Int J Clin Exp Med 2017;10(9):13123-13132
www.ijcem.com /ISSN:1940-5901/IJCEM0049270

Original Article

Dan-Zhi-Yin formula against IL-17A/F-induced chemokines via JNK pathway in mouse embryo fibroblasts NIH 3T3 cells

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Received December 11, 2016; Accepted August 20, 2017; Epub September 15, 2017; Published September 30, 2017

Abstract: Dan-Zhi-Yin formula (DZY) is a traditional Chinese medicine formula consisting of 8 herbs, which has been used to treat chronic pelvic inflammation and pelvic pain. The aim of the present study was to observe the mechanism of DZY on IL-17A/F-induced chemokines in the mouse embryonic fibroblasts (NIH 3T3). The viability of cells was determined by MTT assay. We evaluated the expression of the chemokines monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-2 (MIP-2) by ELISA and real-time RT-PCR. The phosphorylation of JNK was assessed by Cytometric Bead Array. IL-17RA and IL-17RC were inhibited by small interfering RNA (siRNA). Our study demonstrated that IL-17A/F were able to increase the gene levels and protein secretion of MCP-1 and MIP-2. However, the DZY could disrupt the expression of MCP-1 and MIP-2 stimulated by IL-17A/F. We observed that IL-17A/F could induce JNK activation and that pharmacological inhibitors of JNK (SP600125) blocked the IL-17A/F mediated MCP-1 and MIP-2 release. DZY could inhibited JNK expression at each time point, compared with Il-17A/F stimulated group alone. In addition, we also found that NIH 3T3 cells expressed IL-17 receptors. The expression of MCP-1 and MIP-2 stimulated by IL-17A/F could be reduced by inhibiting IL-17RA or IL-17RC expression via siRNA. In conclusion, IL-17A/F could induce the expression of MCP-1 and MIP-2 through JNK pathway in NIH 3T3 cells. However, DZY can inhibit IL-17A/F-induce MCP-1 and MIP-2 expression which might be regulated by JNK pathway. All these above indicate that DZY plays a role in anti-inflammatory process in NIH 3T3 cells.

Keywords: IL-17A/F, DZY, JNK, IL-17RA, IL-17RC, mouse embryo fibroblasts

Introduction

IL-17 (or IL-17A) family members include five additional cytokines (IL-17B, IL-17C, IL-17D, IL-17E, and IL-17F) [1, 2], which is mainly produced by naive $CD4^+$ and activated $CD8^+$ T cells [3]. Previous studies have elaborated that IL-17 induces secretion of inflammatory cytokine in various type of cells, such as macrophages, keratinocytes [4], epithelial cells [5], and fibroblasts [6]. Thus, more studies paid attention to the effect of IL-17A in the pathogenesis of various inflammatory diseases including inflammatory bowel disease, rheumatoid arthritis, sclerosis, and airway inflammation [7].

The IL-17A had been reported to be related to severity of pelvic cavity inflammation [8]. Qiu et al. found that IL-17A increased monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein (MIP-2) and tissue inhibitor of metalloproteinase-1 (TIMP-1) in fibroblastoid L929 cells [9]. However, few evidences are found to establish how IL-17A induce the pelvic inflammation. IL-17F has the 50% amino acid sequence homology to IL-17A [10]. IL-17A and IL-17F have the considerable overlap in the biological process [11]. Previous studies have usually evaluated the inflammatory role of IL-17 *in vitro* using embryonic fibroblasts of NIH 3T3 cells [12, 13].

Herbs have been widely used for 2000 years in China with high efficiency and low toxicity. Compared with single-component drug, the Traditional Chinese medicine (TCM) decoction exhibits a multitargets advantage. Dan-Zhi-Yin (DZY) is composed of Salvia miltiorrhiza Bge, Liguisticum chuanxiong Hort, Morus alba L, Dipsacus asper Wall, Forsythia suspense Vahl,

Table 1. The composition of Da-Zhi-Yin (DZY)

Latin name	Chinese name	Grams
Salvia miltiorrhiza Bge.	Danshen	10
Morus alba L.	Sangzhi	10
Liguisticum chuanxiong Hort.	Chuanxiong	6
Forsythia suspensa Vahl	Lianqiao	10
Dipsacus asper Wall	Xuduan	15
Litchi chinensis Sonn.	Lizhihe	10
Rhizoma corydalis	Yuanhu	10
Cyperus rotundus L.	Xiangfu	6
Total		77

Table 2. The primer sequences used for real-time PCR assay

Primer name	Primer sequences	Length (bp)
MCP-1 upstream	GAAGCTGTAGTTTTTGTCACCAAGC	97
MCP-1 downstream	GGTTCCGATCCAGGTTTTTAATGT	
MIP-2 upstream	TCAATGCCTGAAGACCCTGC	189
MIP-2 downstream	GCCTTGCCTTTGTTCAGTATCT	
GAPDH upstream	CGTGTTCCTACCCCCAATG	126
GAPDH downstream	GCCCAAGATGCCCTTCAGT	

Litchi chinensis sonn, rhizome corydalis, and Cyperus rotundus L. As shown in **Table 1**. In TCM theory, DZY is involved in removing the stasis and dredging collaterals, which are the principles for pelvic inflammatory disease (PID) [14]. Our previous studies had shown that DZY could decrease the level of VEGF-A/C/D expression in the cytoplasm of endometrial glandular epithelial cells [15] and reduce the expression of VEGF (vascular endothelial growth factor), angiopoietin-2 (ang-2) and osteopontin in the upper genital tract and played a positive role in the anti-inflammatory process in pelvic inflammation mouse model [16]. In the pathological process of PID, some pro-inflammatory cytokines can promote the proliferation of fibroblast, which secretes the extracellular matrix such as collagen and elastin resulting in collagenous adhesions [17]. So, the fibroblast plays a great role in the prognosis of PID. Although the DZY had an anti-inflammatory role in pelvic inflammatory disease, the related pharmacological mechanism is still not very clear. Thus, in our present study, we used the mouse embryonic fibroblast NIH 3T3 cells to explore the potential molecular mechanism of DZY, which may provide more evidences on the treatment of pelvic inflammatory disease.

Materials and methods

Regents and antibodies

Recombinant Mouse IL-17A/F and MTT cell proliferation/viability Assay kits were acquired from R&D Systems (Minneapolis, MN, USA). The inhibitor of JNK (SP600125) was the product of Selleck Chemicals (Houston, TX, USA). Antibodies against JNK and phospho-JNK were obtained from Thermo Fisher (MA, USA). Mouse/Rat CCL2/JE/MCP-1 Quantikine ELISA Kit and Mouse CXCL2/MIP-2 Quantikine ELISA Kit were the products of Abcam (Massachusetts, US). BD Cytometric Bead Array (CBA) Cell Signaling Master Buffer Kit and Phospho JNK1/2 (T183/Y185) Flex Set were obtained from the BD biosciences (NJ, USA). Prime Script™ RT reagent Kit with gDNA Eraser, and SYBR® Premix Ex Taq™ II (Tli RNaseH Plus), DL 2,000 DNA Marker were purchased from TaKaRa (JAPAN). Mouse IL-17RA small interfering RNA (siRNA), IL-17RC (siRNA) were obtained from Dharmacon (Lafayette, CO).

Preparation of DZY

The crude drugs of DZY were provided by the pharmacy of Dong fang Hospital of Beijing University of Traditional Chinese Medicine. First, eight herbs of DZY (77 g) were soaked in 800 ml distilled water for 30 min and boiled for 40 min. Then, the drug solution was filtered, and the filter residue was boiled in 800ml distilled water for another 40 min and the solution was filtered again. Both filtrates were homogenized and concentrated by heating to 85 ml liquid. The concentration of the crud drug was 0.9 g/ml, and then stored at -4°C until required.

High performance liquid chromatography (HPLC) and fingerprint analysis

3 ml DZY (0.9 g/ml) was dissolved with 250 ml ultrafiltered water, and filtered through a 0.45 μm membrane filter under vacuum. The filtrates were dried in a rotary evaporator. The dried powder was solubilized in 1 ml ultrafiltered water. The extract of DZY was analyzed with HPLC system. The HPLC system was composed of waters pump (2695), photodiode Array Detector (2998), and Diamosil C18 column (5 μm, 4.6 × 250 mm). The mobile phase was consist-

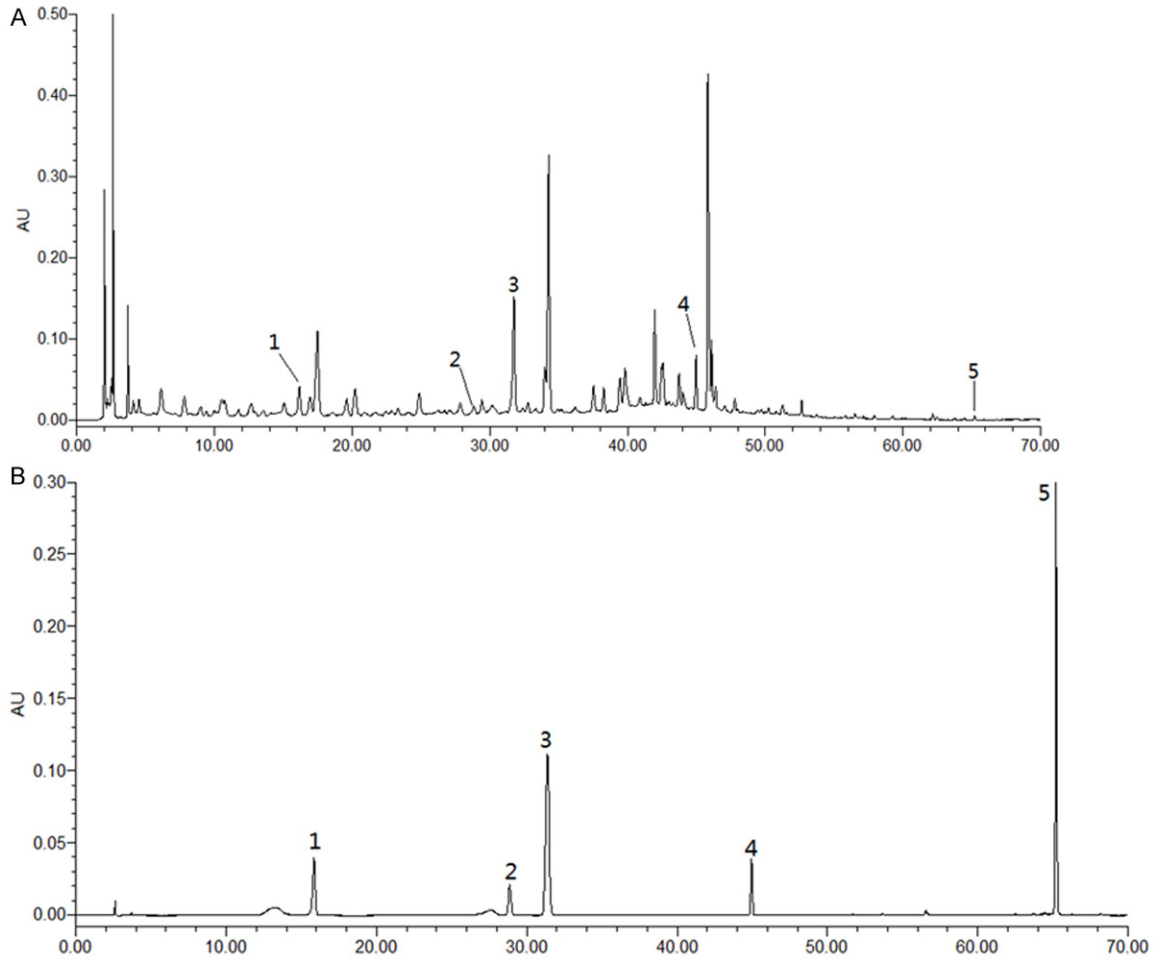

Figure 1. The HPLC-based fingerprint of DZY. A. DZD of 0.9 g/ml; B. The reference compounds. 1. Mulberroside A; 2. Tetrahydropalmatine; 3. Ferulic acid; 4. Phillyrin; 5. Tanshinone IIA.

ed of acetic acid water (A) and acetonitrile (B). The linear gradient was 5%-95% for 0 min-70 min. The flow rate was kept 1.0 ml/min, and injection volume was 10 μl. The analytes were detected at 280 nm.

Cell culture and treatment

Mouse embryo fibroblasts NIH 3T3 cells (from cell culture center of Peking Union medical college) were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA), penicillin (100 U/mL), and streptomycin (100 μg/mL) at 37°C in a humidified atmosphere under 5% CO_2. To induce inflammation, NIH 3T3 cells were exposed to IL-17A/F. When cells reached 70%-90% confluence, they were incubated in 10% FBS-DMEM containg 100 ng/ml IL-17A/F. The NIH 3T3 cells were treated with various concentrations (25, 50, 100 μg/ml) of DZY for 24 h.

Cell viability assays

After various treatments, the viability of cells was determined by MTT assay. In brief, cells were seeded on the 96-well culture plates overnights, then different concentrations of DZY for 24 h. After that, discarding the original culture medium, 100 μL fresh culture medium and 10 μL MTT was added to each well for 4 h at 37°C. 100 μL SDS (10%) was added to solubilize the cells. At last, the OD value of each well was detected at 570 nm using a microplate reader (BioTek, VT, USA). Cell viability (%) was expressed as a percentage relative to the untreated control cells.

Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) kits purchased from Abcam (Massachusetts, US) were used to quantitatively determine the specific concentrations of MCP-1, and MIP-2 in

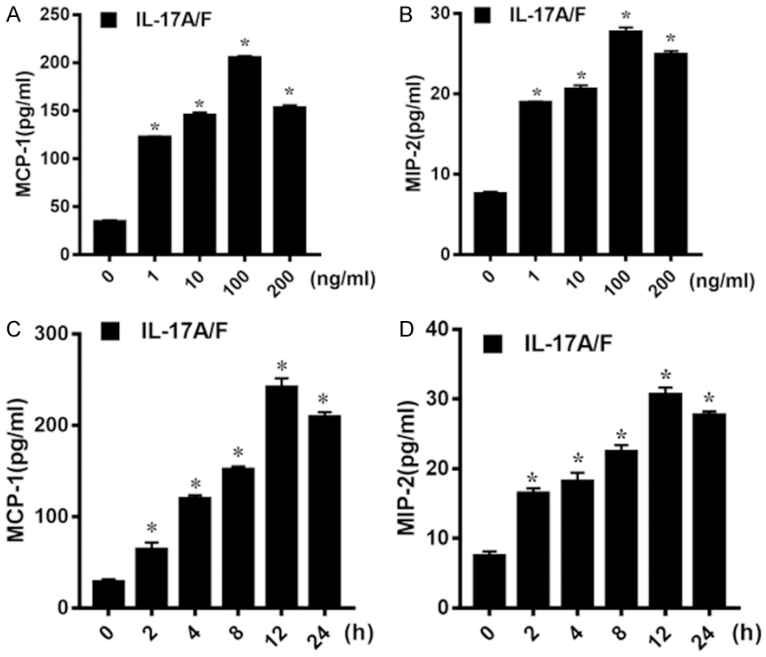

Figure 2. Concentration and time dependence of IL-17A/F-induced MCP-1 and MIP-2 protein expression in mouse embryonic fibroblasts. A and B: The dose response of IL-17A/F on MCP-1 and MIP-2 protein secretion. Mouse embryonic fibroblasts were cultured in 6-well plates and treated with the blank control (IL-17A/F, 0 ng/ml) or IL-17A/F (1, 10, 100, 200 ng/ml) for 24 h. C and D: Mouse embryonic fibroblast were incubated without or with IL-17A/F (100 ng/ml) for the various lengths of time point. The supernatants of cultured mouse embryonic fibroblasts were harvested and analyzed by ELISA. Values are means ± SD, *P<0.05 compared with baseline concentration and time.

the culture supernatants according to manufacturer's instruction.

Western blot

After treatment, the cells were harvested and lysed in RIPA buffer (Sigma, Cat, USA) for 30 min. The lysate was centrifuged at 16000 × g for 20 min at 4°C. Protein concentration of cell samples was assessed via the BCA protein assay reagent kit (Sigma, Cat, USA). For Western blotting, protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 10% gel), and transferred to nitrocellulose membranes (MilliporeCorp, Billerica, MA, USA) by electroblotting at 4°C. The membranes were blocked with 5% skim milk in Tris-buffered saline plus 0.02% Tween-20 (TBST) for 30 min at room temperature and then incubated at 4°C overnight with the following primary antibodies, including anti-JNK (diluted 1:1000) and anti-p-JNK (diluted 1:1000). Following incubation with primary antibodies, the blots were washed four times with TBST and incubated for 1 h at room temperature with goat anti-mouse or antirabbit HPR-conjugate antibody at 1:2000 dilution in TBST containing 5% skim milk. Equal loading was confirmed using GAPDH (diluted 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA). The results were visualized using a chemiluminescence detection kit (Pierce ECL Western Blotting Substrate Detection System; Thermo Fisher Scientific).

Real-time PCR

The mRNA levels of mouse MCP-1, MIP-2, IL-17RA, and IL-17RC were quantitated by Real-time PCR. In brief, total RNA was extracted by Trizol reagent (Tian Gen Biotechnology Inc.). The Nano Drop spectrophotometer was used to measure Total RNA concentration via densitometry (260/280 nm). cDNA was synthesized using an RT kit (Takara). The mRNA expression was detected by a SYBR Premix Ex Taq Kit (Takara) and an ABI PRISM7500 real-time PCR system (Life Technologies), with GAPDH as internal controls. Sequences of the upstream and downstream PCR primer was shown in **Table 2**.

Cytometric bead array (CBA) for quantification of phosphor JNK

Cells were exposed to IL-17A/F or DZY (50 μg/ml) for different times (0-180 min), washed and lysed by 1 × Denaturation Buffer. The cell samples were denatured in boiling water, and collected at -70°C. BCA protein assay reagent kit (Sigma, Cat, USA) was selected to measure protein concentration. Cell Samples were centrifuged at 14000 rpm (3 min) to pellet the debris and diluted by the Assay Diluent at 1:5 before use. The BD CBA Cell Signaling Flex Set Standards (Phospho JNK1/2 (T183/185)) was serially diluted. All samples were incubated with the mixed Capture Beads for 3 hours, and the

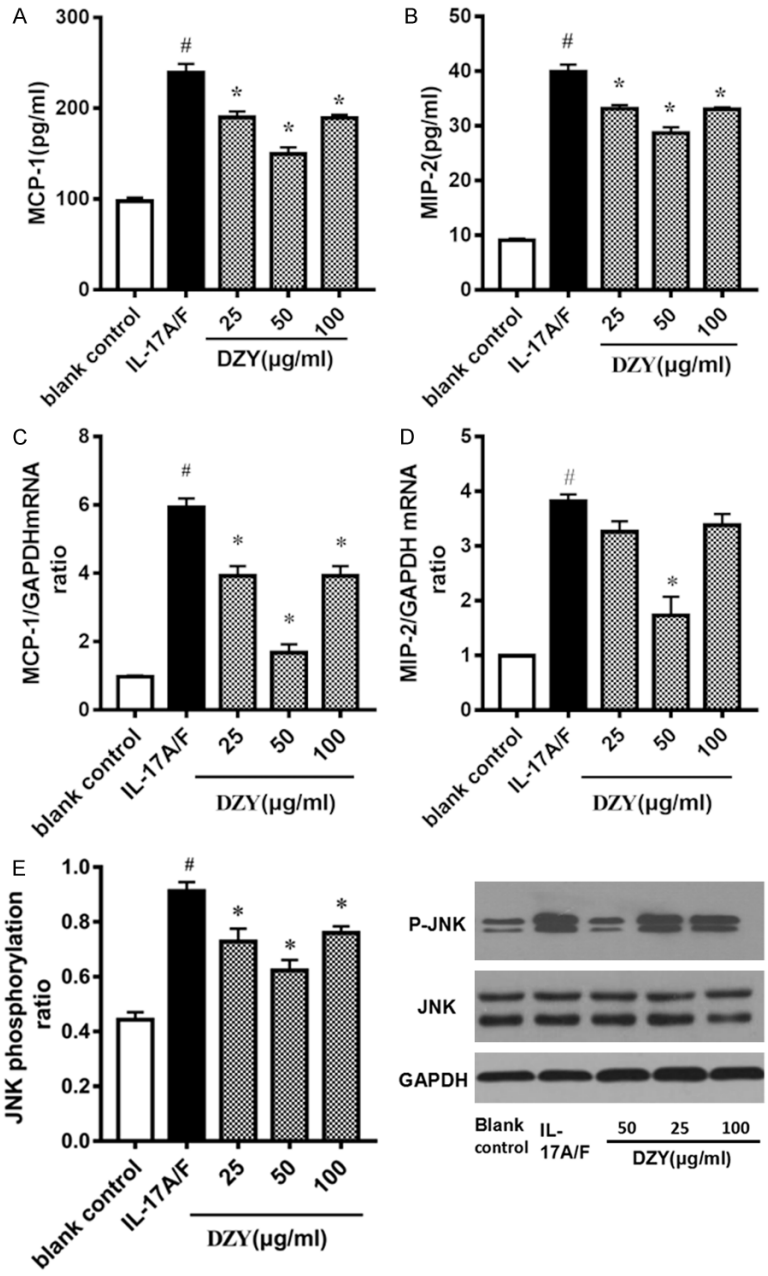

Figure 3. The diferent concentration of DZY meidated the expression of MCP-1, MIP-2 and the JNK phosphorylation. A and B: The MCP-1 and MIP-2 production; C and D: The MCP-1 and MIP-2 mRNA production; E: The JNK phosphorylation ratio. #P<0.05 compared with blank control; *P<0.05 compared with IL-17A/F-stimulated group.

PE Detection Reagent for 1 hour. Samples was washed and centrifuged at 200 g (5 min). Supernatant was discarded, and 300 μl wash buffer was added to resuspend the beads. All samples were acquired on the flow cytometer, and data was analyzed by using FCAP Array software.

Small interfering RNA (siRNA) transfection

Predesigned Acell SiRNA duplexes specially prepared for IL-17RA and IL-17RC were obtained from Dharmacon. The mouse embryo fibroblasts were transfected with control siRNA or target siRNA (IL-17RA siRNA and IL-17RC siRNA) according to the manufacturer's instructions. The NIH 3T3 cells were incubated in 6-well plates to a concentration of 1×10^5 cells/well before transfection. Cells transfection were conducted using 0.5 μg IL-17RAsi RNA or IL-17RC siRNA or control siRNA respectively. After 48 h transfection, above cells were treated with IL-17A/F for 12 h. Cells were harvested for analysis.

Statistical analysis

Data are presented as mean ± standard deviation (SD). The SPSS 18.0 software was used to perform all the analysis. Comparisons between two groups were performed to use the Student's t-test and between multiple groups using one-way analysis of variance (ANOVA). The values of $P<0.05$ were considered statistically significant.

Results

Identification of DZY

As shown in **Figure 1**, the compounds of DZY were identified by HPLC-based fingerprint including tetrahydropalmatine, ferulic acid, mulberroside A, phillyrin, tanshinone IIA.

Effect of IL17A/F on MCP-1 and MIP-2 secretion in NIH 3T3 cells

Elevated levels of MCP-1 and MIP-2 protein secretion were measured in the mouse embry-

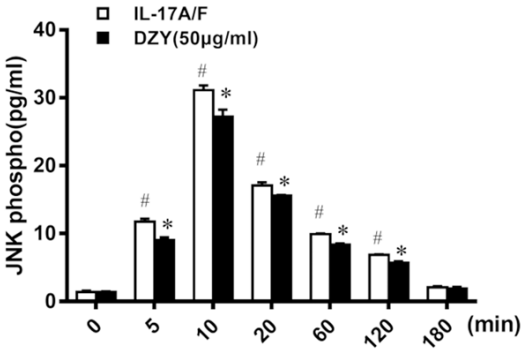

Figure 4. Cytometric Bead Array analyzed the JNK phosphorylation in mouse embryonic fibroblasts stimulated with IL-17A/F or IL-17A/F+DZY (50 μg/ml). #P<0.05 compared with baseline time in IL-17A/F group; *P<0.05 represented the IL-17A/F+DZY group compared with IL-17A/F group in each time-point.

onic fibroblasts exposed to elevating concentrations of IL-17A/F by ELISA techniques. As shown in **Figure 2A** and **2B**, IL-17A/F significantly increased MCP-1 and MIP-2 levels in mouse embryonic fibroblast, and the effect of IL-17A/F reached a peak at 100 ng/ml, which was selected as the standard concentration in the further studies. Additionally, the mouse embryonic fibroblasts were exposed to IL-17A/F (100 ng/ml) at increasing time periods (0 h, 2 h, 4 h, 8 h, 12 and 24 h) to detect the protein expression of MCP-1 and MIP-2. We also found the production of MCP-1 and MIP-2 reached a maximal effect at 12 h, and then declined (**Figure 2C** and **2D**).

Effect of DZY on viability of NIH-3T3 cells

MTT assay was performed to investigate cytotoxic potential of DZY on mouse embryo fibroblasts. Cells were treated with various concentration of DZY (12.5~200 μg/ml) for 24 h. DZY, at concentrations of 200 μg/ml, significantly suppressed the cell viability, therefore we choose 100 μg/ml of DZY for the following experiments.

Effect of DZY on NIH 3T3 following IL17-A/F stimulation

IL-17A/F induced the production of MCP-1 and MIP-2 compared with the blank control group. When mouse embryonic fibroblasts were pretreated with DZY (25, 50, 100 ug/ml) and stimulated with IL-17A/F (100 ng/ml) for 24 h, an effective down regulation of MCP-1 and MIP-2 production was observed (**Figure 3A** and **3B**). As shown in **Figure 3C** and **3D**), IL-17A/F induced the MCP-1 and MIP-2 mRNA expression and different concentration of DZY inhibited the MCP-1 and MIP-2 mRNA expression. We also found that MCP-1 and MIP-2 mRNA expression reached a minimum level at dose of 50 μg/ml. According to the Western blot result, IL-17A/F increased the JNK phosphorylation ratio in mouse embryonic fibroblast (**Figure 3E**). While, DZY inhibited the JNK phosphorylation.

Effect of IL17-A/F and DZY on JNK activation in NIH 3T3 cells

To identify the JNK signal transduction mechanism involved in IL-17A/F-induced mouse embryonic fibroblast, we used Cytometric Bead Array to analyze. It has been showed that the phosphorylation of JNK was elevated as early as 5 min and continued to rise, peaked at 10 min, and returned to baseline at 180 min thereafter. Meanwhile, DZY inhibited the JNK expression at each time-point (**Figure 4**).

IL-17A/F mediated MCP-1 and MIP-2 -dependent JNK pathway in NIH 3T3 cells

To investigate whether the effect of IL-17A/F was mediated through activation of the JNK pathway, the MCP-1 or MIP-2 production were examined in the mouse embryonic fibroblasts by ELISA. Cells were pretreated with JNK inhibitors (SP600125, 10 μM). As a shown in **Figure 5**, treatment of cells with JNK inhibitors caused strong attenuation of the IL-17A/F-induced MCP-1 and MIP-2 levels.

Effect of the administration of SiRNA for IL-17A/F on NIH 3T3 cells

IL-17RA and IL-17RC siRNA were used to confirm whether the secretion of MCP-1 and MIP-2 stimulated by IL-17A/F was conducted through IL-17RA or IL-17RC pathway. Firstly, the results showed that the IL-17RA and IL-17RC mRNA expression down regulated significantly in mouse embryonic fibroblast compared with that stimulated with blank control (**Figure 6A** and **6B**). Secondly, we found that IL-17RA and IL-17RC siRNA could down-regulate the corresponding expression of IL-17RA and IL-17RC mRNA significantly (**Figure 6C** and **6D**). Next, we observed the relationship between down-

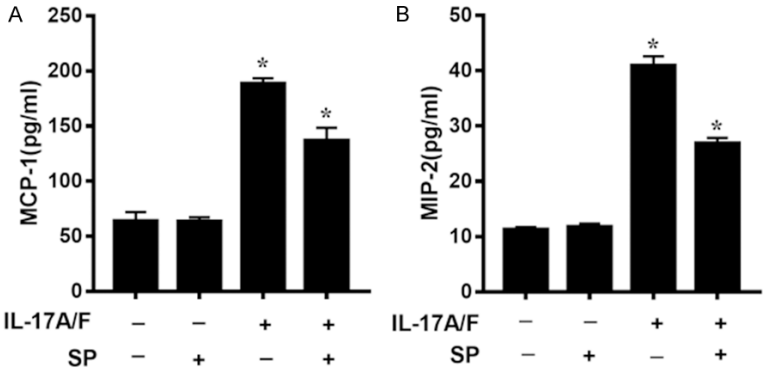

Figure 5. MCP-1 and MIP-2 production induced by IL-17A/F is inhibited by SP. mouse embryonic fibroblasts were pre-incubated with SP followed by stimulation with IL-17A/F (100 ng/ml) for 24 h. *P<0.05 compared with cultured without SP.

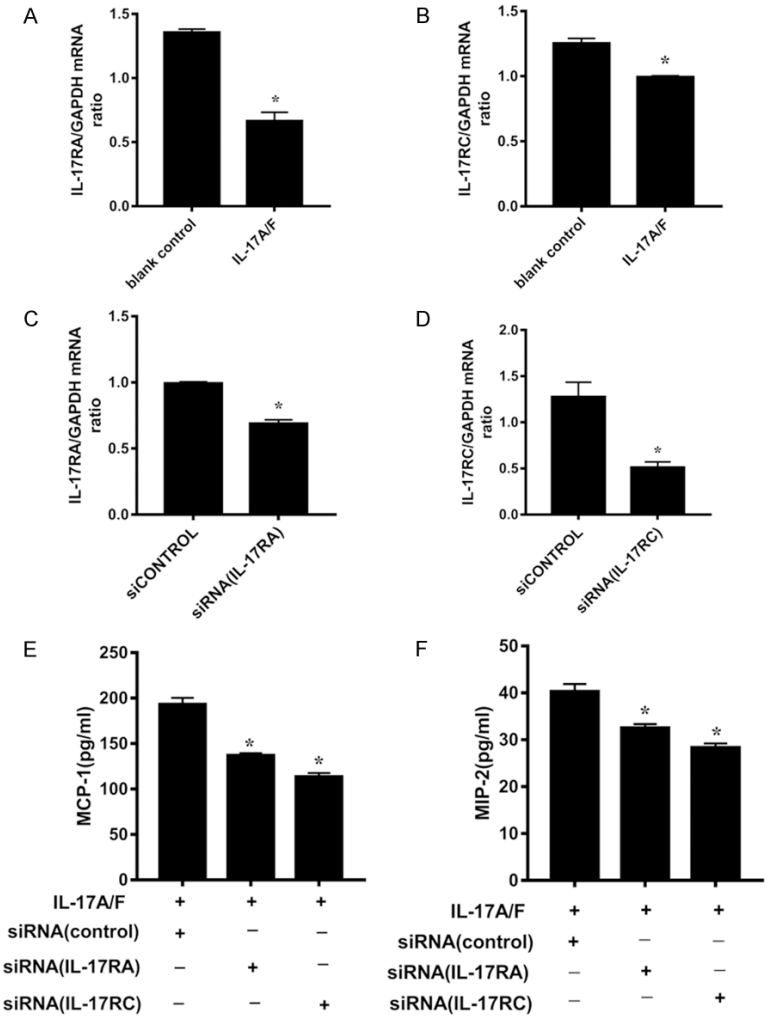

Figure 6. The mRNA levels of IL-17RA and IL-17RC and the receptor-specific effect of IL-17A/F on the expression of MCP-1 and MIP-2 in mouse embryonic fibroblast. A and B: IL-17RA and IL-17RC mRNA expression with or without IL-17A/F induced were observed by RT-PCR. *P<0.05 compared with cultures without the IL-17A/F; C and D: IL-17RA and IL-17RC was downregulated via transfection with RNA interference. Cells were transfected with 0.5 μg IL-17RA, IL-17RC, or control siRNA. The IL-17RA and IL-17RC gene expression were detected by RT-PCR. *P<0.05 compared with control; E and F: Cells were transfected with 0.5 μg IL-17RA, IL-17RC, or control siRNA. After 24 h of transfection, cells were treated with IL-17A/F (100 ng/ml) for 12 h. The expression of MCP-1 and MIP-2 were measure by ELISA. Values are means ± SD. *P<0.05 compared with cultures with no siRNA.

regulating interleukin receptors (IL-17A and IL-17F) and the expression of pro-inflammatory cytokines (MCP-1 and MIP-2). The secretion of MCP-1 and MIP-2 was decreased obviously after knocking-down of IL-17RA or IL-17RC using siRNA technology (**Figure 6E** and **6F**).

Discussion

In the study, we applied IL-17A/F to stimulate the Mouse embryo fibroblasts NIH 3T3 cells and the results showed the production of MCP-1 and MIP-2 increased significantly. Moreover, the two chemokines mediated by IL-17A/F reached maximum at a dose of 100 ng/ml and at 12 h. However, when exposed to DZY formula, the IL17A/F-driven MCP-1 and MIP-2 production decreased strikingly. The CBA results showed activation of JNK was at 5 min, and the level of phosphorylated JNK reached maximum at 10 min and fell down to baseline at 180 min. In addition, we also found that mouse embryo fibroblasts NIH/3T3 cells expressed IL-17 receptors. Knocking down either IL-17RA or IL-17RC via siRNA led to significant decrease of

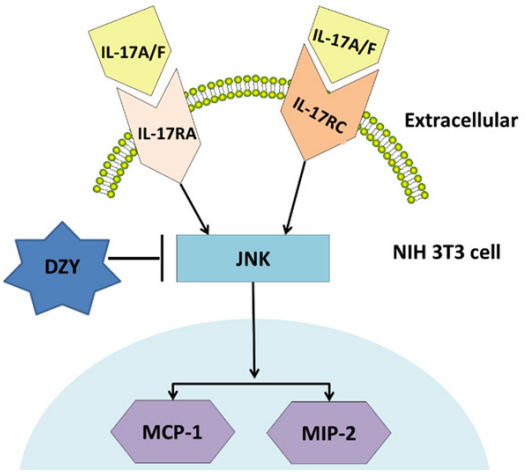

Figure 7. Summary of anti-inflammatory activity of DZY in IL-17A/F-stimulated in NIH 3T3 cells.

MCP-1 and MIP-2 secretion stimulated by IL-17A/F. The mechanism was illustrated in **Figure 7**.

It has been reported that IL-17A could induce the expression of MCP-1 and MIP-1α in the mouse mesangial cells [18]. Together with their data, our study showed that IL-17A/F also could induce the expression of MCP-1 and MIP-2 in mouse embryonic fibroblasts. Our study is the first to report that the IL17A/F regulated the production of MCP-1 and MIP-2 in mouse embryonic fibroblasts. The elevation of MCP-1 and MIP-2 expression stimulated by IL-17A and IL-17F has previously been reported by another study in mouse mesangial cell [19]. Therefore, it appears that IL-17A/F may play role on different host cells.

DZY was formulated in conformity to classic formula principle. *Salvia miltiorrhiza Bge.* and *Morus alba L.* are the main components of DZY. The main active components of *Salvia miltiorrhiza Bge* is Tanshionone IIA which could inhibit the vascular smooth muscle cell proliferation, collagen synthesis and migration in systemic sclerosis patients stimulated by IL-17A [20]. The remaining herbs reduce the anti-inflammatory effect together [21-26]. The Chinese herb formula is thought to increase therapeutic efficacy through multi-targets and multi-biological pathway.

It has been reported that IL-17F-induced IL-6 and IL-8 expression via ERK1/2 pathway but not JNK pathway in HUVEC [27]. In addition, Another study [19] reported that IL-17A and IL-17F induced the expression of MCP-1 and MIP-2 via phosphorylation of ERK1/2 and P38 in mouse cultured mesangial cells. However, our study showed that IL-17A/F induced the production of MCP-1 and MIP-2 via JNK pathways in mouse embryonic fibroblasts. The above inconsistent results may be due to the different role of IL-17 on different cell line.

In our study, two receptors (IL-17RA and IL-17RC) were identified in mouse embryonic fibroblasts. But, we observed that the two receptors did not upregulate following IL17A/F stimulation. It is reported [28] that IL-17A/IL-17F treatment caused decrease in the production of IL-17RA and IL-17RC using flow cytometry. While, IL-17F increased the production of IL-17RC via western blot in human eosinophils instead of IL-17A. We used RNA interference to further understand IL-17 receptors to the signaling of IL-17A/F. Our study demonstrated that down regulation of IL-17RA or IL-17RC expression resulted in a significant reduction of MCP-1 and MIP-2 in mouse embryonic fibroblasts. Next, it is necessary for investigators to clarify the relationship between IL-17A/F and IL-17 receptors using anti-mouse IL-17RA or IL-17RC agonist.

In conclusion, the discovery of our study is that IL-17A/F induce the expression of MCP-1 and MIP-2 through JNK pathway in mouse embryonic fibroblasts. However, DZY can inhibit IL-17A/F-induced MCP-1 and MIP-2 expression, which might be regulated by JNK pathway.

Acknowledgements

The authors thank Xin Gong and Yulong Zhuang of Beijing University of Chinese medicine for their help in cell culture. The study was supported by a grant by National Natural Science Foundation of China [81373677].

Disclosure of conflict of interest

None.

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