Paeonol Inhibits the Development of Rheumatoid Arthritis Through the Formyl Peptide Receptor 2

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Abstract

Rheumatoid arthritis (RA) is a refractory systemic autoimmune disease associated with synovial inflammation. Previous studies postulate that paeonol has good anti-arthritis effects on RA. However, its systematic description remains unknown. Herein, we used bioinformatics tools to evaluate the mechanism of paeonol in arthritis systematically. A macrophage model was employed to study the differentially expressed genes between the inflammation and normal group, revealing 169 inflammation-related genes. Another 275 key genes affected by paeonol were identified in the same model. Three key genes, FPR2, Cd83, and Cfb, were obtained after combining the two data sets. Paeonol inhibited the release of inflammatory factors and the proliferation of synovial. However, its inhibitory effect was blocked by Fpr2 blocker WRW4. In summary, paeonol can inhibit the development of arthritis through FPR2. This provides new scope for the design and development of FPR2 ligands.

1. Introduction

Rheumatoid arthritis (RA) is an autoimmune synovial disease caused by genetic and environmental factors. It is often accompanied by systemic immune disorders[1, 2]. Methotrexate (MTX) constitutes the first-line therapy in rheumatoid arthritis (RA), yet approximately 30% of the patients do not benefit from MTX. In RA, fibroblast-like synoviocytes (FLS) has unique invasive behavior and plays a major role in the pathogenesis and progression of diseases[3, 4]. Macrophages are engaged in the destructive osteoclast formation process and are a key source of inflammatory cytokines IL-1β, IL-6, and TNF-α in rheumatoid arthritis tissue[5, 6]. The RA disease is often explored using the LPS-induced RAW264.7 cell model and FLS [7].

Paeonol has a wide range of anti-inflammatory activities, reduces inflammatory cytokines (IL-1 and IL-6), and reduces inflammatory responses by inhibiting the NLRP3 signaling pathway[8]. The relationship between cardiovascular disease and arthritis in RA patients is stronger than that between traditional cardiovascular risk factors[9]. Paeonol also possesses cardioprotective effects and significant potential as an adjuvant drug for arthritis[10, 11]. Currently, there lacks an overall evaluation of the pharmacological effects of paeonol. However, advancements in technology and bioinformatics techniques have enabled a comprehensive understanding of biological processes[12]. Herein, we collected macrophage control, inflammation, and paeonol treatment groups' datasets to analyze their differential genes. The analysis revealed three genes; FPR2, Cd83, and Cfb.

The formyl peptide receptor (FPR) is a G protein-coupled receptor (GPCRs). FPRs, especially FPR2, play a crucial role in maintaining the balance of inflammatory response[13]. FPR is expressed in various cells, with neutrophils and macrophages having the strongest expression[14]. Many studies postulate that the FPR ligand may be a novel therapeutic drug for treating inflammation and bone injury associated with RA[15]. Novel formylpeptide receptor (FPR) agonists and pyridinone, and pyridinone stents have potential in RA treatment[16]. FPR agonist Cpd43 reduces osteoclast formation and inflammation in RA mouse models. It also exhibits anti-inflammatory effects in related human cells[15, 17]. Cognizant of this, we

hypothesized that paeonol attenuate LPS-induced inflammatory responses through Fpr2. The effect of paeonol on the function of Fpr2 was thus tested to verify the hypothesis. The effect of inflammation inhibition was explored on the cellular model of rheumatoid arthritis.

2 Materials And Methods

2.1 Collection of data sets

RAW264.7 cell and LPS-induced RAW264.7 cell model-related data sets were downloaded from the public microarray database, Gene Expression Omnibus database (GEO; http://www.ncbi.nlm.nih.gov/geo/), which provide complete microarray data[18]. The various data sets had varying publicization dates. The GSE86588(GPL1261 (Mouse430_2) Affymetrix Mouse Genome 430 2.0 Array) data set was publicized on Oct 01, 2017, the GSE76563(GPL10787 Agilent-028005 SurePrint G3 Mouse GE 8x60K Microarray) on Feb 24, 2016, and the GSE21841(GPL18802 (MoGene-2_0-st) Affymetrix Mouse Gene 2.0 ST Array) on May 15, 2010. The GSE9632(GPL2995 ABI Mouse Genome Survey Microarray) data set was obtained from the RAW264.7 cell group treated with paeonol.

2.2 Differential gene analysis

The differential gene analysis process mapped the probes in each data set to a matching gene symbol. Empty probes and probes mapping multiple genes were removed based on the analysis platform of each expression profile. The average value was considered as the gene expression value when multiple probes matched the same gene symbol. Standardized preprocessing of each data set was also done before analysis. The three data sets were grouped and combined based on the positive and negative characteristics of treated with LPS or not (Supplementary 1). Standardized and difference analyses were then carried out for the combined matrix.

Differential analysis of the combined expression matrix using the R-pack "limma" revealed 162 differentially expressed genes, including 150 up-regulated genes and 12 down-regulated genes. The screening conditions were log Fold Change = 1.5 and P-value = 0.05.

2.3 GO & KEGG analysis

The GO function and KEGG pathway enrichment analyses of the differentially expressed genes were performed using the cloud platform (Oebiotech).

2.4 STRING analysis

The STRING tool (https://string-db.org/) was used for protein-protein interaction (PPI) analysis and the Cytoscape software for optimization.

2.5 Cell culture

The RAW264.7 cell line was obtained from the American Type Culture Collection (ATCC) and cultured in the RPMI 1640 Medium (HyClone, USA) at 37°Cand 5% CO_2. The FLS cell line was purchased from

Guandao Biotech and cultured in DMEM medium at 37℃ and 5% CO_2. Both culture media were supplemented with 10% Fetal Bovine Serum (Lonsera, Uruguay).

2.6 Cell counting kit-8 (CCK-8) assay

FLS cells were seeded in a 96-well plate at 2×10^3 cells per well and cultured in a complete medium. The cells were then treated with TNF-α 10 ng/mL (Sigma-Aldrich, USA) for 12 hours when they attained adherence to the plate walls. The medium was then replaced with a fresh medium, and paeonol and WRW4 were added at varying concentrations. The CCK-8 kit (Beyotime, China) was then used to determine the cells' absorbance at a wavelength of 460 nm in a microplate reader after 48 hours.

2.7 Quantitative real-time polymerase chain reaction (qRT-PCR)

The cells' total RNA was extracted using the TRIzol Reagent (Invitrogen™) followed by reverse transcription of the RNA into cDNA using the PrimeScript™ RT reagent Kit with gDNA Eraser (RR047, Takara). The TB Green Kit (TB Green® Fast qPCR Mix, RR430, Takara) was then employed for qRT-PCR on a CFX96 Touch Quantitative Fluorescence PCR System (Bio-Rad). The qRT-PCR conditions were 30 cycles of 95℃ for 15s, 60℃ for 30s. A melt curve analysis was performed at the end of the reaction to check the presence of primers-dimers. The relative expression of the genes was calculated using the 2^-ddCt method based on three independent experiments. Each sample had three replicates. The primers were designed using the Primer6.0 software, and their specificity was analyzed through the BLAST method. The genes name and primer sequences used are listed in Supplementary 2.

2.8 Detection of intracellular concentration of Ca^{2+}

Cells (1000) were first seeded into each well and then treated with TNF-α for 12h at 37℃. They were then probed with FLUO-4AM (S1060, Beyotime) at 37℃ and treated with paeonol and WRW4 inhibitors, respectively. FLIPR Penta High-Throughput Cellular Screening System was finally used to detect the calcium ions present in the cells.

2.9 Data analysis

Data were analyzed using the Prism 7.0 software (GraphPad Software, San Diego, CA, USA) and presented as means ± standard deviation of three independent experiments. P-values less than 0.05 ($P < 0.05$) indicated significant differences between groups.

3 Results

3.1 Screening of DEGs in macrophage inflammatory models

Herein, joint statistical analysis was performed on the GEO data set of the two RAW264.7 cell groups: LPS and control group. There were 9 up-regulated and 160 down-regulated DEGs (Supplementary 3). Figure 1A and B are a volcano map and a heatmap drawn from the 169 DEGs, respectively. DEGs such as

Edn1, Ccrl2, Cmpk2, Saa3, Cxcl2, and Cxcl11 up-regulated by LPS in the RAW264.7 cells were detected by qRT-PCR (Fig. 1C).

GO (Gene Ontology) functional enrichment analysis of the 169 genes (Fig. 1D) revealed that the Biological Process (BP), Cellular Component (CC), and Molecular Function (MF) of the DEGs were closely related to the level of inflammation. The Kyoto Encyclopedia of Genes and Genomes (KEGG) functional analysis revealed that the DEGs were mainly enriched in inflammation-related pathways, especially the level of cellular inflammation (Fig. 1E).

3.2 The network action of paeonol

GEO2R was used to analyze the DEGs affected by paeonol in the GEO9632 dataset. The analysis revealed that 275 genes were affected by paeonol treatment. Figure 2A and B show the volcano map and the KEGG enrichment analysis of the 275 genes, respectively (Supplementary 4). Paeonol treatment mainly affected the biological process of "amacrine cell differentiation" and the cell components of the "plasma membrane." Fig. 2C shows the PPI analysis of the 275 genes affected by paeonol treatment.

3.3 Paeonol can affect calcium influx in FLS cells through FPR2

Venn mapping of the 275 genes affected by paeonol and 169 genes associated with inflammation was first done, which narrowed down to three proteins FPR2, Cd83, and Cfb (Fig. 3A and 3B). Previous studies postulate that various FPR2 ligands alleviate RA inflammation and bone injury[19]. Cognizant of this, we hypothesized that paeonol played a role as an FPR2 ligand. As such, the effect of paeonol on Ca^{2+} was detected to verify whether paeonol could inhibit inflammation through FPR2. The calcium influx reached its peak in 120–140 seconds with a relative intensity of 860 units when the concentration of paeonol reached 20 μM. When the concentration of paeonol reached 100 μM, the relative intensity of the peak value reached 1180 units. Conversely, the value of units only 800 as WRW4 (10 μM) added with paeonol (100 μM). Calcium influx was blocked when we interfered with WRW4, a selective inhibitor of FPR2. Notably, paeonol activated Ca^{2+} influx through Fpr2, thus inhibiting inflammation.

3.4 Paeonol inhibits rheumatoid arthritis through Fpr2

We further tested the response of paeonol to FLS inflammation to verify whether paeonol inhibited rheumatoid arthritis through Fpr2. TNF- α induces inflammation in FLS, which is usually used as a cell model of RA[7]. Herein, the effects of paeonol and WRW4 on inflammatory factors in the FLS model were observed through qRT-PCR assays. Paeonol inhibited the production of FLS inflammatory cytokines induced by TNF-α and blocked the anti-inflammatory effect induced by WRW4 (Fig. 3A). These results suggested that the anti-inflammatory effects of paeonol were partially dependent on the activation of the FPR2 protein. Cognizant of this, we examined the effects of paeonol and WRW4 on the proliferation of FLS. Paeonol inhibited the abnormal proliferation of FLS. However, this inhibitory effect was blocked by WRW4. In summary, paeonol inhibits the occurrence and development of rheumatoid arthritis through the production of Fpr2 receptors.

4 Discussion

We collected all the data sets of LPS-induced macrophages in the current GEO database for obtain more realistic analysis results. Analysis of the GEO dataset revealed that LPS induces the DEGs in macrophage inflammatory response. Some of these genes were verified by qRT-PCR at the mRNA level. These DEGs represent the characteristic genes that induce inflammation in LPS, and provide clues for future research on the mechanism of inflammation.On this basis, we want to study the influence of paeonol in this model. The biological processes affected by paeonol were mainly "amacrine cell differentiation" and "plasma membrane." PPI analysis of the DEGs further provided clues to the mechanism of paeonol in diseases. All these studies remind us that the effect of paeonol on inflammation may be related to the cell membrane system.

The inflammation-related genes FPR2, Cd83, and Cfb were affected by paeonol in the macrophage model. The FPR2 receptor is associated with various inflammatory diseases, especially chronic inflammatory diseases. However, the mechanism of FPR2 remains unclear[20]. FPR2 activator inhibits cellular inflammatory response by increasing calcium influx. The influxion of Ca^{2+} can be detected by fluorescence when FPR2 is activated, and this activation is blocked by WRW4 inhibitors. FPR2 ligands can induce calcium influx by activating FPR2, which inhibits inflammation[21]. In this study paeonol could promote calcium influx in FLS cells. However, this trend was blocked by WRW4, a selective inhibitor of FPR2. Therefore, we believe that paeonol may act as a similar FPR2 activator to promote the influx of Ca^{2+} through direct or indirect effects. In future studies, optimizing the structure of paeonol to find more anti-inflammatory drugs is of significance for the development of subsequent target drugs

In arthritis research, the most important thing is to look at the inflammatory effect of drugs on synovial cells. Consistent with previous research, Paeonol inhibited IL-6, IL-1β and TNF-α production and mRNA expression[22]. WRW4 blocked the inhibition of inflammatory cytokines in FLS cells induced by paeonol. This finding suggests that paeonol affects the verification response of FLS cells through FPR2 receptors. Rheumatoid arthritis is characterised by synovial inflammation and proliferation of FLS[23]. We also studied the inhibitory effect of paeonol on the abnormal proliferation of FLS because of the abnormal proliferation of FLS cells in RA disease. Paeonol also inhibited the abnormal proliferation of FLS. However, this inhibitory effect was blocked by WRW4. In conclusion, paeonol can inhibit the inflammatory level of rheumatoid arthritis and the abnormal proliferation of fibroblasts.

There remain insufficiencies in this research. In this study, the way of paeonol activate the downstream signaling pathway has not been detailed. As well, the effect of paeonol on FPR2 has not been extensively studied in experimental animals.

Abbreviations

RA, Rheumatoid arthritis; FPR2, Formyl Peptide Receptor 2; GPCRs, G protein-coupled receptor; FLS, fibroblast-like synoviocytes

Declarations

Authors' contributions:

Yubao Shao performed the statistical analysis and wrote the manuscript. Jing Ye and Dahai Zhao extracted peripheral blood from the patients. Lanxin Bao and Wenhao Li performed co-cultured cells experiments and submitted the manuscript. Jinchen Dai, Mengmeng Chen and Taorong Wang performed the experiments. Xiaoyu Chen designed the study and revised the manuscript.

Consent for publication:

Not applicable

Ethics approval and consent to participate:

The experimental protocol was established, according to the ethical guidelines of the Helsinki Declaration and was approved by the Human Ethics Committee of Biomedical Ethics Committee of Anhui Medical University. Written informed consent was obtained from individual or guardian participants.

Declaration of competing interest:

All authors have no conflicts of interest to disclose.

Availability of data and materials:

All data and some of the materials relevant to the current study are available from the corresponding author on reasonable request.

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Figures

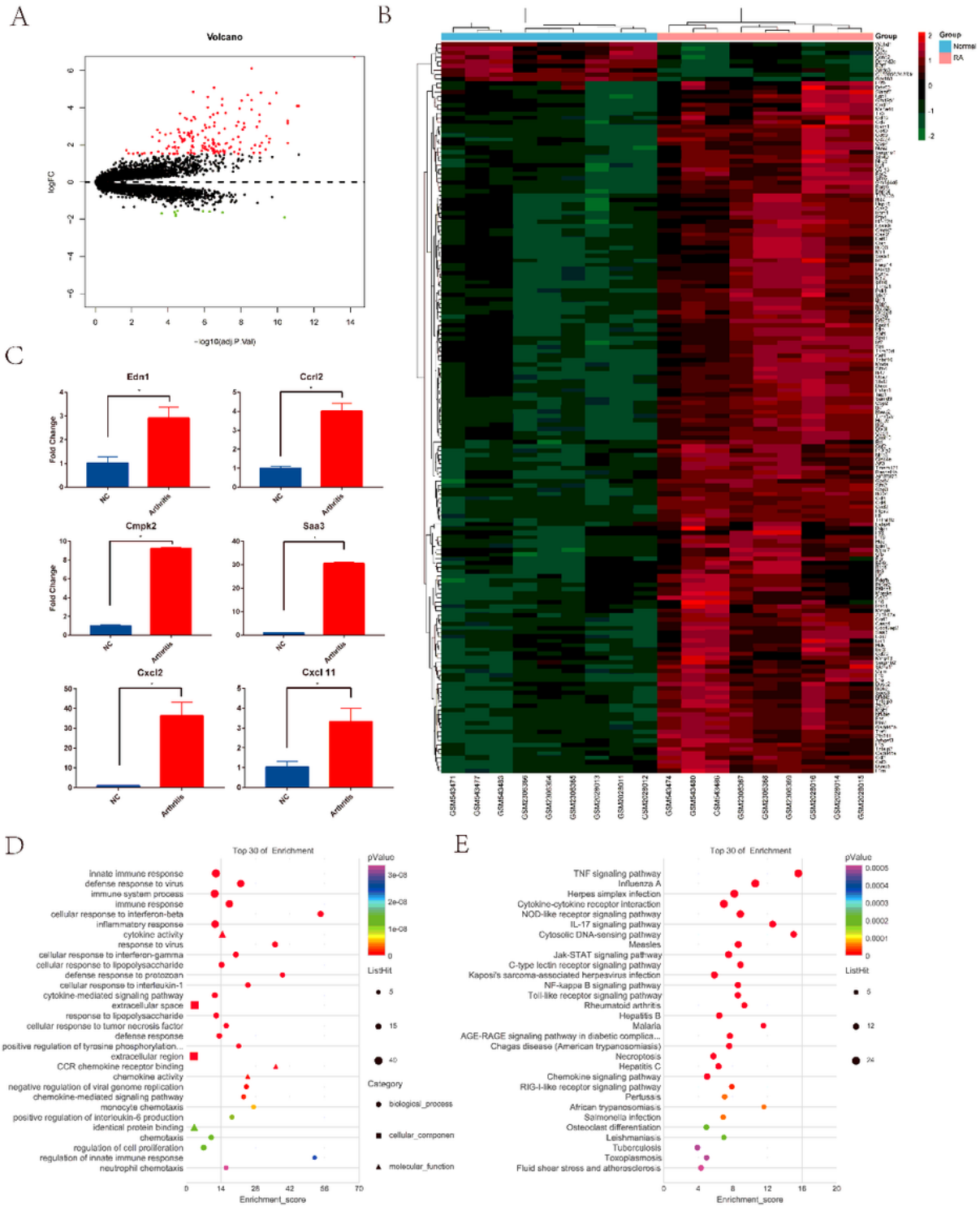

Figure 1

Differentially expressed genes in the LPS-RAW264.7 model (A) Volcano plots constructed using fold-change values and adjusted P. The red point in the plot represents the over-expressed mRNAs, while the blue point indicates the down-expressed mRNAs in the LPS-RAW264.7 model with statistical significance. (B) Hierarchical clustering analysis of mRNAs, which were differentially expressed between inflammatory and normal macrophage. (C) QRT-PCR assay detected some DEGs in B in macrophages with LPS or not.

*P<0.05. (and n = 3, mean ± SD) (D) GO enrichment analysis of DEGs: The gradual color represents the log FC. The genes were ordered according to their log FC values setting gene. (E) KEGG enrichment analysis of the pathways: The gradual color represents the P-value, the size of the black spots represents the gene number.

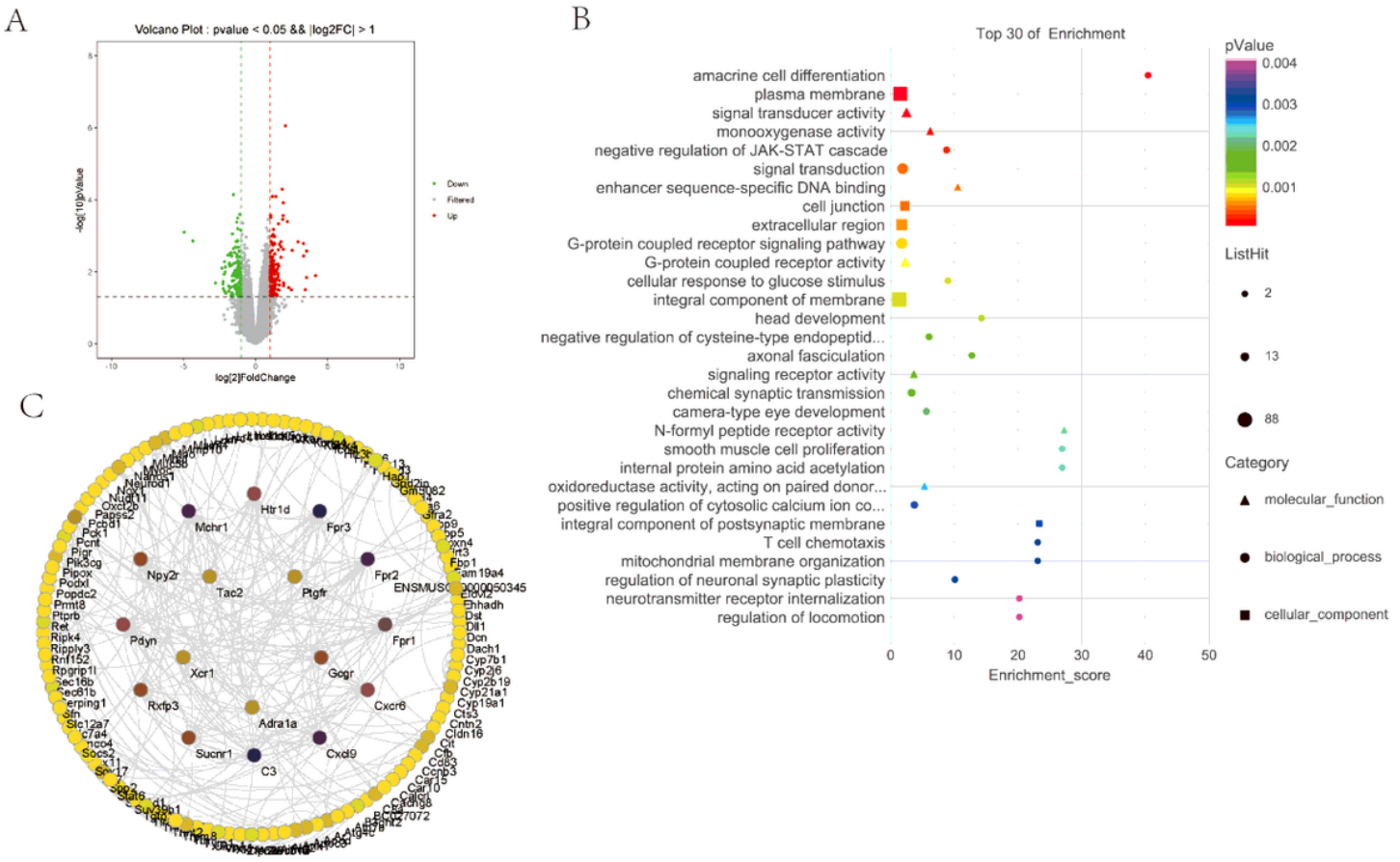

Figure 2

DEGs in macrophages treated and not treated with paeonol (A) Volcano plots were constructed using fold-change values and P-value. The red point in the plot represents the up-regulated mRNAs, while the blue point indicates the down-regulated mRNAs by paeonol. (B) GO enrichment analysis of DEGs: The gradual color represents the log FC. The genes were ordered according to their log FC values setting gene. (C) PPI network of DEGs; Degree of color manifest number of nodes.

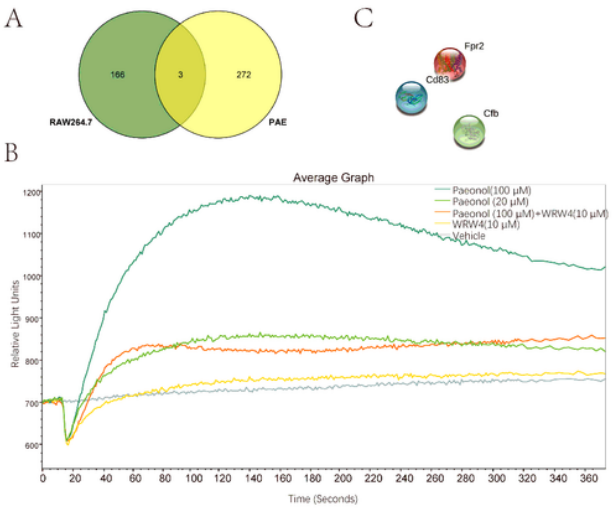

Figure 3

Paeonol depends on FPR2 to inhibit inflammatory responses (A). The qRT-PCR assay detected cytokines in macrophages treated with LPS, paeonol OR WRW4, respectively. (B). The concentration of intracellular Ca2+ in FLS. Y-axis represents the relative intensity of light, and the X-axis is time. The colored lines show groups treated with a different combination.

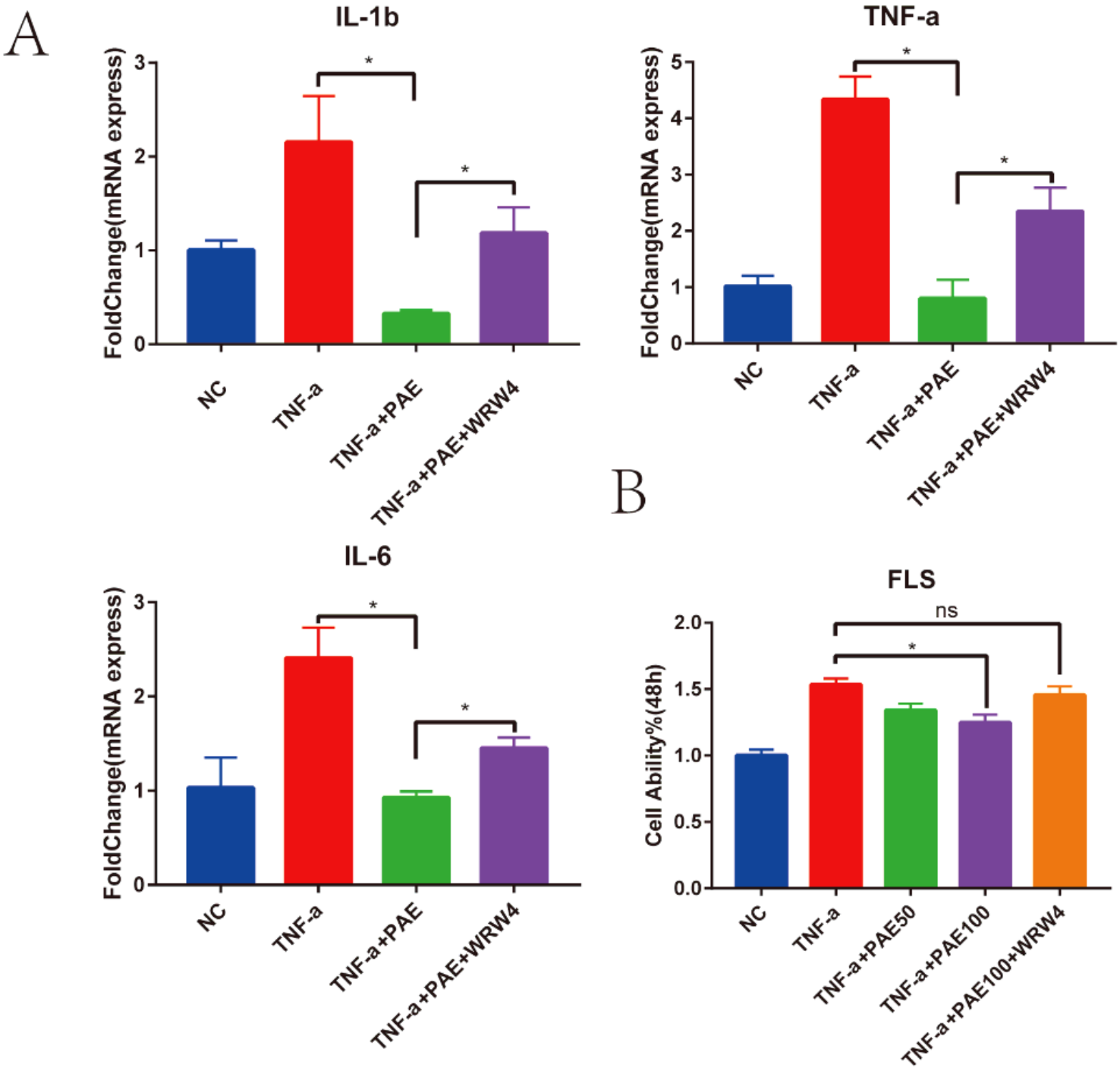

Figure 4

Paeonol has a good anti-inflammatory effect in FLS (A). The qRT-PCR assay detected the inhibitory effect of paeonol on IL-1β of FLS. *P<0.05. (and n = 3, mean ± SD) (B). The inhibitory rate of paeonol against TNF-α-induced abnormal proliferation of synovial cells. *P<0.05 (n = 5, mean ± SD)

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