

Ubiquitin D promotes the progression of rheumatoid arthritis via activation of the p38 MAPK pathway

HONG CHEN^{1*}, LIJU TAO^{1*}, JUHUA LIANG², CHUNFENG PAN¹ and HUA WEI³

¹Department of Rheumatology and Immunology; ²Laboratory Department; ³Department of General Practice, Affiliated Hospital of Youjiang Medical University for Nationalities, Baise, Guangxi 533000, P.R. China

Received July 14, 2022; Accepted November 18, 2022

DOI: 10.3892/mmr.2023.12940

Abstract. Ubiquitin D (UBD), a member of the ubiquitin-like modifier family, has been reported to be highly expressed in various types of cancer and its overexpression is positively associated with tumor progression. However, the role and mechanism of UBD in rheumatoid arthritis (RA) remain elusive. In the present study, the gene expression profiles of GSE55457 were downloaded from the Gene Expression Omnibus database to assess differentially expressed genes and perform functional enrichment analyses. UBD was overexpressed by lentivirus transfection. The protein level of UBD, p-p38 and p38 in RA-fibroblast-like synoviocytes (FLSs) were examined by western blotting. Cell Counting Kit-8 and flow cytometry assays were used to detect the functional changes of RA-FLSs transfected with UBD and MAPK inhibitor SB202190. The concentrations of inflammatory factors (IL-2, IL-6, IL-10 and TNF- α) were evaluated using ELISA kits. The results revealed that UBD was overexpressed in RA tissues compared with in the healthy control tissues. Functionally, UBD significantly accelerated the viability and proliferation of RA-FLSs, whereas it inhibited their apoptosis. Furthermore, UBD significantly promoted the secretion of inflammatory factors (IL-2, IL-6, IL-10 and TNF- α). Mechanistically, elevated UBD activated phosphorylated-p38 in RA-FLSs. By contrast, UBD overexpression and treatment with the p38 MAPK inhibitor SB202190 not only partially relieved the UBD-dependent effects on cell viability and proliferation, but also reversed its inhibitory effects on cell apoptosis. Furthermore, SB202190 partially inhibited the effects of UBD overexpression on the enhanced secretion of inflammatory

factors. The present study indicated that UBD may mediate the activation of p38 MAPK, thereby facilitating the proliferation of RA-FLSs and ultimately promoting the progression of RA. Therefore, UBD may be considered a potential therapeutic target and a promising prognostic biomarker for RA.

Introduction

Rheumatoid arthritis (RA) is a long-term, complex inflammatory relapsing autoimmune disorder in which the immune system mistakenly attacks the joints. RA has a prevalence of ~1% of the global population (1). RA is characterized by inflammation of the synovial joints, which eventually leads to cartilage damage and bone destruction (2). The pathogenesis of RA is not fully known due to the lack of knowledge regarding the etiology of this disease (3). The preliminary event in RA pathogenesis is believed to be the presence of immune complexes in the bloodstream, which is considered the pre-articular phase, during which the generation of autoantibodies against host tissue occurs (4). Notably, certain serological markers can be detected to diagnose disease initiation during this phase, such as citrulline antibodies (5). Subsequently, the transition phase occurs, during which a number of autoantibodies are produced and autoantigens are present in the articular joints. Autoantigens bind to the Fc receptor γ of IgG antibodies to activate the innate immune reaction via sentinel cells (6). Usually, dendritic cells (DCs) are activated as the first line of defense, which bind to autoantigens to induce the proliferation and differentiation of antigen-specific T cells (7,8). Activated DCs increase major histocompatibility complex II co-stimulatory surface molecules CD80/86 to activate the production of cytokines from naive T cells (9). Activated T-helper (Th) cells can activate B cells to produce autoantibodies, such as rheumatoid factor and anti-citrullinated protein antibodies, through plasma cells and can be carried over through different pathways for hyperplastic synovium, cartilage degradation and bone destruction (10-12). Certain molecules, such as microRNA (miR)-24, miR-125A-5p and miR-146a, have been identified as biomarkers for RA that could increase diagnostic accuracy (6,13). Unfortunately, there is currently no effective treatment that can be used to cure RA in the clinic. Medications that are currently used for the treatment of RA also induce prominent side effects alongside their clinical efficacy (14). For example, gastrointestinal adverse

Correspondence to: Professor Hua Wei, Department of General Practice, Affiliated Hospital of Youjiang Medical University for Nationalities, 18 Zhongshan Second Road, Youjiang, Baise, Guangxi 533000, P.R. China
E-mail: 515095448@qq.com

*Contributed equally

Key words: rheumatoid arthritis, ubiquitin D, p38 MAPK, proliferation, apoptosis

reactions such as abdominal pain, nausea, vomiting and diarrhea are common. Furthermore, drug resistance is another serious problem affecting RA treatment (15). Therefore, it is necessary to develop novel drugs or therapeutic strategies for RA.

Ubiquitin D (UBD), also known as FAT10, is a ubiquitin-like protein modifier that is mainly expressed in the tissues and organs of the immune system, including the thymus and lymph nodes (16). UBD expression has been shown to be positively regulated by interferon- γ and TNF- α (17,18). UBD may serve a significant role in immunomodulation, including antigen presentation, immune response and antiviral infection (19). Emerging evidence has confirmed that UBD is involved in a number of regulatory functions, including the cell cycle, apoptosis, autophagy, DNA repair and tumorigenesis (20). High UBD expression has been identified in a variety of tumor tissues, such as liver cancer (21), colorectal cancer (22) and breast cancer (23). Growing evidence has indicated that UBD has a pro-malignant role, due to its overexpression in a broad spectrum of tumor tissues. Notably, forced UBD expression has been reported to be associated with epirubicin resistance and the poor prognosis of triple-negative breast cancer (23). Furthermore, patients with UBD-positive colon cancer have a significantly higher recurrence rate and poorer disease-free survival than those with low UBD expression after radical surgery (24).

Notably, the role of UBD in RA remains to be elucidated. Therefore, the present study aimed to investigate the expression of UBD in RA samples from the Gene Expression Omnibus (GEO) database, and determine the enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways related to the aberrantly expressed UBD. Furthermore, the present study aimed to explore the effects of UBD on the proliferation, apoptosis and inflammatory cytokine production of RA-fibroblast-like synoviocytes (FLS), which have been revealed to play a pathogenic role in RA (25,26), as well as to confirm the underlying mechanism of UBD in RA.

Materials and methods

Identification of DEGs from GEO datasets of RA. To identify differentially expressed genes (DEGs) in RA, the GEO was used to assess RA data. The GSE55457 gene expression profiles were downloaded from the GEO database [<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE55457>; GPL96 platform, Affymetrix Human Genome U133A Array] (27). The GSE55457 dataset contains data from 79 samples, including 20 healthy control individuals, 33 patients with RA and 26 patients with osteoarthritis. The LIMMA Bioconductor package (<http://www.bioconductor.org/>) was used to identify DEGs by comparing the expression values between RA and normal tissue samples. A classical unpaired Student's t-test was used to identify DEGs that were statistically significant ($P < 0.05$ and $|\log_2FC| \geq 2$). Subsequently, to present significant DEGs, volcano plots were plotted using R software (version 3.4.0; <https://www.r-project.org/>).

KEGG pathway and gene ontology (GO) analysis of DEGs. In the present study, the clusterProfiler Bioconductor package (<https://bioconductor.org/packages/release/bioc/>

<http://clusterProfiler.html>) was used to perform the KEGG pathway analysis of the DEGs. The GO enrichment analysis (<https://david.ncifcrf.gov/>) was conducted via R software using the package 'GO plot' to explore the functions of the DEGs. $P < 0.05$ was considered to indicate a statistically significant difference.

Cell culture. Normal human FLSs (cat. no. 408K-05a) were obtained from Cell Applications, Inc. and the MH7A human RA-FLS cell line (cat. no. C0878) was purchased from Shanghai Guandao Biological Engineering Co., Ltd. Normal FLSs and RA-FLSs were incubated in DMEM (cat. no. 12430054) supplemented with 10% fetal bovine serum (FBS; cat. no. 10100147) and 1% penicillin/streptomycin (cat. no. 15070063) (all from Gibco; Thermo Fisher Scientific, Inc.). Normal FLSs and RA-FLSs were grown in 75-cm² flasks at 37°C in an incubator containing 5% CO₂. MAPK inhibitor SB202190 (10 μ M; cat. no. HY-10295; MedChemExpress) was used to treat RA-FLSs for 1 h at 37°C based on previous studies (28,29).

Vector construction and lentiviral infection. The UBD overexpression lentiviral vector (GV492) was purchased from Shanghai GeneChem Co., Ltd., and constructed based on the full-length coding protein sequences of human UBD (GenBank accession number NC_000006.12). A 3rd generation vector system was used. Lentiviral vector (5 μ g) was transfected into 293T cells (cat. No. CRL-3216; American Type Culture Collection) cultured on six-well plates with Lipofectamine[®] 3000 (Invitrogen; Thermo Fisher Scientific, Inc.). A total of 30 μ g of plasmids were used for lentivirus packaging, and the ratio of lentiviral plasmid: GV492: Lipofectamine[®] 3000 was 2:1:1. The UBD overexpression lentivirus (Lv-UBD) and blank GV492 plasmid vector (NC) lentivirus was obtained after 293T cells were cultured at 37°C for 4 days. RA-FLSs were then infected with the lentiviral vectors at an MOI of 20 for 6 h followed by replacement with fresh medium. RA-FLSs were grown for 48 h and subsequently treated with puromycin (PURO; 1 μ g/ml; InvivoGen) for 72 h to select transfected clones. The infected cells were then collected 96 h after infection to determine the infection efficiency.

Small interfering RNA (siRNA) transfection. UBD siRNA and control siRNA (a non-targeting siRNA-scrambled sequence) were constructed by Guangzhou Anernor Biotechnology Co., Ltd. The siRNA sequences were as follows: UBD siRNA 1#, 5'-ACCCATATGACAGCGTGAAAA-3'; UBD siRNA 2#, 5'-CCCATATGACAGCGTGAAAA-3'; UBD siRNA 3#, 5'-CAGCGTGAAAAAATCAAAGA-3' and control siRNA, 5'-UUCUCCGAACGUGUCACGUTT-3'. SiRNAs (10 μ g) were transfected into 5x10⁶ RA-FLSs using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) for 24 h at 37°C according to the manufacturer's protocols. The mRNA and protein expression levels of UBD were measured at 48 h post-transfection by reverse transcription-quantitative PCR (RT-qPCR) and western blotting, respectively.

RT-qPCR. RA-FLSs were collected, centrifuged at 1,000 x g for 5 min at 4°C, the supernatant was removed and 1 ml SuPerfecTRI[™] Total RNA Isolation Reagent

(cat. no. 3101-100; Shanghai Pufei Biotechnology Co., Ltd.) was added to the cell pellet to extract the total RNA. The concentration and quality of RNA were determined using an ND-2000 Spectrophotometer (NanoDrop; Thermo Fisher Scientific, Inc.). The extracted RNA was reverse transcribed into cDNA using the Promega M-MLV kit (cat. No. M1705; Promega Corporation). The samples were incubated for 60 min at 37°C. After heat inactivation of reverse transcriptase (95°C, 2 min), the first-strand cDNA was stored until use at -20°C. Subsequently, qPCR was performed with the KAPA SYBR FAST qPCR kit (Kapa Biosystems; Roche Diagnostics) using a SimpliAmp™ PCR System (Thermo Fisher Scientific, Inc.). The relative mRNA expression levels of each sample were calculated using the $2^{-\Delta\Delta C_q}$ method (30). The primer sequences were as follows: UBD, forward 5'-ATGCTTCCTGCCTCTGTGTG-3', reverse 5'-TGCCCTTTCTGATGCCGTAA-3'; and GAPDH, forward 5'-CTGACTTCAACAGCGACACC-3' and reverse 5'-GTGGTCCAGGGGTCTTACTC-3'.

Western blotting. Proteins were extracted from RA-FLSs using ice-cold RIPA lysis buffer (cat. no. P0013B; Beyotime Institute of Biotechnology) and protein concentration was assessed using the BCA method (cat. no. 23225; Thermo Fisher Scientific, Inc.). Proteins (5 µg) were mixed with 2X SDS sample buffer, separated by SDS-PAGE on 12% gels and transferred onto PVDF membranes, which were incubated with 5% non-fat milk for 2 h at room temperature. The membranes were then incubated overnight at 4°C with mouse anti-UBD (1:1,000; cat. no. ab168680; Abcam), rabbit anti-phosphorylated (p)-p38 (1:1,000; cat. no. ab178867; Abcam), rabbit anti-p38 (1:1,000; cat. no. ab170099; Abcam) and mouse anti-GAPDH (1:5,000; cat. no. 60004-1-Ig; ProteinTech Group, Inc.). Horseradish peroxidase-conjugated goat anti-mouse IgG (1:5,000; cat. no. BA1051; Boster Biological Technology) and horseradish peroxidase-conjugated mouse anti-rabbit IgG (1:5,000; cat. no. BM2006; Boster Biological Technology) were used as secondary antibodies to incubate the membranes for 1.5 h at room temperature. Immunoreactive protein bands were detected using the ECL hypersensitive chemiluminescence kit (cat. no. P0018M; Beyotime Institute of Biotechnology) with the Odyssey Scanning System (version 3.0; LI-COR Biosciences). ImageJ (version 1.8.0; National Institutes of Health) was used for semi-quantification.

Cell counting kit 8 (CCK-8) assay. RA-FLSs were seeded at a density of 2,000 cells/well in 96-well plates and were cultured in an incubator at 37°C with 5% CO₂ for 5 consecutive days. Subsequently, 10 µl CCK-8 solution (cat. no. 96992; MilliporeSigma) was added to each well and incubated for 3 h at 37°C. The optical density (OD) value at 450 nm was measured using a Spectrafluor microreader plate (Molecular Devices, LLC). These experiments were repeated three times.

ELISA. RA-FLSs were cultured in DMEM containing 10% FBS for 24 h. ELISA was performed in accordance with the instructions of the ELISA kits. In brief, the supernatant was collected after centrifugation at 1,500 g for 20 min at 4°C to detect the levels of IL-2, IL-6, IL-10 and TNF-α. IL-2 (cat. no. EH2IL22), IL-6 (cat. no. EH2IL6), IL-10 (cat. no. EHIL10) and TNF-α (cat. no. BMS223HS) ELISA kits were purchased

from Thermo Fisher Scientific, Inc. The calibration curves were plotted and the OD values of samples were calculated from the standard curve for three assays.

EdU incorporation assay. RA-FLS proliferation was evaluated by assessing DNA synthesis using an EdU incorporation assay (Click-iT™ EdU Cell Proliferation Kit for Imaging, Alexa Fluor™ 488 dye; cat. no. C10337; Invitrogen; Thermo Fisher Scientific, Inc.). UBD siRNA- and control siRNA-treated RA-FLSs were incubated with 10 nM EdU for 6 h at 37°C. Subsequently, RA-FLSs were harvested and fixed with fixation buffer for 15 min at room temperature. After washing twice with 2 ml permeabilization/washing buffer, the cells were incubated with Click-iT EdU reaction cocktail for 30 min at room temperature. After washing, the EdU-positive RA-FLSs were detected using a flow cytometer (BD LSR II; BD Biosciences) and the data acquired with BD FACSDiva 8.0.1 software (BD Biosciences).

Apoptosis analysis. The apoptotic RA-FLSs were measured using Annexin V and PI staining (cat. no. V13242; Thermo Fisher Scientific). RA-FLSs were washed twice with ice-cold PBS and centrifuged at 300 x g for 5 min at 4°C. Subsequently, RA-FLSs were resuspended in 195 µl Annexin V-FITC/PI binding buffer. Annexin V-FITC (5 µl) and PI (10 µl) were supplemented following incubation in the dark for 30 min at 4°C. Apoptosis was analyzed using a flow cytometer (BD LSR II). A total of 10,000 events were collected per sample, and data were acquired and processed using CXP analysis software (version 2.0; Beckman Coulter, Inc.). Total apoptosis was considered the sum of early- and late-stage apoptosis.

Statistical analysis. Statistical analysis was performed using GraphPad Prism software (8.0; GraphPad Software, Inc.). Each experiment was repeated three times. Data are presented as the mean ± standard deviation. Unpaired Student's t-test was used for two-group comparisons and one-way ANOVA followed by Tukey's post hoc test was used for multiple comparisons. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

UBD is significantly increased in RA. In the present study, microarray data for RA were retrieved from the GEO. The DEGs between patients with RA and healthy controls were identified from the GSE55457 dataset. A heatmap of the DEGs is shown in Fig. 1A. The results demonstrated that GSN, ZBTB7C, FKBP5, RPS4Y1 and FOSB were significantly decreased, whereas CXCL13, CXCL9, CXCL10 and UBD were significantly increased in patients with RA. UBD mRNA level was further confirmed to be markedly upregulated in patients with RA compared with the healthy controls (Fig. 1B).

The DEGs were then subjected to KEGG pathway enrichment and GO analysis. The results revealed that the downregulated DEGs were enriched in pathways including 'transcriptional misregulation in cancer', 'TNF signaling pathway', 'relaxin signaling pathway', 'regulation of lipolysis in adipocytes', 'osteoclast differentiation' and 'MAPK signaling pathway', whereas the upregulated DEGs were enriched in

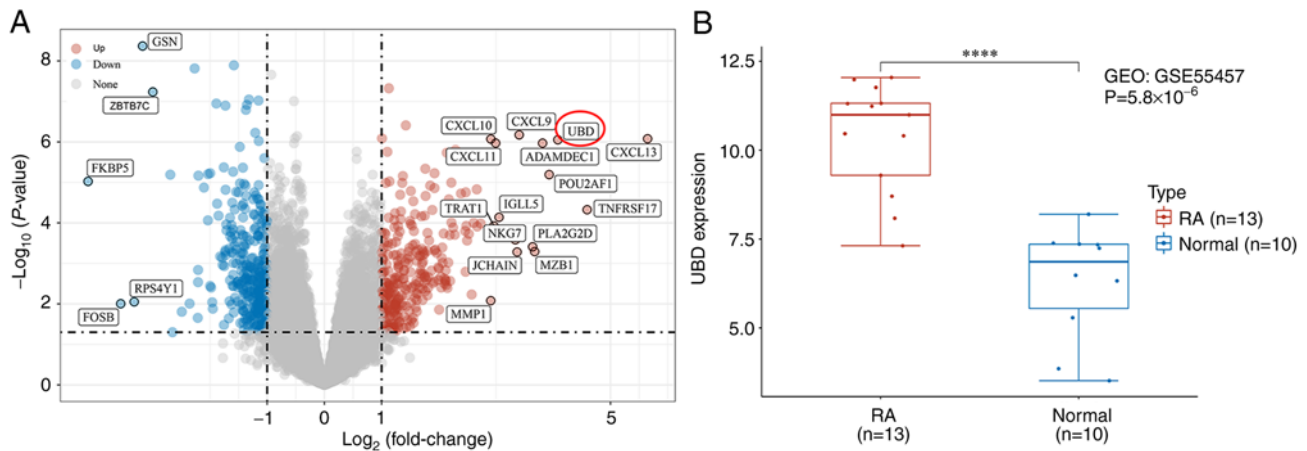


Figure 1. Expression of UBD is significantly increased in RA samples. (A) Differentially expressed genes between RA and normal control samples were selected by volcano plot filtering (adjusted $P < 0.05$ and $\log_2 \text{FC} > 2$). (B) Expression levels of UBD in the GSE55457 dataset. **** $P < 0.0001$. GEO, Gene Expression Omnibus; RA, rheumatoid arthritis; UBD, ubiquitin D.

pathways including ‘viral protein interaction with cytokine and cytokine receptor’, ‘Toll-like receptor signaling pathway’ and ‘Th17 cell differentiation’ (Fig. 2A). The aforementioned results demonstrated that UBD was overexpressed in RA tissues and might be associated with MAPK pathway. GO enrichment analysis revealed that the upregulated genes were enriched in terms including ‘regulation of leukocyte cell-cell adhesion’, ‘regulation of cell-cell adhesion’ and ‘regulation of T cell activation’, whereas the downregulated genes were enriched in terms including ‘rhythmic process’, ‘response to transforming growth factor beta’ and ‘response to peptide hormone’ (Fig. 2B).

Elevated UBD increases the expression of p-p38. The mRNA expression levels of UBD in normal FLSs and RA-FLSs were measured by qPCR. UBD expression levels were significantly increased in RA-FLSs compared with those in normal FLSs (Fig. 3A). RA-FLSs were subsequently transduced with NC lentivirus or UBD-expressing lentivirus for 96 h, and the overexpression of UBD was confirmed by qPCR and western blot. A significant increase was identified in the mRNA and protein expression levels of UBD in the Lv-UBD group compared with those in the uninfected cells or NC group (Fig. 3B and C). By contrast, the mRNA and protein expression levels of UBD were significantly decreased in the specific siRNA-transfected RA-FLSs compared with in those transfected with the scramble siRNA (Fig. 3D and E). Furthermore, UBD overexpression significantly promoted the protein expression levels of p-p38 (Fig. 3F), whereas siRNA-mediated UBD silencing significantly downregulated p-p38 expression (Fig. 3G). These results suggested that upregulated UBD may activate the p38 MAPK pathway in the progression of RA.

UBD regulates RA-FLS cellular processes via the p38 MAPK pathway. To further assess the relationship between UBD and MAPK pathways, the p38 MAPK inhibitor SB202190 was used to treat RA-FLSs. Overexpression of UBD significantly increased RA-FLSs viability, whereas SB202190 administration suppressed the promoting effect of UBD on RA-FLSs activity (Fig. 4A). In addition, UBD overexpression resulted

in a significant increase in the secretion of IL-2, IL-6, IL-10 and TNF- α , whereas the administration of SB202190 blocked the elevated secretion of IL-2, IL-6, IL-10 and TNF- α caused by UBD overexpression (Fig. 4B). In addition, overexpression of UBD enhanced the proliferation of RA-FLSs, whereas SB202190 treatment suppressed the promoting effect of UBD on RA-FLSs proliferation, as confirmed by flow cytometry (Fig. 4C). By contrast, UBD silencing significantly suppressed RA-FLSs proliferation (Fig. 4D). Notably, cell apoptosis was significantly suppressed by UBD overexpression, whereas SB202190 administration alleviated the inhibitory effect of UBD overexpression on cell apoptosis (Fig. 4E). By contrast, UBD silencing significantly promoted RA-FLSs apoptosis (Fig. 4F). These results revealed that UBD increased the viability, the release of proinflammatory factors and the proliferation of RA-FLSs, whereas treatment with the p38 MAPK inhibitor SB202190 exerted the opposite effect on RA-FLSs, thus indicating that UBD regulated the cellular processes of RA-FLSs via the p38 MAPK pathway.

Discussion

Bioinformatics is an interdisciplinary field combining molecular biology and information technology, which is widely used to explore and reveal the molecular mechanism of diseases (31). In the present study, data from patients with RA, which is a type of inflammatory arthritis of unknown etiology, were obtained from the GEO (GSE55457) and were analyzed using bioinformatics tools. A series of DEGs were identified between RA and healthy control samples, among which chemokines related to Th1 cells (CXCL9, CXCL10), and T follicular helper and B cells (CXCL13), were significantly increased in RA tissues, indicating the important roles of these chemokines in the progression of RA, which is in accordance with previous reports (32–34). However, the role of another significantly upregulated gene, UBD, which may have a role in the development of RA remains to be elucidated.

UBD, an 18 kDa protein comprising 165 amino-acid residues, is an immune system protein that is strongly induced by proinflammatory cytokines (17). UBD is the only modifier

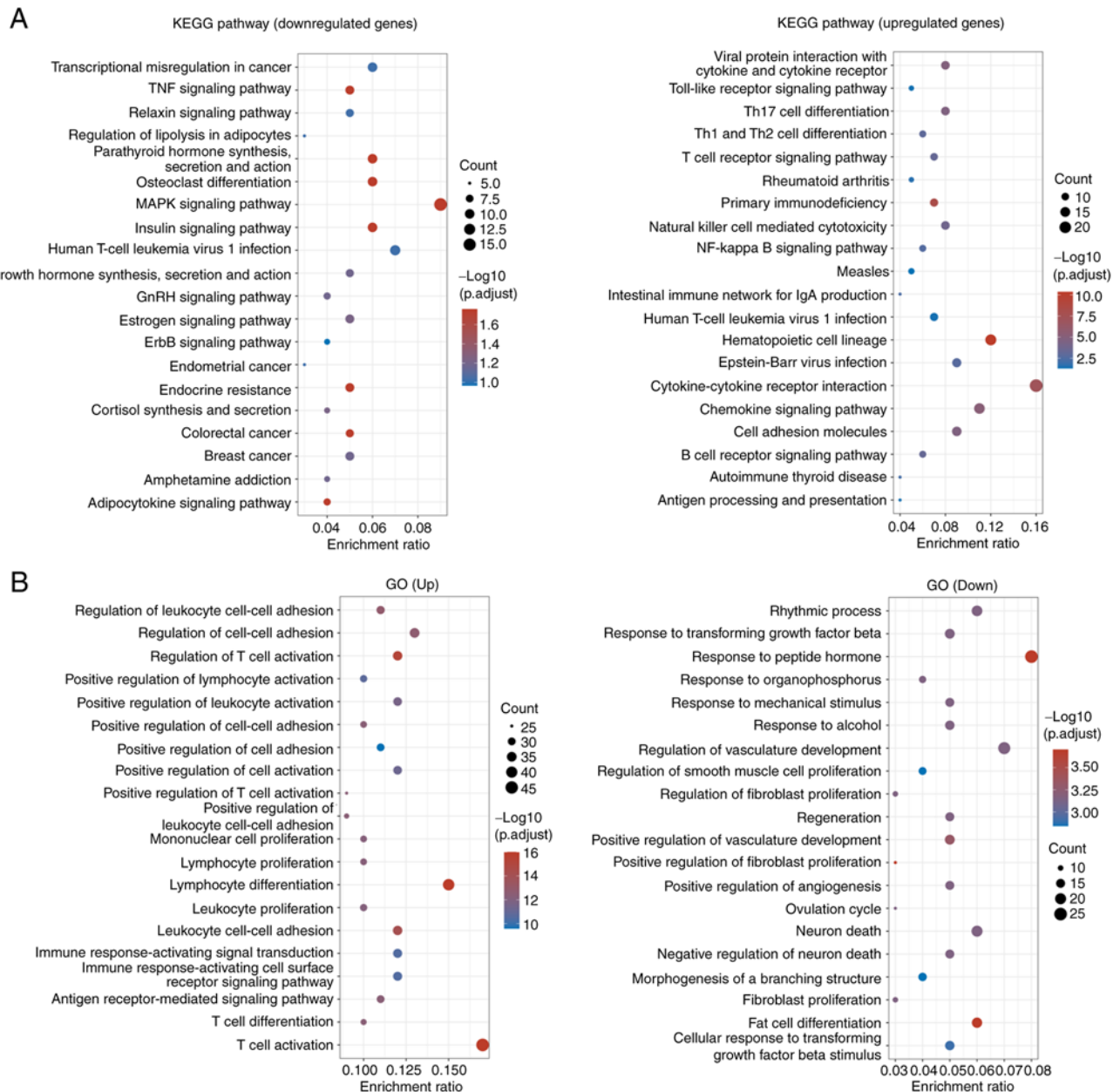


Figure 2. KEGG and GO analyses. (A) KEGG pathway and (B) GO analyses of the differentially expressed genes between the rheumatoid arthritis and normal control samples. GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

of all ubiquitin-like modifiers that acts as an autonomous transferable signal for degradation by the 26S proteasome, which can occur independently of ubiquitin (35). While UBD is primarily stimulated by proinflammatory cytokines within the tumor microenvironment, a growing number of studies has confirmed that the pro-malignant ability of UBD itself largely underlies its upregulation in tumor tissues (16,36). Upregulation of UBD has been confirmed in various types of cancer where it promotes cell migration, invasion and metastasis formation (37-39). In addition, the expression of UBD has been reported to be markedly increased during the maturation of DCs and epithelial cells within the medulla of the thymus where it regulates T-cell selection (40). In the present study, UBD was confirmed as one of the most upregulated genes in RA samples from a GEO dataset, indicating that UBD may have an important role in the progression of RA. However, the

expression of UBD in RA clinical samples was not pursued further due to the lack of clinical samples.

UBD has been reported to accelerate cell viability and proliferation, and to suppress cell apoptosis (39,41-43). In the present study, the biological function of UBD in RA was further investigated. UBD overexpression significantly increased the viability and proliferation of RA-FLSs, and inhibited their apoptosis. A causal link between inflammation and the development of RA is generally accepted (44). IL-2, IL-6, IL-10 and TNF- α serve important roles in RA pathogenesis, participating in Th1-mediated processes, and causing cartilage and bone destruction (45-48). The present study confirmed that overexpression of UBD significantly induced the secretion of IL-2, IL-6, IL-10 and TNF- α in RA-FLSs. These results suggested that UBD may be related to the progression of RA by regulating proliferation, apoptosis

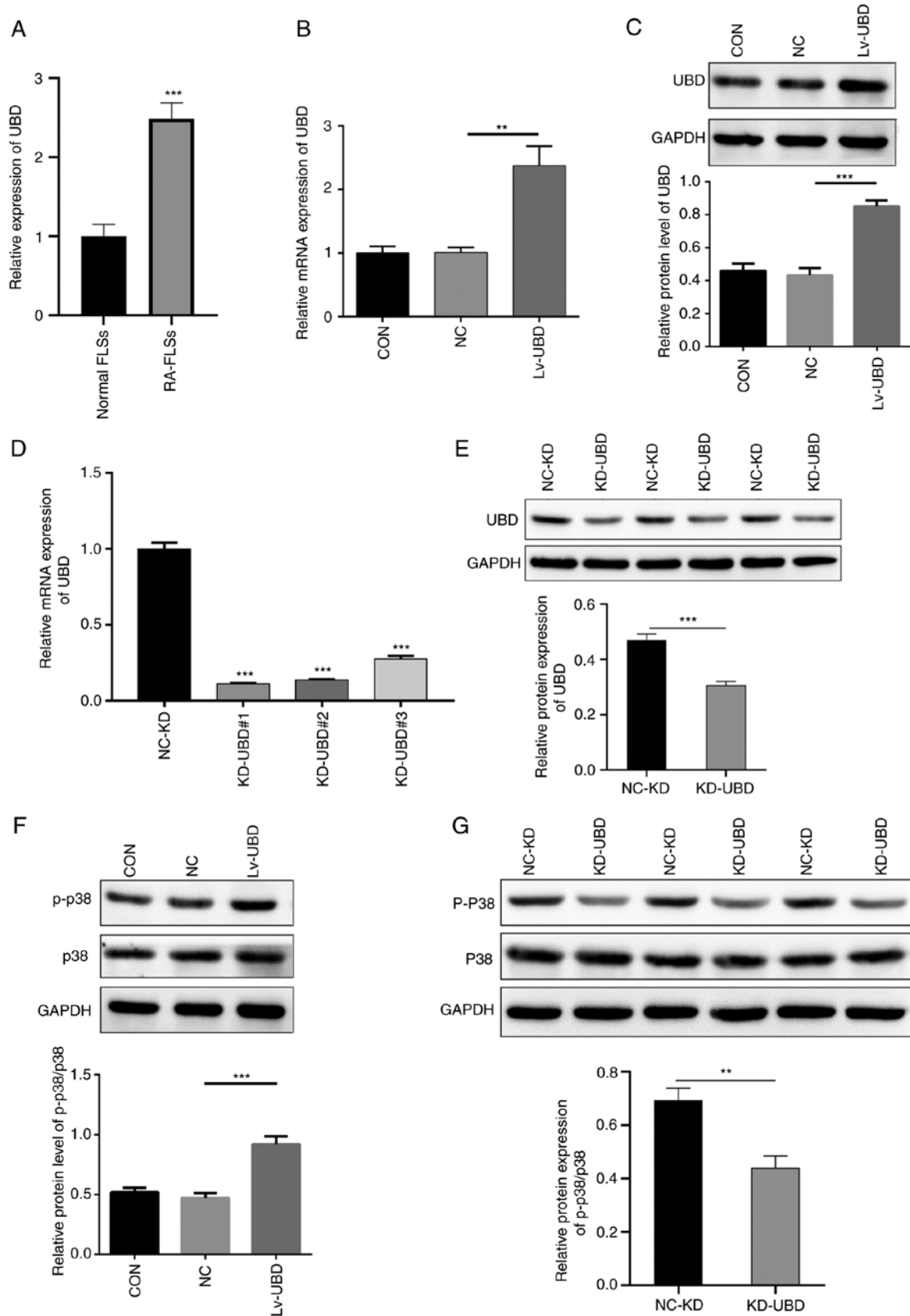


Figure 3. UBD activates the p38 MAPK pathway. RA-FLSs were transduced with a UBD overexpression lentiviral vector or were transfected with UBD siRNAs. (A) mRNA expression levels of UBD in normal-FLSs and RA-FLSs was detected using qPCR. (B) mRNA and (C) protein expression levels of UBD in RA-FLSs transduced with a UBD overexpression lentiviral vector were examined by qPCR and western blotting, respectively. (D) mRNA and (E) protein expression levels of UBD in RA-FLSs transfected with UBD siRNAs were examined by qPCR and western blotting, respectively. Protein expression levels of p-p38 and p38 in RA-FLSs with UBD (F) overexpression and (G) KD, as determined by western blotting. Quantification of gene and protein expression was normalized to GAPDH. Data are presented as the mean \pm SD (n=3). **P<0.01, ***P<0.001, as indicated or vs. Normal FLSs or NC-KD. FLSs, fibroblast-like synoviocytes; KD, knockdown; NC, negative control; p-, phosphorylated; qPCR, quantitative PCR; RA, rheumatoid arthritis; siRNA, small interfering RNA; UBD, ubiquitin D.

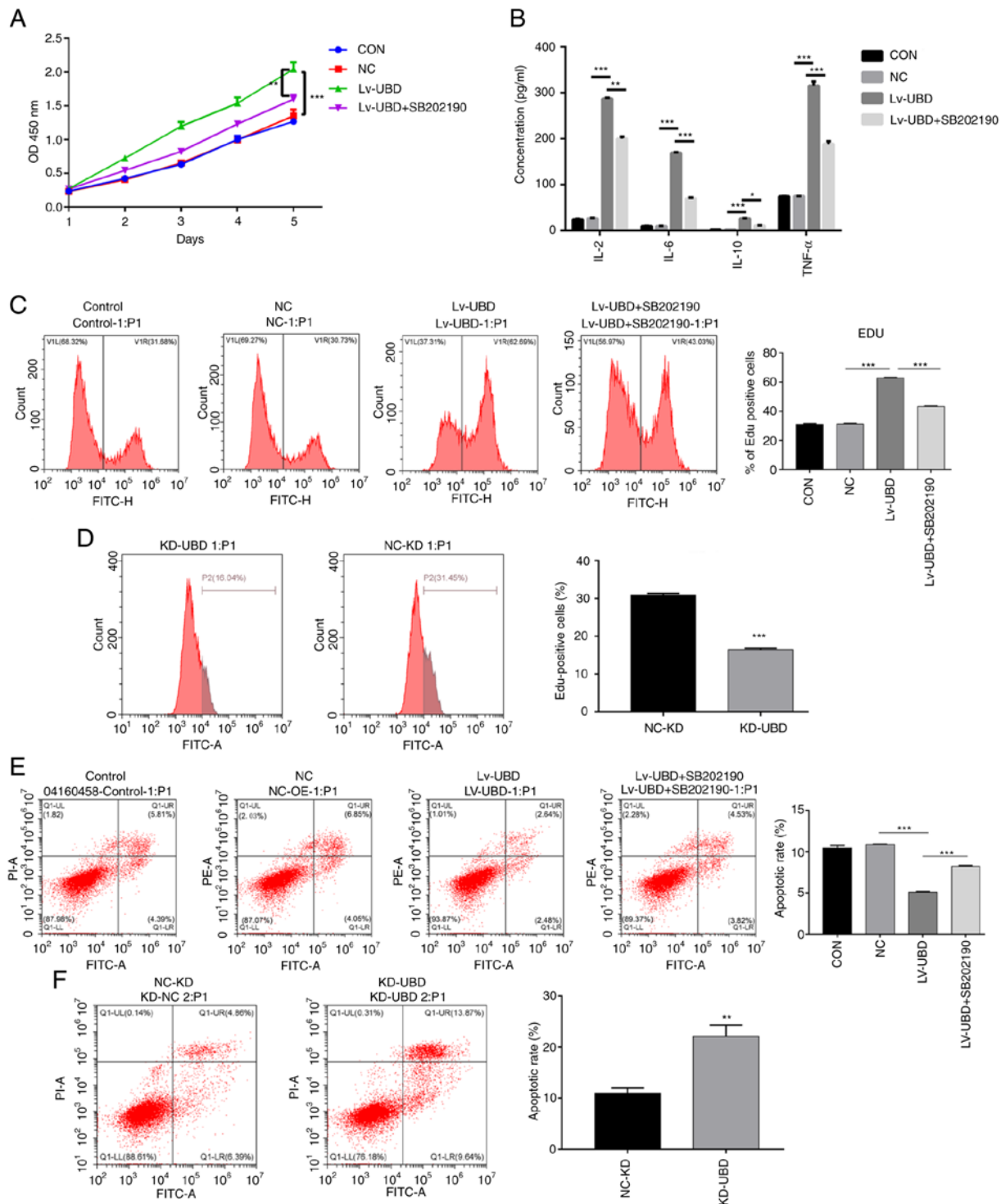


Figure 4. UBD regulates the biological processes of RA-FLSs by targeting the p38 MAPK pathway. RA-FLSs transduced with a UBD overexpression lentiviral vector or UBD siRNA were treated with or without the p38 MAPK inhibitor SB202190. (A) Viability of RA-FLSs was detected by Cell Counting Kit-8. (B) Levels of IL-2, IL-6, IL-10 and TNF- α were examined by ELISA. (C and D) Proliferation of RA-FLSs was evaluated by assessing DNA synthesis using the EdU incorporation assay. (E and F) Apoptosis of RA-FLSs was detected by flow cytometric analysis. Data are presented as the mean \pm SD (n=3). *P<0.05, **P<0.01, ***P<0.001, as indicated or vs. NC-KD. FLSS, fibroblast-like synoviocytes; KD, knockdown; NC, negative control; OD, optical density; RA, rheumatoid arthritis; siRNA, small interfering RNA; UBD, ubiquitin D.

and the secretion of inflammatory factors. Autoantibodies serve essential biological roles in the progression of RA (49). However, the present study mainly demonstrated the function of UBD in RA-FLSs at the cellular level, which is often poorly reflective of the *in vivo* situation; thus, autoantibody testing was not performed in the current study.

UBD modulates various signaling pathways involved in tumor development, such as NF- κ B and Wnt/ β -catenin signaling pathways (16,50). Notably, UBD accelerates the progression of oral squamous cell carcinoma via NF- κ B signaling (50). Elevated UBD expression has been shown to drive the invasion and metastasis of hepatocellular

carcinoma cells by binding to β -catenin to prevent its ubiquitylation and degradation (51). In addition, UBD can directly bind to target genes, including MAD and p53, to promote cell proliferation, metastasis and migration via their regulation (38,39). In the present study, KEGG pathway analysis identified that the DEGs in RA samples were enriched in pathways including 'TNF signaling pathway', 'relaxin signaling pathway', 'osteoclast differentiation' and 'MAPK signaling pathway'. Furthermore, UBD overexpression significantly promoted the protein expression levels of p-p38, whereas silencing of UBD markedly inhibited p-p38 expression, indicating that UBD may activate the p38 MAPK pathway in the progression of RA. To the best of our knowledge, the relationship between UBD and p38 MAPK has not been previously reported. To further investigate the targeting regulatory relationship between UBD and p38 MAPK, the p38 MAPK inhibitor SB202190 was used to treat RA-FLSs. Notably, the application of SB202190 partially relieved the UBD-dependent enhancing effects on cell viability and proliferation, as well as the inhibitory effect on cell apoptosis. In addition, treatment with SB202190 significantly blocked the enhancing effects of UBD overexpression on the secretion of inflammatory factors. Taken together, these results suggested that UBD may be a crucial pathogenic factor for RA by activating the p38 MAPK pathway, which provides additional opportunities for the intervention of RA.

In conclusion, the expression levels of UBD were significantly increased in RA. Notably, the results revealed the important role of UBD in RA, and also identified the novel mechanism that UBD may regulate the biological and inflammatory processes in RA by targeting p38 MAPK. Collectively, the present study provided novel insights into the pathogenesis of RA and the potential of UBD as a therapeutic target against RA.

Acknowledgements

Not applicable.

Funding

This work was supported by the Scientific Research Project of Guangxi Zhuang Autonomous Region Administration of Traditional Chinese Medicine (grant no. gzzc2019145), and the Scientific Research Project of Guangxi Zhuang Autonomous Region Health Committee (grant no. z20200163).

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

HC and HW designed the study. HC and LT performed all of the experiments, interpreted the data and prepared the manuscript. JL and CP analyzed the data. All authors read and approved the final manuscript. HC and HW confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Approved by the Medical Ethics committee of the Affiliated Hospital of Youjiang Medical University for Nationalities, Baise, Guangxi, approval number YYFY-LL-2022-96.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

- O'Neil LJ, Barrera-Vargas A, Sandoval-Heglund D, Merayo-Chalico J, Aguirre-Aguilar E, Aponte AM, Ruiz-Perdomo Y, Gucek M, El-Gabalawy H, Fox DA, *et al*: Neutrophil-mediated carbamylation promotes articular damage in rheumatoid arthritis. *Sci Adv* 6: eabd2688, 2020.
- Smith MH and Berman JR: What is rheumatoid arthritis? *JAMA* 327: 1194, 2022.
- Lee SH, Kwon JY, Kim SY, Jung K and Cho ML: Interferon-gamma regulates inflammatory cell death by targeting necroptosis in experimental autoimmune arthritis. *Sci Rep* 7: 10133, 2017.
- Shah N, Jadidi S and Bajaj P: Correction: A review of non-surgical pain management in osteoarthritis. *Cureus* 13: e45, 2021.
- Mekic M and Hadzigraphic E: Anti-cyclic citrullinated peptide antibody as a predictor of rheumatoid arthritis complications. *Med Arch* 74: 183-186, 2020.
- Zhou Y, Abraham S, Andre P, Edelstein LC, Shaw CA, Dangelmaier CA, Tsygankov AY, Kunapuli SP, Bray PF and McKenzie SE: Anti-miR-148a regulates platelet Fc γ RIIA signaling and decreases thrombosis in vivo in mice. *Blood* 126: 2871-2881, 2015.
- Patin EC, Thompson A and Orr SJ: Pattern recognition receptors in fungal immunity. *Semin Cell Dev Biol* 89: 24-33, 2019.
- Schmid E, Bhandaru M, Nurbaeva MK, Yang W, Szteyn K, Russo A, Leibrock C, Tian L, Pearce D, Shumilina E and Lang F: SGK3 regulates Ca(2+) entry and migration of dendritic cells. *Cell Physiol Biochem* 30: 1423-1435, 2012.
- Jeon JH, Lee BC, Kim D, Cho D and Kim TS: Hydrophilic astragalin galactoside induces T helper type 1-mediated immune responses via dendritic cells. *Int J Mol Sci* 19: 3120, 2018.
- Kuwabara T, Ishikawa F, Kondo M and Kakiuchi T: The role of IL-17 and related cytokines in inflammatory autoimmune diseases. *Mediators Inflamm* 2017: 3908061, 2017.
- Wu S, Wang Y, Zhang J, Han B, Wang B, Gao W, Zhang N, Zhang C, Yan F and Li Z: Efficacy and safety of rituximab for systemic lupus erythematosus treatment: A meta-analysis. *Afr Health Sci* 20: 871-884, 2020.
- Wang F, Shi WX, Chen J, He K and Fang W: Clinical therapeutic effects of combined diacerein and glucosamine in the treatment of osteoarthritis: A protocol for systematic review and meta-analysis. *Medicine (Baltimore)* 100: e27583, 2021.
- Murata K, Furu M, Yoshitomi H, Ishikawa M, Shibuya H, Hashimoto M, Imura Y, Fujii T, Ito H, Mimori T and Matsuda S: Comprehensive microRNA analysis identifies miR-24 and miR-125a-5p as plasma biomarkers for rheumatoid arthritis. *PLoS One* 8: e69118, 2013.
- Donlin LT: Inching closer to precision treatment for rheumatoid arthritis. *Nat Med* 28: 1129-1131, 2022.
- Lourenzi FM, Jones A, Pereira DF, Santos JHCAD, Furtado RNV and Natour J: Effectiveness of an overall progressive resistance strength program for improving the functional capacity of patients with rheumatoid arthritis: A randomized controlled trial. *Clin Rehabil* 31: 1482-1491, 2017.
- Aichem A and Groettrup M: The ubiquitin-like modifier FAT10 in cancer development. *Int J Biochem Cell Biol* 79: 451-461, 2016.
- Raasi S, Schmidtke G, de Giuli R and Groettrup M: A ubiquitin-like protein which is synergistically inducible by interferon-gamma and tumor necrosis factor-alpha. *Eur J Immunol* 29: 4030-4036, 1999.

18. Liu YC, Pan J, Zhang C, Fan W, Collinge M, Bender JR and Weissman SM: A MHC-encoded ubiquitin-like protein (FAT10) binds noncovalently to the spindle assembly checkpoint protein MAD2. *Proc Natl Acad Sci USA* 96: 4313-4318, 1999.
19. Battaglia A, Fossati M, Buzzonetti A, Scambia G and Fattorossi A: A robust immune system conditions the response to abagovomab (anti-idiotypic monoclonal antibody mimicking the CA125 protein) vaccination in ovarian cancer patients. *Immunol Lett* 191: 35-39, 2017.
20. Tanyi JL and George E: Personalized vaccination against ovarian cancer: What are the possibilities? *Expert Rev Vaccines* 17: 955-958, 2018.
21. Pajsens ST, Leffers N, Daemen T, Helfrich W, Boezen HM, Cohlen BJ, Melief CJ, de Bruyn M and Nijman HW: Antigen-specific active immunotherapy for ovarian cancer. *Cochrane Database Syst Rev* 9: CD007287, 2018.
22. Kalli KR, Block MS, Kasi PM, Erskine CL, Hobday TJ, Dietz A, Padley D, Gustafson MP, Shreeder B, Puglisi-Knutson D, *et al*: Folate receptor alpha peptide vaccine generates immunity in breast and ovarian cancer patients. *Clin Cancer Res* 24: 3014-3025, 2018.
23. Han T, Liu Z, Li H, Xie W, Zhang R, Zhu L, Guo F, Han Y, Sheng Y and Xie X: High expression of UBD correlates with epirubicin resistance and indicates poor prognosis in triple-negative breast cancer. *Onco Targets Ther* 8: 1643-1649, 2015.
24. Yan DW, Li DW, Yang YX, Xia J, Wang XL, Zhou CZ, Fan JW, Wen YG, Sun HC, Wang Q, *et al*: Ubiquitin D is correlated with colon cancer progression and predicts recurrence for stage II-III disease after curative surgery. *Br J Cancer* 103: 961-969, 2010.
25. Bottini N and Firestein GS: Duality of fibroblast-like synovocytes in RA: Passive responders and imprinted aggressors. *Nat Rev Rheumatol* 9: 24-33, 2013.
26. Bartok B and Firestein GS: Fibroblast-like synoviocytes: Key effector cells in rheumatoid arthritis. *Immunol Rev* 233: 233-255, 2010.
27. You R, Liu S and Tan J: Screening and identification of osteoarthritis related differential genes and construction of a risk prognosis model based on bioinformatics analysis. *Ann Transl Med* 10: 444, 2022.
28. Huang WC, Liou CJ, Shen SC, Hu S, Hsiao CY and Wu SJ: Luteolin attenuates IL-1 β -induced THP-1 adhesion to ARPE-19 cells via suppression of NF- κ B and MAPK pathways. *Mediators Inflamm* 2020: 9421340, 2020.
29. Yang L, Yang H, Chu Y, Song Y, Ding L, Zhu B, Zhai W, Wang X, Kuang Y, Ren F, *et al*: CREPT is required for murine stem cell maintenance during intestinal regeneration. *Nat Commun* 12: 270, 2021.
30. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 25: 402-408, 2001.
31. Liang X, Zhu W, Lv Z and Zou Q: Molecular computing and bioinformatics. *Molecules* 24: 2358, 2019.
32. Pandya JM, Lundell AC, Andersson K, Nordström I, Theander E and Rudin A: Blood chemokine profile in untreated early rheumatoid arthritis: CXCL10 as a disease activity marker. *Arthritis Res Ther* 19: 20, 2017.
33. Tsai CH, Chen CJ, Gong CL, Liu SC, Chen PC, Huang CC, Hu SL, Wang SW and Tang CH: CXCL13/CXCR5 axis facilitates endothelial progenitor cell homing and angiogenesis during rheumatoid arthritis progression. *Cell Death Dis* 12: 846, 2021.
34. Zhong H, Xu LL, Bai MX and Su Y: Effect of chemokines CXCL9 and CXCL10 on bone erosion in patients with rheumatoid arthritis. *Beijing Da Xue Xue Bao Yi Xue Ban* 53: 1026-1031, 2021 (In Chinese).
35. Schmidtke G, Kalveram B and Groettrup M: Degradation of FAT10 by the 26S proteasome is independent of ubiquitylation but relies on NUB1L. *FEBS Lett* 583: 591-594, 2009.
36. Xiang S, Shao X, Cao J, Yang B, He Q and Ying M: FAT10: Function and relationship with cancer. *Curr Mol Pharmacol* 13: 182-191, 2020.
37. Lee CGL, Ren J, Cheong ISY, Ban KHK, Ooi LLPJ, Yong Tan SY, Kan A, Nuchprayoon I, Jin R, Lee KH, *et al*: Expression of the FAT10 gene is highly upregulated in hepatocellular carcinoma and other gastrointestinal and gynecological cancers. *Oncogene* 22: 2592-2603, 2003.
38. Theng SS, Wang W, Mah WC, Chan C, Zhuo J, Gao Y, Qin H, Lim L, Chong SS, Song J and Lee CG: Disruption of FAT10-MAD2 binding inhibits tumor progression. *Proc Natl Acad Sci USA* 111: E5282-E5291, 2014.
39. Su H, Qin M, Liu Q, Jin B, Shi X and Xiang Z: Ubiquitin-like protein UBD promotes cell proliferation in colorectal cancer by facilitating p53 degradation. *Front Oncol* 11: 691347, 2021.
40. Buerger S, Herrmann VL, Mundt S, Trautwein N, Groettrup M and Basler M: The ubiquitin-like modifier FAT10 is selectively expressed in medullary thymic epithelial cells and modifies T cell selection. *J Immunol* 195: 4106-4116, 2015.
41. Xiao Y, Diao Q, Liang Y, Peng Y and Zeng K: MicroRNA-24-1-5p promotes malignant melanoma cell autophagy and apoptosis via regulating ubiquitin D. *Mol Med Rep* 16: 8448-8454, 2017.
42. Taniai E, Yafune A, Hayashi H, Itahashi M, Hara-Kudo Y, Suzuki K, Mitsumori K and Shibutani M: Aberrant activation of ubiquitin D at G2 phase and apoptosis by carcinogens that evoke cell proliferation after 28-day administration in rats. *J Toxicol Sci* 37: 1093-1111, 2012.
43. Brozzi F, Gerlo S, Grieco FA, Juusola M, Balhuizen A, Lievens S, Gysemans C, Bugliani M, Mathieu C, Marchetti P, *et al*: Ubiquitin D regulates IRE1 α /c-Jun N-terminal kinase (JNK) protein-dependent apoptosis in pancreatic beta cells. *J Biol Chem* 291: 12040-12056, 2016.
44. Wang H, Tu S, Yang S, Shen P, Huang Y, Ba X, Lin W, Huang Y, Wang Y, Qin K and Chen Z: Berberine modulates LPA function to inhibit the proliferation and inflammation of FLS-RA via p38/ERK MAPK pathway mediated by LPA $_1$. *Evid Based Complement Alternat Med* 2019: 2580207, 2019.
45. Graßhoff H, Comdühr S, Monne LR, Müller A, Lamprecht P, Riemekasten G and Humrich JY: Low-dose IL-2 therapy in autoimmune and rheumatic diseases. *Front Immunol* 12: 648408, 2021.
46. Lauper K, Ludici M, Mongin D, Bergstra SA, Choquette D, Codreanu C, Cordtz R, De Cock D, Dreyer L, Elkayam O, *et al*: Effectiveness of TNF-inhibitors, abatacept, IL6-inhibitors and JAK-inhibitors in 31 846 patients with rheumatoid arthritis in 19 registers from the 'JAK-pot' collaboration. *Ann Rheum Dis* 81: 1358-1366, 2022.
47. Wang J, Liu J, Wen JT and Wang X: Correlation between circRNA0003353 in peripheral blood mononuclear cells and immune inflammation in rheumatoid arthritis patients with damp heat obstruction syndrome. *Sichuan Da Xue Xue Bao Yi Xue Ban* 53: 437-443, 2022 (In Chinese).
48. Alturaiki W, Alhamad A, Alturaiqy M, Mir SA, Iqbal D, Bin Dukhyil AA, Alaidarous M, Alshehri B, Alsagaby SA, Almalki SG, *et al*: Assessment of IL-1 β , IL-6, TNF- α , IL-8, and CCL 5 levels in newly diagnosed Saudi patients with rheumatoid arthritis. *Int J Rheum Dis* 25: 1013-1019, 2022.
49. van Delft MAM and Huizinga TWJ: An overview of autoantibodies in rheumatoid arthritis. *J Autoimmun* 110: 102392, 2020.
50. Song A, Wang Y, Jiang F, Yan E, Zhou J, Ye J, Zhang H, Ding X, Li G, Wu Y, *et al*: Ubiquitin D promotes progression of oral squamous cell carcinoma via NF-kappa B signaling. *Mol Cells* 44: 468-480, 2021.
51. Yuan R, Wang K, Hu J, Yan C, Li M, Yu X, Liu X, Lei J, Guo W, Wu L, *et al*: Ubiquitin-like protein FAT10 promotes the invasion and metastasis of hepatocellular carcinoma by modifying β -catenin degradation. *Cancer Res* 74: 5287-5300, 2014.



This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0) License.