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Original article

Regulatory T cell/Th17 balance in the pathogenesis of paediatric Behçet disease

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Abstract

Objectives. Behçet disease (BD) is a chronic systemic inflammatory disorder of unknown aetiology. The aim of this study was to determine the orientation of T cell subpopulations in paediatric BD and more precisely to look for a regulatory T lymphocyte (Treg)/Th17 imbalance.

Methods. T cell subpopulations were analysed by flow cytometry in the peripheral blood of paediatric patients with acute BD (aBD; $n = 24$), remitting BD (rBD; $n = 12$) and in healthy controls (HCs; $n = 24$). Tregs ($CD4^+CD25^{hi}CD127^{-/lo}Foxp3^+$), activated Tregs (GITR, LAP, CTLA-4 and HLA-DR expression), $CD4^+$ and $CD8^+$ T cells producing IFN-γ (Th1 and Tc1) or IL-17 (Th17 and Tc17) under polyclonal (OKT3/IL-2) or antigenic (*Streptococcus sanguis* KTH-1 peptides and heat shock protein 60) stimulation were enumerated.

Results. Th17 (1.9- and 5.1-fold) and Tc17 (4.0- and 2.0-fold) frequency under mitogenic stimulation was significantly increased in aBD and rBD patients as compared with HCs. Th17 frequency under antigenic stimulation was also higher in patients than in HCs. The percentage and number of Tregs and activated Tregs in patients and in HCs were similar. However, when Tregs were removed, antigen-driven differentiation into Th1 and Th17 was significantly boosted in BD but not in HC $CD4^+$ T cells.

Conclusion. There is a bias towards Th17 polarization in aBD and rBD in children. Although we did not observe an increase in the number of Tregs in these patients, their Tregs limit $CD4^+$ T cell differentiation into Th1 and Th17 cells. Thus, in paediatric BD, Tregs seem to incompletely counterbalance a Th17 orientation of the Th cell response.

Key words: Behçet disease, Th17, Th1, regulatory T cells, vasculitis, paediatrics

BASIC SCIENCE

Rheumatology key messages

- Paediatric BD is characterized by a normal Treg number and by Th17 polarization.
- Tregs were functional and strongly controlled specific Th17 responses, especially to streptococcal antigens.
- However, they were unable to counterbalance the Th17 response during BD flare.

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Introduction

Behçet disease (BD) is a chronic systemic inflammatory disorder at the crossroads between autoimmune and autoinflammatory syndromes [1]. It is characterized by recurrent attacks involving oral and genital ulcers and the ocular, intestinal, vascular and/or nervous systems [2, 3]. The pathogenesis of BD is still poorly characterized. Certain infectious (*Streptococcus sanguis*) and environmental factors are reported to trigger symptomatology in individuals with particular genetic variants [2]. Among these variants, HLA-B51 is the genetic factor most strongly associated with BD [4]. In addition, more recently, genome-wide association studies have identified a link with the IL-23R and IL-12RB2 loci [5, 6].

Many streptococcal peptides have been proposed to play important roles as extrinsic factors in BD pathogenesis, such as those issued from Bes-1 gene-encoded protein in *S. sanguis*, which are highly homologous with the retinal protein Brn3b and human heat shock protein 60 (HSP-60) [7, 8]. Various authors have argued that BD symptoms could be due to such cross-reactivities [9, 10]. Moreover, as memory T cells and polymorphonuclear leucocytes are found within vasculitic lesions in BD patients, those cells could also play a role in BD pathophysiology [11]. A study performed in adult BD patients found a marked increase in Th17 cells and a decrease in the frequency of $CD4^+Foxp3^+$ Tregs in peripheral blood that were both induced by IL-21 production and correlated with BD activity [12]. However, this study did not assess the effect of Tregs on Th17 or Th1 differentiation, especially in response to specific stimuli such as *S. sanguis* peptides. Here we determine the nature of T cell polarization in BD and test the hypothesis of a Treg/Th17 imbalance in paediatric BD (PBD).

Methods

Patients

The study population consisted in 36 paediatric patients $\leq$18 years of age fulfilling the international criteria for BD [13]. Patients were from the CéréMAI Reference Center for paediatric autoinflammatory and rheumatic diseases in France. Patients with Behçet-like symptoms, especially with recurrent fever, high CRP, young age, gastrointestinal and CNS findings were checked for autoinflammatory diseases and not enrolled in our study. BD patients were enrolled prospectively after informed consent from the legal guardian [14]. The patients were divided into two groups: patients in acute BD (aBD) attack (at least one symptom of BD at inclusion; $n = 24$) and patients in BD remission (rBD) who presented no symptom since at least 3 months after the last BD attack ($n = 12$). Both populations were compared with 24 healthy controls (HCs).

Enumeration of Tregs and other blood cell lines

Foxp3 expression in $CD25^+CD127^{-/lo}CD4^+$ lymphocytes was analysed by flow cytometry after cell-surface staining of Ficoll-isolated peripheral blood mononuclear cells (PBMCs) with anti-CD3-ECD, anti-CD4-PCy7 (Beckman Coulter, Villepinte, France), anti-CD25-PE (BD Biosciences, Le Pont-De-Claix, France) and anti-CD127-FITC (eBioscience, Paris, France) followed by cell permeabilization (Foxp3 Staining Buffer Set; eBioscience, San Diego, CA, USA) and intracellular staining with anti-Foxp3-APC (clone PCH 101; APC anti-human Foxp3 Staining Set; eBioscience). The following labelled antibodies were used in five-colour combinations for flow cytometric analysis of other blood cells: CD8-PCy7, CD8-APC, CD16-FITC, CD19-ECD, CD27-PE, CD28-FITC, CD45RO-FITC, CD45RA-APC, CD56-PE, HLA-DR-PCy7 (Beckman Coulter), CD38-PCy7, CD56-FITC, CD62L-FITC (BD Biosciences), CCR7-PE (R&D Systems, Lille, France). Data were acquired using a Navios flow cytometer and analysed with the Kaluza analysis software (Beckman Coulter) [15, 16].

The blood Th1 and Th17 cell count was analysed as follows: PBMCs were stimulated for 4 h with phorbol 12-myristate 13-acetate (PMA) and ionomycin (Sigma-Aldrich, St. Louis, MO, USA) in the presence of brefeldin A (BD Pharmingen, San Diego, CA, USA), stained with IFN-γ-FITC (BD Biosciences), IL-17A-Alexa Fluor 647 (eBioscience) and IL-21-Alexa Fluor 647 (BioLegend, Saint Quentin en Yvelines, France), and acquired as described above.

Lymphocyte blood cells ($CD3^+$, $CD4^+$, $CD8^+$ T lymphocytes, $CD19^+$ B lymphocytes and $CD3^-CD56^+$ NK cell) counts (cells/μl) were analysed from fresh blood samples using CYTO-STAT tetraCHROME kits with Flowcount fluorescent beads as an internal standard and tetra CXP software with a FC500 cytometer, according to the manufacturer's instructions (Beckman Coulter).

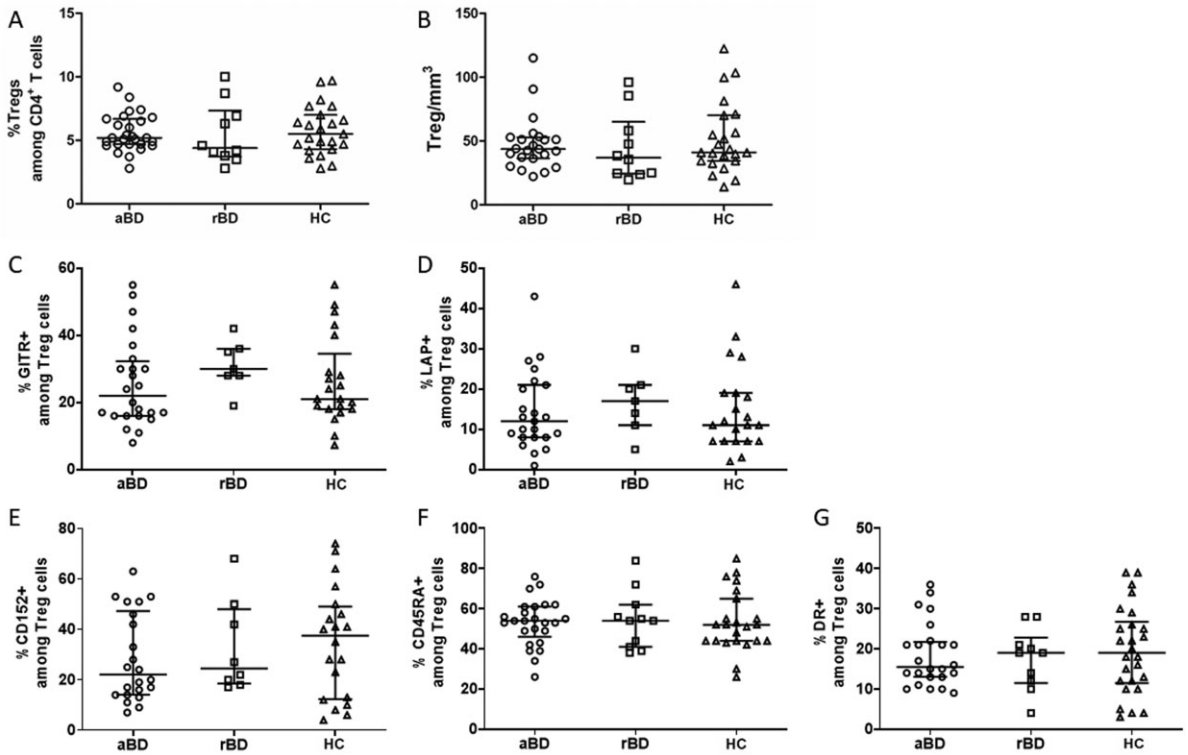
Assessment of Treg functionality

The responses of $CD4^+$ and $CD8^+$ T lymphocytes of BD patients to polyclonal stimulation by OKT3/IL-2 [Orthoclone (Janssen-Cilag, Beerse, Belgium); Proleukin (Novartis, Basel, Switzerland)] and specific antigen stimulation were compared with those from HCs. The antigens were from *S. sanguis* KTH-1 peptides: BES-$1_{229–243}$, BES-$1_{373–385}$ or peptides homologous with the retinal protein Brn3b and human HSP-$60_{336–351}$ (produced by PolyPeptide Group, Strasbourg, France) [7].

PBMCs from BD patients and HCs were isolated with Ficoll density gradient centrifugation and seeded at 8×10^5 cells/well in 48-well plates. Cells were stimulated *in vitro* with BES-1, Brn3b and HSP60 peptides (20 μg/ml) in Roswell Park Memorial Institute culture medium containing, 10% human AB serum, penicillin/streptomycin and 2 mM L-glutamine. After 72 h, cells were collected and restimulated for 4 h at 37°C in 5% carbon dioxide with 50 ng/ml of PMA and 1 mM ionomycin (Sigma-Aldrich) in the presence of a protein transport inhibitor (Golgi plug; BD Biosciences) containing brefeldin A. After stimulation, culture supernatants were collected and kept frozen at −80°C until cytokine determination using ELISA and Luminex technology (Invitrogen, Cergy Pontoise, France). Cells were harvested and stained with the following antibodies: CD3-PE, CD3-PC7, CD4-ECD, CD8-FITC and CD8-PC7 (Beckman Coulter). Cells were then permeabilized with Cytofix/Cytoperm buffer (BD Pharmingen), stained with IFN-γ-FITC (BD Pharmingen), IL-17A-Alexa Fluor 647 (eBiosciences), IL-21-Alexa Fluor 647 (BioLegend) and Perforin-PE (BD Pharmingen). Data were acquired using a Navios flow cytometer and analysed with the Kaluza analysis software (Beckman Coulter).

To assess the regulatory effect of Tregs on effector T cells, Tregs were removed from PBMCs as follows: freshly isolated PBMCs were stained with a biotin-

Fig. 1 Tregs characterization in BD patients and controls



Multicolour flow cytometry analysis of circulating Tregs in aBD patients ($n = 24$), rBD patients ($n = 12$) and HCs ($n = 24$). Tregs were identified as $CD25^{hi}CD127^{-/lo}Foxp3^{+}$ cells among $CD4^{+}$ T cells and are expressed as (A) a percentage of the total $CD4^{+}$ T cells and (B) absolute number. (C–G) Expression of functional markers on Tregs and proportions of naïve Tregs. Data are shown as median (IQR). $^{*}P < 0.05$, $^{**}P < 0.01$. Data corresponding to Fig. 1 are in Supplementary Table S2, available at Rheumatology online.

coupled anti-CD127 antibody (eBiosciences) and then incubated with anti-biotin microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). $CD127^{+}$ cells were isolated using magnetic separation with MS columns (Miltenyi Biotec). Purified $CD127^{+}$ cells (Treg-free PBMCs) were cultured and stimulated as previously described. We verified that the effect of this sorting was specific: an isotype control instead of the anti-CD127 antibody had no effect on immune activation.

Statistical analysis

T lymphocytes and other cell lines were expressed as absolute values and percentages. Tregs were expressed as the percentage of $CD4^{+}$ T cells. For functional tests, results were expressed as the percentage of cells secreting IL-17 and IFN-γ. Data are presented as median [interquartile range (IQR)]. The normal distribution of each set of variables was analysed by the D'Agostino and Pearson omnibus normality test. According to their non-parametric distribution, groups were compared using the Kruskall–Wallis test. Non-parametric multicomparison tests (Kruskall–Wallis) were performed to compare three or more groups and then a Mann–Whitney–Wilcoxon test was performed to compare groups 2×2. The comparison tests of groups 2×2 were performed only when the comparison of the three groups by Kruskall–Wallis test was significant ($P < 0.05$). In this case a penalization of the alpha risk by the method of Benjamini and Hochberg was applied. When only two groups were compared 1×1, a Mann–Whitney–Wilcoxon test were performed as appropriate. *P*-values <0.05 were considered significant. Statistical analyses were performed using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, USA).

Ethical approval

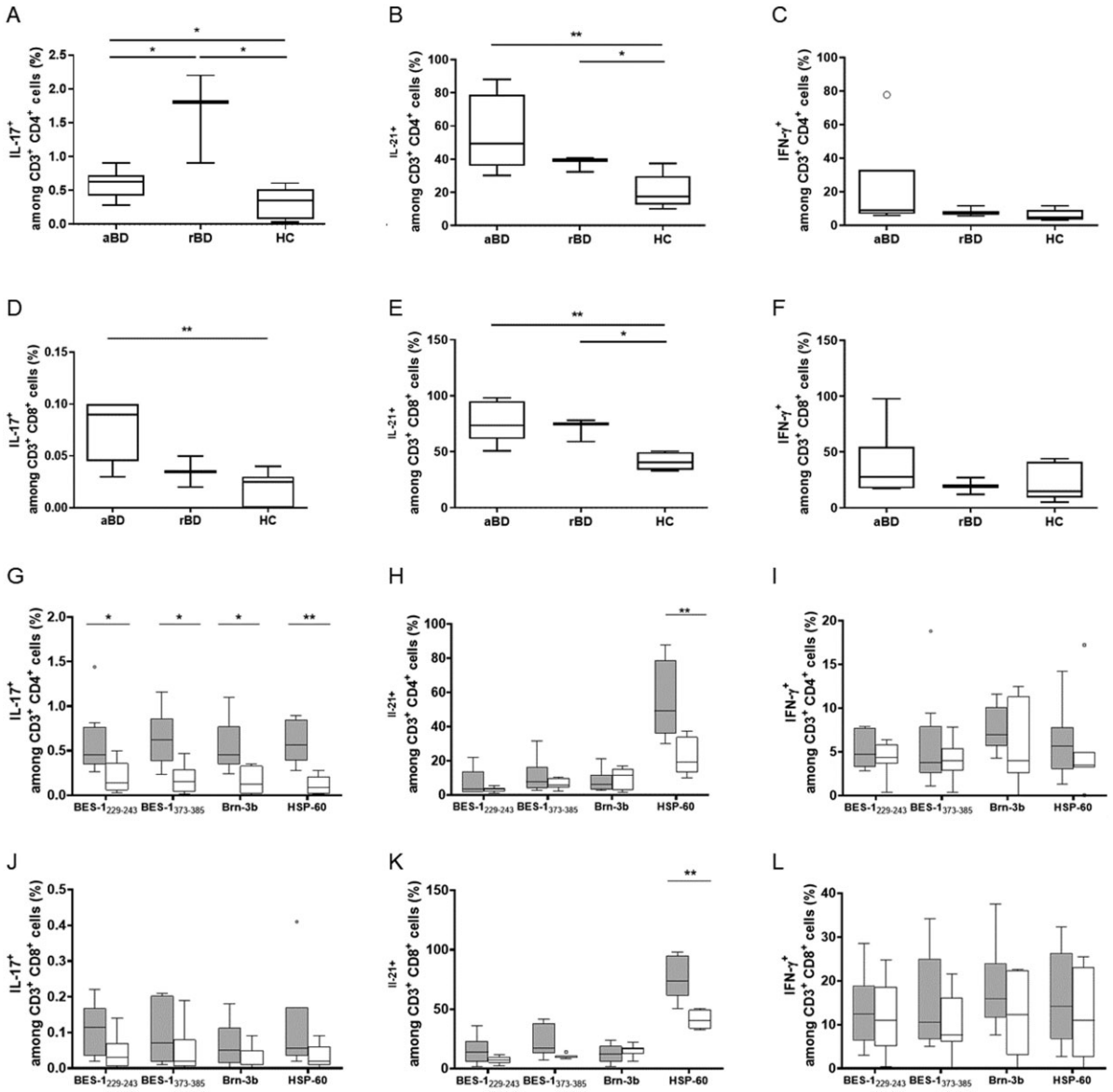
The study was approved by the ethical committee of Ile de France 3 (0811721).

Results

Studied population

In total, 24 aBD patients and 12 rBD patients were compared with 24 HCs. All data are reported as median (IQR). The three groups were comparable according to sex and age (Supplementary Table S1A, available at *Rheumatology* online). The characteristics of the

FIG. 2 Increased Th17 effectors in BD patients upon polyclonal and specific stimulation



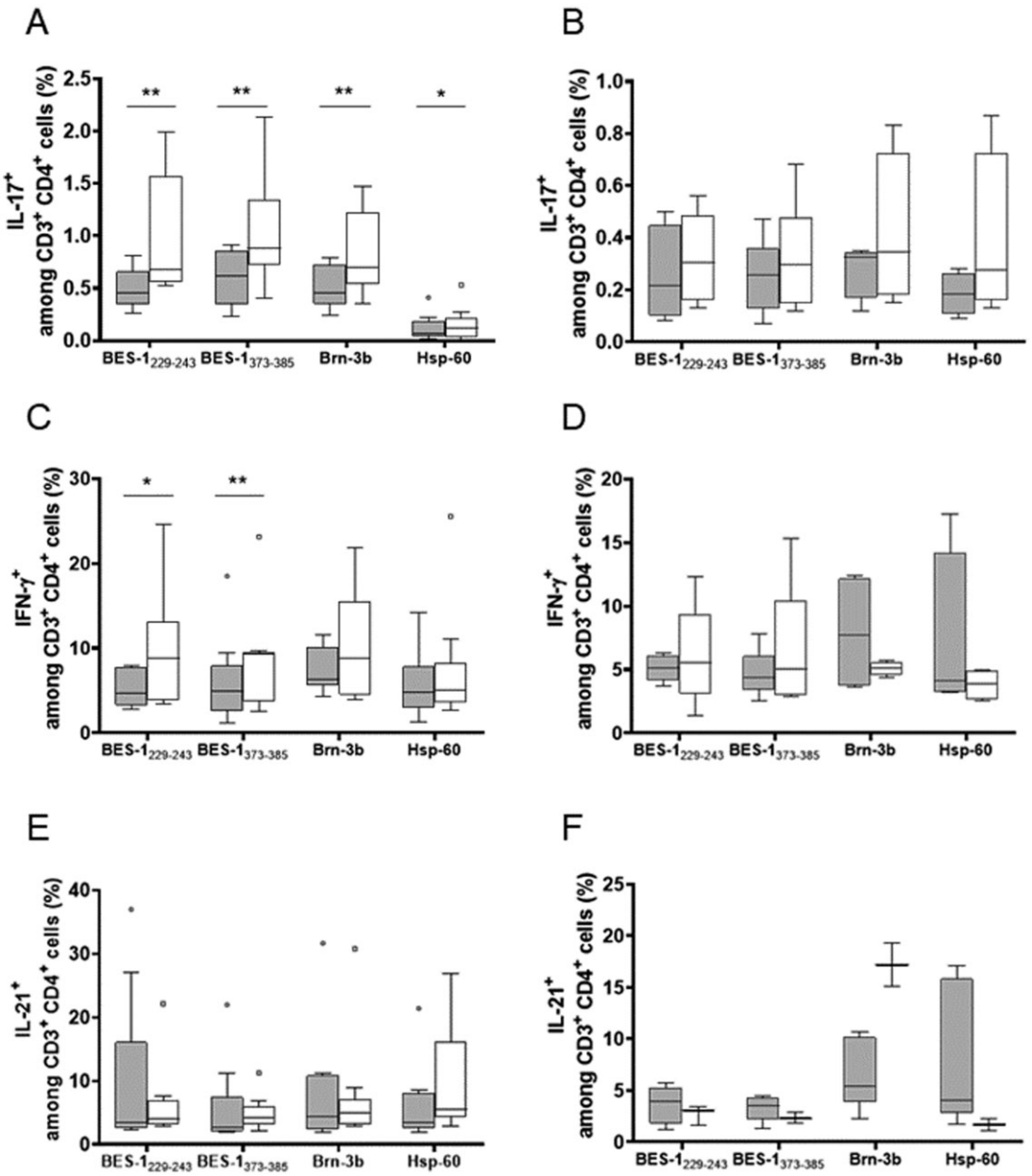
Upon polyclonal stimulation: frequencies of IL-17A, IL-21-producing and IFN-γ (**A–C**) CD4$^+$ and (**D–F**) CD8$^+$ T cells from six aBD patients (closed bar), three rBD patients (grey bar) and eight HCs (open bar). Upon specific stimulation: frequencies of IL-17A, IL-21-producing and IFN-γ-producing (**G–I**) CD4$^+$ and (**J–L**) CD8$^+$ T cells from nine BD patients (closed bar) and nine HCs (open bar). Data are shown as median (IQR). Differences between groups were evaluated with non-parametric Mann–Whitney U-test. *$P < 0.05$, **$P < 0.01$. Data corresponding to Fig. 2 are in Supplementary Table S3A, available at Rheumatology online.

patients' symptoms and treatment at inclusion are summarized in Supplementary Table S1B, available at *Rheumatology* online. Ten aBD patients received no treatment (42%), 11 (46%) were treated with colchicine alone ($n = 7$) or associated with an immunosuppressive agent (Azathioprine/corticosteroids). Five rBD patients had no treatment at inclusion and the remaining patients were on colchicine alone ($n = 2$) or associated with corticosteroids and Mycophenolate mofetil or Azathioprine.

Tregs in BD patients

There were no significant differences considering percentages [5.2% (2.00) *vs* 4.4% (3.63) *vs* 5.5% (2.70)] and absolute numbers [cells/mm^3: 43.7 (16.49) *vs* 37.0 (40.50) *vs* 41.0 (35.70)] between aBD patients, rBD patients and HCs, respectively (Fig. 1 and Supplementary Table S2, available at *Rheumatology* online). The proportions of naïve Tregs (CD4$^+$CD25^{hi}CD45RA$^+$ cells) as well as the expression of functional markers on Tregs (glucocorticoid-induced TNF

Fig. 3 Evaluation of Th effector response after Tregs depletion upon specific stimulation



Frequencies of IL-17A, IFN-γ and IL-21-producing $CD4^+$ T cells (closed bar) and Treg-depleted $CD4^+$ T cells (open bar) from (**A, C** and **E**) nine BD patients and (**B, D** and **F**) six HCs. Data are shown as median (IQR). Differences between groups were evaluated with a non-parametric Wilcoxon rank test. $^*P < 0.05$, $^{**}P < 0.01$. Data corresponding to Fig. 3 are in Supplementary Table S3B, available at *Rheumatology* online.

receptor (GITR), latency-associated peptide (LAP), CTLA-4 and Human Leukocyte Antigen DR isotype (HLA-DR)) were similar in the three groups (Fig. 1 and Supplementary Table S2, available at *Rheumatology* online).

Other blood cell subsets counts

Absolute counts of $CD3^+$, $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells as well as for their repartition in naïve and memory subsets and the expression of the activation markers HLA-DR, CD25 and CD28 were also comparable in all groups (Supplementary Table S2, available at *Rheumatology* online). The rBD group also showed fewer NK cells compared with other groups (Supplementary Table S2, available at *Rheumatology* online). The absolute number of B cells did not differ between groups. However, a decreased percentage of naïve and an increased percentage of switched memory

B cells were observed in patients with aBD ($P = 0.04$ and $P < 0.001$, respectively) compared with HCs (Supplementary Table S2, available at *Rheumatology* online).

Assessment of lymphocyte responses to stimulation and Treg functionality

The T lymphocyte responses of nine aBD and nine rBD patients to polyclonal stimulation (anti-CD3/IL-2) were compared with those of eight HCs. As shown in Fig. 2 and Supplementary Table S3A, available at *Rheumatology* online, aBD and rBD patients showed, compared with HCs, a significantly higher percentage of Th17 [0.63% (0.310), 1.81% (1.300) and 0.35% (0.440), respectively, Fig. 2A] and Tc17 cells [0.09% (0.060), 0.04% (0.030) and 0.03% (0.030), respectively, Fig. 2D], as well as IL-21-secreting $CD4^+$ [49.3% (42.90), 39.4% (8.14) and 17.6% (17.20), respectively, Fig. 2B] and $CD8^+$ [73.4% (33.72), 74.7% (19.19) and 40.4% (15.76), respectively, Fig. 2E] T cell response rate. No difference was observed for Th1 and Tc1 response (Fig. 2C and F).

Specific stimulation with streptococcal antigen (*S. sanguis* KTH-1: peptide BES-$1_{229–243}$, BES-$1_{373–385}$) and homologous peptide of retinal antigen Brn-3b and HSP-$60_{336–351}$ of the same nine aBD patients and eight HC PBMCs was then performed (Fig. 2 and Supplementary Table S3A, available at *Rheumatology* online). Compared with HCs, the responses in BD patients were mainly Th17 and Tc17 (Fig. 2G and J) rather than Th1 and Tc1 (Fig. 2I and L). Increased percentages of IL-21-secreting $CD4^+$ and $CD8^+$ T cells were observed in the BD group upon HSP-$60_{336–351}$ stimulation, but not with the other antigens (Fig. 2H and K).

As blood samples are quantitatively limited in children, the functionality of Tregs was studied via negative selection. Thus we compared the response to antigenic stimuli of PBMCs *vs* Treg-depleted PBMCs. In this assay, the increase in the effector T cell response after removing Tregs reflects the inhibitory effect of Tregs on effector cells. These tests were performed on samples of nine aBD patients and six HCs (Fig. 3 and Supplementary Table S3B, available at *Rheumatology* online). As most of the rBD patients in our cohort were on immunosuppressive drugs or colchicine, we did not select them for these experiments.

After removing Tregs ($CD127^{-/lo}$), there was an increase in IFN-γ^+CD4^+ T cell response to BES-$1_{229–243}$ and BES-$1_{373–385}$ stimulation in BD patients (respectively, 1.82- and 1.42-fold) (Fig. 3C) and an increase in IL-17^+CD4^+ T cell response to all the peptides tested in BD patients (respectively, 1.94- and 1.72-fold) (Fig. 3A) but not in HCs (Fig. 3B and D). Neither BD patients nor HCs showed any alteration in IL-21^+CD4^+ T cell response upon stimulation (Fig. 3E and F).

In summary, Th1 responses to streptococcal antigens were similar in BD patients and HCs. However, Th17 responses to polyclonal and specific stimulations were significantly higher in aBD patients compared with controls. When Tregs were removed from T cells, specific T cell responses were markedly increased in BD patients compared with controls.

Discussion

Th1, Th17 and Tregs seem to play key roles in adult BD pathogenicity [11, 12]. To test whether this is also the case in children, we analysed these $CD4^+$ T cell subpopulations during acute or remittent PBD. For this we measured the frequency of circulating Tregs presenting or not with cell surface activation markers. We also monitored the induction of Th1 and Th17 cells under *in vitro* polyclonal or antigenic stimulation in the presence or absence of Tregs.

We observed an increase in $CD45RA^-$ Tregs in rBD patients as compared with controls and with aBD patients. Regarding naïve/resting $CD45RA^+$ and memory/activated $CD45RA^-$ Tregs [17], we showed a higher frequency of activated Tregs in rBD patients. It is tempting to speculate that this link might be causative, i.e. the remission might be due to an overactivity of Tregs. In contrast, we did not observe any overexpression of three other cell surface markers previously linked to Treg activity: (GITR, LAP and HLA-DR). GITR-expressing Tregs have been shown to be fully active and expanded in SLE patients, particularly in those with inactive disease [18]. Likewise, HLA-DR [19] and LAP [20] were shown to be cell surface markers of Treg functionality. Yet we did not observe neither GITR, LAP or HLA-DR overexpression on Tregs from PBD patients. It would be interesting to test whether other cell surface markers of Treg activity, such as the recently described sialyl Lewis x [21], are also overexpressed during BD remission.

A second finding of our work is the Th17 polarization in active BD that is seen under polyclonal as well as streptococcal *in vitro* stimulation. This observation supports the involvement of Th17 cells in BD. Several authors have reported the high proportion of peripheral T cells able to produce IL-17 under mitogenic or antigenic stimulation in adults with active BD [22–24]. Moreover, genome-wide association studies have established associations with a variant located in close proximity to the gene encoding the receptor for IL-23, a major inducer of Th17 differentiation [5, 6, 25]. This variant resulted in higher IL-23 receptor expression and, under *in vitro* activation via CD3 and CD28, in higher IL-17 production [26]. Moreover, sera from BD patients were shown to orientate normal primary $CD4^+$ T cells towards Th17 differentiation [12] and to induce Foxp3 degradation [27], potentially participating in the Th17/Treg imbalance.

In our study, the elevated presence of a Th17 subpopulation in active BD is further emphasized when Tregs are removed from the culture in the presence of a bacterial antigen. This observation is another argument for an inhibitory effect of Tregs, an effect that could be insufficient during flares but efficient during remissions. Of note, the negative selection of Tregs also uncovers a Th1 polarization. These findings concur with previous

reports showing the concomitant involvement of Th1 cells in BD [28, 29].

Our observations that *S. sanguis* peptides induce Th1 and Th17 responses in BD children are a further argument for a role of streptococcal antigens in this disease. Previously, indirect evidence, such as the presence of streptococcal species, of antibodies specific for the BES-1 protein of *S. sanguis* or of hypersensitivity to streptococcal skin tests [30], was already suggestive of such a role. Our data now provide more direct proof.

Altogether, our data support a model whereby a Th1/Th17 polarization, possibly driven by bacterial components in individuals with a favourable genetic background, such as the rs17375018 variant in the *IL-23R* gene [31], fuels paediatric BD recurrences until the inhibitory effect of Tregs leads to remission. Given the role of IL-21 in Th17 differentiation and in Treg suppression, this gives further credit to IL-21 blockade as a strategic approach. Our findings need to be confirmed in an independent study.

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A.F. analysed the results and wrote the first draft of the article. T.A.T. and M.R. conceived the design of the study, supervised immune monitoring, analysed results and participated in the first draft of the article. A.H. and G.C. performed the experiments and analysed data. A.L. performed statistical analysis of the data. T.A.T, D.S. and I.K.-P. were clinical investigators of the study. P.C. helped analyse results and write the article. All authors approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

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Data availability statement

The data underlying this article are available in the article and its online supplementary material.

Supplementary data

Supplementary data are available at *Rheumatology* online.

References

1 McGonagle D, McDermott MF. A proposed classification of the immunological diseases. PLoS Med 2006;3:e297.

2 Sakane T, Takeno M, Suzuki N, Inaba G. Behçet's disease. N Engl J Med 1999;341:1284–91.

3 Saadoun D, Wechsler B, Desseaux K *et al.* Mortality in Behçet's disease. Arthritis Rheum 2010;62:2806–12.

4 Ohno S, Ohguchi M, Hirose S *et al.* Close association of HLA-Bw51 with Behçet's disease. Arch Ophthalmol 1982;100:1455–8.

5 Remmers EF, Cosan F, Kirino Y *et al.* Genome-wide association study identifies variants in the MHC class I, IL10, and IL23R-IL12RB2 regions associated with Behçet's disease. Nat Genet 2010;42:698–702.

6 Mizuki N, Meguro A, Ota M *et al.* Genome-wide association studies identify IL23R-IL12RB2 and IL10 as Behçet's disease susceptibility loci. Nat Genet 2010;42:703–6.

7 Kaneko F, Oyama N, Yanagihori H *et al.* The role of streptococcal hypersensitivity in the pathogenesis of Behçet's disease. Eur J Dermatol 2008;18:489–98.

8 Kulaber A, Tugal-Tutkun I, Yentur SP *et al.* Pro-inflammatory cellular immune response in Behçet's disease. Rheumatol Int 2007;27:1113–8.

9 Lehner T, Lavery E, Smith R *et al.* Association between the 65-kilodalton heat shock protein, *Streptococcus sanguis*, and the corresponding antibodies in Behçet's syndrome. Infect Immun 1991;59:1434–41.

10 Tanaka T, Yamakawa N, Koike N *et al.* Behçet's disease and antibody titers to various heat-shock protein 60s. Ocul Immunol Inflamm 1999;7:69–74.

11 Hirohata S. Histopathology of central nervous system lesions in Behçet's disease. J Neurol Sci 2008;267:41–7.

12 Geri G, Terrier B, Rosenzwajg M *et al.* Critical role of IL-21 in modulating TH17 and regulatory T cells in Behçet disease. J Allergy Clin Immunol 2011;128:655–64.

13 International Study Group for Behçet's Disease. Criteria for diagnosis of Behçet's disease. Lancet 1990;335:1078–80.

14 Brustle A, Heink S, Huber M *et al.* The development of inflammatory T(H)-17 cells requires interferon-regulatory factor 4. Nat Immunol 2007;8:958–66.

15 Saadoun D, Rosenzwajg M, Joly F *et al.* Regulatory T-cell responses to low-dose interleukin-2 in HCV-induced vasculitis. N Engl J Med 2011;365:2067–77.

16 Rosenzwajg M, Churlaud G, Mallone R *et al.* Low-dose interleukin-2 fosters a dose-dependent regulatory T cell tuned milieu in T1D patients. J Autoimmun 2015;58:48–58.

17 Miyara M, Yoshioka Y, Kitoh A *et al.* Functional delineation and differentiation dynamics of human $CD4^+$ T cells expressing the FoxP3 transcription factor. Immunity 2009;30:899–911.

18 Nocentini G, Alunno A, Petrillo MG *et al.* Expansion of regulatory $GITR^+CD25^{low/-}CD4^+$ T cells in systemic lupus erythematosus patients. Arthritis Res Ther 2014;16:444.

19 Baecher-Allan C, Wolf E, Hafler DA. MHC class II expression identifies functionally distinct human regulatory T cells. J Immunol 2006;176:4622–31.

20 Tran DQ, Andersson J, Hardwick D *et al.* Selective expression of latency-associated peptide (LAP) and IL-1

receptor type I/II (CD121a/CD121b) on activated human FOXP3^{+} regulatory T cells allows for their purification from expansion cultures. Blood 2009;113:5125–33.

21 Miyara M, Chader D, Sage E *et al.* Sialyl Lewis x (CD15s) identifies highly differentiated and most suppressive FOXP3^{high} regulatory T cells in humans. Proc Natl Acad Sci USA 2015;112:7225–30.

22 Takeuchi M, Usui Y, Okunuki Y *et al.* Immune responses to interphotoreceptor retinoid-binding protein and S-antigen in Behçet's patients with uveitis. Invest Ophthalmol Vis Sci 2010;51:3067–75.

23 Chi W, Zhu X, Yang P *et al.* Upregulated IL-23 and IL-17 in Behçet patients with active uveitis. Invest Ophthalmol Vis Sci 2008;49:3058–64.

24 Hamzaoui K, Bouali E, Ghorbel I *et al.* Expression of Th-17 and RORγt mRNA in Behçet's disease. Med Sci Monit 2011;17:CR227–34.

25 Jiang Z, Yang P, Hou S *et al.* IL-23R gene confers susceptibility to Behçet's disease in a Chinese Han population. Ann Rheum Dis 2010;69:1325–8.

26 Jiang Z, Hennein L, Tao Y, Tao L. Interleukin-23 receptor gene polymorphism may enhance expression of the IL-23 receptor, IL-17, TNF-α and IL-6 in Behçet's disease. PLoS One 2015;10:e0134632.

27 Yang XO, Nurieva R, Martinez GJ *et al.* Molecular antagonism and plasticity of regulatory and inflammatory T cell programs. Immunity 2008;29:44–56.

28 Raziuddin S, al-Dalaan A, Bahabri S, Siraj AK, al-Sedairy S. Divergent cytokine production profile in Behçet's disease. Altered Th1/Th2 cell cytokine pattern. J Rheumatol 1998;25:329–33.

29 Hamzaoui K, Hamzaoui A, Guemira F *et al.* Cytokine profile in Behçet's disease patients. Relationship with disease activity. Scand J Rheumatol 2002;31:205–10.

30 Lehner T. The role of heat shock protein, microbial and autoimmune agents in the aetiology of Behçet's disease. Int Rev Immunol 1997;14:21–32.

31 Yalcin B, Atakan N, Dogan S. Association of interleukin-23 receptor gene polymorphism with Behçet disease. Clin Exp Dermatol 2014;39:881–7.