The potential role of leflunomide in inhibiting vascular fibrosis by down-regulating type-II macrophages in Takayasu's arteritis

X. Cui[1], X. Kong[1], R. Chen[1], L. Ma[1,2], L. Jiang[1,2]

[1]Department of Rheumatology, Zhongshan Hospital, Fudan University, Shanghai;
[2]Evidence-Based Medicine Centre, Fudan University, Shanghai, China.

Xiaomeng Cui, PhD, MD
Xiufang Kong, PhD, MD
Rongyi Chen, PhD, MD
Lili Ma, PhD, MD
Lindi Jiang, PhD, MD

Please address correspondence to:
Lindi Jiang,
Department of Rheumatology,
Zhongshan Hospital,
Fudan University,
No. 180 Fenglin Road,
Shanghai 200032, China.
E-mail: zsh-rheum@hotmail.com

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ABSTRACT

Objective. *Inflammatory fibrosis of aortic lesions promoted by type II macrophages (M2) is one of the most serious incidents in Takayasu's arteritis (TAK), and the currently available therapies can not effectively block the inflammatory fibrosis. Here we explored whether leflunomide (LEF) could improve the fibrosis by down-regulating M2 in TAK.*

Methods. *Peripheral blood mononuclear cells (PBMCs) from 16 TAK patients were treated by leflunomide, and the ratio of M1/M2 macrophages and apoptosis of M2 were detected by flow cytometry. Supernatant levels of cytokines and chemokines secreted by M2 were measured by ELISAs. mRNA expression of profibrotic factors in M2 were analysed by real time PCR. Western blotting was used to analyse the activation of signal transducer activator of transcription (STAT)-6.*

Results. *LEF could inhibit M2 polarisation by curtailing STAT6 phosphorylation. LEF could also promote apoptosis of M2 and reduce the release of M2-derived CCL22 as well as the expression of profibrotic cytokines including CCL22 and TGF-β in M2.*

Conclusion. *LEF could potentially reduce vascular fibrosis by down-regulating the number and function of M2, which, eventually, could alleviate inflammatory fibrosis of aortic lesions in TAK patients.*

Introduction

Takayasu's arteritis (TAK) is a chronic inflammatory disease characterised by progressive inflammatory fibrosis in the aorta and its major branches (1). Progressive inflammatory fibrosis results in ischaemia and dysfunction of target organs and, eventually, death (2, 3). Therefore, inhibition of inflammatory fibrosis of arterial walls in TAK patients is important. However, currently treatments could not block inflammatory fibrosis of TAK effectively so far. Glucocorticoids were the first-line medicine for TAK treatment, which could alleviate inflammation, but could not prevent vascular fibrosis subsequent to inflammation. Structural impairment on radiographic and histopathological evaluation was still found in patients receiving glucocorticoids, even though inflammation had been controlled very well (4-6). Similar results have also been observed in the patients treated with methotrexate (MTX) (7). Therefore, it is crucial to find proper treatments to block the vascular fibrosis in patients with TAK.

As an anti-inflammatory reagent, leflunomide (LEF) has been widely used in rheumatoid arteritis treatment but not been generally recommended for TAK treatment so far (8). Recently, studies have shown that LEF might have more potential efficacy in TAK treatment. Keser and Aksu recommended LEF as alternative treatment, especially for refractory TAK cases (9). Another study demonstrated that LEF could maintain disease remission for more than 12 months in about 50% patients (10). Moreover, we have found that in TAK patients who received LEF for 12 months, progression of vascular stenosis in TA was delayed or even improved (11). Taken together, these data suggest that LEF might alleviate fibrosis of aortic wall. Previous animal studies show that leflunomide could attenuate organ fibrosis in lung, heart and liver (12-14). Leflunomide could alleviate bleomycin-induced pulmonary fibrosis, inhibit FYN/AKT signalling pathway in cardiac fibrosis, and inhibit the expression of NF-κB in fibrotic liver tissue. Given the critical role of chronic fibrosis in the progression of TAK, understanding the mechanism of the anti-fibrosis role of LEF might benefit TAK patients.

Monocytes/macrophages play important roles in the pathogenesis of inflammatory diseases (15). According to the function and phenotype, macrophages can be divided into type I (M1) and type II (M2). The former are known as "classical" macrophages and mainly promote inflammation (15). In contrast, M2 macrophages are known as "alternative" macrophages and are involved in fibrosis subsequent to inflammation in the chronic phase of inflammatory disease (15). Under the chronic inflammation microenvironment, M2 further divided into the subsets M2a-d according to differentiation-dependent stimulatory factors (16). $CD68^{+}CD163^{+}$M2a cells, promoted by IL-4 and/or IL-13 *in vitro*, are the main subset of M2 macrophages in development of fibrosis in inflammatory diseases. M2a cells could produce extracellular matrix, enhance collagen deposition, and induce the expression of C-C motif chemokine ligand (CCL)-22, CCL17 and IL-10 etc. (17, 18). In mice suffering from endometrial fibrosis, M2a macrophages have been shown to increase the extent of fibrotic injury, and to be closely related to kidney and myocardial fibrosis (19, 20). CCL22, also known as "macrophage-derived chemokine", is a chemokine produced mainly by M2 macrophages, and has been shown to be closely associated with fibrosis in rheumatoid arthritis (21). In addition, M2a macrophages have been shown to express the profibrotic genes *LGALS3*, *TGFB1* and *PDGFB* (22), which are related to the profibrotic cytokines galectin3, transforming growth factor (TGF)-β and platelet-derived growth factor (PDGF)-β, respectively. Previously, we found abundant macrophages in inflammation- and fibrosis-affected areas of aortic lesions (23), which suggested that targeting macrophages, especially M2a subset, were involved in the pathogenesis of inflammatory fibrosis in TAK.

In the present study we aim to evaluate whether LEF could affect the differentiation and function of M2a macrophages derived from the peripheral blood mononuclear cells (PBMCs) of TAK patients. We found that LEF has the potential ability in alleviating vascular fibrosis by downregulate M2a in TAK. LEF could not only reduce the number of M2a by inhibiting the polarisation and promoting apoptosis simultaneously, but also down-regulate the fibro-genic factors expression in M2a cells. Thus, LEF might be recommended as anti-fibrosis therapy in TAK patients with long-term disease course, which will benefit for TAK patients' treatment greatly.

Materials and methods

Ethical approval of the study protocol

The research protocol was approved by the Ethics Committee of Zhongshan Hospital of Fudan University (B2016-168; Shanghai, China). The study protocol conformed to the tenets of the Helsinki Declaration. All patients provided written informed consent to participate in our study.

Patients

TAK patients hospitalised within the Department of Rheumatology in Zhongshan Hospital of Fudan University from 2017 to 2018 were enrolled in this study (Supplementary Table S1). The TAK diagnosis was made according to the classification criteria for TAK set by the American College of Rheumatology in 1990. Pathology specimens were obtained from Zhongshan Hospital for further analyses.

Staining (haematoxylin and eosin (H&E), Masson's trichrome and immunofluorescence)

Blood-vessel specimens from four TAK patients were fixed in 10% neutral-buffered formalin for 24h at room temperature, and then embedded in paraffin. The procedures used to carry out immunofluorescence staining have been described previously (24). Antibodies specific to CD68, CD86, and CD163 (Abcam, Cambridge, UK) were used to identify macrophage phenotypes (as shown in Suppl. Table S3) (25, 26). Tissue sections were also stained with H&E and Masson's trichrome for each patient according to the manufacturers' instructions.

Cell cultures and differentiation to M2a macrophages

Human peripheral blood mononuclear cells were isolated from blood samples of TAK patients by lysing red blood cells with Red Cell Lysis Buffer (Beyotime Technology, Shanghai, China). This was followed by isolation of monocytes with a Human Pan Monocyte Isolation kit according to manufacturer (Miltenyi Biotec, Bergisch Gladbach, Germany) instructions. Blood samples from different patients were processed and analysed separately. Cells were cultured at a $1\times10\text{^}5/cm^2$ in RPMI1640 medium (Thermos Fisher Scientific, Waltham, MA, USA) supplemented with 5% fetal bovine serum, penicillin (100U/ml) and streptomycin (100μg/ml) in an incubator maintained at 37°C in an atmosphere of 5% CO_2. For differentiation of monocytes into macrophages, macrophage colony-stimulating factor (M-CSF; 20 ng/mL; R&D Systems, Minneapolis, MN, USA) was added to the culture media and incubation for 5 days followed. Additional stimuli (all of which were from R&D Systems) were added for macrophages polarisation after 5 days: lipopolysaccharide (LPS; 50 ng/mL) and IFN-γ (20 ng/mL) or IL-4 (10ng/mL) and IL-13 (10ng/mL).

The human acute monocytic leukemia cell lines THP-1 was purchased from the Chinese Academy of Science (Shanghai, China). This cell line was cultured in RPMI1640 medium supplemented with 10% fetal bovine serum, penicillin and streptomycin. For differentiation into macrophages, cells were incubated with phorbol 12-myristate 13-acetate (100nM; Sigma-Aldrich, Saint Louis, MO, USA) for 24 h, followed by incubation in RPMI1640 medium for 24 h. The same stimuli mentioned above were added to the culture media for macrophages polarisation. To simulate ischaemia and a hypoxia microenvironment in TA, fetal bovine serum in culture media was reduced to 7% when THP-1 cells were differentiated to macrophages.

LEF treatment

LEF can undertake its biological function only after it has been transformed into A77 1726, an active metabolite of LEF (27, 28). Accordingly, the dose conversion of A77 1726 and LEF is 50μg/mL of A77 1726 in plasma if patients receive 20mg of LEF per day

(29). The clinical dose of LEF is 5-20 mg/day (7), so the dose of A77 1726 used in our study was 15-60μg/mL.
A77 1726 was purchased from Cinkate (Suzhou, China). With the stimuli for polarisation described above, cells were incubated with A77 1726 at the concentrations and time indicated. Glucocorticoid and methotrexate were purchased from Selleck Chemicals (Houston, TX, USA). The clinical dose of glucocorticoid is usually 1 mg/kg per day, and the concentration converted to 1 μg/ml for cell-culture *in vitro* accordingly. The clinical dose of methotrexate ranges from 7.5 to 25 mg/week, and the average Cmax of methotrexate is about 22.7ng/ml (30) which converted to 50nM for cell culture. In our study, the dose of methotrexate used was 50-100nM. AS1517499 (Selleck Chemicals), an inhibitor of STAT6, was incubated with macrophages 30 min before polarisation and used as the positive control.

Enzyme-linked immunosorbent assay (ELISA)
Macrophages differentiated from human monocytes were incubated with polarisation stimuli with or without A77 1726 for 24h. Cell-culture supernatants of cells stimulated with IL-4 and IL-13 were collected to measure concentrations of IL-10, CCL22, TGF-β and PDGF by using ELISA kits according to manufacturer (R&D systems) instructions. A standard curve was created for each plate and used to calculate the absolute concentrations.

Analyses of macrophage phenotypes
To assess the surface expression of antigens in differentiated macrophages, macrophages derived from human monocytes were incubated with stimuli for polarisation with or without A77 1726 for 24 h. Cells were detached by incubation in ice-cold phosphate-buffered saline (PBS) for 30 min. After detachment, macrophages were stained with antibodies for 15 min at room temperature. Fluorescence-conjugated monoclonal antibodies specific to human phycoerythrin (PE)-cy7 CD14, fluorescein isothiocyanate (FITC) CD16, PE CD68, Percp (Peridinin Chlorophyll-a Protein)-cy5.5 human leukocyte antigen-DR isotype (HLA-DR) and Allophycocyanin (APC) CD163 (BD Pharmingen, San Diego, CA, USA) were used. Flow cytometry was carried out using a FASCAria™ II cytometer (BD Pharmingen), and the data was analysed using FlowJo software v. 10 (FlowJo, Ashland, OR, USA). We used CD68, HLA-DR and CD163 as markers for M1 and M2 macrophages (Suppl. Table S3) (18, 31).

Apoptosis analyses
Macrophages derived from human monocytes were incubated with stimuli for polarisation with or without A77 1726 for 24 h and then detached, as described above. After detachment, cells were stained using a FITC Annexin V Apoptosis Detection kit according to manufacturer (BD Pharmingen) instructions. After 15 min of staining at room temperature, flow cytometry was done immediately using a FASCAria™ II cytometer (BD Pharmingen).

Real-time polymerase chain reaction (PCR)
Real-time PCR was done as described previously (24). Briefly, the total RNA of macrophages was extracted with TRIzol™ (Invitrogen, Carlsbad, CA, USA). cDNA was synthesised using a reverse transcription kit (Takara Bio, Shiga, Japan). Gene expression was measured by real-time quantitative PCR using the SYBR Green (Yeasen, Shanghai, China) method and normalised to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Data were calculated by the $^{\Delta\Delta}$CT method. The primer sequences were from Primer-BLAST (Suppl. Table S4).

Western blotting
Protein immunoblotting was done as reported previously (24). Briefly, cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA) at 100 V for 90 min. STAT6 phosphorylation was analysed using specific antibody (Cell Signaling Technology, Danvers, MA, USA). After washing with Tris-buffered saline and Tween 20 (TBST), PVDF membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin-G at room temperature for 1 h followed by washing with TBST. Target proteins were examined using an electrochemiluminescence detection reagent (Yeasen) with a LAS-3000 detection systems (Tanon, Shanghai, China). Band intensities were detected using ImageJ (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis
All experiments were repeated at least three times. Data are the mean ± standard deviation. Data were analysed by one-way ANOVA followed by a subgroup analysis using Bonferroni multiple comparisons. Prim 6 (GraphPad, San Diego, CA, USA) was used for data analyses. $p<0.05$ was considered significant.

Results

The number of M2 macrophages increased in the adventitia of arterial walls of TAK patients
In order to explore the phenotype of macrophages in the arterial walls of patients with TAK, we collected the arterial tissues from four patients with TAK. Inflammation and fibrosis was analysed by staining (H&E and Masson's trichrome), and co-localised M1 and M2 macrophages was analysed by immunofluorescence staining. Detailed data of patients are shown in Supplementary Table S2.
Staining using H&E and Masson's trichrome revealed severe inflammatory injury (Fig. 1a) and collagen-fiber hyperplasia (Fig. 1b) in adventitia of TAK arteria. Immunofluorescence staining showed that, in tissues with typically adventitia inflammation, M1 macrophages with CD68 and CD86 co-expression accumulated in regions with infiltration of pro-inflammatory cells (Fig. 1a). However, in tissues with severe proliferation of collagenous fibers in the tunica media of arteries, the number of M2 macrophages with CD68 and CD163 co-expression increased and most of them accumulated in the adventitia (Fig. 1b). The distinguishing distribution of different macrophage phe-

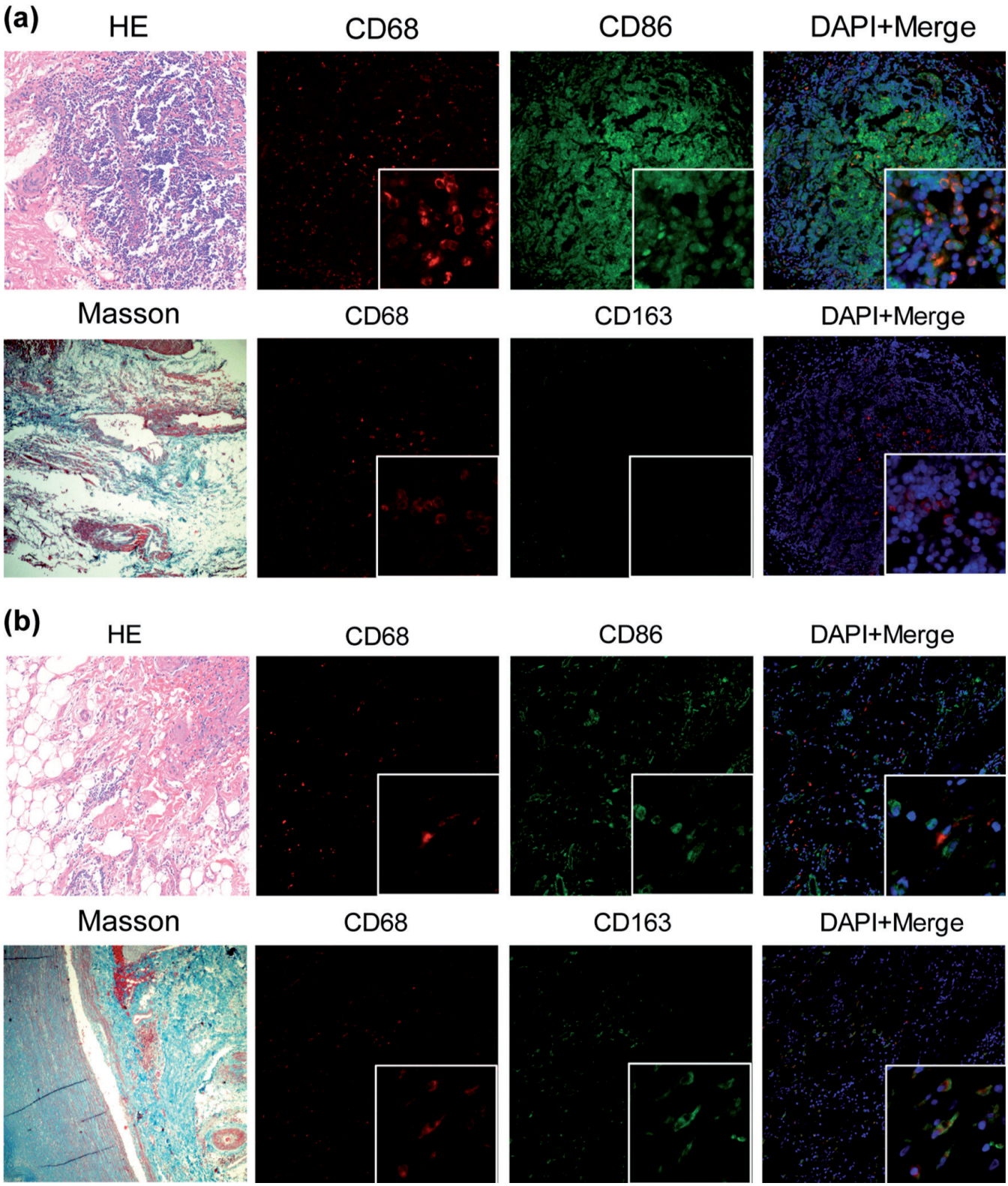

Fig. 1. Macrophages were increased and accumulated in the adventitia of arteries of TAK patients. Staining (H&E, Masson's trichrome and immunofluorescence) shows: (**a**) distribution of M1 macrophages expressing CD68 and CD86, and M2 macrophages expressing CD68 and CD163, in the inflammation sites of the adventitia (magnification: ×10 and ×40) and (**b**) distribution of M1 macrophages expressing CD68 and CD86, and M2 macrophages expressing CD68 and CD163, in the adventitia without obvious inflammation of proinflammatory cells (magnification: ×10 and ×40).

notypes in the adventitia of arteries of TAK patients suggested that M1 and M2 macrophages could have different roles in TAK pathogenesis. In the late stage of TAK, although obvious inflammation in the adventitia has been controlled, M2 macrophages persist, and could be associated with collagen proliferation and fibrosis in the vessel wall.

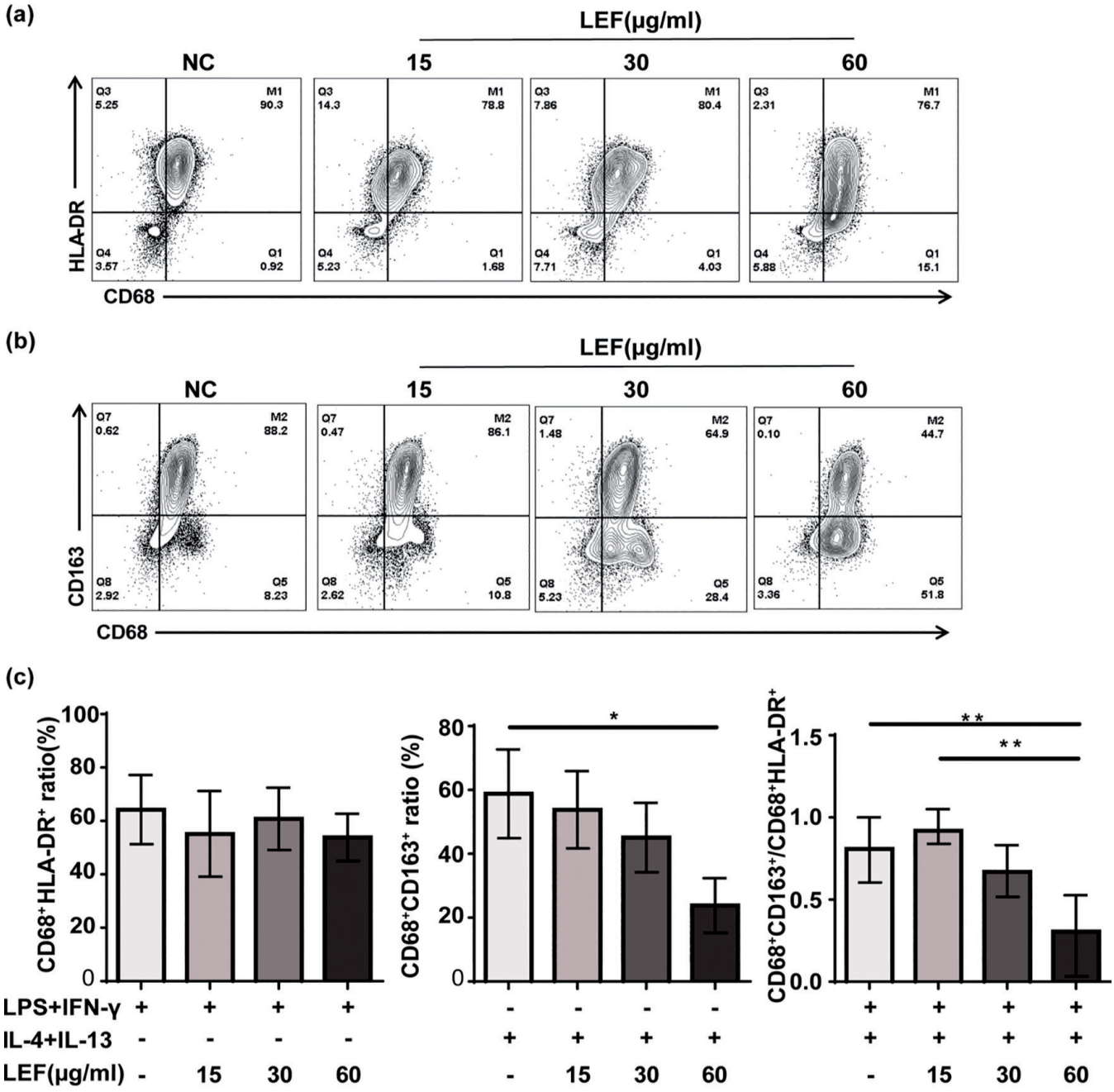

Fig. 2. LEF inhibits polarisation of M2 macrophages in TAK. (**a**) Expression of $CD68^+HLA\text{-}DR^+$cells in human macrophages incubated with LPS and IFN-γ with/without intervention of LEF (A771726 with a concentration of 15, 30 or 60μg/mL for 24 h). (**b**) Expression of $CD68^+CD163^+$ cells in human macrophages incubated with IL-4 and IL-13 with/without intervention of LEF (A771726 with a concentration of 15, 30 or 60μg/mL for 24 h). (**c**) The ratio of $CD68^+HLA\text{-}DR^+$ and $CD68^+CD163^+$ cells in human macrophages under intervention of LEF (A771726 with a concentration of 15, 30 or 60μg/mL for 24 h), and the ratio between $CD68^+HLA\text{-}DR^+$ and $CD68^+CD163^+$ cells with individual intervention.
*p<0.05; **p<0.01 (n=16). LEF: leflunomide.

LEF inhibits M2 polarisation in TAK

We next explored the effect of LEF on M2 macrophages in TAK patients. We collected monocytes from 16 patients with TAK. Detailed demographic data of these patients are shown in Supplementary Table S1. Among the patients, 37.5% were with active TAK, about half were taking glucocorticoids with dosage >15 mg/d, and half were receiving LEF treatment. After 5 days of culture with M-CSF, macrophages were co-cultured with LEF (15, 30 and 60μg/mL) for 24h, together with stimuli to induce polarisation. The effect of LEF on monocyte polarisation was analysed by flow cytometry. The number of M2 (but not M1) macrophages in TA patients was decreased significantly (p=0.03), suggesting that polarisation of M2 macrophages of PBMs derived from TAK patients was inhibited (Fig. 2a-b). Further analyses using MCS-F plus IL-4 and IL-13 revealed that LEF could inhibit polarisation of M2a macrophages in a dose-dependent manner (Fig. 2c). Glucocorticoids and metho-

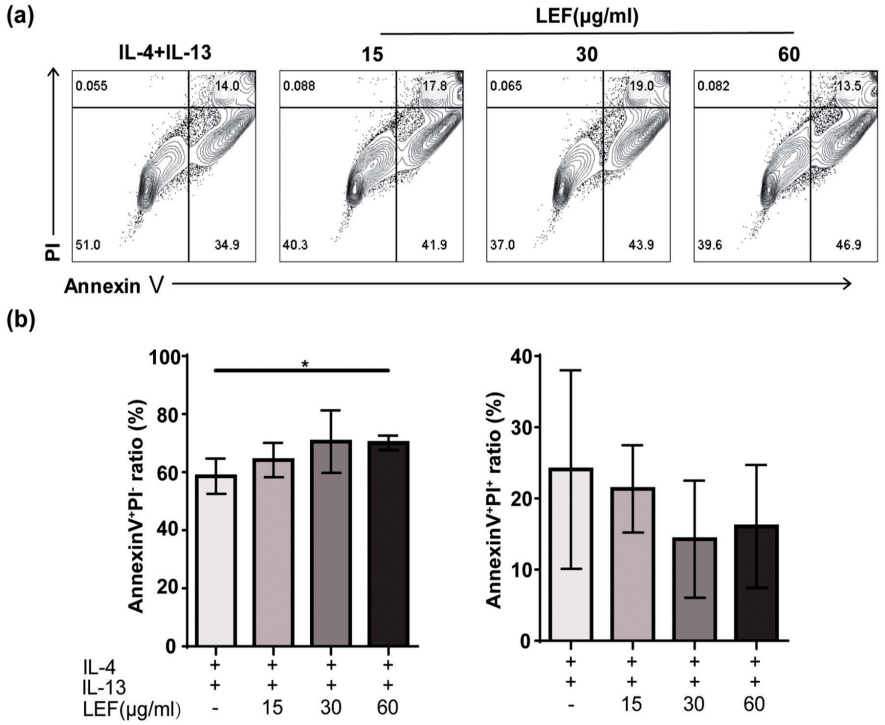

Fig. 3. LEF promotes apoptosis of M2a macrophages in TAK. (**a**) Expression of Annexin V and PI on the cell surfaces of macrophages under intervention of LEF (A771726 with a concentration of 15, 30 or 60μg/mL for 24 h). (**b**) The ratio of Annexin V^+PI^- and Annexin V^+PI^+ cells in macrophages under the intervention of LEF (A771726 with a concentration of 15, 30 or 60μg/mL for 24 h).
*$p < 0.05$ (n=4). LEF: leflunomide.

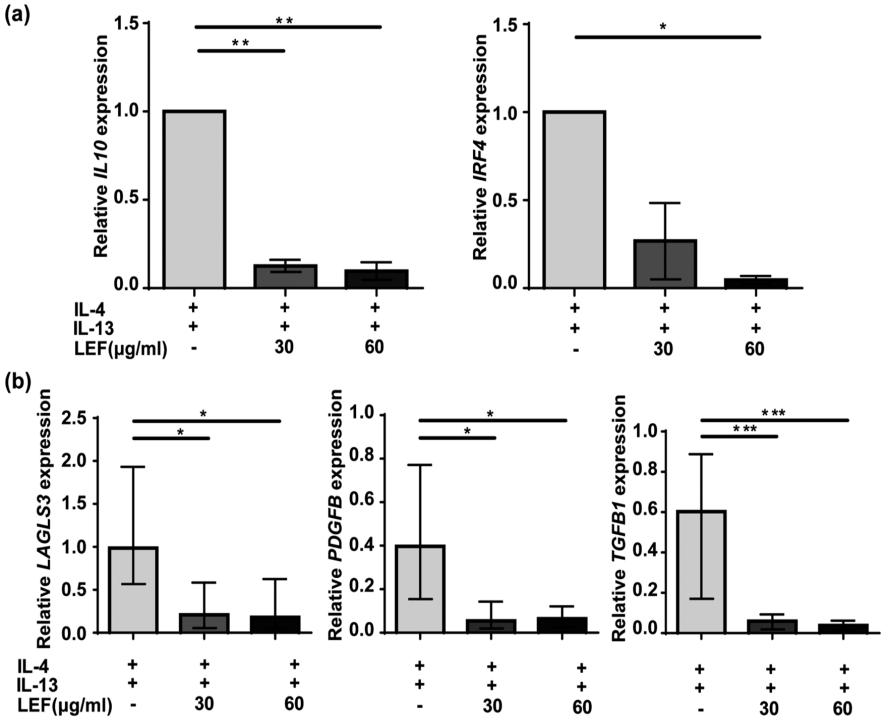

Fig. 4. LEF inhibits expression of profibrotic genes in M2 macrophages derived from THP-1 cells. (**a**) Relative expression of the mRNA of *IL10* and *IRF4* in M2 macrophages co-cultured with LEF (A771726 with a concentration of 15, 30 or 60μg/mL for 4 h). (**b**) Relative expression of the mRNA of *LAGLS3*, *PDGFB* and *TGFB1* in M2 macrophages co-cultured with LEF (A771726 with a concentration of 15, 30 or 60μg/mL for 4 h).
*$p<0.05$; ***$p<0.001$ (n=5). LEF: leflunomide.

trexate, as drugs commonly used for TAK treatment, were also analysed for their effects on monocyte polarisation. The number of M2 macrophages only decreased when co-cultured with high dosage of methotrexate (Suppl. Fig. S1). Monocytes from patients with different disease activity and different treatment regimens showed similar changes when cultured *in vitro*.

LEF promotes M2a apoptosis in TA

Given that LEF could inhibit the polarisation of M2a macrophages in TAK, we further evaluated the effect of LEF on apoptosis of M2a macrophages. Macrophages were co-cultured with different doses of LEF in the presence of IL-4 and IL-13, and the population of M2a macrophages was detected by flow cytometry. Only a proportion of M2a macrophages undergoing early apoptosis (Annexin V^+Propidium iodide $(PI)^-$ cells) increased significantly (p=0.01) in number upon LEF treatment (Fig. 3a, b), suggesting that LEF could induce apoptosis in M2a macrophages derived from TAK patients.

LEF inhibits expression of profibrotic genes in M2 like cells from THP-1 cells

Whether LEF affects the pro-fibrotic function of M2a macrophages needs to be further analysed. Hence, we further examined expression of fibrosis-related genes in the M2a cells derived from THP-1 cell line (a widely used human cell line in macrophage research) (32). By mimicking the condition of TAK, THP-1 cells were polarised into M2a macrophages in the presence of IL-4 plus IL-13 and treated with LEF in a low-nutrient environment for 4 h. Subsequently, expression of the M2a macrophage-related genes *IRF4* and *IL10* were measured by real-time PCR. We found that expression of these two genes was down-regulated significantly (p<0.01 and =0.03, respectively) (Fig. 4a), suggesting that LEF inhibited polarisation of THP-1 cells to M2a macrophages significantly.

To evaluate the pro-fibrotic function of M2a macrophages, we further examined the relative expression of the mRNA of the profibrotic genes *LAGLS3*, *PDGFB* and *TGFB1* expressed in THP-1 cell-

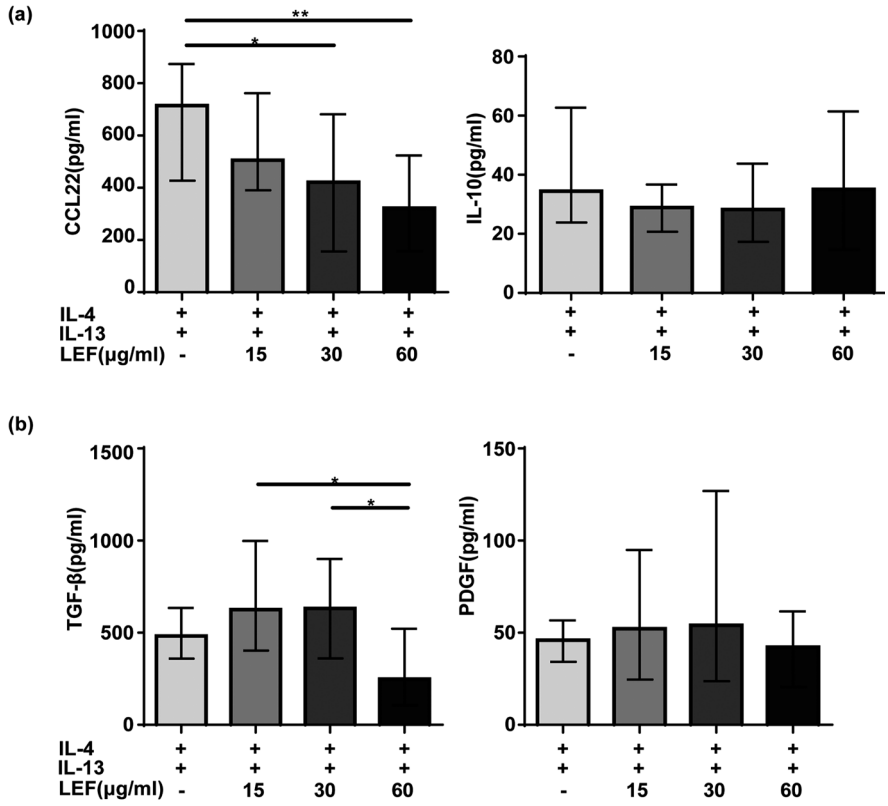

Fig. 5. LEF inhibits secretion of inflammation- and fibrosis-associated cytokines and chemokines from M2a macrophages in TAK. (**a**) Levels of CCL22 and IL-10 in cell culture medium supernatants of PBM-derived M2a macrophages under intervention of LEF (A771726 with a concentration of 15, 30 or 60μg/mL for 24 h). (**b**) Levels of TGF-β and PDGF in cell-culture medium supernatants of PBM-derived M2 macrophages under intervention of LEF (A771726 with a concentration of 15, 30 or 60μg/mL for 24 h).
**p*<0.05; ***p*<0.01 (n=16). LEF: leflunomide.

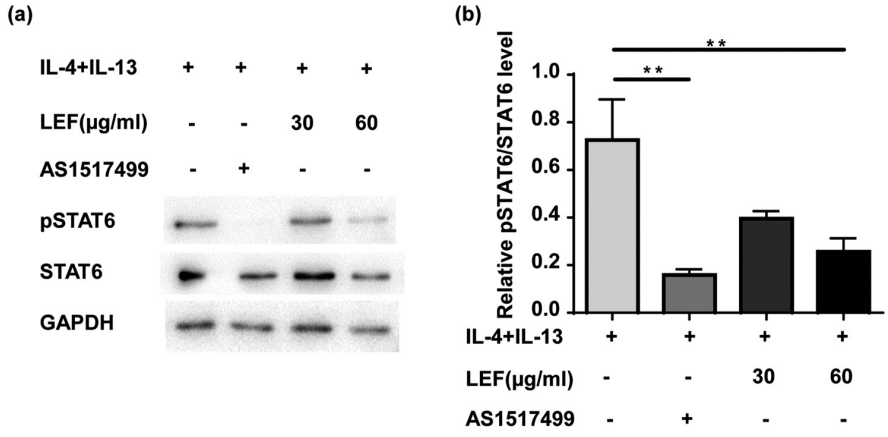

Fig. 6. LEF inhibits polarisation of M2 macrophages by inhibiting STAT6 phosphorylation.
(**a**) Expression of total STAT6, phosphorylated STAT6 and GAPDH proteins in M2 macrophages co-cultured with LEF (A771726 with a concentration of 30 or 60μg/mL for 12 h) and the STAT6 inhibitor AS1517499 (100nM) as a positive control, for 12 h. (**b**) Relative expression of phosphorylated STAT6 protein compared with total STAT6 in M2 macrophages co-cultured with LEF (A771726 with a concentration of 30 or 60μg/mL for 12 h) and AS1517499 (100nM) for 12 h.
***p*<0.01 (n=3). LEF: leflunomide.

induced M2a macrophages. Expression of all of these genes was decreased significantly upon LEF intervention (all $p<0.01$) (Fig. 4b). Relative expression of *LAGLS3*, *PDGFB* and *TGFB1* in LEF (30 μg/mL)-stimulated cells was <30% of that expressed in cells incubated only with IL-4 and IL-13. These results suggested that LEF could inhibit vascular fibrosis in TAK by regulating expression of the profibrotic genes in M2 macrophages.

LEF inhibits expression of chemokines and cytokines of M2a macrophages

Based on the results stated above, the secretory function of M2a macrophages was also explored. ELISAs showed that CCL22 expression in the supernatants of M2a macrophages derived from the PBMs of TA patients decreased significantly ($p<0.01$) in a dose-dependent manner when co-cultured with LEF (Fig. 5a). Expression of TGF-β (a profibrotic cytokine expressed by *TGFB1*) was also reduced significantly in the supernatants of M2a macrophages ($p=0.02$), but that of PDGF did not change (Fig. 5b). Also, the level of TGF-β secreted by M2a macrophages decreased in a dose-dependent manner when M2a macrophages were treated with LEF. These data suggested that, in TAK patients, LEF inhibits the number and biologic function of M2a macrophages by curtailing production of the cytokines and chemokines associated with inflammation and fibrosis.

LEF inhibits polarisation of M2a macrophages by inhibiting activation of STAT6

Based on the inhibition of polarisation of M2a macrophages caused by LEF in TAK patients, we examined the polarisation-related signalling pathways in M2 macrophages derived from THP-1 cells, and co-cultured cells with LEF or AS1517499 for 12 h. Western blotting revealed that expression of phosphorylated-STAT6 decreased significantly ($p<0.01$) in M2 macrophages co-cultured with LEF (Fig. 6a-b). These data suggested that LEF could inhibit polarisation of M2a macrophages by inhibiting STAT6 phosphorylation.

Discussion

The arterial-wall fibrosis could not been effectively controlled by current reagents in TAK patients (8, 33). Due to the anti-inflammatory function, LEF has been used widely as a disease-mod-

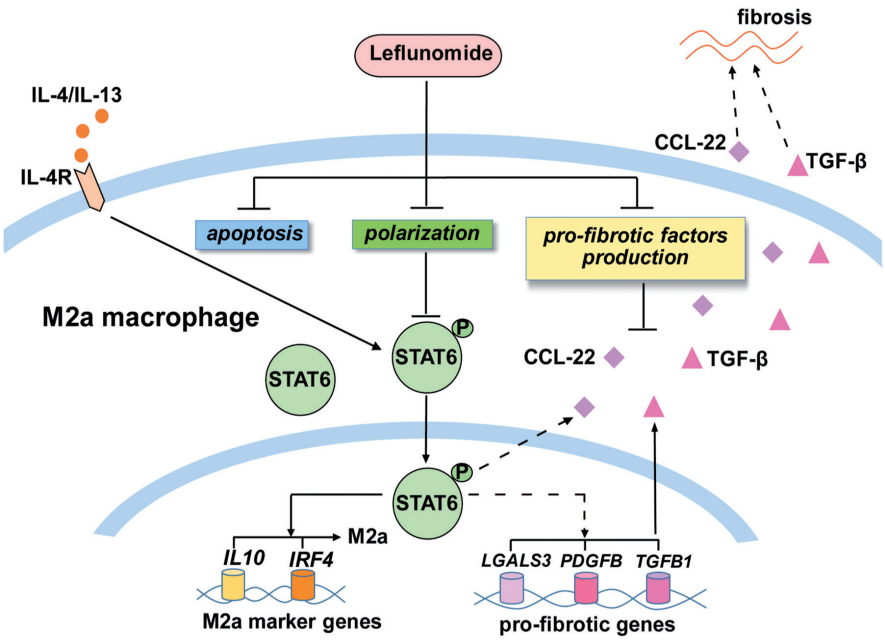

Fig. 7. Research summary. Leflunomide could regulate the number and function of M2a cells in Takayasu's arteritis through multiple targets, including restriction of M2a polarisation by inhibiting STAT6 phosphorylation and reducing expression of IL-10 and IRF4, promotion of apoptosis, inhibition of profibrotic genes expression, as well as reduction of production of profibrotic chemokines. Through these functions, leflunomide could prohibit the involvement of M2a in vascular remodelling.

ifying anti-rheumatic reagent (34, 35), and has been recommended as an alternative treatment for refractory TAK (9). Some studies have shown that LEF can reduce fibrosis (12, 13). Hence, exploring whether LEF can alleviate inflammatory fibrosis in TAK patients and the underline mechanism is important. M2a macrophages have important roles in the pathology of fibrosis (36). Hence, we assessed the effect of LEF on number and function of M2a macrophages.

First, we found that in tissues with severe proliferation of collagenous fibers in the tunica media of arteries, the number of M2 (but not M1) macrophages increased significantly, which is consistent with our previous study. In the previous study, we have found that the subsets of macrophages in arterial wall shifted in different phases of treatment. Before treatment, M1 macrophages indeed filtrated in all the layers while few M2 were observed. In contrast, after treatment, although M1 cells significantly reduced, M2 macrophages increased and mainly infiltrated in the outer layer (data not shown). These observations suggest that down-regulation of M2 macrophages might have a potential role in alleviating the fibrosis observed in TAK.

Secondly, we demonstrated that the number of IL-10-producing $CD68^{+}CD163^{+}$ M2a macrophages were reduced remarkably upon LEF incubation *in vitro*. Previous study found that, in mice suffering from overproduction of hydrogen peroxide, LEF could inhibit expression of a marker of M2a macrophages, CD23 and its associated genes *ym1* and *fizz1* (37). However, the effect of LEF on human M2a development has not been reported before. Here, we found that LEF could down regulate the polarisation of M2a macrophages in humans with a dose-dependent manner. Especially, even in a relatively low dose, LEF could reduce number and function of M2a *in vitro*. Moreover, by comparison with MTX, which is widely used in TAK treatment, we found that MTX could reduce polarisation of M2a macrophages only in high dose, which exceeds the safe range. As shown in the present study, LEF could inhibit M2a polarisation with a safe dose (equally to no more than 20mg daily), which indicates the advantage of LEF on regulating M2a comparing with MTX for TAK treatment.

Furthermore, Montagna *et al*. showed that LEF can promote the apoptosis of immune cells (including macrophages) by activating the Fas/FasL system (38). Hence, we explored the regulatory effect of LEF on apoptosis of M2a macrophages. We found that LEF could reduce the number of M2a macrophages by inducing apoptosis of M2a. Thus, LEF inhibit M2a by down regulating polarisation but promoting apoptosis.

M2 macrophages have been reported to promote fibrosis by expressing the profibrotic genes *LGALS3*, *TGFB1* and *PDGFB*[22], which express the fibrosis-related proteins galectin3, TGF-β and subunit B of PDGF, respectively (39-41). Indeed, we found that LEF could inhibit not only the expression of these profibrotic genes, but also the production of their related proteins. Studies have focused on the inhibitory effect of LEF on fibrosis by reducing production of collagen I, hydroxyproline and TGF-β in a hepatic-fibrosis model in mice (14) or inhibiting production of matrix metalloproteinases in the fibroblasts of patients suffering from rheumatoid arthritis (42). However, we are the first to show that LEF could inhibit expression of PDGF-BB, galectin-3 and *TGFB* mRNA directly in M2a macrophages. Since the effect of promoting fibrosis in M2, our study reveals that LEF would be effective in TAK treatment by inhibiting not only differentiation of M2 macrophages, but also the expression of pro-fibrotic molecules directly.

CCL22 is a chemokine produced mainly by M2 macrophages. CCL22 can bind to CC chemokine receptor-4 on fibroblasts, and promote the migration and growth of fibroblasts, as well as IL-6 secretion (21). Studies have shown that CCL22 expression in bronchoalveolar lavage fluid is increased significantly in patients with idiopathic pulmonary fibrosis (43). It has been recognised that LEF can inhibit secretion of the proinflammatory cytokines IL-6 and TNF-α by macrophages (44). In patients with rheumatoid arthritis, serum levels of CCL22 are decreased significantly upon LEF treatment and, in the human tumour cell line Caco-2, *CCL22* expression is inhibited significantly upon LEF treatment (45). How-

ever, whether LEF can stop/reduce M2 macrophages producing CCL22 is not known. We demonstrated that LEF can inhibit CCL22 production by M2a macrophages. In our previous study, we have shown that increase expression of IL-6 in the aorta can promote adventitial fibrosis, and that abnormal activation of fibroblasts has an important role in the fibrotic process seen in TAK (23). Thus, the present study supplied new evidence that LEF can block the interaction of macrophages and fibroblasts in the aortic walls of TAK patients by affecting CCL22 production.

M2 macrophages polarisation is associated with activation of multiple signalling pathways. Upon stimulation of IL-4 and IL-13, the janus kinase-1/2/3-Stat6 signalling pathway is activated in the cytoplasm of macrophages, with increasing IRF-4 expression, next followed by an increase in production of IL-10 (18). Vergadi *et al*. showed that the phosphoinositide 3-kinase/protein kinase B signalling pathway also has a role in polarisation of M2 macrophages (46). Our study showed that STAT6 phosphorylation in macrophages as well as the mRNA expression of IRF-4 and IL-10 were inhibited significantly by LEF, which were in accordance with the work of He *et al*. (37). These results suggest that LEF can affect multiple targets of differentiation at RNA and protein levels to inhibit polarisation of M2 macrophages.

Conclusions

Our study suggests that LEF could regulate the number and function of M2a macrophages in TAK by: (i) restriction of polarisation of M2a macrophages by inhibiting STAT6 phosphorylation; (ii) promotion of apoptosis of M2a macrophages; (iii) inhibition of production of fibrosis-associated chemokines by M2a macrophages; (iv) reduction of expression of profibrotic genes (Fig. 7). Treatment with LEF in TAK can prevent vascular fibrosis by modulating M2a macrophages function. Moreover, our data support that LEF might be commended as one of the first-line medicine in TAK treatment, due to its role not only in anti-inflammation, but also in anti-fibrosis.

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