

# Andrographolide affects Th1/Th2/Th17 responses of peripheral blood mononuclear cells from ulcerative colitis patients

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**Abstract.** Ulcerative colitis (UC) is a chronic, idiopathic, inflammatory bowel disease of the colon. T cell responses have been associated with the pathology of UC. *Andrographis paniculata* (AP) extract has been previously reported as an effective treatment of UC. The present study aimed to explore the effects of andrographolide, the primary active component of AP, on the T cell responses of patients with UC. Peripheral blood mononuclear cells (PBMCs) were isolated from patients with UC and treated with various concentrations of andrographolide (0, 10, 20 and 30  $\mu\text{g/ml}$ ). Andrographolide decreased interferon  $\gamma$ , interleukin (IL)-23 and IL-17A, however it increased IL-4 in a dose-dependent manner, as indicated by ELISA assay. Andrographolide treatment resulted in a decreased percentage of T helper (Th)1 and Th17 cells and an increased proportion of Th2 cells, as demonstrated by flow cytometry analysis. T-bet (a Th1-specific transcription factor) and RAR-related orphan receptor  $\gamma\text{T}$  (key transcription factor of Th17 cells) expression was decreased, but GATA-3 (Th2 lineage-specific transcription factor) expression was increased following andrographolide treatment as indicated by western blot analysis. These results demonstrated the inhibitory effects on Th1/Th17 responses and the promoting effects on Th2 responses of andrographolide. Experiments on IL-23-treated PBMCs from healthy donors revealed similar effects of andrographolide on Th1/Th2/Th17 responses. In summary, these results suggest that andrographolide may be an effective candidate for the treatment of IL-23-mediated diseases.

## Introduction

Ulcerative colitis (UC) is a chronic, idiopathic, inflammatory bowel disease (IBD) of the colon (1,2). The primary symptoms

of UC are abdominal pain and bloody diarrhea (3). The direct causes of UC are unknown, but immune system dysfunction has been implicated in the pathogenesis of UC (4). T cell responses have been intensively explored in UC. Upon activation, naive CD4<sup>+</sup>T cells differentiate into T helper 1 (Th1), Th2 and Th17 cells. The mucosal levels of interferon  $\gamma$  (IFN $\gamma$ , a Th1 cell produced cytokine) was increased in patients with UC compared with those in normal control (5). Interleukin-4 (IL-4, a Th2 cell produced cytokine) was more frequently detected in UC than in inflammatory controls (6). By using an oxazolone colitis model, Fuss *et al* (7), demonstrated that UC has an increased Th2-oriented immune response. IL-17 (a Th17 cell produced cytokine) mRNA was increased in biopsy specimens from UC (8,9). IL-23, the key cytokine that promotes Th17 cells to produce IL17 (10), differentially regulates the Th1/Th17 balance in UC (11).

Approximately 50% of patients with UC can be treated with a number of medications, including 5-aminosalicylic acid (ASA) drugs, such as sulfasalazine and mesalamine (2,3,12). Patients who fail to respond to 5-ASA drugs are treated with steroids (13), azathioprine (14) and infliximab (15), which may have serious toxicity (16). Additional medical therapies for patients failing 5-ASA drugs are needed. *Andrographis paniculata* (AP), an important herbal medicine, has been used to treat inflammatory and infectious diseases (17-19). Andrographolide is the main active component of AP. AP extracts and andrographolide possess immunostimulatory (20,21), anti-cancer (20,22), antiviral (23) and antibacterial activities (24). AP extract (HMPL-004) showed similar efficacy to mesalamine for UC (25,26). Andrographolide sulfonate, a derivative of andrographolide, could inhibit Th1/Th17 responses and improve experimental colitis (27). However, whether andrographolide affects the T cell responses of UC patients has not been explored.

In the present study, peripheral blood mononuclear cells (PBMCs) isolated from UC patients were treated with various concentrations of andrographolide. Then, the effects of andrographolide on Th cell differentiation were investigated. Further experiments with PBMCs from healthy donors confirmed these findings.

## Materials and methods

*Isolation, culture and treatment of PBMCs.* The present study received ethical approval from the Ethics Committee

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of Zhejiang Hospital. Blood samples were collected from 3 UC patients and 3 age-match healthy donors after written informed consent was obtained from all participants. Clinicopathological data of UC patients are listed in Table I. PBMCs were freshly isolated from blood samples by gradient centrifugation with lymphocyte cell separation media (Cedarlane Laboratories, Ontario, Canada) and grown in RPMI-1640 (HyClone; GE Healthcare Life Sciences, Logan, UT, USA) containing 10% fetal bovine serum (FBS; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and penicillin/streptomycin. The PBMCs were maintained in a 37°C incubator with 5% CO<sub>2</sub>.

PBMCs from UC patients were randomly treated with dimethyl sulfoxide (DMSO; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and 10, 20 or 30 µg/ml of andrographolide (AG; Sigma-Aldrich; Merck KGaA). PBMCs from healthy donors were randomly divided into three groups: Control group, treated with DMSO; IL-23 group, treated with 50 ng/ml IL-23 (Sigma-Aldrich; Merck KGaA); and IL-23+AG group, treated with 20 µg/ml andrographolide for 2 h and then treated with 50 ng/ml IL-23. After 48 h of culture, the culture media were collected for enzyme-linked immunosorbent assay (ELISA), and PBMCs were harvested for flow cytometry and Western blot analysis.

**ELISA assay.** The culture media were collected and the concentrations of IFN $\gamma$ , IL-4, IL-23 and IL-17A were measured by using commercial ELISA kits (Bio-swap, Shanghai, China) according to the manufacturer's instructions. Optic densities were measured at 450 nm, and the concentrations of cytokines were calculated according to a standard curve.

**Cytokine staining and flow cytometry.** The treated PBMCs were centrifuged at 1,000 rpm for 10 min. The pellet was resuspended in cultured media supplemented with PMA (100 ng/ml; Sigma-Aldrich; Merck KGaA)/ionomycin (1 µg/ml; Sigma-Aldrich; Merck KGaA) and monensin (1 µg/ml; Shanghai Aladdin Bio-Chem Technology Co., Shanghai, China) and plated onto 24-well plates (0.5x10<sup>5</sup> cells/well). After incubation at 37°C for 4 h, the PBMCs were collected, resuspended in phosphate-buffered saline (PBS) and labeled with anti-CD4-FITC (BioLegend, Inc., San Diego, CA, USA) for 1 h at 4°C. Subsequently, the cells were fixed with 2% formaldehyde and permeabilized with 0.1% Triton X-100 in PBS. Intracellular cytokine staining was then performed with anti-IFN $\gamma$ -APC, anti-IL-4-PE or anti-IL-17A-PE (BioLegend, Inc.) for 1 h. The cells were detected by using flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA). The proportions of CD4+IFN $\gamma$ + cells, CD4+IL-4+ cells and CD4+IL-17A+ cells (right upper quadrant) in CD4+ cells (right upper and lower quadrant) were calculated.

**Western blot analysis.** PBMCs was lysed in RIPA buffer and then centrifuged at 12,000 rpm for 20 min. The supernatant was collected, and the protein concentrations were quantified by using a BCA method. An equal amount of protein (30 µg) from each sample was loaded onto 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred onto a nitrocellulose blotting membrane (EMD Millipore,

Table I. Clinicopathological data of patients with UC (n=3).

Characteristic	Patient data
Sex	Male
Mean age at operation (years)	32
Duration of disease (years)	4.8
Extent of disease	Total colitis

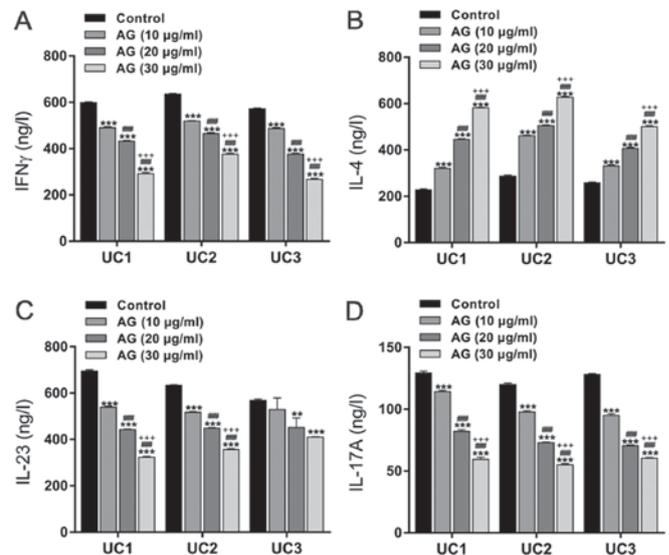


Figure 1. Effects of AG on the release of cytokines in PBMCs from patients with UC. PBMCs from 3 UC patients (UC1, UC2 and UC3) were randomly treated with 10, 20 or 30 µg/ml of AG. Cells treated with DMSO served as negative controls. After 48 h of treatment, the culture media were collected, and the concentrations of (A) IFN $\gamma$ , (B) IL-4, (C) IL-23 and (D) IL-17A were assessed by ELISA assay. \*\*P<0.01 and \*\*\*P<0.001 vs. the control; ##P<0.001 vs. AG (10 µg/ml); ###P<0.001 vs. AG (20 µg/ml). AG, andrographolide; PBMC, peripheral blood mononuclear cells; UC, ulcerative colitis; IFN, interferon; IL, interleukin.

Billerica, MA, USA). Following incubation with 5% skim milk at 4°C for 1 h, the membranes were incubated with anti-T-bet (cat. no. Ab91109, 1:500; Abcam, Cambridge, MA, USA), anti-GATA-3 (cat. no. Ab106625, 1:1,000; Abcam), anti-ROR- $\gamma$ t (cat.no. Ab78007, 1:1,500; Abcam) and anti-GAPDH (cat. no. 5174, 1:2,000; Cell Signaling Technology, Danvers, MA, USA) antibodies at 4°C overnight. Then, the membrane was washed three times with TBST buffer and incubated with horseradish peroxidase conjugated secondary antibody (Beyotime Institute of Biotechnology, Shanghai, China) for 1 h. Immunoreactive bands were detected using an ECL detection kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and semi-quantified by ImageJ software (<http://rsb.info.nih.gov/ij/>, National Institutes of Health, Bethesda, MD, USA).

**Statistical analysis.** Data are expressed as the means  $\pm$  standard deviation (SD). Statistical analysis was performed with GraphPad Prism software (v6.0, San Diego, CA, USA). One-way analysis of variance with a Tukey's post hoc test was performed. P<0.05 was considered to indicate a statistically significant difference.

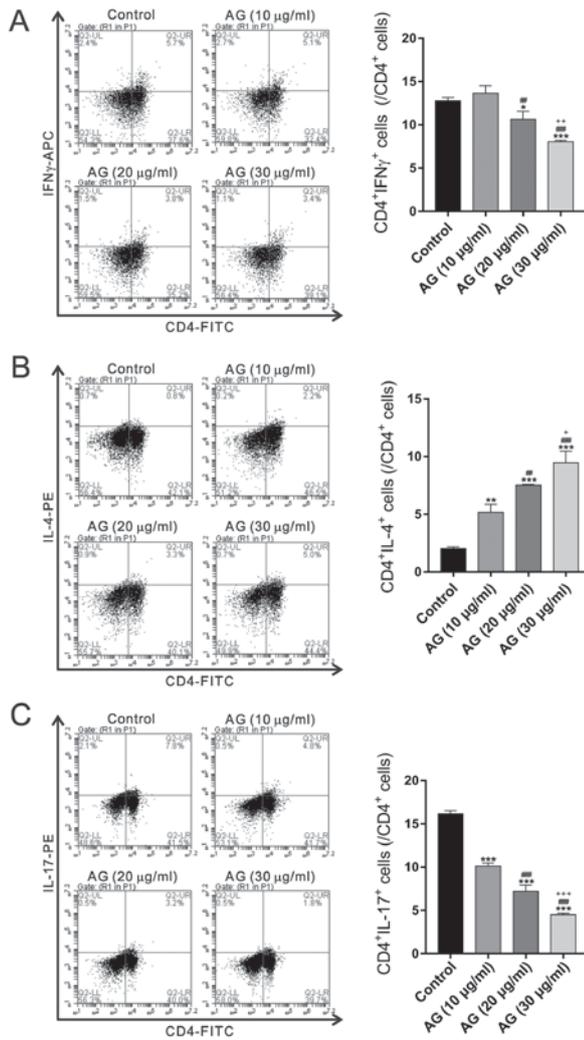


Figure 2. Effects of AG on the Th cell subset in PBMCs from UC patients. PBMCs were treated with DMSO or various concentrations of andrographolide for 48 h. The proportion of (A) CD4<sup>+</sup>IFN $\gamma$ <sup>+</sup>, (B) CD4<sup>+</sup>IL-4<sup>+</sup> and (C) CD4<sup>+</sup>IL-17A<sup>+</sup> cells in CD4<sup>+</sup> cells was detected by flow cytometry. The experiments were performed in PBMCs from 3 UC patients and similar results were obtained. Representative results are shown. \*P<0.05, \*\*P<0.01 and \*\*\*P<0.001 vs. the control; #P<0.01 and ###P<0.001 vs. AG (10  $\mu$ g/ml); \*P<0.05, \*\*P<0.01 and \*\*\*P<0.001 vs. AG (20  $\mu$ g/ml). Th, T helper; CD, cluster of differentiation; IL, interleukin; AG, andrographolide; PBMC, peripheral blood mononuclear cells; UC, ulcerative colitis.

**Results**

*Effects of andrographolide on the release of IFN $\gamma$ , IL-4, IL-23 and IL-17A in PBMCs from UC patients.* To examine the effect of andrographolide on the production of Th cell-specific cytokines, PBMCs were isolated from three UC patients and treated with 10, 20 or 30  $\mu$ g/ml of andrographolide. The concentrations of cytokines in the culture media were determined by an ELISA assay. After 48 h of treatment, andrographolide decreased IFN $\gamma$ , IL-23 and IL-17A but increased IL-4 in a dose-dependent manner (Fig. 1).

*Effects of andrographolide on Th cell subset in PBMCs from UC patients.* To analyze the effect of andrographolide on subtypes of Th cell populations, PBMCs treated with

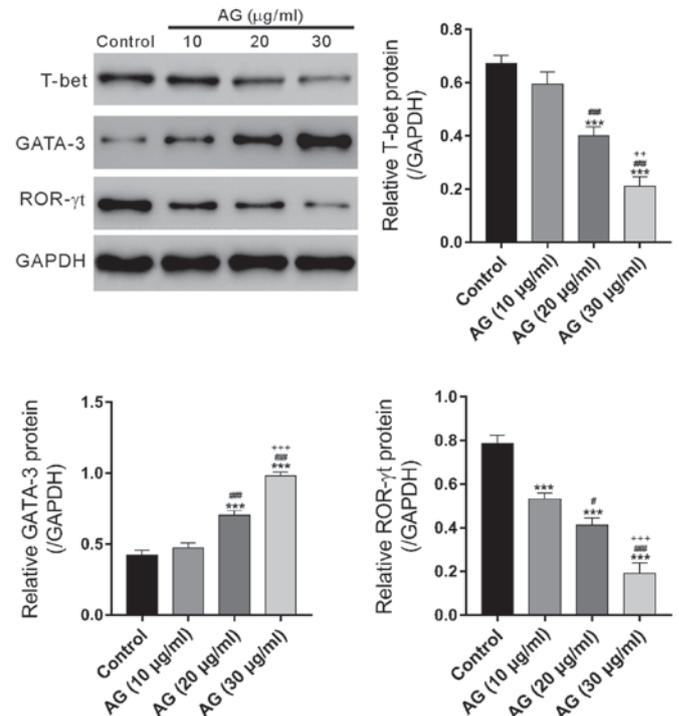


Figure 3. Effects of AG on the protein levels of T-bet, GATA3 and ROR- $\gamma$ t in PBMCs from patients with UC. PBMCs were treated with DMSO or various concentration of andrographolide for 48 h. The protein levels of T-bet, GATA3 and ROR- $\gamma$ t were detected by western blot analysis. The experiments were performed in PBMCs from three UC patients and similar results were obtained. Representative blots are shown. \*\*\*P<0.001 vs. the control; #P<0.05 and ###P<0.001 vs. AG (10  $\mu$ g/ml); \*\*P<0.01 and \*\*\*P<0.001 vs. AG (20  $\mu$ g/ml). ROR, RAR-related orphan receptor; AG, andrographolide; PBMC, peripheral blood mononuclear cells; UC, ulcerative colitis.

andrographolide were stained with IFN $\gamma$ , IL-4 and IL-17A in CD4<sup>+</sup> T cells, which are the respective signature cytokines of Th1, Th2 and Th17 cells. Andrographolide treatment resulted in a decreased percentage of Th1 and Th17 cells and an increased proportion of Th2 cells (Fig. 2).

*Effects of andrographolide on the protein levels of T-bet, GATA3 and ROR- $\gamma$ t in PBMCs from UC patients.* The protein expression levels of the transcription factors, T-bet, GATA-3 and ROR- $\gamma$ t, of the T lymphocytes were measured, and the results showed that T-bet and ROR- $\gamma$ t expression was decreased (n=3); however, GATA-3 expression was increased after andrographolide treatment (n=3, Fig. 3).

*Effects of andrographolide on IL-23-treated PBMCs from healthy donors.* We next explored the effects of andrographolide pretreatment on IL-23-treated PBMCs from healthy donors. As shown in Fig. 4, IL-23 treatment significantly increased the concentrations of IFN $\gamma$ , IL-23 and IL-17A but decreased the concentrations of IL-4. IL-23 exposure caused a notable increase in the percentages of IFN $\gamma$ +CD4<sup>+</sup> cells and IL-17+CD4<sup>+</sup> but a decrease in the percentages of IL-4+CD4<sup>+</sup> cells. Additionally, IL-23 treatment significantly increased the protein levels of T-bet and ROR- $\gamma$ t but reduced GATA-3 expression. Pretreatment with andrographolide significantly rescued the effects of IL-23 on PBMCs.

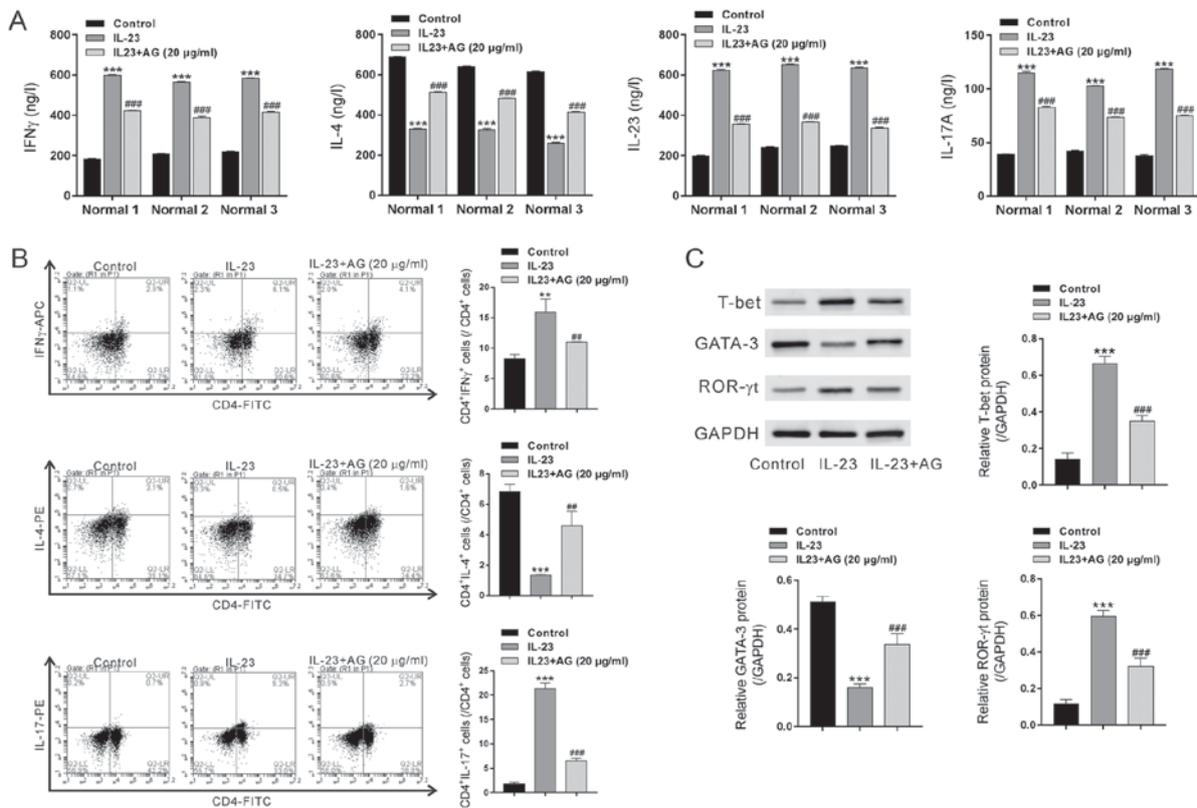


Figure 4. Effects of AG on IL-23 treated PBMCs from healthy donors. PBMCs from healthy donors were randomly divided into three groups: Control group treated with DMSO; IL-23 group treated with 50 ng/ml IL-23; and IL-23+AG group treated with 20  $\mu$ g/ml AG for 2 h and then treated with 50 ng/ml IL-23. (A) After 48 h of treatment the culture media were collected for ELISA assay. (B) The proportion of CD4<sup>+</sup>IFN $\gamma$ <sup>+</sup>, CD4<sup>+</sup>IL-4<sup>+</sup> and CD4<sup>+</sup>IL-17A<sup>+</sup> cells in CD4<sup>+</sup> cells was detected by flow cytometry. (C) The protein levels of T-bet, GATA3 and ROR- $\gamma$ t were detected by western blot analysis. \*\*\**P*<0.001 vs. the control; \*\**P*<0.01 and \*\*\**P*<0.001 vs. the IL-23 group. IL, interleukin; PBMC, peripheral blood mononuclear cells; UC, ulcerative colitis; ROR, RAR-related orphan receptor; AG, andrographolide; CD, cluster of differentiation.

## Discussion

Th1, Th2 and Th17 immune responses have been associated with the pathology of UC (5-7). AP extract (HMPL-004) was efficient for UC treatment (25,26). In the present study, we examined whether andrographolide, the main active component of AP, affected T cell responses of UC patients.

First, PBMCs isolated from UC patients were treated with various concentrations of andrographolide. The concentrations of IFN $\gamma$  (a Th1 cell produced cytokine), IL-23 and IL-17A (Th17 cell produced cytokine) in the culture medium, the percentages of Th1 and Th17 cells, and the protein levels of T-bet (a transcription factor directing Th1 lineage commitment (28)) and ROR- $\gamma$ t (Th17 lineage-specific transcription factor (29)) were significantly decreased by andrographolide treatment. These data suggested that andrographolide could inhibit Th1/Th17 response. These findings were consistent with those of a previous study of andrographolide sulfonate in mice (27). In contrast, the concentrations of IL-4 (a Th2 cell produced cytokine) in the culture medium, the percentages of Th2 cells, and the protein levels of GATA-3 (Th2 lineage-specific transcription factor (30)) were significantly increased by andrographolide treatment. The present study demonstrated that andrographolide could inhibit Th1/Th17 responses and enhance the Th2 response of PBMCs from UC patients.

Recently, increasing evidence has established correlative links between the association of IL-23/IL-17 axis and the

frequency of several human autoimmune or immune-mediated inflammatory diseases, such as Crohn disease, psoriasis and spondyloarthritis (31-33). Then, we treated PBMCs from healthy donors with IL-23 to induce a Th17 response. IL-23 treatment significantly increased Th1/Th17 responses but decreased the Th2 response, as indicated by the concentrations of specific cytokines, the percentages of Th cell subsets, and the levels of specific transcription factors. More importantly, andrographolide pretreatment rescued the effects of IL-23. These data suggested that andrographolide might effectively treat other IL-23-mediated diseases. Further studies are needed to investigate the therapeutic effects of andrographolide on such diseases.

In conclusion, the present study explored the effects of andrographolide on the Th1/Th2/Th17 responses of PBMCs from UC patients and IL-23-treated-PBMCs from healthy donors. These results suggest that andrographolide can be an effective candidate for the treatment of IL-23-mediated diseases.

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### Availability of data and materials

All data generated or analyzed during this study are included in this published article.

### Authors' contributions

QZ and PZ conceived and designed the study. QZ, JZ, XC, YF, WW, FZ and QH performed the experiments. QZ and PZ wrote the manuscript. All authors read and approved the final manuscript.

### Ethics approval and consent to participate

The present study received ethical approval from the Ethics Committee of Zhejiang Hospital and written informed consent was obtained from all participants.

### Consent for publication

Written informed consent was obtained from all participants for the publication of their data and any accompanying images.

### Competing interests

The authors declare that they have no competing interests.

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