Pronounced interferon-alpha production from plasmacytoid dendritic cells in patients with Behçet's disease following CpG D ODN stimulation

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Received on February 13, 2009; accepted in revised form on June 3, 2009.

Clin Exp Rheumatol 2009: 27 (Suppl. 53): S37-S42.

Key words: Interferon-alpha, plasmacytoid dendritic cells, CpG D ODN, Behçet's disease.

Competing interests: none declared.

ABSTRACT

Objective. *Several lines of evidence point to a polarized T-helper-1 (Th1) immune response in Behçet's disease (BD). Interferon (IFN)-alpha which has an ability to promote strong Th1 type immune response has been shown to increase in patients with BD. In order to clarify if plasmacytoid dendritic cells (pDCs) abnormally respond to a stimulus in patients with BD, we investigated the levels of intracellular IFN-alpha and beta in pDCs with or without CpG D ODN stimulation.*

Methods. *The study population consisted of 8 patients with clinically active BD, 8 ankylosing spondilitis (AS) patients having active disease and 11 healthy volunteers. pDC subsets in peripheral blood mononuclear cells (PBMCs) cultures were analysed by flow cytometry.*

Results. *The percentage of IFN-alpha+ pDCs in unstimulated PBMCs cultures from patients BD was significantly higher (p=0.036) than in AS and HC. But this difference disappeared in stimulated PBMCs cultures (p=0.167). The mean fluorescence intensity (MFI) of IFN-alpha+ pDCs in stimulated PBMCs cultures of BD patients was significantly higher than those from patients with AS and HC. The percentage of IFN-beta+ pDCs in unstimulated PBMCs cultures from patients with BD and AS was significantly higher (p=0.004) than in HC. But this difference was not significant in stimulated PBMCs cultures (p=0.694). When compared to healthy subjects, the MFI of IFN-beta + pDCs in unstimulated and stimulated PBMCs cultures from patients with BD and AS was not different (p=0.287, p=0.152, respectively). In patients with BD, the percentage and MFI of IFN-alpha+ pDCs were higher (p=0.012 for all) in stimulated PBMCs cultures as compared to unstimulated ones.*

Conclusion. *We suggest that increased frequency of IFN-alpha+ pDC in BD patients and the higher sensitiveness of these cells to CpG D ODN stimulus contribute to high serum IFN-alpha levels found in these patients which eventually resulted in Th1 type immune response.*

Introduction

Behçet's disease (BD) is a chronic systemic inflammatory disease characterized by oral and genital ulcers and by cutaneous, ocular, arthritic, vascular, and neurological involvement (1). Although several humoral and cellular abnormalities of immune system have been reported since 1937 when it is first described, the etiology and pathogenesis of the disease still remains uncertain (2). There are several lines of evidence including, increased serum levels of interleukin (IL)-2 (3), interferon (IFN)-gamma (4), and high serum pro-inflammatory cytokines levels, such as IL-1beta, IL-6, tumor necrosis factor (TNF)-alpha, IL-8 (5, 6), and IL-18 (7) in BD patients with active disease which point to a polarized T helper-1 (Th1) immune response. Indeed, a preferential skewing of T cells in active BD patients towards an IFN-gamma and IL-2 producing Th1 phenotype has been previously demonstrated by intracellular cytokine staining (8). Furthermore, studies conducted in mucocutaneous lesions of BD patients suggest a direct role of Th1 type immune response in the pathogenesis of mucocutaneous lesions (9, 10). However, it is not yet clear which factors are involved in the proposed Th1 mediated pathogenesis of BD. Development of a Th1 and/or Th2 immune response depends on several genetic and environmental factors including, the nature of the antigenic stimulus, the cytokine microenvironment, subset of dendritic cells (DCs) and related mediators produced by these cells, as well as factors confined to T cells (11).

Dendritic cells have the unique ability to initiate and regulate immune responses, and thus one of the critical determinants for polarizing naïve T cells into Th1 or Th2 cells (12). In humans, two distinct subsets of DCs, myeloid DCs (mDCs) and plasmacytoid DCs (pDCs) have been identified. A myeloid pathway generates both Langerhans cells (LCs) which express Langerin, are found in stratified epithelia such as the skin, and interstitial (int)DCs which express DC-SIGN, are found in all other tissues. It also generates mDCs circulating in the blood. Upon inflammation monocytes can yield in mDCs expressing CD11c. A lymphoid pathway generates pDCs, which specifically express BDCA2 and secrete large amounts of IFNs after viral infection and TLR triggering (13).

Type I IFNs, which use an IFNAR receptor composed of IFNAR1 and IFNAR2 chains, contain 13 IFN-alpha, 1 IFN-beta, 1 IFN-ω, 1 IFN-ε, 1 IFN-κ. (14).

Type I IFNs are produced by many cell types in response to viral infection. While pDCs are defined as the major producer of IFN-alpha and IFN-beta (but at much lower levels relative to IFN-alpha) in respect to both viral infection and TLR triggering, they also promote a strong release of all IFN subtypes. However, mDCs release only IFN-beta and IFNs-λ upon TLR-4 and TLR-3 stimulation. Type I IFNs play a major role in the innate immune response because of their potent antiviral activities. They are currently defined as "danger" molecules signaling the presence of a wide variety of microbial agents and establishing an important link between innate and adaptive immunity (14). In addition, types I IFNs have a dual effect on Th1 type immune response with different ways. They can promote Th1-type responses through their direct effect in inducing T cell IFN-gamma secretion (15, 16). In contrast, type I IFNs can hinder interleukin (IL)-12 p70 secretion by monocyte-derived dendritic cells (DC), and consequently inhibit the differentiation of naive Th cells into IFN-gamma secreting Th1 cells (17, 18). Nagai *et al*. have shown that this opposite effect depends on the time of IFN-beta exposure to monocyte-derived dendritic cells. If IFN-beta is added during TNF-alpha-mediated DC maturation, it may promote Th1 polarization by increasing DC IL-12 p70 secretion, through enhancement of autocrine-acting IFN-gamma production by the DC. But if IFN-beta is supplemented during naive Th cell stimulation it may inhibit Th1 cell generation by blocking the IFN-gamma-induced signals required for optimal CD40L-induced DC IL-12 p70 secretion (19, 20).

Kötter and co-workers reported increased serum levels of IFN-alpha in a group of BD patients with ocular involvement (21). We have recently demonstrated that in BD, the percentage of pDCs was reduced in peripheral blood, and that this was associated with increased production of IFN-alpha and decreased production of IFN-beta (22). Based on these results, we suggested that increased IFN-α along with decreased IFN-β might play a role in Th1 type response in BD. In order to clarify if pDCs abnormally respond to a stimuli in patients with BD, we investigated the levels of intracellular IFN-alpha and beta in pDCs which are cultured overnight with or without CpG D ODN. We aimed to stimulate TLR-9 which are expressed only by B cells and pDC but not mDCs. As a TLR-9 trigger, we prefered CpG D ODN, also reffered to CpG A ODN by some authors (23), which contains a single palindromic CpG motif linked to a poly(G) tail at the 3'end, and trigger pDCs to produce high levels of IFN-alpha but fail to stimulate B cells (24).

Material and methods

Patients and controls

The study population consisted of 8 patients with BD fulfilling the criteria of the International Study Group for BD. All patients had clinically active disease at the time of the sampling showing at least 2 clinical manifestations of BD, including oral ulcer, genital ulcer, ostiofolliculitis, uveitis, and vessel involvement. Eight patients who fulfilled the Modified New York Criteria for AS with active disease (BASDAI score ≥4) served as disease controls. Eleven healthy volunteers composed of medical students were also participated. All patients and control subjects gave informed consent and the work was approved by Gulhane School of Medicine Local Research Ethics Committee.

Flow cytometric analysis

Ten ml of blood was drawn into the tubes containing sodium citrate which were then transferred into the blue-top plastic tubes for PBMC isolation using *Histopaque* 1077 (Sigma Cat no: 1077-1 USA). Cell suspension containing 1 x 10^6/ml PBMCs were then incubated overnight with (stimulated) or without (unstimulated) CpG D ODN at 37°C in 7% CO_2. At the end of the incubation period, intracellular transport and transfer of intracellular cytokines outside of the cell was blocked by treating cells with BFA (Brefeldin A Cat no: 347688 Becton Dickinson Immunocytometry Systems San Jose CA 95131USA) for 2 hours at 37°C.

Following incubation, cell surface staining was performed using the following panel of monoclonal antibodies, lineage cocktail (lin 1) FITC (cat no: 340546), CD123 PE (Cat no: 340545), Anti HLA DR Per CP (Cat no: 347402), CD83 APC (Cat no: 551073), CD86 APC (Cat no: 555660), Mouse IgG1 PE, Mouse Igg2a PE(Cat no: 349053) "Dickinson Immunocytometry Systems San Jose CA 95131USA". After the incubation with monoclonal antibodies, cells were underwent fixation (Cellfix rilmissolusyon Ref no: 340181 Becton Dickinson Benelux NV Erembodegem, Belgium) and permeabilization (FACS Permeabilizing solution 2 Cat no: 347692, Becton Dickinson Immunocytometry Systems San Jose CA 95131, USA) procedures. Cells were then incubated with IFN a FITC (Product number: 2112-3, IFN B FITC (Product number: 21400-3) and appropriate isotypic controls (Mouse IGG1 FITC Cat no: 345815, Becton Dickinson Immunocytometry Systems San Jose CA 95131, USA). Cells were washed in cold PBS and then fixed using Cellfix solution containing 1% paraformaldehyte. Until analysis, cells were remained at 2-8°C in the dark for up to 24 hours. At the end of cell surface and intracellular staining procedures, samples were processed by a FACS Canto flow cytometer (Becton

Dickinson Immunocytometry Systems San Jose CA 95131, USA) with at least 50.000 gating events for each sample. Results are expressed as the percentage, absolute number, and mean fluorescence intensity (MFI) of the IFN-alpha and IFN-beta producing cells.

Statistical analysis

All values were presented as median (range) unless otherwise specified. For tests of normality Kolmogorov-Smirnov test was used. Kruskal-Wallis test and Mann-Whitney U-tests were used for multiple groups and for the evaluation of the differences between the two groups, respectively. To investigate the relations among the variables, Spearman rank correlation test or Pearson correlation test was used where appropriate. The criterion for statistical significance was set at $p<0.05$. Statistical analyzes was performed with the Statistical Package for the Social Sciences for Windows (version 11.0, SPSS Inc., Chicago, IL).

Results

Clinical characteristics

No difference was found between the study groups and healthy controls (HC) with regard to the age, sex, and total WBC count (data not shown).

The percentage and MFI of IFN-alpha and beta+ pDCs

We analyzed the percentage and MFI of IFN-alpha and beta+ pDCs in unstimulated and stimulated PBMCs cultures from patients with BD an AS, and HC. The percentage of IFN-alpha+ pDCs in unstimulated PBMCs cultures from patients BD was significantly higher (p=0.036) than in AS and HC. But this difference disappeared in stimulated PBMCs cultures (p=0.167) although there was a higher percentage of IFN-alpha+ pDCs from patients with BD compared to those from AS and HC (Table I and Fig. 1). In addition, the MFI of IFN-alpha+ pDCs in unstimulated PBMCs cultures from patients with BD was higher than those from AS and HC, but this difference again was not statistically significant (p=0.068). Furthermore, the MFI of IFN-alpha+ pDCs in stimulated PBMCs cultures from patients with BD was significantly higher than those from patients with AS and HC (Table I and Fig. 2).

The percentage of IFN-beta+ pDCs in unstimulated PBMCs cultures from patients BD and AS was significantly higher (p=0.004) than in HC (Table I and Fig. 3). But this difference was lost in stimulated PBMCs cultures (p=0.694). When compared to healthy subjects, the MFI of IFN-beta+ pDCs in unstimulated and stimulated PBMCs cultures from patients with BD and AS was not different (p=0.287) and (p=0.152), respectively.

The effect of CpG D ODN stimulation

We compared the percentage and MFI of IFN-alpha and beta+ pDCs between unstimulated and stimulated PBMCs cultures from patients with BD and AS, and HC in each group. In patients with BD, the percentage and MFI of IFN-alpha+ pDCs were higher (p=0.012 for all) in stimulated PBMCs cultures than in unstimulated ones. In contrast, there was no difference between unstimulated and stimulated PBMCs cultures with respect to the percentage and MFI of IFN-beta+ pDCs (Table I).

In patients with AS, the percentage, but not MFI, of IFN-alpha+ pDCs was higher (p=0.015) in stimulated PBMCs cultures as compared to unstimulated ones. We could not find any difference between unstimulated and stimulated PBMCs cultures according to both the percentage and MFI of IFN-beta+ pDCs.

In healthy subjects, the percentage, but not MFI, of IFN-alpha+ pDCs and IFN-beta+ pDCs were higher (p=0.013 and p=0.021 respectively) in stimulated PBMCs cultures than in unstimulated ones.

Discussion

Though Type I IFNs are being secreted as a response to viruses by various types of cells, pDCs are the major well-known cells that secrete IFN. It has been shown that Type I IFNs activate adaptive immunity and thus act in various immune-mediated diseases, in addition to their role in the natural immune response by their potent antiviral activity (13). We had found in a previous study that serum IFN-alpha levels were significantly higher in BD patients

Table I. Comparisons of the levels of INF-α + and INF-β+ plasmacytoid dendritic cells in mononuclear cell culture stimulated with or without CpG D ODN, among Behçet's patients, the patients with ankylosing spondylitis, and healthy controls.

	Patients with BD (n=8)			Patients with AS (n=8)			HC (n=11)			BD *vs.* AS *vs.* HC p^{**}	
	US	S	p^{*}	US	S	p^{*}	US	S	p^{*}	US	S
IFNα⁺ PDC (%)	2.0 (5.3)≠	4.8 (8.2)	0.012	0.4 (2.5)	2.2 (5.3)	0.015	0.4 (3.1)	2.7 (21.8)	0.013	0.036	0.167
IFNα⁺ PDC (MFI)	348 (152)	528 (235)≠	0.012	396 (466)	411 (506)	0.214	72 (551)	187 (582)	0.131	0.068	0.006
IFNβ⁺ PDC (%)	2.2 (4.0)‡	2.5 (4.9)	0.123	1.9 (7.2)‡	2.2 (5.4)	0.061	0.06 (8.3)	3.2 (11.7)	0.021	0.004	0.694
IFNβ⁺ PDC (MFI)	433 (889)	513 (336)	0.123	682 (793)	666 (765)	0.198	229 (651)	352 (671)	0.182	0.287	0.152

*The differences between two groups were evaluated by Mann-Whitney U-test.
**Multiple comparisons were done by using Kruskal-Wallis test.
≠significant difference (<0.05) compared to patients with AS and HC
‡significant difference (<0.05) compared to HC ↑

BD: Behçet's disease; AS: ankylosing spondylitis; HC: healthy control; PDC: plasmacytoid dendritic cell; IFN: interferon; MFI: mean fluorescence intensity; CSN: culture supernatant; US: unstimulated; S: stimulated.

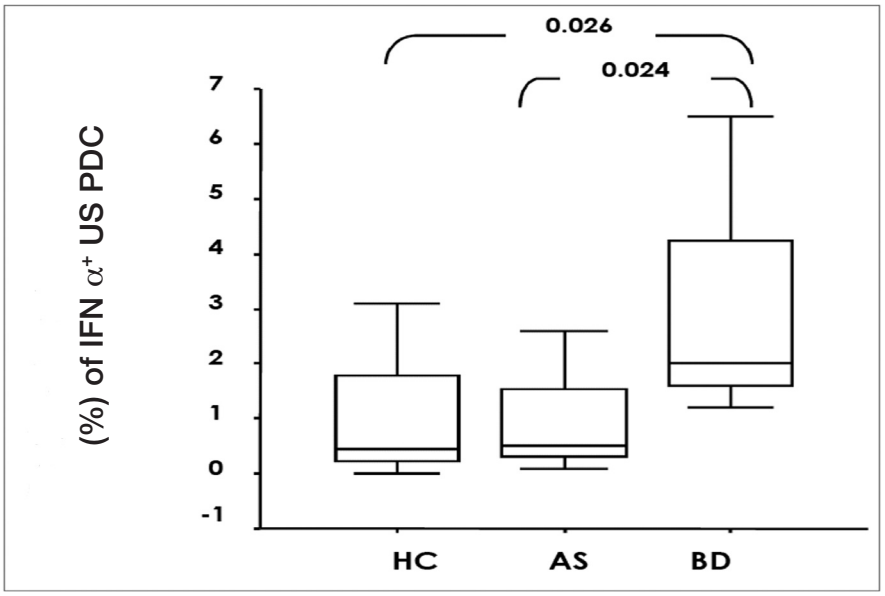

Fig. 1. Comparison of the percentage of IFN-alpha+plasmacytoid dendritic cells in PBMCs cultures without CpG D ODN from the patients with Behçet's disease and ankylosing spondylitis and healthy controls. Boxes show the ranges of 1st and 3rd quartiles and extreme values. Horizontal bars represent median values. The differences between two groups were evaluated by Mann-Whitney U test. *P*-values were indicated above the boxes when a level of significance <0.05 was reached in comparisons of study groups.
The percentage of IFN-alpha+PDC in unstimulated cultures from Behçet's patients is higher than those from AS and HC. But this diference was lost in stimulated cultures.
The MFI of IFN-alpha+PDC in unstimulated cultures from Behçet's patients and patients with AS is higher but not statistically significant than those from HC. But in stimulated cultures, The MFI of IFN-alpha+PDC from Behçet's patients is statistically significantly higher than those from AS and HC.

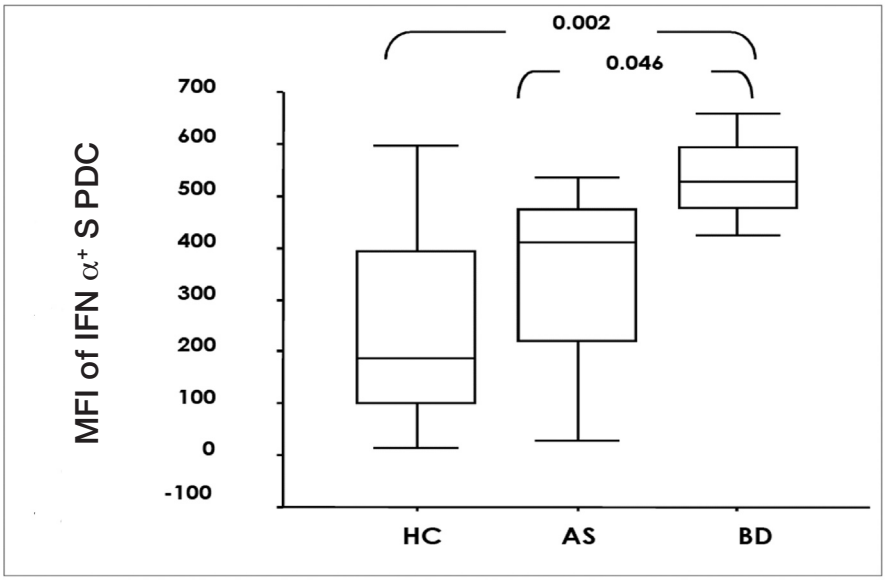

Fig. 2. Comparison of the MFI of IFN-alpha+plasmacytoid dendritic cells in PBMCs cultures with CpG D ODN from the patients with Behçet's disease and ankylosing spondylitis and healthy controls. Boxes show the ranges of 1st and 3rd quartiles and extreme values. Horizontal bars represent median values. The differences between two groups were evaluated by Mann-Whitney U-test. *P*-values were indicated above the boxes when a level of significance <0.05 was reached in comparisons of study groups.
The percentage of IFN-alpha+PDC in unstimulated cultures from Behçet's patients is higher than those from AS and HC.But this diference was lost in stimulated cultures.
The MFI of IFN-alpha+PDC in unstimulated cultures from Behçet's patients and patients with AS is higher but not statistically significant than those from HC. But in stimulated cultures, The MFI of IFN-alpha+PDC from Behçet's patients is statistically significantly higher than those from AS and HC.

than in AS patients as well as in healthy controls. In addition, serum IFN-alpha levels were correlated with the disease activity. In contrary, serum levels of IFN-beta, which has quite similar aminoacid sequence to that of IFN-alpha and is secreted from the same types of cells, mainly pDCs and by similar stimuli which act via IFNAR, were significantly lower in BD patients than in AS patients and in healthy controls (22). These findings suggested that in patients with BD, there can be a functional disorder against stimuli in pDCs, which are the main source of type I IFNs. In the present study, we therefore have evaluated intracellular cytokine levels in pDCs and their response to the stimuli. For this purpose, we performed PBMCs cultures in BD and AS patients, and in healthy controls. Since it is a receptor being expressed only in pDC and B cells, we decided to perform the stimuli in PBMCs cultures through TLR-9. There are several types of CpG ODNs that can activate B cells and pDCs. CpG D ODN (also referred to as A class (23), contain a single palindromic CpG motif linked to a poly(G) tail at the 3' end. CpG D ODN trigger pDCs to produce high levels of IFN-alpha but fail to stimulate B cells (23). CpG K ODN, referred to as B class by some investigators (23, 24), typically express multiple CpG motifs but lack a poly(G) tail. K ODN stimulate B cells to produce IgM and IL-6 while triggering pDC to produce TNF-alpha rather than IFN-alpha (24, 25). Despite these differences in cellular specificity and functional activity, both K- and D-class CpG ODN are recognized by the same intracytoplasmic receptor (TLR 9) and signal through a conserved TLR9-MyD88 pathway (26). Gürsel *et al.* have shown that this difference is dependent on CXCL16 chemokine, which expressed in pDCs but not expressed in B cells, binds to the cellular surface and acts like a scavenger receptor (27). The same authors shown that CXCL16 expressed in cellular surfaces of pDCs increases the binding, uptake and stimulating activity of CpG D ODN. They also showed that this interaction causes CpG D ODN to be accumulated in the pre- endosome instead of the lysosome

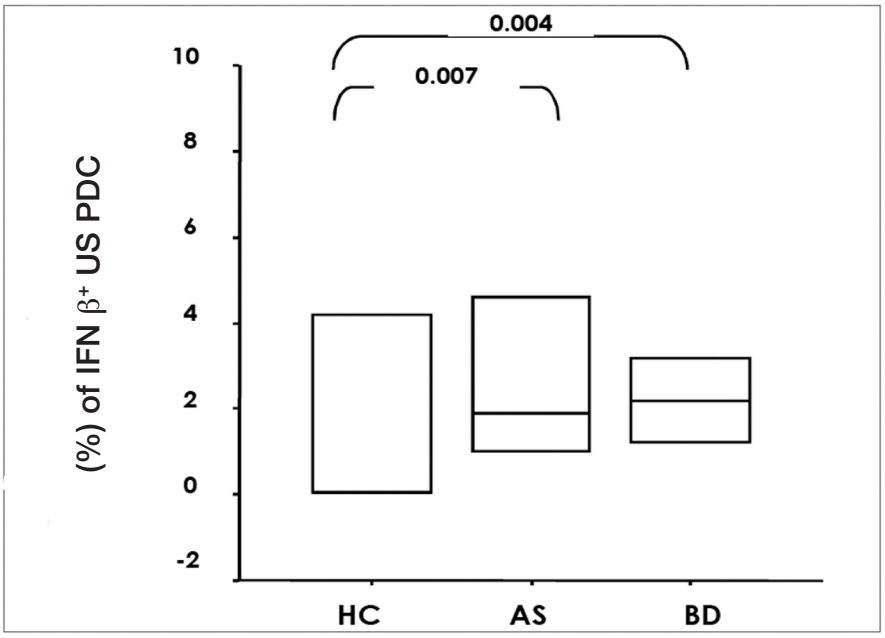

Fig. 3. Comparison of the percentage of IFN-beta+plasmacytoid dendritic cells in PBMCs cultures without CpG D ODN from the patients with Behçet's disease and ankylosing spondylitis and healthy controls. Boxes show the ranges of 1st and 3rd quartiles and extreme values. Horizontal bars represent median values. The differences between two groups were evaluated by Mann-Whitney U-test. *P*-values were indicated above the boxes when a level of significance <0.05 was reached in comparisons of study groups.
The percentage of IFN-beta+PDC in unstimulated cultures from HC is lower than those from AS and BD. But this diference was lost in stimulated cultures.

and IRF7 activation, thus directing pDC to produce IFN-alpha. In contrast, classical TLR9-MyD88 signal pathway shows activity in lysosomal vesicles and causes IRF5 activation and TNF-alpha secretion by pDC. In the present experiment, we used CpG D ODN as a TLR-9 trigger, since it has been shown to be more specific for pDCs.

We found in this study that in patients with BD, the percentage of IFN-alpha+ pDC and their MFIs in PBMCs cultures which were not stimulated with CpG D ODN were higher than in AS patients and in control group. MFIs of IFN-alpha+ pDCs in PBMCs cultures which were not stimulated with CpG D ODN in BD and AS patients were found to be higher as compared to healthy controls, while this difference was not statistically significant. These results are in line with the studies of Kotter and al. and our previous study in which serum IFN-alpha levels were found to be higher in BD patients (21, 22). The percentage of IFN-alpha+ pDC in PBMCs cultures stimulated by CpG D ODN in BD patients were higher in comparison to those in AS patients and control group, however the difference was not statistically significant. On the other hand, the level of MFIs of IFN-alpha+ pDC in PBMCs cultures stimulated by CpG D ODN in BD patients were significantly higher than those of AS patients and in healthy individuals. These results suggest that pDCs of BD patients cause more IFN-alpha secretion via stimulation of their TLR-9 receptors by an exogen or may be an endogen stimulant and this may explain high serum IFN-alpha levels detected in patients with active disease.

We found both in patient groups as well as healthy controls that, when PBMCs cultures stimulated and unstimulated by CpG D ODN are compared within their groups, proportion of IFN-alpha+ pDC increased significantly in all groups. However, MFI levels of IFN-alpha+ pDCs were found to be increased only in BD patients. Data of the present study clearly show that BD patients respond to CpG D ODN stimulus more noticeably than AS patients and healthy controls. However, results of the present study do not consistent with the study performed by Plskova *et al*. (28). These authors reported that there was neither increase in the number of pDCs as a response to CpG D ODN stimulus nor IFN-alpha production in their study group. However, there are some important differences between our study and theirs. Plskova et al studied non-infectious uveitis patients other than BD. Furthermore all patients included in the study of Plskova *et al*. were those patients who were in remission with IFN-alpha treatment. In contrast, we have included active Behçet's patients who hadn't been taking any treatment. Therefore, it is not so convenient to compare the data of these two studies.

In our study, we found that percentage of IFN-beta+ pDC and their MFIs in unstimulated cultures of PBMCs of BD and AS patients were higher in comparison to those of the control group. This difference was significant only in the percentage IFN-beta+ pDC. These results did not consistent with the ones of our previous studies in which we found that serum IFN-beta levels in BD patients were lower than in AS patients and in healthy controls (22). According to these results it can be speculated that low serum levels of IFN-beta do not rely on the lack of pDC secretion. There was no difference between the proportion of IFN-beta+ pDC and their MFIs in PBMCs cultures stimulated by CpG D ODN of the patients and control groups.

When comparing stimulated and unstimulated cultures of PBMCs in patient groups and healthy controls, we found that IFN-beta+ pDC rates increased significantly only in healthy individuals. We also found that in all groups, MFI levels of IFN-beta+ pDCs were not affected by CpG D ODN stimulus. We did not find any study in the literature to compare our results regarding IFN-beta+ pDC. The results regarding IFN-beta+ pDC were inconsistent with IFN-alpha+ pDC and no difference was found between the study groups. We suggest that two reasons are responsible for this. Firstly, pDC secrete IFN-beta in much lower levels than IFN-alpha. Taking into consideration that even pDC which are found in the periferic blood in very low rates are

very difficult to be examined by flow cytometry, IFN-beta+ pDCs are much more difficult to be examined. Therefore, it could not have been possible to show the difference between the study groups. Secondly, CpG D ODN had been used in the previous studies only to stimulate IFN-alpha secretion from pDCs. Probably, IFN-beta secretion from pDCs is stimulated by other pathways which makes CpG D ODN is not an optimal stimulant for IFN-beta secretion.

In summary, as reviewed by Coccia recently, it has been found that IFN-alpha causes monocytes transform into myeloid dendridic cells and contributes to Th1 polarisation by enabling myeloid dendridic cell maturation (14). Nagai *et al*. showed in their studies that exposure to IFN-beta especially during mDC development, led these cells to Th1 type immune response, while naive T cell stimulation resulted in Th2 type immune response (19, 20). We suggest that increased frequency of IFN-alpha+ pDC in BD patients and the higher sensitiveness of these cells to CpG D ODN stimulus contribute to high serum IFN-alpha levels found in these patients which eventually resulted in Th1 type immune response. We further speculate that, probably naive T cell stimulus might be operative in suppressing the clinical findings obtained with IFN-alpha treatment by inducing Th2 type immune response.

Acknowledgement

This study was supported by the Society for Education and Research in Rheumatology (RAED).

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