Critical role of IL-21 in modulating T_H17 and regulatory T cells in Behçet disease

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Background: Behçet disease (BD) is a chronic systemic inflammatory disorder of unknown etiology.
Objective: To determine the nature of T cells driving inflammatory lesions in BD.
Methods: T cell homeostasis and cytokines production were analyzed in peripheral blood and brain inflammatory lesions from 45 adult patients with BD (active and untreated BD [n = 25] and patients in remission [n = 20]) and 20 healthy donors, using Luminex, flow cytometry, immunohistochemistry, and immunofluorescence analysis.
Results: We found a marked increase in T_H17 cells and a decrease in the frequency of $CD4^+$ forkhead box $P3^+$ regulatory T cells (Tregs) in peripheral blood that were induced by IL-21 production and that correlate with BD activity. The addition of serum from patients with active BD in a sorted $CD4^+$ T cells culture of healthy donors induced a significant and dose-dependent production of IL-17A and a decrease in forkhead box P3 expression. We demonstrated the presence of IL-21– and IL-17A–producing T cells within the cerebrospinal fluid, brain parenchyma inflammatory infiltrates, and intracerebral blood vessels from patients with active BD and central nervous system involvement. The stimulation of $CD4^+$ T cells with IL-21 increased T_H17 and T_H1 differentiation and decreased the frequency of Treg cells. Conversely, IL-21 blockade with an IL-21R-Fc restored the T_H17 and Treg homeostasis in patients with BD.
Conclusion: We provided here the first evidence of the critical role of IL-21 in driving inflammatory lesions in BD by promoting T_H17 effectors and suppressing Treg cells. IL-21 represents a promising target for novel therapy in BD.
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Key words: *Behçet disease, T_H17, T_H1, regulatory T cells, IL-21, vasculitis, autoimmunity*

Behçet disease (BD) is a chronic systemic inflammatory disorder at the crossroad between autoimmune and autoinflammatory syndromes.[1] It is characterized by recurrent episodes of oral and genital ulcers, uveitis, and central nervous system (CNS) involvement.[2,3] Although the pathogenesis of BD remains poorly characterized, it is currently thought, as with many autoimmune or autoinflammatory syndromes, that certain infectious (in particular, *Streptococcus sanguis*) and/or environmental factors are able to trigger symptomatology in individuals with particular genetic variants.[2] In common with ankylosing spondylitis and psoriatic arthropathy, BD shares MHC class I association. HLA-B51 is the most strongly associated known genetic factor to BD.[4] However, it accounts for less than 20% of the genetic risk, which indicates that other genetic factors remain to be discovered. Recently, genome-wide association studies from Japan and Turkey identified an association at IL23R and IL12RB2 locus.[5,6] The implication of T cells and polymorphonuclear leukocytes is supported by pathological studies showing perivascular infiltration of memory T cells and polymorphonuclear leucocytes within vasculitic lesions in patients with BD who have arterial and CNS involvement.[7] However, the nature of T cells driving inflammatory lesions remains elusive.

Here, we first demonstrated the promotion of T_H17 responses and the suppression of regulatory T cells (Tregs) that were induced by IL-21 production and that correlate with BD activity. We demonstrated the presence of IL-21– and IL-17A–producing T cells within the cerebrospinal fluid (CSF), brain parenchyma inflammatory infiltrates, and intracerebral blood vessels from patients with active BD (aBD) and CNS involvement. The stimulation of $CD4^+$ T cells with IL-21 increased T_H17 and T_H1 differentiation and decreased the frequency of Treg cells. Conversely, IL-21 blockade with an IL-21R-Fc restored the T_H17 and Treg cells' homeostasis in patients with BD. Our findings suggest that IL-21 exerts a critical role in the pathogenesis of BD, thus providing a promising target for novel therapy.

METHODS

Patients

The study population consisted of 45 consecutive adult patients (25 men and 20 women; mean age, 40 years; range, 23-72 years) fulfilling the international criteria for BD.[8] Patients were divided into two groups: patients with untreated aBD (n = 25) and patients in remission of BD (rBD; n = 20).

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Abbreviations used
aBD: Active Behçet disease
BD: Behçet disease
CNS: Central nervous system
CSF: Cerebrospinal fluid
Fc: Fragment constant
FoxP3: Forkhead box P3
HD: Healthy donor
rBD: Behçet disease in remission
TCM: Central memory T cells
Treg: Regulatory T cell

Patients with aBD were defined as patients with severe posterior or pan-uveitis and/or CNS involvement, in the absence of corticosteroids or immunosuppressant agents. Patients with rBD were defined by the absence of severe clinical manifestation and increased inflammatory parameters. Patients in remission were on corticosteroids (mean dosage, 5.2 mg/d; n = 12), immunosuppressant agents (n = 7), or were left untreated (n = 6). Blood samples from healthy donors (HDs) were obtained from Etablissement Français du Sang (Hôpital Pitié-Salpêtrière). The study was performed according to the Helsinki declaration. All donors gave informed consent.

Analysis of cell surface markers and forkhead box P3 expression

PBMCs were stained with the following conjugated mAbs at predetermined optimal dilutions for 30 minutes at 4°C: CD3-ECD, CD4-PCy7, CD4-ECD, CD8-PCy7, CD8-APC, CD10-APC, CD16-FITC, CD19-ECD, CD27-PE, CD28-FITC, CD45RO-FITC, CD45RA-APC, CD56-PE, HLA-DR-PCy7 (Beckman Coulter, Villepinte, France), CD25-PE, CD38-PCy7, CD56-FITC, CD62L-FITC, IgD-FITC (BD Pharmingen, Le Pont-De-Claix, France), CCR7-PE (R&D Systems, Lille, France), and CD127-FITC (eBioscience, Paris, France). Intracellular detection of forkhead box P3 (FoxP3) was performed on fixed and permeabilized cells using an appropriate buffer (eBioscience). Data were acquired using a Navios flow cytometer and analyzed with the CXP analysis software (Beckman Coulter).

Analysis of cytokine production

PBMCs from the 45 patients with BD and the 20 HDs and CSF mononuclear cells from 3 patients with active CNS involvement were stimulated for 4 hours with phorbol 12-myristate 13-acetate (PMA) and ionomycin (Sigma-Aldrich) in the presence of brefeldin A (BD Pharmingen). Cells were then permeabilized with a Cytofix/Cytoperm buffer (BD Pharmingen); stained with IFN-γ-FITC (BD Pharmingen), IL17A-Alexa Fluor 647 (eBioscience), and IL-21-Alexa Fluor 647 (Biolegend, Saint Quentin en Yvelines, France); and acquired. Fresh PBMCs from patients with aBD and HDs were also cultured in a serum-free medium (X-vivo 20; Lonza, Levallois-Perret, France) supplemented with 2% penicillin-streptomycin with various conditions: medium alone and anti-CD3/CD28 mAbs. After 5 days of culture, cells were collected and restimulated for 4 hours with PMA/ionomycin, and an analysis of intracellular IL-17A and IFN-γ production and FoxP3 expression was performed. Cytokine levels in serum and culture supernatants were measured by using ELISA and Luminex (Invitrogen, Cergy Pontoise, France). IL-21 was also measured in the CSF from 8 patients with BD with active CNS involvement, 8 patients with BD without CNS involvement, and 3 patients with inactive connective tissue disorders. The IL-21 ELISA had a threshold sensitivity of 16 pg/mL.

Purification of $CD4^+$ T lymphocytes from patients with BD

Peripheral total $CD4^+$, $CD4^+CD25^-$, $CD4^+CD25^+$, and $CD4^+CD25^{++}$ T cells were isolated from PBMCs by using immunomagnetic depletion (Miltenyi Biotec, Paris, France) and FACSAria sorting (BD Biosciences) with a purity of each population being more than 97%. Purified $CD4^+$ T cell populations were cultured in X-vivo 20 supplemented with 2% penicillin-streptomycin (1×10^6 cells/mL) and stimulated in 48-well plates coated with anti-CD3/CD28 mAbs with various conditions: anti-CD3/CD28 alone, recombinant human IL-21 (50 ng/mL; BioVision, Lyon, France), and recombinant human IL-21R/fragment constant (Fc) chimera (100 μg/mL, R&D Systems). FoxP3 expression was analyzed by using flow cytometry as previously described, and intracellular cytokine production was analyzed after restimulation with PMA/ionomycin and flow cytometry.

TABLE I. Phenotypical analysis of T, B, and NK cell subsets in patients with Behçet disease and healthy donors

Measurement	HD	aBD	rBD
$CD4^+$ T cells (n, %)			
Total	65.8 (11.2)	54.1 (15.0)*	60.8 (15.6)
Naive	28.4 (13.2)	29.4 (16.0)	29.6 (18.9)
CM	23.3 (10.7)	27.2 (17.1)	17.3 (11.5)
TEM	36.4 (13.3)	31.8 (16.8)	35.5 (20.2)
tTEM	12.0 (7.0)	11.9 (15.1)	17.6 (14.4)
HLA DR^+	24.8 (13.3)	20.9 (17.6)	25.4 (15.4)
$CD25^-$	66.8 (15.0)	69.7 (9.8)	67.9 (9.6)
$CD25^+$	28.8 (14.3)	25.4 (10.4)	26.9 (9.1)
$CD25^{++}$	1.9 (0.8)	3.6 (1.8)†	3.6 (1.5)‡
$CD8^+$ T cells (n, %)			
Total	29.2 (9.5)	34.9 (12.4)	29.7 (8.8)
Naive	25.5 (14.5)	40.3 (17.4)†	28.8 (16.3)
CM	5.8 (4.0)	9.1 (8.0)	4.4 (3.5)
TEM	29.3 (11.0)	17.4 (8.6)†	25.4 (18.1)
tTEM	39.4 (19.9)	33.2 (18.6)	41.4 (19.8)
HLA DR^+	52.7 (19.5)	45.1 (19.7)	50.6 (20.1)
$CD25^-$	90 (11.4)	85.1 (12.9)	85.8 (10.6)
$CD25^+$	10.0 (11.4)	13.3 (9.9)	13.9 (10.3)
Ratio CD4/CD8	2.6 (1.3)	1.9 (1.0)*	2.4 (1.0)
$CD19^+$ B cells (n, %)			
Total	9.6 (4.6)	8.6 (5.4)	8.1 (4.3)
Immature	4.4 (3.2)	3.1 (3.3)	3.5 (4.4)
Naive	45.1 (21.3)	45.5 (20.0)	41.3 (20.5)
MZ	26.3 (18.1)	26.4 (14.7)	30.2 (18.2)
CS	19.9 (12.1)	23.1 (12.7)	23.2 (12.0)
Plasmablast	0.7 (0.4)	1.3 (1.1)	0.8 (0.6)
$CD3^-CD56^+$ NK cells	10.8 (7.7)	7.6 (5.1)	6.7 (4.3)

CM, Central memory subset ($CD45RA^-CD62L^+$); *CS,* class switched memory subset (IgD^-CD27^+); *MZ,* marginal zone subset (IgD^+CD27^+); Naive B cells (IgD^+CD27^-); Naive subset ($CD45RA^+CD62L^+$); *NK,* natural killer ($CD3^-CD56^+$); *TEM,* effector memory subset of T cells ($CD45RA^-CD62L^-$); *tTEM,* terminally differentiated EM T cells ($CD45RA^+CD62L^-CD27^-CD28^-$). Comparisons were performed between patients with aBD and HDs and patients with rBD and HDs.
*$P < .05$.
†$P < .01$.
‡$P < .001$.

Immunohistochemical analysis

$IL\text{-}21^+$, $IL\text{-}17A^+$, chemokine (C-C motif) ligand (CCL) 20^+, and chemokine (CXC motif) ligand (CXCL) 8^+ cells were detected on fixed, paraffin-embedded samples from 2 patients with BD with active CNS involvement (necropsic samples) and 2 normal controls (from the Banque d'échantillons biologiques de recherche en neurologie). Dewaxed slides were submitted to antigen retrieval by heating in a citrate buffer with pH 6.0. Before incubation with primary antibodies, the Fc receptor was blocked with 2% BSA. Slides were incubated overnight with polyclonal goat antihuman IL-17A (dilution 1:20; R&D Systems), polyclonal rabbit IL-21 (dilution 1:20; 500-P191; PeproTech, Neuilly sur Seine, France), polyclonal goat antihuman CXCL8 (dilution 1:20, R&D Systems), and polyclonal rabbit