INTRODUCTION

Behçet’s disease (BD) is a heterogeneous multiorgan disorder of unknown etiology. This disease typically manifests as recurrent oral and genital ulcersations and uveitis that are variably accompanied by symptoms affecting the skin, large vessels, gastrointestinal system, and central nervous system (CNS). The precise mechanisms of tissue destruction in BD have not been fully elucidated. A number of viruses, including hepatitis viruses, parvovirus B19, and herpes simplex virus, have been implicated. Neurological involvement in BD, namely, neuro-BD (NBD), causes

ABSTRACT

Behçet’s disease (BD) is a multisystemic perivasculitis with recurrent oral aphthous ulcers, genital ulcers, and eye involvement. Neurological involvement in BD patients is referred to as neuro-BD (NBD). The previous studies found that several cytokines and transcription factor activation pathways for Th17 cells (interleukin [IL]-17 and IL-26) were significantly implicated in NBD manifestation. Recently, IL-9 has been confirmed to play a significant role in the central nervous system. The aim of the present study was to investigate in the cerebrospinal fluid (CSF) the role of IL-9 and its eventual associated with Th17 (RORγt) and regulatory T (Treg, Foxp3) cells in NBD and in control diseases: Non-inflammatory neurological disease (NIND) and headache attributed to BD (HaBD). We examined the expression of CSF IL-9 level by enzyme-linked immunosorbent assay and Western blot. CD4+ IL-9+ cells were counted by immunostaining. CSF mRNA expression of transcription factors of IL-9 (PU.1 or IRF4), IL-17 (RORγt), and Treg cells (Foxp3) was studied using real-time polymerase chain reaction in NBD patients. We found that NBD has increased CSF IL-9 level compared to HaBD and NIND patients. Association was found between PU.1 transcription factor and CD4+ IL-9+ in NBD. Positive correlation was observed between PU.1, IRF4 mRNA, and RORγt contrasting with the absence of association with Foxp3 in NBD. After treatment, 22 NBD patients went at the remission phase. At this stage, an increased CSF level of IL-9 and Foxp3 was observed contrasting with a significant regression of RORγt. These results suggested first that IL-9 could be associated with the inflammatory process during NBD activity. Th9 cells were associated with NBD and could be an important mediator suppressing inflammation. More arguments are needed to support this hypothesis.

Key words: Foxp3, Neuro-Behçet disease, RORγt, Th9 cells, transcription factors

INTRODUCTION

Behçet’s disease (BD) is a heterogeneous multiorgan disorder of unknown etiology. This disease typically manifests as recurrent oral and genital ulcerations and uveitis that are variably accompanied by symptoms affecting
devastating CNS complications and is present in 5%–30% of patients with BD.\cite{1-4} Immunological and molecular pathways are involved in NBD and include the release of interleukin (IL)-1, IL-6 and IL-8, IL-17, IL-26, tumor necrosis factor-α, and interferon-γ into cerebrospinal fluid (CSF).\cite{15,16} which reflects a non-specific inflammatory pattern that is compatible with autoimmune disease pathways. NBD is categorized into two main groups: Parenchymal including brainstem, hemispheric, spinal, and meningoencephalitic involvement; and non-parenchymal including dural sinus thrombosis, and arterial occlusion or aneurysms. CSF analysis has different patterns in parenchymal and non-parenchymal subdivisions of NBD.\cite{19-21}

The knowledge of new cytokines involved in inflammatory and anti-inflammatory processes in the nervous system, exploration of certain cytokines remains insufficient in NBD patients. Recent studies have shown an important role for Th9 cells in the CNS of several diseases.\cite{12-14} IL-9 is a pleiotropic cytokine that has documented effects on lymphocytes and central nervous cells.\cite{15,16} The role of Th9 cells in multiple sclerosis (MS) has been largely investigated in the mouse model of experimental autoimmune encephalomyelitis (EAE). Data from this study suggested that the mechanisms of EAE induction by Th9 cells differed from the mechanisms of Th1 and Th17 cells.\cite{17} The high levels of T helper cells and related cytokines and chemokines found in CNS lesions and in CSF must contribute to the breakdown of the blood–brain barrier (BBB), the activation of resident astrocytes and microglia, and the outcome of neuroinflammation.\cite{17} In the human system, Ruocco et al. analyzed the effect of IL-9 in MS by correlating the levels of IL-9 in the CSF of MS patients at the diagnosis and during the course of disease. Interestingly, they found that IL-9 levels in the CSF were inversely correlated with indexes of inflammatory activity, neurodegeneration, and progression of MS-associated disability.\cite{18}

Although IL-9 was considered as a cytokine of Th9 cells, other Th subsets were also reported with the production of IL-9, including Th2, Th17, and Tregs.\cite{18} IL-9 has been implicated in the clearance of extracellular pathogens, including parasites, inflammatory allergic responses, and anti-tumor immunity.\cite{19} Although the mechanisms underlying their formation are still being characterized, their differentiation is induced at least in part through signals received from the cytokines TGF-β1 and IL-4, which promote the expression of downstream Th9-associated transcription factors PU.1 and IRF4.\cite{20} Overexpression of PU.1 upregulates the secretion of IL-9.\cite{20} TH9 cells share developmental requirements with TH2 cells, including a dependence on both IL-4 and STAT6 signaling and the subsequent induction of GATA-binding protein 3.

We tried to identify the role and interplay between Th-9, Th-17, and Treg cells and their lineage-defining cytokines in modulating inflammation or homeostasis.

### MATERIALS AND METHODS

#### Ethical approval

This investigation was performed at the Medicine University of Tunis (collaboration between the Department of Immunology and Basic Science, Tunis, Tunisia and the Department of Neurology, Shiraz University of Medical Sciences, Shiraz, Iran). The working procedures conform to the ethical standards of the National Research Committee and the 1964 Helsinki Declaration. The work was approved by the ethical committee of the review board of Tunis Medicine University. Informed consent was obtained from all participants.

#### Patient

Neuro- Behçet (NBD): All studied BD patients fulfilled the diagnostic criteria of the International Study Group for BD.\cite{22} Thirty patients (25 males and 5 females, mean age: 42.7 years) diagnosed as BD with neurological involvement and have underwent lumbar puncture which were included in this study. Twenty patients had parenchymal involvement and 10 had non-parenchymal brain disease. The CSF samples were obtained at the active stage of the disease (at the onset of a neurological attack) in 22 patients before high-dose corticosteroid treatment was given. The characteristics of patients are reported in Table 1. Seven NBD patients were treated by azathioprine, methotrexate, and corticosteroids during 8–10 months and were considered by physician in the remission stage. Since the ethics committee did not allow us to obtain the CSF, however, 5 mL of blood was obtained and serum IL-9 and IL-17 cytokines were tested with the expression of RORγt and Foxp3 mRNA in the PBMCs. Headache attributed to BD (HaBD) was considered for those patients who experienced at least five attacks of headache with moderate-to-severe intensity that occurred for the 1st time with close temporal relationship with evolution of the disease or only associated with exacerbations of BD. This type of headache should neither be accompanied by any focal or diffuse neurological signs nor fulfilling the International Headache Society criteria for primary headaches.\cite{23} A second control group includes patients with non-inflammatory neurological disorder (NIND) such as stroke or dementia.

#### CSF

CSF samples were obtained by NBD, HaBD, and NIND and were processed at 4°C immediately after spinal tap. Then, samples were immediately centrifuged at 800 rpm (100 x (g)/min at 4°C for 5 min, and the supernatants were stored at −80°C until analysis.

#### Flow cytometry analysis

Cells were stimulated with 50 ng/ml phorbol myristate acetate (Sigma-Aldrich, St. Louis, MO), 1 mg/mL ionomycin (Sigma-Aldrich), and 10 mg/mL Golgi Stop (BD Biosciences,
San Jose, CA) at 37°C under 5% (v/v) CO₂ for 6 h then stained with fluorescein isothiocyanate-labeled anti-CD4 antibodies (BD Biosciences) and fixed and permeabilized using Fix/Perm solution (eBioscience, San Diego, CA), according to the manufacturer’s instructions. The cells were then incubated with phycoerythrin (PE)-labeled anti-IL-9 antibodies (BD Biosciences). The percentages of cytokine secreting CD4⁺IL-9⁺ T cells were determined by flow cytometry using a FACSCalibur instrument (Becton-Dickinson, Franklin Lakes, NJ, USA); these data were evaluated using FlowJo software 7.6 (TreeStar Inc., San Carlos, CA). Isotype controls were included for compensation and to confirm antibody specificity.

**Enzyme-linked immunosorbent assay (ELISA)**

CSF samples from NBD, HaBD, and NIND patients were collected and immediately immersed in melting ice, and allowed to clot 1 h before centrifugation. All the CSF samples were stored at −80°C before use. All the procedures used were standardized. CSF IL-9 levels were measured using specific ELISA kits (R&D Systems, Minneapolis, MN, USA). Each sample was tested in duplicate. The results were expressed

<table>
<thead>
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<th>No.</th>
<th>Age (years)/sex</th>
<th>Systemic involvements</th>
<th>Central nervous system features</th>
<th>Treatment at time of CSF sampling</th>
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OU: Oral ulcers; GU: Genital ulcers; A: Arthritis; S: Skin lesions (erythema nodosum, acneiform nodules, pseudofolliculitis, and popular lesions); U: Uveitis; GIS: Gastrointestinal system lesions (abdominal pain and occult blood); CNS: Central nervous system.
pNBD: Parenchymal NBD; npNBD: Non-parenchymal NBD; PSL: Prednisolone; CP: Cyclophosphamide; CyA: Cyclosporine; Col: Colchicine. CSF: Cerebrospinal fluid
as pg/mL and the detection limit of this assay was 0.5 pg/ml. The protein levels of serum IL-17 were measured according to the manufacturer’s instructions. Each sample was tested in duplicate. The sensitivity for detecting IL-17 Quantikine ELISA kits (eBioscience) was 15 pg/mL.

RNA extraction and real-time polymerase chain reaction (RT-PCR) analysis
RNA samples were extracted by TRIzol reagent (Invitrogen), according to the manufacturer’s instructions and as we recently reported.⁷⁸ cDNA was prepared using the iScript cDNA Synthesis Kit (Bio-Rad). The primers were analyzed with the following primer sequences (all 5′ to 3′) as follows:

For IL-9: Forward 5′ GGGC ATCAGAGACAACAT-3′, reverse 5′-GGAGGACGAGAGACAAAGCA-3′; PU.1: Forward 5′-CTCACCTCTGCAACAGC-3′, reverse 5′-TTCTTACACTTGCTGCTT TT-3′; IRF4: Forward: 5′-ACA GCG CCT GGC CTA TTT TG-3′, reverse 5′-TGCTGGCATCGGTTTCG; Foxp3: Forward: 5′-GTGGGGACAAGTCGTCTG G-3′, reverse: 5′-AGTGCTGGCATCGGTTTCG; β-actin: Forward 5′-AGATT ACTGCTCT-3′, reverse 5′-GCTG ATCCACATCTGCTGGAA-3′ were used as an endogenous reference. RT-PCR amplification reactions were prepared with the SYBR Green PCR Kit (Bio-Rad) and performed using the ABI 7500 Fast RT-PCR System (Applied Biosystems). PCR products were verified by melting curve analysis. Relative expression levels of target genes were calculated by normalization to b-actin values using the 2-ΔΔct method.

Statistical analysis
Results were expressed as mean ± standard deviation and were assessed by the Mann–Whitney U-test. Correlations between different variables were analyzed using Spearman’s rank correlation. \( P < 0.05 \) was considered statistically significant.

RESULTS

Th9 cells are overexpressed in the CNS of patients with NBD
To address whether IL-9 expression is associated with immune alterations of NBD, we used flow cytometry to examine IL-9 production in NBD patients and in the disease control group (NIND and HaBD). We found that IL-9 expression was markedly increased in NBD as compared to NIND and HABD [Figures 1a-c]. NBD patients exhibited a significant increase in CD4⁺ IL-9⁺ cells (1.75 ± 0.39%) compared to NIND (0.86 ± 0.14%) and HaBD (0.68 ± 0.29%) patients [Figure 1a and c]. Western blotting analyses showed higher IL-9 protein expression in NBD than the NIND and HaBD patients [Figure 1b]. In CSF fluid, IL-9 concentrations were elevated in NBD (2.27 ± 0.40 pg/mL; \( P < 0.0001 \)) relatively to NIND (0.94 ± 0.32 pg/mL) and HaBD (0.82 ± 0.18 pg/mL) [Figure 1d]. These results suggest that IL-9...
signaling may be a key regulator of immune dysfunction in NBD patients. Two major points have to be treated: First, a comparative study of IL-9 mRNA level between NBD patients and control diseases to confirm the overexpression of Th9 cells, and second, it would be interesting to determine the impact of transcription factors of Th9 (PU.1, IRF-4) cells in NBD and its association Th17 (RORγt) and Treg (Foxp3) transcription factors.

**IL-9 mRNA expression in neuro-BD**

IL-9 mRNA expression was investigated in the peripheral blood cells of NBD patients compared to HaBD and NIND. CD3⁺ T cells were investigated for mRNA expression. NBD patients expressed important levels of IL-9 mRNA compared with HaBD and NIND patients [Figure 2a]. IL-9 mRNA was significantly correlated with CD4⁺IL-9⁺ cells (r = 0.512; P = 0.764) [Figure 2b]. No significant correlations were observed in the control diseases.

**IL-9 interplays with RORγt and Foxp3 mRNA expression in active neuro-BD**

NBD patients were investigated for the mRNA transcription factors of Th9 (PU.1, IRF4), Th17 (RORγt), and Treg (Foxp3) cells. As PU.1 (2.94 ± 0.66) and IRF-4 (2.89 ± 0.53; P = 0.764) were similarly expressed in NBD, we established the correlations between PU.1 and the transcription factors of RORγt and Foxp3. Significant correlation was observed between RORγt and PU.1 (r = 0.552; P = 0.0015) contrasting with a negative correlation between PU.1 and Foxp3 (r = -0.542; P = 0.002) [Figure 3]. PU.1 was antagonistically related to Foxp3.

**Increased expression of CSF levels of IL-9 and treg cells after treatment**

Twenty-two NBD patients treated by azathioprine, methotrexate, and corticosteroids (during 8–12 months) were considered in the remission stage by physician (having lost all their clinical symptoms), were investigated for the expression of Th9, Th17, and Treg parameters [Figure 4].

CSF expressed more important level of IL-9 (3.43 ± 0.66 pg/mL) than in the active stage (2.75 ± 0.50 pg/mL; P = 0.0005). CD4⁺ IL-9⁺ was also found increased (remission: 2.87 % ± 0.52 % vs. active stage: 1.75% ± 0.39%; P = 0.0007). At the mRNA level, we observed increased expression of both PU.1 (remission: 3.60 ± 0.75 v.s. active: 3.10 ± 0.67; P = 0.0256) and Foxp3 (remission: 2.60 ± 0.54 v.s. active: 1.83 ± 0.38; P = 0.0001). However, a significant decrease of RORγt was depicted (remission: 2.73 ± 0.78 vs. active: 3.31 ± 1.03; P = 0.039) between active and remission NBD stage.

**DISCUSSION**

The involvement of Th9 cells in NBD has been demonstrated based on the increased expression of mRNA and protein IL-9 expression in the CSF. Th9 cells were similarly expressed in pNBD and npNBD patients. The present study indicates that IL-9 production was significantly increased in NBD patients compared to NIND and HaBD. Significant correlation was observed between IL-9 mRNA expression and CD4⁺IL-9⁺ protein level in NBD. Likewise, CSF-CD4⁺IL-9⁺ cells were associated to the expression of PU.1 transcription factor. Th9 transcription factors, PU.1 and IRF4, were inversely correlated with the expression of Foxp3 and positively associated with RORγt. The medical treatment given to the patients was mostly benign. It has always been observed that the remission phase was accompanied by an improvement of clinical and immunological parameters. The mRNA Foxp3 expression increased contrasting with a decreased RORγt, indicating an eventual gradual recovery of immune homeostasis. This could suggest that an eventual plasticity exits between Th9, Th17, and Treg cells.
IL-9 was considered as a cytokine of Th9 cells, other Th subsets were also reported with the production of IL-9, including Th2, Th17, and Treg cells.\cite{18,24} The regulatory network of transcription factors in Th9 cells is quite intricate since a large proportion of the transcription factors expressed in Th9 cells is also expressed in other Th subsets. These transcription factors have divergent roles in different cells. For example, PU.1 is involved in the differentiation of both Th9 and Th2 cells, induces the expression of GATA3 after phosphorylated by IL-4 signaling. The primary function of IL-4R-STAT6-GATA-3 in Th9 cells is to counteract the TGF-β-induced Foxp3 expression, while the same axis is responsible for inducing the expression of IL-4 in Th2 cells.\cite{25,26} PU.1 transcription factor is highly expressed in Th9 cells and the overexpression of PU.1 upregulates the secretion of IL-9.\cite{21}

IL-9 signaling is mediated by STAT5 transcription factor as recently reported by Elyaman et al.\cite{27} The effect of IL-9 appears to involve brain mast cells.\cite{28} IL-9 is important for T-cell activation and differentiation in autoimmune inflammation of the CNS and that IL-9-mediated Th17-cell differentiation triggers complex STAT signaling pathways.\cite{12} The role of Th9 cells has been largely investigated in the mouse model of EAE. Jager et al. reported that Th9 cell recipients possessed fewer infiltrates of lymphocytes in the meninges compared to EAE developed by Th1 and Th17 cells, thus suggesting that the mechanisms of EAE induction by Th9 cells differed from the mechanisms of Th1 and Th17 cells.\cite{17} IL-9 and its receptor (IL-9R) are both expressed in CNS of MS patients (MS).\cite{29} IL-9 decreases the activation state and promotes the anti-inflammatory properties of human macrophages.\cite{29} This mechanism may contribute to the beneficial effects of IL-9 that is observed in MS and may be therapeutically potentiated by modulating IL-9 expression in MS. In MS and experimental autoimmune encephalomyelitis, Yoshimura et al. reported that IL-9 controls CNS inflammation by modulating dendritic cells.\cite{30} IL-9 reduces autoimmune neuroinflammation by suppressing GM-CSF production by CD4+ T cells through the modulation of DCs.\cite{30} Such immunological mechanisms are probably seen in NBD.

In another study from Tan et al., IL-9 receptors (IL-9R) were expressed on astrocytes and the number of IL-9 protein was increased in peripheral blood mononuclear cells from patients with ischemic stroke.\cite{31} The authors detected the effect of IL-9 on astrocytes using an anti-IL-9-neutralizing antibody to treat rats with experimental stroke. The authors concluded that astrocyte-conditioned medium treated with IL-9 aggravated the disruption of the BBB accomplished by the degradation of tight junction proteins in endothelial cells.

In human studies, children with autism spectrum disorder (ASD) had increased IL-9 positive in CD4+ cells.\cite{32} In the same study, mRNA and protein expression for IL-9, JAK1, pJAK1, STAT5, and pSTAT5 were also significantly elevated in ASD.\cite{32} These results suggested that IL-9 cytokine and JAK-STAT activation signaling have an essential role in immune dysfunction in ASD. According to these recent publications, we find that the results obtained in humans and in experimental studies are contradictory due to the contradictory role of IL-9 in the central nervous system.

Treg cells play a critical role in directing and regulating the dynamic plasticity required for balancing Th17/Treg ratio to suppress inflammation.\cite{33} Recent analysis of transcription factor in NBD patients revealed an increase in the
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RORC/FOXP3 (Th17/Treg) ratios. Our present data could suggest that Th9 cells could involve a possible transitory impairment of Treg cells contrasting with high expression of RORγt. Under the effect of treatment, Th9 cells could act during inflammation through the interplay of cytokines (IL-4 and TGFβ) and through the plasticity between helper (Th) to restore immune homeostasis. This could be explained by the increased expression of Foxp3 at the detriment of RORγt. However, the plasticity between Th9, Th17, and Treg cells remains to be demonstrated. This might play a role in CSF-NBD permitting activation of harmful T-cell subpopulations. This promises new insights into strategies for balancing immune defense with restraints on immune-mediated tissue injury and raises new questions regarding the stability of epigenetic modifications that accompany induction of cytokine gene expression during T-cell lineage development.

Our study has a major limitation. All our NBD patients were studied in active stage. During treatment modalities based on azathioprine or methotrexate and corticosteroids (after 8–10 months), only 22 patients of them went in remission stage and were investigated in their CSF. More investigation was needed in this context, and a complete study at the level of the CSF during the active and remission phases of the disease would be essential. If this becomes clear, an important conclusion would be favorable for possible treatment.

CONCLUSION

Now, an extensive array of signaling molecules and transcription factors involved in Th9 cell differentiation has been reported in inflammatory neurological diseases. However, it is difficult to obtain a comprehensive understanding of the transcriptional regulation of the Th9 subset and its correlations with Foxp3 and RORγt due to the ambiguity and overlap of crucial cytokines required for Th9 transcriptional regulation with other T helper subsets. To use Th9 cells in clinic, much more effort will be required to obtain a better understanding of Th9 cell development and function in auto-inflammatory disease like NBD.

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DISCLOSURES

All the authors declare have no financial conflicts of interest.

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