

# Ex vivo and in vitro production of pro-inflammatory cytokines in Blau syndrome

P. Galozzi<sup>1</sup>, O. Negm<sup>2,3</sup>, E. Greco<sup>1</sup>, N. Alkhattabi<sup>2</sup>, A. Gava<sup>1</sup>, P. Sfriso<sup>1</sup>, L. Fairclough<sup>2</sup>, I. Todd<sup>2</sup>, P. Tighe<sup>2</sup>, L. Punzi<sup>1</sup>

<sup>1</sup>Rheumatology Unit, Department of Medicine - DIMED, University of Padova, Italy;

<sup>2</sup>School of Life Sciences, Department of Immunology, University of Nottingham, Nottingham, United Kingdom;

<sup>3</sup>Medical Microbiology and Immunology Department, Faculty of Medicine, Mansoura University, Egypt

## SUMMARY

The objective was to study both *ex vivo* and *in vitro* secretion of pro-inflammatory cytokines in patients affected by Blau syndrome (BS) and carrying p.E383K mutation in the *CARD15/NOD2* gene associated with the disease.

For *ex vivo* studies, peripheral blood mononuclear cells (PBMCs), serum from three patients and healthy controls have been collected. PBMCs have been cultured in the presence or absence of inflammatory enhancers, such as lipopolysaccharide (LPS) and muramyl dipeptide (MDP). The levels of interleukin (IL)-1 $\beta$ , IL-6, IL-8, tumor necrosis factor (TNF)- $\alpha$  and interferon (IFN)- $\gamma$  were assayed by either immunoassay or array-based system. For *in vitro* studies, different constructs were created cloning human *wild-type* and p.E383K-mutated *NOD2* cDNA into the expression vector pCMV-Tag2c. HEK293 cell lines were stably transfected, cultured with or without MDP and IL-8 level was assayed in their supernatants. Statistical analysis in both studies was performed using non-parametric tests.

Both *ex vivo* and *in vitro* studies have not identified a significant increase in secretion of the analyzed pro-inflammatory cytokines. p.E383K-mutated *NOD2* transfected cells express low level of IL-8. The *ex vivo* basal level results from both serum and PBMCs supernatants present similar levels of IL-1 $\beta$ , IL-6, TNF- $\alpha$  and IFN- $\gamma$  in patients and controls. The presence of the stimulant agents (LPS and MDP), either individual or paired, does not lead to significant increases in all cytokines concentrations in patients compared to controls.

Taken together, the *ex vivo* and *in vitro* data suggest that there is not a primary mediation of IL-1 $\beta$  and other pro-inflammatory cytokines in BS patients carrying p.E383K.

**Key words:** Pro-inflammatory cytokines, Blau syndrome, Autoinflammatory disease, Interleukin-1 $\beta$ .

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## INTRODUCTION

Blau syndrome (BS, MIM #186580) is a rare autoinflammatory granulomatous disorder, dominantly inherited, described in 1985 as a chronic inflammatory syndrome characterized by granulomatous dermatitis, symmetric arthritis and recurrent uveitis (1). The eye involvement is the most relevant morbidity of BS, presenting in particular as granulomatous uveitis that can evolve into cataract and band keratopathy. Skin manifestations are described as papulonodular, tender, brownish rash or multiple, firm, subcutaneous plaques only apparent on palpation. The most frequent joint involvement in BS is inflammatory

arthritis, with moderate redness, warmth and tenderness, associated with synovial cysts and tenosynovitis with a chronic evolution (2). Due to the rarity and variable expressivity of BS, no studies on the optimal treatment have been made yet. Low-dose glucocorticoids are generally acceptable at the quiescent stage (3), although biologic anti-cytokine agents (infliximab and anakinra) may be a promising therapeutic approach in refractory cases (4).

The gene responsible for BS was identified in 2001 in the caspase recruitment domain 15 gene (*CARD15/NOD2*), mapped in the chromosomal region 16q12.1-13 (5). Since then, several cohorts of BS patients have been genotyped, leading to identification

Corresponding author:  
Galozzi Paola  
UOC di Reumatologia  
Dipartimento di Medicina - DIMED  
Via Giustiniani, 2 - 35128 Padova, Italy  
E-mail: paola.galozzi@unipd.it

of different *CARD15/NOD2* mutations from distinct ethnic origins. Until now, 17 missense substitutions have been observed associated with BS. Among those, the mutations affecting the highly evolutionary conserved Arginine residue at codon 334 could be counted as mutational hot spot (6-8). In 2005, our group has identified the mutation p.E383K in an Italian family affected by BS (9). Mutations in *CARD15/NOD2* have also been associated with a higher risk of Crohn's disease (MIM #266600), a multifactorial inflammatory bowel disorder characterized by transmural granulomatous inflammation of the gastrointestinal tract (10). *CARD15/NOD2* gene encodes a multi-domain protein of 1040 amino acids, called nucleotide oligomerization domain 2 (NOD2), presenting a three-domain structure composed of two N-terminal effector regions CARD, a central activator NACHT, and nine C-terminal pathogen-sensing leucine-rich repeats (LRRs) (11). NOD2 is a member of the NOD-like receptor family (NLR), highly expressed in monocytes and granulocytes. There, it plays an important role in innate immune defence as intracellular receptor for bacterial products, *i.e.* muramyl dipeptide (MDP) (12). Upon recognizing MDP, NOD2 triggers host defence response by inducing nuclear factor (NF)- $\kappa$ B activation through a cascade of phosphorylations. Once activated (NF)- $\kappa$ B, it translocates to the nucleus and starts the transcription of pro-inflammatory genes (13). The BS-associated mutations are reported to be related *in vitro* with elevated basal and MDP-induced NF- $\kappa$ B activity, supporting a gain-of-function theory and suggesting a spontaneous release of pro-inflammatory cytokines, firstly interleukin (IL)-1 $\beta$  (14). Despite the fact that BS is genetically well-characterized, few studies in the literature have analyzed the cytokines profile in BS. Elevated serum levels of IL-1 $\beta$ , IL-6 and tumor necrosis factor (TNF)- $\alpha$  in BS patients have been reported by Arostegui et al. (15). However, different other studies could not confirm an increase in pro-inflammatory cytokines levels (16-18). Based on this controversy, we analyzed

both *ex vivo* and *in vitro* the cytokines profile in BS patients carrying p.E383K mutation.

## ■ MATERIALS AND METHODS

### **Subjects**

Three subjects with the classical manifestation of BS and 5 gender- and age-matched healthy controls were included in this study. The three BS patients belong to the same Italian family (Fig. 1), the only one carrying the p.E383K mutation (9). The proband (II4), now 61 years old, was referred to our unit in 1984 and diagnosed as BS on the basis of the described criteria (19). Main clinical signs were widespread papules and firm subcutaneous nodules at the upper and lower limbs, severe bilateral uveitis, and symmetrical arthritis of the small joints of the hands. At the time of this study, the proband presented arthralgia of the lower limbs treated with methylprednisolone 4 mg/day and granulomatous uveitis evolved into cataract and band keratopathy. The daughter (III2), now 36 years old, showed similar, although less severe, skin manifestations, uveitis and arthritis. Her clinical state at the time of this study was good, while she was assuming methylprednisolone 4 mg/day. The grand-daughter (IV1), now 5 years old, has not developed any clinical BS manifestation yet.

### **Collection of serum and stimulated peripheral blood mononuclear cell**

After informed consent, serum and blood samples were obtained from BS patients and healthy controls. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-density gradient separation and seeded at a density of 10<sup>6</sup> cell/mL in RPMI media containing 1% Glutamine and 10% FCS. Cells were cultured for 24 h in an atmosphere of 95/5% air/CO<sub>2</sub> at 37°C either without stimulation or in the presence of muramyl dipeptide [10  $\mu$ g/mL] (Sigma-Aldrich, St. Louis, MO, USA), purified lipopolysaccharide from *E. Coli* 055:B5 [100 ng/mL] (Sigma-Aldrich) or a combination of both.

### Construction of NOD2-encoding plasmids

Expressing plasmid pCMV-Tag2c encoding amino-terminally FLAG-tagged NOD2 wild-type (kindly provided by Dr T. Kufer) was the starting point for obtaining mutated NOD2 expressing plasmids. p.E383K mutation was introduced by using QuikChangeXL site-directed mutagenesis kit (Stratagene). All constructs were subjected to full-length sequencing.

### Stable transfection into HEK293 cells and stimulation

Human HEK293 cells were cultured in DMEM High glucose 90% medium (Life Technology, Carlsbad, CA, USA) supplemented with 0.1% Gentamycin, 10% FCS and 2% L-glutamine. Stable transfections were performed by using *Lipofectamine*<sup>TM</sup> 2000 (Life Technologies), according to the manufacturer's directions. After 48 h the transfected cell lines were selected adding 0.8 mg/mL G418 (Life Technology). A control cell line was generated by transfecting an empty mock vector.

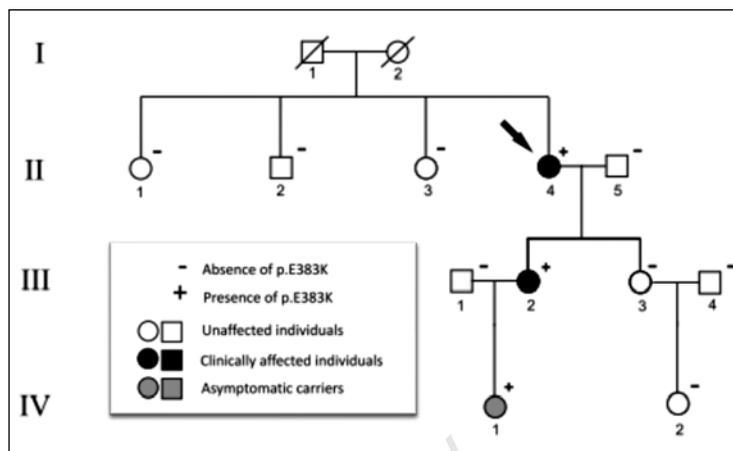
Transfected HEK293 cells were cultured for 7 and 24 h either with or without muramyl dipeptide stimulation [10 µg/mL] (Sigma-Aldrich).

### Cytokine analysis

Human IL-1β, IL-6, IL-8, TNF-α and IFN-γ in PBMCs supernatants were measured by immunoassays using commercial kits (IMMULITE, Siemens, Berlin, Germany) according to the manufacturer's instructions.

In serum samples, the cytokines were assayed using an array-based system. Briefly, capture antibodies dilutions [100 µg/mL] were prepared in a 384-well plate and spotted on poly-l-lysine slides by MicroGrid II robot (BioRobotics Inc., Ramsey, MN, USA).

After creating separated wells with proper slide holders, the printed slides were blocked with I-Block solution (Applied Biosystem, Waltham, MA, USA) and incubated for 1 h with serum samples, along with a cocktail of antibodies standards to enable quantification. A mix of biotinyl-



**Figure 1** - Pedigree structure of the proband and her family. Filled-in symbols indicate clinically affected family members, both proband (subject II-4) and her daughter (subject III-2). Shaded symbol represents proband's granddaughter (subject IV-1), an asymptomatic carrier of the p.E383K mutation. Absence (-) or presence (+) of p.E383K mutation is indicated.

ated detection antibodies was added then to each block and incubated for 1 h. Serum samples need an amplification step to improve the signal sensitivity (TSA, Bio-rad Lab., Hercules, CA, USA). Streptavidin-conjugated cy5 at 1:1000 is finally incubated for 15 min, then the slides are dried by centrifugation and scanned at 635nm with Axon 4200AL scanner.

In surnatants of NOD2-transfected HEK293 cells, IL-8 was measured by the same array-based system described above, without the amplification step.

All experiments were made in duplicate.

### Data processing and statistics

The immunoassay data were used for statistical comparisons between the means of data obtained from experimental groups and those from control groups, using analysis of variance with Fisher's post hoc (GraphPad Prism 5).

Cy5 fluorescence, gained from the microarray system, was instead quantified using GenePix Pro-6 software (Axon Instrument, Union City, CA, USA), subtracted from the background and used for computing average and standard deviation. The concentrations of each cytokine were calculated based on a standard curve of known

amounts of standard cytokines. Between-group comparisons were performed with non-parametric tests (Kruskal-Wallis and Mann-Whitney) (GraphPad Prism 5). Statistical significance was defined as two-sided  $P$ -value  $< 0.05$ .

## ■ RESULTS

To define the biological effects of p.E383K BS-associated mutation, we examined both *ex vivo* and *in vitro* the ability of this mutation to produce cytokines in the presence or absence of activating stimuli.

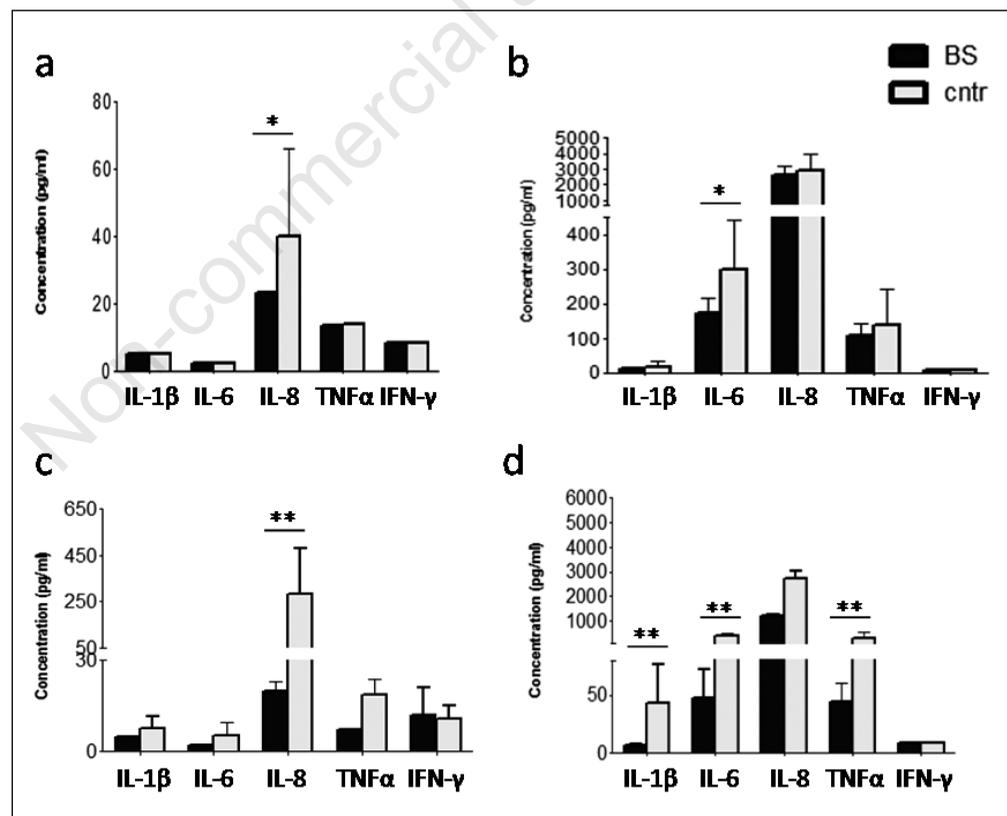
### *Ex vivo* cytokines profile

As presented in Figure 2a, no statistical differences in cytokine levels were detected between patients and controls, ex-

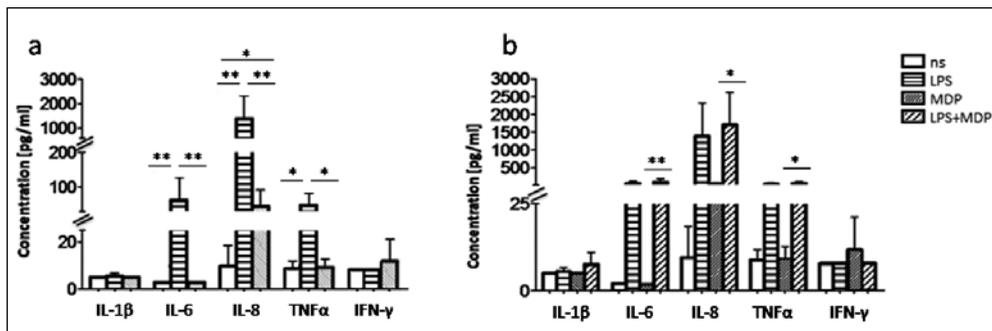
cept for IL-8 that was secreted at higher titre ( $P < 0.05$ ) by healthy controls in the absence of stimulation. When stimulated with the TLR agonist [lipopolysaccharide (LPS)], BS PBMCs released similar or statistically lower levels of cytokines compared to healthy controls, such as for IL-6 ( $P < 0.05$ ) (Fig. 2b).

Despite these results, the cytokines levels raised significantly in patients after LPS stimulation, as expected (Fig. 3a). IL-6, IL-8 and TNF- $\alpha$  concentrations were highly released after LPS stimulation ( $P < 0.005$ ,  $P < 0.005$  and  $P < 0.05$  respectively), with the exception of IL-1 $\beta$  and IFN- $\gamma$ .

Even the NOD2 agonist (MDP) did not stimulate a high cytokine release in patients compared to healthy controls. A significant decrease, indeed, was seen



**Figure 2** - Cytokines levels of peripheral blood mononuclear cells surnatants obtained from Blau syndrome patients (black) and healthy controls (grey) in the absence (a) or presence of lipopolysaccharide (b), muramyl dipeptide (c) and both stimulants (d) after 24 h. The data shown are means of data from duplicate experiments and the error bars indicate standard deviation. \* $P < 0.05$ ; \*\* $P < 0.005$ . IL, interleukin; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; IFN- $\gamma$ , interferon- $\gamma$ .



**Figure 3** - Cytokines levels in peripheral blood mononuclear cells surnatants obtained from Blau syndrome patients after 24 h. a) Comparison between individual stimuli and basal level; b) Comparison between double stimulation and muramyl dipeptide (MDP) stimulation. The data shown are means of data from duplicate experiments and the error bars indicate standard deviation. \* $P < 0.05$ ; \*\* $P < 0.005$ . ns, not significant; LPS, lipopolysaccharide; IL, interleukin; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; IFN- $\gamma$ , interferon- $\gamma$ .

in patients ( $P < 0.005$ ) for IL-8 (Fig. 2c). Evaluating only BS patients (Figure 3a), it was notable that only IL-8 levels increased after MDP stimulation ( $P < 0.05$ ), while levels of all other cytokines were similar to the non-stimulated ones or statistically lower than LPS stimulated ones ( $P < 0.005$  for IL-6 and TNF- $\alpha$ ). Moreover, this study failed to identify an increase in cytokine secretion in patients following double stimulation. IL-1 $\beta$ , IL-6 and TNF- $\alpha$  showed higher concentration in controls ( $P < 0.005$ ) (Fig. 2d), whereas, considering only patients, a synergistic effect was detected in IL-6, IL-8, and TNF- $\alpha$  production ( $P < 0.005$ ,  $P < 0.05$ ,  $P < 0.05$  respectively) (Fig. 3b).

The *ex vivo* results of serum samples at basal level (Fig. 4) reflect those presented above about PBMCs surnatants, *i.e.* no differences between patients and controls.

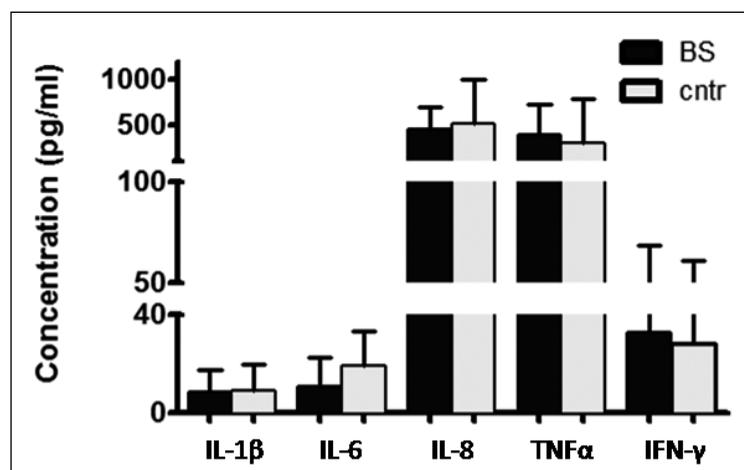
#### *In vitro* cytokines profile

Analyzing the data obtained after 7 h (Fig. 5a), we noted that in the absence of stimulation the levels of IL-8 in cells expressing *wild-type* NOD2 were statistically higher ( $P < 0.05$ ) than those obtained from p.E383K-mutated cells. When added MDP at the cell cultures, no differences were spotted between p.E383K and *wild-type* NOD2 transfected cells. A similar trend for both cell lines was observed for IL-8 secretion after 24 h (Fig. 5b).

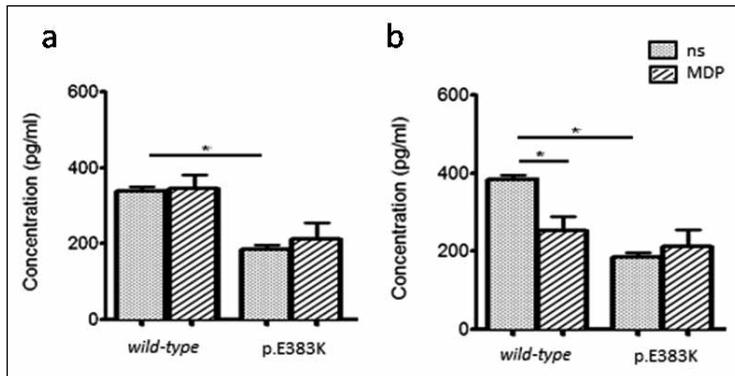
## DISCUSSION

The *CARD15/NOD2* gene encodes the intracellular detector NOD2 that mediates NF- $\kappa$ B pathway activation in response to invasive bacterial infections. A distinct group of mutations in this gene causes, in heterozygous state, the rare autoinflammatory disease named Blau syndrome.

In 2005, our group has identified the mutation p.E383K in an Italian family affected by BS (9). This mutation is located in the



**Figure 4** - Cytokines concentrations in serum obtained from Blau syndrome (BS) patients (black) and healthy controls (grey) at basal level. The bars indicate the means of data, whereas the error bars indicate standard deviation. No significant differences are observed. cntr, control; IL, interleukin; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; IFN- $\gamma$ , interferon- $\gamma$ .



**Figure 5** - Interleukin-8 levels of surnatants obtained from NOD2 transfected HEK293 cells stimulated or not with muramyl dipeptide (MDP) for 7 (a) and 24 (b) h. The data shown are means of data from duplicate experiments and the error bars indicate standard deviation. \* $P < 0.05$ . ns, not significant.

central domain and changes Glutamate 383 to Lysine. To date, no functional studies are presented in literature concerning this mutation. Its pathogenicity was supported by indirect pieces of evidence, such as its dominantly inheritance and its conservation among species.

The autoinflammatory nature of BS is related with its associated mutations. The gain of function hypothesis of BS mutations may suggest a spontaneous release of more cytokines, such as IL-1 $\beta$  (15). Moreover, several studies published in the literature have stated the importance of pro-inflammatory cytokines in driving the inflammation in different autoinflammatory conditions. Most of these studies underline the prominence of the IL-1 $\beta$  levels and its role in inflammasome activation and regulation, in particular on autoinflammatory diseases associated pyrin, NLRP12 and cryopyrin (NLRP3) proteins (20, 21). This has led to a breakthrough in therapy, with IL-1 inhibitors producing rapid and sustained amelioration of symptoms.

Moreover, also IL-6 is likely to be involved in the pathogenesis of some autoinflammatory diseases, due to high serum levels in periodic fevers and complex autoinflammatory disorders (Adult-Onset Still disease and Behçet disease) (22-24). Furthermore, IL-8 and TNF- $\alpha$  are known to increase in the presence of autoinflammatory conditions, such as TNF receptor-associated pe-

riodic syndrome, Behçet and Adult-Onset Still diseases (25-27). However, recent works from the literature observed no evidence of excess pro-inflammatory cytokines such as IL-1 $\beta$  and TNF- $\alpha$  in PBMCs from BS patients compared to healthy controls (16, 17). In addition to these, Son and colleagues confirmed *ex vivo* that BS is not associated with increased basal levels of TNF- $\alpha$  (18).

We analyzed the up-regulation of pro-inflammatory cytokines production *ex vivo* and *in vitro* in p.E383K mutation carriers. Due to the small dimension of our BS family, we included individuals taking systemic anti-inflammatory medications in this study. However, the extracted PBMCs were less likely to be exposed to drugs during *in vitro* culturing, because cells underwent vigorous washing steps during the isolation process. This indicated that the observed cellular response was not influenced by patients treatment, but reflected the biologic response related to p.E383K mutation.

Our *ex vivo* data from patients PBMC and serum showed similar levels for IL-1 $\beta$ , IL-6, IL-8, TNF- $\alpha$  and IFN- $\gamma$  production compared to healthy controls. The concentration values, instead, were generally higher in serum samples than PBMCs surnatants, as expected. LPS or MDP stimulation of PBMCs showed similar or lower levels of these cytokines than in healthy controls. Furthermore, no synergistic cytokine release was observed in patients compared to controls when muramyl dipeptide was used in combination with TLR agonist. In addition, our *in vitro* results did not show increased level of IL-8, both at basal and after MDP stimulation in p.E383K-mutated compared to *wild-type* NOD2.

Taken together, these findings suggest that an exaggerated NOD2 response leading to pro-inflammatory release is not a direct mechanism explaining the pathophysiology of BS. Moreover, the reduced response upon bacterial stimulation may limit the clearance by the host, contributing afterward to persistent inflammation and other clinical manifestations.

Focusing on IL-1 $\beta$ , its slightly attenuated response in our BS subjects suggests that,

in contrast with cryopyrinopathies and other hereditary periodic fever diseases, IL-1 $\beta$  do not primarily mediate the disease and IL-1 $\beta$  blocking therapy may not be an eligible treatment. In literature, some authors report the use of anakinra, IL-1 receptor antagonist, in some BS cases with variable results, in particular regarding ocular morbidity (4, 15).

Levels of the other cytokines analyzed were also not raised in p.E383K carriers. A case report of a BS patient, carrying a different mutation from the one we analyzed, in which IL-6 and IL-8 were slightly elevated has been described (28). This may suggest a possible role of NOD2 in influencing the cytokines production, maybe related to differences in amino acid mutations.

The observed low level of TNF- $\alpha$  is consistent with the reported low clinical efficacy of TNF blocking therapies (infliximab and etanercept) in BS associated with recurrent infections (4, 16).

The similar levels of IFN- $\gamma$  between our BS patients and the controls need further investigations. IFN- $\gamma$  level would be expected to be elevated in BS patients due to its involvement in granulomas formation. Moreover, Rosenzweig et al. have demonstrated in a murine model a relation between NOD2 activation and IFN- $\gamma$  increased production within the eye (29), thus suggesting that dysregulation of IFN- $\gamma$  may occur in patients with BS and contribute to the granulomatous nature of the disease. Concerning our divergent result, a possible explanation may lie in the particular mutation we have analyzed or in the cells type we have collected.

Furthermore, the almost comparable cytokine levels between the affected subjects suggest that different disease states presented at the time of this study do not have visible effect on the results.

In summary, this study is the first that characterizes both *ex vivo* and *in vitro* cytokines profile in BS subjects carrying p.E383K mutation. Our results are consistent with those reported in literature by Martin et al. (16) and Son et al. (18), whilst their studies were based on p.R334W/Q mutated NOD2. Although our analysis suffers from a num-

ber of limitations, in particular the small sample size, our data may be the starting point for studying the functional effects of p.E383K-mutated NOD2 in BS.

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