Original Article

CD4+ T cells from behcet patients produce high levels of IL-17

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Abstract

Purpose: To investigate the role of interleukin (IL)-17-producing CD4+ T cells in Behcet disease (BD).

Methods: Blood samples were drawn from eight BD patients with active uveitis, eight BD patients with inactive uveitis and eight normal controls, respectively.PBMCs were prepared from heparinized blood by Ficoll-Hypaque density-gradient centrifugation. Peripheral CD4+ T cells were purified by Human CD4 Microbeads (MACS). The purity rate of CD4+ T cells was detected using flow cytometry. Purified CD4+ T cells were stimulated with or without anti-CD3 and anti-CD28 antibodies in the presence or absence of recombinant-IL-23 (rIL-23) or recombinant-IL-12 (rIL-12) for 72 hours. The concentrations of IL-17, IFN-γ and IL-4 in the collected supernatants from CD4+ T cells were measured using a Duoset ELISA Development kit.

Results: The results showed that the levels of IL-17 and IFN-γ observed in active BD patients were significantly higher as compared with those in inactive patients and normal controls. There was no significant difference concerning IL-4 production between BD patients and normal controls. rIL-23 significantly augmented the production of IL-17 by CD4+ T cells from both BD patients and normal controls. Both rIL-23 and rIL-12 could increase IFN-γ production by CD4+ T cells from BD patients and normal controls. Moreover, the effect of rIL-12 was more robust compared with that of rIL-23. Neither rIL-23 nor rIL-12 exerted any effect on IL-4 production.

Conclusion: rIL-23 can promote the production of IL-17 by CD4+ T cells in BD patients. The upregulated IL-17 levels may be related with the intraocular inflammation of Behcet patients. (***Eye Science 2011; 26: 65-69***)

Keywords: behcet disease; IL-23; IL-17; IFN-γ

Introduction

Behcet disease (BD) is a chronic, systemic, relapsing inflammatory disease mainly featured as four major manifestations including recurrent uveitis, oral aphthae, genital ulcers, and skin lesions[1]. Although the pathogenesis of BD is still unclear, several reports have suggested that autoimmunity may play a crucial role[2,3]. In view of the confirmed role of autoimmune mechanism in BD rather than other uveitis, therefore BD patients were involved in this study as research objects. Previous studies suggested that BD is predominated by a Th1 type immune response.Increased Th1-associated cytokines such as IFN-γ, IL-12 and tumor necrosis factor (TNF)-α have been documented in BD patients[4,5]. However, treatment with IFN-α and anti-TNF-α could only partially prevent the recurrence of BD[6]. Therefore, other factors seem to be involved in the development of BD.

Recent studies have found a new subset of CD4+ T helper (Th) cells that selectively produce IL-17 and play a critical role in the pathogenesis of autoimmune and chronic inflammatory disorders, which has

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been defined as Th17 cells[7]. IL-17 is an important proinflammatory cytokine and is upregulated in certain inflammatory diseases such as rheumatoid arthritis, inflammatory bowel disease (IBD), multiple sclerosis[8-10]. IL-23, a novel member of the IL-12 family, has been shown to play a pivotal role in the development and maintenance of autoimmune diseases[11,12]. Although several cytokines, such as IL-23 and IL-6, have been demonstrated that can induce the differentiation of Th17 cells. Accumulated evidence suggests that IL-23 is the most important effector involved in the maintenance of Th17 cells. Therefore, our study was designed to investigate the role of IL-17-producing $CD4^+$ T cells in the development of BD and the effect of IL-23 in this subset cells in BD.

Materials and methods

Patients' information

Sixteen BD patients referred to our hospital from June 2009 to October 2010, aged 35 years old in average and eight healthy individuals, aged 37 years old in average, were included in this study. The diagnosis of BD was based on the diagnostic criteria de signed by the International Study Group for BD[11]. Eight BD patients showed active recurrent intraocular inflammation.These patients had not received any immunosuppressive agents for over two months, who discontinued immunosuppressive treatment for personal matters in local hospitals and resumed this therapy after diagnosis in our hospital. Blood samples were collected from all BD patients with active uveitis and normal controls. These patients showed active recurrent intraocular inflammation evidenced by dust keratic precipitates (100%), flare and cells in the anterior chamber (100%), vitreous cells (50%) and retinal vasculitis observed clinically or disclosed by fluorescein angiography (100%). The extraocular manifestations were recurrent oral aphth ous lesions (100%), multiform skin lesions (62.5%), recurrent genital ulcers (37.5%) and arthritis (25%). Eight patients showed inactive intraocular inflammation after receiving prednisone combined with cyclosporin A treatment for more than three months. Blood samples were obtained from BD patients without active uveitis at least 2 months after the termination of all medications. The experimental design was described in detail below. All procedures followed the tenets of the Declaration of Helsinki, and informed consents were obtained from all BD patients and normal controls.

Antibodies and agents

Human CD4 Microbeads (MACS) and MS purified column were purchased from Miltenyi Biotec (Germany). Anti-CD3 mAb, anti-CD28 mAb and anti-CD4-PE mAb were purchased from eBiosciece (USA). rIL-23, rIL-12, IL-17 Duoset ELISA kit, IFN-γ Duoset ELISA kit and IL-4 Duoset ELISA kit were obtained from R&D Systems (USA). Complete RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS) (Hy-Clone Laboratories, Logan, UT), 0.1% 2-ME, 100 U/mL penicillin and 100 lg/mL streptomycin (GIBCO, USA).

Methods

PBMCs were prepared from heparinized blood by Ficoll-Hypaque density-gradient centrifugation. Peripheral $CD4^+$ T cells were purified by Human CD4 Microbeads according to the manufacturer's instructions. $CD4^+$ T cells were stimulated with or without anti-CD3 (OKT_3, 5 μg/ml) and anti-CD28 antibodies (1 μg/ml) in the presence or absence of rIL-23 (50 ng/ml) or rhIL-12 (1 ng/ml) for 72 hours at a concentration of 1×10^6 cells/ml. The concentrations of IL-17, IFN-γ and IL-4 in the collected supernatants of $CD4^+$ T cells were measured using a Duoset ELISA Development kit with detection limit of 15 pg/ml.

Statistical analysis

All data were expressed as mean ± SD. Statistical analysis was performed using Student t-test. A level of $P<0.05$ was considered statistically significant.

Results

The purification of $CD4^+$ T cells

Purified $CD4^+$ T cells by MACS were stained using CD 4-PE mAb. The purification rate of $CD4^+$ T cells was detected by flow cytometry (FACSCalibur, BD Bioscience, Mountain View, USA).The results showed that the purification rate of $CD4^+$ T cells was over 98% every time (Figure 1), suggesting that the purified $CD4^+$ T cells can be used for cell culture.

The expression of IL-17 in the supernatants of $CD4^+$ T cells from all subjects

It has been demonstrated that IL-17-producing $CD4^+$ T helper cells (Th17 cells) play a crucial role

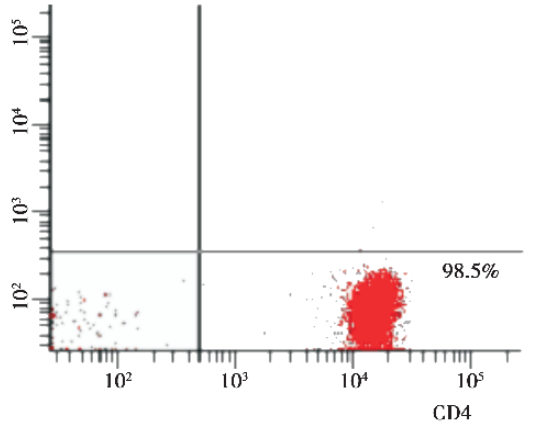

Figure.1 $CD4^+$ T cells were purified by MACS. The results showed that the purification rate of $CD4^+$ T cells was 98.5%.

in the development and maintenance of autoimmune diseases.IL-17 is the pivotal factor of Th17 subset cells.Therefore, this study investigated the IL-17 producing $CD4^+$ T cells in BD patients. We found that IL-17 levels in the supernatants of un-stimulated $CD4^+$ T cells were undetectable or at a low level in BD patients and normal controls. The production of IL-17 was strikingly increased upon stimulation with anti-CD3 and anti-CD28 antibodies both in BD patients and normal controls.Anti-CD3 and anti-CD28-stimulated $CD4^+$ T cells produced a larger amount of IL-17 in BD patients with active uveitis as compared with that in BD patients without active uveitis ($P<0.001$) and normal controls ($P<0.001$) (Figure 2).

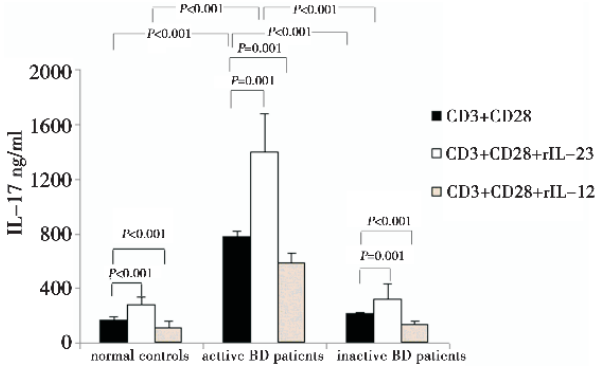

Figure.2 The production of IL-17 by activated $CD4^+$ T cells from BD patients with or without active uveitis and normal controls in the presence or absence of rIL-23 and rIL-12. Isolated $CD4^+$ T cells were cultured with anti-CD3 (5 mg/ml) and anti-CD28 antibodies (1 mg/ml) in the presence or absence of rIL-23 (50 ng/ml)and rIL-12 (1 ng/ml)for 72 hours, the levels of IL-17 in the supernatants of cultured cells were detected with ELISA kit.

The production of IFN-γ and IL-4 in the supernatants of $CD4^+$ T cells from all subjects

IL-17 is the most important effector of Th17 subset cells, thus IFN-γ and IL-4 are the key cytokines of classic Th1 and Th2 helper subset cells respectively. An increased level of IL-17 in $CD4^+$ T cells from active BD patients was noted in above study. Therefore we further investigated the levels of classic IFN-γ and IL-4 in BD patients. IFN-γ and IL-4 were undetectable or detected at a low level in the supernatants of inactivated $CD4^+$ T cells from both BD patients and normal controls.Upon stimulation with anti-CD3 and anti-CD28 antibodies, the production of IFN-γ by $CD4^+$ T cells was markedly increased both in BD patients and normal controls. Such increases were significantly higher in BD patients with active uveitis than those in BD patients without active uveitis and normal controls (Figure 3). The amount of IL-4 yielded by activated CD4+ T cells has no significant difference among active BD patients, inactive BD patients and normal controls (Table 1).

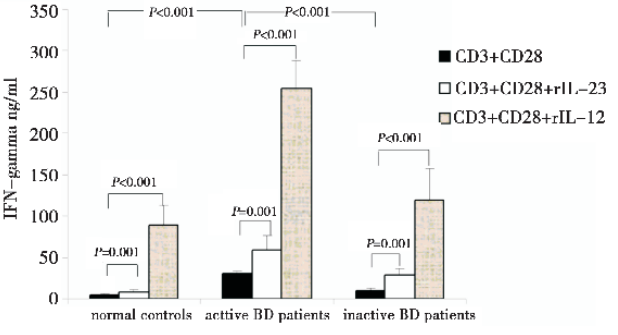

Figure.3 The production of IFN-γ by activated $CD4^+$ T cells from BD patients with or without active uveitis and normal controls in the presence or absence of rIL-23 and rIL-12. Isolated $CD4^+$ T cells were cultured with anti-CD3 (5 mg/ml) and anti-CD28 antibodies (1 mg/ml) in the presence or absence of rIL-23 (50 ng/ml)and rIL-12(1 ng/ml) for 72 hours, the levels of IFN-γ in the supernatants of cultured cells were detected with ELISA kit.

Effects of IL-23 and IL-12 on the production of IL-17 by $CD4^+$ T cells

In above study, we have demonstrated that the production of IL-17 by $CD4^+$ T cells is upregulated in active BD patients. Accumulated evidence showed that IL-23 is the most important factor in the differ-

Table 1 The production of IL-4 by activated CD4+ T cells in the presence or absence of rIL-23 and rIL-12(mean±SD)(pg/ml)

Groups	CD3+CD28	CD3+CD28+rIL-23	CD3+CD28+rIL-12
normal controls A	12.93±18.91	10.56±14.54	13.10±15.66
inactive BD patients B	8.34±11.43	8.27±11.33	12.81±15.12
active BD patients C	18.47±11.43	12.72±20.05	12.81±15.12

Note: A vs B $P>0.05$, A vs C $P>0.05$, B vs C $P>0.05$

entiation and maintenance of IL-17-producing CD4+ T cells. Therefore, we further investigated the effect of IL-23 on IL-17-producing CD4+ T cells in BD patients. We utilized the stimulator of classic Th1 cells, IL-12, as a control. Purified CD4+ T cells were cultured with anti-CD3 and anti-CD28 antibodies in the presence or absence of rIL-23 (50 ng/ml) or rIL-12(1 ng/ml)for 72 hours. The results showed that rIL-23 significantly promoted activated CD4+ T cells to produce IL-17, while rIL-12 inhibited its production.In addition, activated CD4+ T cells from BD patients with active uveitis produced more IL-17 than those from BD patients with inactive uveitis and normal controls in the presence of rIL-23 (Figure 2). We further detected the effect of IL-23 on the production of Th1 cytokine-IFN-γ and Th1 cytokine-IL-4 by CD4+ T cells. Our results showed that rIL-23 could also increase IFN-γ production by activated CD4+ T cells and the effect of rIL-12 was more sig nificant than that of rIL-23 (Figure 3). However, both rIL-23 and rIL-12 exerted no effect on IL-4 production (Table 1).

Discussion

The present study showed that CD4+ T cells from active BD patients produced excessive IL-17 and IFN-γ. However, there was no significant difference on the production of IL-4 among BD patients with or without active uveitis and normal controls. It was also demonstrated that IL-23 could promote IL-17 production by CD4+ T cells, while IL-12 inhibited its production. In addition, IL-23 stimulated a considerably higher production of IL-17 by activated CD4+ T cells of BD patients with active uveitis than those of BD patients with inactive uveitis and normal controls. Both IL-23 and IL-12 promoted IFN-γ production by CD4+ T cells, IL-12 possessed a stronger stimulatory effect on IFN-γ production. IL-23 and IL-12 had no effect on IL-4 production. All these findings suggest that IL-23 may induce CD4+ T cells from active BD patients to produce excessive IL-17, which mediates the development and maintenance of BD.

In recent studies using some autoimmune disease models such as experiment autoimmune encephalomyelitis (EAE) and collagen-induced arthritis (CIA), IL-17-producing CD4+ T cells are highly pathogenic[13,14]. Several reports also found that IL-17 level is elevated in some human diseases such as multiple sclerosis, arthritis, inflammatory bowel disease and Vogt-Koyanagi-Harada disease[8–10,15]. CD4+ T helper cells play an essential role in the development of autoimmune diseases. Traditionally, T helper cells were divided into Th1 and Th2 cells. Previous studies showed that the production of Th1 cytokines is upregulated in some autoimmune disease. But autoimmune diseases were easily to be induced in animal models, once Th1 cytokines being blocked, suggesting that Th1 cells and cytokines are not necessarily involved in the development of these diseases. Recent studies demonstrated that IL-23 induces a highly pathogenic IL-17-producing CD4+ T cells-Th17 cells, which mediates the development of some autoimmune diseases. Therefore, we explored the role of IL-17-producing CD4+ T cells in BD. In this study, we purified the CD4+ T cells and analyzed the production of IL-17 by CD4+ T cells from BD patients and normal controls. Our results showed a significantly elevated IL-17 production by CD4+ T cells in active BD patients. Therefore these excessive IL-17-producing CD4+ T cells in BD patients may be actively associated with the activity of ocular inflammation in active BD patients.

As IFN-γ, Th1 cytokine, was considered as an important mediator participating in the development of autoimmune diseases, previous study was performed to detect the expression of Th1 cytokine, and also analyze the expression of IL-4, Th2 cy-

tokine. Our results showed the amount of of IFN-γ produced by $CD4^+$ T cells from BD patients with active uveitis was significantly upregulated. However, the production of IL-4 by $CD4^+$ T cells had no obvious change. As IFN-γ had transitory effect in autoimmune diseases, it may play a role in the early-stage development of BD. IL-4 had no effect on BD.

It has been demonstrated that IL-23 plays an essential role in the differentiation and maintenance of Th17 cells[16]. Therefore, we further investigated the effect of IL-23 on IL-17-producing $CD4^+$ T cells in BD patients, using IL-12, a stimulator of Th1 cells, as a control. Our findings showed that IL-23 could induce the production of IL-17 by $CD4^+$ T cells, while IL-12 exerted an inhibitory effect upon IL-17 production.All these results suggested that IL-23 may be the key mediator for the excessive IL-17-producing $CD4^+$ T cells in active BD patients.

In conclusion, our study showed an elevated production of IL-17 and IFN-γ by $CD4^+$ T cells. IL-23 increased the production of IL-17 by $CD4^+$ T cells and IL-12 decreased the frequencies of IL-17-pro ducing $CD4^+$ T cells in BD patients with active uv eitis. All these results suggest that the IL-23/IL-17 pathway is associated with active uveitis in BD patients and that this pathway may be involved in the pathogenesis of BD. Corresponding studies on the manipulation of this pathway may warrant its role and also provide a strategy for the treatment of BD.

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