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Novel NLRP3/cryopyrin mutations and pro-inflammatory cytokine profiles in Behçet's syndrome patients

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Abstract

Behçet's syndrome (BS) is a systemic inflammatory disorder with unknown etiology. Features of both innate and adaptive immunity have been claimed in the pathogenesis of BS. To test the possible dysregulation of the NLRP3/cryopyrin (Nod-like receptor with a pyrin domain 3) inflammasome, as a result of mutation(s), we performed single-strand conformation polymorphism analyses and/or sequencing of all the coding regions and intron–exon boundaries of NLRP3/cryopyrin and ASC (apoptosis-associated speck-like protein containing CARD) genes from Turkish BS patients and healthy controls. At the same time, we determined pro-inflammatory cytokine secretion profiles of peripheral blood cells in response to LPS treatment using ELISA. BS patients with vascular involvement showed significantly increased levels of TNF-α release at 2-, 4- and 8-h post-treatment and significantly increased IL-1β levels were detected at 2 h ($P = 0.005$) and 4 h ($P = 0.025$) ($n = 10$). We identified four mutations in the NLRP3/cryopyrin gene, V200M ($n = 3/104$) and T195M ($n = 1/104$), in BS patients but none in control samples. No mutations were detected in the ASC gene. The effect of these NLRP3/cryopyrin mutants on ASC speck assembly and IL-1β secretion was tested and the V200M mutant was shown to induce IL-1β secretion. Thus, it is likely that certain mutations in NLRP3/cryopyrin in combination with yet unknown other factors may contribute to the pro-inflammatory cytokine profiles in BS patients.

Keywords: ASC, autoinflammation, IL-1β, NLR, TNF-α

Introduction

Behçet's syndrome (BS) is a systemic inflammatory disease with manifestations including recurrent oral and genital ulcerations, and vasculitis involving the skin, mucosa, joints, eyes, veins, arteries, nervous and gastrointestinal systems. While some groups included BS among autoimmune disorders, the absence of specific antibodies and the episodic nature of the clinical findings led some authors to include BS among autoinflammatory diseases (1). A recent review claimed that both innate and adaptive immune responses play substantial parts in the pathogenesis of the disease (2). Elevated levels of acute phase proteins and recurrent inflammatory attacks are associated with increased secretion of pro-inflammatory cytokines including IL-1β, TNF-α and IL-6. Both in the active and inactive BS patients, increased pro-inflammatory cytokine secretion and superoxide generation have been reported (3, 4).

Inflammasomes are multiprotein complexes controlling IL-1β secretion. The NLRP3/cryopyrin (Nod-like receptor with a pyrin domain 3) inflammasome includes ASC (apoptosis-associated speck-like protein containing CARD) (TMS1/PyCard) and procaspase-1. Interestingly, it has been shown that oligomers of ASC can form specks, presumably pyroptosome complexes that can activate caspase-1 in response to pro-inflammatory stimuli (5).

IL-1β is a significant pro-inflammatory cytokine contributing to the establishment of the symptoms in autoinflammatory diseases and the NLRP3/cryopyrin inflammasome is the major regulator of caspase-1 activation leading to the cleavage of pro-IL-1β and the secretion of the mature cytokine (6). We hypothesized that IL-1β may be the earliest cytokine to be secreted; initiating the pro-inflammatory

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state in BS attacks and that mutations in inflammasome components might contribute to de-regulated IL-1β secretion.

In order to establish the general cytokine secretion profiles of newly diagnosed and untreated BS patients, we performed a time-dependent release study of IL-1β, TNF-α and IL-6 for the first time. Furthermore, we analysed for the first time the cytokine release profiles of BS patients falling into the mucocutaneous, vascular and uveitis/ocular involvement subtypes. Simultaneously, all of the coding regions and intron–exon boundaries of the inflammasome components, NLRP3/cryopyrin and ASC, were screened for the presence of mutations.

Methods

Patients and controls for peripheral blood stimulation and ELISA study

Newly diagnosed BS patients, who fulfilled International Study Group (ISG) criteria published in *The Lancet* (7) and had active disease, were included in the peripheral blood stimulation and ELISA study. These patients were not using any medications at the time of blood collection and had not used immunosuppressives in the past. This group was defined as the functional study cohort. There were 51 patients (34 men, 17 women, mean age 31.7 ± 6.5) in this group. Among these, 29 had mucocutaneous, 12 had eye and 10 had vascular involvement. One of the patients with eye disease also had neurological disease. All of the patients with eye involvement had active uveitis with a drop of at least two lines on the Snellen chart in visual acuity. Patients in the vascular group had deep vein thrombosis and/or pulmonary artery aneurysms. Mucocutaneous patients had only skin and mucosa lesions.

Thirty-seven healthy controls (22 men, 15 women, mean age 29.1 ± 9.2) were also included. Both the controls and patients in this group were also included in the genetic screen.

Patients and controls for genetic study

A total of 50 patients who fulfilled ISG criteria and 50 controls (30 men, 20 women, mean age 27.4 ± 11) were screened for mutations in all nine coding exons of NLRP3/cryopyrin and three exons of ASC genes. Only for the exon 3 of the NLRP3/cryopyrin, which is a hot spot for mutations in several autoinflammatory disorders, we increased the number of patients to 104 (72 men, 32 women, mean age 34.3 ± 7.1).

Among these, 43 had mucocutaneous, 39 had eye, 6 had neurological and 19 had vascular involvement. Two of these patients had both eye and vascular involvement and one had both neurological and eye involvement.

This study was approved by the Bogazici University Institutional Review Board for Research with Human Subjects. Informed consent was obtained from all patients and control subjects.

Cell culture and ELISA

Blood samples were collected into EDTA-Vacutainer tubes (BD, Plymouth, UK) and within 2 h diluted 1:3 with prewarmed RPMI supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine and antibiotics (Gibco, Grand Island, NY, USA). The diluted blood was incubated in 48-well tissue culture plates (0.5 ml per well) (TPP, Trasadinge, Switzerland) with 100-ng ml^{-1} ultra-pure LPS (*Escherichia coli* 0111:B4; Alexis Biochemicals, San Diego, CA, USA), at 37°C in a CO_2 incubator (5%) for the indicated time points. Untreated cells were collected at the same time and tested at the 24-h time point. At the end of incubation, cells were transferred to microfuge tubes and centrifuged (Spectrafuge 16M Microcentrifuge; Labnet, Woodbridge, NJ, USA) at 3000 r.p.m. for 3 min and supernatants were aliquoted and frozen for determination of cytokine secretion. IL-1β, TNF-α and IL-6 levels in the supernatants were measured by a commercially available ELISA kit (R&D Biosystems, Minneapolis, MN, USA) according to the manufacturer's instructions. Standard curves of known concentrations of recombinant human cytokines were used to calculate the cytokine concentration (pg ml^{-1}) in each individual assay.

Genomic DNA isolation and screening of mutations/polymorphisms

A modified version of salting-out method was used for the extraction of genomic DNA from whole blood samples. All coding regions including exon–intron boundaries of NLRP3/cryopyrin (11 fragments), pyrin (two PCR reactions for exon 2 and exon 10) and ASC (three fragments) were amplified by PCR using specific primers. Primer pairs are available upon request. Sequencing was done in MGH DNA Core Facility, Boston, MA, USA; Macrogen Inc., Seoul, South Korea and Iontek Inc., Istanbul, Turkey.

Statistical analysis

SPSS version 18 (IBM Corporation, Armonk, NY, USA) was used for all statistical analyses. Data were given as mean and standard deviation. Statistical analysis for IL-1β, TNF-α and IL-6 in untreated cells or those at, 2 h, 4 h, 8 h and 24 h was analysed by independent samples *t*-test (two tailed) and analysis of variance for comparison of the means in patient and controls. Each treatment was performed in triplicates. The results were considered significant when the *P* value was <0.05.

Site-directed mutagenesis

NLRP3/cryopyrin V200M mutation was generated by PCR amplification of pcDNA3-NLRP3/cryopyrin wild-type (WT)-FLAG (a gift from Gabriel Nunez, University of Michigan, Ann Arbor, MI, USA) with two sets of primers: F1 (5′-AAGAGATGAGCCGAAGT GGG-3′)/R1 (5′-CTTAATGGGACTCATGGGGCTCTCACACG-3′) and F2 (5′-CGTGTGAGAGCCCCATGAGTCCCATTAAG-3′)/R2 (5′-CAAACTGGAAAGGAAGAAGACG-3′). R1 and F2 primers were complementary to each other and contained the underlined mutant nucleotides. Resulting PCR products were mixed in the same amounts after gel purification and amplified with F1/R2 primers to obtain a final product containing the mutations. This fragment and pcDNA3-WT NLRP3/cryopyrin-FLAG vector were digested by using *Pml*I and *Sac*II restriction enzymes, ligated using T4 DNA ligase and transformed into competent *E. coli* TOP 10. Positive colonies for the mutation were selected by colony PCR and confirmed by sequencing. All restriction enzymes and T4 DNA ligase were purchased from New England Biolabs (Ipswich, MA, USA).

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A similar protocol was applied for the generation of T195M mutant. In this case, F1 (5′-AAGAGATGAGCCGAAGTGGG-3′)/R1 (5′-GGGGCTCTCACACATCTTGGTCTTGC-3′) and F2 (5′-GCAAGACCAAGATGTGTGAGAGCCCC-3′)/R2 (5′-CAAACTGGAAAGGAAGAAGACG-3′) primer sets were used. The final product was digested with *Pml*I and *Sac*II enzymes and re-ligated into pcDNA3-NLRP3/cryopyrin-FLAG vector. All primers were purchased from MGH Harvard DNA Core Facility.

Generation of pLenti-Ef1a-EGFP-ASC

The ASC gene cDNA was cloned from the pcDNA3-ASC plasmid (a gift from Prof. Gabriel Nunez, University of Michigan) into the pEGFP-C3 (Clontech, Mountain View, CA, USA) vector by restriction digestion with *Hin*dIII-*Eco*RI enzymes. The resulting enhanced green fluorescent protein (EGFP)-ASC fragment was cloned into the *Nhe*I-*Eco*R I double-digested pLenti-Ef1a-AKIL vector, which has a polylinker suitable for cloning from pcDNA3 and Clontech vector series (including *Nhe*I, *Hin*dIII, *Xba*I, *Eco*RI, *Not*I, *Eco*RV, *Nde*I restriction sites).

Lentivirus production

HEK293FT cells in 10-cm plates (TPP) were transfected with 4-μg pLenti-Ef1a-EGFP-ASC, packaging vectors pCMVdeltaR8.74 and pMD2.G (contains vesicular stomatitis virus-glycoprotein) by the calcium phosphate method. Two days after transfection, supernatants containing virus were filtered with 0.45-μm filters, aliquoted and frozen at −80°C.

Transduction and stable line production

HEK293FT cells were plated 1 day before transduction. Virus-containing vials were thawed at room temperature and mixed with polybrene (Sigma-Aldrich, Taufkirchen, Germany) at a final concentration of 4 μg ml^{-1}. Media on cells were removed and virus-containing medium was added dropwise; 5 h later, the cells' media were changed. One week later, the transduced HEK293FT cells were serially diluted and transferred into 96-well plates (TPP) so that one cell or less could fall into each well. One of the subclones, EGFP-ASC_C8 was chosen for its high of GFP^+/GFP^- cell ratio (90%) and low frequency of spontaneous speck formation (<1%).

Transfection of EGFP-ASC stable lines with FLAG-NLRP3/cryopyrin variants, quantification of ASC specks and IL-1β secretion

EGFP-ASC HEK293FT stable lines were plated on 6-well plates (TPP). On the next day, cells were transfected with either 20 ng of pcDNA3-FLAG-NLRP3/cryopyrin _WT, _V200M, _T195M or _R260W or empty pcDNA3, with 50-ng caspase-1 and 500-ng IL-1β vectors. Transfection was performed in triplicate wells for each construct and the experiment was replicated three times.

Five images were captured from each well at random locations with an inverted fluorescence microscope (n = 15) (Zeiss Observer Z1; Carl Zeiss Microscopy, Thornwood, NY, USA). All images used for speck counting were taken under identical conditions. Cells were imaged with a ×10 objective. EGFP-ASC specks in the entire visual field were counted. The two-tailed unpaired Student's t-test was employed to compare speck counts.

Additionally, IL-1β release by these cultured cells was measured in triplicates by ELISA using a commercially available IL-1β ELISA kit (R&D Biosystems).

EGFP-ASC HEK293FT cells were cultured for 24 h after transfection in DMEM without FBS. Then the supernatants were collected and the proteins were precipitated with trichloroacetic acid by incubation on ice for 10 min, followed by a wash with acetone. Then the protein precipitates were dissolved in 50 μl 0.5% NP40 lysis buffer and loaded on SDS-PAGE. The proteins were transferred to a polyvinylidene difluoride membrane in wet transfer for 2 h at 100 V in 4°C. Following 1 h blocking with 5% BSA, the membrane was incubated overnight at 4°C with 1:1000 anti-IL-1β antibody (Cell Signaling; 2022) in 5% BSA. Then the membrane was incubated for 2 h at room temperature in 1:2000 anti-rabbit secondary antibody (Cell Signaling; 7074) in 5% BSA and visualized using Lumi-Light Western Blotting Substrate (Roche Applied Sciences; 12015200001).

Results

Elevated levels of TNF-α secretion detected in LPS-stimulated BS patients' cells

Peripheral blood cells collected from BS patients, with active lesions who had not received any treatment at the time of blood collection (functional study group), and healthy controls were stimulated with LPS to establish time-dependent IL-1β, TNF-α and IL-6 secretion profiles (Fig. 1A–C). Mean IL-1β, TNF-α and IL-6 levels secreted at 2, 4, 8 and 24 h after stimulation with LPS, among vascular, mucocutaneous and ocular BS patients, and healthy controls are given in Table 1 and shown in Fig. 1D.

BS patients with vascular involvement had significantly higher IL-1β levels at 2 and 4 h after LPS stimulation ($P < 0.001$, $F_{3df} = 6.59$ and $P = 0.039$, $F_{3df} = 2.92$, respectively) and higher TNF-α levels at 2 and 8 h after LPS stimulation ($P = 0.033$, $F_{3df} = 3.05$ and $P = 0.022$, $F_{3df} = 3.38$, respectively), compared with mucocutaneous BS, ocular BS and healthy control groups. The levels among the mucocutaneous BS, ocular BS and healthy control groups were similar for these time points ($P = 0.58$, $F_{2df} = 0.58$; $P = 0.166$, $F_{2df} = 1.84$; $P = 0.383$, $F_{2df} = 0.956$; $P = 0.214$, $F_{2df} = 1.28$, respectively).

When the total group of BS patients was compared with healthy controls, IL-1β and IL-6 levels were not different at any of the time points after LPS stimulation. The BS group had a trend for higher TNF-α secretion at 2 h (251 ± 173 SD versus 178 ± 147 SD, $P = 0.048$), 4 h (280 ± 181 SD versus 200 ± 164, $P = 0.046$) and 8 h (240 ± 178 versus 169 ± 146, $P = 0.056$) after stimulation. The values for mean IL-1β, TNF-α and IL-6 levels secreted at 2, 4, 8 and 24 h after stimulation with LPS for the total BS group versus healthy controls are given in Table 2.

The IL-6 levels in the ocular subgroup were higher than the other groups, starting from 4 h post-treatment. However, the difference did not reach statistical significance, probably due to the limited number of patients in this subgroup (n = 7) (Fig. 1D).

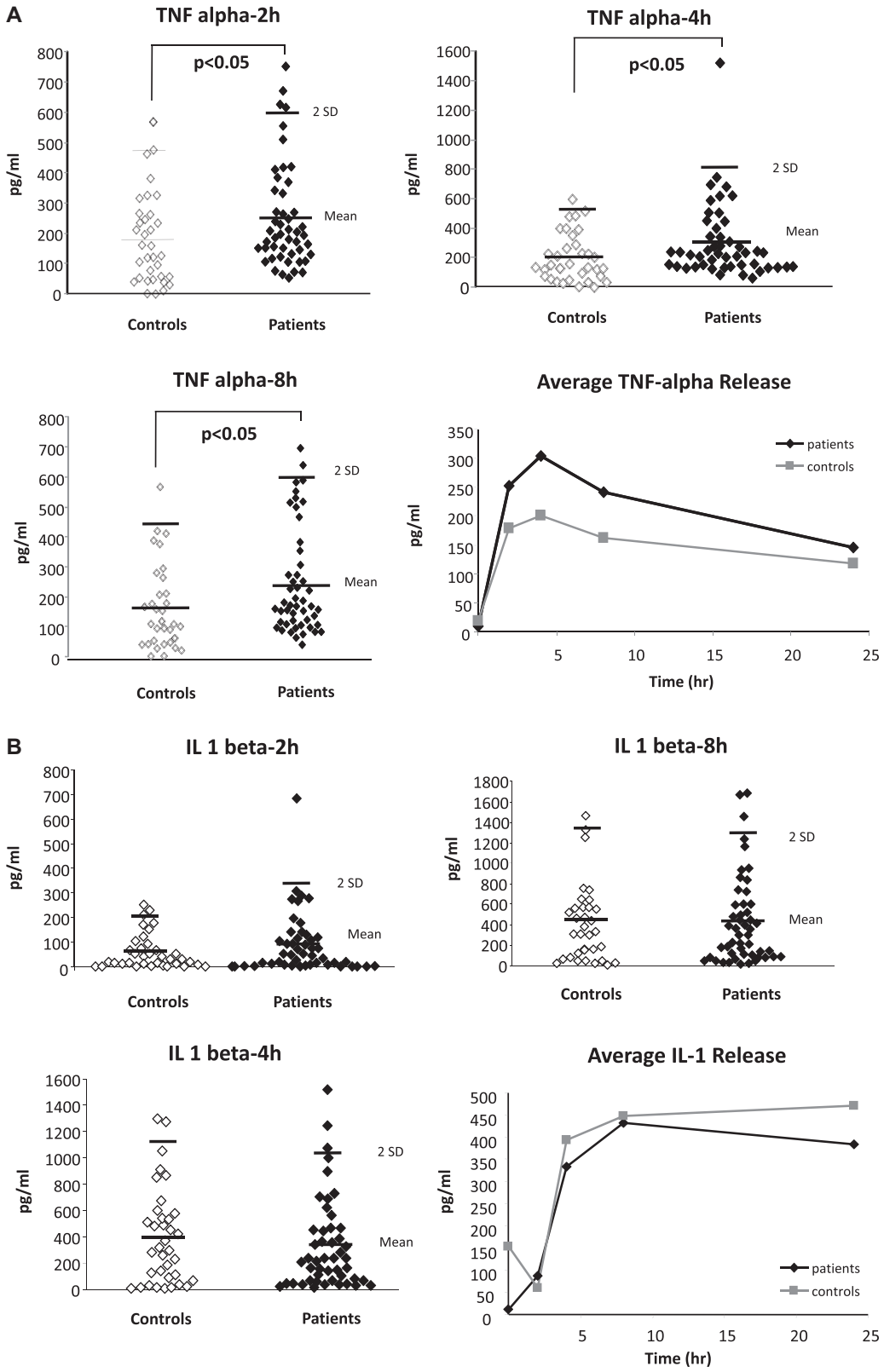
The IL-1β and IL-6 levels in a few untreated samples were higher in some healthy controls, but the cytokine levels in

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untreated samples were not compared since these samples were studied 24 h after blood collection, which might have resulted in spontaneous cytokine release.

Novel mutations identified in the NLRP3/cryopyrin gene

Screening of all nine exons including intron–exon boundaries of the NLRP3/cryopyrin gene resulted in the identification of a



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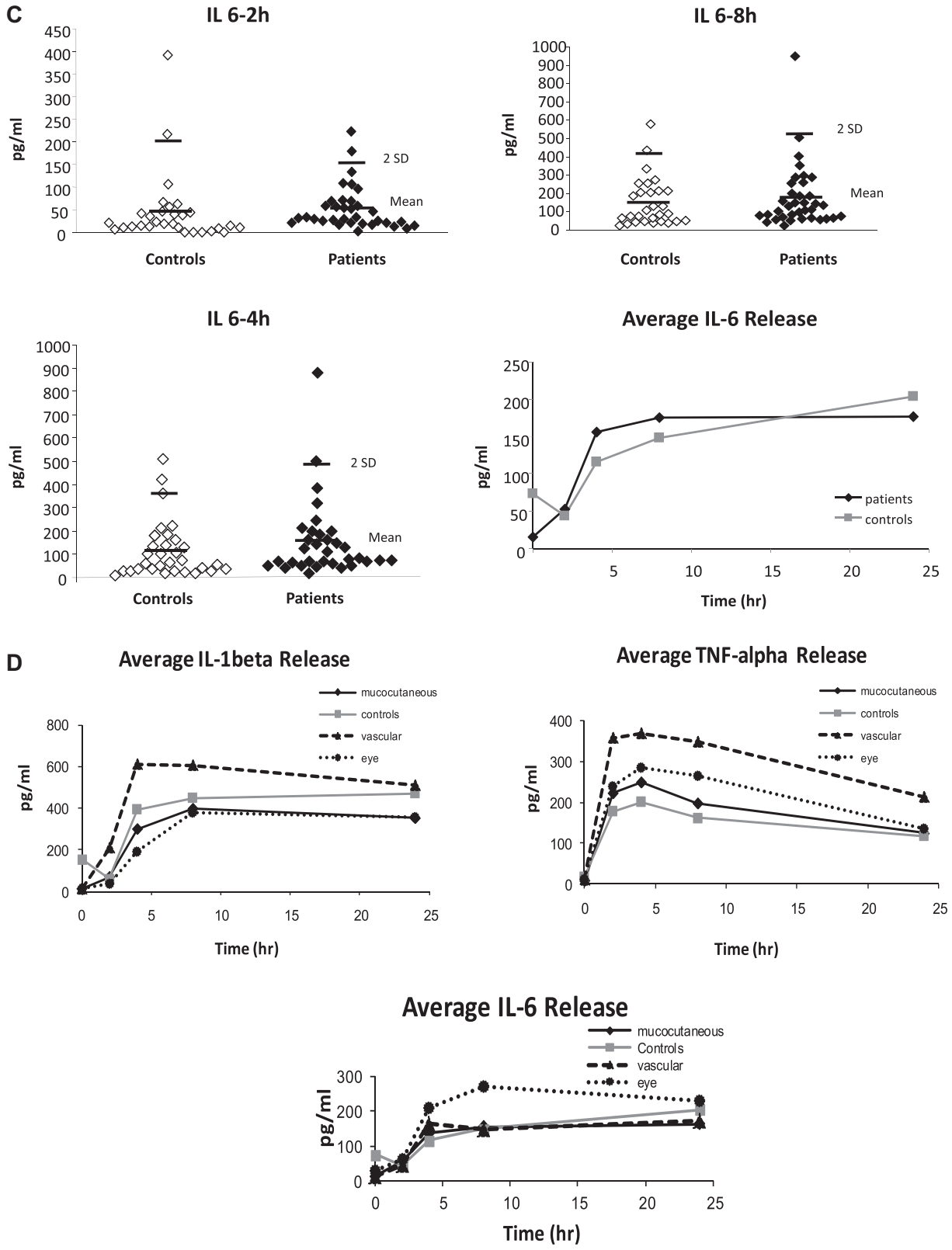


Fig. 1. IL-1β, TNF-α and IL-6 secretion from LPS-stimulated blood cells in BS patients and healthy controls. (A) TNF-α (patients $n = 51$, controls $n = 34$), (B) IL-1β (patients $n = 51$, controls $n = 36$) and (C) IL-6 (patients $n = 37$, controls $n = 30$) release from BS patients' and healthy controls' whole blood samples after LPS treatment (100 ng) analysed at 2-, 4-, 8- and 24-h post-stimulation. Untreated control at 0 h was collected together with 24-h treatment samples. First bar shows mean and the second mean ± 2 SD. (D) Average TNF-α (vascular $n = 10$, eye $n = 12$ and mucocutaneous $n = 29$), IL-1β (vascular $n = 10$, eye $n = 12$ and mucocutaneous $n = 29$) and IL-6 (vascular $n = 6$, eye $n = 7$ and mucocutaneous $n = 21$) release in BS patient subgroups compared with healthy controls.

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Table 1. Cytokine secretion profiles of BS patient subgroups

Time	IL-1β			TNF-α			IL-6		
	Mucocutaneous (n = 29)	Eye (n = 12)	Vascular (n = 10)	Mucocutaneous (n = 29)	Eye (n = 12)	Vascular (n = 10)	Mucocutaneous (n = 21)	Eye (n = 7)	Vascular (n = 6)
Untreated	10 ± 18	12 ± 17	12 ± 28	8 ± 22	12 ± 25	11 ± 26	12 ± 18	28 ± 52	13 ± 32
2 h	68 ± 98	37 ± 43	210 ± 187**	223 ± 161	238 ± 153	358 ± 207*	53 ± 46	59 ± 82	47 ± 30
4 h	300 ± 315	188 ± 178	611 ± 474*	250 ± 161	285 ± 205	369 ± 202	136 ± 105	211 ± 330	164 ± 132
8 h	399 ± 436	381 ± 407	606 ± 461	198 ± 152	264 ± 206	348 ± 189*	154 ± 94	271 ± 338	147 ± 128
24 h	352 ± 391	354 ± 386	509 ± 489	124 ± 117	137 ± 123	213 ± 137	163 ± 107	229 ± 259	170 ± 147

IL-1β, TNF-α and IL-6 secretion from LPS-stimulated blood cells in subgroups of BS patients. Results are given as means in pg ml^{-1} ± standard deviations.
*P value <0.05, **P value <0.001.

Table 2. Cytokine secretion profiles of BS patients and healthy controls

Time	IL-1β		TNF-α		IL-6	
	Patients (n = 51)	Controls (n = 36)	Patients (n = 51)	Controls (n = 34)	Patients (n = 37)	Controls (n = 30)
Untreated	11 ± 20	154 ± 226	10 ± 23	18 ± 22	16 ± 31	74 ± 104
2 h	88 ± 125	60 ± 72	252 ± 173*	178 ± 148	53 ± 49	45 ± 78
4 h	333 ± 350	393 ± 363	303 ± 25*	200 ± 164	156 ± 166	119 ± 123
8 h	432 ± 433	449 ± 449	241 ± 179*	162 ± 142	175 ± 174	149 ± 133
24 h	384 ± 407	470 ± 469	145 ± 125	117 ± 118	177 ± 152	204 ± 206

IL-1β, TNF-α and IL-6 secretion from LPS-stimulated blood cells in BS patients and healthy controls. Results are given as means in pg ml^{-1} ± standard deviations.
*P value <0.05.

novel *T195M* mutation in one and *V200M (V198M)* mutation in three patients among 104 BS patients, whereas no mutations were found in 49 healthy controls (Fig. 2A). Patients bearing the V200M mutation are from either the eye, mucocutaneous or neurological subgroups, whereas the patient with T195M belongs to the mucocutaneous subgroup.

In order to check whether the V200M mutation exists in healthy family members, we sequenced the available genetic material from two brothers and a grandmother in one BS patient carrying the mutation and who had eye involvement. The V200M mutation was not detected in the tested healthy individuals (Fig. 2B).

Only one of the V200M carrier patients in the mucocutaneaous subgroup was suitable for the functional cytokine study, since the other two were already being treated for the disease. In this patient, IL-1β secretion levels were higher than the average values for the whole functional study group at 2 and 4 h and only at 2 h compared with the mucocutaneous subgroup (Table 3).

Interestingly, the Q705K (rs35829419) (HapMap-CEU %11, Hapmap-JPT %0) polymorphism in the NLRP3/cryopyrin gene appears with an increased frequency in both Turkish controls (n = 16/116) and BS patients (n = 13/109). Other polymorphisms especially in the NOD/NACHT/NAD domain of NLRP3/cryopyrin were found as a result of sequencing (Table 4). When analysed statistically, the frequency differences in patients and controls do not appear to be significant. However, the H260H (1526 C→ T) polymorphism that we find in 1/49 controls has not been reported to date. In our study, we did not find changes in any of the three exons and intron–exon boundaries of the ASC in 50 BS patients and 50 unrelated controls tested.

Besides NLRP3/cryopyrin exons, we wanted to see whether mutations in the inflammasome regulatory protein PYRIN may be involved in BS, so we sequenced a subset of patients with high IL-1β secretion levels and/or carrying a mutation in NLRP3/cryopyrin for *MEFV* exon 10 (n = 26). Five patients have the M694V mutation (19.2%), previously linked with familial Mediterranean fever, one has M680I and another one has A744S. The V200M patient with neurological disease also has a MEFV M694V mutation.

Mutant NLRP3/cryopyrin's effect on ASC speck formation

We studied whether V200M and T195M NLRP3/cryopyrin over-expressing cells are more prone to ASC speck formation in comparison with WT NLRP3/cryopyrin-expressing cells. EGFP-ASC stable HEK293FT cells were transfected with equal amount of FLAG-NLRP3/cryopyrin_WT, _V200M, _T195M or _R260W plasmids and the number of speck formation in five representative visual fields from three different wells were counted under identical settings of the inverted fluorescent microscope (Fig. 3A and B).

As expected, we observed that transfection of 20 ng of WT NLRP3/cryopyrin induced speck formation (average number of specks per visual field= 17.6 ± 5.6) significantly, compared with control empty pcDNA3-transfected cells (9.2 ± 2.4,

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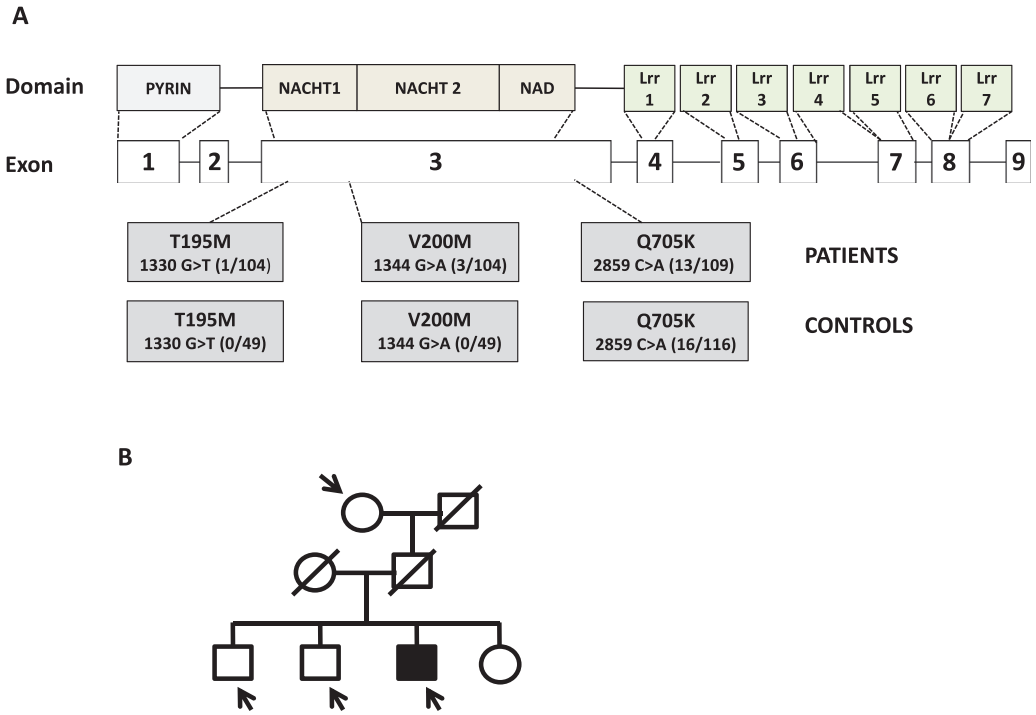


Fig. 2. Novel T195M mutation and other genetic changes were identified in the NLRP3/cryopyrin gene. (A) Genetic mutations and polymorphism found in BS patients and healthy controls. (B) Genetic analysis of a patient family with V200M mutation.

Table 3. Cytokine secretion profile of BS patient with V200M mutation

	IL-1β	TNF-α	IL-6
Untreated	27.2 ± 3.9	6.9 ± 0.5	18.1 ± 0.6
2 h	289.2 ± 21.8	342.7 ± 20.9	30.8 ± 2.2
4 h	343.6 ± 24.8	274.8 ± 39.9	50.9 ± 5.9
8 h	303.0 ± 48.8	194.4 ± 3.4	55.3 ± 10.4
24 h	341.4 ± 39.8	74.0 ± 27.1	48.7 ± 5.6

IL-1β, TNF-α and IL-6 secretion from LPS-stimulated blood cells in a patient with V200M mutation. Results are given as means of triplicates readings in pg ml^{-1} ± standard deviations.

$P < 0.0001$). On the other hand, transfection of R260W mutant NLRP3/cryopyrin, previously reported to cause spontaneous IL-1β release in over-expression studies, increased speck numbers significantly (109.4 ± 48.3 specks) compared with WT NLRP3/cryopyrin-transfected cells. Besides, T195M mutant NLRP3/cryopyrin-transfected cells have a slight but significant decrease in the number of specks (12 ± 6) in comparison with WT NLRP3/cryopyrin-transfected cells, whereas V200M mutant NLRP3/cryopyrin-transfected cells did not form a significantly higher number of specks (25.3 ± 13.4) (Fig. 3B). NLRP3/cryopyrin expression was verified for each condition (Fig. 3D). These data suggest that, unlike R260W, the T195M and V200M mutant NLRP3/cryopyrin proteins do not have a pronounced positive effect on ASC oligomerization.

Mutant NLRP3/cryopyrin's effect on IL-1β secretion

Any effect of NLRP3/cryopyrin mutants on IL-1β secretion was tested in the EGFP-ASC HEK293FT stable line (Fig. 3C). As expected, transfection of the R260W mutant increased IL-1β secretion significantly (196 ± 33.6 pg ml^{-1}, $P < 0.0001$) compared with WT (24.77 ± 2.57 pg ml^{-1}; 0.81 ± 1.2 pg ml^{-1} for empty plasmid-transfected cells, Fig. 3C). V200M also increased IL-1β release (65.93 ± 6.9 pg ml^{-1}, $P < 0.0001$), whereas the T195M mutation decreased IL-1β release (8.01 ± 6.21 pg ml^{-1}, $P < 0.0001$) in comparison with WT NLRP3/cryopyrin.

Secreted mature IL-1β was also tested by western blotting (Fig. 3D) and results correlate with ELISA results. Furthermore, the IL-1β secretion pattern is in line with the ASC speck formation.

Discussion

Our study shows that BS patients with vascular involvement have higher IL-1β and TNF-α levels after stimulation with LPS, compared with BS patients with eye involvement, BS patients with only mucocutaneous involvement and healthy controls (Fig. 1D). Cytokine levels of IL-1β and TNF-α in BS patients with eye and mucocutaneous involvement were similar to healthy controls (Fig. 1D). These findings reflect the clinical observation that BS patients with vascular involvement are the ones who have fever and other constitutional symptoms, especially during active disease. Moreover, elevation of acute phase reactants is also a feature of vascular BS, which alarms the clinician to this type of involvement.

There are several findings indicating the presence of different pathogenetic mechanisms underlying different types of organ involvement in BS. The presence of various symptom clusters in BS, such as the association of deep vein thrombosis and superficial thrombophlebitis or the association of acne, arthritis and enthesopathy, raises the possibility

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Table 4. Single nucleotide polymorphisms in NLRP3/cryopyrin exon 3 for BS patients and healthy controls

Nucleotide changes	Patients				Controls				Total population			
	Homozygote WT	Heterozygote	Homozygote variant	Total variant	Homozygote WT	Heterozygote	Homozygote variant	Total variant	Homozygote WT	Heterozygote	Homozygote variant	Total variant
p.T221T 1409 ACC>ACT rs7525979	82.7% (86/104)	16.35% (17/104)	0.96% (1/104)	17.3% (18/104)	83.67% (41/49)	16.32% (8/49)	0% (0/49)	16.32% (8/49)	83% (127/153)	11.11% (17/153)	0.65% (1/153)	16.99% (26/153)
p.A244A 1478 GCG>GCA rs3806268	18.27% (19/104)	52.89% (55/104)	28.85% (30/104)	81.73% (85/104)	12.25% (6/49)	69.39% (34/49)	18.37% (9/49)	91.84% (45/49)	16.34% (25/153)	58.17% (89/153)	25.49% (39/153)	83.66% (128/153)
p.H260H 1526 CAC>CAT New	100% (104/104)	0% (0/104)	0% (0/104)	0% (0/104)	97.96% (48/49)	2.04% (1/49)	0% (0/49)	2.04% (1/49)	99.35% (152/153)	0.65% (1/153)	0% (0/153)	0.65% (1/153)
p.R262R 1532 CGA>CGG rs4925543	0.96% (1/104)	3.85% (4/104)	95.19% (99/104)	99.04% (103/104)	0% (0/49)	6.12% (3/49)	93.88% (46/49)	100% (49/49)	0.96% (1/153)	4.58% (7/153)	94.77% (145/153)	95.42% (146/153)
p.D312D 1682 GAC>GAT rs143840033	97.22% (105/108)	2.78% (3/108)	0% (0/108)	2.78% (3/108)	98% (49/50)	2% (1/50)	0% (0/50)	2% (1/50)	97.47% (154/158)	2.53% (4/158)	0% (0/158)	2.53% (4/158)
p.P342P 1772 CCC>CCT rs41311573	98.15% (106/108)	1.85% (2/108)	0% (0/108)	1.85% (2/108)	98% (49/50)	2% (1/50)	0% (0/50)	2% (1/50)	98.1% (155/158)	0% (0/158)	1.9% (3/158)	1.90% (3/158)
p.L346L 1784 CTG>CTA rs144616577	98.15% (106/108)	1.85% (2/108)	0% (0/108)	1.85% (2/108)	96% (48/50)	4% (2/50)	0% (0/50)	4% (2/50)	97.47% (154/158)	2.53% (4/158)	0% (07158)	2.53% (4/158)
p.L413L 1983 CTG>TTG rs148478875	98.15% (106/108)	1.85% (2/108)	0% (0/108)	1.85% (2/108)	100% (50/50)	0% (0/50)	0% (0/50)	0% (0/50)	98.73% (156/158)	1.27% (2/158)	0% (0/158)	1.27% (2/158)
p.S436S 2054 TCC>TCT rs34298354	64.82% (70/108)	26.85% (29/108)	8.33% (9/108)	35.19% (38/108)	58% (29/50)	40% (20/50)	2% (1/50)	42% (21/50)	62.66% (99/158)	31% (49/158)	6.33% (10/158)	37.34% (59/158)
p.H465H 2141 CAC>CAT rs111400208	99.07% (107/108)	0.93% (1/108)	0% (0/108)	0.93% (1/108)	100% (50/50)	0% (0/50)	0% (0/50)	0% (0/50)	99.37% (157/158)	0.63% (1/158)	0% (0/158)	0.63% (1/158)

Single-nucleotide polymorphisms in BS patients and healthy controls.

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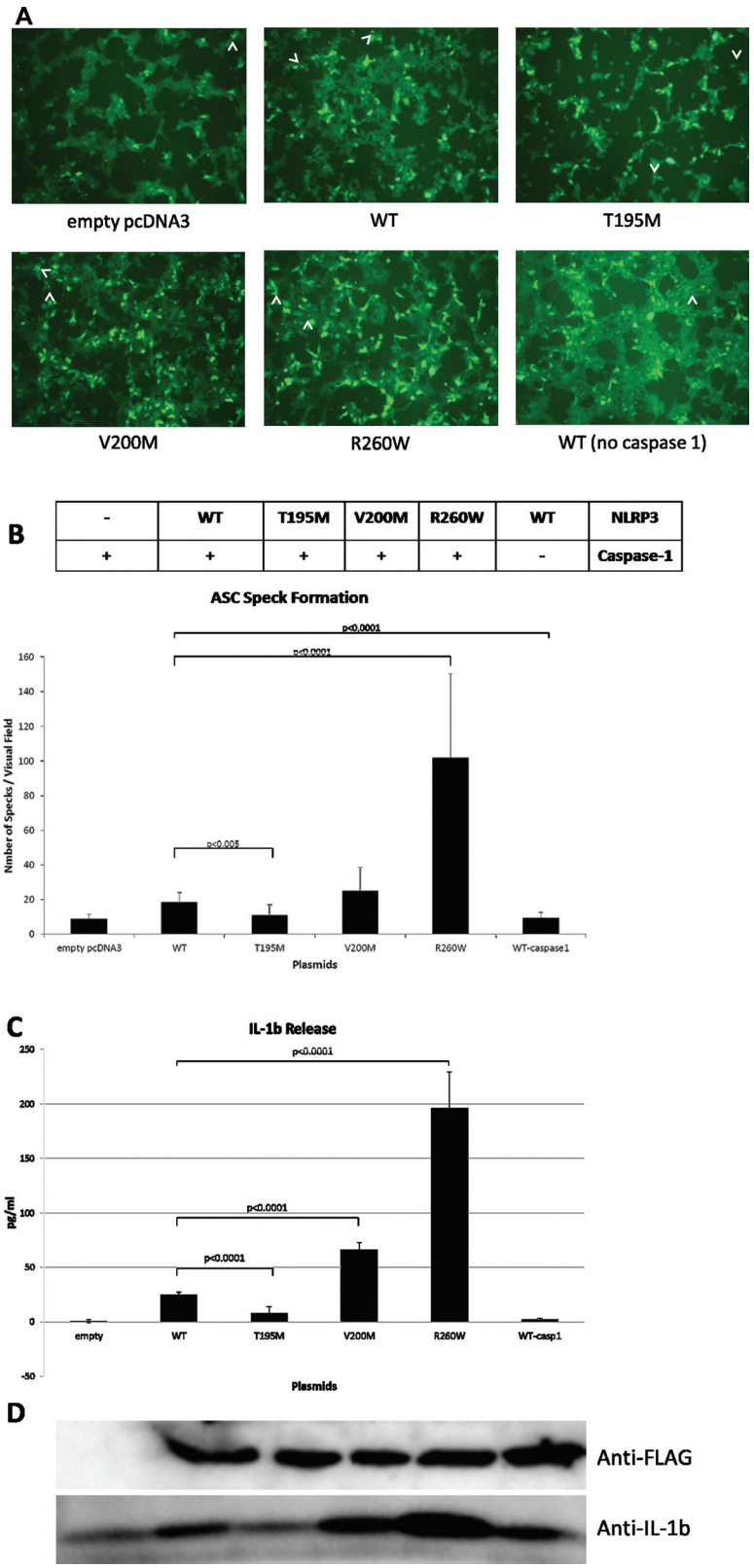


Fig. 3. The effects of NLRP3/cryopyrin mutations on inflammasome activation. (A) ASC speck formations. Inverted fluorescent microscopy images of stable ASC-EGFP-expressing HEK293FT cells transfected with empty vector, FLAG-NLRP3/cryopyrin_WT, _V200M, _T195M, _R260W and _WT in the absence of caspase-1. ASC specks are shown with a white arrow. The images represent cells transfected with 20 ng of the corresponding NLRP3/cryopyrin, 50-ng caspase-1 and 500-ng IL-1β plasmids. (B) Quantification of ASC speck numbers. (C) Measurement of IL-1β release by ELISA. (D) Western blot of secreted mature IL-1β protein.

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that the pathogenesis of BS involves more than one biologic pathway (8). This is also supported by evidence from drug trials. Colchicine, which is an effective drug in skin lesions and arthritis of BS patients, has no efficacy in eye or vascular involvement. The difference in cytokine secretion profiles among different types of BS patients in this study is more evidence for the presence of different mechanisms in the pathogenesis of BS.

There are controversial studies regarding the serum IL-1β levels (9–11) in BS patients some of which report normal and some elevated levels. Mege *et al.* (3) found increased TNF-α, IL-1β, IL-6 and IL-8 levels in culture conditions after stimulation with LPS 16 h post-treatment, whereas Slobodin *et al.* (12) reported that TNF-α production after LPS stimulation was similar in BS patients and healthy controls. The advantage of our study is that we included only patients with active lesions at the time of blood collection, who had not received any medication including colchicine. Moreover, analyzing clinical subgroups separately gave us the opportunity to evaluate the role of each cytokine in each type of involvement.

Another novelty brought by this paper is that we studied the kinetics of cytokine secretion in BS patients and compared this to healthy controls. This is important because not only the level of inflammation, but also the timing is thought to be important in BS. A typical example for this is the pathergy phenomenon, where the reaction is clinically indistinguishable from healthy controls in the beginning. However, after 24 h, the reaction begins to subside in healthy controls, in contrast to BS patients where the inflammation is prolonged up to 72 h. This was also shown in the tissue level by skin biopsies of the needle prick sites at 0, 8 and 48 h after the prick. Cellular infiltration patterns, cytokine and chemokine levels were similar in BS patients and healthy controls for the first 8 h. However, after 8 h, increased influx of mature dendritic cells, monocytes and lymphocytes as well as increased cytokine and chemokine levels continued only among BS patients (13). Our study shows that whole blood cytokine levels after LPS stimulation are higher, especially for the vascular BS patients, but the cytokine release kinetics of IL-1β, TNF-α and IL-6 are parallel in BS patients and healthy controls (Fig. 1D).

A randomized controlled trial and several open studies showed that TNF-α antagonists are effective in the management of BS. Especially, patients with eye, neurologic and gastrointestinal involvement, resistant to other drugs have benefitted from TNF-α antagonists. Case series showing efficacy of IL-1 antagonists have also been published. The elevated IL-6 levels that we have observed in ocular BS patients may be important with this respect. Elevated IL-6 levels were previously shown in cerebrospinal fluid and serum of BS patients with neurologic involvement, another subgroup of patients that may sometimes be associated with ocular involvement. These findings point out to the possibility of using IL-6 antagonists in such patients (Fig. 1D).

IL-1β is a key cytokine in autoinflammatory disorders. Dominant mutations in the inflammasome component NLRP3/cryopyrin have been documented to cause higher levels of IL-1β secretion. For instance, the R260W mutant NLRP3/cryopyrin has been reported to cause spontaneous IL-1β release by monocytes isolated from Muckle–Wells syndrome (MWS) patients (14).

We have found three patients carrying the V200M NLRP3/cryopyrin mutation and a novel T195M NLRP3/cryopyrin mutation all in the heterozygous state (Fig. 2A). V200 and T195 are conserved among primates and higher mammals such as cows, but not in mice and rats. For one of the V200M mutation carriers, genetic material from healthy family members was tested negative for this mutation. V200M has been reported in one study to have an incidence of 1/97 for BS patients (15). None of these previous reports examines the functional importance of the V200M for inflammasome assembly. Interestingly, a clinical case report about a British family with three MWS patients carrying the heterozygous V200M mutation gave a complete response to Anakinra treatment (16). This finding supports the fact that V200M might be important in the disease pathogenesis. However, the low incidence in our study and presence of the mutation in the healthy Caucasian populations with an allele frequency of 0.0074 (17) make it impossible to draw strong conclusions about its significance in BS pathogenesis.

We investigated the functional consequence of the V200M and T195M mutant NLRP3/cryopyrin on inflammasome assembly and activation via the speck formation and IL-1β secretion assays. The V200M mutant tends to show a positive effect on the formation of specks, albeit not significantly (Fig. 3A and B), but the significant effect of this mutant on IL-1β secretion was confirmed by ELISA and western blot analyses in a more solid and quantitative manner (Fig. 3C and D). Although this increase in IL-1β secretion was not as pronounced as R260W's effect, still V200M appears to be a moderately activating mutation.

Recently, the MEFV M694V mutation has been shown to be associated with BS in the Turkish population (18). PYRIN has been previously shown to activate NLRP3/cryopyrin inflammasome and combination of PYRIN and NLRP3/cryopyrin mutant proteins did not interfere with inflammasome activation (19). The presence of both NLRP3/cryopyrin V200M and MEFV M694V mutations in one of the four patients carrying NLRP3/cryopyrin mutations suggests that mutations of inflammasome components and/or regulators, such as NLRP3/cryopyrin and PYRIN, may have a combinatorial effect on the cytokine release profiles of BS patients. It was shown very recently that treatment of BS patients suffering from acute intraocular inflammation with XOMA 052, a humanized anti-IL-1β antibody, resulted in reduction of inflammation in all seven patients, spotlighting the importance of the IL-1β pathway in BS pathogenesis (20).

Overall, our data show that NLRP3/cryopyrin and/or ASC by themselves are unlikely to be responsible for BS pathogenesis or to account for the observed a cytokine profiles. It is clear that BS is a complex disorder with possible involvement of multiple genes and pathways (such as MEFV, HLA-B51, MICA, etc.) along with a contribution from environmental factors. Mutations in proteins, such as TLR4 and TLR2, known to regulate the inflammation process via the NF-κB pathway, controlling TNF-α and pro-IL-1β levels, were recently shown to be associated with BS (18, 21). Besides Toll-like receptors, other complexes controlling caspase-1 and caspase-5 activation such as AIM-2, NLRC4/Ipaf and other NLRP inflammasomes are still plausible mutation targets that may predispose individuals to develop BS.

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