Serum IL-33 levels and skin mRNA expression in Behçet's disease

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ABSTRACT

Objective. Behçet's disease (BD) is an autoimmune/inflammatory disease characterised by abnormal production of proinflammatory cytokines. Interleukin-33 (IL-33) is a novel cytokine of the IL-1 cytokine family that has been recently implicated in several inflammatory and autoimmune diseases and the association of IL-33 with BD has remained unknown. Here we document for the first time, IL-33 level and its association with BD.

Methods. Serum IL-33 levels were measured in 46 BD patients (20 patients in active stage) and compared to multiple sclerosis (MS), rheumatoid arthritis (RA) patients and to healthy controls. In parallel, the transcription factor NF-κB that mediates IL-33 transcription was also measured. IL-33 mRNA was also quantified in freshly isolated PBMCs and in skin biopsies by real-time RT-PCR analysis. IL-6 and IL-17 were measured by ELISA.

Results. Serum IL-33 level was significantly higher in active BD patients [159.65 ± 61.7 pg/mL] compared to inactive BD patients [85.57 ± 21.07 pg/mL] (p<0.0001) and healthy controls [70.03±25.95 pg/mL] (p<0.0001). Active BD patients expressed lower IL-33 levels than the control disease group, RA and MS patients [p=0.00021]. The serum IL-33 level in active BD patients was corroborated by IL-33 mRNA expression in fresh PBMC. Patients with active BD with retinal vasculitis showed the highest serum IL-33 level. We further stimulated cultured PBMCs with phorbol myristate acetate (PMA) and ionomycin and macrophages with LPS for 24 h. Following stimulation the levels of IL-33 were increased similarly in PBMC [92.35±24.81 pg/ml] and macrophages [93.10±21.58 pg/ml] in active BD patients compared to healthy controls. NF-κB DNA binding activity was significantly increased in PBMCs of active BD patients particularly in LPS-stimulated macrophages compared to healthy controls. IL-33 mRNA expression in the skin lesions of patients with active BD was significantly increased compared to that in healthy skin biopsies [p=0.00016]. A significant relationship was found between the levels of IL-33 and IL-17 [r=0.533; p=0.0024] and IL-33 and IL-6 [r=0.661, p=0.0015] in 20 active BD patients.

Conclusions. Elevated IL-33 level in active BD patients was found to correlate with disease activity. Targeting IL-33 should be approached with caution.

Introduction

The interleukin-1 (IL-1) family of cytokines are important mediators of inflammatory disorders and are important therapeutic targets, through the use of neutralising monoclonal antibodies (1). The most recently discovered member of the IL-1 family is IL-33 (IL-F11) (2). Interleukin-33 exhibits structural similarity to IL-18 and is synthesised as a 30 000 molecular weight precursor protein that lacks a signal peptide. IL-33 is proposed as the first line of defense against microbes that shape adaptive immune response (3). IL-33 is emerging as a new regulator of immune responses and inflammatory vascular diseases. It is known as "nuclear factor from high endothelial venules" (NF-HEV) (4-5).

IL-33 was identified as the ligand for the orphan receptor, ST2 (IL-1RL1). ST2 molecule is a member of the IL-1 receptor family that exists in two forms: a transmembrane full-length form (ST2L) and a soluble secreted form (sST2) due to differential splicing of ST2 mRNA (6). IL-33 appears to be a cytokine with dual function, acting both as a traditional cytokine through activation of its receptor and as an intracellular nuclear factor with transcriptional regulatory properties (7). Recent studies emphasise the importance of IL-33 in amplifying innate immunity and pos-

sibly serving as an alarmin to activate the immune system following cell necrosis or apoptosis (8). IL-33 transcription is mediated by NF-κB activation secondary to Toll-like receptor (TLR) signalling or stimulation with TNF-α or IL-1β. IL-33 transduces its effects through the classical IL-1 signalling machinery, including activation of NF-κB and MAPKs (2). IL-33 is widely expressed in cells of haematopoietic origin, particularly in restricted populations of professional antigen presenting cells (2).

Recent evidence suggests a role for IL-33 in several rheumatological diseases, including rheumatoid arthritis (RA) (9-10), systemic lupus erythematosus (SLE) (11). IL-33 mRNA and protein is also substantially higher in the skin lesions of patients with atopic dermatitis (12). Recently Fujita *et al.* reported that IL-33 is a potent inducer of IL-17F in bronchial epithelial cells via the activation of the ST2-ERK1/2-MSK1 signalling pathway (13).

Behçet's disease (BD) is a systemic vasculitis with unknown aetiology. Immune dysregulation with active neutrophils, supposedly triggered by infectious agents, contribute to disease pathogenesis in addition to genetic predisposition (14-16). BD as many autoimmune diseases are considered to be T cell regulated diseases; further classified as Th1-mediated diseases, with Th1-like diseases featuring a high production of IFN-γ. Th1 as well as Th17 cytokines play crucial roles in BD inflammatory responses (17-20). Our study aims to examine serum levels of IL-33 and IL-33 mRNA expression in peripheral blood. The relation to disease activity and clinical association in patients with active BD patients was also investigated.

Materials and methods
Patients and controls
This study was approved by the Institutional Review Board. Informed consent was obtained from patients prior to enrolling them into the study. The patients' group consisted of 46 BD patients (active BD: 20, inactive BD: 26). Active BD patients fulfilled the International Study Group (ISG) criteria for

Behçet's disease (21). Patients with BD (14 females and 32 males) and controls disease were recruited from Charles Nicolle Hospital (Ophtalmology department, Tunis) and the Mongi Slim Rheumatology Department (Marsa). The mean age was 38.63 ± 6.12 years (range: 26–48 years). Patients with inactive BD did not received corticosteroïds within 7 weeks. All active BD patients received corticosteroid treatment at the dose of 3 to 5 mg/ day. Ten patients with BD were studied for serum IL-33 level at active and inactive BD stages.

Clinical characteristics of active BD patients are summarised in Table I. Mean disease duration was 9.45±5.36 years. Behcet's disease activity index (BDAI) was also performed on our patients according to the method presented by Bhakta *et al.* (22) and Lawton (23). Patients who had no symptoms related to BD in a four-week period, or less than two symptoms with a healing process and an overall wellbeing status, were grouped in inactive BD.

Active BD patient with skin lesions are summarised in Table I. The skin lesions were scored using the system of Diri *et al.* (24) (0=no lesions; 1=1-5 lesions; 2=6-10 lesions; 3=11-15 lesions; 4=16-20 lesions; and 5=more than 20 lesions).

Control group was composed of 18 multiple sclerosis (MS) patients in relapse (12 males, 6 females, mean age: 37.50±4.20 years) and 20 rheumatoid arthritis (RA) patients in active stage (16 men and 4 women; mean age: 39±4.7 years). MS and RA were investigated as control diseases. They are known as inflammatory disease, where adaptive and innate immunity play important roles. The inflammatory process in RA and MS as well as BD, was characterised by increased production of Th17 and Th2 cytokines (18). BD and the two control diseases progressed as acute and remission phase. RA was diagnosed by the 1987 revised classification criteria of the American College of Rheumatology (25) and MS diagnosis was established according to Poser's criteria (26). A cohort of age- and sex-matched 32 healthy individuals served as the healthy control (26

men and 6 women; aged from [25–43 years; (mean age: 33.9±7.54 years)]. None of the controls enrolled in this study had ongoing infections, either viral or bacterial.

Peripheral blood mononuclear cells (PBMCs) isolation and expansion
Patients and normal subjects donated 40 mL of blood, which was collected in heparinised tubes. Lymphocytes were isolated from PBMC by Ficoll Hypaque gradient centrifugation (Histopaque; Sigma Aldrich, The Netherlands). For the *in vivo* studies several samples of the freshly isolated cells were either resuspended in STAT-60 (Tel-Test, Friendswood, TX) for RNA analysis, RIPA buffer for protein analysis (27), or fixed for intracellular flow cytometry analysis. For the *in vitro* studies, part of the PBMCs was cultured for 10 days in RPMI 1640 media with 20 units/mL IL-2 (R&D Systems), and 10% fœtal bovine serum. After one week, cells were treated with cytokines and analysed as outlined in the text. Cultured PBMCs were stained with antibodies against CD3, CD19, and CD14, which were used for differentiating T-cells, B-cells, and monocytes respectively. The proportion of cell types making up the PBMCs of BD patients and healthy controls were 78% T-cells, 5% B-cells, 7% monocytes, and 14% of negative cells). A majority of negative cells probably represent natural killer (NK) cells based on their scatter properties (28). Seven mL of the PBMC was washed twice with HBSS. Adherent monocytes were cultured for one week in RPMI media, 15% fetal bovine serum, and 50 ng/mL GM-CSF (29). Medium was replenished every 3 days and non-adherent cells were removed at the second feeding (at 6 days). For experiments, cells were cultured for an additional day in the absence of GM-CSF, washed twice with PBS, and adherent cells were lysed in STAT-60 (Tel-Test, Friendswood, TX) for RNA analysis or in RIPA buffer (30) for protein analysis. Flow cytometry cells were detached by incubation in 5.0 mM EDTA for 10 min at 37°C. The method consistently yielded more than 95% pure macrophages assessed by both morphological criteria

Table I. Clinical manifestations of 20 active Behçet's disease.

	Age/sex	Clinical manifestations	Scored skin lesion
1	32/M	U, OAU, GU, AE, A, Pulm	[0]
2	46/M	U†, OAU, GU, AE, EN, A, Pulm, (+) pathergy	[5]
3	40/M	U†, OAU, GU, AE, EN, A, CNS, (+) pathergy	[1]
4	26/F	U†, OAU, GU, AE, EN, (+) pathergy	[5]
5	30/M	U†, OAU, GU, AE, EN, A, Pulm, (+) pathergy	[5]
6	36/M	OAU, GU, AE, A, Pulm, (+)pathergy	[2]
7	45/M	U†, OAU, GU, AE, EN, Pulm, (+) pathergy	[5]
8	42/M	U†, OAU, GU, AE, A, (+) pathergy	[4]
9	30/M	U, OAU, GU, AE, A, (+) pathergy	[1]
10	40/F	U, OAU, GU, AE, A	[0]
11	45/M	U, OAU, GU, AE, A	[0]
12	43/M	U†, OAU, GU, AE, EN, CNS, (+) pathergy	[3]
13	41/F	U, OAU, GU, AE, EN, A, (+) pathergy	[2]
14	44/M	U, OAU, GU, AE, EN, Pulm, (+) pathergy	[2]
15	41/M	U, OAU, GU, AE, EN, A, (+) pathergy	[1]
16	39/M	U, OAU, GU, AE, EN, A, (+) pathergy	[1]
17	48/M	U, OAU, GU, AE, A	[0]
18	42/M	U, OAU, GU, AE, A, (+) pathergy	[2]
19	46/M	U, OAU, GU, AE, A	[0]
20	40/M	U, OAU, GU, AE, A	[0]

A: arthritis; AE: acneiform eruption; EN: erythema nodosum lesions; Ul: ulcerated skin lesions; OAU: oral aphthous ulcers; GU: genital ulcers; U: uveitis, (U†):with retinal vasculitis; CNS: central nervous system; Pulm: pulmonary manifestations; CRP: C-reactive protein (mg/L) [mean ± SD: 7.47±2.76 *vs*. 1.2±0.6 in the healthy controls].

as described (31) and expression of the myeloid lineage marker CD14 as determined by flow cytometric analysis. Seven millilitres of peripheral blood were collected from patients and controls, and stored at -70°C for subsequent measurement of IL-33.

Enzyme-linked immunosorbent assay (ELISA) for IL-33, IL-17 and IL-6
The IL-33, IL-17, and IL-6 concentrations in diluted serum and synovial fluid were measured by sandwich ELISA. Briefly, 4 μg/ mL of monoclonal capture antibody (R&D Systems, Minneapolis, MN, USA) were added to a 96-well plate (Nunc, Rochester, NY, USA) and the plate was incubated for 2 hr at room temperature. The plate was then incubated in blocking solution comprising phosphate-buffered saline (PBS) containing 1% bovine serum albumin and 0.05% Tween 20 for 2 hrs at room temperature. All sera samples were diluted 1:2 by using PBS containing 1% bovine serum albumin and 0.05% Tween 20. The test samples and standard recombinant IL-33, IL-17, and IL-6 (R&D Systems) were added to the plates, and the plate was incubated for 2 hr at room temperature. The plate was washed four times with PBS containing Tween 20,

200 ng/mL of biotinylated detection monoclonal antibodies (R&D Systems) was added, and the plate was incubated for 2 hrs at room temperature. The plate was washed, streptavidin–alkaline-phosphatase (Sigma-Aldrich, St Louis, MO, USA; diluted 1:2,000) was added, and the reaction was allowed to proceed for 2 hr at room temperature. The plate was washed four times, and 1 mg/mL of *p*-nitrophenyl phosphate dissolved in diethanolamine (both from Sigma-Aldrich) was added to induce the color reaction, which was stopped by adding 50 μL of 1 N NaOH. The optical density at 405 nm was measured on an automated microplate reader. A standard curve was drawn by plotting optical density *versus* the log of the concentrations of IL-33, IL-17, and IL-6.

RNA isolation from skin biopsies
We examined skin biopsies from the 20 patients with active BD and 10 controls (biopsy samples removed for other reasons than inflammatory diseases). All of the BD lesions were active at the time of biopsy (Table I). After a clinical evaluation, 4-6 mm sections of skin were snap-frozen in liquid nitrogen and stored at -80°C until used. In two patients 2 mm punch biopsies were taken

from the lesions. The specimens were fixed in formalin and embedded in paraffin, sections of the paraffin blocks were stained with hematoxylin and eosin, and a pathologist who was blinded to the patient data examined the biopsies. Total RNA from the skin lesions was extracted as we have recently reported (33).

Real-time RT-PCR
Total RNA was isolated using STAT-60 RNA isolation kit (Tel-test). RNA was quantified spectrophotometrically and 0.5 μg of total RNA was converted into cDNA. Briefly, to generate cDNA 0.5 μg total RNA and random primers (Invitrogen, Carlsbad, CA) were incubated at 72°C for 10 min. Reverse transcription was performed using the Superscript II RT enzyme (Invitrogen, Carlsbad, CA) and following the manufacturer's specifications. cDNA was diluted to 200 μL with water and 4 μL was used for quantitative real time PCR using a SYBR Green kit (Abgene, Epson, UK). The following forward and reverse primers were used at 10 nM. IL-33: AAGGCAAAGCACTCCA-CAGT and GAAAGAAGTTTGCC-CCATGT, GAPDH: ACC ACCATG-GAGAAGGC and GGCATGGACT-GTGGTCATGA, and β-Actin: AG-GCACCAGGGCGT GAT and GCC-CACATAGGAATCCTTCTGAC. The PCR parameters were 15 min for 95°C, 35 cycles at 95°C for 15 s, and 60°C for 1 min in an ABI Prism 700 thermocycler (Applied Biosystems, Foster City, CA). Serial dilutions of cDNA containing a known copy number of each gene were used in each quantitative PCR run in order to generate a standard curve relating copy number to threshold amplification cycle (32). Gene expression levels were calculated during the logarithmic amplification phase by determining the initial mRNA copy number using the standard curve. Amplification of each gene specific fragment was confirmed both by examination of melting peaks and by agarose gel electrophoresis.

NF-κB DNA-binding activity assay
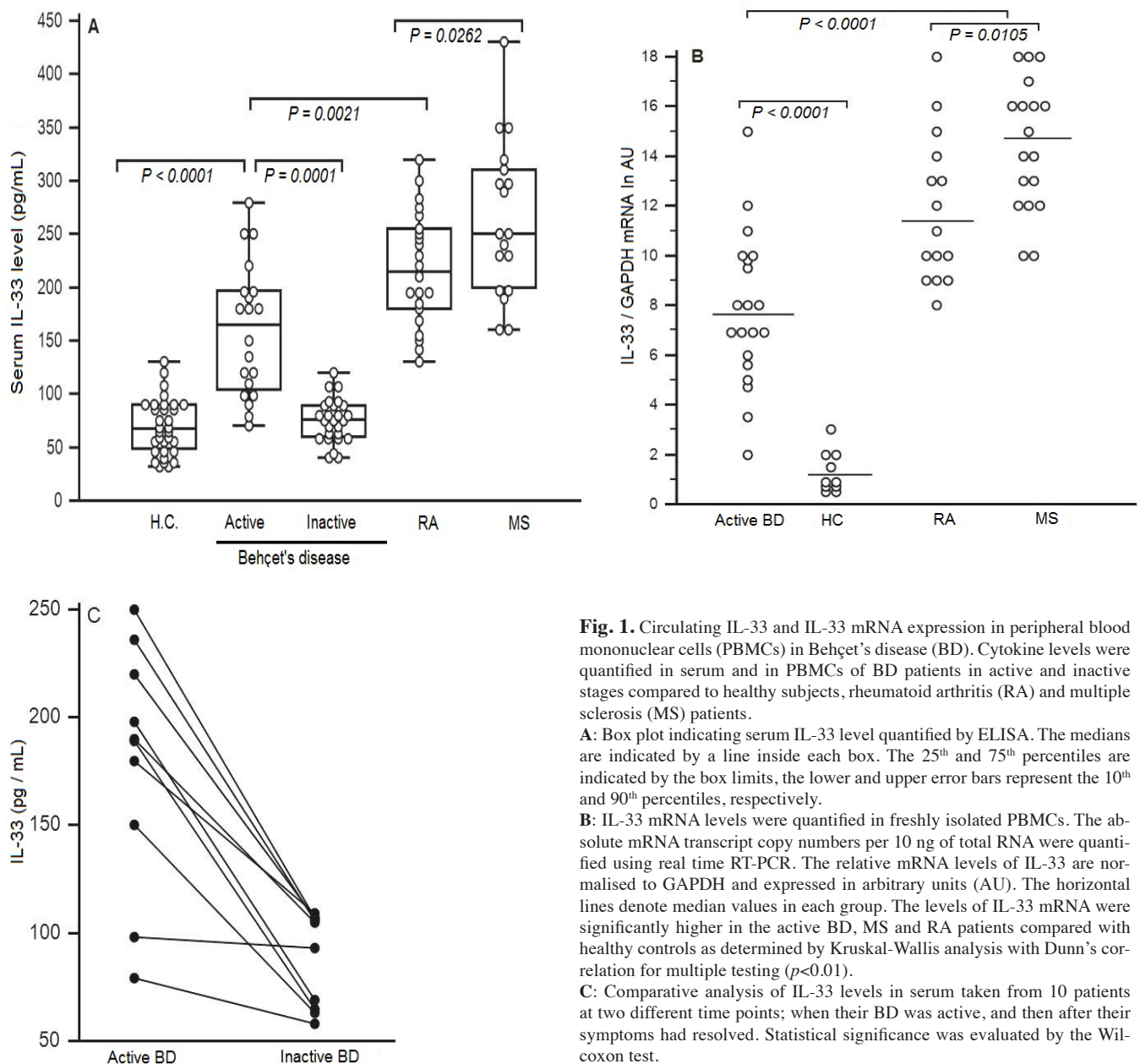NF-κB DNA-binding activity was analysed using the TransAMNF-κB p65 transcription factor assay kit (Active

Fig. 1. Circulating IL-33 and IL-33 mRNA expression in peripheral blood mononuclear cells (PBMCs) in Behçet's disease (BD). Cytokine levels were quantified in serum and in PBMCs of BD patients in active and inactive stages compared to healthy subjects, rheumatoid arthritis (RA) and multiple sclerosis (MS) patients.

A: Box plot indicating serum IL-33 level quantified by ELISA. The medians are indicated by a line inside each box. The 25th and 75th percentiles are indicated by the box limits, the lower and upper error bars represent the 10th and 90th percentiles, respectively.

B: IL-33 mRNA levels were quantified in freshly isolated PBMCs. The absolute mRNA transcript copy numbers per 10 ng of total RNA were quantified using real time RT-PCR. The relative mRNA levels of IL-33 are normalised to GAPDH and expressed in arbitrary units (AU). The horizontal lines denote median values in each group. The levels of IL-33 mRNA were significantly higher in the active BD, MS and RA patients compared with healthy controls as determined by Kruskal-Wallis analysis with Dunn's correlation for multiple testing ($p < 0.01$).

C: Comparative analysis of IL-33 levels in serum taken from 10 patients at two different time points; when their BD was active, and then after their symptoms had resolved. Statistical significance was evaluated by the Wilcoxon test.

Motif, Carlsbad, CA) following the manufacturer's instructions and as previously described (34). Briefly, nuclear extracts were prepared from freshly isolated PBMCs that were isolated from normal subjects and active BD patients. Protein levels of the nuclear extracts were quantified with the Bradford assay (Pierce Chemicals, Rockford, IL) and 10 μg was incubated in a 96-well plate coated with oligonucleotide containing the NF-κB consensus-binding sequence 5′-GGGACTTTCC-3′. Bound NF-κB was then detected by a p65-specific primary antibody. An HRP-conjugated secondary antibody was then applied to detect the bound primary antibody and provided the basis for colorimetric quantification. The enzymatic product was measured at 450 nm with a reference wavelength of 650 nm by a microplate reader. To quantify the amount of NF-κB, serial dilutions of purified p65 recombinant protein (20 ng–0.16 ng) were measured to provide a calibration curve between p65 binding and absorbance. The specificity of the assay was further tested by the addition of wild type or mutated NF-κB consensus oligonucleotide in the competitive or mutated competitive control wells before the addition of nuclear extracts.

The addition of the wild-type NF-κB consensus oligonucleotide completely abolished NF-κB binding.

Statistical analysis
All data are reported as (mean ± standard deviation). Differences between groups were analysed by using the Wilcoxon signed-rank test or the Mann-Whitney U-test. Correlation was assessed using Spearman rank test. A p-value <0.05 was considered significant.

Results
IL-33 is highly expressed in BD patients
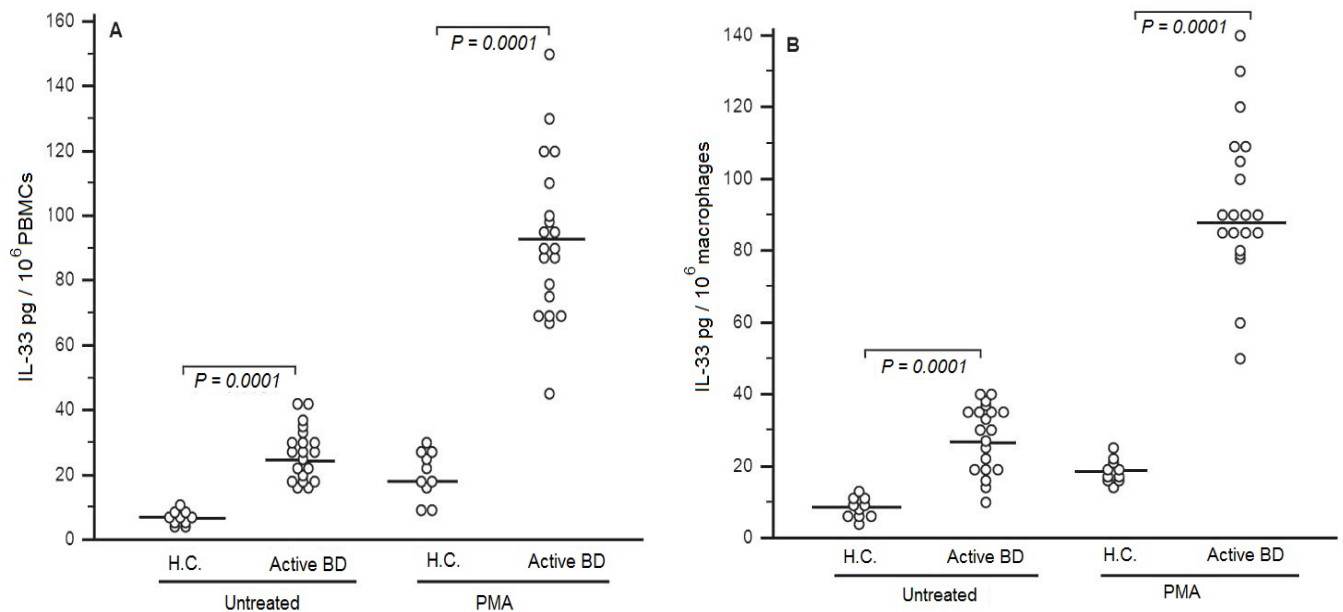The levels of serum IL-33 protein and

Fig. 2. IL-33 levels in the supernatants of cultured PBMCs and macrophages of active BD patients.
A: PBMCs were cultured in the presence of IL-2 for one week and then stimulated with phorbol myristate acetate and ionomycin (PMA) for 24 h or media alone. Then IL-33 level was quantified in the supernatants by ELISA. **B**: Adherent monocytes were expanded in the presence of GM-CSF for one week. Macrophages were then stimulated for 24 h with LPS and secreted IL-33 protein levels were determined with ELISA.

IL-33 mRNA expression from PBMC were measured in BD patients, RA patients, MS patients and healthy subjects (Fig. 1A). The average level of IL-33 was in healthy subjects (70.03±25.95 pg/mL). Significantly higher level of IL-33 was observed in BD patients (112.130±60.13 pg/mL; $p<0.0001$) compared to healthy controls. Active BD patients expressed higher levels of IL-33 (159.65±61.76 pg/ml) than inactive patients (75.57±20.34 pg/ml; $p<0.0001$). Significant difference was obtained between RA patients (218.0±53.15 pg/ml) and MS patients (264.05±72.67 pg/ml; $p=0.0262$). Both control disease group expressed higher IL-33 levels than active BD patients ($p=0.00021$). Active BD patients with retinal vasculitis expressed the highest (205.27±41.08 pg/ml; 135–279 pg/ml; $p<0.0001$) serum IL-33 level than patients without this symptom (103.88±24.27 pg/ml; rage: 70–150 pg/ml). No other correlation was observed between IL-33 level and clinical manifestations in BD patients.
The level of IL-33 was compared before and after treatment in 10 patients with BD. Figure 1C shows single value of IL-33 before and after treatment for each patient. The serum levels of IL-33 decreased after treatment in patients with BD.

In order to corroborate the ELISA findings and examine whether IL-33 was transcriptionally regulated, we also quantified IL-33 mRNA in freshly isolated PBMCs by real-time RT-PCR in 10 healthy controls, 20 active BD patients, 18 MS patients and 15 RA patients (Fig. 1B). Active BD patients demonstrated 6 fold higher level of IL-33 compared to healthy subject PBMCs (1.29±0.81; $p<0.0001$). IL-33 mRNA expression was highly expressed in MS patients (14.44±2.61; $p<0.0001$) and in RA patients (11.80±2.95; $p<0.0005$) when compared to active BD patients (7.80±3.05). Low significant differences were observed between MS and RA patients ($p=0.0105$).

IL-33 expression in cultured lymphocytes and macrophages of active BD patients
The expression of IL-33 in cultured PBMCs was examined from 20 active BD patients and 10 healthy controls (Fig. 2A-B). PBMCs were cultured in the presence of IL-2 for one week to expand the lymphocyte population. The proportion of cell making up the PBMCs of BD patients and healthy controls (as reported in materials and methods) were 78% T cells, 5% B cells and 3% monocytes. We

further stimulated cultured PBMCs with phorbol myristate acetate (PMA) and ionomycin for 24 h. Following stimulation, the levels of IL-33 measured by ELISA were increased, and importantly PBMCs from active BD patients secreted significantly higher levels (92.35±24.81 pg/ml) compared to healthy controls (20.40±7.32 pg/ml; $p<0.0001$) (Fig. 2A).
Additionally, we examined the expression of IL-33 in macrophages of normal subjects and active BD patients (Fig. 2B). Blood monocytes were expanded in the presence of GM-CSF for one week resulting in more than 95% adherent macrophages determined by morphology in microscope and CD14 staining. Macrophages were then stimulated with LPS for 24 h and secreted IL-33 protein levels were determined with ELISA. The stimulated macrophages from active BD patients secreted significantly higher levels of IL-33 (93.10±21.58 pg/ml) compared to macrophages from healthy controls (18.80±3.29 pg/ml; $p<0.0001$).
Importantly, the stimulated macrophages and PBMCs from active BD patients secreted significantly higher levels of IL-33 compared to macrophages and PBMCs from healthy controls.

NF-κB expression activation correlates with IL-33 levels

To examine possible mechanisms of elevated IL-33 in leukocytes of active BD patients, we quantified the activation of the transcription factor NF-κB, which has been shown previously to mediate IL-33 induction (2, 35). Nuclear extracts from freshly isolated PBMCs were prepared and allowed to bind an NF-κB consensus oligonucleotide sequence. Bound NF-κB was then detected by a p65 (RelA)-specific antibody and quantified based on a calibration using purified p65 recombinant protein. NF-κB DNA binding activity was significantly increased in PBMCs of active BD patients compared to healthy controls (Fig. 3A). LPS stimulation significantly induced NFκB activation and was significantly more elevated in macrophages from active BD patients compared to healthy controls (Fig. 3B).

NF-κB DNA binding activity was significantly increased in PBMCs of active BD patients (2.53±0.59) compared to healthy controls (0.67±0.32; $p<0.0001$).

Thus, NF-κB activation correlated with IL-33 mRNA expression in fresh PBMC (r=0.495; $p=0.0263$), and LPS stimulated PBMC (r=0.491; $p=0.0279$), suggesting a possible transcriptional mechanism by which IL-33 is elevated in active BD patients.

IL-33 mRNA expression in skin lesions in active BD patients

In patients with active BD as reported in Table I, the skin lesions were scored as 0: absent; 1–5: 2; 6–10: 3; 11–15; 4: 16–20; and 5: >20 in number and the scores were correlated with serum IL-33 levels. A significant positive correlation was observed between serum IL-33 levels and skin lesions (r=0.587; $p=0.0064$) (Fig. 4).

IL-33 mRNA expression in the skin lesions of patients with active BD was significantly increased compared to that in healthy skin biopsies ($p=0.00016$) (Fig. 5).

Correlation of IL-33 with inflammatory cytokines IL-6 and IL-17

IL-6 and IL-17 levels were measured in sera of patients with active BD.
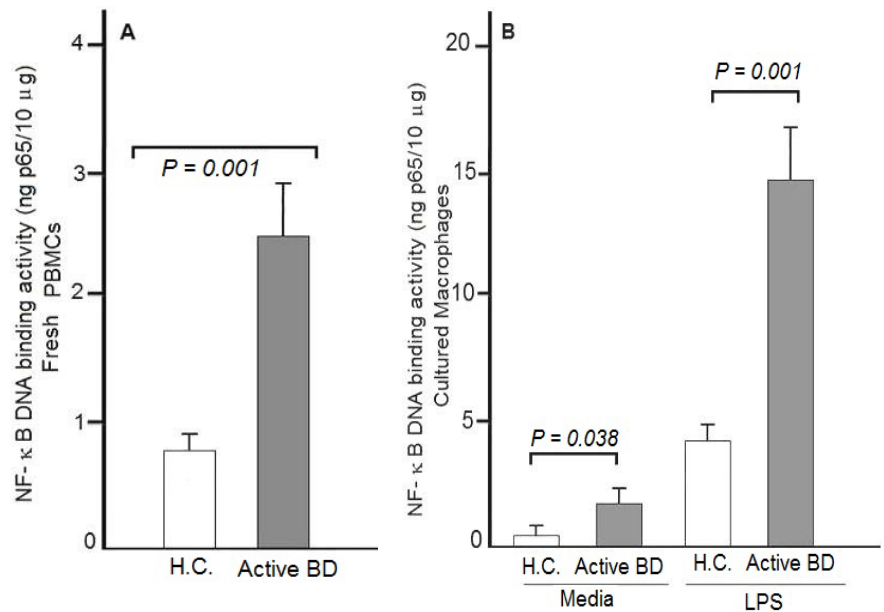

Fig. 3. NF-κB activation in PBMCs from active Behçet's disease (BD) patients and healthy controls (HC). Nuclear extracts were isolated from freshly isolated PBMCs or cultured macrophages and allowed to bind an NF-κB consensus-binding sequence. Bound NF-κB was then detected by a p65 specific antibody and quantified based on a calibration curve generated by using a purified p65 recombinant protein. The NF-κB DNA binding activity is reported as ng of bound p65 protein per 10 μg of total protein in nuclear extracts.
A: NF-κB activation in freshly isolated PBMCs from BD patients and healthy controls.
B: NF-κB activation in cultured macrophages from BD patients and healthy controls following treatment with media alone or LPS.
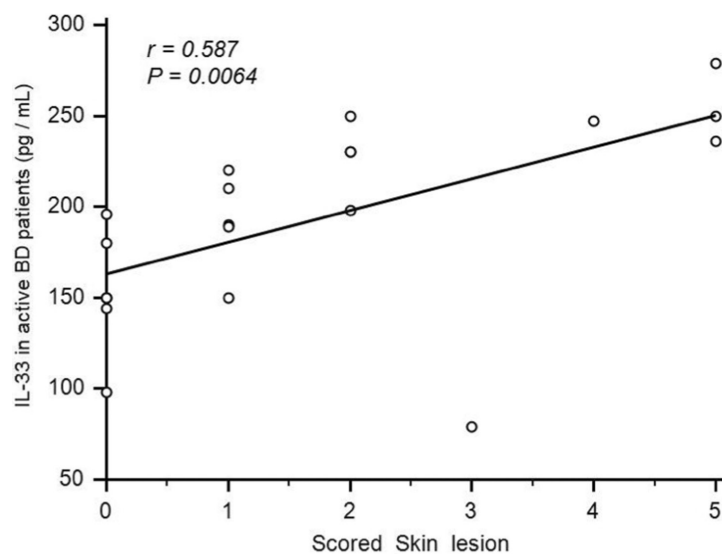

Fig. 4. Correlation between scored skin lesions (X axis) and serum IL-33 in active Behçet's disease (BD) patients (Y axis). The results demonstrate a positive correlation (r=0.587; $p=0.0064$).

High levels of IL-6 (BD: 21.7±4.6 pg/mL; healthy controls: 6.8±0.7 pg/mL; $p=0.0001$) and IL-17 (BD: 17.63+6.18 pg/mL; healthy controls: 8.75+2.84 pg/mL $p=0.0001$) were observed in active BD patients. A significant positive correlation was expressed between serum IL-17 and IL-33 level (r=0.533; $p=0.0024$) and between IL-33 and IL-6 (r=0.661, $p=0.0015$) in active BD patients.

Discussion

This is the first report studying the expression of the cytokine IL-33 in BD. We have shown that active BD patients expressed elevated levels of IL-33 in
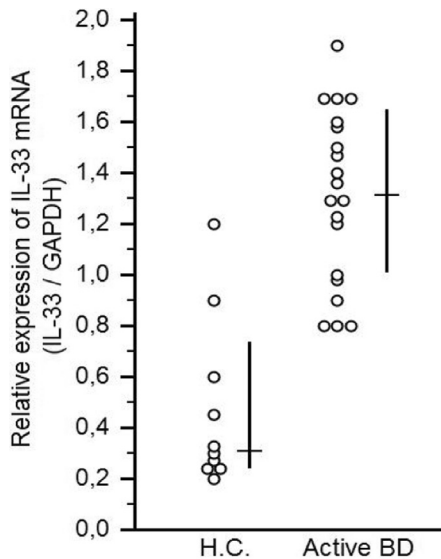
Fig. 5. IL-33 mRNA expression in skin lesions from patients with active BD was measured using a real-time reverse transcription-polymerase chain reaction. Histologic examination of affected skin from these patients revealed predominantly perivascular infiltration or leukocytoclastic vasculitis (data not shown). IL-33 mRNA expression relative to a calibrator (one of the control samples) was expressed as $2^{-\Delta\Delta XT}$ (fold) and is indicated by the solid circles. Data are presented as medians and 25th-75th percentiles. Statistical comparisons between pairs of groups were made using the Mann-Whitney U-test. A p-value less than 0.05 was considered to be statistically significant.

serum and in freshly isolated PBMCs compared to healthy controls. Patients with active BD expressed lower levels of IL-33 than MS and RA patients, studied as control diseases. Skin lesions from BD patients exhibited increased IL-33 mRNA expression compared to biopsies from healthy skins. Recently it was reported that IL-33 receptor antibody attenuates severity of experimental arthritis, accompanied by a significant reduction of IL-17 (36). We reported significant increase of IL-33 level in active BD with retinal vasculitis.

IL-2-expanded PBMCs and GM-CSF-cultured macrophages led to a significantly higher secretion of IL-33 in active BD patients compared to healthy controls. These data suggest a possible role for IL-33 in the immune response activation in BD patients. It has been speculated that IL-33 may be released as a result of cell damage, which alerts the immune system to 'danger', but otherwise remains in the nucleus of living cells where it limits the immune response (37). IL-33 is a dual-role cytokine. It can not only promote but also reduce inflammation depending on the tissue environment (37). IL-33 contributes to development of Th1-type of immune response as well as enhanced IL-1 and IL-18 secretion. Raised levels of several pro-inflammatory cytokines have been reported in BD, which can lead to elevated transcription factors including NF-κB in blood leukocytes (38-39). NF-κB is a family of critical transcriptional factors involved in the regulation of a large variety of inflammatory responses and apoptosis. Importantly, NF-κB contributes to the regulation of the apoptosis-related factors and death receptors leading to apoptosis resistance of T cell subsets in BD patients (39). NF-κB activity may play a fundamental role in the pathogenesis of BD, and its down-regulation by thalidomide as reported by Todaro *et al.* could outline an alternative therapeutic approach for the treatment of BD (39). These findings contribute to the delineation of the intracellular mechanism whereby BD T lymphocytes are resistant to cell death (39).

IL-33 has been shown to act on monocytes, dendritic cells, and macrophages to induce the expression of cytokines, chemokines, and other proinflammatory genes (40). *In vitro* stimulated macrophages from BD patients produced higher IL-33 levels compared to macrophages healthy controls. A diversity of cell types have been reported to express IL-33 mRNA including smooth muscle cells, epithelial cells, fibroblasts, keratinocytes, dendritic cells and activated macrophages (2).

Serum IL-33 levels were correlated with the skin lesions in BD patients. Skin lesions expressed high IL-33 mRNA compared to healthy skin. Pushparaj *et al.* reported (12) that IL-33 expression in the nucleus is significantly upregulated in inflamed skin. Damaged skin in response to an allergen and inflammation lead to cell necrosis and release of biologically active IL-33 (41-42). IL-33 can potently activate innate immune cells leading to release of biologically active mediators such as VEGF, histamine, prostaglandin E2 (PGE2) and chemokine, thus recruiting neutrophils. IL-33 mRNA and protein is also substantially higher in the skin lesions of patients with atopic dermatitis compared with non-inflamed skin samples (12). Our result has to be integrated with caution in the immune skin lesions of BD patients as reported Melikoglu *et al.* (42). We provide evidence for up-regulated expression of IL-33 mRNA expression in PBMCs and in skin lesions of BD patients. It is possible that IL-33 is released as an alarmin by keratinocytes and/or endothelial cells as a result of tissue damage. These data suggest an important interplay between the immune system, structural cells of the skin and migration of peripheral blood inflammatory cells through endothelial cells.

Importantly, in the present report we demonstrate that NF-κB activation correlates with IL-33 expression suggesting possible mechanisms of the transcriptional upregulation of IL-33 in leukocytes of active BD patients.

We found that both IL-6 and IL-17 expressed significant positive relationships with IL-33 in the sera of active BD. Other cytokines such as IL-1 and TNF-α established significant correlations with IL-33 (10-11). Xu *et al.* (43) showed that IL-33 increases the levels of IL-1β, IL-6, IL-13, and chemokines produced *in vitro* by bone marrow-derived mast cells from wild-type. Palmer *et al.* (36) investigation in RA immunity, reported that an anti-ST2 blocking antibody inhibited IL-33-induced IL-6 secretion. These results suggest that increased IL-33 leads to upregulation of inflammatory cytokines such as IL-1β and IL-6 in patients with RA. Therefore, we think that serum level of IL-33 could partially reflect BD disease. Recently we reported that BD patients expressed NOD2 as a result of lung inflammation in active BD patients (44). TLRs and NOD2 synergise for the induction of proinflammatory cytokines (44). Inflammasomes are cytoplasmic

multiprotein complexes that mediate the maturation of the proinflammatory cytokines IL-1β, IL-18, and possibly IL-33 by controlling the activation of the inflammatory caspases-1 and -5 (45). Further research should be performed to ascertain the utility of IL-33 as biomarker for assessing disease activity in BD and to clarify the interactions between IL-33 and others mediators.

IL-33 may be involved in the trafficking of T cells to an inflammatory site (46). Implication of IL-33 in the trafficking of inflammatory cells could be associated with the pathogenesis of BD at the same level than Th2 and Th17 cells. Taken together, this study provides first observations on the increased serum IL-33 levels and the high IL-33 mRNA expression in PBMCs and in skin lesions. The targets and kind of cells for liberated IL-33 in inflammatory BD sites (lung, central nervous system, intestine) is unanswered and have to be clarified. In the same way one of our study limitations is lack of cross sectional design. We did not estimate IL-33 level and laboratory characteristics. Finally more important sample size has to be investigated in future work.

IL-33 is a double-edged sword (47), and targeting IL-33 should be approached with caution. Further studies are needed to define the role of IL-33 and IL-33 receptors (sST2 and ST2L) in BD. Anti-IL-33 therapy may lower TNF-α and IL-17 production and benefits autoimmune/inflammatory diseases. Despite limitations, more studies should be conducted to explore potential therapeutic value of anti-IL-33 therapy in BD patients.

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