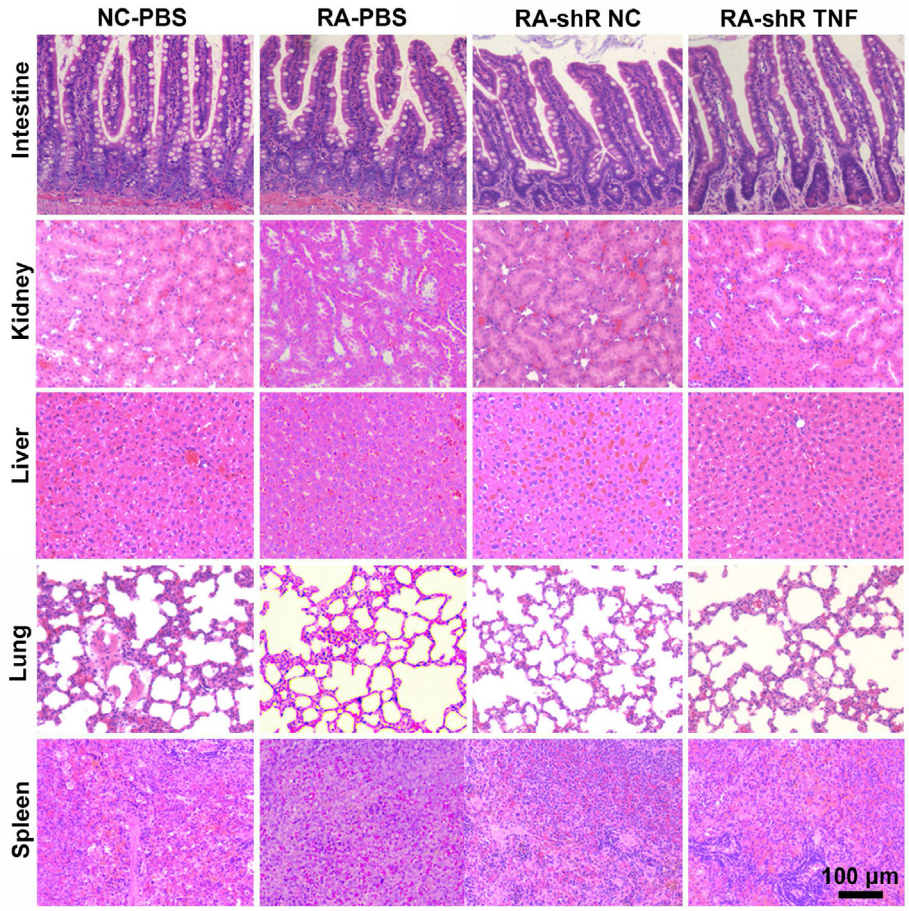


Fig. 8. Biosafety assessment of yeast/shRNA drug delivery systems on histological staining level. After 12 days of oral administration of recombinant yeast/shRNA, intestine, kidney, liver, lung and spleen of rats were collected for hematoxylin-eosin (H&E) staining. Compared with the control groups (NC-PBS and RA-PBS), no significant difference in tissue integrity, cell structure and morphology have been found in RA-shR NC and RA-shR TNF group (n = 6).

yeast-mediated small interfering RNA drugs reduce RA immunoinflammatory status by improving gut microbiota microecology when used in RA therapy and warrants further exploration and study.

In conclusion, we developed a novel approach for the treatment of RA by delivering TNF-α shRNA *via* orally administered recombinant yeast. Compared with existing TNF-α inhibitors for clinical application, this method not only has an ideal therapeutic effect and can regulate systemic immune inflammation, but also is more convenient and safe. Therefore, yeast-mediated oral delivery of shRNA, as a new strategy for arthritis gene therapy warrants further in-depth exploration and application on other targets.

Author contributions

Nan Hu, Li Zhu, Lan He and Zhiming Hao performed the *in vitro* experiments. Nan Hu, Li Zhu, Li Zhang, Jing Wang, Yanhua Wang and Jing Luo performed the *in vivo* experiments. Nan Hu and Long Zhang prepared the figures and wrote the manuscript. Long Zhang designed and drafted the work. All authors discussed the data and final approval of the version published.

Credit author statement

Ceptualization: Long Zhang; **Data curation**: Hu Nan, Zhu Li, Zhang Li, Wang Yanhua, Luo Jing, He Lan, Zhao Zhiming; **Formal analysis**: All authors; **Funding acquisition**: Hu Nan and Zhang Long; **Investigation**, **Methodology**: Zhu Li, Zhang Li, Wang Yanhua, Luo Jing; **Project administration**, **Resources**: All authors; **Software**, **Supervision**: Zhang Long; Validation: All authors; **Visualization**: Hu Nan, He Lan, Zhao Zhiming; **Writing – original draft**: Long Zhang and Hu Nan; **Writing – review & editing**: All authors.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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