Therapeutic value of curcumin on the initiation and development of inflammation in Takayasu's arteritis mediated by HSP65-induced CCL2 overexpression

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Abstract

Background

Takayasu's arteritis (TAK) is a chronic inflammatory disease characterized by macrophage infiltration. During the active stage, aorta adventitial fibroblasts (AAFs) proliferate excessively and produce numerous pro-inflammatory factors in the adventitia, the primary target of TAK therapy. Although the monocyte chemokine, CCL2, may contribute to macrophage infiltration in TAK arteries, whether there is an associated relationship with HSP65 remains unknown. Moreover, there are no reports of the activation of AAFs to produce inflammatory factors involvement in TAK pathogenesis. TAK treatment is associated with several challenges and contradictions. Curcumin is a traditional Chinese medicine with anti-inflammatory effects; however, its effectiveness for TAK and the underlying mechanism remains unclear.

Methods

We first verified high HSP65 expression in the aortic adventitia of TAK patients by IHC. mRNA-seq was used to profile differentially expressed genes (DEGs) between AAFs stimulated by HSP65 with or without pretreatment with curcumin, and untreated AAFs. The key chemokine, CCL2, screened by mRNA-seq was detected in the adventitia of the TAK aorta, and its correlation with HSP65 expression was analyzed by double-labelled immunofluorescence. We subsequently explored how HSP65 affected the production of inflammatory factors by AAFs at a cellular level and the related signaling pathway. We explored whether curcumin could hinder this process and verified the effect of curcumin on the level of serum CCL2 in patients with TAK.

Results

HSP65 was highly expressed in the adventitia of TAK arteries. The DEG analysis showed a key role of CCL2. The expression of CCL2 in the adventitia of TAK arteries was significantly higher than that of healthy subjects, and was correlated with HSP65. HSP65 facilitated the production of CCL2, IL-6, and IL-1 β by AAFs via activation of the TLR4-JAK2/AKT/STAT3 pathway, among which the change in CCL2 was the greatest. Curcumin treatment reversed HSP65-induced CCL2 upregulation in vitro, which was more obvious than that of MTX and tofacitinib. Finally, curcumin significantly downregulated the level of serum CCL2 in TAK patients.

Conclusion

HSP65 initiates and promotes TAK inflammation by upregulating CCL2 in AAFs through the JAK2/AKT/STAT3 pathway, whereas curcumin could reverse this process and slow the initiation and development of TAK.

Background

Takayasu's arteritis (TAK) is a chronic inflammatory disease, which often occurs in women of childbearing age^[1]. Although it has a higher morbidity in Japan, China, and other Asian countries, the morbidity in other countries throughout the world has also increased annually^[2]. TAK preferentially affects the aorta and its primary branches^[3] during the active period. The pathological feature is the infiltration of numerous inflammatory cells with granulomatous formation in the adventitia and media, where aorta adventitial fibroblasts (AAFs) do not only excessively proliferate to narrow the aorta wall but also produce several pro-inflammatory factors (e.g., IL-6, the main target of TAK treatment)^[4]. Although the precise etiology remains largely unknown, macrophage infiltration plays an extremely important role in TAK pathogenesis^[5]. Our previous work has confirmed the accumulation of macrophages in the adventitia of arteries of TAK patients^[6]. CCL2 is a chemokine that attracts monocytes into inflammatory tissues and may play an important role in macrophage infiltration^[7]. It has been well documented that CCL2 can be secreted by various immune and tissue cells^[8]. However, it is extremely important to explore whether and how the AAFs are activated to produce CCL2 since there are more AAFs than macrophages in the local arteries during TAK development.

It has been well-established that the infection has been considered to be an important factor in triggering the immune response to TAK, particularly *Mycobacterium tuberculosis* (M. TB) infection. The prevalence of TAK is significantly higher in countries and regions where tuberculosis (TB) is prevalent. Moreover, several studies have explored the potential role of M.TB infection in the pathogenesis of TAK^[9, 10, 11]. The 65 kDa heat shock protein (HSP65) is a small molecular antigen derived from M.TB, which has been reported to be associated with a variety of autoimmune diseases^[12, 13, 14], including TAK. The expression of HSP65 in the aorta of TAK patients is higher than that of healthy controls, which may mediate the T cell immune response^[15]. HSP65-reactive T cells, as well as anti-HSP65 IgG antibodies were noted, suggesting infection-induced autoimmunity in TAK^[16]. While HSP65 activates various natural and adaptive immune cells^[17], whether HSP65 can activate AAFs to produce inflammatory factors, especially CCL2, has not been previously reported.

Traditionally, active TAK is treated using glucocorticoids combined with immunosuppressive agents^[18]. However, relapses occur in a considerable proportion of patients who have achieved remission, especially when the dose of glucocorticoids is reduced, resulting in the development of vessel damage^[19]. Curcumin is a small polyphenolic compound extracted from the rhizome of the plant, *Curcuma longa*, which has traditionally been used for pain and wound-healing. Recent studies have revealed some new pharmacological effects of curcumin (e.g., anti-inflammation, anti-oxidation, and anti-cancer activity), without obvious side effects^[20]. Curcumin inhibits the production of cytokines (i.e., IL-1 β , IL-6, IL-12, TNF- α , and IFN- γ) in immune cells by regulating the JAK/STAT, AP-1, and NF- κ B signaling pathways to ameliorate autoimmune disease (e.g., multiple sclerosis, rheumatoid arthritis, type 1 diabetes, and

inflammatory bowel disease) [21]. However, whether and how curcumin could inhibit the expression of inflammatory factors in TAK treatment remains unknown.

In the current study, we first explored the mechanisms of inflammatory factors produced by AAFs triggered by the TB-related molecule, HSP65. Next, we compared the inhibition efficacy of curcumin with tofacitinib and methotrexate (MTX) on the proinflammatory factors produced by AAFs. Furthermore, we analyzed the level of proinflammatory factors in the serum before and after treatment with curcumin.

The results showed that HSP65 could promote CCL2, IL-1 β , and IL-6 production in AAFs. Curcumin primarily inhibits CCL2 production compared with two other reagents (tofacitinib and MTX). The patients who received curcumin treatment exhibited a significant reduction in the level of serum CCL2, which was accompanied by a decrease in the Kerr scores.

Materials And Methods

Human subjects and ethical considerations

Eight patients with TAK who had undergone surgical treatment were recruited from Zhongshan Hospital, Fudan University from January 1, 2010 to July 31, 2015. All patients were diagnosed according to the American College of Rheumatology 1990 (ACR 1990) criteria. The clinical characteristics were collected prior to surgery and presented in Supplementary Table I. In addition, normal aortic tissues were obtained from six donors for heart or kidney transplantation with matching ages.

A total of 16 patients diagnosed with TAK were enrolled in the curcumin treatment study, whose characteristics clinical were shown in Supplementary Table II. The inclusion criteria were as follows: i) over 14 years old; ii) patients with abnormal erythrocyte sedimentation rate (ESR), symptoms, or imaging progression; iii) no adjustment of immunosuppressants in the preceding one month; and iv) signing of an informed consent form. Exclusion criteria consisted of: i) presence of a recent active infection (e.g., tuberculosis); ii) patients with organ failure; iii) patients allergic to curcumin; (iv) subjects who had used other traditional Chinese medicine in the past month; (v) women pregnant, lactating, or preparing for pregnancy.

The protocol used in this study was approved by the Ethics Committees of Zhongshan Hospital and conforms to the ethical guidelines of the 1975 Declaration of Helsinki. All patients provided written informed consent for inclusion in this study.

Cell culture and reagents

AAFs were purchased from ScienCell Research Laboratories (Cat No.6120) and grown in Fibroblast Medium-2 (FM-2, ScienCell, Cat No. 2331) supplemented with 5% fetal bovine serum (FBS, ScienCell, Cat No.0025), 1% Fibroblast Growth Supplement-2 (FGS-sf, ScienCell, Cat No.2382), and a 1% Penicillin/Streptomycin Solution (P/S Solution, ScienCell, Cat No.0503). HSP65 was cloned, expressed

and purified from *Mycobacterium tuberculosis* virulent strain H37Rv. EtEraser Endotoxin Removal Kit (Xiamen Bioendo Technology Co.,Ltd.) was used to remove the endotoxin in HSP65. In this study, 1 µg/mL HSP65 was used to stimulate AAFs, and the level of residual Lipopolysaccharide (LPS) was determined by Endpoint Chromogenic LAL Assays (Xiamen Bioendo Technology Co.,Ltd.), which was <0.0625 EU/mL. A group named BC was set to avoid the potential effect caused by the residual LPS in HSP65 by adding 0.0625 EU/mL extra LPS. The total RNA, cell culture supernatants, and non-phosphorylated total proteins were collected after 12 h, whereas the expression of phosphorylated signaling pathway proteins were detected within 2 h after stimulation (0, 15, 30, 60, 90, and 120 min).

mRNA-seq analysis

Total RNA of AAFs stimulated by purified HSP65 (1 µg/mL) with or without pretreatment with curcumin (10 µM), as well as AAFs without any treatment was extracted for mRNA transcriptome sequencing, each group was performed in triplicate. For all samples, the original gene sequence was counted with StringTie software (Johns Hopkins University; UT Southwestern Medical Centre, USA), and the gene expression was calculated via fragments per kilobase of transcript per million fragments mapped (FPKM). FPKM ≥ 0.1 indicates that the transcript is expressed. DESeq2 software was used to screen the differentially expressed genes (DEGs) among different sample groups. The genes that met the screening criteria of $|\log_2\text{FoldChange}| \geq 1$ and $\text{FDR} \leq 0.05$ were the DEGs between the two groups. $\log_2\text{FoldChange}$ was used to range the screened DEGs.

Immunohistochemical staining

After deparaffinizing and hydrating the samples, the arterial tissue sections were repaired with a citric acid buffer solution, treated with 3% H₂O₂ for 25 min at room temperature (RT) protected from light to block the endogenous peroxidase activity. The sections were then incubated in 5% BSA at RT for another 30 min. The slices were subsequently incubated with the following diluted primary antibodies overnight at 4°C: HSP65 (Cell Signal Technology), CCL2 (Abcam), IL-6 (Abcam) and IL-1 β (Servicebio, China), then reacted with a secondary antibody conjugated with HRP (Yesen) for 1 h at RT. The slices were developed with DAB reagent and counterstained with hematoxylin. Photographs of random sites were captured under high-power 400 $\times$ magnification with Leica QWin Plus v3 software with identical setting parameters. Positive staining was measured using Image J 1.8u software (National Institutes of Health, USA). Integrated optical density of the positive stains in each photograph was measured, and the area fraction (%) of the positive stains was calculated.

Double-labelled immunofluorescence

The first few steps of performing immunofluorescence on paraffin sections were the same as those of Immunohistochemical staining, except that each section was simultaneously incubated with two of the following primary antibodies: HSP65 (Cell Signal Technology) and α -smooth muscle actin (α -SMA, Abcam), HSP65 and CCL2 (Abcam), HSP65 and IL-6 (Abcam), HSP65 and IL-1 β (Abclonal), or α -SMA and TLR4 (Servicebio), then 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 RT 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 by an Olympus FV3000 laser confocal microscope. The area fraction of positive staining was measured using Image J 1.8u software.

Drugs and small molecule inhibitors

Curcumin powder (Sigma-Aldrich, Cat No.C7727) was first dissolved in 13.57 mL DMSO to prepare a 100 mM storage solution. Prior to use, the storage solution was diluted with DMSO into a 2, 10, 20, 40, 60, 80 mM working solution. AG490 (Selleck, S1143), a selective JAK2 inhibitor, was dissolved in DMSO into a 100 mM working solution. Tofacitinib (Selleck, S2789) was dissolved in DMSO into a 500 µM working solution before use. MTX (Selleck, S1210) was dissolved in DMSO into a 100 µM working solution. The final concentrations of AG490, tofacitinib, and MTX were 50 µM, 250 nM, and 50 nM, respectively. All drugs had no significant cytotoxicity at the concentration used (cell viability>95%). DMSO was added in non-intervention groups to avoid the potential deviation due to its cytotoxicity.

Cell viability and proliferation assay

AAFs were plated at a density of 10^4 cells/well with 100 µL of the whole culture medium in 96-well culture plates and cultured in an incubator (37°C, 5%CO₂). After adhering to the well, the cells were treated with different concentrations of curcumin (1, 10, 20, 30, 40, 50, and 60 µM) for 12 h. We washed the cells with D-PBS (Gibco), and added 10 µL of Cell Counting Kit-8 (CCK8) solution (Dojindo, JAPAN) to each well and incubated the plates in the incubator for 2 h. Finally, the absorbance at 450 nm was measured using a FlexStation3 Multi-Mode Microplate Reader (Molecular Devices, USA). Six replicates were performed in each experiment.

RT-qPCR

Total RNA was extracted from AAFs using TRIzol reagent (Sigma). The PrimeScript RT reagent kit (Takara, Cat No. RR036A) was used for reverse-transcription. The cDNA template was further amplified with Hieff® qPCR SYBR Green Master Mix (Yesen, China, Cat No.11202ES03) and gene-specific primers (Table I). Three replicates were set for each primer in each group. The relative mRNA expression was normalized to β-actin and reported as $2^{-\Delta\Delta C_t}$.

Western blot and antibodies

The total proteins were extracted from AAFs using a lysis buffer containing RIPA buffer (Beyotime, China), 10% PhosSTOP (Thermo), and 1 mM phenylmethanesulfonylfluoride (Beyotime). Next, 10% and 12% SDS-PAGE were used to separate the proteins, and then the proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore). The PVDF membrane was blocked in TBS/Tween

with 5% BSA and subsequently incubated with the following primary antibodies: p-JAK1, JAK1, p-JAK2, JAK2, p-JAK3, JAK3, p-AKT (Thr308), AKT, p-STAT3, STAT3 (Cell Signal Technology), IL-1 β (Abclonal, China, Cat No.A11369), TLR4 (Santacruz), and β -actin (Abcam). Next, we incubated the membrane with an HRP-conjugated secondary antibody (Yesen). Ultimately, a super ECL Detection Reagent Kit (Yesen, Cat No.36208ES60) was used to visualize the target protein band.

Treatment

TAK patients were treated with curcumin granules (Yifang Pharmaceutical Co., Ltd., Guangdong, China) at a dose of 15 g/day, p.o. (equivalent to approximately 45 mg/kg/day curcumin) while maintaining the original treatment for three months. The patient serum was collected at the beginning of the study and after three months and stored at -80°C after separate packaging.

Enzyme linked immunosorbent assay (ELISA)

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

Statistical analysis

PCR, western blot and Immunohistochemical staining were expressed by mean $\pm$ SD values and analyzed by the student t-test. Data regarding patient' characteristics was expressed as the mean $\pm$ SD values. Paired t test (For data that conform to normal distribution) or Wilcoxon test (For data that did not conform to normal distribution) were used to analyze the differences of indicators of patients before and after curcumin treatment. Pearson and Spearman correlation analysis was also performed. All statistical analyses were performed using GraphPad prism 8.2.1 (GraphPad Software Inc., USA). All experiments have been performed in triplicate. $p < 0.05$ indicating statistical significance.

Results

TAK patients exhibit high HSP65 expression in the aortic adventitia

To gain clinical insight into the role of HSP65 in the pathogenesis of TAK, we first detected the expression of HSP65 in the aortic adventitia. It was found that HSP65 expression in the aortic adventitia of TAK patients was significantly higher than that in the healthy controls (Figure 1A). Of interest, HSP65 was primarily expressed around fibroblasts in the stroma between inflammatory cells, which displayed large, oval nucleus. In addition, the number of inflammatory cells that infiltrated the adventitia was positively correlated with HSP65 expression (Figure 1B). Double-labelled immunofluorescence staining revealed the co-localization of HSP65 and α -SMA, and further analysis showed a positive correlation between the expression of these two molecules in the TAK aorta (Figure 1C).

mRNA-seq analysis of AAFs revealed CCL2 to be a key inflammatory factor participating in HSP65-induced inflammation

HSP65 has been identified to exhibit high levels of expression in TAK arteries¹⁴. Moreover, our previous work has confirmed that aortic adventitia fibroblasts represent the key tissue cells involved in the pathogenesis and progression of TAK. However, it remains unknown how HSP65 activates adventitia fibroblasts and initiates inflammation and whether curcumin can alleviate vascular inflammation of TAK. To address this issue, we first profiled the global expression of mRNA in AAFs stimulated with purified HSP65 (1µg/mL) with or without curcumin pretreatment (group HSP65 or group HSP65 + CC), as well as untreated AAFs (group NC). As a result, we identified 1176 DEGs between the HSP65 and NC groups, among which 812 genes were upregulated. A total of 3797 genes were simultaneously downregulated in the HSP65 + CC group compared with 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 1D). Figure 1E shows the top 30 genes which were ranked based on the log2FoldChange. Among the inflammatory chemokines, CCL2 and CXCL6 attracted our attention. Since curcumin down-regulates CCL2 more significantly than CXCL6 and our previous work⁶ has also confirmed the important role of macrophages in the pathogenesis of TAK. Thus, we focused on CCL2 for further study. Since IL-1β and IL-6 are also among the DEGs mentioned above, they were detected synchronously.

High CCL2 expression in the aortic adventitia of TAK patients is correlated with HSP65

The expression of CCL2, IL-6, and IL-1β in the aortic adventitia was significantly increased in patients with TAK, especially CCL2 (Figure 2A-B). We then evaluated the co-expression of HSP65 and pro-inflammatory factors using double-labelled fluorescence staining. The results showed that HSP65 was co-localized with the three pro-inflammatory factors, CCL2, IL-6, and IL-1β, respectively (Figure 2C, Supplementary Figure 1A-B). There was a positive correlation between the expression of HSP65 and CCL2 (Figure. 2C), whereas there was no significant correlation between HSP65 expression and the other two factors (Supplementary Figure 1C). Subsequently, AAFs were stimulated with purified HSP65 (1µg/mL) for 12h, to varying degrees, gene expressions of the three pro-inflammatory factors were all up-regulated, among which CCL2 gene expression was increased most dramatically (Figure 2D).

HSP65 increased pro-inflammatory factor production in AAFs through the TLR4/JAK2-STAT3 pathway

HSP65 has been reported to be a toll-like receptor TLR-4 ligand^[22]. Nevertheless, it is unclear whether HSP65 transmits antigen signals into the AAF cytoplasm through TLR4. TLR4 was detected on the surface of fibroblasts, which was shown by the co-localization of TLR4 and α-SMA in tissues (Figure 3A). Simultaneously, AAFs stimulated by HSP65 expressed higher levels of TLR4 than that of untreated AAFs in vitro (Figure 3B). The levels of JAK2, AKT, and STAT3 phosphorylation in the AAFs were markedly upregulated following HSP65 stimulation (Figure 3C-D), while the expression of p-JAK1 and p-JAK3 did not significantly change (Supplementary Figure 2A-C). When the AAFs were pretreated with Resatorvid, a

TLR4 inhibitor, the level of p-JAK2, p-AKT, and p-STAT3 expression was remarkably reduced (Figure 3E). Similarly, blocking TLR4 also reduced the secretion of CCL2, IL-6 and IL-1 β , as well as the production of mature-IL-1 β by AAFs following HSP65 stimulation (Figure 3F, Supplementary Figure 3A). Therefore, these results suggest that HSP65 transmits signals into the cytoplasm of AAFs through TLR4, and promotes the production and release of pro-inflammatory factors by activating the JAK2/AKT/STAT3 signaling pathway.

Curcumin reversed the inflammatory response initiated by HSP65 in vitro

Curcumin has been shown to play an anti-inflammatory role in several inflammatory diseases; however, whether curcumin blocks the initiation of inflammation in the arterial wall of TAK has not been reported. The IC₅₀ of curcumin to AAFs was 38.48 Mm. We used curcumin to treat the cells with a concentration of 5 μ M and 10 μ M, under which the concentration of the cell survival rate was higher than 95% (Figure 4A). Following treatment with curcumin, the mRNA expression of all three pro-inflammatory factors in the AAFs was downregulated (Figure. 4B), and the level of secreted CCL2 and IL-6, as well as cleaved mature IL-1 β was dramatically reduced compared with those stimulated with HSP65 alone (Figure 4C-E). Moreover, curcumin reversed the JAK2, AKT, and STAT3 phosphorylation induced by HSP65 (Figure 4F-G), which suggests that curcumin can reverse the inflammatory response initiated by HSP65 by preventing activation of the JAK2/AKT/STAT3 pathway.

Curcumin significantly inhibited CCL2 secretion compared to tofacitinib and MTX

To explore the advantages of curcumin on the treatment of TAK, we established different intervention groups. AAFs pretreated with curcumin, JAK2 inhibitor (AG490), tofacitinib (JAK1 and JAK3 inhibitor), and MTX were stimulated with HSP65 for 12 h. We found that AG490 had the greatest significant inhibitory effect on the secretion of each pro-inflammatory factors. The inhibitory effect of tofacitinib on IL-6 secretion was better than that of curcumin and MTX, while the inhibitory effect of curcumin on CCL2 secretion was better than that of tofacitinib and MTX (Figure 5A-C, Supplementary Figure 3B). CCL2 is an important monocyte chemokine, and the above results suggest that curcumin primarily inhibits CCL2 production by AAFs. Such production may lead to the reduction of monocyte recruitment and colonization, thus inhibiting the progression of inflammation in TAK arteries.

Curcumin significantly reduced the serum CCL2 concentration of TAK patients

Compared with the baseline levels, the serum CCL2 concentration of the TAK patients was significantly decreased after the curcumin was added for three months, from a mean value of 52.42 ± 34.41 to 49.66 ± 16.58 ; $p = 0.04$ (Figure 5D and Supplementary Table II). There were no statistically significant changes in the serum IL-6 or IL-1 β concentrations; however, the level of IL-6 was decreased from a mean value of 38.01 ± 11.61 to 35.27 ± 8.64 (Figure 5E-F; Supplementary Table II). The ESR did not change significantly, which was likely related to the dose, the duration of treatment, and the sample size. The Kerr score, which was related to disease activity, was decreased to some extent (Figure 5G and Supplementary Table II).

Discussion

Although the pathogenesis of TAK remains unclear, M. TB infection is known to play an important role. Recent studies have confirmed that patients with TAK have a higher proportion of TB infection^[10,11]. The M.TB antigen, HSP65, was identified in TAK artery tissue and the sites of increased HSP65 expression also displayed γ - δ T cell infiltration, which suggested that HSP65 could be directly recognized by these cytotoxic cells^[15,23]. Moreover, elevated levels of IgG antibodies against HSP65 could be detected in the peripheral blood of patients with TAK^[16], which indicated 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.

We confirmed that HSP65 expression in the aortic adventitia of TAK patients was significantly higher than that of healthy individuals, and the sites of high HSP65 expression also showed severe inflammatory infiltration. It is important to note that HSP65 was preferentially expressed in the interstitium surrounding fibroblasts in inflammatory granulomas. Thus, we speculated that HSP65 likely plays a role in initiating the immune response by affecting the function of fibroblasts. It has been established that the M.TB HSP65 and human HSP65 sequences are highly homologous, and the associated T cell reactivity is highly similar. Therefore, we first explored the effect of HSP65 on the initiation of inflammation in AAFs by profiling the global mRNA expression. The results of the DEG analysis suggested that CCL2 was a key chemokine. We further detected a substantially higher level of CCL2 expression in the aorta of TAK patients compared to that of healthy subjects. Moreover, there was a positive correlation between HSP65 expression and CCL2, indicating that CCL2 was released following HSP65 stimulation. CCL2 is a member of the CC chemokine family that is chemotactic to monocytes and memory T lymphocytes^[24,25]. After being recruited to local tissues, monocytes differentiate into macrophages subtypes that release various inflammatory factors or play a role in tissue remodeling^[26,27]. A recent study found that the insufficient synthesis of an anti-inflammatory gene in monocyte-derived macrophages was associated with increased susceptibility to TAK^[28]. Moreover, our recent work detected a higher level of serum CCL2 in TAK patients compared to that in healthy controls (data not shown). Therefore, CCL2 mediated recruitment of monocytes to the vasculature represents an important pathological process in TAK.

IL-1 β is a potent pro-inflammatory cytokine that functions by inducing cyclooxygenase-2 production and participating in the activation of various immune cells^[29]. Although there is no significant increase in IL-1 β in the serum of TAK patients, this may be due to the fact that IL-1 β is an intense inflammatory mediator that is briefly expressed during the acute period, which is difficult to capture. Our previous work has shown that IL-6 can promote fibrotic remodeling of the TAK vascular wall by activating the JAK/STAT signaling pathway in fibroblasts or regulating fibroblast autophagy^[30,31]. The present results showed that HSP65 was co-expressed with IL-6 and IL-1 β in the aorta of TAK patients, and the mRNA expression of CCL2, IL-6, and IL-1 β in the AAFs was significantly up-regulated when stimulated with HSP65 in vitro.

This suggested that HSP65 initiated inflammation in the adventitia via activating AAFs to produce inflammatory mediators.

Toll-like receptor is a key component of innate immunity, a primitive immunity characterized by the rapid recognition of bacterial and other motifs as dangerous. Extracellular HSP65 is considered to be a TLR4 ligand, which can activate intracellular inflammatory signaling pathways through TLR4 [22, 32]; however, no studies have reported the interaction between HSP65 and TLR4 in AAFs and TAK. To further explore the underlying mechanism of HSP65 in initiating vascular inflammation, we detected the level of JAK1-3, AKT, and STAT3 phosphorylation in AAFs stimulated with HSP65. It was found that the level of p-JAK2, p-AKT, and p-STAT3 expression was significantly higher than that in the untreated group, and the expression of TLR4 was increased. In contrast, blocking TLR4 decreased the expression of p-JAK2, p-AKT, and p-STAT3, as well as the production of CCL2, IL-6, and IL-1 β . This means that HSP65 promotes the production of pro-inflammatory factors by activating the JAK2/AKT/STAT3 signaling pathway in AAFs. In addition, TLR4 is likely the key receptor for transducing the HSP65 antigen signal into cells.

Curcumin is a natural extract derived from the rhizome of the *Curcuma longa* plant, which was traditionally used for the treatment of various cancers and inflammation [33]. Most in vitro and animal studies have reported that curcumin exerts an anti-inflammatory effect by down-regulating NF- κ B and the MAPK signaling pathway. In addition, curcumin exerts both anti-tumor and anti-inflammatory effects by inhibiting cyclooxygenase-2 [20]. Recently, the therapeutic value of curcumin in autoimmune diseases has attracted increased attention. Zengjie Fan et al. [34] injected a new drug composed of hyaluronic acid/curcumin (HA/Cur) nanomicelles into the arthritic ankle joint RA rats, which significantly reduced arthritis and production of IL-1 and TNF- α . Moreover, in systemic lupus erythematosus (SLE), curcumin has been shown to modify the function of dendritic cells (DCs) and various T lymphocyte subsets [35]. However, although several studies have reported the anti-inflammatory effect of curcumin in vasculitis [36, 37], the probable underlying mechanism remains unexplored. This study demonstrated that curcumin could reduce the production and release of CCL2, IL-6, and IL-1 β in AAFs through inhibiting the activation of JAK2/AKT/STAT3 signaling pathway at a low dose. Among the three identified inflammatory factors, curcumin exerted the most significant inhibitory effect on CCL2, which was two-fold greater than the control group. It is important to note that curcumin is similar to the JAK2 inhibitor, AG490, in its capacity to inhibit the CCL2 expression. In addition, both curcumin and AG490 were more effective than MTX and tofacitinib (JAK1/JAK3 inhibitor). This finding confirmed that CCL2 was the primary regulatory target of curcumin, of which the key mechanism was a blockade of JAK2 phosphorylation.

Finally, to explore the potential clinical value of curcumin, we evaluated the regulatory effect of curcumin on serum cytokines levels, ESR, and Kerr scores in TAK patients. The results showed that the mean value of the serum CCL2 and IL-6 levels was decreased to varying degrees, and the change in the level of CCL2 was statistically significant. Although other cytokines were not significantly downregulated, cytokine and ESR upregulation only occurred in some patients. Therefore, we speculated that this effect was likely attributed to the small sample size. The ratios of a Kerr score greater than 0 and 1 were decreased to

some degree, suggesting that curcumin had a certain alleviating effect on mild disease activity in these TAK patients, or that curcumin could maintain these patients in the stable phase of TAK.

It has been well-established that curcumin prevents macrophages from differentiating into the M1 subtype, which represents the inflammatory subtype^[38]. Our previous work has found that the M2 macrophage subtype correlates with the fibrosis of the vascular wall^[6]. This study demonstrated that CCL2 played a key role in the early and acute stages of TAK, which represents an important chemokine of monocyte-macrophages. The importance of CCL2 suggests that monocyte recruitment and differentiation in the local tissues is important for the pathogenesis of TAK, and that curcumin treatment can hinder this process. Therefore, curcumin can inhibit the formation of both M1 and M2 macrophages in the arteries, which can both inhibit inflammation and slow the progression of fibrosis. The small sample size and short observation time are the main limitations associated with this study. According to these preliminary results, the sample size should be expanded to further explore how curcumin affects vascular inflammation and the imaging progress in TAK.

Conclusion

In conclusion, HSP65 upregulates CCL2 via activating TLR4-JAK2/STAT3 pathway in aorta adventitial fibroblasts, which plays a key role in triggering inflammation in TAK. Curcumin hinders the initiation and development of inflammation in TAK by reducing HSP65-induced CCL2 production (Fig. 6). This study provides mechanistic evidences according which curcumin can be considered as a new therapeutic drug for controlling the inflammation of TAK.

Abbreviations

TAK: Takayasu's arteritis; AAFs: Aorta adventitial fibroblasts; M. TB: *Mycobacterium tuberculosis*; TB: tuberculosis; HSP65: 65 kDa heat shock protein; DEGs: Differentially expressed genes; MTX: methotrexate; ACR, American College of Rheumatology; ESR: erythrocyte sedimentation rate; RT: Room temperature; ELISA, Enzyme linked immunosorbent assay; SLE, systemic lupus erythematosus; DC, dendritic cells; α -SMA: alpha-smooth muscle actin; CC: Curcumin; NC: Negative control; BC: Basic control; HC: Healthy control; Res: Resatorvid.

Declarations

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Authors' contributions:

LDJ and LLM conceived and designed the study. YJW, XYF, RYC, YS performed the experiments. WSY and QRH contributed statistical analysis. All authors read and approved the final manuscript.

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Availability of data and materials:

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

Ethics approval and consent to participate:

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 for inclusion in this study.

Consent for publication:

All authors consented to the publication of this manuscript.

Competing interests:

The authors declare that there are no competing interests

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Tables

Table I. Primer sequences of the detected genes

Gene symbol	Forward primer (5'-3')	Reverse primer (5'-3')
IL-1 β	GCGGCATCCAGCTACGAATCTC	AACCAGCATCTTCCTCAGCTTGTC
IL-6	GGTGTTGCCTGCTGCCTTCC	GTTCTGAAGAGGTGAGTGGCTGTC
CCL2	GAAAGTCTCTGCCGCCCTTC	TGATTGCATCTGGCTGAGCG
β -actin	GGCACCACACCTTCTACAATGAGC	GATAGCACAGCCTGGATAGCAACG

Figures

Figure 1

HSP65 is highly expressed in the aortic adventitia of TAK patients and CCL2 is the key inflammatory gene participating in HSP65-induced inflammation. A, The level of HSP65 expression in the aortic adventitia of healthy controls ($n = 6$) and TA patients ($n = 8$). B, Correlation analysis of HSP65 expression and the inflammatory scores. Definition of inflammation score: 1 point for less than 100 inflammatory cells per high magnification visual field ($400\times$); 2 points for 100 - 200 cells; 3 points for 200 - 300 cells; and 4

points for more than 400 cells ($r = 0.8838$, $p < 0.0001$). Spearman correlation analysis was performed. C, Double-labelled immunofluorescence images of HSP65 and α -SMA and correlation analysis of HSP65 and α -SMA expression ($r = 0.4675$, $p = 0.0213$). For the correlation analysis, three high magnification visual fields were randomly selected for each patient ($n = 8$). Pearson correlation analysis was performed. D, Among the 812 genes upregulated by HSP65 stimulation, 215 DEGs overlapped with the 3797 genes that were downregulated following curcumin pretreatment. E, The top 30 genes which were ranked based on the log2FoldChange (group HSP65 vs. group NC). Error bar represents SD; *** $p < 0.001$.

Figure 2

High CCL2 expression in the aortic adventitia of TA patients correlated with HSP65. A-B, The level of CCL2, IL-6, and IL-1 β expression in the aortic adventitia of healthy controls and TA patients. C, Double-labelled immunofluorescence images of HSP65 and CCL2. Correlation analysis of HSP65 and CCL2 expression ($n = 8$; $r = 0.7593$; $p < 0.0001$). Spearman correlation analysis was performed. D, mRNA expression of CCL2, IL-6, and IL-1 β in AAFs stimulated with HSP65 (1 $\mu\text{g/mL}$) for 12 h. The LPS content in the BC group was equal to that in the HSP65 group (0.0625 EU/mL). Experiments were repeated 3 $\times$ with similar results. Error bar represents SD; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Figure 3

HSP65 increased the production of pro-inflammatory factors in AAFs via the TLR4/JAK2-STAT3 pathway. A, Double-labelled immunofluorescence images of TLR4 and α -SMA (n=8). B, The level of TLR4 protein expression in unstimulated AAFs or following stimulation with HSP65. LPS (10 ng/mL) was used as a positive control. C-D, The level of JAK2, AKT, and STAT3 phosphorylation at 0, 15, 30, 60, 90, and 120 min following HSP65 stimulation (1 μ g/mL). E, The level of JAK2, AKT, and STAT3 phosphorylation at 90 min following stimulation with HSP65, LPS (10 ng/mL), or HSP65 combined with Resatorvid (10 μ M). F, The concentration of CCL2, IL-6 and IL-1 β in the AAFs culture supernatants following stimulation for 12 h. Error bar represents SD. All experiments have been performed in triplicate. *p < 0.05; **p < 0.01; ***p < 0.001; ns, not significant.

Figure 4

Curcumin (CC) reversed the HSP65-induced inflammatory response by inhibiting the JAK2/AKT/STAT3 signal pathway in AAFs. A, The IC50 of curcumin to AAFs is 36.48 μ M. B, The mRNA expression of CCL2, IL-6, and IL-1 β in AAFs following stimulation with HSP65 (1 μ g/mL) alone or HSP65 combined with CC (5 μ M and 10 μ M) for 12 h. C-D, The concentration of CCL2 and IL-6 in the AAFs culture supernatants following stimulation with HSP65 (1 μ g/mL) alone or HSP65 combined with curcumin for 12 h. E, The protein levels of pro-IL-1 β and mature IL-1 β in AAFs following stimulation with HSP65 alone or HSP65 combined with curcumin for 12 h. F-G, The level of JAK2, AKT, and STAT3 phosphorylation at 90 min after stimulation with HSP65 alone or HSP65 combined with curcumin. Error bar represents SD. All experiments have been performed in triplicate. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

Figure 5

The effects of curcumin compared with Tofacitinib and MTX and curcumin reduced the serum CCL2 level of TA patients (n=16). A-C, The concentrations of CCL2, IL-6 and IL-1 β in AAFs culture supernatants following stimulation with HSP65 (1 μ g/mL) alone or HSP65 combined with curcumin (CC) (5 μ M), AG490 (50 μ M), tofacitinib (250 nM), and MTX (50 nM) for 12 h. All experiments have been performed in triplicate. D, TA patients were treated with curcumin granules at a dose of 15 g/day, p.o. (equivalent to approximately 45 mg/kg/day curcumin) while maintaining the original treatment for three months. The serum CCL2 concentration was significantly lower than that at baseline ($p = 0.04$). E-G, There was no significant changes in the serum IL-6 ($p = 0.18$) and IL-1 β ($p = 0.13$) concentrations or ESR ($p = 0.49$) between baseline and after three months. Error bar represents SD; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns, not significant.

Figure 6

A cellular model illustrating the mechanisms involved in this study. HSP65 promotes monocytes recruitment by upregulating CCL2 in AAFs through the JAK/STAT3 pathway; curcumin reverses this process through blocking the phosphorylation of JAK2.

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