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Activated phenotype of circulating neutrophils in familial Mediterranean fever

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ABSTRACT

Familial Mediterranean fever (FMF) is autoinflammatory disorder, characterized by MEFV gene mutations and recurrent episodes of fever and serosal or synovial inflammation. Neutrophils are the predominant effector cells of acute inflammatory attacks in FMF; however pathogenic role and molecular phenotype of these cells remain largely unknown. To gain insight into the processes that contribute to the self-directed autoinflammation we characterized expression of a spectrum of genes involved in regulation of inflammation in unstimulated and LPS-activated neutrophils from FMF patients. Expression of 12 candidate immune genes encoding for inflammation-related molecules was assessed by quantitative RT-PCR in freshly isolated and LPS-stimulated peripheral polymorphonuclear neutrophils from fifteen FMF patients in attack-free period and ten healthy volunteers as controls. The relative expression was calculated using the second derivative method; the target gene expression was normalized to the expression of RPL32 gene. FMF neutrophils were characterized by up-regulated baseline gene expression of c-FOS (9.5-fold, $p < 0.05$), IL-8 (12-fold, $p < 0.05$), MMP9 (8-fold, $p < 0.01$), TLR2 (7-fold, $p < 0.05$) compared to the neutrophils from control subjects, a trend was also evident towards increased caspase-1 expression (3-fold, $p = 0.09$). Discriminant analysis clustered the patient and control subjects into two distinct groups (Wilks's lambda = 0.165, $p = 0.042$). Further, LPS-induced alterations of expression profiles were shared between FMF and healthy neutrophils, the profile consisting namely of up-regulated IL-1β, TLR4, IL-8, and TNFAIP6 transcripts. Present study demonstrates distinct expression patterns of pre-activated neutrophils during attack-free period of FMF when compared to neutrophils from healthy controls. Furthermore, our data emphasize the importance of host-derived ligands in activation of FMF neutrophils.

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Introduction

Familial Mediterranean fever (FMF, MIM249100) is one of the most common hereditary autoinflammatory syndromes predominantly affecting populations from the eastern Mediterranean basin, particularly Jews, Armenians, Arabs and Turks. FMF is an autosomal recessive disease characterized by recurrent episodes of fever and polyserositis (Masters et al. 2009; Simon and van der Meer 2007). FMF is caused by mutations in the *MEFV* gene, encoding the pyrin protein (The International FMF Consortium 1997; The French FMF Consortium 1997), which is expressed primarily in neutrophils, eosinophils, monocytes, dendritic cells, synovial fibroblasts, but not in lymphocytes (Centola et al. 2000; Diaz et al. 2004). It has been demonstrated that the wild-type pyrin modulates caspase-1 and interleukin (IL)-1β activation, exerting both pro- and anti-inflammatory effects, depending on experimental conditions (Yu et al. 2006; Chae et al. 2006, 2011; Papin et al. 2007). Pyrin is likely a part of regulatory pathway of inflammation and physiologically keeping inflammation under control by modulating the immune response (Yu et al. 2006). Nevertheless, the mechanisms of immune system regulation by pyrin have been not completely understood.

Dysfunction of the innate immune system is the central issue in self-reactive autoinflammatory diseases such as FMF. FMF is caused by abnormal activation of polymorphonuclear neutrophils (PMNs) and monocytes, and unprovoked influx of these cells into the affected sites (Ben-Chetrit and Levy 1998; Stojanov and Kastner 2005). Sterile effusions in the serosal cavities of individuals experiencing attacks are almost exclusively composed of activated PMNs (Sohar et al. 1967; Heller et al. 1966). The important role of PMNs

Abbreviations: FMF, familial Mediterranean fever; IL, interleukin; PMNs, polymorphonuclear neutrophils; LPS, lipopolysaccharide; TNF, tumor necrosis factor; HMGB, high mobility group box protein; MMP, matrix metalloproteinase; TNAIP6, TNF-a-induced protein 6; TLR, Toll-like receptor; DA, discriminant analysis; PCA, principal component analysis.

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in the pathogenesis of FMF is further supported by the effectiveness of colchicine, a suppressor of PMNs adhesion, chemotaxis and phagocytosis, in the prophylaxis of attacks and amyloidosis in FMF-affected subjects (Keller et al. 1984). In contrast to other inflammation-associated diseases, the inflammatory attacks of FMF almost never result in tissue destruction, suggesting an accelerated rate of neutrophil apoptosis (Ozen et al. 2001).

As the regulator of innate immunity, pyrin could be involved in the orchestration of both pathogen and danger immune recognition through its interactions with NALP3 (formerly known as cryopyrin) inflammasome (Papin et al. 2007). *MEFV* mutations may lead to the heightened responsiveness of NALP3 inflammasome, which can be activated in response to a very diverse range of ligands (Martinon et al. 2004; Mariathasan et al. 2006). Here, contribution of PMNs to the initiation of attacks should be also considered. Lipopolysaccharide (LPS) is a well known potent neutrophil priming agent which is capable of inducing a strong inflammatory response by recruiting circulating PMNs (Guthrie et al. 1984), as well as one of the important activators of the NALP3 inflammasome (Mariathasan and Monack 2007). In the present study, we used an *in vitro* model of LPS-mediated neutrophil activation to determine whether neutrophils from FMF patients have heightened sensitivity towards low level bacterial endotoxin (10 ng/ml).

Despite the fact that neutrophils are predominant effector cells of acute inflammation in FMF, there has been no evidence of their pathological effect at the transcriptional level. The only available study has demonstrated elevated mRNA levels of IL-1β, IL-6, IL-8 and tumor necrosis factor (TNF)α in circulating leukocytes from FMF patients in attack-free period (Notarnicola et al. 2002). It is unclear whether there is a peripheral activation of PMNs in FMF patients between acute flares, and also if FMF neutrophils are activated *via* alternative signalling pathways. To address these questions we assessed differential gene expression in circulating PMNs at baseline level as well as in PMNs stimulated *in vitro* with LPS in attack-free patients with FMF in comparison to healthy subjects. Among the candidate genes, a total of 12, those encoding for pro- and anti-inflammatory molecules, as well as transcription factor were selected. Specifically, the mRNA expression profiles were analyzed for IL-1β and its receptor antagonist IL-1RA, IL-8 and its receptor CXCR2, caspase-1 and caspase-3, c-FOS, high mobility group box protein 1 (HMGB1), matrix metalloproteinase 9 (MMP9), TNF-a-induced protein 6 (TNAIP6/TSG-6), Toll-like receptors (TLR) 2 and TLR4.

Materials and Methods

Subjects

Two groups of volunteers were recruited for the study and signed informed consent was obtained from all subjects who provided blood samples. The study was approved by the Ethical Committee of the Institute of Molecular Biology of the NAS RA (IRB IORG0003427). The patient group included 15 FMF patients in remission period (11 male and 4 females; aged from 14 to 41 years, mean age of 23.4 years). The clinical diagnosis of FMF and the disease period were based on Tel-Hashomer criteria (Livneh et al. 1997), and genetic confirmation of the *MEFV* mutations carrier status was performed at the Centre of Medical Genetics (Yerevan, Armenia). All FMF patients were under colchicine treatment. The control group included 10 sex- and age-matched healthy individuals without any family history of FMF (6 male and 4 females; aged from 22 to 39 years, mean age of 26.6 years). All patients and healthy volunteers were of the same ethnicity (Armenian).

Preparation of peripheral/circulating neutrophils

Peripheral blood samples were collected in tubes containing EDTA and processed within 2 h after collection. Neutrophils were isolated by density centrifugation using the Histopaque-1077 gradient technique (Sigma, St. Louis, MO) according to the manufacturer's protocol. Contaminating erythrocytes were removed from the granulocyte fraction by brief hypotonic lysis. Granulocyte purity, as determined by counting of cytospin preparations stained with May-Grundwald Giemsa, was always greater than 95%. Viability of neutrophils, assessed by trypan blue exclusion, was greater than 95% immediately after purification.

Neutrophils (5×10^6 cells/ml) were cultured at 37 °C in RPMI 1640 supplemented with 10% fetal calf serum, 2 mmol/L L-glutamine, 1 mM sodium pyruvate, 5 mM HEPES, 100 units of penicillin, 100 μg of streptomycin in the absence or presence of 10 ng/ml LPS (*Escherichia coli* 026:B6) in a total volume of 1 ml for 4 h. After stimulation or directly after isolation, neutrophils were washed once with cold PBS, and stored in 150 μl RNAlater (Qiagen, Hilden, Germany) at −20 °C until use.

RNA extraction and RT-QPCR

Total RNA was isolated using the RNA/DNA/protein purification kit (Norgen, Thorold, Canada) according to the manufacturer's protocol. All samples were treated with RNaseOUT™ Recombinant Ribonuclease Inhibitor (Invitrogen, Carlsbad, CA, USA). Total RNA was extracted from cells for each time-point group. Total RNA was converted to double-stranded cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science, IN, USA) in a 20 μl reaction volume according to the manufacturer's protocol. PCR mixes were prepared as follows: 5 μl of cDNA calculated on input total RNA for each individual gene was added to 20 μl PCR-Mix (Abgene, Epsom, United Kingdom). The final concentrations of each component: 900 nM of each sense and antisense primers and 100 nM LNA probe (for Roche probes), 500 nM of primers and 250 nM of probe (for probes from Applied Biosystem and IDT); 3.5 mM $MgCl_2$ (for PSMB2, probes from Applied Biosystem and IDT) or 7.5 mM $MgCl_2$ (for Roche probes); 200 μM each dNTPs, 1U Thermo-Start TAQ polymerase, 1 × Thermo-Start Buffer (Abgene). cDNA was stored at −20 °C before further use. After initial denaturation (one cycle at 94 °C for 15 min), 40 cycles amplification (94 °C for 45 s, 60 °C for 30 s) were performed on RotorGene 3000 system (Corbett Research, Sydney, Australia).

The primer sequences, probes, and amplicon sizes for investigated genes are listed in Table 1. The RPL32 gene was used as a reference gene for normalization of targeted mRNAs quantification data in circulating neutrophils (Zhang et al. 2005; Kriegova et al. 2008), a human universal reference RNA (Stratagene, La Jolla, CA, USA) was used as calibrator. The calibrator with total concentration of 1.25 ng RNA/reaction was assessed in quadruplicates (Kriegova et al. 2008). Relative expression was calculated using second derivative method (Rotor Gene software 6.1.71, Corbett Research).

Statistical analysis

Statistical analyses were performed using the statistical software Graph Pad Prism 5.01 (Graph Pad Software; San Diego, CA). The significance of differences between two groups was evaluated using Mann–Whitney *U*-tests. The principal component analysis, discriminant analysis were performed using the SPSS 20.0 package (SPSS Inc., Chicago, IL, USA). $p < 0.05$ was considered as statistically significant.

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