$CD4^+CD25^+$ regulatory T cells in patients with Behçet's disease

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ABSTRACT

Objective. *To investigate whether the $CD4^+CD25^+$ regulatory T cell (Treg) population, which plays important role in autoimmune diseases is related to the pathophysiology of Behçet's disease (BD).*

Methods. *Forty-two patients with BD (20 patients in active disease) fulfilling the criteria of the International Study Group of BD. Twenty age-matched healthy controls were studied. We analyzed $CD4^+CD25^{+/high}$ T cells and the mRNA expression of Foxp3, cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), and transforming growth factor β (TGF-β) in BD. We have studied the ability of $CD4^+CD25^+$ (Treg) to regulate proliferation of $CD4^+CD25^-$ T cells during active BD stage.*

Results. *Active BD patients had significantly higher $CD4^+CD25^{+/high}$ T cells, as compared with BD in the remission stage, and healthy controls. There was no significant differences in the CD4+ $CD25^{+/high}$ T cells expression between healthy controls and remission BD. In active BD, mRNA for Forkhead box p3 (Foxp3) and cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) were highly expressed when compared to remission BD and healthy controls. There was no differences in the mRNA expression for TGF-β in active BD, remission BD and healthy controls. Functionally, $CD4^+CD25^{+/high}$ T cells in active BD were impaired in their proliferative responses and could suppress the proliferation of their $CD4^+CD25^-$ counterparts.*

Conclusion. *These data demonstrate that $CD4^+CD25^+$ Treg cells, with the potential to regulate suppression of effector T cells, were increased in the peripheral circulation of active BD patients. The role of $CD4^+CD25^{+/high}$ T cells in the regulatory process of the inflammation in active BD, could be taken in account.*

Introduction

Behçet's disease (BD) is a multisystem inflammatory disease, characterized by recurrent attacks of uveitis, oral aphthous, genital ulcerations, skin and joint lesions of variable severity, complicated by central nervous manifestations (1, 2). BD could be in part considered as an autoimmune disease (3). The aetiology of BD remains unknown, but the most widely held hypothesis of disease pathogenesis is that a profound inflammatory response is triggered by an infectious agent in a genetically susceptible host. Investigation of the aetiology of BD has focused predominantly on herpes simplex virus immunopathology, streptococcal infection and autoimmunity to oral or cross-reactive microbial antigens (4, 5). The human 60-kD HSP are involved in the development of BD and suggested an activated innate immunity (6, 7).

$CD4^+CD25^+$ T cells, which constitute 3% to 7% of peripheral $CD4^+$ T cells in human subjects, maintain immunologic self-tolerance (8-10). Removal of this population from normal rodents leads to the spontaneous development of various autoimmune diseases. Recent reports have documented that $CD4^+$ $CD25^+$ regulatory T (Treg) cells play critical roles not only in preventing autoimmunity but also in controlling various immune reactions against bacteria, viruses, fungi, and intracellular parasites (11-14). In the periphery, $CD4^+CD25^+$ regulatory T cells constitutively express a variety of phenotypic markers that has allowed a further characterization and subdivision of this cell population: glucocorticoid-induced TNFR (GITR) (15), OX40 (16), CD103 (17), and CTLA-4 (CD152) (18-19). However, their precise role in the predominantly contact-dependent activity of $CD4^+CD25^+$ regulatory T cells remains controversial and the topic of ongoing investigations. It is

Table I. Characteristics of 20 active patients with Behçet's disease (BD). Overall burden of the disease manifestations in BD patients. Patients received colchicine, steroids/cyclosporine. Five patients with oral ulcer, genital ulcer, uveitis and vasculo-symptoms were newly diagnosed.

	Number of patients (%)	
Major criteria		
Oral ulcer	20	(100)
Genital ulcer	20	(100)
Skin lesions (erythema nodosum, folliculitis)	15	(75)
Uveitis	20	(100)
Minor criteria		
Arthritis	18	(90)
Vasculo-symptoms (*)	20	(100)
Intestinal symptoms	08	(40)

*cerebral vasculitis (n = 7), pulmonary aneurysms (n = 4), retinal vasculitis (n = 9).

also important to note that these markers, similar to expression of CD25, are differentially regulated upon activation of T cells and thus do not permit a categorical distinction between regulatory and recently activated T cells. Therefore, identification of Foxp3 as a signature transcription factor for development and function of $CD4^+CD25^+$ regulatory T cells in mice appeared to offer a solution for this dilemma (20-22). Yet, a recent report demonstrated that activation of human T cells, in contrast to mouse T cells, upregulates expression of Foxp3 and induces regulatory activity in $CD4^+CD25^-$ T cells (23). Although discrepancies between activation-induced Foxp3 expression in murine and human $CD4^+CD25^-$ T cells, potentially the consequence of naive *vs* memory T cell populations investigated (23), remain to be studied in greater detail. The work by Walker *et al.* supports the contention that the particular phenotype of regulatory T cells may arise at least via two pathways, thymic selection, and peripheral T cell activation. The importance of latter pathway is further documented by the intriguing observation that TCR activation of murine $CD4^+CD25^-$ T cells in the presence of transforming growth factor-β (TGF-β) induced CD25, CTLA-4 as well as Foxp3 expression and was associated with the acquisition of *in vitro* and *in vivo* regulatory activity by these cells (24).

We undertook this study to explore the presence and characteristics of $CD4^+$ $CD25^+$ Treg cells in patients with BD. We show that the frequency of $CD4^+$ T cells expressing $CD25^{+/high}$ is increased significantly in active BD compared to the remission BD stage. No significant difference was observed in the numbers of $CD4^+$ $CD25^{+/high}$ T cells in peripheral blood from remission BD patients and healthy controls. Phenotypically, $CD4^+$ $CD25^{+/high}$ T cells express high levels of mRNA expression of Foxp3 and CTLA-4. Importantly, isolated $CD4^+$ $CD25^{+/high}$ cells showed the classic functional characteristic of Treg cells, they suppressed the proliferation of $CD4^+$ $CD25^-$ T cells.

Table II. Characteristics of 22 remission patients with Behçet's disease (BD). Overall burden of the disease manifestations in BD patients. Patients were treated: colchicine (oral ulcer, genital ulcer); steroids and colchicine (uveitis, arthtritis).

	Number of patients (%)	
Major criteria		
Oral ulcer	7	(31.8)
Genital ulcer	2	(9.0)
Skin lesions (erythema nodosum, folliculitis)	2	(9.0)
Uveitis	2	(9.0)
Minor criteria		
Arthritis	1	(4.5)
Intestinal symptoms	2	(9.0)

Materials and methods

Patients and control subjects

We studied 42 patients with BD (20 patients in active stage), and 20 healthy controls. The mean age of active BD patients (3 females and 17 men) was 32 years (range, 27-52 years) and the mean duration of disease was 74 months (range 10-168 months). The mean age of patients in remission BD (2 females and 20 men) was 42 years (range 35-47 years) and the mean duration of the disease was 128 months (range 22-176 months). All patients (5 females and 37 males) fulfilled the criteria defined by the International Study Group for diagnosis of Behçet's disease (25). Disease activity was evaluated according to the published criteria (26). Clinical features of the patients with active and remission stages are given in Table I and Table II. Tables describe overall burden of the disease manifestations in BD patients. Active BD patients (15/20 patients) were always treated with steroids and colchicine. Five patients with active disease were newly diagnosed and received treatment before venipuncture. No patient was twice studied both during active and remission stages. Twenty healthy volunteers matched for age and sex (17 men and 3 women; age range 32-60 years, mean 42 years) were included as control subjects, none of them had evidence of acute infection or chronic disease (e.g., other autoimmune or atopic disorders). Venous blood samples (20 ml) were collected aseptically into tubes with anti-coagulant. The design of the study was approved by our National Ethics Committee.

Flow cytometry

Flow cytometry was performed by using an EPICS XL (Immunotech Coulter), as described previously (27). The forward and side light scatter gate was set to analyze viable cells and to exclude background artifacts. Multicolor staining was carried out with fluorescein isothiocyanate-, phycoerythrin-, or phycoerythrin-cyanin 5·1-conjugated monoclonal antibodies against CD25, CD3, and CD4 (R&D Systems Europe Ltd). Three-color flow cytometric analysis was performed on cells within the lymphocyte light scatter gate by using forward and side scatters. The percentage of $CD4^+CD25^+$ T cells was determined by the ratio of $CD4^+CD25^+$ T cells in lymphocytes. A flow cytometric enumeration and gating strategy of $CD4^+CD25^+$ T cells was represented in Figure 1A. The $CD4^+$ $CD25^{high}$ fraction was determined by fluorescence intensity > 10^2, with the same setting used in all BD patients and healthy controls. The gating strategy is shown for the analysis of $CD4^+$ $CD25^{low}$ (R_3) and CD4+$CD25^{high}$ (R_4) in one patient with active BD and in one patient in the remission stage.

Cell isolation

Peripheral blood lymphocytes (PBL) were prepared by centrifugation over Ficoll-Hypaque gradients (Pharmacia, Uppsala, Sweden) and $CD4^+$ cells were purified by negative selection, by depletion of cells expressing CD8, CD11b, CD16, CD19, CD36, and CD56 with the $CD4^+$ No-touched T cell isolation kit (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany).

After isolation of $CD4^+$ cells, $CD25^+$ cells were stained with PE-coupled anti-CD25 mAb and purified after the addition of anti-PE-coupled magnetic beads (Miltenyi Biotec). Starting with $2x10^8$ peripheral blood lymphocytes, typically $3x10^6$ $CD4^+CD25^+$ cells were obtained with a purity ranging from 90% to 94%. In patients and healthy controls, CD25 Microbeads (Miltenyi) were used to further isolate $CD25^+$ and $CD25^-$ cells from $CD4^+$ cell preparations. The purity of the cell preparations was always analysed using FACS. Cells obtained using this method were typically > 98% $CD3^+$ $CD4^+$ cells. The purity of $CD25^+$ fraction was 80%. CD25 depleted cells contained less than 3% of contaminating $CD25^+$ cells. An anti-CD69 antibody (BD Pharmingen) was used to evaluate the activation status of the population sorted. $CD4^+$ $CD25^{+/high}$ from both BD patients and healthy controls did not express CD69 indicating that the population we sorted was not contamined by recently activated T cells.

Proliferation assays

$CD4^+$ $CD25^{+/high}$ and $CD4^+$ $CD25^-$ T cells ($5\text{-}7.5x10^4$/well) were cultured in triplicate in 200 µl RPMI 1640 (GIBCO BRL, Grand Island, NY, USA) supplemented with 10% heat inactivated AB-serum (GIBCO BRL, Grand Island, NY, USA), 0.1 m M 2-mercaptoethanol (Sigma, St Louis, MO, USA), 10 m M HEPES (GIBCO BRL) and 100 µg/ml gentamicin in flat-bottom 96-well microtitre plates (Costar® 3599, Costar, Cambridge, MA, USA) for 4 days. Anti-CD3 (1 µg/ml) + anti-CD28 (10 µg/ml) MoAbs were added in the beginning of the culture. Tritiated thymidine (1.0 µCi/well) was added 16-18 h before terminating the culture. The cells were harvested to glass fibre filters with a 96-well harvester (Tomtec, Orange, CT, USA). The amount of incorporated ^{3}H-thymidine was determined by liquid scintillation spectroscopy (β-counter, Canberra Packard Ltd., Pangbourne, UK). Assays analysing the inhibitory capacity of $CD4^+$ $CD25^+$ T cells were performed under the same conditions. In these assays $5x10^4$ $CD4^+$ $CD25^-$ T cells were cultured in triplicate with or without $1.25x10^4$ or $2.5x10^4$ $CD4^+$ $CD25^{+/high}$ T cells and anti-$CD3^+$ anti-CD28 MoAbs in U-bottomed 96-well microtitre plates (Nunclon®, InterMed/Nunc, Denmark).

Quantitative reverse transcriptase-polymerase chain reaction by using the TaqMan method

Total RNA was extracted from cells by using an RNA extraction kit (Qiagen, Valencia, Calofirnia, USA), according to the manufacturer's instructions. A high-molecular-weight carrier, ethachinmate (Qiagen Gene), was added to RNA solution before the isopropyl alcohol precipitation to increase the recovery. The cDNA was synthesized with a first-strand cDNA synthesis kit (Amersham Pharmacia Biotech) by using random hexamers. Foxp3 mRNA expression was analyzed by using the Assays-on-Demand Gene Expression product (Applied Biosystems). This product consists of a 20x mix of unlabeled polymerase chain reaction (PCR) primers and TaqMan MGB probe (FAM dye-labeled). Primer sequences for Foxp3 were: Foxp3 forward; 5'-TCATCCGCTGGGCCATCCTG-3, and Foxp3 reverse; 5'-GTGGAAACC-TCACTTCTTGGTC-3' (SuperArray Bioscience Corporation, GEArray S Series Human Autoimmune and Inflammation Responses. HS-602.3, USA). The PCR primers and TaqMan probes of CTLA4 and TGF-β were designed with the assistance of the computer program PRIMER EXPRESS (PE Biosystems) on the basis of information obtained from the GenBank database. Nucleotide sequences of PCR primers and TaqMan probe were as follows (Assays-on-demand gene expression assays; Applied Biosystem): CTLA4 forward primer (F), 5'-CAG-TGGAAATCAAG TGAACCTCAC-3'; CTLA4 reverse primer (R), 5'-GCACGGTT CTGGATCAATTACA-3'; CTLA4 TaqMan probe, 5'-TGGA-GCTCAT GTACCC ACCGCC ATAC-TA-3'; TGF-β F, 5'-ATTGCTTC AGC TCCA CGGA-3'; TGF-β R, 5'-CCCG-GGTTATGCTGGTTGTAC-3'; TGF-β TaqMan probe, 5'-CAGCTGTACATT GACTTCCGCA AGGACCT-3' Each TaqMan probe was labeled at the 5' end with the reporter dye molecule FAM (6-carboxyfluorescein; emission I, 538 nm). A TaqMan β-actin control reagent kit was used as an internal control. These TaqMan probes were labeled with the quencher flour TAMRA (6-carboxy-tetramethyl rhodamine; emission I, 582 nm) at the 3' end through a linker arm nucleotide.

The mRNA expression levels of the targeted and β-actin genes were quantified by using an ABI PRISM 7700 sequence detector (PE Biosystems). Briefly, a master mixture containing all reagents for PCR was prepared at a final concentration of 1x TaqMan universal PCR master mix (PE Biosystems). PCR primers and TaqMan probes were added at the final concentrations of 200 nmol/L and 100 nmol/L, respectively, in a final volume of 25 µL. For Foxp3, 900 nmol/L PCR primer and 250 nmol/L TaqMan probes were used. For β-actin, 50 nmol/L of both PCR primers and TaqMan probe were used. Primers used for β-actin (sense 5'-AGCCTCGCCTTTGCCGA-3'; antisense 5'-CTGGTGCCTGGGGCG-3'). The PCR conditions were as follows: 50°C for 2 minutes and 95°C for 10 minutes, followed by 50 cycles of amplification at 94°C for 15 seconds and 60°C for 1 minute. During each cycle of the PCR, the 5' to 3' exonuclease activity of Ampli-Taq Gold DNA polymerase cleaves the TaqMan probe, thereby increasing the fluorescence of the reporter dye at the appropriate wavelength. The increase in fluorescence is proportional to the concentration of template in the PCR mixture. The relative units of each gene expression were defined by standard curves, which were drawn by the expression levels in serially diluted cDNA of phytohemagglutinin-stimulated mononu-

clear cells derived from the buffy coat. The value of each gene expression was divided by that of β-actin to calculate the relative amount of the gene in cells. All analyses were carried out in duplicate and were repeated for confirmation.

Statistical analysis

The differences in percentages of $CD4^+CD25^{+/high}$ cells and mRNA expression levels between each group were analyzed by using the Wilcoxon test. The Mann-Whitney *U* test was used to analyze the significance of the differences. Differences were considered to be significant when the *P* value was less than 0.05.

Results

$CD4^+$ $CD25^+$ T cells in BD

We first analyzed the proportions of $CD4^+CD25^{+/high}$ cells in the peripheral blood of 42 patients with BD (20 in active stage) by using flow cytometry. As shown (Fig. 1A, 1B) the proportions of $CD4^+CD25^{+/high}$ cells in active BD were significantly higher (median: 9; 25 percentile: 7; and 75 percentile: 12) than those in healthy controls (median: 4.5; 25 percentile: 3.575; and 75 percentile: 6) and BD patients in the remission phase (median: 3; 25 percentile: 2; and 75 percentile: 4.25). Patients in remission BD exhibited similar proportions of $CD4^+CD25^+$ than healthy controls. In patients with active BD, there was no difference between treated patients (colchicine, steroids, cyclosporine) and newly diagnosed active BD patients. A typical pattern of $CD4^+CD25^{+/high}$ T cell expression from one patient with active BD and one patient in remission stage are illustrated in Figure 1A.

Foxp3 mRNA expression in BD

To further characterize regulatory T (Treg) cells in patients with BD, the mRNA expression levels of Foxp3 was analyzed in purified $CD4^+CD25^{+/high}$ T cells in 10 active BD, in 12 remissions BD as well as in 10 healthy controls. A significant increase in Foxp3 mRNA expression was observed in freshly and unstimulated $CD4^+CD25^{+/high}$ T cells of patients with active BD (median: 7.5; 25 percentile: 6; and 75 percentile: 8.75) (Fig. 2) compared with remission BD (median: 1; 25 percentile: 0.25; and 75 percentile: 1) and healthy controls (median: 1.45; 25 percentile: 0.5; and 75 percentile: 2). No significant difference was observed between healthy controls and BD in remission stage. No difference was observed in the Foxp3 expression between treated and newly diagnosed active BD patients (P = 1).

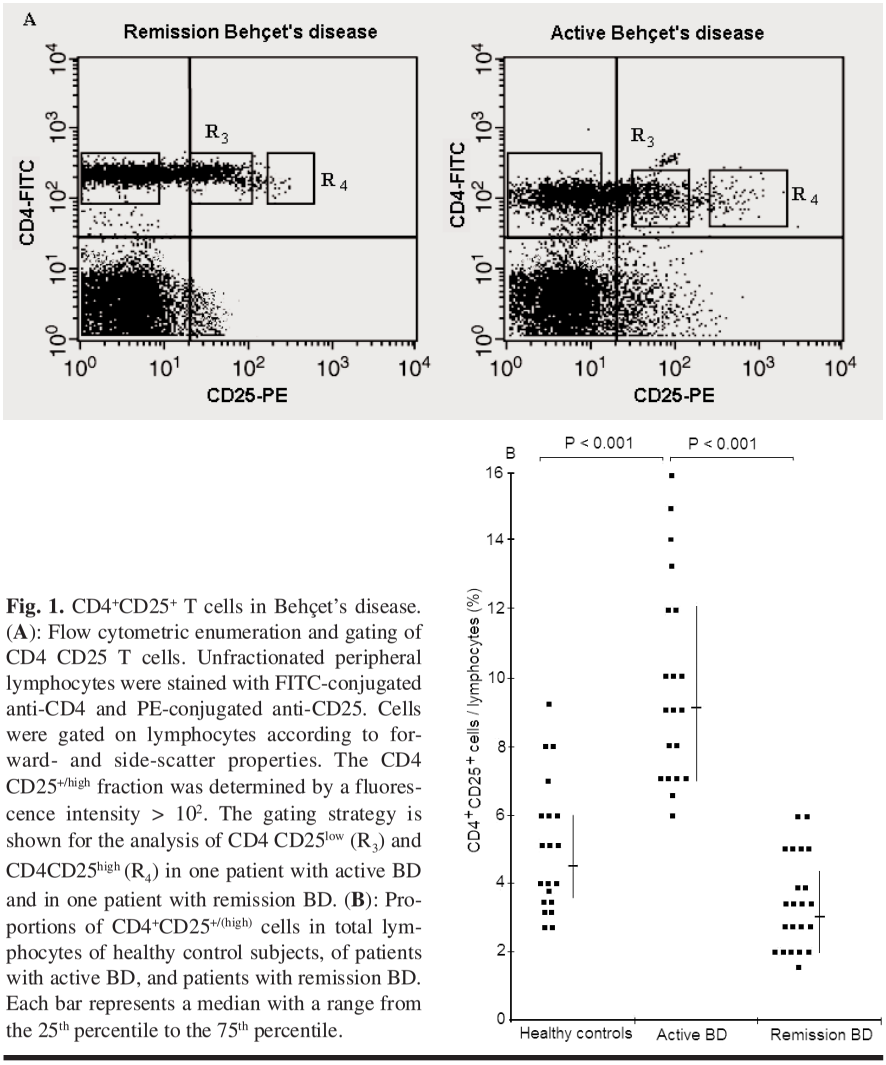

Fig. 1. $CD4^+CD25^+$ T cells in Behçet's disease. (**A**): Flow cytometric enumeration and gating of CD4 CD25 T cells. Unfractionated peripheral lymphocytes were stained with FITC-conjugated anti-CD4 and PE-conjugated anti-CD25. Cells were gated on lymphocytes according to forward- and side-scatter properties. The CD4 $CD25^{+/high}$ fraction was determined by a fluorescence intensity > 10^2. The gating strategy is shown for the analysis of CD4 $CD25^{low}$ (R_3) and $CD4CD25^{high}$ (R_4) in one patient with active BD and in one patient with remission BD. (**B**): Proportions of $CD4^+CD25^{+/(high)}$ cells in total lymphocytes of healthy control subjects, of patients with active BD, and patients with remission BD. Each bar represents a median with a range from the 25th percentile to the 75th percentile.

CTLA-4 and TGF-b mRNA expression in BD

Relative CTLA-4 mRNA expression levels on purified $CD4^+CD25^{+/high}$ T cells were significantly higher in active BD patients (median: 4; 25 percentile: 3; and 75 percentile: 4.75; P < 0.01) than those in healthy controls (median: 1.25; 25 percentile: 0.7; and 75 percentile: 2) and recovered to the level of healthy controls at the remission phase (median: 2; 25 percentile: 1; and 75 percentile: 2) (Fig. 3A). No difference was observed in the CTLA-4 mRNA expression between treated and newly diagnosed active BD patients.

We also analyzed the mRNA expression of TGF-β, which is not specifically expressed on $CD4^+CD25^{+/high}$ regulatory T cells (28). Relative TGF-β mRNA expression levels at the active BD stage (median: 2; 25 percentile: 1.25; and 75 percentile: 3) and remission BD stage were not significantly different (median: 1.75; 25 percentile: 0.25; and 75 percentile: 3.5; P = 0.006) (Fig. 3B). No differences were observed between BD patients in the active and remission stages compared to healthy controls (median: 1.5; 25 percentile: 1; 75 percentile: 3).

$CD4^+$ $CD25^{+/high}$ T cells inhibit the proliferation of $CD4^+$ $CD25^-$ cells

Hyporesponse to TCR-mediated stimu-

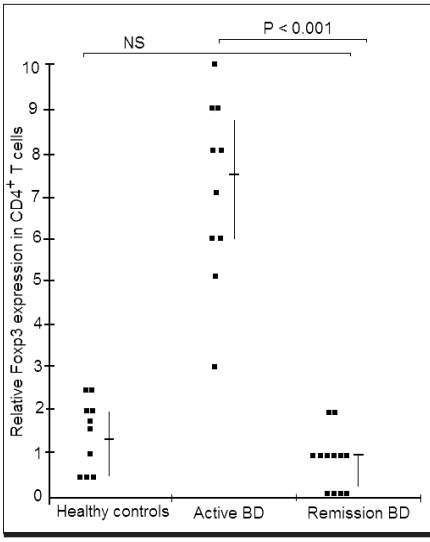

Fig. 2. Relative Foxp3 expression in Behçet's disease (BD). Foxp3 expression was determined in cDNA samples by means of the real-time reverse transcriptase PCR method by using β-actin as an endogenous reference gene. Shown is the analysis of freshly and purified $CD4^+CD25^{+/high}$ cells from samples obtained from healthy control (n = 10), patients with active BD (n = 10), and remission BD (n = 12). Each bar represents a median with a range from the 25th percentile to the 75th percentile. NS: not significant.

lation and mitogens is one characteristic of $CD4^+$ $CD25^+$ Treg cells (8). Therefore, we studied in 10 patients with active BD, the proliferation of sorted $CD4^+CD25^{+/high}$ and $CD4^+$ $CD25^-$ T cells in response to anti-$CD3^+$ anti-CD28 MoAbs. Significant, proliferative responses of $CD4^+$ $CD25^-$ cells were observed, whereas the proliferation of $CD4^+CD25^{+/high}$ T cells was almost undetectable in all samples studied (Fig. 4A) and reduced significantly in relation to that of $CD4^+$ $CD25^-$ cells in all the samples ($P < 0.001$). As one of the main functions of T_{reg} cells is to inhibit the activation of T cells, we studied whether enriched $CD4^+$ $CD25^{+/high}$ T cells are able to inhibit the proliferation of their $CD4^+$ $CD25^-$ counterparts. As shown in Figure 4B, the presence of $CD4^+CD25^{+/high}$ T cells clearly reduced the proliferative response of $CD4^+$ $CD25^-$ T cells to anti-$CD3^+$ anti-CD28 MoAbs in all samples tested. The degree of suppression was cell ratio-dependent, but difference was not significant (ratios $CD25^-$: $CD25^{+/high}$, 2 : 1 and 4 : 1). These data confirm that $CD4^+CD25^{+/high}$ T cells in active BD show the *in vitro* functional activities of Treg cells.

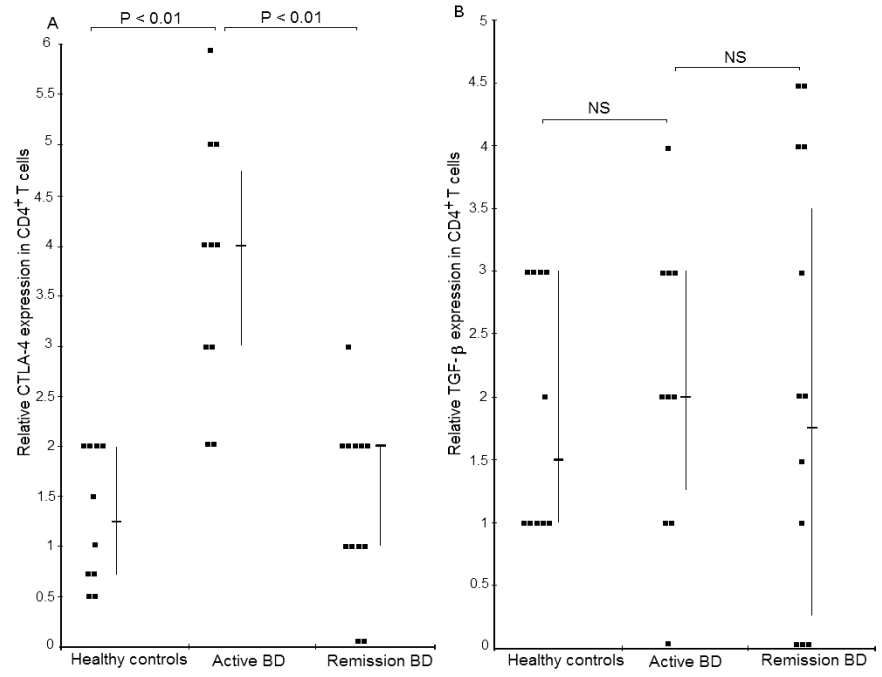

Fig. 3. Relative CTLA-4, and TGF-β mRNA expression at the active and remission BD: CTLA4 (**A**), and TGF-β (**B**). mRNA expression levels of these genes were determined in cDNA samples by using a real-time reverse transcriptase PCR method, with β-actin as an endogenous reference gene. Shown is the analysis of freshly isolated $CD4^+CD25^{+/high}$ cells from samples from healthy control (n = 10), patients with active BD (n = 10), and remission BD (n = 12). Each bar represents a median with a range from the 25th percentile to the 75th percentile. NS: not significant.

Discussion

Behçet's syndrome is a multisystemic inflammatory disorder with numbers of immunological alterations suggesting at least in part an autoimmune pathogenesis. Autoantibodies have been reported against mucosal cells (29), endothelial cells (30), and lymphocytes (31). In the hope to explore regulating pathways in BD, we focused on $CD4^+CD25^+$ T cells. Our study demonstrates increased $CD4^+CD25^{+/high}$ T cells in active BD compared to remission BD and healthy controls. $CD4^+CD25^{+/high}$ cells have been the object of numerous studies because their function appears critical in maintaining self tolerance. Baecher-Allan *et al.* (8) demonstrated that the regulatory $CD4^+$ T cells in peripheral blood of healthy human subjects preferentially reside within the $CD4^+CD25^{high}$ T-cell population. In mice, the *forkhead/* winged helix family protein Foxp3 has recently been shown to be expressed predominantly in Treg cells and to be critical for their generation and function (32). It is suggested that Foxp3 and CTLA-4 are associated with regulatory T cells (18, 19, 22). In this study we have demonstrated in active BD, that Foxp3, and CTLA-4 mRNA expression were increased in purified $CD4^+$ $CD25^{+/high}$ T cells, compared to healthy controls and patients in remission BD. Taken together, our results indicated that $CD4^+CD25^+$ regulatory T cells were increased transitory during inflammatory manifestations (clinical exacerbation) and recovered normal values at the remission phase. The suppressive function of $CD4^+$ $CD25^{+/high}$ T cells in active BD was confirmed by the observation that these cells were hyporesponsive and inhibited the proliferation of $CD4^+$ $CD25^-$ T cells after polyclonal stimulation.

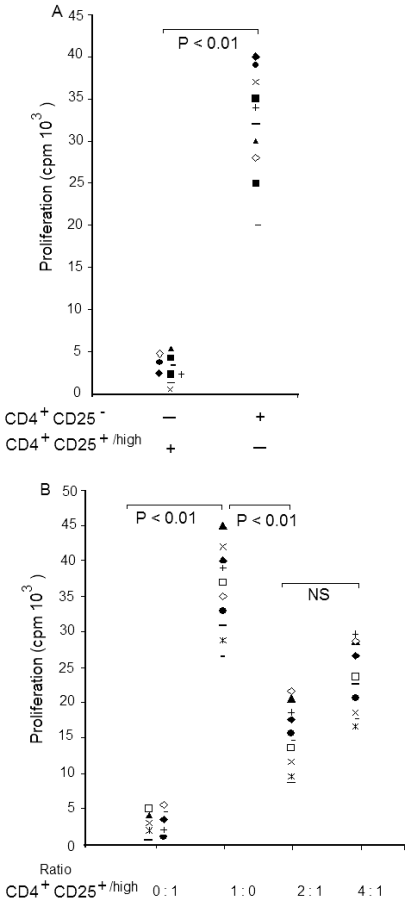

Fig. 4. Inhibition of $CD4^+$ $CD25^-$ T-cells proliferation after addition of $CD4+CD25^{+/high}$ T cells in ten patients with active Behçet's disease. (**A**): $CD4^+CD25^{+/high}$ and $CD4^+$ $CD25^-$ cells ($5\text{-}7.5x10^4$ cells/well) were stimulated with anti-CD3 (1 µg/ml) + anti-CD28 (10 µg/ml) MoAbs and the proliferative response was measured by tritiated thymidine (^3H) incorporation after 4 days of culture. (**B**) $CD4^+$ $CD25^{+/high}$ T cells are able to suppress the proliferation of their $CD4^+$ $CD25^-$ counterparts. $CD4^+$ $CD25^-$ cells ($5x10^4$ cells/well), $CD4^+$ $CD25^+$ cells ($5x10^4$ cells/well) and co-cultures of both $CD4^+$ $CD25^-$ ($5x10^4$ cells/well) and $CD4^+$ $CD25^+$ cells ($2.5x10^4$ or 1.25×10^4 cells/well) from ten active BD patients were stimulated with anti-CD3 (1 µg/ml) and anti-CD28 (10 µg/ml) MoAbs for 4 days. NS: not significant.

There have been several reports demonstrating the relationship between $CD4^+CD25^+$ regulatory T cells and human inflammatory diseases, including noninfectious uveitis and rheumatoid arthritis (33-36). They showed increases of the $CD4^+CD25^+$ regulatory T-cell proportion in peripheral blood or its presence in target organs. In the opposite Kukreja *et al.* (37) have reported the depletion of $CD4^+CD25^+$ T cells in human immune-mediated diabetes. Nevertheless, they did not analyze the $CD4^+CD25^+$ regulatory T cell-specific markers Foxp3. Therefore it is not clear whether the decreased $CD4^+CD25^+$ T cells represented the regulatory T-cell proportion. In the same way, Furino *et al.* (38) reported decreased $CD4^+CD25^+$ regulatory T cells in the acute phase of Kawasaki disease, correlated with a decrease in Foxp3. The precise role of Foxp3 in the development and/or function of Treg cells remain to be elucidated. Thorstenson and Khorusts (39) have generated $CD25^+$ T cells in the DO11.10 TCR transgenic mouse via oral or *i.v.* administration of peptide Ag and demonstrated that the resulting $CD25^+$ T cells are anergic and have Treg properties *in vitro* and *in vivo* (39). Upon adoptive transfer, the expression of CD25 in donor-derived cells is not stable, with $CD4^+CD25^+$ cells appearing in $CCD4^{+-}$ $CD25^-$ T cell-injected animals and *vice versa*. The maintenance of CD25 expression by $CD4^+CD25^+$ cells depends on IL-2 secreted by co-transferred $CD4^+CD25^-$ or by Ag-stimulated T cells in peripheral lymphoid organs (40).

The present study shows that patients with active BD, remission BD and healthy subjects expressed similar levels of TGFβ mRNA expression. Treg activity generated by $CD25^+$ cells did not require TGF-β (23, 41). Whatever, the engagement of CTLA-4 was further more, found to be linked to the production of TGF-β (42). The role of TGF-β in the context of Treg cells has been recently described to be in the induction phase of regulatory process and not necessarily in the suppression itself (41). $CD4^+CD25^+$ peripheral blood T cell compartment does not exclusively include TGF-β producing Th_3 subtype of regulatory T cells, which is suggested to be antigen-induced regulatory T cells at the tissue site of inflammation (43-45). $CD4^+$ T cells that primarily produce TGF-β appear to be a unique T-cell subset that includes mucosal helper T-cell function and downregulated properties for Th_1 and other immune cells and that have been termed Th_3 cells (44). Th_3-type regulatory cells that secrete TGF-β have been described in human conditions (44). As outlined by Prud'homme GJ and Piccirillo CA (43), TGF-β is produced at high levels by CNS including neurons, and that circulating or locally produced TGF-β can bind to a wide variety of tissue matrix proteins, which creates a tissue reservoir of this cytokine (46). The intensity of CTLA-4 expression has been correlated to the suppressive capacity of Treg cells (47). $CD4^+$ $CD25^+$ Treg cells constitutively express CTLA-4 (47). Signalling through CTLA-4 has been suggested to be essential for the suppressive function of Treg cells.

Recent evidence indicates that $CD4^{+-}$ $CD25^+$ regulatory T cells can also modulate immune responses against pathogenic microbes. Hori *et al.* (48) have demonstrated that transfer of T cells depleted of $CD4^+CD25^+$ regulatory T cells elicited severe pneumonitis in mice with severe combined immunodeficiency (SCID) that were chronically infected with *Pneumocystis carinii*, whereas transfer of T cells not depleted for regulatory cells did not. Similarly, inflammatory bowel disease in mice with SCID was induced by the transfer of T-cell suspensions depleted of $CD4^+CD25^+$ T cells (49), although transfer of nondepleted T-cell suspensions produced no inflammatory bowel disease. Furthermore, mice with SCID raised in germ-free conditions did not have the disease. These findings suggested that $CD4^+CD25^+$ regulatory T cells act to suppress hyperimmune responsiveness against microbes.

BD was associated with autoimmune and/or viral aetiology (4). The aetiology of BD remains unknowns with major contributors to inflammatory conditions (IL-6, TNF-α, IFN-γ, …) and $CD4^+$ $CD25^+$ Treg cells implication should be performed with caution. Investigation of the aetiology of BD has focused on HSV_1 (5, 50) and microbial antigens (4, 51). It was shown that microbial induction of Toll pathway in dendritic cells blocked immunosuppression by $CD4^+CD25^+$ Tregs, which was in part dependent on IL-6 (52). These "danger" signals could therefore override immunosuppression by Tregs, thus allowing immunity to infection to occur. Such disturbance in

BD process could contribute to the development of inflammation.

The ability of $CD4^+CD25^+$ regulatory T cells to down-regulate immune responses to pathogens argues against their function being inhibited by inflammatory signals (53). On the contrary, local $CD4^+CD25^+$ regulatory T cells homeostasis and function may even be enhanced following infection reactivation, hyperstimulation by HSP, as found in active BD. More investigations are needed in BD to discuss such hypothesis.

In active BD, $CD4^+CD25^+$ T cells may directly or indirectly inhibit the expansion of rival T cells by secretion of inhibitory cytokines. Interleukin-10 (IL-10) have been implicated in T-cell regulation (54, 55). Active BD was characterized by a higher increase of IL-10 compared to remission BD (56, 57) inducing the equilibrium of the immune system and the avoidance of immune pathology that the exuberant expansion of effector T cells is kept in check.

In this study we have demonstrated the presence of $CD4^+CD25^+$ regulatory T cells in active BD. Defining the position of $CD4^+CD25^+$ regulatory T cells in BD is a challenge. Regulatory role of $CD4^+CD25^{+/high\ T}$ cells in Th1 (IFN-γ) production and correlation of these cells with HSP production is under investigation. Further studies are needed to reveal the exact role of Treg cells in BD and to identify the factors regulating their function and accumulation in the peripheral circulation. BD is a multisystemic disease, where blood could reflect certain immune dysregulations. The characterization of the $CD4^+CD25^{+/high}$ T cells appears central to defining aberrancies in autoimmunity, transplantation, cancer and chronic inflammation, continued exploration of this population will uncover potential regulatory pathways in the treatment of multiple immunopathological conditions as suspected in BD.

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