IFN-α blocks IL-17 production by peripheral blood mononuclear cells in Behçet’s disease

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Abstract

Objectives. IFN-α has been used to treat patients with Behçet’s disease (BD). Recent studies have implicated the IL-23/Th-17 pathway in the pathogenesis of BD. In this study, we investigated whether IFN-α could affect this pathway.

Methods. Peripheral blood mononuclear cells (PBMCs) obtained from patients with active BD and controls were cultured alone or with IFN-α and the levels of IL-17 and IL-10 in the supernatants were measured by ELISA. Similar experiments were performed with isolated CD4⁺ T cells from controls. The levels of phosphorylated STAT1 (p-STAT1), p-STAT2, p-STAT3 and p-STAT5 in CD4⁺ T cells from controls cultured with or without IFN-α were also evaluated by ELISA. Furthermore, an experiment using anti-IL-10 was performed to examine underlying mechanisms of action of IFN-α.

Results. Significantly higher levels of IL-17 and IL-10 were observed in the supernatants of PBMCs from BD patients as compared with controls. IFN-α significantly decreased IL-17 production by PBMCs from both patients and controls. On the other hand, IFN-α increased IL-10 production by PBMCs from patients and controls. Similar findings were obtained when using CD4⁺ T cells from controls, IFN-α significantly increased p-STAT2 expression in control CD4⁺ T cells. Anti-IL-10 antibody was able to neutralize the inhibitory effect of IFN-α on IL-17 by 35% as compared with controls.

Conclusions. In vitro experiments showed that IFN-α could inhibit IL-17 expression and increased IL-10 production by PBMCs and CD4⁺ T cells. The inhibitory role of IFN-α on IL-17 was partly mediated by IL-10. IFN-α activity was mediated via STAT2 phosphorylation.

Key words: Behçet’s disease, IFN-α, IL-17, IL-10.

Introduction

Behçet’s disease (BD) is one of the common uveitis entities in the countries along the old silk road, such as Japan, Turkey, Israel and China [1, 2]. It is characterized by recurrent oral aphthae, genital ulcers, skin lesions, uveitis and often leads to visual loss [3]. Since the mid-1980s, recombinant IFN-α has been used in the treatment of BD [4, 5] with very promising results [6]. A Phase III clinical trial comparing IFN-α and CSA in the treatment of BD is ongoing (Clinical trials.gov identifier NCT00167583). The mechanisms whereby IFN-α exerts its immunomodulatory effects in BD are, however, not completely understood and were the subject of the study presented here.

We recently provided strong evidence implicating the IL-23/Th-17 pathway in the pathogenesis of the active uveitis observed in the course of BD [7] and hypothesized that the effective treatment of BD patients with IFN-α might be mediated via this pathway. Recent studies have shown that Type I IFNs including IFN-α could inhibit IL-17 production by peripheral blood mononuclear cells (PBMCs) [8], and that it was able to stimulate IL-10 production by activated CD4⁺ T cells [9]. IL-10 is a cytokine of T-regulatory Type 1 cells and could inhibit the production of IFN-γ [10] and antigen presentation [11]. It has also been reported that IL-10 could restrain the pathogenic role of Th17 cells [12]. However, it is not yet known whether IFN-α could inhibit IL-17
production directly or indirectly through promoting IL-10 expression. This study was designed to investigate whether IFN-α could inhibit IL-17 production by PBMCs in BD and which mechanism was involved in this regulation. Our results showed that IFN-α could significantly inhibit IL-17 production in association with increased IL-10 production by PBMCs from both BD patients and controls and similar findings were observed using isolated CD4+ T cells from controls. Interestingly, we found that the inhibitory role of IFN-α on IL-17 was partially mediated by IL-10. Our results also showed that recombinant human interferon (rhIFN-α) could up-regulate the level of phosphorylated STAT2 (p-STAT2) in CD4+ T cells.

Materials and methods

Subjects

Eight patients with active BD (5 men and 3 women) with an average age of 36.5 years and 23 healthy controls (17 men and 6 women) with an average age of 35.4 years were included in this study between September 2008 and April 2009. The diagnosis of BD was made according to the criteria of the International Study Group for BD [13]. All of these patients showed recurrent uveitis and had active uveitis as evidenced by dust keratic precipitates (100%), flare and cells in the anterior chamber (100%), vitreous cells (37.5%) and retinal vasculitis observed clinically or disclosed by fluorescein angiography (100%). The extraocular manifestations were recurrent oral aphthous ulcers (100%), multif orm skin lesions (50%), recurrent genital ulcers (37.5%) and arthritis (37.5%). No immuno- suppressive agents were used for these patients at least 1 week before visiting us and before blood sampling. Written informed consent was obtained from all patients and healthy controls. This project followed the tenets of the Declaration of Helsinki and was approved by the ethics committee of Chongqing Medical University.

Cell isolation and culture

PBMCs were isolated from blood by Ficoll–Hypaque density-gradient centrifugation. Peripheral CD4+ T cells of controls were isolated from PBMCs by human CD4 microbeads (Miltenyi Biotec, Palo Alto, CA, USA) according to the manufacturer’s instructions. The purity of CD4+ T cells was detected using FACS Calibur and CellQuest software (BD Company, Franklin Lakes, NJ, USA) after staining with anti-human CD4 antibodies. The result showed that the purity was >90% in each experiment. PBMCs were cultured with or without rhIFN-α 2a (PBL Biomedical Lab, Piscataway, NJ, USA), rhIL-10 (R&D Systems, Minneapolis, MN, USA) or anti-IL-10 (R&D Systems) at a concentration of 1 × 10^6 cells/ml in combination with anti-CD3 (5 μg/ml) and anti-CD28 antibodies (1 μg/ml) (eBioscience, San Diego, CA, USA) for 72h. CD4+ T cells (1 × 10^6 cells/ml) were cultured with or without rhIFN-α 2a (1 μg/ml) in combination with anti-CD3 (5 μg/ml) and anti-CD28 antibodies (1 μg/ml) (Miltenyi Biotec) for 72h. Supernatants from these cell cultures were used for detecting the concentration of IL-17 and IL-10. In order to detect the levels of p-STAT, CD4+ T cells (1 × 10^6 cells/ml) were cultured with rhIFN-α 2a (1 μg/ml) for 15 min in complete Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, Grand Island, NY, USA) and then used for protein isolation or ELISA.

Protein isolation and quantitation

PBMC cultures from BD patients as compared with controls. No significant difference was observed concerning the inhibitory rate of rhIFN-α 2a on IL-17 production between patients and controls. However, rhIFN-α 2a induced a significantly higher IL-10 level in PBMC cultures from BD patients as compared with controls. No significant difference was observed concerning the IL-10 level in the supernatants of PBMCs from BD patients and controls incubated in the absence of IFN-α (Fig. 1C). Experiments using isolated CD4+ T cells from healthy controls showed that rhIFN-α 2a also significantly inhibited IL-17 production and promoted IL-10 production (Fig. 2). IL-17 and IL-10 levels were much higher in experiments using isolated CD4+ T cells from healthy individuals as compared with those using PBMCs.

Results

rhIFN-α 2a could inhibit IL-17, but increased IL-10 production by PBMCs or CD4+ T cells from BD patients and controls

Stimulation of PBMCs with anti-CD3 and anti-CD28 antibodies resulted in the production of IL-17 (Fig. 1A). IL-17 levels in the cell culture supernatants of PBMCs from BD patients were significantly higher than those obtained from controls. Addition of rhIFN-α 2a to this cell culture model revealed a significantly decreased IL-17 production and an increase in the IL-10 production (both P < 0.001) (Fig. 1A–C). There was no significant difference concerning the inhibitory rate of rhIFN-α 2a on IL-17 production between patients and controls. However, rhIFN-α 2a induced a significantly higher IL-10 level in PBMC cultures from BD patients as compared with controls. No significant difference was observed concerning the IL-10 level in the supernatants of PBMCs between BD patients and controls incubated in the absence of IFN-α (Fig. 1C). Experiments using isolated CD4+ T cells from healthy controls showed that rhIFN-α 2a also significantly inhibited IL-17 production and promoted IL-10 production (Fig. 2). IL-17 and IL-10 levels were much higher in experiments using isolated CD4+ T cells from healthy individuals as compared with those using PBMCs.
Anti-IL-10 antibody partially neutralized the inhibitory effect of rhIFN-α 2a on IL-17 production by PBMCs from active Behçet’s patients. A significant decrease of IL17 production by PBMCs was observed in the presence of IFN-α, which was associated with an elevated IL-10 level (P = 0.001, 0.026, respectively). To investigate the role of IL-10, we incubated PBMCs in the presence of IFN-α and an antibody against IL-10. In the presence of an antibody against IL-10, the inhibitory effect of rhIFN-α 2a on IL-17 production (P = 0.042) was partially blocked in a dose-dependent manner (Fig. 3A and B). However, this antibody failed to completely reverse the role of rhIFN-α 2a even at a concentration as high as 20 μg/ml. rhIFN-α 2a could up-regulate the level of p-STAT2 in CD4+ T cells from controls.

We subsequently examined which STAT was involved in the aforementioned inhibitory effect of rhIFN-α 2a on IL-17 production using PBMCs from healthy controls. The results showed a significantly increased level of p-STAT2 in the CD4+ T cells after stimulation with rhIFN-α 2a (P = 0.034; Fig. 4). A basal level of p-STAT1, p-STAT3 and p-STAT5 was found in CD4+ T cells cultured alone. Expression of p-STAT1, p-STAT3 and p-STAT5 was increased, but did not reach a significant difference upon exposure to rhIFN-α 2a (data not shown).

Discussion

In the present study, we found that IFN-α 2a could inhibit IL-17 expression and increased IL-10 production by PBMCs from BD patients and controls. Further experiments using isolated CD4+ T cells from healthy controls showed similar results and furthermore showed that an
IFN-α is mainly produced by fibroblasts. Both IFN-α and IFN-β molecules share the same receptor consisting of a dimer of IFNAR1 and IFNAR2. The different IFN-α/β subtypes interact with different sites on the receptor explaining the intensity of the biological effects of the various Type I IFNs. Receptor density on various cell types provides further explanation for the diversity of responses [15]. Upon binding, signal transduction is mediated via a STAT1–STAT2–IRF9 complex. IFN-γ belongs to the Type II IFNs and is produced by activated T cells and NK cells and uses other receptors and signal transduction pathways than the Type I IFNs.

Our results concerning the mechanism of action of IFN-α are consistent with those reported earlier in patients with other autoimmune diseases [16]. Zhang et al. [16] showed that IFN-α inhibited the differentiation of Th17 cells and production of IL-17 through up-regulating TLR7 expression in dendritic cells from patients with multiple sclerosis. It was reported that treatment with IFN-α could induce an increased frequency of CD4+CD25(high) T cells in PBMCs from MS patients [17]. However, Seya and coworkers [18] found that IFN-α from poly I:C-stimulated dendritic cells (DCs) inhibited the expansion of CD4+CD25(FoxP3) Treg cells. Whether a down-regulatory effect of IFN-α on IL-17 production, by T cells, as observed in BD, is associated with an increased CD4+CD25(FoxP3) Tregs needs to be further studied. IFN-α has been successfully used for the treatment of BD [19] and hepatitis [20]. Earlier findings from our group showed that the levels of IL-23 and IL-17 were elevated in BD patients with active uveitis and suggested that the IL-23/IL-17 pathway is associated with the active intraocular inflammation in BD patients [7]. These findings may explain the success of IFN-α treatment in BD and suggest that it may also be used for other autoimmune diseases mediated by IL-17.

IL-10 is an anti-inflammatory cytokine involved in the down-regulation of autoimmune disease [12]. In our BD patients, we observed a stronger induction of IL-10 as compared with controls following incubation of PBMCs with IFN-α, a finding that has also been reported earlier

**Fig. 3** Anti-IL-10 antibody could partially neutralize the inhibitory effect of rhIFN-α 2a on IL-17 production by PBMCs from BD patients (n = 8). PBMCs from BD patients were cultured with or without rhIFN-α 2a, rhIL-10 or anti-IL-10 antibody in combination with anti-CD3 and anti-CD28 antibodies for 72 h. Concentrations of IL-17 in the supernatants were detected using ELISA. (A) Anti-IL-10 antibody at a concentration of 10 μg/ml could partially neutralize inhibitory role of rhIFN-α 2a on IL-17 production. (B) Dose-related effect of anti-IL-10 antibody. Bla means PBMCs were cultured without rhIFN-α. Data are expressed as mean (s.o.).

**Fig. 4** The levels of p-STAT2 in CD4+ T cells stimulated with IFN-α from controls (n = 5). CD4+ T cells from controls were cultured with or without rhIFN-α 2a for 15 min. The protein levels of p-STAT2 in these cells were evaluated. Bla means CD4+ T cells were cultured without rhIFN-α. Data are expressed as mean (s.o.), RFUs: relative fluorescence units.

anti-IL-10 antibody was able to partially reverse the inhibitory effect of IFN-α 2a on IL-17 expression. IFN-α significantly up-regulated the level of p-STAT2 in CD4+ T cells. Our study suggests that the inhibitory effect of IFN-α on IL-17 production may be partially mediated by IL-10 and indicates that IFN-α exerts its possible immunoregulatory role by signal transduction involving STAT2.

The IFNs are a family of proteins with an important role in protection against viral infections, tumour growth, inflammation and angiogenesis [14]. The IFNs are divided into three classes whereby the first two are most important from an immunological point of view. In humans, the main Type I IFNs consist of IFN-α (with 13 subtypes) and IFN-β (1 subtype). IFN-α is produced in large amounts by plasmacytoid dendritic cells, whereas IFN-β is mainly produced by fibroblasts. Both IFN-α and IFN-β molecules share the same receptor consisting of a
by others [21–23]. This result seems to suggest that a latent negative regulatory mechanism already exists in these patients but that it is not triggered and is insufficient to suppress the overwhelming ongoing Th17 response. In this study, we investigated whether IFN-α could exert its role through up-regulation of this cytokine. The results showed that IFN-α induced both PBMCs and CD4+ T cells to produce IL-10. We questioned whether IFN-α could inhibit IL-17 expression through an up-regulation of IL-10. Interestingly, we did find that anti-IL-10 antibody blocked the inhibition of IL-17 production promoted by IFN-α. However this antibody, even at a concentration as high as 20 μg/ml, only reversed the role of IFN-α by 35%. These results suggest that the function of IFN-α is, at least partially mediated by IL-10. We performed these experiments using PBMCs from healthy control patients and ideally the data should also be confirmed using cells obtained from BD patients. Other cytokines such as IL-27 that are known to be induced by IFN-α and that can block the differentiation of Th precursor cells into Th17 cells may also be involved but were not included in our study [24, 25]. Our experiments using isolated CD4+ T cells from healthy controls showed a much higher IL-17 and IL-10 level in the culture supernatants as compared with experiments with the same number of PBMCs. The absolute amount of CD4+ T cells is much higher in such cultures and indicates that these cells are probably the main source of IL-17 and IL-10 in this in vitro model. On the other hand, γδ T cells have also been shown to produce IL-17 and may play an important role in experimental uveitis in animal models [26]. Further studies are needed to establish which T-cell subpopulations are subject to IFN-α regulation.

Our results are consistent with an in vivo study by Forrester et al. [19]. They found that IFN-α up-regulated the percentages of CD4+IL-10+ T cells in association with an amelioration of active intraocular inflammation in patients with refractory uveitis. A number of mechanisms involved in the therapeutic effect of IFN-α have been proposed [6]. Pirhonen et al. [24] found that IFN-α was able to up-regulate the gene expression of IL-27, a negative regulator of Th17 cells. Kotter et al. [27] found that IFN-α significantly down-regulated the percentages of NK cells, CD8+γδ T cells and CD3+γδ T cells and up-regulated the levels of TNF-α, sTNF-RII, soluble intercellular adhesion molecule-1 (sICAM-1) and soluble vascular cell adhesion molecule-1 (sVCAM-1) in BD patients. This list is not all inclusive and many other effects of Type I IFNs on adaptive immunity have been mentioned [14]. Which of the above mechanisms plays the most important role in the observed beneficial effects of IFN-α in the treatment of BD remains to be elucidated.

In this study, we found that rhIFN-α could up-regulate the level of P-STAT2 in isolated CD4+T cells. However, it is not clear how STAT2 affects IL-17 or IL-10 production. It has been reported that STAT2 could stimulate production of IL-21 [28], which has been shown to stimulate IL-10 production [29]. On the other hand, STAT2 has been shown to promote production of IL-1Ra, which is able to suppress the differentiation of Th17 cells through inhibition of IL-1β [30]. The aforementioned studies may provide, at least partially, an explanation concerning the mechanisms of STAT2 induction by IFN-α as observed in the present study.

In conclusion, our study showed that IFN-α could down-regulate IL-17 expression in association with an up-regulation of IL-10 in BD patients and controls. The mechanism by which IFN-α exerts its inhibitory effect on IL-17 was partially mediated by IL-10 and was associated with an up-regulation of the level of p-STAT2.

**Rheumatology key messages**

- The study revealed a mechanism by which IFN-α controlled the inflammation of BD.
- The study suggests IFN-α may be used for the diseases mediated by IL-17.

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