Curcumin alleviates inflammation in Takayasu's arteritis by blocking CCL2 over-expression in adventitial fibroblasts

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ABSTRACT

Objective. *Takayasu's arteritis (TAK) is a chronic inflammatory disease with several challenges in treatment. Curcumin is known for its anti-inflammatory effects, whereas its effect in the treatment of TAK remains unclear. In this study, we aimed to investigate the effect of curcumin in the treatment of TAK and its underlying mechanisms.*

Methods. *16 TAK patients were treated with curcumin granules at a dose of 15 g/day for three months. Kerr score was explored to assess disease activity. Serum levels of inflammatory factors were measured by ELISA. Immunohistochemical and immunofluorescence staining were used to detect the expression of CCL2 (also known as MCP-1) in aortic adventitia. RT-qPCR, ELISA and western blot were used to determine the regulatory effect of curcumin on CCL2 expression in aortic adventitia fibroblasts (AAFs) and its mechanism.*

Results. *Curcumin treatment significantly lowered Kerr score and the levels of serum CCL2 in TAK patients. The expression of CCL2 in TAK aortic adventitia was increased and colocalised with CD68. Serum levels of CCL2 was increased in subjects with Kerr score ≥2. After curcumin treatment, the changes in CCL2 were positively associated with the changes in IL-6. In further analysis, it showed that CCL2 was co-localised with CD90 and α-SMA, markers of adventitia fibroblasts. In vitro, HSP65, an agonist of TLR4, could induce CCL2 expression in AAFs via phosphorylating and activating the JAK2/AKT/STAT3 pathway. Nevertheless, curcumin could reverse the HSP65-induced CCL2 upregulation through restraining JAK2/AKT/STAT3 pathway. The inhibitory effect of curcumin on the JAK2/AKT/STAT3 pathway was even more obvious than that of methotrexate and tofacitinib.*

Conclusion. *Curcumin alleviated inflammation in TAK by downregulating CCL2 overexpression in AAFs through inhibiting the JAK2/AKT/STAT3 signaling pathway.*

Introduction

Takayasu's arteritis (TAK) is a chronic inflammatory disease mainly affecting the aorta and its primary branches (1). Although it mainly affects Asians, the prevalence of TAK has increased worldwide (2). Generally, TAK is treated with glucocorticoids (GCs) combined with immunosuppressive agents (3); however, disease relapse may occur in a considerable proportion of patients with remission, especially during tapering of GCs, and result in the development of vessel damage (4). In recent years, biological agents and small molecular inhibitors, such as tocilizumab and tofacitinib, have also shown their efficacy in TAK, but limited to small sample sizes (5).

The pathological characteristic of TAK is the infiltration of various inflammatory cells with granulomatous formation and subsequent fibrosis of the aorta wall. Among the infiltrated inflammatory cells, macrophages play an extremely important role, including releasing multiple pro-inflammatory factors to induce inflammation and vascular fibrosis (6, 7). Thus, it is very important to investigate the factors involved in the macrophage immigration into the aorta wall and further to find reagents for inhibiting the infiltration of macrophages.

C-C motif chemokine ligand 2 (CCL2), also known as monocyte chemoattractant protein-1 (MCP-1), is a macrophage chemotactic factor and plays an important role in macrophage infiltration (8). Savioli *et al*. have found that serum concentrations of CCL2 were increased in patients with TAK (9). Nevertheless, where the CCL2 comes from and whether CCL2 plays a role in the patho-

genesis of TAK and its upstream regulations remain unclear.

In TAK, vascular lesion is generally thought to start from adventitia (6), which is characterised by numerous fibroblasts. During the pathogenesis of TAK, aorta adventitial fibroblasts (AAFs) do not only proliferate excessively and produce numerous extracellular matrix (10) but also produce several pro-inflammatory factors (11). However, whether and how AAFs involves in the production of CCL2 in TAK is never reported so far.

Curcumin is a small polyphenolic compound extracted from the rhizome of the *Curcuma longa* plant, and exhibits several pharmacological functions, such as anti-inflammation, anti-oxidation, anti-cancer activity, etc. (12). Clinically, curcumin has been used in a variety of inflammatory diseases including rheumatoid arthritis, multiple sclerosis, inflammatory bowel disease, systemic lupus erythematosus, and TAK (13-16).

As curcumin was reported to reduce CCL2 expression in a variety of cell types (17-19). For example, curcumin was reported to inhibit LPS-induced inflammation in rat vascular smooth muscle cells *in vitro* via ROS-relative TLR4-MAPK/NF-κB pathways (17). Another report described that curcumin could inhibit MCP-1 expression by suppressing the c-Jun N-terminal kinase pathway in macrophages (18). These indicated that curcumin alleviated inflammation with multiple targets signaling pathway. However, whether curcumin could inhibit CCL2 production in AAFs and whether it is related to alleviating inflammation in TAK remains unknown.

In this study, we first determined the effect of curcumin on disease activity and the levels of serum CCL2 of TAK patients. Then we explored the relationship between CCL2 and inflammation in TAK, as well as the mechanism of CCL2 overexpression in AAFs. Finally, the underlying mechanisms for curcumin in regulating CCL2 expression was investigated *in vitro*.

Materials and methods

Human subjects

TAK was diagnosed according to the American College of Rheumatology 1990 (ACR 1990) criteria. 16 patients with TAK were enrolled from October 1, 2018 to October 1, 2019. The inclusion criteria were as follows: i) over 14 years of age; ii) patients with active disease as assessed by elevated erythrocyte sedimentation rate (ESR), symptoms, or imaging progression; iii) no adjustment of immunosuppressants in the last month before enrollment. The exclusion criteria consisted of: i) presence of a recent active infection; ii) organ failure; iii) allergy to curcumin; (iv) treatment with other traditional Chinese medicine in the last month; (v) pregnant, lactating, or preparing for pregnancy. According to the advice of Traditional Chinese medicine experts, patients were treated with curcumin granules (Yifang Pharmaceutical Co., Ltd., Guangdong, China) at a dose of 15 g/day, p.o. for three months, with the original treatment maintaining. Serum was collected from each patient at baseline and three months after and stored at -80°C after equal aliquoted.

Aortic tissues were obtained from 8 TAK patients who had undergone surgical between 1 January 2010 and 31 July 2015 and 6 age-matching controls who underwent heart or kidney transplantation in Zhongshan Hospital.

The protocol used in this study was approved by the Ethics Committees of Zhongshan Hospital (Approval No. B2016–168) and conforms to the ethical guidelines of the 1975 Declaration of Helsinki. All patients provided written informed consent prior to inclusion in this study.

Immunohistochemical staining and double-labelled immunofluorescence

After deparaffinising and hydrating the samples, the arterial tissue sections were repaired with a citric acid buffer solution, treated with 3% H_2O_2 for 15 min at room temperature to block endogenous peroxidase activity. The sections were then incubated in 5% BSA at room temperature for another 30 min. The slices were subsequently incubated with the following diluted primary antibodies overnight at 4°C: CCL2 (Abcam, Cat no. ab9669), CD68 (Abcam, Cat no. ab213363), CCR2 (Proteintech, Cat Nno. 16153-1-AP) and then reacted with a secondary antibody conjugated with HRP (Yesen) for 1 h at room temperature. The slices were developed with DAB reagent and counterstained with haematoxylin. Photographs of random sites were captured under high-power 400× magnification with Leica QWin Plus v3 software using identical setting parameters. Positive staining was measured using Image J 1.8u software (National Institutes of Health, USA). The integrated optical density of the positive stains was measured in each photograph, and the area fraction (%) of the positive stains was calculated.

For double-labelled immunofluorescence, section was simultaneously incubated with two of the following primary antibodies: CCL2 and CD90 (Abcam, Cat no. 133350), CCL2 and alpha-smooth muscle actin (α-SMA, Abcam, Cat no. ab5694), CCR2 and CD68 (Abcam, Cat no. ab955), or α-SMA and TLR4 (Servicebio, Cat no. GB11519). The samples were subsequently reacted with species-specific Alexa Fluor® 488 AffiniPure Goat Anti-Rabbit IgG (H+L) and Alexa Fluor 594 AffiniPure Goat Anti-Mouse IgG (H+L), or Alexa Fluor® 488 AffiniPure Goat Anti-Mouse IgG (H+L), and Alexa Fluor 594 AffiniPure Goat Anti-Rabbit IgG (H + L) (Yesen) for 1 h at room temperature in the dark. Finally, the sections were sealed with an antifade Mounting Medium with DAPI (Beyotime). Photographs of random sites were captured under high-power magnification using an Olympus FV3000 laser confocal microscope. The area fraction of positive staining was measured using Image J 1.8u software.

Cell culture and reagents

Aortic adventitia fibroblasts (AAFs) were purchased from ScienCell Research Laboratories (Cat no.6120) and grown in fibroblast medium-2 (ScienCell, Cat no. 2331) supplemented with 5% fetal bovine serum (ScienCell, Cat no. 0025), 1% fibroblast growth supplement-2 (ScienCell, Cat no.2382), and a 1% penicillin/streptomycin solution (ScienCell, Cat no.0503). 65kD heat shock protein (HSP65) was cloned, expressed, and purified from the *Mycobacterium tuberculosis* virulent strain

H37Rv in a BSL-2 laboratory. An EtEraser Endotoxin Removal Kit (Xiamen Bioendo Technology Co., Ltd.) was used to remove the endotoxin in HSP65. The level of residual lipopolysaccharide (LPS) was determined using an Endpoint Chromogenic LAL Assay (Xiamen Bioendo Technology Co., Ltd.), to be <0.0625 EU/mL. Finally, 1 μg/mL HSP65 was used to stimulate AAFs. The basic control group (BC) was added with 0.0625 EU/mL LPS to avoid the potential effect caused by residual LPS in HSP65. The total RNA, cell culture supernatants, and non-phosphorylated total proteins were collected after 12 h; the expression of phosphorylated signaling pathway proteins were detected within 2 h after stimulation (0, 15, 30, 60, 90, and 120 min).

Enzyme linked immunosorbent assay (ELISA)

The concentrations of IL-1β, IL-6, and CCL2 were determined in the cell culture supernatants or serum of patients using a Human IL-1β Quantikine ELISA Kit (R&D Systems, Cat no. DLB50), Human IL-6 Quantikine ELISA Kit (R&D Systems Cat No.D6050), Human MCP-1/CCL2 ELISA Kit (Boster, China, Cat no. EK0441), respectively. Procedures were conducted in accordance with the manufacturer's instructions.

Reagents

Curcumin (Sigma-Aldrich, Cat no. C7727), AG490 (Selleck, S1143), tofacitinib (Selleck, S2789), and methotrexate (MTX, Selleck, S1210) were all dissolved in DMSO according to the manufacture's instruction. Curcumin was further diluted with DMSO into a 2, 10, 20, 40, 60, or 80 mM working solution. The final concentrations of AG490, tofacitinib, and MTX were 50 μM, 250 nM, and 50 nM, respectively. None of the drugs displayed significant cytotoxicity at the concentration used (cell viability >95%). Control groups were added with the same volume of DMSO.

Statistical analysis

Normal distributed data were expressed as the mean ± SD. Whereas, skewed distributed variables were shown as the median (interquartile range). The differences between groups were analysed by Student's t-test or paired t-test for normally distributed data. Wilcoxon test were explored for skewed data. Pearson correlation analysis or Spearman's bivariate correlation analysis were performed to study the association between the changes in IL-6 and he changes in CCL2. Experiments using cells were conducted in triplicate. A value of $p<0.05$ was indicated statistical significance. All statistical analyses were performed using GraphPad Prism 8.2.1 (GraphPad Software Inc., USA).

Table I. Clinical characteristics of the enrolled patients in the curcumin treatment study (n=16).

Parameters	Curcumin treatment (7.5 g bid, n=16)		
	Baseline	3 months later	*p*-value
Demographics			
Sex (female, %)	15 (93.8%)		
Age (year)	30.44±4.94		
Imaging classification			
I (n, %)	2 (12.5%)		
IIa (n, %)	1 (6.3%)		
IIb (n, %)	3 (18.8%)		
IV (n, %)	2 (12.5%)		
V (n, %)	8 (50%)		
Treatment			
GC dosage (mg)	13.75 (8.50-18.75)	12.25 (10.00-15.00)	0.50
Combination therapy	Tacrolimus (1); LEF (8); AZA (2); MMF (2); Rapamycin (2); HCQ (2)	Tacrolimus (1); LEF (5); AZA (1); MMF (2); Rapamycin (2); HCQ (2)	
Laboratory tests			
Hb (g/L)	123.563 ± 19.43	120.438 ± 17.74	0.18
WBC ($\times10^9$/L)	8.89 ± 2.21	9.46 ± 1.97	0.13
PLT ($\times10^9$/L)	262.31 ± 72.13	254.44 ± 82.06	0.32
ESR (mm/H)	11.00 (5.50-24.75)	15.00 (3.50-35.75)	0.49
CRP (mg/L)	3.30 (0.85-13.78)	5.40 (0.85-16.70)	0.14
CCL2 (pg/ml)*	50.23 ± 28.55	42.22 ± 20.57	**0.03**
Disease activity index			
Kerr score ≥1 (n, %)*	12 (75.0%)	5 (31.2%)	**0.03**
Kerr score ≥2 (n, %)	7 (43.7%)	3 (18.7%)	0.28

*Hb: haemoglobin; WBC: white blood cell; PLT: platelets; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein.
¥LEF: leflunomide; AZA: azathioprine; MMF: mycophenolate mofetil; HCQ: hydroxychloroquine.
‖Paired t test (for data that conform to a normal distribution) or Wilcoxon test (for data that did not conform to a normal distribution) were used to analyse the differences in patient indicators before and after curcumin treatment.
p-values: comparison of the baseline and after three months.
p-values <0.05 were considered significant.

Results

Curcumin alleviates disease activity in patients with TAK

To explore the effect of curcumin on TAK, 16 patients were treated with curcumin, the clinical characteristics of these patients were shown in Table I. No obvious side effects were observed and no discomfort was reported among enrolled patients. After three months treatment with curcumin, serum levels of c reactive protein and ESR were similar with baseline, however the proportion of patients with Kerr score ≥1 decreased significantly (75.0% *vs.* 31.2%; $p=0.03$) (Table I).

In an effort to explore the possible mechanisms through which curcumin alleviated disease activity in TAK, a series of serum inflammatory factors were detected. It was showed that, after three months treatment with curcumin, serum levels of IL-6 and IL-1β were similar with baseline (data not shown). Whereas, the levels of serum CCL2 were significantly reduced (50.23±28.55 *vs.*

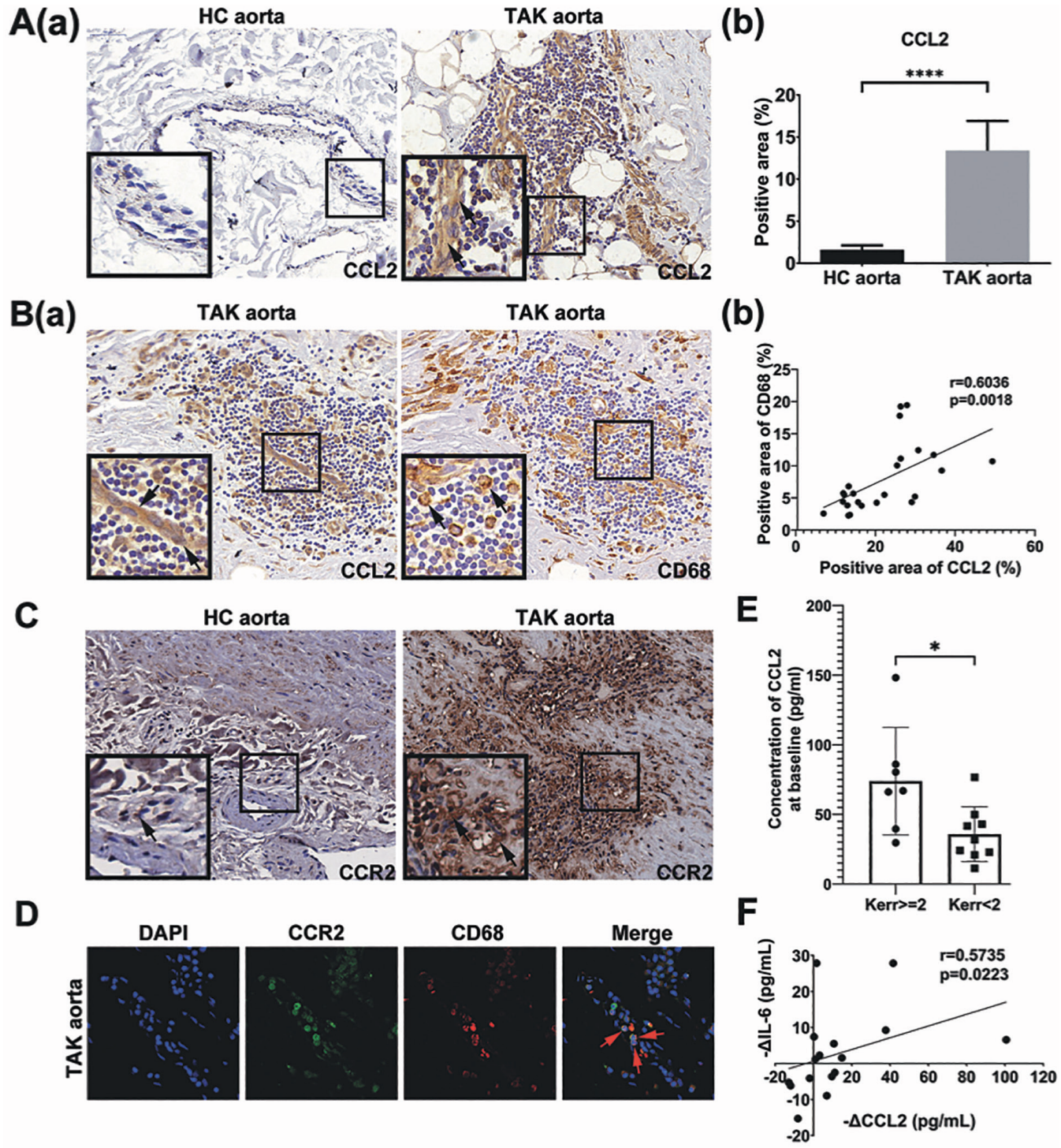

Fig. 1. Associations between CCL2 expression and inflammation in TAK. **A)** CCL2 expression in the aortic adventitia of TAK patients (n=8) and healthy controls (n=6). **B)** (a) Serial sections underwent immunohistochemical staining for CCL2 and CD68; (**b)** Spearman correlation analysis of CLL2 and CD68 expression (r=0.6036; *p*=0.0018). For the correlation analysis, three high magnification visual fields were randomly selected for each patient. **C)** CCR2 expression in the aortic adventitia of TAK patients (n = 8) and healthy controls (n=6). **D)** Double-labelled immunofluorescence images of CCR2 and CD68 (n=8). **E)** The serum concentrations of CCL2 at baseline in the Kerr score ≥2 and Kerr score <2 groups. The age (31.00, 26.00-36.00 vs. 30.00, 27.50, 33.00, *p*=0.53) and gender (n% female, 85.7% vs. 100.0%; *p*=0.17) of the two groups of patients were matched. **F)** The Spearman correlation analysis of -ΔIL-6 and -ΔCCL2 (r=0.5735; *p*=0.0223).Δ= 3-month values - baseline values. The error bars represent SD; **p*<0.05; *****p*<0.0001.

42.22±20.57 pg/mL; *p*=0.03) (Table I). These results indicated that curcumin may act through CCL2 to alleviate TAK activity.

Associations between CCL2 expression and inflammation in TAK

In order to explore the changes of CCL2 in TAK, aortic tissues were obtained from 8 TAK patients and 6 healthy controls. The preoperative clinical characteristics of the TAK patients were presented in Supplementary Table S1.

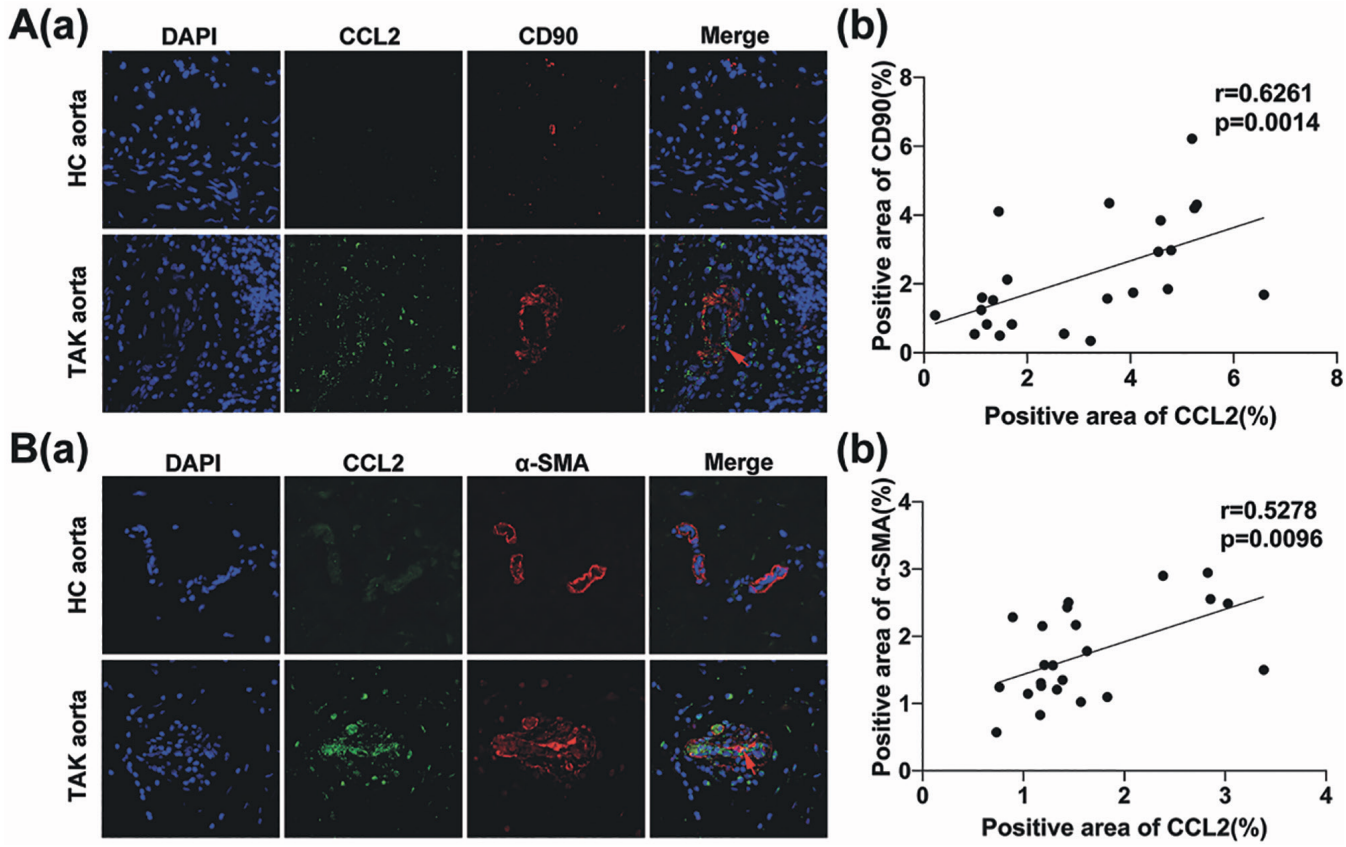

Fig. 2. CCL2 is mainly derived from AAFs. **A)** (a) Double-labelled immunofluorescence images of CCL2 and CD90; (b) a Spearman analysis of CCL2 and CD90 expression (r=0.6261; *p*=0.0014). **B)** (a) Double-labelled immunofluorescence images of CCL2 and α-SMA; (b) Pearson correlation analysis of CCL2 and α-SMA expression (r=0.5278; *p*=0.0096). For the correlation analysis, three high magnification visual fields were randomly selected for each patient (n=8).

It was showed that CCL2 expression were significantly higher in the adventitia of TAK patients than healthy controls (Fig. 1A). It was worth noting that CCL2 is mainly distributed in the areas with severe inflammation. More importantly, CCL2 was co-expressed with CD68, a marker of macrophage (Fig. 1B). Chemokine (C-C motif) receptor 2 (CCR2) is a key function receptor for CCL2, its binding with CCL2 on monocytes and macrophages mediates chemotaxis and migration induction (20). CCR2 expression was found enhanced in the TAK adventitia (Fig. 1C). In further analysis, the expression of CCR2 was co-localised with CD68 (Fig. 1D). This result highly suggested that the chemotaxis of macrophages to the aorta wall of TAK could be associated with the activation of CCL2/CCR2 axis.

At baseline, patients with a Kerr score ≥2 had higher level of serum CCL2 than patients with a Kerr score <2 (Fig. 1E). After treatment with curcumin, serum levels of CCL2 were decreased in 12 subjects (Supplementary Table S2), we further divided these 16 patients into two groups: CCL2 reduced group (n=12) and non-reduced group (n=4). After 3 months treatment, the serum levels of IL-6 were significantly downregulated in CCL2 reduced group (Suppl. Table S2). Compared to the CCL2 non-reduced group, CCL2 reduced group had more subjects achieving reductions in serum IL-6 levels. At 3 months, the changes in IL-6 and IL-1β from baseline (ΔIL-6 and ΔIL-1β) were much lower in the CCL2 reduced group (Table II). ΔIL-6 were positively associated with changes in CCL2 (r=0.5735; *p*=0.02) (Fig. 1F).

Table II. Comparison of changes in the levels of serum IL-6 and IL-1β between the CCL2 reduced and CCL2 non-reduced groups.

	CCL2 reduced (n=12)	CCL2 non-reduced (n=4)	*p*-value
IL-6 downregulated (%)	75.0% (9/12)	0.0% (0/4)	**0.019**
ΔIL-6 (pg/mL)	-3.90 (-8.81, 1.57)	5.71 (4.38, 13.00)	**0.008**
IL-1β downregulated (%)	75.0% (9/12)	25.0% (1/4)	0.118
ΔIL-1β (pg/mL)	-3.99 (-5.11, 1.31)	3.04 (0.07, 4.32)	**0.039**

*Δ=3-month values - baseline values. ¥*p*-values: comparison of the CCL2 reduced and CCL2 non-reduced groups. *p*-values <0.05 were considered significant.

These results suggested that expression of CCL2 was enhanced in TAK and the increased expression of CCL2 was associated with the inflammatory factors in the TAK.

CCL2 is mainly derived from AAFs and regulated by HSP65.

To further explore the sources of elevated CCL2 in TAK, double-labelled

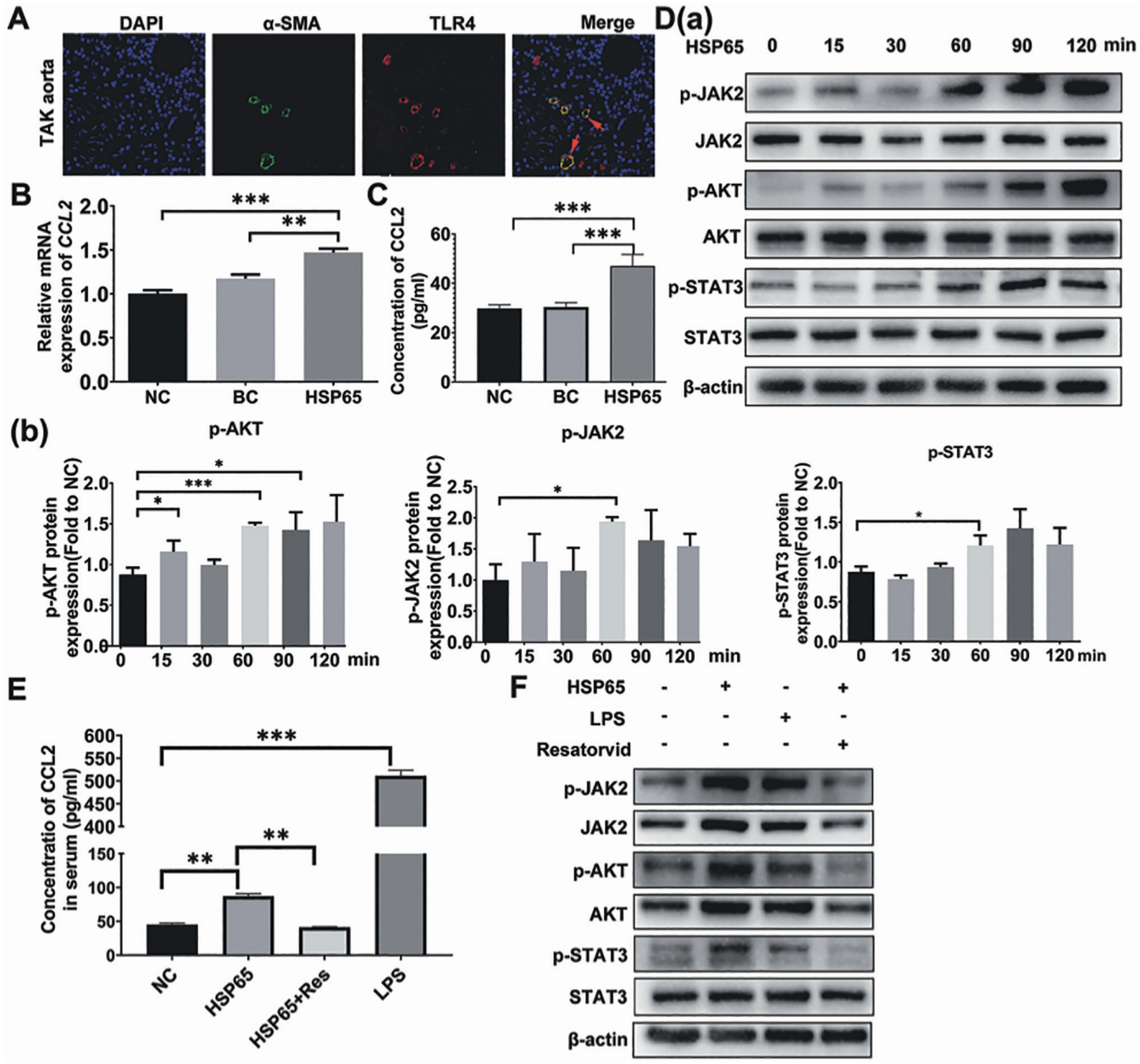

Fig. 3. HSP65 increased the production of CCL2 in AAFs via the TLR4-JAK2/AKT/STAT3 pathway. **A)** Double-labelled immunofluorescence images of TLR4 and α-SMA (n=8). **B)** The level of CCL2 mRNA expression in AAFs stimulated with HSP65 (1 μg/mL) for 12 h. **C)** the concentration of CCL2 in the supernatants of AAFs following stimulation with HSP65 for 12 h. The LPS content in the basic control (BC) group was equal to that in the HSP65 group (0.0625 EU/mL). **D)** The level of JAK2, AKT, and STAT3 phosphorylation at 0, 15, 30, 60, 90, and 120 min following HSP65 stimulation. **E)** The concentration of CCL2 in the AAFs culture supernatants following stimulation with HSP65, LPS, or HSP65 combined with Resatorvid (10 μM) for 12 h. LPS (10 ng/mL) was used as a positive control. **F)** The level of JAK2, AKT, and STAT3 phosphorylation at 90 min following stimulation with HSP65, LPS, or HSP65 combined with Resatorvid. The error bar represents SD. Experiments were repeated 3× with similar results. *p<0.05; **p<0.01; ***p<0.001.

immunofluorescence staining was performed, it was showed that CCL2 was co-expressed with CD90$^+$, α-SMA$^+$, markers of adventitia fibroblasts (Fig. 2). These results indicated that elevated expression of CCL2 in TAK may derive from AAFs.

CCL2 expression was regulated by the activity of the TLR4 signaling pathway (21, 22). HSP65, an agonist of TLR4 reported been expressed in aortic tissue of TAK (23), was used to study the possible upstream regulation of CCL2 in AAFs. We detected TLR4 and used its inhibitor (Resatorvid) to confirm that HSP65 functioned through the expression of TLR4 in AAFs. Immunofluorescence detection revealed that the expression of TLR4 co-localised with α-SMA on the surface of active fibroblasts in aortic adventitia (Fig. 3A). After 12 h treatment with HSP65, the mRNA expression and supernatant levels of CCL2 were both substantially upregulated (Fig. 3B-C) with enhanced phosphorylation of JAK2, AKT, and STAT3 (Fig. 3D), whereas the phosphorylation levels of JAK1 and JAK3 did not differ significantly (Suppl. Fig. S1). Resatorvid reduced the enhanced ex-

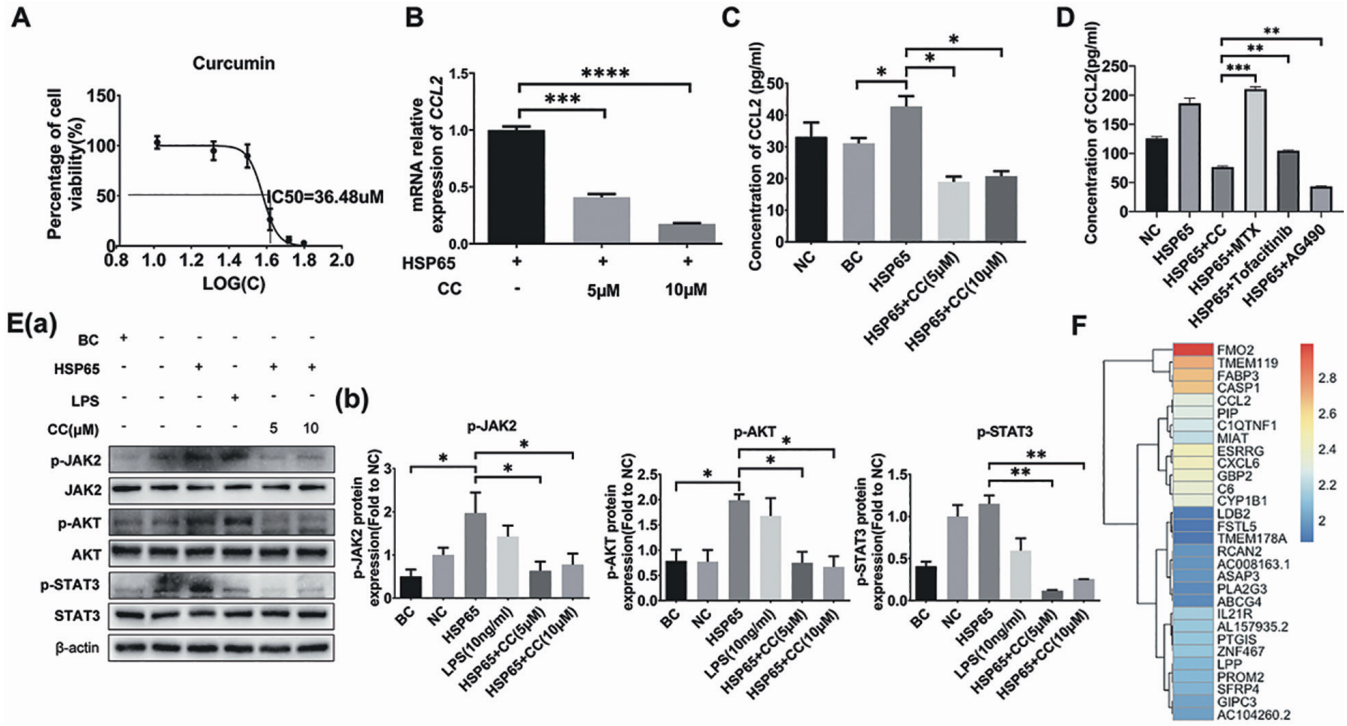

Fig. 4. Curcumin (CC) inhibits the HSP65-stimulated upregulation of CCL2 by blocking the activation of JAK2/AKT/STAT3 pathway**. A**) The IC50 of curcumin for AAFs is 36.48 μM. **B)** The level of CCL2 mRNA expression in AAFs following stimulation with HSP65 (1 μg/mL) alone or HSP65 combined with CC (5 μM and 10 μM) for 12 h. **C)** The concentration of CCL2 in the AAF culture supernatants following stimulation with HSP65 alone or HSP65 combined with CC for 12 h. **D)** The concentration of CCL2 in the AAF culture supernatants following stimulation with HSP65 (1 μg/mL) alone or in combination with CC (5 μM), AG490 (50 μM), tofacitinib (250 nM), and methotrexate (50 nM) for 12 h. **E)** The level of JAK2, AKT, and STAT3 phosphorylation at 90 min after stimulation with HSP65 alone or in combination with curcumin. **F)** The top 30 genes ranked based on the $\log_2$ fold-change. The error bar represents SD. All experiments have been performed in triplicate. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

pression of CCL2 and phosphorylation of JAK2, AKT, and STAT3 stimulated by HSP65 in AAFs (Fig. 3E, F). These results suggest that enhanced expression of CCL2 in AAFs was regulated by HSP65 possibly through TLR4-JAK2/AKT/STAT3 pathway.

Curcumin inhibits the HSP65-stimulated upregulation of CCL2 by blocking the activation of JAK2/AKT/STAT3 pathway

The IC50 of curcumin in AAFs was 38.48μM. Curcumin was used to treat AAFs with a concentration of 5 μM and 10 μM, under which the cell survival rate was higher than 95% (Fig. 4A). Following curcumin treatment, the enhanced mRNA expression and supernatant level of CCL2 stimulated by HSP65 were reversed (Fig. 4B-C). Increased level of TGF-β in the supernatant of AAFs was also found after HSP65 treatment and decreased by curcumin (Suppl. Fig. S2). To explore the advantages of curcumin on the treatment of TAK, we established different

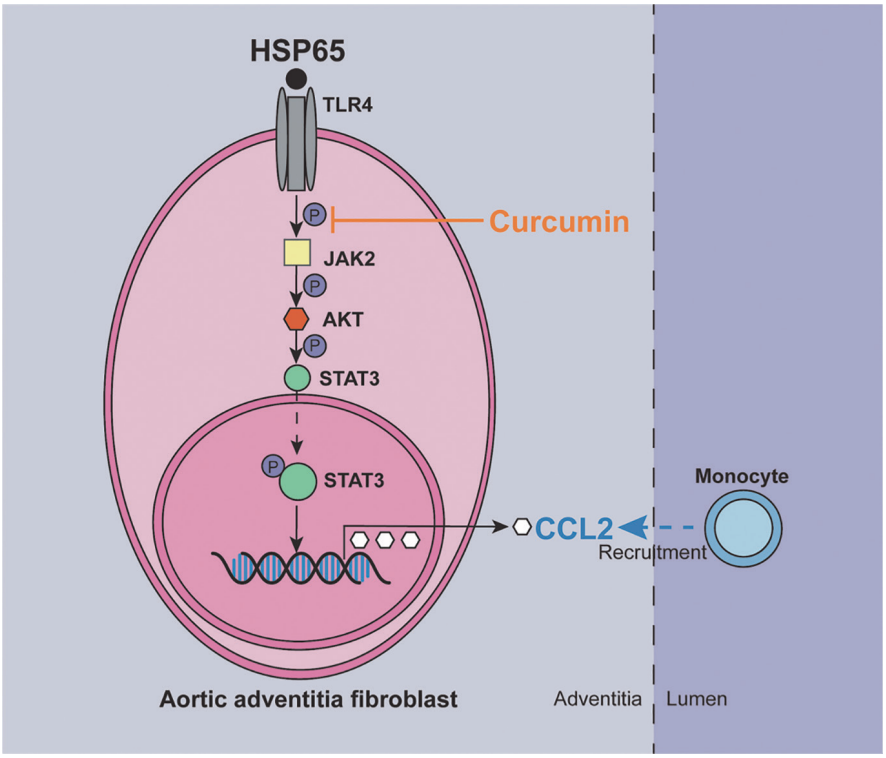

Fig. 5. A cellular model illustrating the mechanisms involved in this study. Curcumin alleviated inflammation in TAK patients by downregulating HSP65-induced CCL2 overexpression in adventitial fibroblasts though blocking activation of the JAK2/AKT/STAT3 signaling pathway.

intervention groups. According to the result, the inhibitory effect of curcumin on CCL2 expression was greater than that of JAK1/JAK3 inhibitor tofacitinib and MTX *in vitro* (Fig. 4D).

To further explore the underlying mechanisms by which curcumin reversed the enhanced expression of CCL2, western blot was performed. It showed that the enhanced phosphorylation of JAK2, AKT, and STAT3 induced by HSP65 was reversed by curcumin (Fig. 5E). These results suggest that, through inhibiting the phosphorylation of JAK2/AKT/STAT3 pathway, curcumin reversed the enhanced expression of CCL2 induced by HSP65.

Finally, we profiled the global mRNA expression in AAFs stimulated with purified HSP65 (1 μg/mL) with or without curcumin pretreatment (group HSP65 or group HSP65 + curcumin), as well as untreated AAFs (group NC). As a result, we identified 1176 DEGs between the HSP65 and NC groups, among which there were 812 upregulated genes. A total of 3797 genes were simultaneously downregulated in the HSP65 + curcumin group compared with that of the HSP65 group. Among the 812 genes upregulated by HSP65 stimulation, 215 DEGs overlapped with the 3797 genes that were downregulated following curcumin pretreatment. Figure 4 F shows the top 30 genes ranked based on the $\log_2$ fold-change, which includes CCL2.

Discussion

Innate immune response plays an impact role in the pathogenesis of TAK (24). As a member of the CC chemokine family, CCL2 exhibits strong monocyte chemotaxis and plays an important role in the innate immune response (25). It has been reported that early CCL2 production in the aortic root and coronary arteries can initiate inflammation in Kawasaki disease (26). In addition, CCL2 is believed to contribute to the pathogenesis of vascular dysfunction (27). A noticeable role of CCL2 in TAK has been proposed by several studies (28, 29). We detected a higher level of CCL2 expression in TAK adventitia, which strongly correlates with activated macrophages. Moreover, the levels of serum CCL2 were associated with TAK activity. These findings indicate that a significantly higher expression of CCL2 might be an important contributor of inflammation in patients with TAK.

Curcumin is a natural extract derived from the rhizome of the *Curcuma longa* plant, which has traditionally been used for the treatment of various cancers and inflammatory conditions(30). Curcumin has previously been reported to exert an anti-inflammatory effect by down-regulating NF-κB and the MAPK signaling pathway, both *in vitro* and animal studies (17). In addition, curcumin exerts both anti-tumour and anti-inflammatory effects by inhibiting cyclooxygenase-2 (12). Recently, the therapeutic value of curcumin for the treatment of various autoimmune diseases has attracted increased attention. Injecting acid/curcumin nanomicelles into the arthritic ankle joint of rheumatoid arthritis (RA) rats has been shown to significantly reduce arthritis and the production of IL-1 and TNF-α (31). In systemic lupus erythematosus, curcumin treatment modified the function of dendritic cells and various T lymphocyte subsets (16). Moreover, curcumin has also been demonstrated to reduce TNF-α-enhanced CCL2 expression in osteoarthritis synovial fibroblasts (32). Although several studies have reported an anti-inflammatory effect of curcumin in vasculitis (33, 34), the underlying mechanism remains unexplored, especially in TAK.

To explore the potential clinical value of curcumin as a treatment for TAK, we evaluated the changes in the levels of serum cytokines and disease activity before and after curcumin treatment. The ratios of Kerr score ≥1 decreased significantly after curcumin administration, suggesting that curcumin had a certain alleviating effect on mild disease activity in these TAK patients, or that curcumin could maintain these patients in a stable phase of TAK. Moreover, the level of serum CCL2 was significantly decreased following curcumin treatment. The analysis demonstrated that the decrease in IL-6 was highly consistent with changes in CCL2. Therefore, CCL2-mediated recruitment of monocytes to the vasculature represents an important pathological process in TAK. We speculated that curcumin can control the active inflammatory situation by targeting CCL2.

We subsequently explored the source of CCL2 and the potential mechanism of its overexpression in TAK aorta adventitia. Although the pathogenesis of TAK remains unclear, *Mycobacterium tuberculosis* (M. TB) infection is known to play an important role. Recent studies have confirmed that patients with TAK have a higher proportion of M. TB infection (35, 36). HSP65 was identified in TAK artery tissue and sites of increased HSP65 expression also displayed γ-δT cell infiltration, suggesting that HSP65 could be directly recognised by these cytotoxic cells (23, 37). Moreover, elevated levels of IgG antibodies against HSP65 could be detected in the peripheral blood of patients with TAK (38), indicating that HSP65 may be a putative antigen responsible for stimulating the immune response. However, previous studies on the ability of HSP65 to promote inflammation have focused on immune cells, and there are no published reports of fibroblasts. HSP65 is a conserved protein of the M. TB cell wall, which can also act as a TLR4 agonist (39).

In vitro, blocking TLR4 decreased the production of CCL2 induced by HSP65, which indicated TLR4 is likely the key receptor required to transduce the HSP65 inflammatory activation signal into the cells. Next, we determined the levels of JAK1-3, AKT, and STAT3 phosphorylation in AAFs following HSP65 stimulation and found that the JAK2/AKT/STAT3 pathway was activated. In addition, Resatorvid, a TLR4 inhibitor, was found to decrease the level of JAK2, AKT, and STAT3 phosphorylation. These results demonstrate that CCL2 production can be stimulated by HSP65 in AAFs via activation of the TLR4-JAK2/AKT/STAT3 pathway.

The findings of this study further showed that treatment with curcumin could reduce the production and release of CCL2 in AAFs through inhibiting activation of JAK2/AKT/STAT3 signaling pathway at a low dose. It was important to note that curcumin was simi-

lar with the JAK2 inhibitor, AG490, in its capacity to inhibit CCL2 expression. In addition, both curcumin and AG490 were found to be more effective than MTX and tofacitinib (JAK1/JAK3 inhibitor). As a first-line treatment for TAK, MTX has the inevitable side effect of inhibiting bone marrow haematopoiesis due to its antifolate effect. Tofacitinib is an effective biological DMARDs recently used in RA, with only a minor number of reports in TAK (40, 41), indicating an important effect of targeting the JAK/STAT pathway as a form of TAK treatment. The safety of curcumin has been well documented, and an oral high-dose curcumin does not cause toxic reactions (30). Our *in vitro* findings demonstrated that CCL2 was the primary regulatory target of curcumin, for which the key mechanism was a blockade of JAK2 phosphorylation, which deserves further *in vivo* confirmation.

This study demonstrates that significantly higher CCL2 expression may be a very important promoter of inflammation in patients with TAK, which is strongly correlated with IL-6 (and IL-1) production and macrophage recruitment. After being recruited into local tissues, monocytes differentiate into macrophage subtypes that release various inflammatory factors or play a role in tissue remodeling (42, 43). Our previous work has reported a pro-fibrotic effect of IL-6 in TAK aortic adventitia (10). We have also found that the M2 macrophage subtype is correlated with fibrosis of the vascular wall (7). It has been well-established that curcumin prevents macrophages from differentiating into the M1 subtype, which represents the inflammatory subtype (44). In the current study, we have reported for first time that CCL2 is significantly decreased, accompanied by a simultaneous reduction in serum IL-6 levels after curcumin treatment in TAK. Together, these findings indicate that curcumin can both inhibit inflammation and potentially slow the progression of fibrosis in the TAK aorta by targeting CCL2. Although the small sample size and short observation time are the main limitations associated with this study, our study provides a possible mechanism for the treatment of TAK with curcumin. These findings provide a reference value for more TAK patients to be treated with curcumin in the future.

In conclusion, significantly higher CCL2 expression might be a very important promoter of inflammation in patients with TAK. Curcumin alleviated inflammation in TAK patients by downregulating HSP65-induced CCL2 overexpression in adventitial fibroblasts though blocking the JAK2/AKT/STAT3 signaling pathway. Our study is the first to indicate that curcumin may act as a common additive in TAK treatment for increasing the therapeutic efficacy targeting the CCL2 inflammatory pathway.

Ethics statement

The experimental protocol used in this study was approved by the Ethics Committees of Zhongshan Hospital (Approval no. B2016-168) and conforms to the ethical guidelines of the 1975 Declaration of Helsinki. All patients provided written informed consent for inclusion in this study.

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