

# Inflammatory cytokines and cells are potential markers for patients with cerebral apoplexy in intensive care unit

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**Abstract.** Cerebral apoplexy is a disease caused by obstruction of the blood circulation in the brain. Evidence has indicated that inflammatory cytokines are implicated in ischaemic cerebral apoplexy and are regarded as a general cardiovascular risk factor, which may be a possible immediate trigger, a component of the response to tissue injury and a therapeutic target. The present study investigated changes of inflammatory cytokines and cells in patients with cerebral apoplexy at the intensive care unit (ICU). The plasma concentrations of inflammatory cytokines, including tumor necrosis factor (TNF)- $\alpha$ , interleukin (IL)-4, IL-6, IL-8, IL-10, IL-1 $\beta$  and IL-17A were evaluated using ELISA. Changes in the plasma concentrations of inflammatory cells were detected by using flow cytometry. The results indicated that serum levels of TNF- $\alpha$ , IL-4, IL-8, IL-1 $\beta$  and IL-17A were upregulated in patients with cerebral apoplexy compared with those in healthy individuals, while those of IL-6 and IL-10 were downregulated. Furthermore, it was demonstrated that the plasma concentration of lymphocytes, granulocytes and mononuclear cells was decreased in patients with cerebral apoplexy in the ICU compared with that in healthy individuals. Of note, humoral as well as cellular inflammatory cytokines were evidently increased in patients with cerebral apoplexy in ICU. In conclusion, the present study provided evidence that inflammatory cytokines and inflammatory cells are upregulated, while anti-inflammatory cytokines are downregulated in patients with cerebral apoplexy in an ICU setting. These results suggest that anti-inflammatory interventions may be beneficial either in the prevention or acute treatment of patients with cerebral apoplexy.

## Introduction

Cerebral apoplexy (also known as stroke or cerebrovascular accident) is an acute medical condition whose major clinical manifestations are brain ischemia and hemorrhagic injury (1,2). Cerebral apoplexy is associated with a higher mortality and morbidity than other types of brain injury, and is mainly divided into hemorrhagic cerebral apoplexy (intracerebral hemorrhage and subarachnoid hemorrhage) and ischemic cerebral apoplexy (cerebral infarction, cerebral thrombosis) (3,4). Studies have indicated that cerebral swelling is of particular concern following cerebral apoplexy, as it is a major cause of mortality and disability in affected patients (5-7). Cerebral apoplexy, which may be one of the potential complications of cerebral infarction, is a potentially fatal condition that has serious consequences even after successful treatment of cerebral infarction (8). Therefore, it is important to identify potential targets for the treatment of cerebral apoplexy, e.g., by exploring additional underlying molecular mechanisms to expand the network of known molecular interactions and thereby provide a new horizon for the development of therapies for human cerebral apoplexy.

Cerebral apoplexy is followed by acute and prolonged inflammatory responses characterized by increased plasma inflammatory cytokine levels and leukocytes (9). Inflammation has an important role in the progression of cerebral apoplexy and has been reported to mediate damage as a potential therapeutic target in acute ischemic cerebral apoplexy (10). Evidence for the epidemiological association of inflammatory markers has accrued in patients with cerebral apoplexy (11). Current and future therapeutic strategies to target inflammation to resolve inflammatory responses in patients with cerebral apoplexy have been reviewed (12). Another review has provided an overview of the impact of systemic inflammation on the susceptibility to cerebral apoplexy and on patient outcome, outlined the potential mechanisms underlying its impact on ischemic brain injury and highlighted strategies for cerebral apoplexy prevention, therapy and prognosis (13). However, to the best of our knowledge, no previous study has performed any systemic investigations on inflammatory cytokines and cells in patients with cerebral apoplexy in an intensive care unit (ICU) setting.

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**Key words:** cerebral apoplexy, intensive care unit, inflammation, cytokines

In the present study, the serum levels of inflammatory cytokines and plasma concentrations of lymphocytes as well as the expression of inflammatory genes were investigated in patients with cerebral apoplexy in an ICU setting. The present study highlights the importance of inflammatory responses in the evaluation of the risk of cerebral apoplexy and suggests that anti-inflammatory interventions may be beneficial for the treatment of patients with cerebral apoplexy.

## Materials and methods

**Ethics statement.** This study was approved by Ethics Committee of Sichuan Provincial People's Hospital (Chengdu, China). A total of 85 patients (mean age, 35.6 years; range, 23-50 years; female:male, 41:44) with cerebral apoplexy at the ICU and 68 healthy individuals (mean age, 37.8 years; range, 24-52 years; female:male, 32:36) were recruited for analysis of inflammatory cells and factors. This study was performed in Sichuan Provincial People's Hospital between May 2014 and July 2016. Patients and healthy volunteers provided written informed consent.

**Flow cytometry.** Peripheral blood was drawn from patients with cerebral hemorrhage in the ICU and total leukocytes were extracted using a Human Leukocyte Extraction kit (cat. no. AM1933M; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Serum levels of lymphocytes, plasmacytes, neutrophils, monocytes, macrophages and mast cells in patients with cerebral apoplexy or healthy volunteers were analyzed by flow cytometry using CellTracker™ Green BODIPY™ Dye (cat. no. C2102; Thermo Fisher Scientific, Inc.) as described previously (14).

**ELISA.** In the present study, commercialized Human ELISA Kits from Thermo Fisher Scientific, Inc. were used to assess TNF- $\alpha$  (cat. no. BMS2034TEN), IL-4 (cat. no. BMS225-2TEN), IL-6 (cat. no. BMS213-2TEN), IL-8 (cat. no. KHC0083), IL-10 (cat. no. KHC0102), IL-1 $\beta$  (cat. no. KHC0019) and IL-17A (cat. no. BMS2017TEN) in the peripheral blood of patients with cerebral apoplexy at the ICU. The ELISAs were performed according to the manufacturer's protocols. The absorbance of the plates was measured at 570 nm using an ELISA reader and finally converted to the concentrations of TNF- $\alpha$ , IL-4, IL-6, IL-8, IL-10, IL-1 $\beta$  and IL-17A.

**Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) assay.** A total of 15 ml peripheral venous blood was obtained from patients with cerebral apoplexy. Human peripheral blood mononuclear cells (hPMCs) were separated by density gradient centrifugation (CsCl). Total RNA was extracted from in hPMCs cells using RNAzol, RNase-free DNase was used to digest total RNA at 37°C for 15 min, and the RNeasy kit was then applied to purify RNA and adjust its concentration to 1  $\mu$ g/ $\mu$ l. A total of 2  $\mu$ g RNA was used as a template to synthesize complementary (c)DNA by reacting it with reverse transcriptase at 37°C for 120 min, at 99°C for 4 min and at 4°C for 3 min using High Capacity cDNA Reverse Transcription kit (cat. no. 4368814; Applied Biosystems; Thermo Fisher Scientific, Inc.) according to manufacturer's protocol. Subsequently, PCR was performed

to amplify the cDNA of tumor necrosis factor (TNF)- $\alpha$ , interleukin (IL)-4, IL-6, IL-8, IL-10, IL-1 $\beta$  and IL-17A with the primers (Invitrogen; Thermo Fisher Scientific, Inc.) listed in Table I to determine the transcription level of mRNA, and  $\beta$ -actin was used as the housekeeping gene of the internal control group. The reaction mixture was as follows: 2  $\mu$ l cDNA synthesized from the RT reaction, 5 pmol of each primer, 25  $\mu$ l of SYBR Green Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) and 23  $\mu$ l water in a total volume of 50  $\mu$ l. The reaction conditions were performed as follows: 95°C for 10 min, and 35 cycles of 95°C for 20 sec and 58°C for 1 min. Subsequently, agarose electrophoresis with 1% ethidium bromide was adopted to assess the PCR-amplified products. Relative mRNA expression changes were calculated using the  $2^{-\Delta\Delta C_q}$  method (15). The results are expressed as the fold change compared with the control.

**Statistical analysis.** Values are expressed as the mean  $\pm$  standard deviation. All data were analyzed with SPSS 17.0 (IBM Corp., Armonk, NY, USA). Comparisons between two groups were performed using Student's t-test.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Characteristics of patients with cerebral apoplexy.** A total of 85 patients with cerebral apoplexy and 68 healthy individuals were recruited for the present clinical study. The mean of age was 35.6 and 37.8 years in cerebral apoplexy patients and healthy individuals, respectively. The numbers of male and female cerebral apoplexy patients and healthy individuals were approximately equal. The characteristics of the patients with cerebral apoplexy are summarized in Table II.

**Analysis of inflammatory cells in serum of patients with cerebral apoplexy.** The changes of inflammatory cells were analyzed in the serum of patients with cerebral apoplexy. It was demonstrated that the plasma concentration of lymphocytes, plasmacytes, neutrophils and monocytes was increased in the patients with cerebral apoplexy compared with that in healthy individuals (Fig. 1A-D). Furthermore, the percentage of macrophages and mast cells was increased in patients with cerebral apoplexy compared with that in healthy individuals (Fig. 1E and F). Taken together, these outcomes suggest that inflammatory cells were upregulated in patients with cerebral apoplexy compared with those in healthy individuals.

**Analysis of inflammatory cytokines in patients with cerebral apoplexy.** To detect the association between inflammation and cerebral apoplexy, the plasma levels of inflammatory factors were measured in patients with cerebral apoplexy in the ICU and compared with those in healthy volunteers as control. It was observed that the serum levels of TNF- $\alpha$ , IL-4, IL-8, IL-1 $\beta$  and IL-17A were upregulated in patients with cerebral apoplexy compared with those in healthy individuals (Fig. 2A-E). It was also demonstrated that the plasma levels of IL-6 and IL-10 were downregulated in patients with cerebral apoplexy in the ICU compared with those in healthy individuals (Fig. 2F and G). These results suggest that inflammatory cytokines in those

Table I. Sequences of primers used for polymerase chain reaction.

Gene name	Sequence
TNF- $\alpha$	Forward, 5'-GGCGATTACAGACACAACCT-3' Reverse, 5'-TCCAGACTTCCTTGAGACA-3'
IL-4	Forward, 5'-CCTCTGTTCTTCCTGCTAG-3' Reverse, 5'-CTCTGGTTGGCTTCCTTC-3'
IL-6	Forward, 5'-GTGAGGAACAAGCCAGAG-3' Reverse, 5'-TGACCAGAAGAAGGAATGC-3'
IL-8	Forward, 5'-TGGCATCTTCACTGATTCTTG-3' Reverse, 5'-TCAGTGCATAAAGACATACTCC-3'
IL-10	Forward, 5'-GCCAGCCCACCTCCACTCC-3' Reverse, 5'-TGGGCTACGTGACCTATGAC-3'
IL-1 $\beta$	Forward, 5'-GTGCTGACGCTAACTGACC-3' Reverse, 5'-GCACCCATGGCAGAAGGAGGAG-3'
IL-17A	Forward, 5'-ATGCACAGCCACCGCGACTT-3' Reverse, 5'-CTTCATGACTGCCTCCAAGTAG-3'
$\beta$ -actin	Forward, 5'-AGCCTTCTCCATGGTCGTGA-3' Reverse, 5'-CGGAGTCAACGGATTTGGTC-3'

TNF, tumor necrosis factor; IL, interleukin.

Table II. Characteristics of patients with cerebral apoplexy at the intensive care unit and the control group.

Parameter	n (%)
Patients	85 (100)
Males	43 (51)
Females	42 (49)
Mean age	35.6
Healthy controls	68 (100)
Males	33 (49)
Females	35 (51)
Mean age	37.8

patients was increased, while anti-inflammatory cytokines were decreased in patients with cerebral apoplexy.

*Analysis of inflammatory gene expression in hPMCs of patients with cerebral apoplexy.* Previous studies have suggested that the expression levels of inflammatory cytokines are correlated with the severity of patients with cerebral apoplexy (16,17). The present study analyzed the expression of inflammatory genes in hPMCs of patients with cerebral apoplexy. The results indicated that the gene expression levels of TNF- $\alpha$ , IL-4, IL-8, IL-1 $\beta$  and IL-17A were upregulated in patients with cerebral apoplexy compared with those in healthy individuals (Fig. 3A-E). The results also demonstrated that the gene expression levels of IL-6 and IL-10 were downregulated in patients with cerebral apoplexy in the ICU compared with

those in healthy individuals (Fig. 3F and G). These and the above results indicate that in cerebral apoplexy, hPMCs produce inflammatory cytokines, which are then secreted into the serum, while anti-inflammatory cytokine production is downregulated.

*Analysis of the imbalance of T helper cell type 1 (Th1)/Th2 cytokines in patients with cerebral apoplexy.* The imbalance of Th1/Th2 cytokines is a crucial indicator and has an essential role in the pathology of cerebral apoplexy in the ICU according to a previous study (18). In the present study, Th1 and Th2 cytokines were therefore quantified in patients with cerebral apoplexy in the ICU. The results demonstrated that patients with cerebral apoplexy presented with lower serum levels of Th1 cytokines compared with those in healthy individuals (Fig. 4A). However, higher serum levels of Th2 cytokines were observed compared with those healthy individuals (Fig. 4B). It was revealed that the balance of Th1/Th2 cytokines was disturbed in patients with cerebral apoplexy compared with that in healthy volunteers (Fig. 4C). Collectively, these results suggest that the balance of Th1/Th2 cytokines is disturbed in patients with cerebral apoplexy.

## Discussion

Inflammatory factors are secreted by inflammatory cells and have been reported to be associated with the morbidity and severity of cerebral apoplexy (19,20). The present study attempted to investigate changes in the inflammatory cytokines and cells in patients with cerebral apoplexy. Previous studies have analyzed the inflammation markers C-reactive protein, IL-6, IL-10, intercellular adhesion molecule-1, vascular cell adhesion molecule-1, matrix metalloproteinase-9 and cellular fibronectin, and predicted the recurrence risk of vascular disease post-cerebral apoplexy (16,21). The present study confirmed the previous results, i.e., that inflammatory cells are upregulated in patients with cerebral apoplexy compared with those in healthy individuals. Furthermore, the balance of Th1/Th2 cytokines was disturbed in patients with cerebral apoplexy, which may be utilized for the prevention or prognosis prediction of cerebral apoplexy.

Inflammatory cytokines are regarded as potential therapeutic targets in the treatment of cerebral apoplexy, cardiovascular and cerebrovascular diseases (22). A study has indicated that a polymorphism in the IL-1 receptor antagonist gene variable number tandem repeat is associated with ischemic cerebral apoplexy in a Chinese Uyghur population (23). The present results indicated that the serum levels of IL-1 $\beta$  and the associated gene expression in hPMCs were upregulated in patients with cerebral apoplexy. Sumbria *et al* (24) suggested that biologic TNF inhibitors may be re-engineered for blood-brain barrier penetration, which indicates that the immunoglobulin G-TNF receptor fusion protein may be regarded as a therapeutic agent after delayed intravenous administration in experimental cerebral apoplexy. In addition, the results in the current study suggested that IL-4 has a neurodegenerative role in the lesion-protection process. In acute cerebral apoplexy, TNF- $\alpha$  and IL-8 were increased and modulation of these cytokines by antiplatelet agents was demonstrated to be beneficial in affected patients (25). In

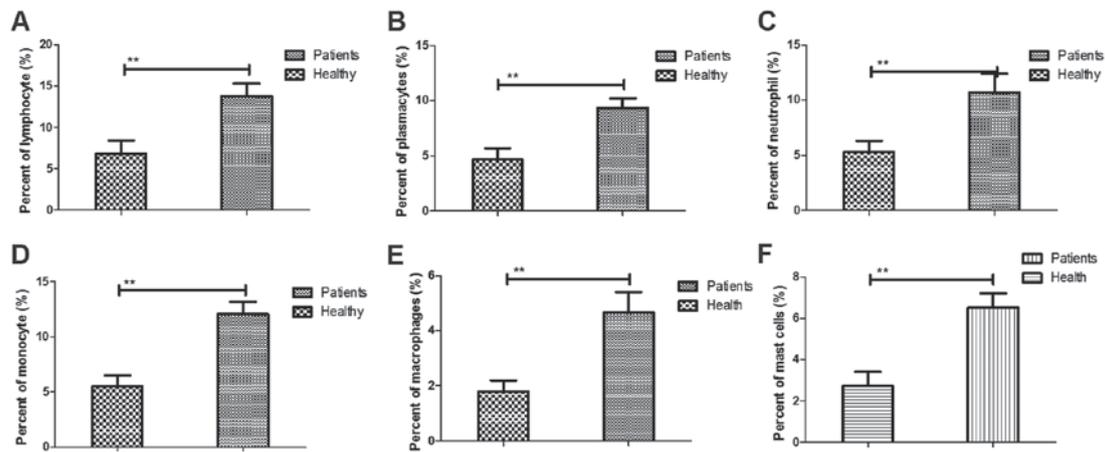


Figure 1. Analysis of inflammatory cells in serum of patients with cerebral apoplexy. Plasma levels of (A) lymphocytes, (B) plasmacytes, (C) neutrophils and (D) monocytes were increased in the patients with cerebral apoplexy compared with those in healthy individuals. (E and F) Patients with cerebral apoplexy had a higher percentage of (E) macrophages and (F) mast cells than healthy individuals. \*\*P<0.01.

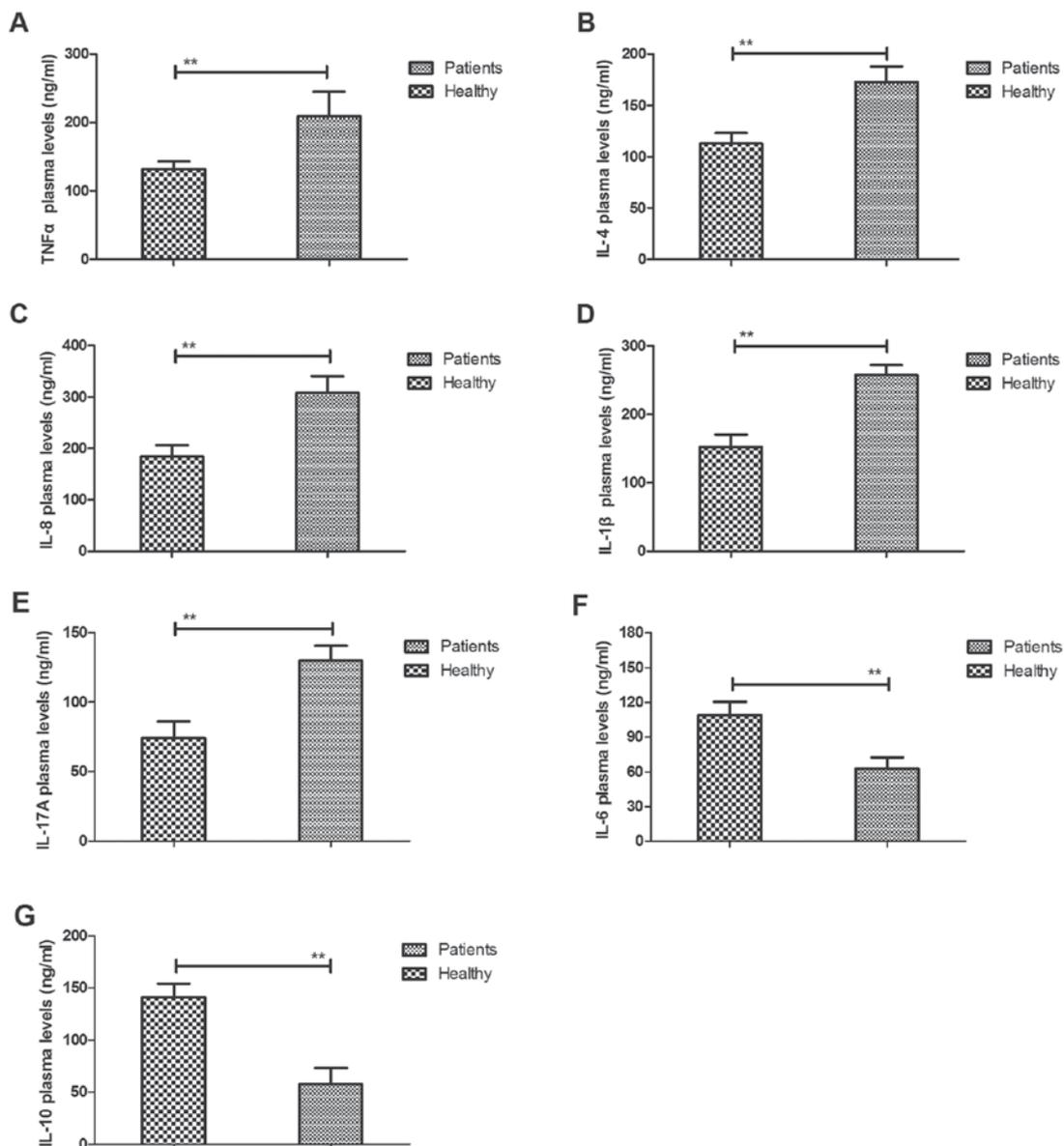


Figure 2. Analysis of inflammatory factors in patients with cerebral apoplexy. (A-E) Patients with cerebral apoplexy had higher serum levels of (A) TNF- $\alpha$ , (B) IL-4, (C) IL-8, (D) IL-1 $\beta$  and (E) IL-17A than healthy individuals. (F and G) Patients with cerebral apoplexy presented with lower serum levels of (F) IL-6 and (G) IL-10 than healthy individuals. \*\*P<0.01. TNF, tumor necrosis factor; IL, interleukin.

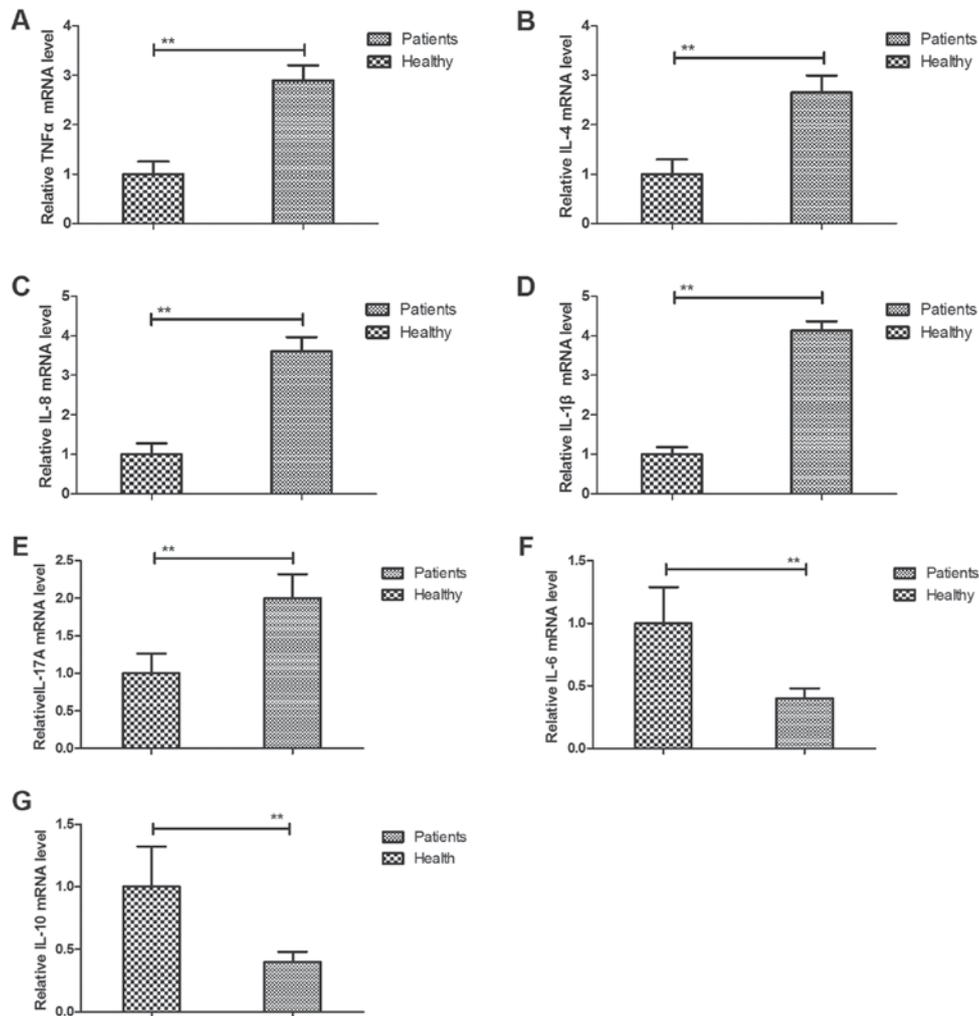


Figure 3. Analysis of the expression of inflammatory genes in hPMCs from patients with cerebral apoplexy. (A-E) Patients with cerebral apoplexy had higher gene expression levels of (A) TNF- $\alpha$ , (B) IL-4, (C) IL-8, (D) IL-1 $\beta$ , (E) IL-17A in hPMCs than in healthy individuals (F and G) Patients with cerebral apoplexy had lower gene expression levels (F) IL-6 (G) and IL-10 than healthy individuals. \*\*P<0.01. TNF, tumor necrosis factor; IL, interleukin.

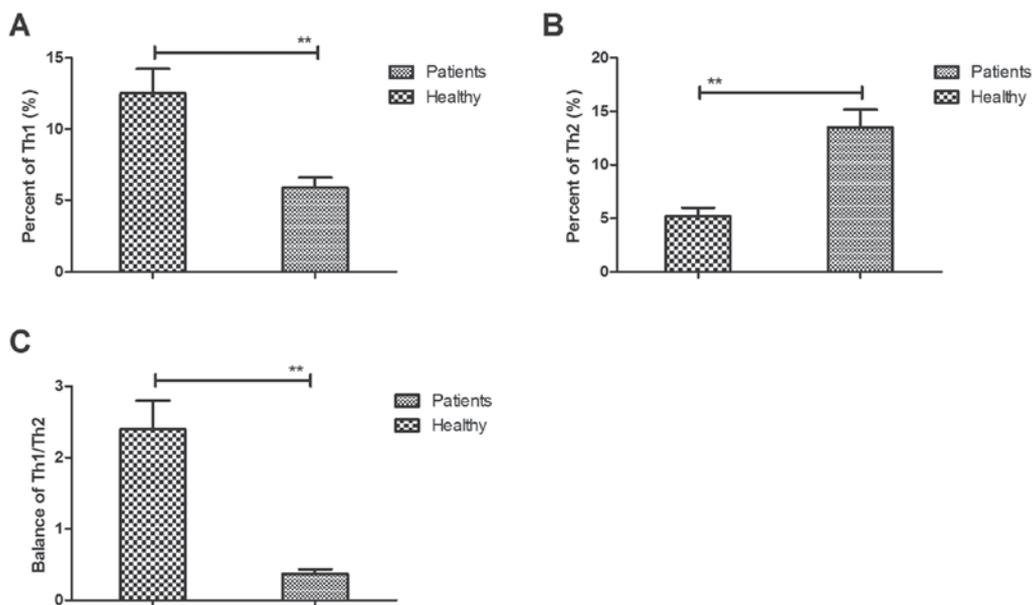


Figure 4. Analysis of the imbalance of Th1/Th2 cytokines in patients with cerebral apoplexy. (A) Patients with cerebral apoplexy presented with lower serum levels of Th1 cytokines compared with those in healthy individuals. (B) Patients with cerebral apoplexy had higher serum levels of Th2 cytokines compared with those in healthy individuals. (C) Patients with cerebral apoplexy had an imbalance of Th1/Th2 cytokines compared with those in healthy volunteers. \*\*P<0.01. Th1, type 1 T helper cell.

addition, immunomodulatory effects of bone marrow stromal cells on IL-17-mediated ischemic cerebral apoplexy were identified in the pathophysiological process of cerebral infarction, and these results may help to understand the roles of cytokines in cerebral infarction (26). The present study indicated that the serum levels of TNF- $\alpha$ , IL-4, IL-8, IL-1 $\beta$  and IL-17A were upregulated in patients with cerebral apoplexy compared with those in healthy individuals, which may be utilized as an approach for the treatment of patients with cerebral apoplexy.

IL-6 is a predictive biomarker for cerebral apoplexy-associated infection and risk of mortality in the elderly after ischemic cerebral apoplexy (27). The present results indicated that the serum levels of IL-6 were lower in patients with cerebral apoplexy. A previous study indicated that the anti-inflammatory IL-10 is upregulated in both hemispheres after experimental ischemic cerebral apoplexy (28). The results of the present study indicate that the gene expression levels in hPMCs and the plasma concentration of IL-6 and IL-10 and were downregulated in patients with cerebral apoplexy in the ICU compared with those in healthy individuals. Attenuating inflammatory responses has been reported to be beneficial in the treatment of cerebral apoplexy (29,30).

In conclusion, the present results indicate that the gene expression and secretion of inflammatory cytokines is upregulated in patients with cerebral apoplexy. Of note, inflammatory cells were upregulated and the balance of Th1/Th2 cytokines was disturbed in patients with cerebral apoplexy compared with that in healthy individuals, which may provide a potential anti-inflammation treatment approach for patients with cerebral apoplexy.

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#### Availability of data and materials

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

#### Authors' contributions

JW performed the majority of the experiments. ZH, SY, CL, HY and DW performed the experiments and analyzed the data. FG designed experiments in the current study.

#### Ethical approval and consent to participate

This study was approved by Ethics Committee of Sichuan Provincial People's Hospital (Chengdu, China). All patients and healthy volunteers provided written informed consent.

#### Consent for publication

All patients have provided written informed consent for publication.

#### Competing interests

The authors declare that they have no competing interests.

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