Expression of Th-17 and RORγt mRNA in Behçet’s Disease

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Source of support: This work was supported by a grant from the “Minister of Science and Technology of Tunisia”. UR/99/08-40

Summary

Background: To investigate plasma IL-17 level and the expression of Th17 cell transcription factor RORγt in the pathogenesis of Behçet’s Disease (BD).

Material/Methods: Blood samples were collected from 73 patients with BD (45 patients were in active stage), 20 systemic lupus erythematosus (SLE) and 12 multiple sclerosis patients (MS). Twelve patients with BD were investigated both in their active and remission stages. Samples were processed to detect IL-17A level in plasma by enzyme-linked immunosorbent assay (ELISA). Related gene expression was assessed by real-time reverse transcription polymerase chain reaction. Function of Th17 cells in active BD patients with erythema nodosum (EN)-like eruption was studied in relation to human umbilical vein endothelial cells (HUVECs).

Results: We demonstrated the presence of Th17 cells and RORγt among the peripheral blood mononuclear cells (PBMC). The percentage of circulating Th17 cells and the ability to produce interleukin-17A (IL-17A) were increased in samples derived from patients with active BD, MS and SLE patients. We observed that IL-17A from patients with active BD could induce adhesion molecule messenger RNA expression in HUVECs.

Conclusions: RORγt determined Th17 cell might be involved with increased IL-17A in BD. Our results indicate that IL-17 contributes to the active proinflammatory pattern that is characteristic of inflammatory diseases and patients with active BD.

key words: Behçet’s Disease • interleukin-17A • RORγt mRNA • Th17 cell

Full-text PDF: http://www.medscimonit.com/fulltxt.php?ICID=881720

Word count: 3032
Tables: 2
Figures: 5
References: 45

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Patients (n=45) 4
8
60
26
71.1
(%)
8.9
88.9
26.6
40
17.8
12
57.8
16

CR228
penicillin, 100 units/ml streptomycin (Sigma-Aldrich), and 10% fetal calf serum (Gibco). PBMCs from patients and healthy controls were incubated for 5 hours with 50 ng/ml PMA and 750 ng/ml ionomycin. Supernatants were then collected for later use.

Analysis of cytokine and transcription factor mRNA expression

Total RNA was extracted using TRIZOL® reagent (Invitrogen) according to manufacturer’s instructions. Complementary DNA (cDNA) samples were synthesized using random hexamer primers and RNase H-reverse transcriptase ((Fermentas)). The reaction system includes 2.5 µl of cDNA template, 12.5 µl of SYBR Green mix (Fermentas), and 8 µl of distilled H₂O, 1 µl (10 µM) of each forward and backward primer set. Real-time PCR Detection System (Fermentas) was used for amplification and employed cycling program as follows: denaturation at 94°C for 2 min; then 40 cycles of denaturation, 15 s at 94°C; annealing 45 s at 59°C and extension 45 s at 72°C; and extension at 72°C for 10 min. The purity of PCR products was assessed by dissociation curve plots. Amplification plots were used to assign values for the “cycle threshold” (Ct) by SLAN software. The differences of gene expression in each sample were evaluated by 2⁻ΔΔCt, ∆ΔCt = (Ct target gene − Ct b-actin) − (Ct negative control − Ct b-actin). The primer pairs used are shown in Table 2.

Flow cytometric analysis

PE-conjugated anti-IL-17 mAbs were purchased from eBiosciences (San Diego, CA). For Th17 detection, whole blood samples (200 µl) were activated with phorbol-12-myristate 13-acetate (PMA, 50 ng/ml) and ionomycin (1 µg/ml) for 5 h. After surface staining for CD3/CD4+ T cells and lysis of red blood cells, the remaining cells were permeabilized and stained with FITC-conjugated anti-human IFN-γ and PE-conjugated anti-human IL-17. The cells were fixed in 1% of paraformaldehyde and flow cytometric analyses were performed using FACSCalibur and CELLQuest software (Becton Dickinson, San Jose, CA).

Immunohistochemical staining

Seven patients with erythema nodosum (EN)-like eruption were studied [27]. Paraffin-embedded, formalin-fixed skin tissue lesions were cut into 5-μm sections and placed on polylysine-coated slides. Goat anti-human IL-17 (R&D Systems, Minneapolis, MN) and biotinylated donkey anti-mouse immunoglobulin (Ig) or biotinylated rabbit anti-goat-Ig (Becton Dickinson, Biopole Lab Tunisia) were used for IL-17 staining. The substrate was 3-amin9-ethyl-carbazole (AEC) followed by counterstaining with hematoxylin for single staining. Quantitative evaluation of lymphocytes was done by analyzing 10 different high-powered fields (hpf, ×400) by two independent observers.

Statistical analysis

Data were tested for normal distribution using Kolmogorov–Smirnov test. Correlation was estimated by Pearson’s (r)
correlation coefficient. Data were analyzed using the Statistical Package for the Social Sciences (SPSS) software (Advanced Statistics, version 17.0), Chicago, IL. A probable value of P<0.05 was considered to be statistically significant.

**Results**

**Plasma IL-17A level in BD and**

Plasma IL-17A level in BD patients, SLE patients, MS patients and normal controls were shown in (Figure 1A). Patients with active BD expressed similar level of IL-17A (20.94±3.96 pg/ml) compared to MS patients (22.45±4.21 pg/ml; P=0.25). However active BD patients expressed low IL-17A level when compared to SLE patients (24.26±3.02 pg/ml; P=0.0015). No significant difference was observed between SLE and BD patients (P=0.170). Significant differences were observed between active BD and remission BD (11.22±3.49 pg/ml; P=0.0001). No differences in IL-17 levels were found between healthy controls (11.045±4.33 pg/ml) and remission BD patients (P=0.858).

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**Figure 2.** Correlation between plasma IL-17 levels in 45 active BD patients and biological parameters CRP and ESR using Pearson’s correlation coefficient. Erythrocyte sedimentation rate (ESR: mm/h) and C-reactive protein (CRP: mg/L) expressed as median (range) values in active BD patients were significantly increased in active BD patients [ESR: 35.76 (2–90); CRP: 107.5 (3–260)] compared to healthy controls [ESR: 6 (2–17); CRP: 3.2 (3–9); P<0.0001]. (A): Positive correlation between plasma IL-17A level and CRP (r=0.687; P=0.0021) in active Behçet’s disease (BD). (B): Significant positive correlation was observed between plasma IL-17A level and ESR (r=0.597; P=0.003).

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**Figure 3.** Increased circulating Th17 frequency is correlated with Behçet’s diseases (BD) activity. (A): Representative figures of Th17 cells from a normal control and one active BD patient. CD4+IL-17+ Th17 were gated from CD3+ T cells. (B): Percentage of Th17/CD4+ cells from 45 active BD patients, 28 remission BD patients, 40 healthy controls (HC), 12 multiple sclerosis (MS) and 20 systemic lupus erythematosus (SLE) patients. The frequencies of Th17 cells in active BD patients and all two patient groups are significantly higher than for normal controls and remission BD patients. The medians are indicated by a line inside each box, the 25th and 75th percentiles by the box limits, the lower and upper error bars represent the 10th and 90th percentiles. (C1): Histologic feature of middle dermis of erythema nodosum (EN)-like eruption taken from active BD patient. Mononuclear cells are mainly infiltrated around and in the walls of vessels in the middle dermis (HE, 200×) [30]. Panels on the right show immunohistochemical histology of the EN-like eruption with a predominance of CD3+ lymphocytes around vessels. (C2): Lesional skin was further stained with IL-17. Bars =100 µm.
Twelve patients with BD were studied for IL-17 levels during active and remission stages (Figure 1B). These patients have oral ulcer, genital ulcer, ocular symptoms and arthritis. During their remission stage, they exhibited a decreased IL-17 level which contrasted with their IL-17A levels during the active stage: (active stage: 20.7±3.16 pg/ml; remission stage: 12.45±3.52 pg/ml; P=0.0001). None of these patients had vascular lesions, CNS involvement or pulmonary manifestation. This result indicates the role of IL-17 levels in the inflammatory process in BD as reported in other inflammatory diseases [28]. Significant correlation were observed between CRP (r=0.687; P=0.0021), ESR (r=0.597; P=0.003) and plasma IL-17 levels (Figure 2A, B).

**Increased circulating Th17 cell frequencies are correlated with disease activity**

To determine whether Th17 cells are present in BD (45 patients with active BD and 28 patients in remission BD), PBMC were isolated and stimulated with PMA and ionomycin in the presence of brefeldin A (Figure 3A, B). We compared the Th17 cell proportions in BD; in healthy controls and in control diseases (MS and SLE patients). There was a significantly higher frequency of circulating Th17 cells in active BD patients (2.87±1.2%) compared to healthy controls (0.64±0.37%; P=0.0001) and remission BD patients (0.75±0.37%; P=0.0001). No Significant differences were observed between active BD patients, MS patients (2.96±1.21%; P=0.52) and SLE patients (2.75±1.31; P=0.47). No difference was also observed between MS and SLE patients (P=0.663).

Th17 cell proportions in 12 BD patients were also compared in their active and remission stages for circulating Th17 cell frequencies. There was a significantly higher frequency of circulating Th17 cells in active BD patients (2.43±0.97%) compared to the same patients in remission stage (0.86±0.52%; P=0.0001).

Using immunohistochemistry, we analyzed erythema nodosum (EN)-like eruption from 7 patients with active BD (Figure 3C) compared to controls (5 biopsy samples removed for other reasons from patients with non specific inflammation). EN-like eruption samples from active BD exhibited typical pathologic changes, with a large number of CD3+ T cells infiltrating, especially around vessels (Figure 3C). To better understand these CD3+ T cells from EN-like eruption samples, staining with antibodies to IL-17A was performed. The results confirmed the presence of an important population of IL-17+ cells infiltrating the BD skin lesions (26.8±2.7%) compared to control biopsies (6.8±1.15%; P=0.001) (Figure 3C).

These data indicated that Th17 cells are present among PBMCs and at sites of inflammation, supporting the high levels of plasma IL-17 levels in active BD, as observed in our control inflammatory diseases SLE and MS patients.

**Relative expression of IL-17 and RoR-γt from PBMC**

There were no significant differences in the mean IL-17 mRNA between active BD patients (15.9±3.4), MS (16.7±8.5) and SLE (16.4±6.5) patients (Figure 4). RoR-γt (RORC) mRNA was expressed in active BD patients (1.78±0.72; P=0.0001) at lower levels than MS (4.5±0.63) and SLE (4.3±0.52) patients. No significant difference was observed between MS and SLE patients (P=0.0652).

**Th-17 cytokine-mediated expression of adhesion molecule mRNA**

To determine whether IL-17A derived from patients with active BD can induced increased expression of adhesion molecule mRNA, we prepared sera from patients with active BD and supernatants from culture of stimulated PBMC from patients with active BD, and compared their effects on the gene expression of adhesion molecules in HUVECs.

PBMCs from patients with active BD (n=40), with MS patients (n=12) and with SLE patients (n=20) and control subjects (n=12) were stimulated for 5 hours with phorbol myristate acetate and ionomycin, and supernatants were collected to detect IL-17A by enzyme-linked immunosorbent assay (Figure 5A). IL-17A secretion from stimulated PBMC from patients with active BD was increased (1092±270 pg/ml) compared with that in samples from healthy controls (580±178 pg/ml; P=0.0001). No significant difference was observed between active BD and MS patients (1130±270 pg/ml; P=0.685). However low significant difference was observed when active BD were compared to SLE patients (1500±310 pg/ml; P=0.0048).

Quantitative reverse transcription-polymerase chain reaction was performed to determine the expression of adhesion molecule mRNA in HUVECs induced by the sera from patients with active BD or supernatants from the culture of
Peripheral blood mononuclear cells (PBMCs) derived from patients with active BD (n=10). The supernatants from culture of PBMC derived from patients with active BD or sera from patients with active BD could promote expression of mRNA for E-cadherin, intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) in HUVECs (Figure 5B). Neutralization of IL-17A in culture medium was able to inhibit the expression of adhesion molecule mRNA. Our data indicate that HUVEC may be responsive to stimulation of IL-17A produced by PBMC derived from active BD patients.

**Discussion**

Th17 cells constitute the third effector arm of Th cells, complementing the Th1 and Th2 lineages. The secretion of IL-17 is a primary defining characteristic of Th17 cells. The orphan nuclear receptor RORγt/RORC2 (mice/humans) is the master transcription factor that can drive Th17 cell differentiation in human. We have found that plasma IL-17 level was significantly increased in active BD, MS and SLE patients. Our data showed that patients with active BD exhibit an increased proportion of Th17 cells compared with remission BD and healthy individuals. IL-17 level was associated with disease activity. A positive correlation was found between CRP, ESR and plasma IL-17 level in active BD patients, which suggested that Th17 cells may sustain inflammation in these patients. Because we detected enhanced IL-17A production in single T cells from patients with active BD, we next examined in vivo concentrations of IL-17A in BD, which was produced at higher levels in patients with active disease. Taken together, these data demonstrate that both the proportion of Th17 cells and the ability to produce IL-17A are enhanced in the setting of active BD despite low expression of RORγt mRNA (compared to MS and SLE patients), suggesting that the population of Th17 cells might be expanded as a result of disease activity. The increased plasma IL-17 level and circulating Th17 cell frequencies in active BD patients was supported by data obtained at the molecular levels (IL-17 mRNA and RORC mRNA) indicating an inflammatory conditions as observed in our control diseases MS and SLE patients.

The inflammatory process observed in active BD was corroborated by the presence of multiple inflammatory mediators in the peripheral circulation and in the inflammatory sites [29–33]. Taken together, these data suggest that expansion of the Th17 cell population is related to distinct cytokine milieu in active BD, confirming the recent finding that the IL-23-IL-17 axis is important for the inflammatory reaction in BD [31]. Increased mRNA expression and protein production of IFN-γ was detected in patients with active BD [4]. IFN-γ might promote the expansion of Th17 cells through the generation of an inflammatory milieu in humans [34].

Previous findings supported the notion that Th1 cytokines could play an important role in inflammation and tissue injury and are correlated with active BD [29,35]. Th17 cells also have a specific role in immune function through coordinated effector cytokine action [36]. IL-17A is a key cytokine produced by Th17 cells that can induce the expression of adhesion molecules in several cell lines [37] and our findings indicate that Th17 cell numbers are correlated with BD disease activity. We hypothesized that Th17 cells might contribute to inflammation due to increased secretion of IL-17A.

Our data showed that increasing gene expression of ICAM-1, VCAM-1, and E-cadherin in HUVECs was induced by supernatants of stimulated PBMCs derived from patients with active BD. Treatment with antibodies to IL-17A suppressed the production of adhesion molecules. Taken together, these data emphasized that IL-17A was present in the sera of patients with active BD, and that the level was elevated in culture supernatants of stimulated PBMCs from these patients.
These results suggest that microenvironmental Th17 cells and IL-17A might be involved in inflammation in active BD. Although a substantial population of IL-17+ cells could be detected in skin lesion from patients with active BD, and IFNγ infiltrated to a lesser extent [38–40]. We should not conclude that Th1 cells were not implicated in such tissue inflammation. Both the Th17 and Th1 cytokines might be involved in the pathogenesis of active BD albeit via different mechanisms. Recent studies showed that Th17 and Th1 cells might be involved at different stages during the development of the inflammation [41]. Th17 cells might be generated more rapidly than Th1 cells during inflammation. Through the potent induction of chemokines and adhesion molecules, Th17 cells might attract Th1 cells to sites of inflammation at later stages of the inflammation process, which could further propagate inflammation and tissue damage [33,39,42,43]. However, sequential involvement and different functions of Th17 and Th1 cells during the development of BD have not been demonstrated. Given that Th17 cells appear at sites of inflammation at the early stage, it is meaningful to inhibit the inflammation by blocking the function of Th17 cells, which might delay or block the subsequent recruiting of Th1 cells.

The decreased levels of Th17 in remission BD compared to active BD could be explained by a probable conversion of Th17 into regulatory T (Treg) cells. Several recent studies have indicated the existence of a close interplay between Treg and Th17 cells in regulating some autoimmune conditions [41]. Differentiation of Treg into Th17 cells involved down-regulation of Foxp3 expression and suppressor functions. Foxp3 has been suggested to inhibit Th17 differentiation by antagonizing RORγt function [45].

**Conclusions**

We cannot draw conclusions regarding the observed expansion of the Th17 cell population as a function of disease flare. Regardless of whether this is a cause or a consequence, the expansion of Th17 cells is related to distinct cytokine environments in active BD that influence the extent inflammation within tissues. IL-17A from patients with active BD can elevate the expression of adhesion molecule mRNA and induces adherence of T cells to HUVEC, which underscores the role of Th17 cells and IL-17A-induced adhesion molecule signaling in vascular inflammation in active BD. Understanding the intricate regulatory mechanisms that govern T cell subsets at the molecular level in BD as well as in inflammatory/autoimmune diseases, will undoubtedly yield therapies for dysregulated immune responses.

**Conflict of interest**

The authors declare no conflict of interest.

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