

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. (J Allergy Clin Immunol 2011;■■■■:■■■-■■■.)

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 leukocytes 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.

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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

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; $n = 20$). 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.

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.

* $P < .05$.

† $P < .01$.

‡ $P < .001$.

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 μ g/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.

Immunohistochemical analysis

IL-21⁺, IL-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

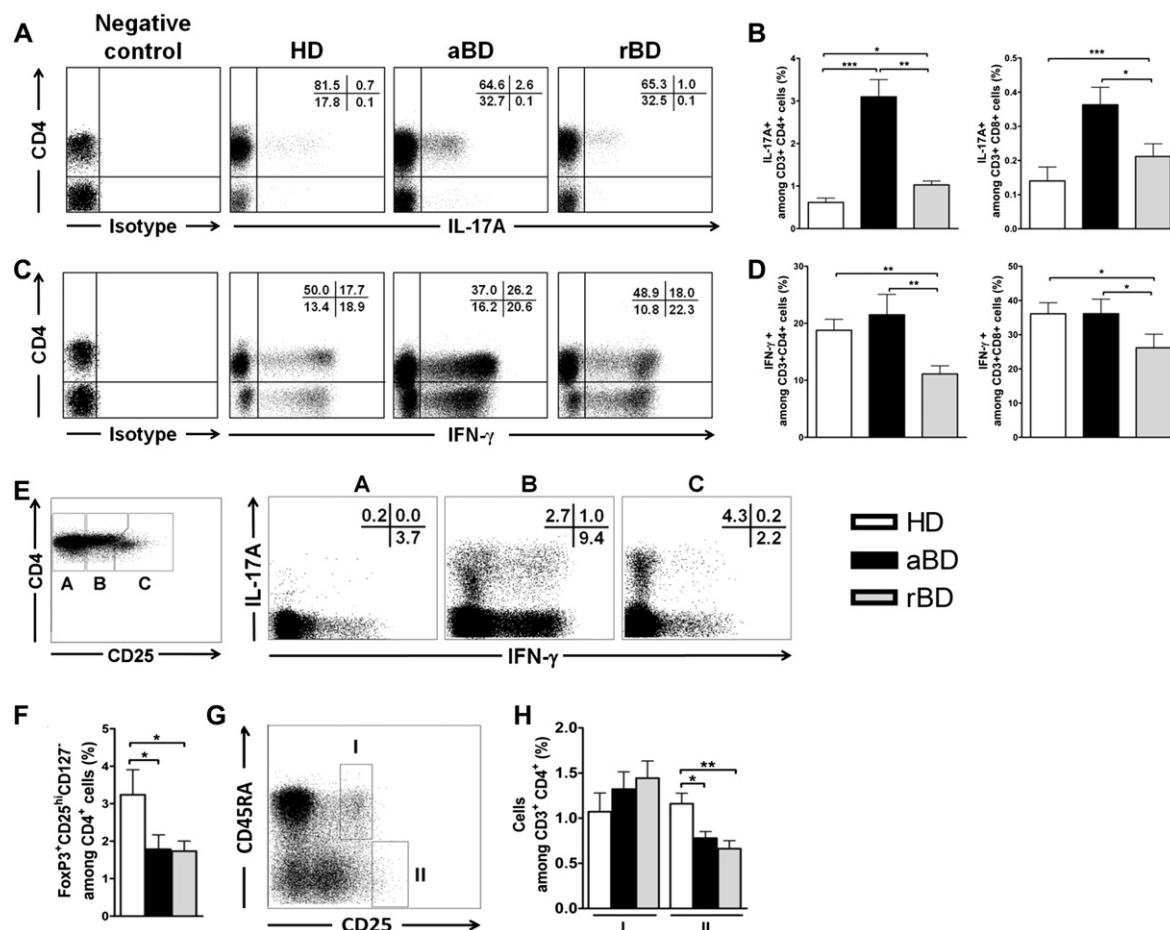


FIG 1. Increased T_H17 effectors and decreased $FoxP3^+CD25^{hi}CD127^-$ Treg cells in BD. Representative dot plots of IL-17A– (A) and IFN- γ (C)–producing $CD4^+$ after gating on $CD3^+$. Frequencies of IL-17A– (B) and IFN- γ (D)–producing T cells from 20 HDs, 25 patients with aBD, and 20 resting patients with BD. Analysis of IL-17A and IFN- γ production by $CD4^+$ T cells according to CD25 expression (E). Frequencies of Treg cells (F) and frequencies (H) of resting (box I) and activated Treg cells (box II). * $P < .05$; ** $P < .01$; *** $P < .001$.

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 antihuman CCL20 (working dilution 1:30; Abcam, Paris, France) antibodies. Antibody binding was visualized with diaminobenzidine tetrahydrochloride (Dako, Trappes, France). For the negative control, proper isotype controls were used. $CD3^+IL-17A^+$ and $CD3^+IL-21^+$ cells were detected on the same samples. Slides were incubated overnight at 4°C with polyclonal goat antihuman IL-17A (dilution 1:10) or polyclonal rabbit antihuman IL-21 (dilution 1:10) and monoclonal mouse antihuman CD3 antibody (Clone F7.2.38, Dako). Slides were then incubated for 1 hour at room temperature with Cy3-conjugated goat antirabbit antibody or biotinylated polyclonal antigoat antibody, then with APC-conjugated streptavidin, and finally with Cy2-conjugated goat antirabbit antibody (working dilution 1:200, Invitrogen), mounted in Fluoromount (Amersham, Orsay, France), and evaluated under fluorescence microscopy.

Data analysis

Data are presented as a mean (SEM) for continuous variables and as a percentage for qualitative variables. The Fisher exact test was used to compare qualitative variables, and nonparametric Mann-Whitney and Wilcoxon tests were used to compare continuous variables, as appropriate. A P value of $<.05$ was considered significant. Statistical analyses were performed using

GraphPad Prism version 4.0 and InStat version 3.0 for Windows (GraphPad Software, San Diego, Calif).

RESULTS

Phenotypical analysis of T cell subsets in patients with BD

To identify perturbations in T cell homeostasis, frequencies of naive, central memory T cells (TCMs), effector memory T cells (TEMs), and terminally differentiated TEM (tTEM) cells were compared among $CD4^+$ or $CD8^+$ T cells subsets from patients with aBD, patients with rBD, and HDs (Table I). No difference was found among $CD4^+$ T cell, B cell, and NK cell subsets. Among $CD8^+$ T cells, naive T cells were increased in patients with aBD compared with HDs and patients with rBD (40.3% vs 25.5% and 28.8%; $P = .007$ and $P = .049$, respectively). In contrast, TEMs were decreased in patients with aBD compared with HDs (17.4% vs 29.3%; $P = .002$).

T_H17 cells are increased in the peripheral blood in BD and correlate with disease activity

We examined the frequency of IL-17A– and IFN- γ –producing T cells after 4 hours of stimulation with PMA and ionomycin

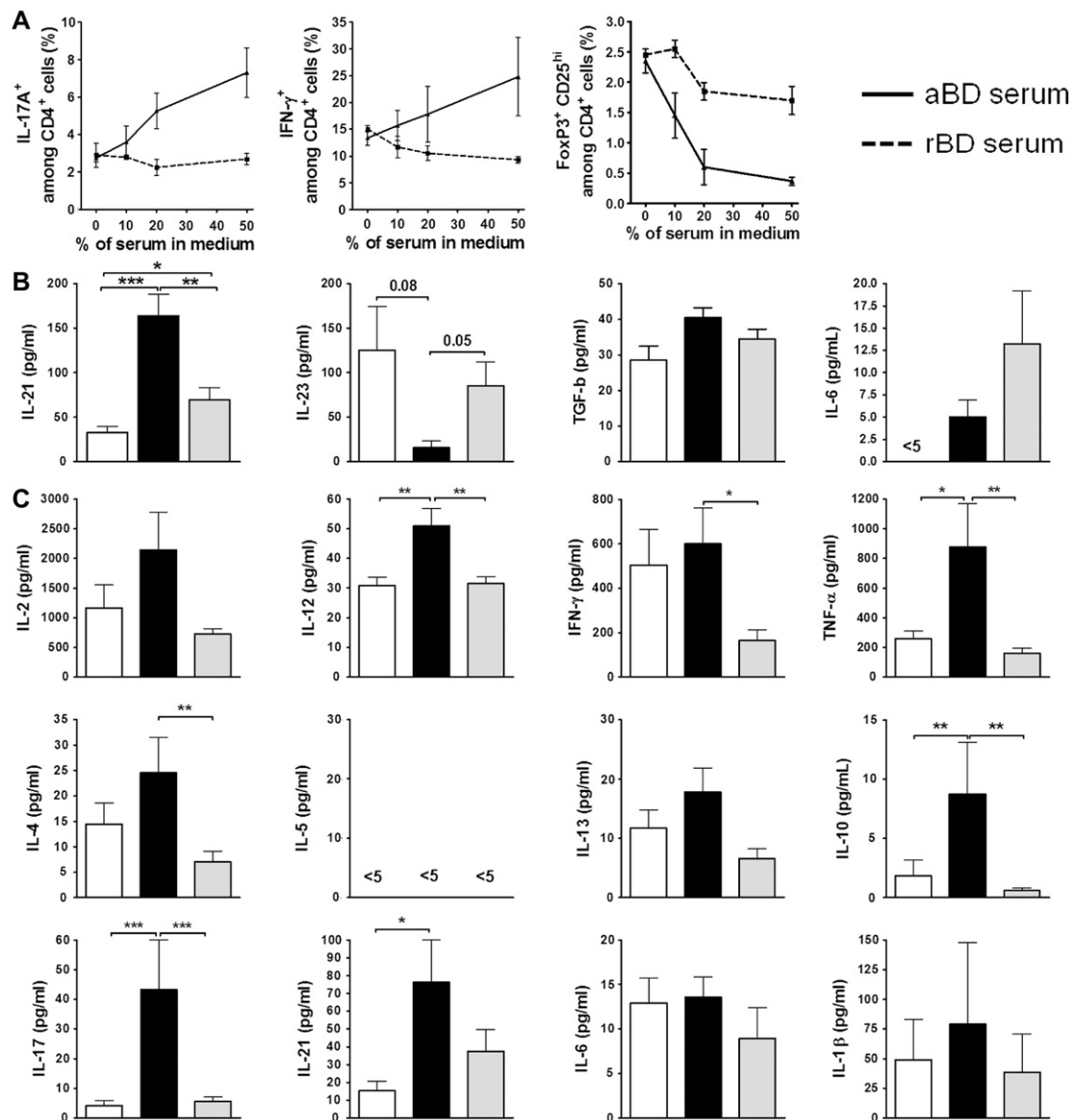


FIG 2. Sera from patients with BD contains increased IL-21 level and modulates predominant T_H17 cytokines modulating T_H1 and T_H17 differentiation and FoxP3 expression in HDs. Frequencies of IL-17A⁺, IFN-γ⁺, and FoxP3⁺ cells among sorted CD4⁺ T cells after 5 days culture with anti-CD3/anti-CD28 and with increasing concentrations of sera (A) in patients with aBD and 20 patients with rBD (3 independent experiments). T_H17 cytokine levels in the sera of HDs, patients with aBD, and those with rBD (B). T_H1, T_H2, and T_H17 cytokine levels in culture supernatants in BD (C). Cytokines were assessed in 20 HDs, 25 patients with aBD, and 20 patients with rBD, except for IL-23 and TGF-β, which were assessed in 10 HDs, 18 patients with aBD, and 18 patients with rBD. **P* < .05; ***P* < .01; ****P* < .001.

(Fig 1). We found a marked enrichment in IL-17A-producing CD45RO⁺CCR6⁺CD4⁺ T cells (T_H17) in patients with aBD compared with those who had rBD and in HDs (3.1% vs 1.0% and 0.6%; *P* < .0001 for both) (Fig 1, A and B), whereas IFN-γ-producing CD4⁺ and CD8⁺ T cells (T_H1) did not differ significantly between patients with aBD and HDs. In contrast, T_H1 cells were increased in patients with aBD compared with patients with rBD (Fig 1, C and D). Immunosuppressant therapy in patients with rBD may explain the decrease in IFN-γ production by T cells. We next examined the IL-17A and IFN-γ production in purified CD4⁺ T cells from patients with BD, according to the CD25 expression, after 5 days of stimulation with anti-CD3/CD28 (Fig

1, E). CD4⁺CD25⁺ T cells (B) were enriched in both IL-17A- and IFN-γ-producing cells, whereas CD4⁺CD25⁺⁺ T cells (C) produced mainly IL-17A (Fig 1, E).

Decreased Tregs in peripheral blood from patients with BD

We next explored a possible defect in T cell regulation associated with the T_H17 and T_H1 imbalance. We found that CD4⁺FoxP3⁺CD25^{hi}CD127⁻ Treg cells were decreased in peripheral blood from patients with aBD and rBD compared with HDs (1.78% and 1.73% vs 3.2%; *P* = .02) (Fig 1, F). We analyzed

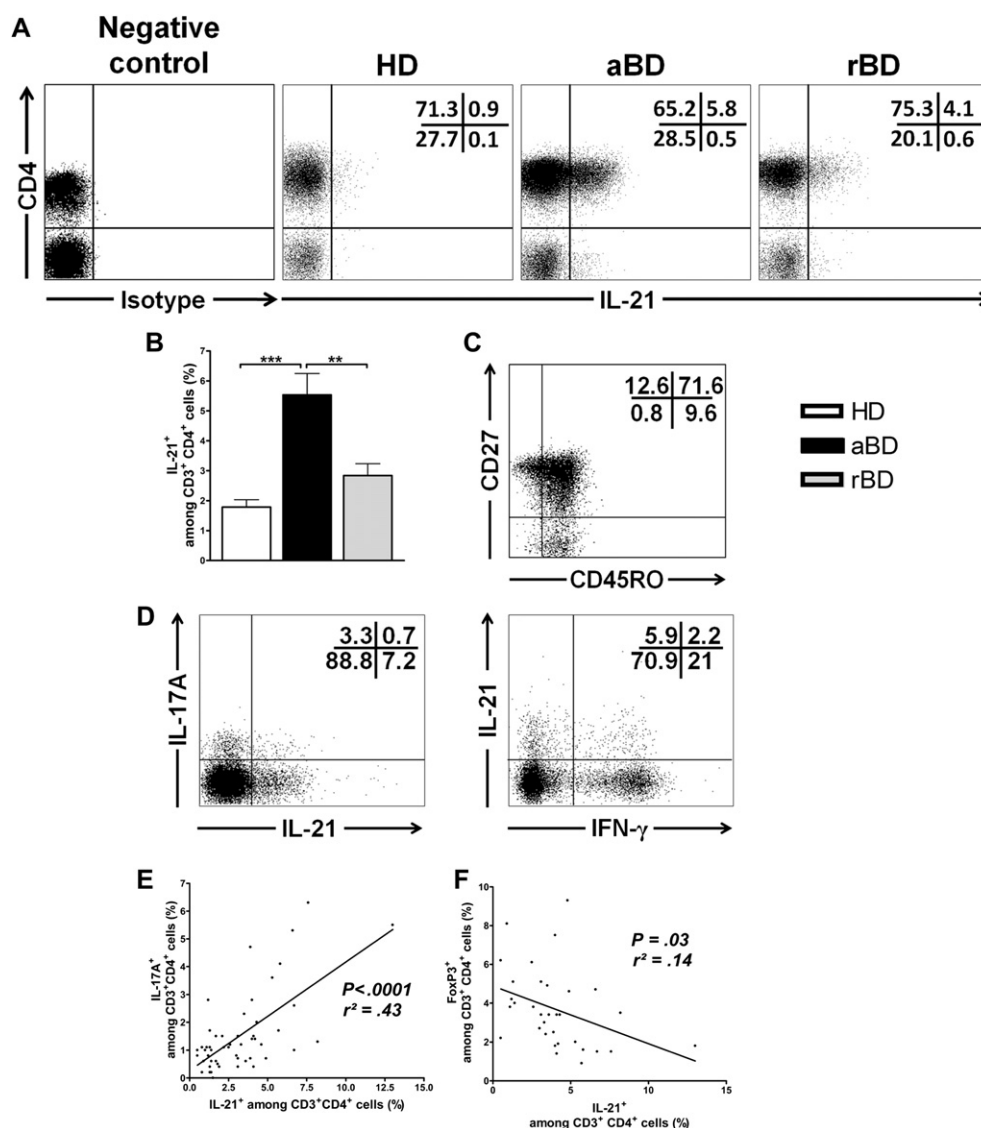


FIG 3. IL-21 is produced by central memory CD4⁺ T cells and correlates with T_H17 response and FoxP3 expression. Dot plots (A) and frequencies of IL-21-producing CD4⁺ T cells (B) after PMA/ionomycin stimulation. IL-21⁺ is produced by central memory CD4⁺ T cells (C). Dot plot of IL-21⁺, IL-17A⁺, and IFN-γ-producing CD4⁺ T cells after PMA/ionomycin stimulation (D). Correlations between IL-21-producing CD4⁺ T cells and T_H17 cells (E) and FoxP3⁺ Tregs (F).

the expression of CD45RA and CD25 among the CD4⁺ T cells in order to better delineate CD45RA⁺CD25⁺⁺ resting Tregs (rTregs) and CD45RA⁺CD25⁺⁺⁺ activated Tregs (aTregs) (Fig 1, G). aTreg cells were decreased in patients with aBD and rBD compared with HDs (0.8% and 0.7% vs 1.2%; $P = .02$ and $P = .014$, respectively) (Fig 1, H).

Sera from patients with BD modulate T_H1 and T_H17 differentiation and FoxP3 expression in healthy controls

We determined whether serum from patients with BD was able to modulate T cell differentiation and FoxP3 expression in healthy controls. The frequency of IL-17A⁺ and IFN-γ-producing T cells and FoxP3 expression were evaluated after 5 days of stimulation of purified CD4⁺ T cells from healthy controls with anti-CD3/

CD28, in the presence of various proportions of serum from patients with aBD and rBD in culture medium. The frequency of IL-17A⁺ and IFN-γ-producing T cells increased in a dose-dependent manner with sera from patients with aBD compared with that from patients with rBD (2.8%, 3.6%, 5.3%, and 7.3% of T_H17 and 13.4%, 15.7%, 17.8%, and 24.8% of T_H1 with 0%, 10%, 20%, and 50% of serum concentrations from patients with aBD compared with 2.9%, 2.8%, 2.3%, and 2.7% of T_H17 and 15.1%, 11.7%, 10.5%, and 9.3% of T_H1 with 0%, 10%, 20%, and 50% of serum concentrations from patients with rBD, respectively). FoxP3 expression decreased similarly in a dose-dependent manner with sera from patients with aBD compared with that from patients with rBD (2.4%, 1.5%, 0.6%, and 0.4% serum concentrations from patients with aBD compared with 2.5%, 2.6%, 1.9%, and 1.7% serum concentrations from patients with rBD) (Fig 2, A).

Increased IL-21 in the serum of patients with BD

Levels of cytokines promoting IL-17A production (IL-21, IL-23, TGF- β , and IL-6) were measured in serum from patients with aBD and rBD and from HDs (Fig 2B). IL-21 level was markedly increased in patients with aBD compared with those who had rBD and in HDs (164 pg/mL vs 69.5 and 32.9 pg/mL; $P = .004$ and $P = .0002$, respectively). In contrast, levels of IL-23 (15.5, 85.5, and 125.4 pg/mL), TGF- β (40.4, 34.4, and 28.5 pg/mL), and IL-6 (13.3, 5.0, and <5 pg/mL) did not differ significantly among patients with aBD, patients with rBD, and HDs. Levels of T_H1 (IL-2, IL-12, IFN- γ , and TNF- α), T_H2 (IL-4, IL-5, and IL-13), and T_H17 cytokines (IL-1 β , IL-6, IL-17A, and IL-21) were also measured in culture supernatants after 4 hours of stimulation with PMA/ionomycin (Fig 2, C). IL-17A and IL-21 levels were markedly increased in patients with aBD compared with patients with rBD and HDs (43.3 pg/mL vs 5.5 and 4.2 pg/mL; $P = .0001$ for both and 76.3 pg/mL vs 37.6 and 15.3 pg/mL; $P = .19$ and $P = .03$, respectively). Similarly, TNF- α (877.2 pg/mL vs 159.2 and 258.6 pg/mL; $P = .002$ and $P = .03$, respectively), IL-12 (50.9 pg/mL vs 31.5 and 30.8 pg/mL; $P = .005$ and $P = .009$, respectively), and IL-10 levels (8.7 pg/mL vs 0.6 and 1.9 pg/mL; $P = .001$ and $P = .006$, respectively) were significantly increased in patients with aBD compared with patients with rBD and HDs. IFN- γ level was higher in patients with aBD compared with patients with rBD (599 pg/mL vs 165 pg/mL; $P = .012$) but not when compared with HDs (599 pg/mL vs 504 pg/mL; $P = .94$). No difference was found between groups for the levels of remaining cytokines (IL-1 β , IL-2, IL-4, IL-5, IL-6, and IL-13) (Fig 2, C).

IL-21 is produced by central memory CD4⁺ T cells and correlates with T_H17 response and FoxP3 expression

Given the increased levels of IL-21 in serum and culture supernatants, we analyzed intracellular production by T cells by using flow cytometry. Levels of IL-21-producing CD4⁺ T cells were markedly increased in patients with aBD compared with patients with rBD and HDs (5.5% vs 2.8% and 1.8%; $P = .002$ and $P < .0001$, respectively) (Fig 3, A and B). IL-21-producing CD4⁺ T cells displayed the phenotype of a TCM, as indicated by the expression of CD45RO and CD27 (Fig 3, C). Most of the IL-21-producing CD4⁺ T cells did not produce IL-17A or IFN- γ (Fig 3, D). Double-positive IL-21⁺ and IL-17⁺CD4⁺ cells were a minor population corresponding to less than 1% of the whole CD4⁺ T cell population. Increase in IL-21-producing CD4⁺ T cells was positively correlated with T_H17 ($r^2 = 0.43$; $P < .0001$) and negatively correlated with FoxP3 Treg cells ($r^2 = 0.14$; $P = .03$) in patients with BD (Fig 3, E and F). In addition, in patients with aBD, IL-21-producing CD4⁺ T cells were inversely correlated with aTreg cells ($r^2 = 0.45$; $P = .009$) but not with rTreg cells ($r^2 = .03$; $P = .38$).

Increased production of IL-21, IL-17A, and IFN- γ in cerebrospinal fluid from patients with active BD with central nervous system flare

Pathological studies support the implication of T cells and polymorphonuclear leukocytes in the inflammatory lesions of patients with BD with CNS involvement. We analyzed the CSF level of IL-6 and IL-21 in patients with BD with (BD CNS⁺) or without (BD CNS⁻) CNS flare (Fig 4, A) and in inactive

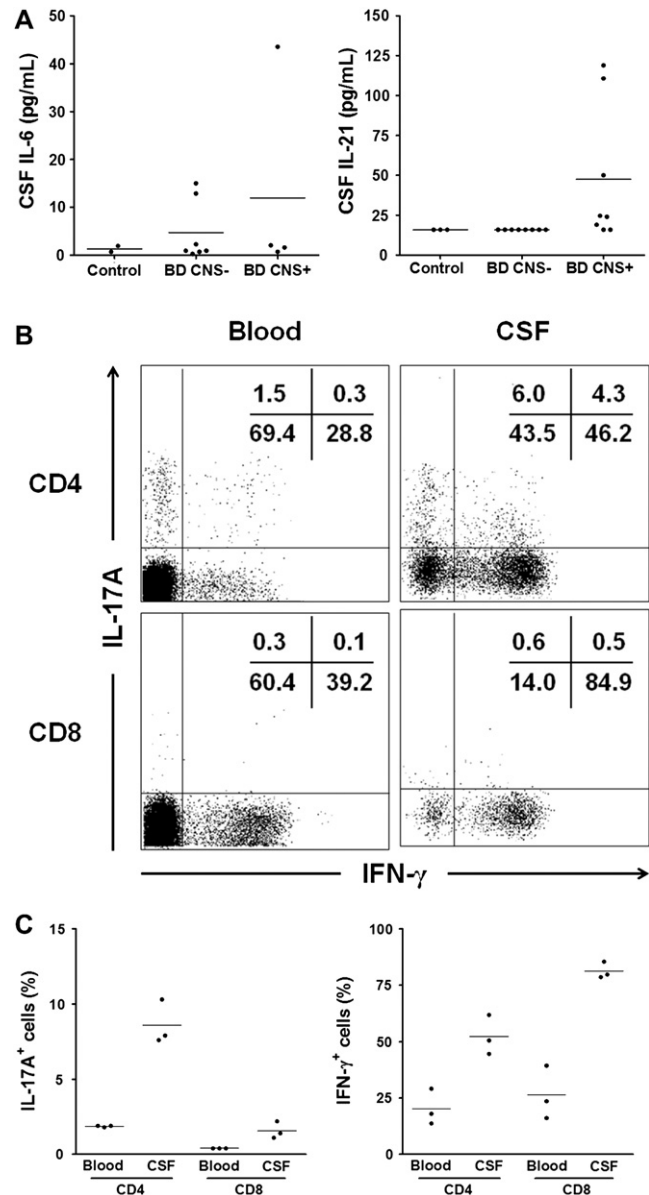


FIG 4. Increased production of IL-21, IL-17A, and IFN- γ in the CSF from patients with BD with CNS flare. IL-6 and IL-21 levels in the CSF from control and patients with BD with (BD CNS⁺) or without (BD CNS⁻) CNS involvement (A). Dot plots (B) and frequencies (C) of IL-17A- and IFN- γ -producing T cells after PMA/ionomycin stimulation in the peripheral blood and CSF from patients with BD with CNS involvement.

controls. Significant levels of IL-21 were detectable only in the CSF from patients with aBD with CNS involvement (47.5 pg/mL in BD CNS⁺ vs undetectable levels in BD CNS⁻ and healthy controls). The IL-6 level was also detectable in the CSF from patients with aBD with CNS involvement (9.6 pg/mL vs 5.9 pg/mL in BD CNS⁻ and 1.3 pg/mL in healthy controls). We compared the frequencies of IL-17A- and IFN- γ -producing T-cells in the peripheral blood and CSF from 3 patients with aBD with CNS involvement (Fig 4, B and C). The levels of IL-17A-producing CD4⁺ and CD8⁺ T cells were dramatically increased in the CSF than in the peripheral blood from patients with aBD (8.6% vs 1.9% of CD4⁺ T cells and 1.6% vs 0.4% of CD8⁺ T cells, respectively). Levels of

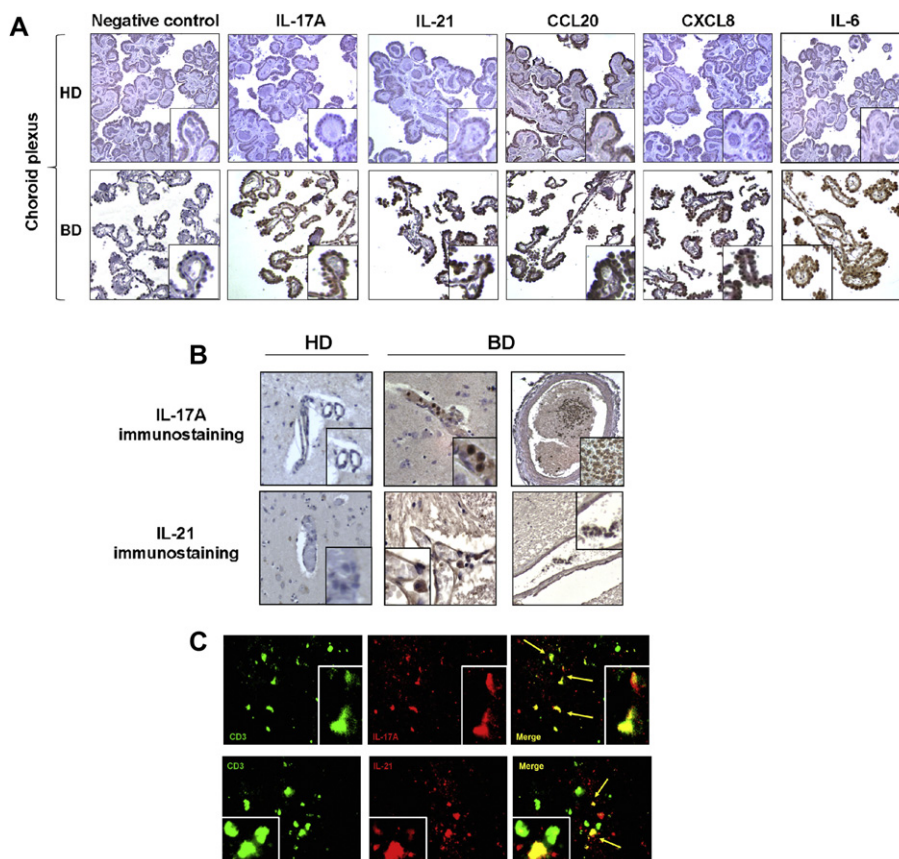


FIG 5. Strong IL-21 and IL-17A expression in CNS inflammatory lesions from patients with BD. Immunohistochemical analysis of IL-21, IL-17A, CCL20, CXCL8, and IL-6 expression in the choroid plexus from patients with aBD and from HDs (A). IL-17A and IL-21 are expressed in intracerebral blood vessels from patients with BD (B). IL-17A and IL-21 are expressed in inflammatory brain lesions and colocalized with CD3 in patients with aBD with CNS involvement (C).

IFN- γ -producing CD4⁺ and CD8⁺ T cells were also dramatically increased in the CSF than in the peripheral blood from patients with aBD (52.3% vs 20.3% of CD4⁺ T cells and 85.4% vs 39.3% of CD8⁺ T cells, respectively).

Strong IL-21 and IL-17A expression within central nervous system inflammatory lesions from patients with active BD

Immunohistochemical analysis of paraffin-embedded necropsic brain tissue specimens from 2 patients with aBD with CNS involvement and 3 normal brain controls was used to investigate the pattern of expression of IL-21, IL-17A, IL-6, CCL20, and CXCL8, which is a potent chemoattractant of T_H17 cells and polymorphonuclear leukocytes. The brain's choroid plexus in patients with BD showed a strong expression of IL-21, IL-17A, IL-6, and CXCL8, as compared with normal brain controls (Fig 5, A). Although a uniform staining of CCL20 by choroid plexus epithelial was noted in normal controls, a higher expression of CCL20 was observed in patients with BD (Fig 5, A). IL-17A and IL-21 were expressed within intracerebral blood vessels and in brain parenchyma infiltrates from patients with BD compared with normal controls (Fig 5, B). Immunofluorescence microscopy analysis of brain parenchyma from patients with BD showed the expression of IL-17A and IL-21, and we observed the colocalization of IL-

17A and IL-21 with CD3, indicating the presence of T_H17 cells and IL-21-producing T cells (Fig 5, C).

IL-21 is critical in modulating T_H17 differentiation and FoxP3 expression in patients with BD

We then stimulated purified CD4⁺ T cells for 5 days with anti-CD3/CD28 with or without human recombinant IL-21 (rHuIL-21). The adjunction of rHuIL-21 increased T_H17 cells (2.1%-2.6% of T_H17 cells in HDs with and without rHuIL-21 and 3.8%-5.7% in patients with aBD; $P = .04$ and $.03$, respectively) and, to a lesser extent, T_H1 cell frequencies (10.9%-12.0% of T_H1 cells in HDs with and without rHuIL-21 and 12.6%-15.8% in patients with aBD; $P = .30$ and $.31$, respectively) (Fig 6, A-C), and it decreased FoxP3 expression (3.3%-1.8% of FoxP3⁺CD25^{hi}CD4⁺ T cells in HD with and without rHuIL-21 and 0.5%-0.3% in patients with aBD; $P = .004$ and $.03$, respectively) (Fig 6, D and E). The effect of rHuIL-21 in promoting T_H17 differentiation was mainly observed on purified CD25⁺ and CD25⁺⁺ CD4⁺ T cells (Fig 6, F).

Lastly, we analyzed the effect of IL-21 blockade by using IL-21R/Fc chimera on T_H17, T_H1, and Treg cells. The adjunction of IL-21R/Fc decreased the proportion of T_H17 (5.2% to 6.8% and 2.6% of IL-17A-producing CD4⁺ T cells with anti-CD3/CD28 alone, anti-CD3/CD28 plus rHuIL-21, and anti-CD3/CD28 plus IL-21R/Fc, respectively) and T_H1 cells (13.2% to 20.8% and

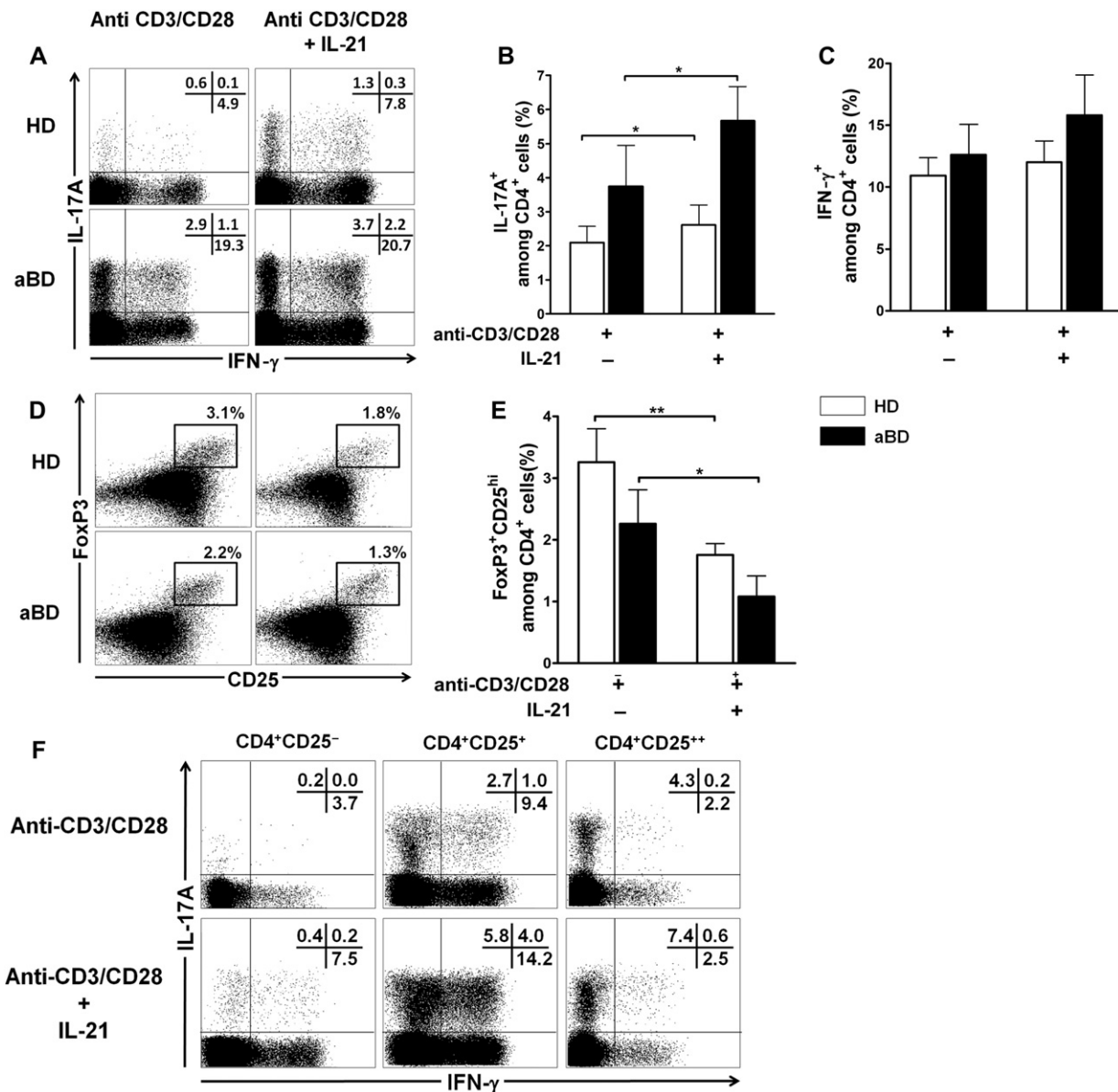


FIG 6. IL-21 is critical in modulating T_H17 response and FoxP3 expression in BD. Dot plots (A) and frequencies of IL-17A (B) and IFN-γ (C)-producing cells and FoxP3⁺ expression (D, E) in purified CD4⁺ T cells in patients with aBD and in HDs after anti-CD3/CD28 stimulation with and without IL-21. IL-21 promotes T_H17 and T_H1 differentiation in CD4⁺CD25⁺ T cells and exclusively promotes T_H17 differentiation in CD4⁺CD25⁺⁺ T cells (F). Histograms are representative of 5 patients in at least 3 independent experiments. **P* < .05; ***P* < .01; ****P* < .001.

11.3% of IFN-γ-producing CD4⁺ T cells, respectively) (Fig 7, A and C) and increased FoxP3⁺ Treg cells (2.5% to 1.5% and 3.4%, respectively) (Fig 7, B and C).

DISCUSSION

Herein, we first demonstrate a marked increase in T_H17 cells and a decreased frequency of Treg cells in peripheral blood that correlate with BD activity. In contrast, the balance between T_H1 and T_H2 differentiation was not tilted. Along this line, mice that were deficient in IRF-4-binding protein, a protein that inhibits IL-17A production by controlling the activity of IRF-4 transcription factor,⁹ rapidly developed a large-vessel vasculitis, sharing

similarities with human BD because of an inappropriate synthesis of IL-17A.¹⁰ The role of T_H17 cells has recently been demonstrated in giant-cell arteritis, another model of human large-vessel vasculitis.¹¹ Strikingly, the addition of serum from patients with aBD in sorted CD4⁺ T cells culture from HDs induced a significant and dose-dependent production of IL-17A and a decrease in FoxP3 expression. We then intended to characterize the mechanism that promotes T_H17 differentiation and suppresses Treg cells. We did not find increased serum levels of T_H17-promoting cytokines such as IL-1β, IL-6, IL-23, and TGF-β in patients with BD compared with HDs. In contrast, serum levels of IL-21 and IL-21-producing CD4⁺ T cells were dramatically increased in peripheral blood from patients with BD.

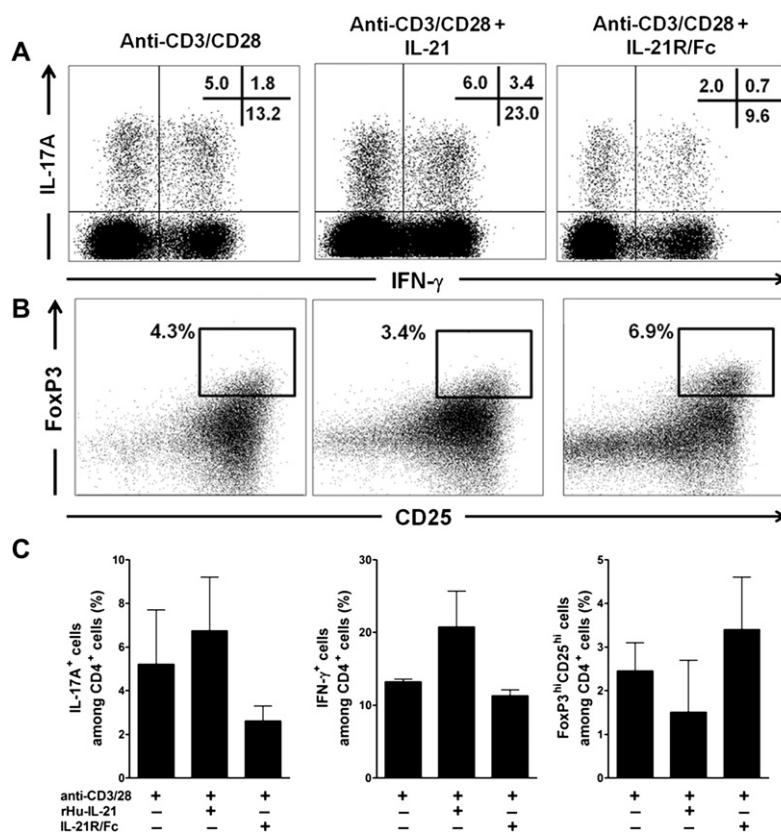


FIG 7. Blockade of IL-21 with IL-21R/Fc restores effector and regulatory T cells homeostasis. Dot plots (**A**, **B**) and histograms (**C**) of IL-17A and IFN- γ production and FoxP3 expression (**B**) in purified CD4⁺ T cells after anti-CD3/anti-CD28 stimulation with and without IL-21 or IL-21R/Fc in patients with aBD. Data are representative of 3 independent experiments in 3 patients with aBD. * $P < .05$; ** $P < .01$; *** $P < .001$.

IL-21 is the most recently identified of the type 1 cytokine-family members.¹² IL-21 is produced by activated CD4⁺ T cells but targets a much broader range of cells.¹³ In patients with BD, IL-21-producing CD4⁺ T cells displayed the phenotype of TCMs. Increased expression of IL-21 has been detected in two mouse models of autoimmunity: the BXS.B6-Yaa⁺/J mouse, which is a model of SLE, and the nonobese diabetic mouse.^{14,15} IL-21 was shown to tilt the balance between Treg cells and T_H17 cells.¹⁶ We observed a decreased frequency of activated/memory Treg cells in patients with BD, whereas the proportion of resting/naive Treg cells was within the normal range. The adjuvant of IL-21 on stimulated purified CD4⁺ T cells decreased FoxP3 expression. Consistent with our findings, IL-21 was recently shown to suppress the conversion of rTreg cells into aTreg cells,^{16,17} supporting the deleterious effect of IL-21 on Treg cells' homeostasis. In addition, mice deficient in IRF-4-binding protein, which rapidly developed a large-vessel vasculitis similar to BD, also showed an inappropriate IL-21 synthesis.¹⁰

We next demonstrated the presence of IL-21- and IL-17A-producing T cells within the CSF, choroid plexus, brain parenchyma inflammatory infiltrates, and intracerebral blood vessels from patients with aBD with CNS involvement. Levels of IFN- γ -producing CD4⁺ and CD8⁺ T cells were also dramatically increased in the CSF from these patients. IL-17A- and IFN- γ -producing T cells are observed in the CSF and inflammatory CNS lesions of patients with multiple sclerosis.^{18,19} Interestingly, we found a strong expression of CCL20, a potent chemoattractant

for T_H17, and CXCL8, known to be a potent chemoattractant for inflammatory polymorphonuclear leukocytes, in the choroid plexus in patients with BD. In addition, the choroid plexus in patients with BD showed strong and uniform expression of IL-21 and IL-17A. IL-21 has proinflammatory effects that result from the induction of the expression of CXCL8 by macrophages. Taken together, our data suggest that IL-21 drives T_H17 effectors' differentiation and chemoattractants for T_H17 and polymorphonuclear leukocytes in CNS inflammatory lesions of patients with BD. Leukocyte entry into the CNS is restricted, in part, because of the blood-brain barrier. However, it is now well established that lymphocytes can also enter the CNS through the choroid plexus.^{20,21} Recently, Reboldi et al provided a molecular and anatomical basis for distinguishing between constitutive and inflammatory pathways of T-cell entry into the CNS, with a critical role for the choroid plexus in the control of immune surveillance of the CNS.²² Their findings in experimental autoimmune encephalitis (EAE) support a 2-step model of EAE pathogenesis in which a first wave of CCR6⁺ T_H17 cells leads to the CCR6-independent recruitment in the CNS of a second wave of T cells, including T_H1 cells and inflammatory leukocytes. Our data in patients with BD with active CNS involvement are consistent with this hypothesis. The presence of T_H1 cells and polymorphonuclear leukocytes, in addition to T_H17 cells, in the brain and CSF of patients with BD supports this hypothesis.²³ IL-17A synergized with TNF- α , a cytokine efficiently targeted in BD,²⁴ for the induction of CXCL8 and CCL20, allows the recruitment of polymorphonuclear leukocytes and T_H17 cells within target tissues.²⁵ A similar

effect of IL-17A, in synergy with TNF- α , on epithelial cells of the choroid plexus in patients with BD could be observed.

Lastly, we showed that IL-21 was critical in modulating T_H17 differentiation and FoxP3 expression in patients with BD. The ad-junction of IL-21 on stimulated purified CD4⁺ T cells increased T_H17 and T_H1 cells' frequencies and decreased FoxP3 expression. The blockade of the IL-21 pathway with IL-21R-Fc restored the balance between Treg cells and T_H17 cells by suppressing IL-17A production and increasing FoxP3 expression by CD4⁺ T cells.

In conclusion, we demonstrate the implication of IL-21 in the peripheral blood and CNS inflammatory lesions of patients with BD. IL-21 modulates T_H17 differentiation and FoxP3 expression and correlates with BD activity. Our findings suggest that IL-21 exerts a critical role in the pathogenesis of BD and represents a promising target for novel therapy.

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Key messages

- IL-21 promotes T_H17 responses and suppresses regulatory T cells in Behçet disease (BD) and correlates with BD activity.
- IL-21- and IL-17A-producing T cells are enriched within inflammatory brain lesions from patients with BD.

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