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Original Research

LPS-induced Src family kinases activity mediates IL-10 production through activation of STAT3 in peripheral blood mononuclear cells of patients with Behçet's Disease

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Abstract: Behçet's disease (BD) is a chronic inflammatory disorder characterized by recurrent oral and genital ulcers, uveitis and skin lesions. Although, the pathogenesis of BD remains poorly understood, excessive or dysregulated cytokine production including IL-10 is associated with BD. Revealing the key molecular mechanism by which IL-10 expression is regulated is crucial to understanding the pathogenesis of BD. The aim of this study was to investigate whether Src family kinases (SFKs) are upstream mediators of STAT3/IL-10 pathway in peripheral blood mono nuclear cells (PBMCs) of active BD patients. Twenty active BD patients and twenty healthy subjects used as control were included in the study. PBMCs were isolated from total blood by density gradient centrifugation. Western blot and ELISA methods were applied to analyze lipopolysaccharide (LPS)-induced SFKs/STAT3/IL10 signaling pathway in BD. Inhibition of SFKs activity suppressed LPS-induced IL-10 production in PBMCs from both controls and active BD patients. Similarly, blockage of STAT3 activation abrogated LPS-induced IL-10 production. However, LPS-induced STAT3 activation required for IL-10 production was found to be dependent on SFKs activity as LPS-induced STAT3 phosphorylation was reduced by the inhibition of SFKs activity in PBMCs of active BD patients. SFKs activity is essential for LPS-induced STAT3/IL-10 pathway in PBMCs of active BD patients. Manipulation of the SFKs activity may offer a novel therapeutic approach for BD.

Key words: Behçet's disease; Src family kinases; IL-10; STAT3; LPS.

Introduction

Behçet's disease (BD) is a chronic relapsing inflammatory disorder characterized by recurrent oral and genital ulcers, uveitis and skin lesions. Multiple organ involvement including skin, eyes, vessels, joints, gastrointestinal and central nervous systems, lung and kidneys occurs in BD (1-3). The pathophysiology of BD is not clearly understood, but it is postulated that immunological abnormalities, environmental factors and infectious agents in genetically susceptible subjects contribute to BD development (4). BD is more common in the countries located along the ancient Silk Road, while it is quite less frequent in United States and Northern European countries. The incidence of BD is reported to be high in Turkey, Japan, China, Middle Eastern and Mediterranean countries (5). The strong association of HLA-51 with increased risk of developing BD is well documented (6-7). In addition, genome wide association studies (GWAS) demonstrated that variants in IL-23R-IL12RB2, and IL-10 are associated with BD susceptibility (8-9).

BD has a complex etiological and genetic background leading to excessive immune and inflammatory response (10). Implication of the aberrant cytokines production is reported in BD pathogenesis, but the underlying immunological mechanism remains largely unknown (11-12). Increased levels of Tnf-α, IL-6, IL-8, IL-10, IL-12 have been reported in BD patients and high levels of these cytokines have been shown to be correlated with disease activity (13-14). For instance, elevated IL-6 level has been demonstrated to be associated with ocular involvement and the course of disease activity (15).

IL-10 is a key immunosuppressive cytokine generated predominantly by monocytes/ macrophages, T and B cell subsets and dendritic cells (16-17). The mode of action for IL-10 is to suppress toll like receptor (TLR) response induced by agonists such as lipopolysaccharide (LPS) (18). LPS is a component of outer membrane of Gram-negative bacteria which interacts with TLR4. LPS/TLR4 signaling can induce the production of pro-inflammatory cytokines (19). IL-10 exerts its immunosuppressive activity by selectively inhibiting excessive production of inflammatory mediators such as cytokines and chemokines critical for the propagation of inflammation (20). Anti-inflammatory response of IL-10 is mediated by the activation of signal transducer and activator of transcription 3 (STAT3) (21). STAT3 is a transcription factor which modulates expression of target genes in response to various cytokines and growth factors and it is critically involved in immune response. Previous studies demonstrated that STAT3 activity is required for LPS-induced IL-10 signaling pathways (18, 22). There is evidence that IL-10 level is increased in active BD patients (14). It has been also shown that STAT3

pathway is upregulated in active BD patients (23, 24). However, molecular signaling cascades controlling the STAT3 dependent production of IL-10 in BD patients are not clearly understood.

Src family kinases (SFKs) are non-receptor tyrosine kinases which play pivotal roles in the regulation of diverse cellular events including cell proliferation, adhesion, migration, survival and immune functions (25,26). SFKs consist of eight members (Hck, Lyn, Fyn, c-Src, Lck, Yes, Blk and Fgr) that are structurally conserved and aberrant expression or activity of SFKs is associated with many types of diseases including cancer and autoimmune diseases (27,28). Several studies demonstrated that SFKs are implicated in LPS-TLR4 mediated signaling pathways leading to the production of cytokines in monocytes/macrophages (29-31). We have also recently reported that Hck, Lyn, Fyn, c-Src, Lck, Yes are highly expressed in PBMCs and LPS-induced Tnf-α production is tightly regulated by SFKs activity in PBMCs of active BD patients (13). STAT3 is known to be a downstream target of SFKs and activation of SFKs by growth factors and cytokines leads to the induction of several signaling pathways including STAT3 (32,33). We and others previously reported that activation of c-Src, a prototype member of SFKs, is highly correlated with phosphorylation level of STAT3 and inhibition of c-Src activity led to reduced STAT3 phosphorylation at tyrosine-705 (34-36). Although it is well established that STAT3 pathway is involved in LPS-induced IL-10 production, but the requirement of SFKs for upregulation of STAT3/IL-10 pathway needs to be investigated in patients with BD. Identifying molecular drivers of the STAT3 activated anti-inflammatory response occurred in patients with BD may enable to develop new therapeutic strategies for BD.

In this study, we set out to determine the immunomodulatory effect of SFKs in LPS-induced STAT3/IL-10 pathway in PBMCs of both controls and active BD patients.

Materials and Methods

Study subjects

This study was approved by the ethics committee of Dicle University Faculty of Medicine. Written informed consent was obtained from all participants. Twenty active BD patients (nine female, eleven male) followed in the rheumatology, dermatology, ophthalmology clinics of Dicle University hospital were recruited. The mean age of active BD patients was 31±5,2. All patients fulfilled the criteria of the International Study Group for the diagnosis of BD (37). 95 % of the patients (n=19) had oral ulcers, 70 % of the patients (n= 14) had genital ulcers, 60 % of the patients (n= 12) had arthritis, 80 % of the patients (n=16) had skin lesions, 50 % of the patients (n=10) had ocular involvement and 65 % of the patients (n=13) had pathergy positivity. BD patients had at least one year of treatment break at the time of blood sampling. Twenty sex and age matched healthy subjects were included as control.

Sample preparation and cell culture

15 ml peripheral blood was drawn into heparin tubes and the blood samples were transported to the laboratory within 1 h at ambient temperature. PBMCs were isolated from fresh blood samples by Ficoll-Paque Plus density gradient centrifugation (GE Healthcare Biosciences AB, Uppsala, Sweden). After PBMCs isolation, the cell pellets were suspended in complete RPMI 1640 growth medium (Life Technologies, UK) containing 10 % heat-inactivated fetal bovine serum (Hyclone, UK), 2mM L-Glutamine (Life Technologies , UK) and 100 units/ml penicillin/streptomycin (Life Technologies , UK). The cells were incubated at 37 ºC in a humidified atmosphere containing 5 % CO2.

ELISA

PBMCs were seeded on a 96-well plates at 5×10^5 cells per well and cultured in 200 µl complete RPMI 1640 medium. The cells were treated with 10 µM PP2, a SFKs inhibitor, for 1 h and 10 µM parthenolide (PTL), a STAT3 inhibitor, for 2 h. DMSO treated cells were used as control. After treatments, the cells were stimulated with 100 ng/ml LPS for 18 h. Supernatants were collected and the levels of IL-10 in supernatants were determined by ELISA kit (BOSTER Immunoleader, USA) according to the manufacturer's instructions. Absorbance was measured at 450 nm on a spectrophotometric plate reader (Multiskan Go, Thermo Scientific). The limit of detection for IL-10 was 7.8 pg/ml.

Western blot analysis

PBMCs were seeded on a 6-well plate at $5x10^6$ cells per well and cultured in 4 ml complete RPMI 1640 medium. The cells were treated with 10 µM PP2 for 1 h and 10 µM PTL for 2 h. DMSO treated cells were used as control. After treatments, the cells were stimulated with 100 ng/ml LPS for 1h. After LPS stimulation the cells were harvested and lysed in RIPA buffer (Sigma Aldrich) containing protease phosphatase inhibitor cocktail (Thermo Scientific) and the concentration of total proteins in cell lysate were determined by BCA protein assay kit according to the supplier's protocol (Thermo Scientific). Equal amounts of protein from each samples (20 µg) were resolved on 10 % TGX stain free gels (Bio-Rad) and electrophoretically transferred onto PVDF membranes (Bio-Rad). The membranes were incubated with 5 % skim milk powder in phosphate-buffered saline containing 0,1 % Tween 20 (PBST) at room temperature for 1 h and probed with primary antibodies at room temperature for 2 h. Primary antibodies were monoclonal anti-c-Src (#2110) and polyclonal anti-phospho-SFKs (Tyr416) (#2101) from Cell signaling, monoclonal anti-STAT3 (#610190) from BD Biosciences, monoclonal anti-phospho-STAT3 (Tyr705) (#ab76315) and monoclonal anti-β-actin (#ab8224) used as a loading control from Abcam. After incubation of the membranes with primary antibodies, the membranes were washed with PBST and then probed with an anti-rabbit (#ab97051) or anti-mouse (#ab9808) horseradish conjugated secondary antibodies (Abcam). The protein bands were developed by enhanced chemiluminescence reagents (Bio-Rad). The intensity of the bands was quantified by Image lab (Bio-Rad).

Statistical analysis

The significance of the results was evaluated by Student's t- test using Sigmaplot 12 software. Data are

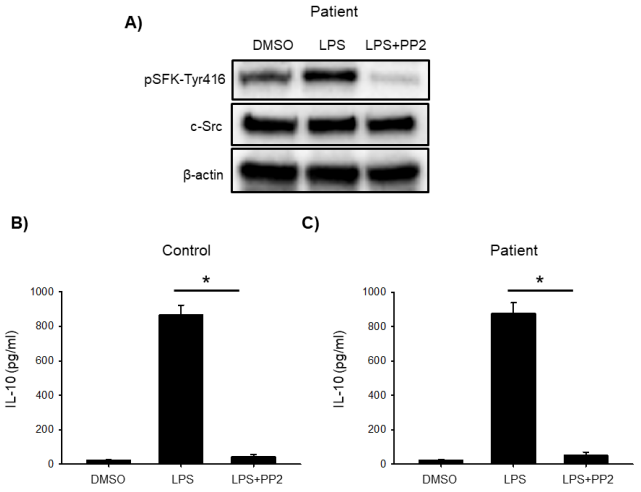

Figure 1. The effect of SFKs activity on LPS-induced IL-10 production in PBMCs of active BD patients. A) PBMCs were treated with 10 μM PP2 for 1h before 100 ng/ml LPS stimulation for 1 h. The cells were treated with DMSO to be used as control. The total amount of c-Src and activity of SFKs were detected by Western blot. β-actin was used as a loading control. B,C) The cultured PBMCs were stimulated with 100 ng/ml LPS for 18 h following treatment with 10 μM PP2 for 1h. Supernatants were collected and IL-10 levels were determined by ELISA. The statistical significance of the data was analyzed by Student's t-test. Results are representative of three independent experiments.

shown as mean (S.D.). $P<0.05$ was considered as statistically significant.

Results

To determine whether SFKs are involved in TLR4 mediated IL-10 production, PBMCs obtained from patients with active BD were treated with PP2, a SFKs specific inhibitor, before LPS stimulation leading to activation of the TLR4 mediated signaling pathway. The Western blots showed that the activity of SFKs measured by phosphorylation level of Tyr-416 was increased in response to LPS stimulation, whereas LPS-induced SFKs activity was blocked by PP2 treatment in the PBMCs of active BD patients (Figure 1A). As we previously showed SFKs members are highly expressed in PBMCs of active BD patients (13), we here showed only the expression level of c-Src, a prototype member of SFKs (Figure 1A). We then investigated whether LPS requires SFKs activity to induce IL-10 production in PBMCs of active BD patients and healthy controls. The ELISA results showed that suppressing the SFKs activity by PP2 led to the inhibition of LPS-induced IL-10 production in the PBMCs of both controls and active BD patients (Figure 1B, C).

We next examined the effect of SFKs activity on the modulation of LPS-induced STAT3 phosphorylation using the strategy of blocking SFKs activity to measure STAT3 phosphorylation (activation) at Tyr-705. The Western blot revealed that LPS stimulation led to the phosphorylation of STAT3, but the inhibition of SFKs activity by PP2 restrained the LPS-induced STAT3 phosphorylation (Figure 2). This result prompted us to consider that LPS-induced STAT3/IL-10 signaling pathway might be regulated by SFKs activity. To address this, we investigated the effect of inhibition of STAT3

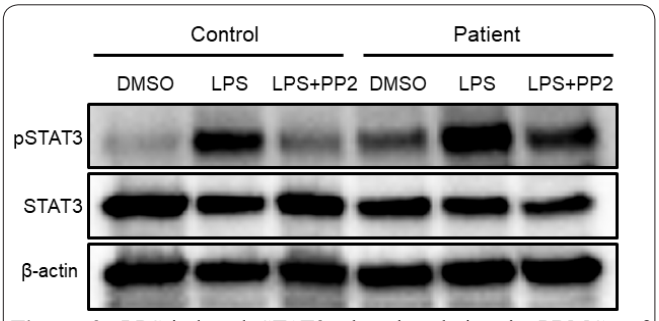

Figure 2. LPS-induced STAT3 phosphorylation in PBMCs of active BD patients is dependent on SFKs activity. PBMCs were stimulated with 100 ng/ml LPS for 1 h following treatment with 10 μM PP2 for 1h. The cells were treated with DMSO to be used as control. Total amount of STAT3 and phosphorylation level of STAT3 at Tyr-705 were detected by Western blot. β-actin was used as a loading control. Data are representative of three independent experiments.

activity on LPS-induced IL-10 increase. The Western blot showed that phosphorylation of STAT3 was elevated upon LPS treatment; however, the LPS-induced STAT3 phosphorylation was blocked by PTL, a STAT3 inhibitor (Figure 3A). We then analyzed the effects of both STAT3 and SFKs activity on LPS-induced IL-10 production. It was found that the inhibition of STAT3 by PTL impaired the LPS-induced IL-10 production in a similar manner with the inhibition of SFKs activity in PBMCs of both controls and active BD patients (Figure 3B, C).

Discussion

Stimulation of PBMCs with LPS leads to the production of IL-10; however, the signaling pathways

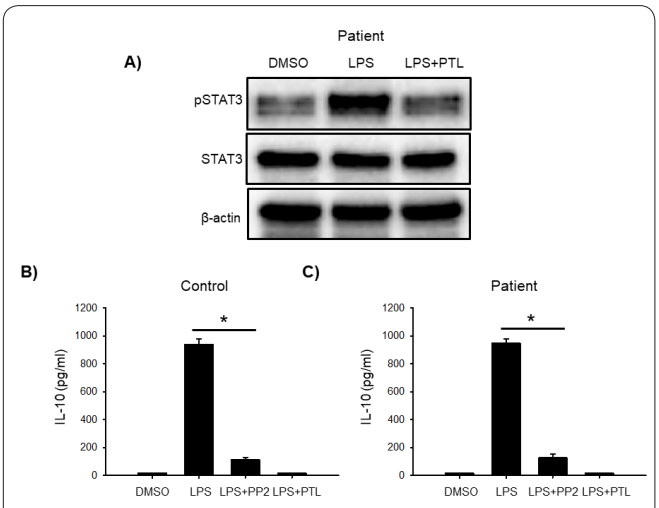

Figure 3. The effect of STAT3 activity on LPS-induced IL-10 production in PBMCs of active BD patients. A) PBMCs were treated with 10 μM PTL for 2 h prior to stimulation with100 ng/ml LPS for 1 h. The cells were treated with DMSO to be used as control. Total amount of STAT3 and phosphorylation level of STAT3 at Tyr-705 were detected by Western blot. β-actin was used as a loading control. B, C) The cultured PBMCs were stimulated with LPS for 18 h following treatment with 10 μM PP2 and 10 μM PTL for 2 h. Supernatants were collected and IL-10 levels were measured by ELISA. The statistical significance of the data was analyzed by Student's t-test. Data are representative of three independent experiments.

governing the IL-10 production in PBMCs of active BD patients remain poorly understood. In this study, we demonstrated the requirement of SFKs activation on LPS-induced IL-10 production in PBMCs of both controls and active BD patients. Our findings suggest that LPS exerts the induction of IL-10 through activation of TLR4/SFKs/STAT3 signaling pathway.

IL-10 is an important cytokine because of its anti-inflammatory and immunosuppressive properties. IL-10 can suppress immune response and inflammation by inhibiting the production of inflammatory mediators including Tnf-α, IL-6 and IL-1 (38). It has been reported that LPS stimulation leads to STAT3 activation by inducing phosphorylation of STAT3 at serine and tyrosine residues (39). The regulation of IL-10 gene by STAT3 has been also reported (22). A study demonstrated that the expression of dominant negative STAT3 in human macrophages affected the majority of IL-10 functions, the study also reported that some aspects of anti-inflammatory activity of IL-10 were regulated by STAT3 independent mechanisms (40). Given that IL-10 is regulated by transcriptional level, revealing the key transcription factor and signaling molecules involved in this pathway will be important to manipulate the expression of IL-10 in various inflammatory disease such as BD. The expression of IL-10 has been shown to be regulated by PBMCs, but the role of SFKs on LPS-induced STAT3/IL-10 signaling pathway in PBMCs of active BD patients remains unknown.

STAT3, a member of STAT family, mediates transcription of the genes regulating cell growth and differentiation induced by various cytokines and growth factors (41,42). STAT3 has been reported to be a substrate of SFKs and SFKs-mediated STAT3 activation is crucial for cell growth (43). Furthermore, SFKs activity was shown to be essential for STAT3 phosphorylation in various cancer cell lines such as lung, colon, breast and ovarian cancer cells (32). However, the requirement of SFKs activity for LPS-induced STAT3 phosphorylation in PBMCs of active BD patients remains elusive. STAT3 is also known to be a downstream target of IL-10 and IL-10 can mediate its production in an autocrine manner via activation of STAT3 (20). A STAT3 binding site was identified in the IL-10 promoter region and activated STAT3 can bind to the IL-10 promoter leading to expression of IL-10 (44). Indeed, IL-10 mRNA expression in the skin of patients with psoriasis was shown to be increased by IL-10 treatment (45). However, blocked of STAT3 was shown to abrogated IL-10 expression in primary human T cells (46).

IL-10 level which plays an immunomodulatory role was elevated in several inflammatory diseases in response to pro-inflammatory cytokine increments. It has been reported that serum level of IL-10 was increased in active BD patients (14). In this study, we found that IL-10 production was increased in response to LPS stimulation, but the inhibition of SFKs activity by PP2 abrogated the LPS-induced IL-10 production. To the best of our knowledge, this is the first study demonstrating that chemical inhibition of SFKs regulates LPS-induced IL-10 production in PBMCs of active BD patients.

In line with previous studies we observed that STAT3 phosphorylation was elevated upon LPS stimulation (18,47). But, we first time reported that LPS-induced STAT3 phosphorylation in PBMCs of active BD patients is dependent on SFKs activity, as chemical inhibition of SFKs activity impaired LPS-induced STAT3 activation. It appears that the signaling pathways controlling the production of IL-10 are cell specific (48). A previous report demonstrated that TLR-mediated IL-10 production by human B cell is governed by the activation of ERK and STAT3 (49). In addition, another study demonstrated that the production of IL-10 in tumor induced-Treg cells is controlled by the co-operation of FOXP3 and STAT3 (50). Based on the data presented in this study, we showed that SFKs activity is absolutely required for LPS-induced IL-10 production and STAT3 is pivotal for the regulation of IL-10 production. Our findings suggest that LPS-induced SFKs activation leads to the phosphorylation and activation of STAT3 and subsequently the translocation of STAT3 to the nucleus which binds to the IL-10 promoter leading to the expression of IL-10 in PBMCs of both controls and active BD patients.

It is important to note that SFKs consist of eight members and PP2 is a selective inhibitor of SFKs members including Src, Lck and Fyn. Therefore, a further study applying siRNA technology is required to investigate which SFKs members are involved in the regulation of SFKs-dependent STAT3/IL-10 pathway in PBMCs of both controls and active BD patients.

Taken together, our results establish a novel role of SFKs for TLR4/STAT3/IL-10 signaling pathway in PBMCs of both controls and active BD patients in response to LPS stimulation. The observation that altered activity of SFKs effects IL-10 production in PBMCs from BD patients offers a new molecular target for drug research in BD. Manipulation of SFKs activities could provide novel therapeutic approaches for BD.

Author's contribution

S. Irtegun-Kandemir: Project development, Data collection, Data analysis, Manuscript writing
M. A. Tekin: Data collection
M. Bozkurt and A.Z. Daglı: Providing patients samples and Data analysis
S. Kalkanlı-Taş: Data collection and analysis

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Conflict of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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