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Triptolide regulates neutrophil function through the Hippo signaling pathway to alleviate rheumatoid arthritis disease progression

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ABSTRACT

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by inflammatory changes in the joints, the etiology of which is unclear. It is now well established that regulated cell death (RCD) and migration of neutrophils play an important role in the pathogenesis of RA. Tripterygium wilfordii Hook.f (TwHF) is a total saponin extracted from the root of Tripterygium wilfordii Hook.f, a plant of the family Wesleyanaceae, which has strong anti-inflammatory and immunomodulatory effects and has been used as a basic drug in the clinical treatment of RA. Despite the good efficacy of TwHF treatment, the mechanism of action of TwHF remains unclear. Several studies have demonstrated that the drug tripterygium glycosides, in which TwHF is the main ingredient, has achieved excellent efficacy in the clinical treatment of RA. Investigations have also found that TwHF can affect cellular RCD, cell migration, cell proliferation, and the apoptosis-related Hippo signaling pathway. In this study, we first analyzed the RCD and migration differences of neutrophils in patients with RA through network pharmacology and transcriptome analysis. Subsequently, we used electron microscopy, immunofluorescence, and other methods to identify the RCD phenotype of neutrophils. In collagen-induced arthritis (CIA) model, we demonstrated that Triptolide (the main active ingredient in TwHF) could alleviate the progression of arthritis by reducing the bone destruction and the infiltration of neutrophils. Furthermore, in vitro experiments showed that Triptolide induced neutrophil apoptosis, inhibited the formation of neutrophil extracellular traps (NETs), and impeded the neutrophil migration process in a Hippo pathway-dependent manner. Taken together, these findings indicate that Triptolide has potential for treating RA and provide theoretical support for the clinical application of TwHF, as a traditional Chinese medicine, in RA.

1. Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by inflammatory changes in the joints, which are clinically manifested by swelling, pain, and even deformity [1]. Studies have shown that the absence or abnormal function of neutrophils in intrinsic immunity significantly affects the RA disease process, which includes neutrophil migration [2], infiltration [3], release of cytoplasmic inclusions [4,5], oxidative stress, and inflammatory factor release processes [6–8]. Numerous studies have confirmed that differences in the manner of neutrophil death similarly affect the disease process. Currently, types of cell death can be classified into accidental cell death (ACD)and regulated cell death (RCD)[9]. RCD is classified into apoptosis, autophagy, and programmed necrosis based on different morphological, biochemical, immunological, and genetic features, and programmed necrosis is further classified into iron death, pyroptosis, necrotizing apoptosis, and neutrophil extracellular traps (NETs) [10]. During homeostasis, non-inflammatory apoptosis is the main mode of

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neutrophil death. However, under pathological conditions, such as inflammation, the type of neutrophil death is altered to involve membrane lysis and extracellular release of damage-associated molecules, which triggers or exacerbates local tissue damage [10–12]. Under such conditions, NETs, as the most representative mode of death in the RCD of neutrophils, represent the main inflammatory necrotic process. Neutrophils can secrete numerous cytokines and chemokines, which cause neutrophil chemotaxis, leading them to migrate to the joint cavity and aggravate the inflammatory response [4]. Among these cytokines, IL-8 (also known as CXCL8), which is secreted by neutrophils and has the strongest chemotactic effect, plays a crucial role in the recruitment and activation of neutrophils.

Tripterygium wilfordii Hook.f (TwHF), a total saponin extracted from the root of the wilfordia plant, is mainly used in the clinical treatment of RA. However, its mechanism of action has not been fully clarified. Previous studies have confirmed that triptolide, the main component of TwHF, can effectively improve RA by downregulating the inflammatory function of neutrophils [13], while triptolide can activate cysteine protease 8 (CASP8) and cysteine protease 3 (CASP3) to induce apoptosis in pancreatic and cervical adenocarcinoma cells [14]. Meanwhile, in a mouse model of osteoarthritis (OA), triptolide has been shown to reduce the inflammatory response by interfering with the release of chemokines and reducing inflammatory cell migration [15]. Tan et al. showed that Triptolide activated the Hippo signaling pathway, attenuated the expression of YAP/TAZ, and blocked the progression of invasive melanoma [16]. The Hippo signaling pathway plays an important role in cell proliferation and apoptosis [7], and Du-Juan Dong's study demonstrated that Hippo pathway activation can deregulate cysteine asparaginase inhibition and thus regulate RCD [17]. Meanwhile, Sun et al. showed that the Hippo signaling pathway could promote the proliferation and migration of esophageal squamous cell carcinoma cells [18]. Therefore, we hypothesized that triptolide, which is an herbal ingredient, could intervene in RA disease progression by affecting the Hippo signaling pathway, causing it to intervene in RCD and neutrophil migration.

2. Materials and methods

2.1. Animals and reagents

Eighteen male mice (6 weeks old, 160–200 g) were provided by Nanjing Model Animal Center. All experimental procedures were reviewed and approved by the Ethics Committee of the First Affiliated Hospital of Baotou Medical College and were conducted in accordance with the Guidelines for the Care and Use System of Laboratory Animals. Triptolide was purchased from Lemaitan Pharmaceuticals; TM-25659 was purchased from MCE; the Annexin V-FITC Apoptosis Detection Kit and BCA Protein Concentration Measurement Kit were purchased from Biyun Tian; anti-caspase-3 and anti-caspase-8 antibodies were purchased from Proteintech; and anti-YAP antibody, anti-TAZ antibody, and anti-rabbit horseradish peroxidase antibody were purchased from Abcam.

2.2. Construction of collagen-induced arthritis (CIA) mice and progress monitoring

The modeling process of CIA mice was performed with reference to the method of Liu [19] et al. Briefly, One hundred microliters of Freund's adjuvant containing 4 mg/ml of *Mycobacterium tuberculosis* and 100 μL of emulsified type II collagen were injected intracutaneously into the root of the tail of DBA/I mice at 2 cm. After 21 days, the process for booster immunization was repeated to establish a CIA mouse model. The mice were randomly divided into control, CIA, and Triptolide (70 μg/kg/d injected every other day, n = 6) groups.Mice were executed after 60 days of drug treatment following successful modelling and were evaluated radiologically. Microcomputed tomography plain films of the hind limbs were obtained using a cone beam scanner (high-resolution in vivo X-ray microtomograph) to assess the imaging progression of the joints of mice. Subsequently, the mice were handled and the hind limbs were isolated for tissue fixation and HE and toluidine blue staining according to a previously reported [20] method.

2.3. Preparation of neutrophils

Venous blood (10 ml) was collected from healthy control or patients with RA in a sodium citrate anticoagulation tube or EDTA anticoagulation tube and mixed well. Neutrophils were sorted using the EasySepTM Neutrophil Sorting Kit.

2.4. Transmission electron microscopy scanning

Neutrophils were collected by adding Triptolide (200 nmol/L) with or without TM-25659 (YAP/TAZ agonist 10 μmol/L) and incubated for 2 h, before adding 2.5 % ambient glutaraldehyde fixative, fixing at room temperature for 5 min, and collecting the suspension in fixative. Subsequently, the cells were subjected to dehydration at room temperature, osmotic embedding, polymerization, ultrathin sectioning, and staining, before observing under a transmission electron microscope.

2.5. Flow cytometry analysis

The collected neutrophils were resuspended in a flow-through tube, and the experimental groups were treated with Triptolide (200 nmol/L) , with or without TM-25659 (YAP/TAZ agonist 10 μmol/L) for 2h. Subsequent treatment and analysis were performed according to the operating instructions of the Annexin V-FITC Apoptosis Detection Kit.

2.6. Immunofluorescence staining to detect NET formation

The collected peripheral blood neutrophils were inoculated into 24-well plates, and the experimental groups were pretreated with Triptolide (200 nmol/L), with or without TM-25659 (YAP/TAZ agonist 10 μmol/L), for 1 h. Subsequently, ionomycin (5 μmol/L) was added to stimulate the cells for 2 h, before fixing the cells with 4 % paraformaldehyde for 30 min and incubating with bovine serum albumin (BSA) at room temperature for 30 min. Subsequently, the primary antibody configured in BSA was added and incubated at 4 °C overnight. Following this, the corresponding secondary antibody was added and incubated for 1 h without light, after which it was rinsed with PBST three times. DAPI was then added to stain the nucleus, and it was again kept out of light for 10 min. After this, the entry of NETs and key proteins in the Hippo signaling pathway (YAP, TAZ) into the nucleus was observed under a fluorescence microscope.

2.7. Measurement of neutrophil chemotaxis function

The collected peripheral blood neutrophils were resuspended, and 600 μl of RPMI1640 medium (containing 10 % fetal bovine serum) was added to the lower layer of the transwell. Then, 200 μl of the cell suspension, along with Triptolide(200 nmol/L), was added to the upper layer, either with or without TM-25659(YAP/TAZ agonist 10 μmol/L), and incubated for 2 h. Following incubation, the chambers were immobilized in 4 % paraformaldehyde for 20 min and then placed in 1 % crystal violet solution for 15 min, the chamber was gently placed on a slide and observed under a microscope.

2.8. Immunoblotting method

Neutrophils were collected and RIPA lysate and PMSF were added to extract protein at a ratio of 100:1. The extracted protein was added to 10 % SDAS-PAGE gel, before transferring to PVDF membrane and incubating with primary antibodies corresponding to YAP, TAZ, CASP8,

CASP3 at 4 °C overnight. Following incubation, the appropriate secondary antibody was added, after which it was incubated again for 1 h without light. The reaction was visualized using SuperSignal™ West Femto Ultrasensitive Substrate.

2.9. Real-time fluorescence quantitative PCR

Peripheral blood neutrophils were collected from healthy controls (HC) and RA patients, and RNA was extracted according to the instructions of the TRIzol reagent. The RNA was reverse transcribed to obtain template cDNA, before adding master mix, primer, enzyme-free water, and template cDNA according to the instructions of the PCR kit. The mRNA expression levels of MST1, LATS1, SAV1, YAP, and TAZ were determined.

2.10. Active ingredient and target screening

Compound components and targets of TwHF were searched for on the Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform (TCMSP (https://tcmspw.com/tcmsp.php)) and screened for active ingredients based on oral bioavailability (OB) $\geq$ 30 % and drug-like properties (DL) $\geq$ 0.18. All targets were corrected using UniProt (https://www.uniprot.org/) to remove non-human targets. The keyword "Rheumatoid arthritis" was analyzed by the GeneCards (https://www.genecards.org) , NCBI (https://www.ncbi.nlm.nih.gov/gene) , OMIM (https://www.omim.org/) , TTD (https://db.idrblab.net/ttd/) , and PharmGkb databases (https://www.pharmgkb.org/) to search for human genes and screen out disease target genes. The screened drug targets and disease targets were taken as intersections to obtain common targets.

2.11. Protein interaction network construction and topology analysis

The drug and disease targets were entered into the STRING platform (https://cn.string-db.org/) , the biological species was set as "Homo sapiens," the confidence level was set at $>$ 0.9, and the protein interaction (PPI) network was constructed. The PPI network was imported into Cytoscape 3.8.0 software, and topology analysis was performed using the NetworkAnalyzer tool. Targets with greater than average values were selected as key targets by degree ranking.

2.12. Molecular docking

The protein structure of the key target was identified using RCSB PDB, and PyMOL was employed to remove its small molecule ligands and water molecules. Protein hydrogenation and charge calculation were completed using AutoDock Tools. The structure of the active ingredient was downloaded from PubChem and AutoDock Tools was used to check the charge balance and rotatable bonds of the small molecules. The range of docking boxes was selected based on the receptor active center. Finally, AutoDock Vina was used to calculate receptor and ligand docking and the structure with the lowest binding free energy (highest binding affinity) in the output was selected.

2.13. Transcriptome sequencing

Neutrophils obtained from peripheral blood samples of patients with rheumatoid arthritis (RA) were subjected to triptolide treatment, followed by transcriptome sequencing and quality control of the sequencing data. Subsequently, differential gene expression analysis was conducted using R language, and Gene Set Enrichment Analysis (GSEA) pathway analysis was performed. The identified target proteins were further analyzed using Cytoscape software for protein interaction network analysis. Finally, changes in gene expression before and after triptolide treatment were determined, with a focus on the expression and enrichment of genes related to apoptosis, programmed necrosis, and migration.

2.14. Statistical analysis

Statistical analyses were performed using GraphPad Prism 9.0. Data from multiple repeat experiments are presented as the means $\pm$ standard deviation (SD). Significant differences among the groups were evaluated by one-way analysis of variance (ANOVA) and Dunnett's *t*-test, and $p < 0.05$ was considered statistically significant. The number of replicates and/or the total number of animals are shown in the figure legends or within the figures.

3. Results

3.1. Neutrophils in patients with RA possess a death phenotype and abnormal migration function

The results of electron microscopy showed that apoptosis was the main cell death pattern of neutrophils in the HC group, which was characterized by cytoplasmic condensation, chromatin margination, and nuclear fragmentation. In contrast, programed necrosis was the main death pattern of neutrophils in the RA group, which was characterized by rupture of the plasma membrane, cytoplasmic translucency, swelling of the organelles, and retention of the nuclear membrane (Fig. 1A). The results of the transwell assay further clarified that neutrophils in patients with RA had increased chemotaxis ability(Fig. 1B) These results suggest that neutrophils from patients with RA have an abnormal death phenotype, suggesting that the balance between apoptosis and programed necrosis is disrupted. Therefore, to clarify the regulatory relationship between apoptosis and programed necrosis, we used the STRING database to visualize the gene interactions of apoptosis and programed necrosis pathways. We found that CASP8, FADD, RIPK1, RIPK3, TNF, and TP53 coexisted in both apoptosis and programed necrosis pathways, suggesting that these genes are able to regulate the RCD process, which determines the propensity for death homeostasis (Fig. 1C).

3.2. Network pharmacological analysis revealed that TwHF affects RCD and migration

Given the established therapeutic efficacy of TwHF in rheumatoid arthritis (RA) alongside the ongoing ambiguity surrounding its precise mechanism of action, our study undertook a preliminary examination of the disease targets influenced by its intervention in RA utilizing a network pharmacological database. This investigation aimed to ascertain whether the therapeutic benefits of TwHF are contingent upon the regulation of programmed cell death and chemotactic ability.

We obtained 131 TwHF targets by searching the TCMSP database. Using the GeneCards, NCBI gene, OMIM, TTD, and PharmGkb databases together with overlapping validation, 4040 disease-related targets were obtained. TwHF potential therapeutic targets were compared with 4040 RA-related candidate targets, and 94 overlapping target genes were targeted(Fig. 1D). We then imported the 94 target proteins into the STRING 11.0 online database for protein association analysis and imported the screened PPI networks into Cytoscape 3.8.0 for topology analysis (Fig. 1E) . The top 19 proteins are shown in Fig. 1F. The following proteins were found to be associated with RCD: CASP8, CASP3, B-lymphoblastoma-2 (bcl-2), tumor protein 53 (TP53), intercellular adhesion molecule 1 (ICAM-1), and TNF. The following proteins were associated with migration: interleukin-8 (CXCL8), serine/threonine kinase 1 (AKT 1), signal transducer and activator of transcription 1 (STAT1), signal transducer and activator of transcription 3 (STAT3), and V-Rel reticuloendothelial hyperplasia virus oncogene homolog A (RELA). This phenomenon implies that TwHF could potentially impact the advancement of rheumatoid arthritis by disrupting regulated cell death and cellular migration.

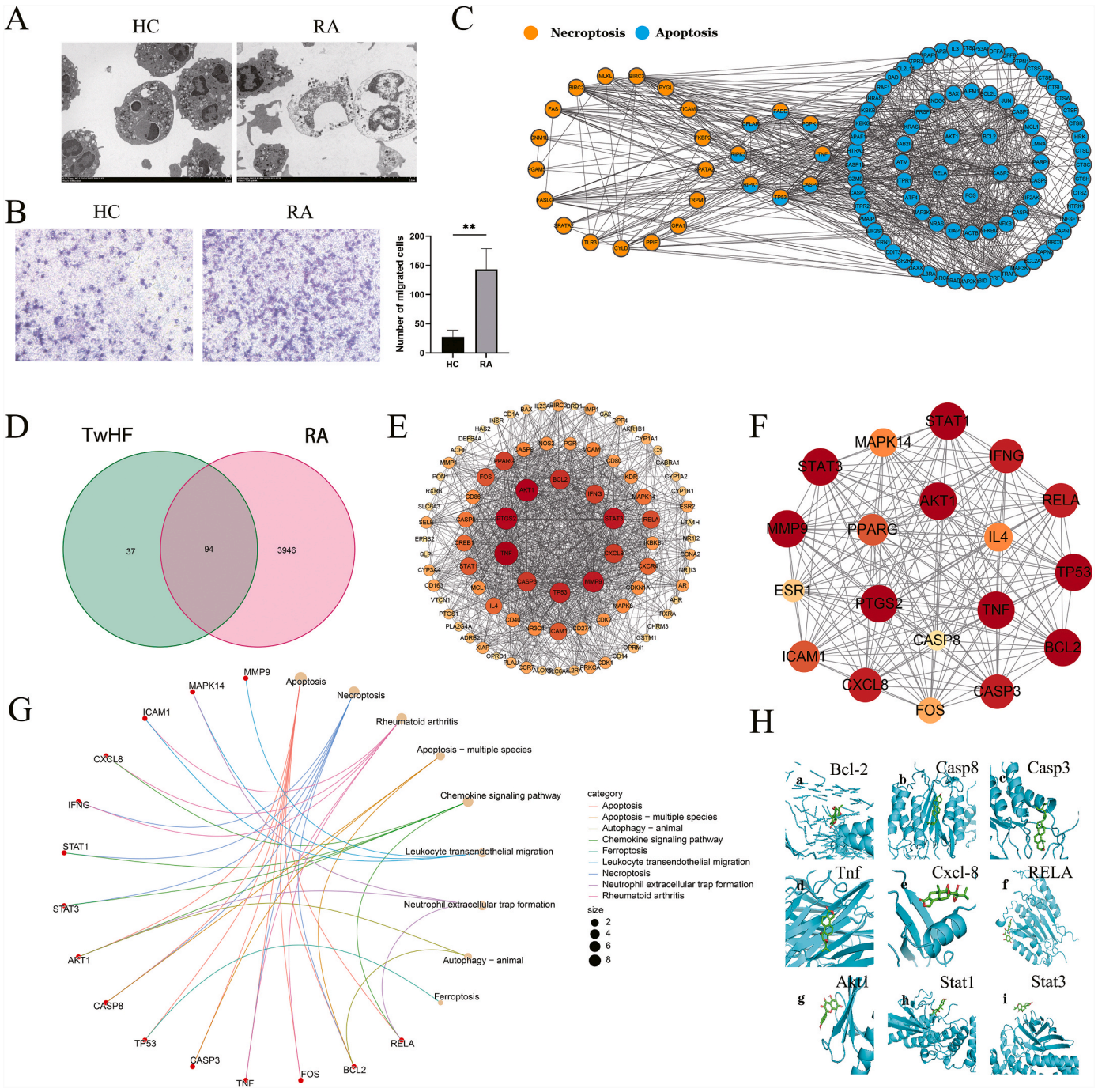

Fig. 1. Neutrophils in patients with RA possess a death phenotype and abnormal migration function and TwHF network pharmacology (A) Electron microscopic observation of the peripheral blood neutrophil death phenotype in patients with RA and healthy controls (HC). (B) Transwell assay showed the migration ability and quantitative analysis of neutrophils in peripheral blood of RA and HCs.(C) Network analysis between apoptosis and programmed necrosis pathway-related genes. (D) Venn diagram of cross-targeting between TwHF and RA. (E) Topological analysis of TwHF therapeutic RA targets. (F) Topological analysis of the first 19 targets of TwHF for RA (different colors represent different levels of importance in the network, with red and yellow representing more and less important, respectively. (G) KEGG pathway enrichment analysis. (H) Molecular docking modeling of TwHF with core targets , interacting with TwHF molecules, represented by the green stick model. The results were expressed as mean ± SD. All experimental results were repeated three times. *p < 0.05; **p < 0.01; ***p < 0.001; ns , not significant. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Nineteen proteins were subjected to KEGG pathway enrichment analysis, and several pathways affecting RA disease were identified, including the apoptotic pathway, programed necrosis pathway, iron death, neutrophil extracellular trap network formation, chemokine signaling pathway, and other pathways related to RCD and migration. Nine KEGG enrichment analyses were performed(Fig. 1G).

In order to more precisely focus on the regulatory cell death (RCD) and chemotactic genes impacted by TwHF , We molecularly docked the core genes related to RCD and migration that are shared by TwHF and RA with the core TwHF targets. The results showed that TwHF can dock well with CASP8, CASP3, bcl-2, TNF, CXCL8, AKT 1, STAT1, STAT3, and RELA, which are closely related to RCD and migration(Fig. 1H).

3.3. Triptolide improves joint inflammation in CIA mice

The in vivo results of Triptolide showed that Triptolide treatment significantly reduced joint swelling and arthritis scores in CIA mice (Fig. 2A and B), which was consistent with the histopathological evaluation and imaging results of the ankle joints of the mice. The results of joint imaging showed more inflammation and bone destruction, as well as more pronounced joint space narrowing and bone erosion(Fig. 2C). The results of HE and TB staining also showed that, compared to the control group, the ankle joints of the mice in the CIA group exhibited increased inflammatory cell infiltration and synovial hyperplasia, together with obvious cartilage erosion and bone destruction(Fig. 2D). Triptolide treatment significantly alleviated these joint symptoms and significantly inhibited the infiltration of joint neutrophils (MPO labeled) in the synovial tissues of CIA mice(Fig. 2E). This finding aligns with our initial hypothesis that triptolide modulates neutrophil migration.

3.4. Transcriptome expression of neutrophils in patients with RA treated with triptolide

To further demonstrate that the therapeutical effect of Triptolide in RA is dependent on the regulation of the RCD process in neutrophils, we

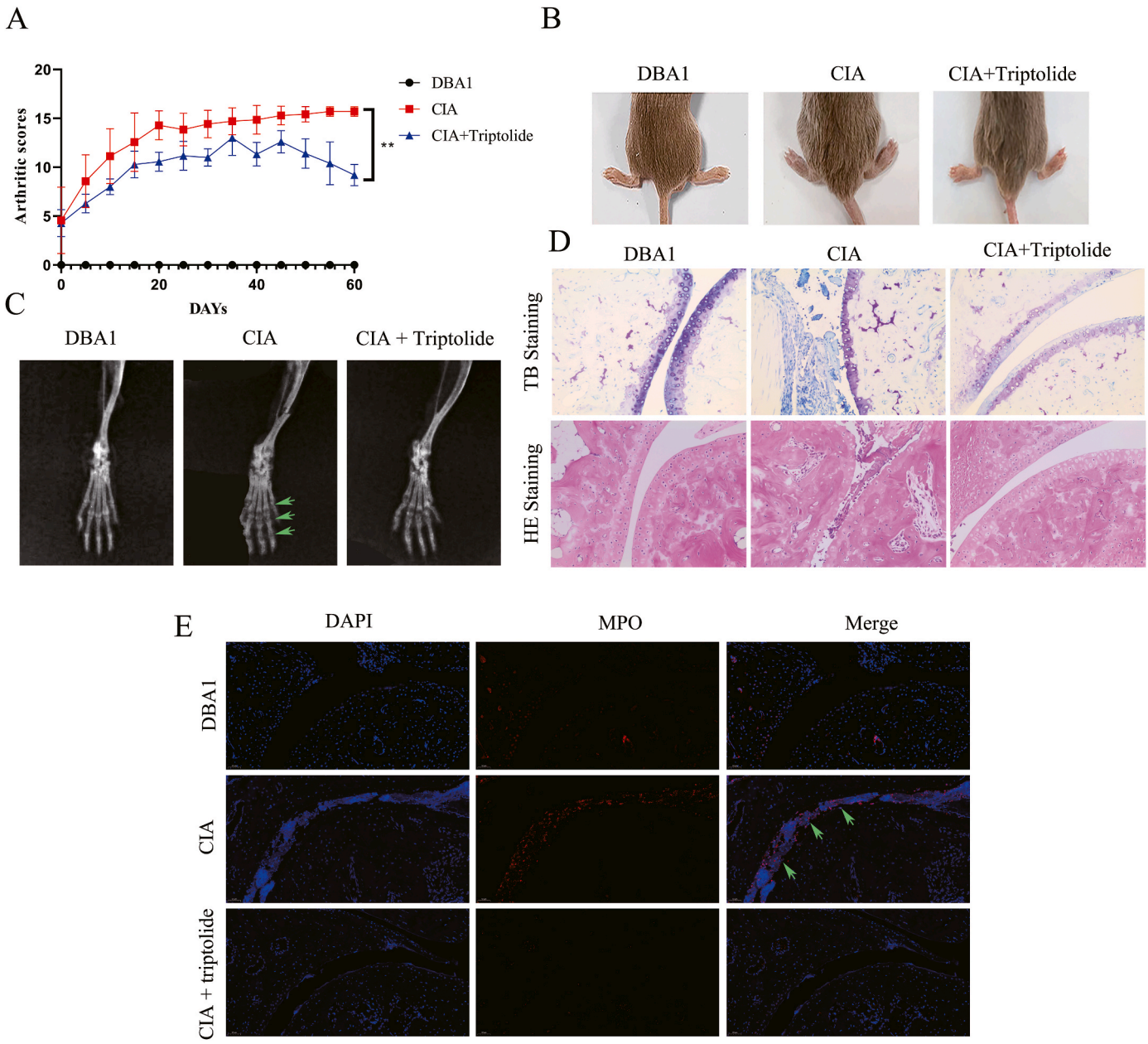

Fig. 2. Triptolide improves arthritis in collagenous arthritis mice
Mice were intraperitoneally injected with Triptolide (at a dose of 70 μg/kg/d per mouse, every other day). (A) Arthritis scores of the mice. (B) Representative images of the joints of each group of mice on the 60th day of treatment. The severity of arthritis was graded on a scale of 0–4. Data were used to compare the Triptolide treatment and collagen-induced arthritis (CIA) groups and are expressed as the SD ± mean (n = 6). (C) Microcomputed tomography flat sections of the ankle hindlimb of mice treated for 60 days to assess bone destruction in mice. (D) H&E staining and toluidine blue staining of ankle joint sections from mice treated for 60 days to detect histopathologic changes in the synovial membrane and bone erosion and destruction. (E) Immunosilver light staining to observe the expression of myeloperoxidase (MPO) in the ankle joints of mice treated for 60 days. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

treated neutrophils from patients with RA with Triptolide and performed transcriptome sequencing analysis. We screened for differential genes using the "DESeq2" package in R language, which showed that 389 differential genes appeared in neutrophils from patients with RA after Triptolide treatment ($P < 0.05$), including 44 upregulated differential genes and 345 downregulated differential genes(Fig. 3A and B). To further explore the specific relationship between the enrichment of 389 differential genes, the C2 KEGG dataset in GSEA software was used to compare the gene enrichment of differentially expressed genes in the Triptolide drug intervention group. The genome screening standard was $P < 0.05$. The results are shown in Fig. 3C, which shows that the differentially expressed genes in the apoptotic, programed necroptosis, and migration-associated pathways were enriched in the Triptolide intervention group. Among them, BCL-2, TP53, CASP3, CASP8, TNF, AKT1, FADD, RIPK1, RIPK3, MLKL, and CXCL8, which are the core genes that control the RCD trend, and the genes related to RCD and migration in the 19 core genes before the network pharmacological screening were enriched in the relevant pathways analyzed by GSEA (Fig. 3D). Triptolide treatment increased the expression of CASP3, and decreased the expression of CXCL8.

3.5. *Triptolide affects the RCD and migration of neutrophils in RA*

To confirm the effect of Triptolide on the RCD process and migration

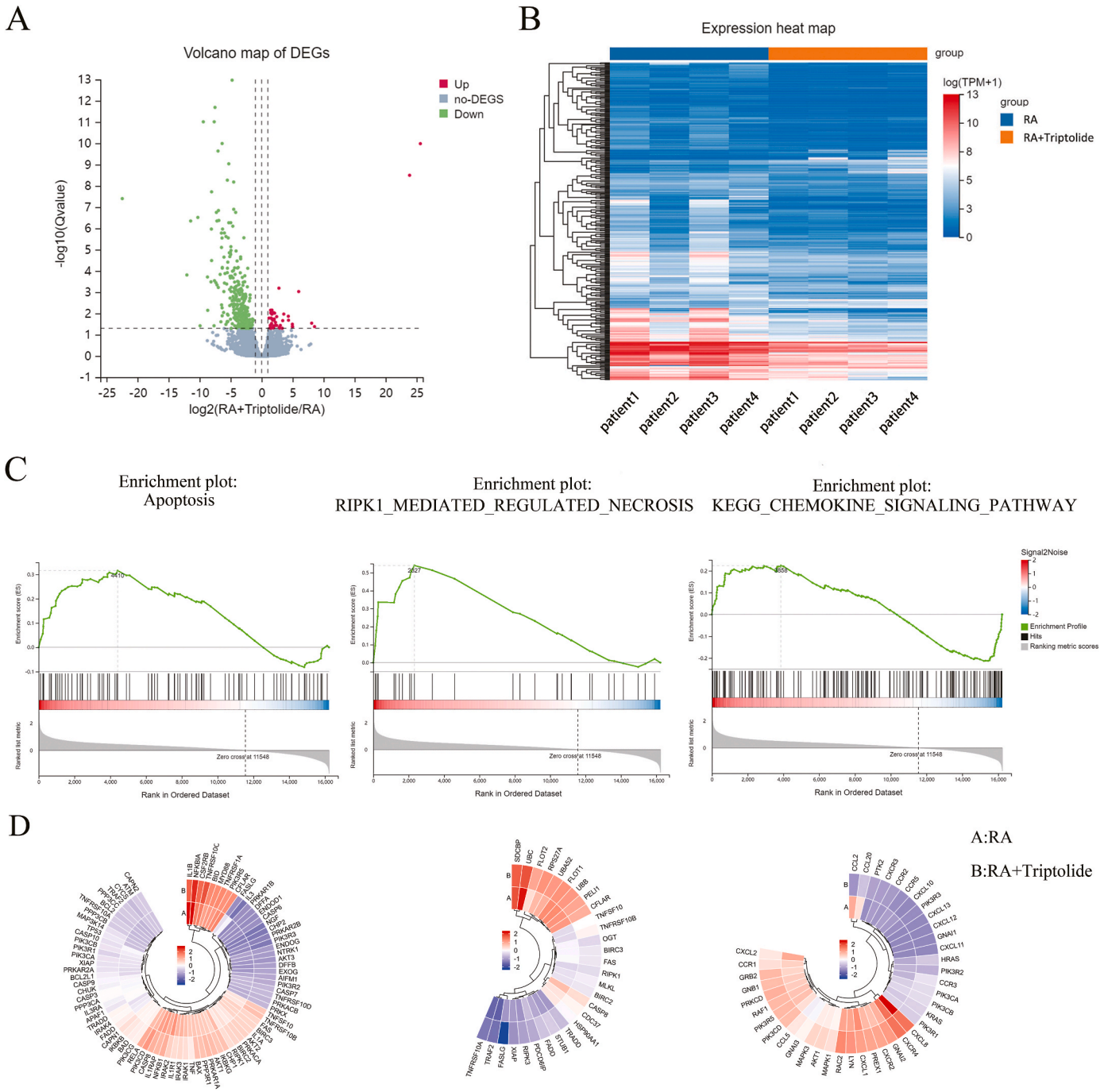

Fig. 3. Transcriptome expression of neutrophils in patients with RA treated with Triptolide (A, B) Volcano and heatmaps showing differentially expressed genes before and after **Triptolide** intervention. (C,D)Graphs showing the enrichment of differentially expressed genes in apoptotic, programmed necrotic, and migratory pathways, as well as the differential expression of related genes on the pathways after Triptolide intervention obtained using GSEA.

ability of neutrophils in patients with RA, we next conducted a series of in vitro experiments using Triptolide intervention on peripheral blood neutrophils. Apoptosis was the predominant death mode of neutrophils after Triptolide intervention, as observed by electron microscopy (Fig. 4A). Meanwhile, flow cytometry staining showed that Triptolide treatment significantly increased the percentage of apoptotic cells (membrane associated protein V+, PI-), with early apoptosis accounting for 7.83 % vs. 17.1%in RA vs. Triptolide, and late apoptosis accounting for approximately 3.59 % vs. 3.01 % in RA vs. Triptolide(Fig. 5F). Moreover, following ionomycin-induced NET formation and Triptolide intervention, we used immunofluorescence staining to observe whether Triptolide could inhibit NETosis. The results showed that neutrophil NETs were significantly reduced after Triptolide intervention(Fig. 4B). Taken together, these results indicate that Triptolide intervention could induce neutrophil apoptosis, inhibit neutrophil NET formation, and regulate the apoptosis–NETosis balance (see Fig. 6.

Given their migratory and invasive properties, neutrophils can migrate to the joint cavity and aggravate the joint reaction. The results of our transwell experiments showed that Triptolide intervention could significantly inhibit the migration of neutrophils(Fig. 4C), which is consistent with our results of the neutrophil-specific staining of synovial membranes of Triptolide-intervened CIA mice(Fig. 2E).

3.6. Triptolide affects the RCD and migration of neutrophils in RA in a Hippo pathway-dependent manner

Recent research has substantiated the pivotal role of the Hippo pathway in the regulation of cell death and migration. Consequently, our initial investigation involved the assessment of differential expression of molecules associated with this pathway in individuals with RA compared to healthy controls using Western blot and qPCR. The results showed that the upstream genes of the proliferation- and apoptosis-related Hippo signaling pathway, including MST1, LAST1, and SAV1, were significantly downregulated compared to those of the HC group, whereas the downstream target genes, YAP and TAZ, were upregulated. This result suggests that the Hippo signaling pathway is turned off in patients with RA, which may explain the imbalance of neutrophil death and abnormal migration(Fig. 5A and B).

To further verify the effect of Triptolide on the effector proteins of the Hippo signaling pathway, YAP and TAZ, we verified the activation of YAP and TAZ by qPCR and immunofluorescence staining. The qPCR results showed that the Hippo signaling pathway was activated and the expression of the downstream target genes YAP and TAZ was reduced after Triptolide intervention compared to that in the RA group(Fig. 5C). Immunofluorescence staining showed that YAP and TAZ entry into the nucleus was significantly reduced after Triptolide intervention (Fig. 5D).

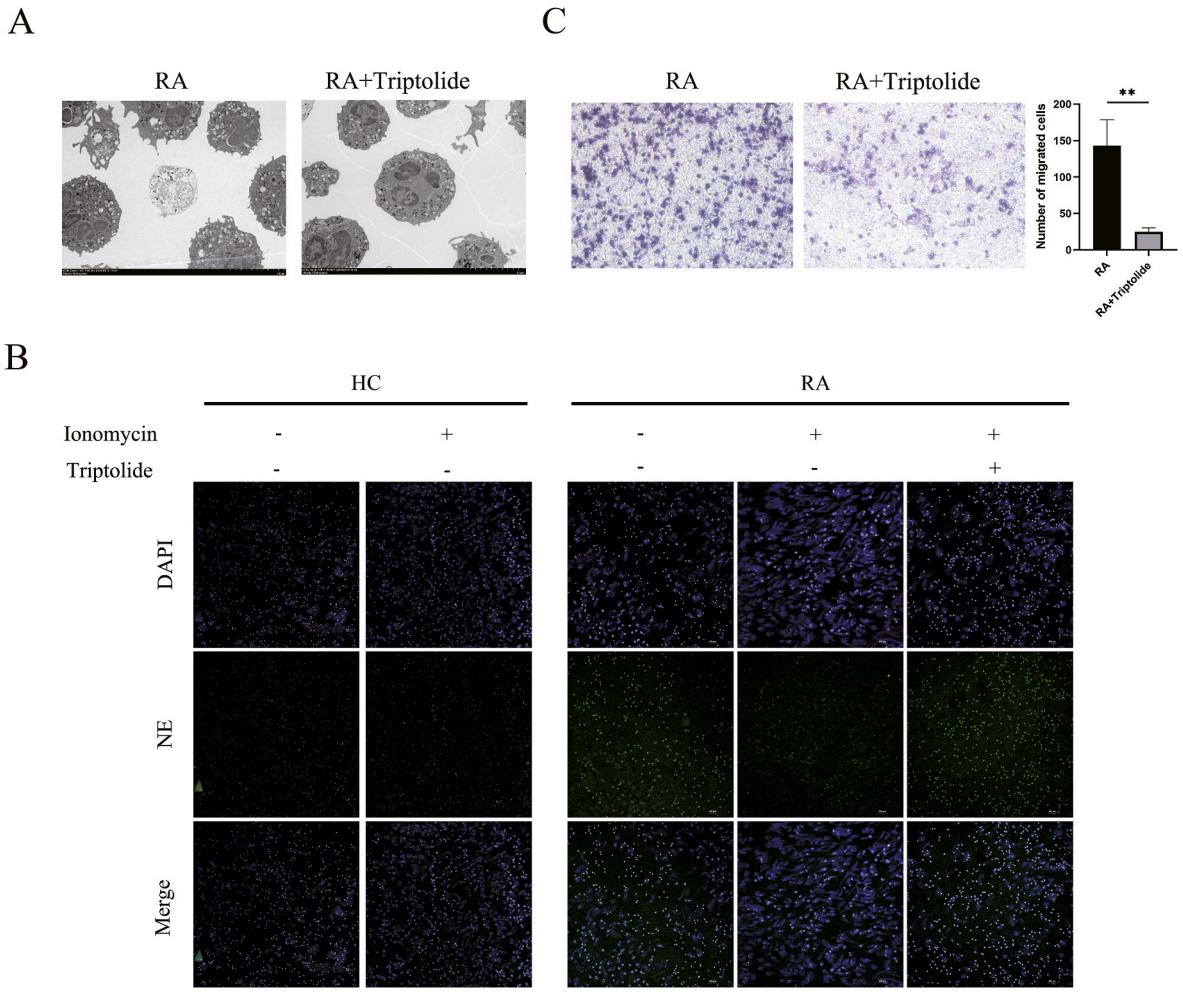

Fig. 4. Triptolide affects the RCD and migration of neutrophils from patients with RA. Neutrophils were stimulated with Triptolide (200 nmol/L) for 2 h and then collected for transmission electron microscopy, immunofluorescence staining, and transwell experiments. (A) Cell death morphology after Triptolide intervention was observed under an electron microscope. (B) Ionomycin-induced NET formation was visualized by staining the cells with DAPI (blue) and anti-NE antibody (green) and observed using a confocal microscope. (C) Observation of the migration ability of peripheral blood neutrophils after intervention with Triptolide using Transwell method and quantitative analysis。The results were expressed as mean ± SD. All experimental results were repeated three times.*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ns , not significant. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

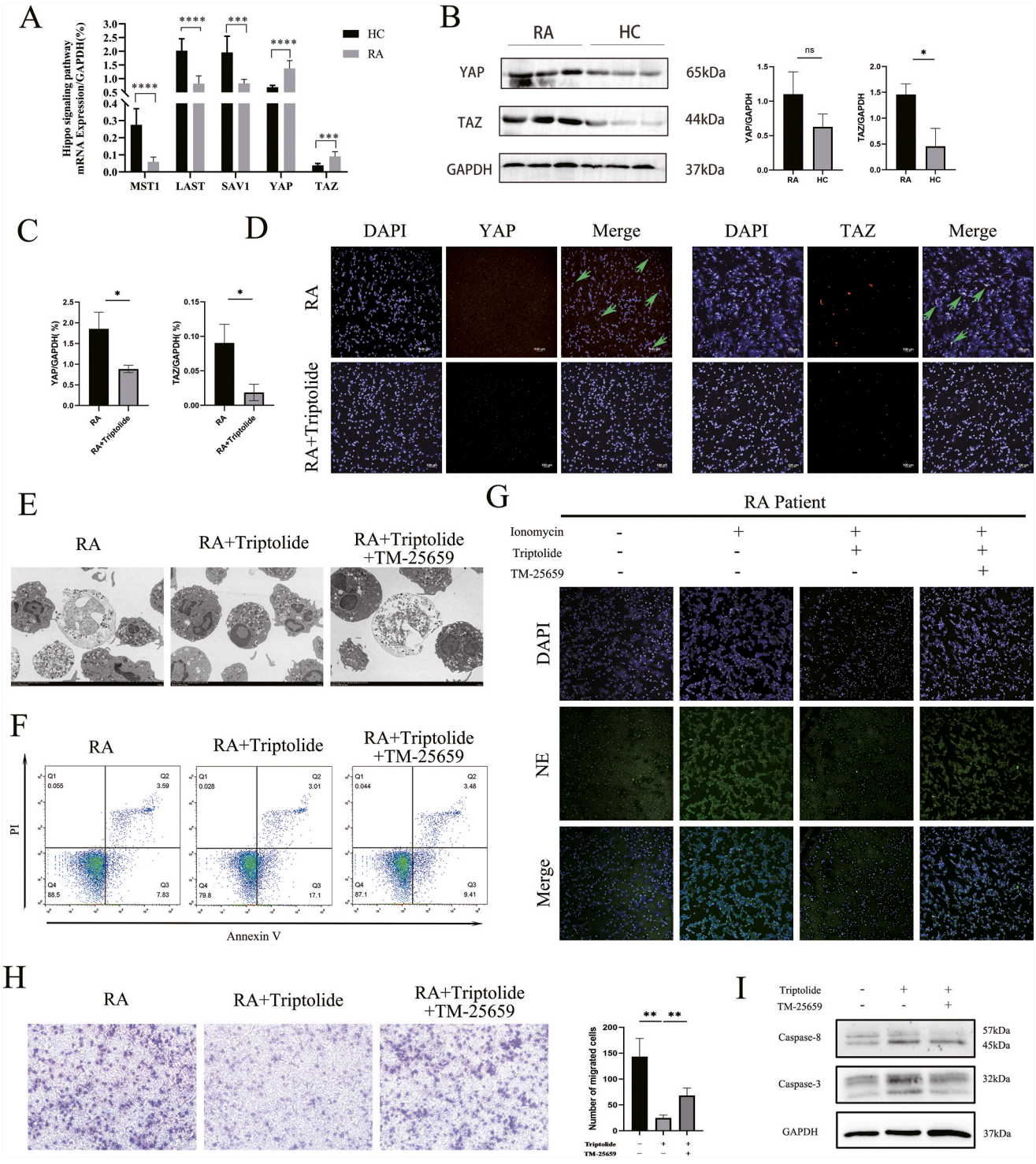

Fig. 5. Hippo pathway-dependent RCD and migration of neutrophils from patients with RA affected by Triptolide. (A, B) Differences in the Hippo signaling pathway between peripheral blood neutrophils in the RA and HC groups. The mRNA levels of proteins upstream and downstream of the Hippo signaling pathway were assessed using qPCR and normalized against GAPDH. Perform protein blotting to analyze the expression of YAP and TAZ and perform quantitative analysis; Neutrophils were stimulated by Triptolide (200 nmol/L) along with TM-25659 for 2 h. (C) The mRNA levels of YAP and TAZ after Triptolide intervention were assessed using qPCR and normalized against GAPDH. (D) Incorporation of YAP and TAZ into the nucleus was visualized by DAPI (blue) and anti-YAP and anti-TAZ antibody (red) cell staining under confocal microscopy. (E) Cell death morphology after the addition of TM-25659 was observed under electron microscopy. (F) The effect of TM-25659 on neutrophil apoptosis was analyzed using flow cytometry. (G) NET formation after the addition of TM-25659 was visualized by DAPI (blue) and anti-NE antibody (green) cell staining using confocal microscopy. (H)Observation of the migration ability of peripheral blood neutrophils after adding TM-25659 using Transwell assay and quantitative analysis (I) Protein blotting analysis of CASP8, CASP3, and its shearsomes.The results were expressed as mean ± SD. All experimental results were repeated three times.*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ns，not significant. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

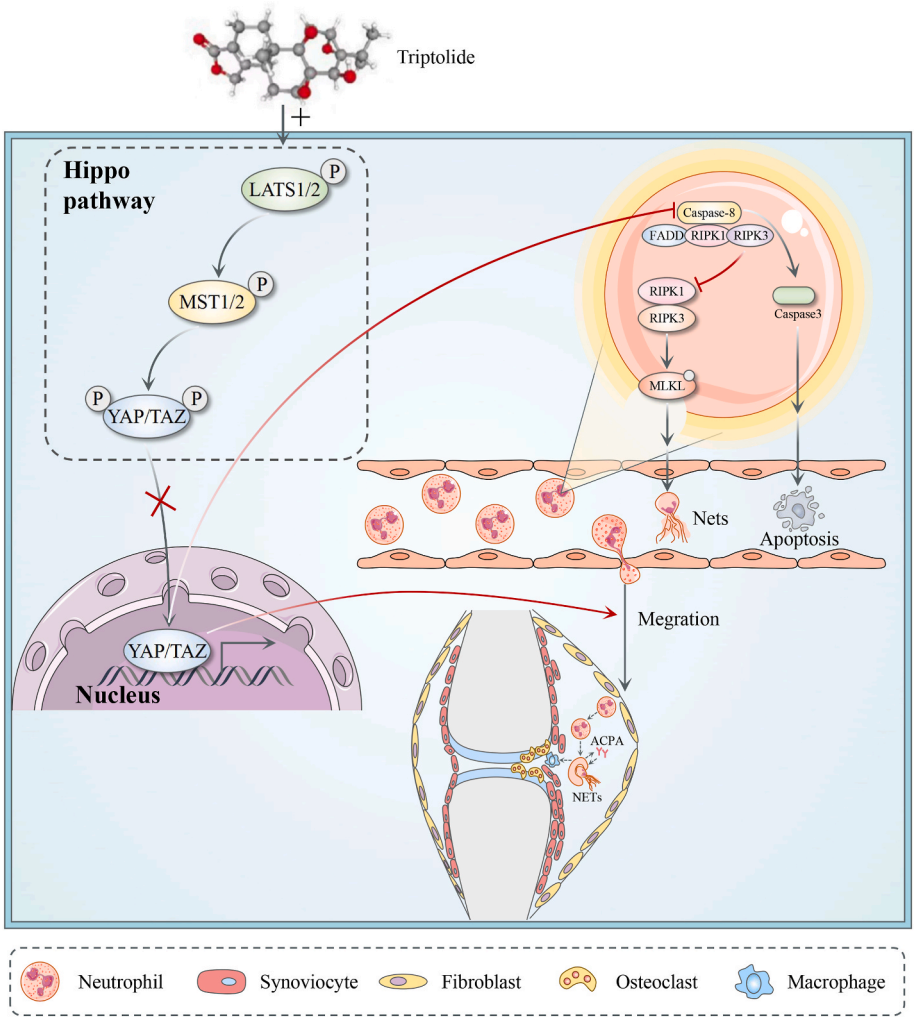

Fig. 6. Demonstration of how Triptolide reduces RA by regulating the neutrophil apoptosis–NETosis balance and migration through the Hippo signaling pathway.

These findings indicate that triptolide is a potent inhibitor of the expression and nuclear translocation of YAP and TAZ, thereby disrupting their regulatory effects on downstream genes.

In addition, after the administration of TM-25659 (YAP/TAZ agonist), both electron microscopy and flow assay showed that neutrophil apoptosis was inhibited(Fig. 5E and F), while immunofluorescence showed that the formation of NETs increased(Fig. 5G). The transwell results suggested that cell migration was significantly more active than that following Triptolide monotherapy intervention, which suggests that the inhibition of the Hippo pathway blocked the therapeutic effect of Triptolide (Fig. 5H).

To further investigate the pathway by which Triptolide regulates the balance of apoptosis–NETosis in neutrophils, we analyzed the expression of core proteins in apoptosis by western blotting. The results are shown in Fig. 5I, After Triptolide intervention, the expression of apoptosis-associated CASP8, CASP3, and their shear body proteins increased. The regulatory effect of Triptolide on the proteins was reversed by the addition of TM-25659, which further verified at the protein level that Triptolide regulates the balance between neutrophil apoptosis and NETosis and that the process is partly through the Hippo signaling pathway.

4. Discussion

Neutrophils are the first responders to the inflammatory response, and neutrophil infiltration to the synovial tissue of RA joints release pro-inflammatory factors that exacerbate joint degeneration [21]. In our experiments, we found several activated neutrophils in the joint cavity of CIA mice. These cells play a key role in the process of cartilage and bone destruction in RA joints, which has been confirmed in the study of H. L. Wright [22].

It has also been confirmed that neutrophil RCD abnormalities play a critical role in the pathogenesis of RA, and previous studies have shown delayed neutrophil apoptosis in the peripheral blood and synovial tissues of patients with RA [22,23]. In contrast, cytokines that may affect delayed neutrophil apoptosis (GM-CSF, TNFα, and IL1β) are highly expressed in RA [24]. In addition, the hypoxic environment in the joints of patients with RA leads to elevated MCL1 expression in neutrophils and delays the apoptotic process [25]. The presence of a large number of neutrophils in the synovial tissues of patients with RA induces NETosis in an inflammatory environment. Citrulline is one of the major factors mediating disease progression in RA, and the accumulation of large numbers of NETs in the synovial tissues of patients with RA further stimulates the formation of anti-citrullinated peptide antibodies (ACPA). Taken together, these results suggest that the mode of neutrophil death has an important impact on RA. Interestingly, we not only found an altered RCD morphology in neutrophils from RA patients, but also observed that the RCD process in RA patients was more inclined towards programmed necrosis. Furthermore, programed necrosis was the primary form of neutrophil death in patients with RA, while apoptosis was the primary form in neutrophils from the HC group. Therefore, we believe that interfering with the neutrophil death mode and migration

process may represent a new tool for the treatment of RA.

Currently, clinical medications for RA mainly include methotrexate, nonsteroidal anti-inflammatory drugs, and biologics against various molecular targets, all of which are known to affect disease progression to a certain extent by intervening in the apoptotic process. Indeed, methotrexate intervention induces caspase pathway activation, which induces the cleavage of DNA fragments mediating the apoptotic process in FLS cells, thereby relieving joint symptoms in patients with RA [26]. In addition, various nonsteroidal anti-inflammatory drugs can effectively inhibit the proliferation and induce apoptosis of synovial cells [27]. As the application of new biological agents becomes increasingly widespread, the mechanism of their intervention becomes clearer. Infliximab, as a monoclonal antibody to TNF-α, has been shown to partially promote the caspase-dependent apoptotic cell death process [28]. In contrast, rituximab, which targets CD20, is involved in the therapeutic process of RA by inducing the apoptotic process of B cells and blocking the antibody-mediated cytotoxicity of B cells [29].

TwHF is the main bioactive ingredient extracted from the root of Tripterygium wilfordii Hook.f; however, the immune mechanism underlying its modulation of neutrophil death mode and migration remains unclear. Here, we used the results of network pharmacology and transcriptome sequencing to investigate the relevant targets and pathways of TwHF for treating RA. The core genes obtained from network pharmacology for TwHF treatment of RA are closely related to RCD and cell migration. We analyzed the transcriptome expression after Triptolide (the main active ingredient in TwHF) intervention in neutrophils, and the results of GSEA showed a significant enrichment of differential genes in the apoptotic, programed necrotic, and migration-related pathways after Triptolide drug intervention. Among them, the pathway-related regulatory genes were consistent with the core genes controlling the RCD trend and those related to RCD and migration by network pharmacological screening.

In this study, we observed that Triptolide could mitigate RA disease progression by regulating the neutrophil apoptosis–NETosis balance and inhibiting neutrophil migration. Normally, the non-cleaved form of neutrophil apoptosis does not induce inflammation; however, in the context of pathology or inflammation, neutrophils that choose the cleaved form of programed death induce inflammation and tissue damage due to the release of intracellular contents, ultimately accelerating the disease process. Although we previously considered neutrophil apoptosis and NETs to be relatively independent death processes, there is growing evidence of crosstalk between the two pathways. CASP8 is thought to play a critical role in the balance between neutrophil apoptosis and NETs [30–32]. CASP8 inhibits programmed necrosis mediated by RIPK1 and RIPK3 [33,34]. Moreover, when CASP8 activity is blocked, RIPK3 and RIPK1 form necrosomes and form kinase structural domain-like (MLKL) phosphorylations that insert themselves into the cell membrane, causing cell swelling and rupture and triggering NETs [35–37]. Our results showed that after Triptolide intervention, neutrophils exhibited increased expression of apoptosis-associated CASP8 and CASP3, together with increased rates of apoptosis. Furthermore, NETs were significantly reduced after Triptolide intervention. We believe that elevated CASP8 binds to RIPK1 and RIPK3 to form a complex, which prevents RIPK1 and PIPK3 from forming a death body alone, and further phosphorylates MLKL to trigger NETs. These results suggest that Triptolide attenuates RA disease progression.

Notably, in addition to the form of cell death, the ability of neutrophils to migrate is another key factor influencing the onset and progression of RA. Neutrophils migrating into the joint cavity release numerous NETs, which promote the production of ACPAs, release immunostimulatory factors such as IL-6 and IL-8, and exacerbate the autoimmune response. In turn, ACPAs stimulate the production of NETs, resulting in a vicious circle [33]. NETs can also stimulate fibroblast-like synoviocytes to release nuclear factor kB receptor activator ligand (RANKL), which in turn promotes the formation and activation of osteoclasts, further aggravating the inflammatory response and bone destruction. Transwell experiments have revealed that the migration ability of neutrophils is significantly diminished after Triptolide intervention and that neutrophil infiltration is significantly reduced in the joints of CIA mice after drug intervention. These results are consistent with those of our network pharmacology and transcriptome sequencing.

In addition, the core kinase of the Hippo signaling pathway, which is involved in organ size regulation, growth and development, and cell proliferation and apoptosis [7], consists of MST1/2 and LATS1/2. Phosphorylated core kinase further phosphorylates the YAP/TAZ complex, which prevents it from entering the nucleus of the cell, inhibits its transcriptional co-activation function, and further inhibits its effects on downstream target genes. When the classical Hippo signaling pathway is blocked, unphosphorylated YAP/TAZ enters the nucleus to exert transcriptional co-activation and binds to TEAD structural domain family transcription factors to regulate the expression of downstream target genes [38]. Recent studies have shown that the Hippo signaling pathway plays a pathogenic role in RA, which may be related to its regulation of cellular RCD and migration. Our study revealed a decrease in the Hippo signaling pathway-associated downstream core proteins YAP and TAZ in neutrophils following Triptolide intervention. While the therapeutic efficacy of Triptolide was diminished with the inclusion of pathway inhibitors, we speculate that the impact of Triptolide on neutrophil regulated cell death (RCD) and migration may be partly reliant on the Hippo signaling pathway.

Although our study provides an in-depth study on the effect of Triptolide on the RA disease process by interfering with neutrophil RCD and migration, there are some limitations that warrant discussion. First, TwHF has achieved good therapeutic efficacy in the clinical treatment of RA, but because it is often used in combination with other drugs, it was not possible to obtain samples of neutrophils from patients treated with monotherapy, and therefore we failed to monitor the effect of TwHF on the neutrophil RCD process in the patients' bodies. The key regulatory genes for this process were not clear. In this research, an analysis of the transcriptome profiles of peripheral blood neutrophils from rheumatoid arthritis (RA) patients before and after triptolide treatment revealed key genes that are significantly linked to the RCD process. This analysis clarified the importance of the abnormal RCD process in neutrophils for the disease progression of RA and identified potential targets among the key regulatory genes.

5. Conclusion

In this study, we found that alteration of the neutrophil death balance and the effect of Triptolide on cell migration may be the underlying factors responsible for its ability to alleviate RA disease progression from the perspective of cell-regulated death. This finding will likely guide the combination of this drug with other RA disease-intervening drugs to maximize its clinical application prospects.

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CRediT authorship contribution statement

Pengyuan Liu: Writing – original draft, Methodology. **Huiyang Liu:** Supervision, Methodology. **Yali Sang:** Methodology. **Lingyan Zhu:** Data curation. **Peiyao Zhang:** Methodology. **Chunyan Pang:** Supervision, Software, Investigation. **Yongfu Wang:** Supervision, Project administration. **Li Bai:** Writing – review & editing, Writing – original draft, Funding acquisition.

Declaration of competing interest

We affirm that all authors concur with the submission and that the material submitted for publication has not been previously reported and is not under consideration for publication elsewhere, and the authors declare that they have no competing financial interests. All authors confirm that clinical samples collected from patients which has signed informed consents, and the Ethics Committee of Baotou Medical College has approved this study.

Data availability

The data that has been used is confidential.

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