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Genetic analysis of C5a receptors in neutrophils from patients with familial Mediterranean fever

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Abstract Familial Mediterranean fever (FMF) is an autoinflammatory disease, characterized by *MEFV* gene mutations and self-limited recurrent episodes of fever and localized serositis. Complement system is a key regulator of the inflammatory process. The aim of this study was to investigate the genetic alterations and mRNA expression pattern of *C5aR* and *C5L2* genes in neutrophils from attack-free FMF patients. No mutations were observed in the two receptors' genes, while the genetic alteration observed in the *C5aR1* gene was identified as N279 K polymorphic variant. Furthermore, lower mRNA expression of *C5L2* gene was observed in neutrophils from FMF patients compared to control subjects. The binding capacity of rhC5a and the ability to produce reactive oxygen species was similar in neutrophils from healthy subjects and FMF patients and independent of the presence of N279 K polymorphism or mRNA expression of *C5L2*.

Keywords Familial Mediterranean fever · C5a · C5aR1 · C5L2

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Introduction

Complement activation and the subsequent generation of the anaphylatoxins C3a and C5a are involved in neutrophil-dependent innate immune responses [1, 2]. C5a plays a critical role in the accumulation of neutrophils at the site of inflammation, acting as a potent chemoattractant [1, 2]. It also regulates several other neutrophil functions, inducing degranulation, enzyme release and the generation of reactive oxygen species (ROS) [1–4]. C5a binds with high affinity to C5aR (C5R1, CD88) a transmembrane receptor linked with intracellular G proteins and mediates an intracellular signaling, resulting in cell activation and the initiation of acute inflammatory responses [3, 4]. Recently, a second receptor for C5a, C5L2, has been described. C5L2 was initially proposed to be a default or scavenger receptor due to undetectable functional responses after ligation of C5a [5]. However, several recent studies propose a functional role for this receptor [6–8]. The expression of C5aR and C5L2 is regulated by inflammatory stimuli. It has been reported that the expression of both receptors is downregulated during sepsis [9, 10]. In addition, TNFa and IL-8 reduces the expression of C5aR [11].

Familial Mediterranean fever (FMF) is an autoinflammatory syndrome characterized by acute febrile inflammatory attacks and serositis [12, 13]. This disorder is associated with mutations in *MEFV* gene, which encodes the protein pyrin. Several point mutations have been

recognized in patients with FMF [13]. Even though it was originally described that the disease was transmitted in the autosomal recessive pattern, there is a subset of FMF patients who are carriers of a single MEFV mutation [14, 15]. Several data imply a critical role for neutrophils in the pathogenesis of the disease. The accumulation of neutrophils at the involved sites during FMF attacks is a hallmark of the disease [16].Of interest, neutrophils are the cellular population that mainly expresses *MEFV* gene [17]. However, there are scarce evidence for the chemotactic potential and the survival of neutrophils during FMF attacks or in attack-free periods.

Considering that neutrophil chemotaxis and influx into the affected sites is observed during FMF attacks and that C5a exerts significant chemotactic activity in neutrophils, we aimed to study whether a possible dysregulation in C5a receptor signaling due to genetic alterations and/or altered gene expression is implicated in the pathogenesis of FMF by affecting neutrophil functions. We investigated the expression pattern and possible genetic alteration of C5R1 and C5L2 receptors. GFP conjugated human C5a was also used to study C5a binding ability. Moreover, ROS production after C5a binding was assessed.

Materials and methods

Patients

A total of 105 clinically diagnosed [12] and genetically confirmed FMF patients [18], homozygous or combined heterozygous for *MEFV* gene mutations in exon 10 (49 female, 56 male; mean age, 26.4 years; range 2–64 years), and 217 healthy volunteers (114 female, 103 male; mean age, 34.5 years, range 18–60 years) were recruited for the purposes of the study. Control group included individuals not describing familial history or symptomatology related to FMF. Informed, written consent has been obtained from every recruited volunteer. The study protocol design was in accordance with the Helsinki Declaration and all procedures were approved by the Institutional Review Board (Scientific Committee of the University Hospital of Alexandroupolis, Greece).

Isolation of peripheral blood cells

Peripheral blood mononuclear cells (PBMCs) were isolated from the aforementioned FMF patients and healthy volunteers using Histopaque-single gradient centrifugation. PBMCs were used for isolation of genomic DNA as previously described [18]. Furthermore, peripheral blood polymorphonuclear cells (PMNs) were isolated by Histopaque-double gradient centrifugation from 12 attack free FMF patients and 10 healthy individuals.

Real-time qRT-PCR

RNA extraction and cDNA synthesis was performed as previously described [19]. Real-time qRT-PCR (rt qRT-PCR) for *C5aR1* and *C5aL2* was performed under the conditions described in Table 1. The GAPDH housekeeping gene was used to normalize the expression levels of target genes. Relative expression levels were determined using the 2^{DDCT} method [20].

Non-Isotopic RNase Cleavage Assay (NIRCA)

In order to investigate the presence of mutations in *C5aR1* and *C5L2*, NIRCA was applied as previously described [16]. In principle, a sense and antisense RNA transcript generated from a wild type control template and a sample template, using the appropriate T7 or SP6 RNA polymerase [16], were hybridised with themselves or cross hybridized (wild type to sample) in a sense-antisense manner. Thus, for each sample, a group of four RNA duplexes was generated and subsequently digested by suitable RNases. This

Table 1 Primer sequences[a] and PCR conditions[b] for real-time qRT-PCR

Gene	Sequence of primers	Position[c]	PCR conditions
GAPDH	FRD: 5′-GGGAAGCTTGTCATCAATGG-3′	295	(1) 52°C for 5 min
	REV: 5′-CATCGCCCCACTTGATTTTG-3′	370	(2) 95°C for 2 min
rt *C5aR1*	FRD: 5′-GCCCAGGAGACCAGAAC-3′	33	(3) 40 cycles of:
	REV: 5′-CCCACCAGGAAGACGAC-3′	201	95°C for 15 s
rt *C5L2*	FRD: 5′-AACCAGAGACACCAGGAG-3′	49	56°C for 40 s
	REV: 5′-CCAGGCAGTCCACAGG-3′	144	(4) 56°C for 5 min
			(5) melting curve

[a] Oligonucleotide primers were designed by Beacon Designer™ ver. 4.0 and synthesized by Invitrogen (50 pmol per reaction)

[b] Real-time PCR was performed using SYBR Green qPCR Master Mix (2 ×) gene expression master mix (Fermentas, St. Leon-Rot, Germany) on a Chromo4™ Real-Time Detector (Bio-Rad, CA, USA)

[c] Positions correspond to mRNA sequences (GAPDH-NM_002046.3, C5aR1-NM_001736.3, C5L2-NM_018485.1)

Table 2 Primer sequences[a] and PCR conditions[b] for NIRCA and RFLP analysis

Gene	Sequence of Primers	Position[c]	PCR conditions
C5aR1 5′ end region[g]	FRD[d]: 5′-GGAGACCAGAACATGAAC-3′	38	(1) 94°C for 3 min
	REV[e]: 5′-TCGTGAGCGTGAGTAG-3′	707	(2) 38 cycles of:
C5aR1 3′ end region[g]	FRD[d]: 5′-GCCTGGATCGCCTGTG-3′	506	94°C for 50 s
	REV[e]: 5′-GCTGTCGCCTACACTGC-3′	1110	57°C for 50 s
			72°C for 50 s
			(3) 72°C for 5 min
C5L2[f] external region	FRD 5′:-CTGGACAAATCTTAACTC-3′	16	(1) 94°C for 3 min
	REV 5′:-TGTTGAATGAAGGAAGG-3′	1202	(2) 35 cycles of:
		51	94°C for 70 s
			57°C for 70 s
			72°C for 70 s
			(3) 72°C for 5 min
			57°C for 50 s
			72°C for 50 s
			(3) 72°C for 5 min
Nested *C5L2* 5′ end region[f,h]	FRD[d]: 5′-CCAGAGACACCAGGAG-3′	51	(1) 94°C for 3 min
	REV[e]: 5′-CCGTAGTCCACCACAC-3′	643	(2) 30 cycles of:
Nested *C5L2* 3′ end region[f,h]	FRD[d]: 5′-CTGCTTCCTGGCTCTC-3′	467[h]	94°C for 50 s
	REV[e]: 5′-ACCCACAATGTCTCTCC-3′	1105	60°C for 50 s
			72°C for 50 s
			(3) 72°C for 5 min

[a] Oligonucleotide primers were designed by Beacon Designer™ ver. 4.0 and synthesized by Invitrogen

[b] PCR was performed using Platinum Taq (Invitrogen, Carslbad, CA)

[c] Positions correspond to mRNA sequences (C5aR1-NM_001736.3, C5L2-NM_018485.1)

[d] The primer has a T7 promoter (TAATACGACTCACTATAGGG) attached to its 5′ end

[e] The primer has a SP6 promoter (ATTTAGGTGACACTATAGGA) attached to its 5′ end

[f] PCR contains 10% DMSO

[g] 5′ end and 3′ end C5aR1 PCRs have an overlapping region (506–707)

[h] 5′ end and 3′ end C5L2 PCRs have an overlapping region (467–643)

method is appropriate for mutation screening on PCR amplicons up to 700 base pairs. For better efficiency of PCR amplification of *C5L2*, a nested PCR was performed. PCR primers and conditions are presented in Table 2. Direct sequencing of PCR products (Macrogen Inc., http://www.macrogen.com/eng/macrogen/macrogen_main.jsp) derived from NIRCA positive samples, 10 negative FMF samples, as assessed by RNases digestion, was performed for *C5aR1* and *C5L2* genes.

Restriction fragment length polymorphism (RFLP) analysis

RFLP analysis was performed on DNA level in sample from 217 healthy individuals and 105 FMF patients, utilizing the PCR primers and conditions for the lower C5aR1 region (Table 2). PCR was performed in a final volume of 50 μl containing 100–300 ng genomic DNA. The amplicon length is 644 base pairs (bp). After PCR, 7 μl of each PCR product was digested in a final volume of 20 μl with restriction enzyme *Acu*I (New England Biolabs, Beverly, MA, US), following manufacturers' instructions. The digestion products were electrophoresed in 2% agarose gel stained with ethidium bromide and visualized under UV lamp. The normal allele yielded 2 fragments of 536 + 69 bp, whereas mutant allele yielded 4 fragments of 536 + 397 + 139 + 69 bp.

GFP-rhC5a binding studies

Recombinant C5a protein linked to GFP was utilized to assess the binding capacity of C5a to its receptors. The isolated neutrophils were resuspended in PBS/1%BSA/0.02%NaN_3 buffer at a final concentration of 3×10^6/ml. Neutrophils were pre-incubated with 25 μM C5aR antagonist-PMX53 or an inactive scrambled peptide-control

PMX53 used as control for 10 min in the dark at room temperature. Afterwards, 250 nM GFP-hC5a was added and samples were incubated for 10 min in the dark. A wash in the same buffer ensued (5 min, 80 × g), cells were resuspended in 500 μl buffer plus 4% formalin and GFP-rhC5a binding was examined using flow cytometry. Differences in binding are presented as Mean Fluorescence Intensity-MFI ratio.

Quantitative oxidative burst activity in peripheral blood neutrophils

To investigate ROS production in neutrophils, the flow cytometry-based Phagoburst assay (ORPENGEN Pharma, Heidelberg, Germany, gmbH) was applied. The respiratory burst activity was quantified according to the manufacturer's instructions and as previously described [21]. MFI was performed in order to quantify ROS production.

Statistical analysis

Values are presented as mean ± SD. Allele frequencies in the two populations were compared using Chi-Square and odds ratio. To compare the differences in expression of C5a receptors a non-parametric Mann–Whitney test was applied. For determination of significance levels in binding capacity of C5a receptors a t test was utilized. The significance level was set to $P < 0.05$. Data were processed using OriginPro8.

Results

Expression of C5a receptors

We initially investigated the expression of *C5aR1* and *C5L2* genes in attack-free FMF patients and healthy subjects. The expression of *C5aR1* gene was not statistically different in FMF patients compared to healthy controls (Fig. 1). However, the expression of *C5L2* gene was significantly downregulated in neutrophils from FMF patients (Fig. 1).

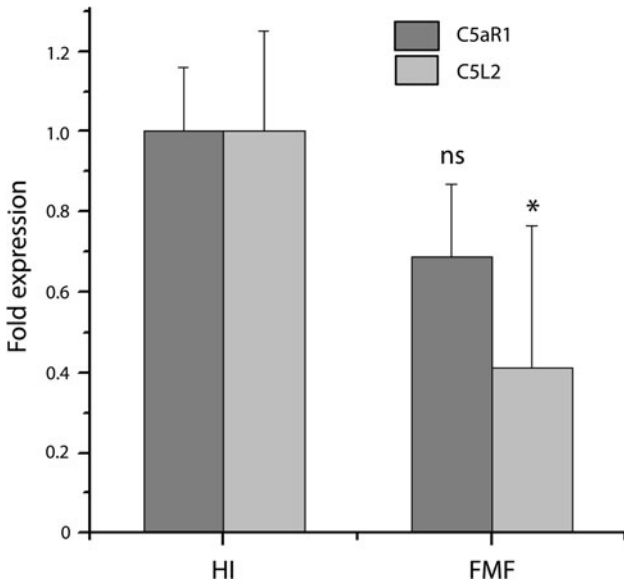

Fig. 1 mRNA transcript levels of C5a receptors. Relative expression of *C5aR1 and C5L2* genes in PMNs derived from FMF patients ($n = 12$) compared to those from healthy individuals ($n = 10$). Data are presented as mean ± SD (*$P < 0.05$, *ns* non-significant)

Genetic analysis of C5a receptors' genes in FMF patients

The non-isotopic RNase cleavage assay (NIRCA) was used to detect mutations in *C5aR1*and *C5L2* genes in RNA samples from 12 patients with FMF. C5aR1 receptor was found mutated in 2 patients with FMF (Fig. 2a). Direct sequencing was applied and revealed the presence of the N279 K polymorphic variant (rs11880097, Fig. 2b) in two positive samples, assessed by NIRCA, and the absence of any mutation in 10 negative samples. RFLP analysis using *Acu*I digestion was further performed to assess the distribution of N279 K polymorphism in the *C5aR1* gene in 217 healthy volunteers and 105 FMF subjects (Fig. 2c). The frequency of this polymorphism was not statistically different between the two groups (9.5% for FMF, 10.7% for healthy individuals; OR: 1.472, 95% CI: 0.697–3.109, $P = 0.308$). The distribution of the polymorphism in FMF patients in association with genotype and clinical presentation is presented in Table 3. There was not observed any association between any specific *MEFV* gene mutation or clinical feature of the disease with the presence of N279 K polymorphism. Moreover, NIRCA analysis and direct sequencing in 12 patients did not reveal any mutation in *C5L2* (data not shown), while the heterozygous nonsense polymorphism 597G > A (rs36046934) was detected in the 2 out of 12 patients.

C5a receptors' functional analysis

Although the expression of C5L2 gene was downregulated in neutrophils from FMF patients compared to healthy controls, the binding capacity of rhC5a was similar between the two groups (Fig. 3a). To assess the implication of *C5aR1* N279 K polymorphism in the binding capacity of C5a to its receptors, neutrophils derived from 8 carriers of *C5aR1* N279 K polymorphism were studied. GFP-rhC5a bound equally to C5aR1 receptor in WT neutrophils and those who carried the polymorphism (Fig. 3a). We further assessed the implication of the polymorphism in the

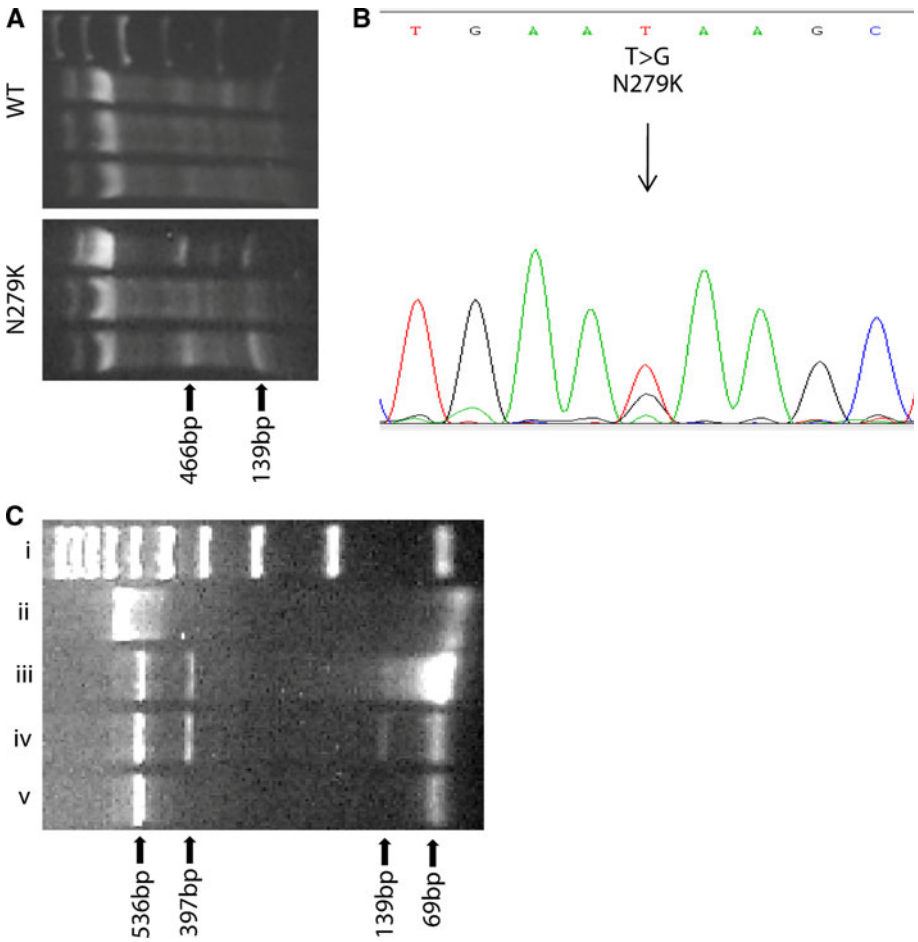

Fig. 2 Genotyping of *C5aR1* gene N279 K mutation by NIRCA and *Acu*I restriction enzyme digestion. **a** NIRCA digestion of normal (WT) and mutant sample (N279 K). **b** Verification of N279 K mutation by direct sequencing. **c** *Acu*I restriction enzyme digestion of *C5αR1* gene. Normal (WT; v) sample is digested by restriction enzyme in two fragments, whereas mutant (N279 K) heterozygote samples give four fragments (iii, iv), as observed on 2% agarose gel (i, DNA ladder; ii, PCR product)

capacity of C5a to induce respiratory burst. ROS production was independent from the *C5aR1* polymorphism (Fig. 3b).

Discussion

The aim of the present study was to investigate the presence of mutations, the mRNA expression patterns and the C5a binding capacity of C5a receptors in neutrophils derived from patients with FMF during remission. The expression level of *C5L2* gene was found to be downregulated in neutrophils from FMF patients. However, the binding capacity of rhC5a on neutrophils was similar in FMF patient and healthy subjects. We further investigated the frequency of the N279 K polymorphic variant of *C5aR1* gene in patients with FMF. The polymorphism was equally distributed in patients and healthy subjects.

Complement system plays a critical role in inflammatory responses and could theoretically participate in the pathogenesis of the inflammatory crises of FMF. The functional role of anaphylatoxin C5a and its receptors has been extensively investigated [1–3]. C5aR1 is a transmembrane receptor that initiates G-protein-coupled signaling via mitogen-activated protein kinase pathways. C5a signaling through C5aR1 leads to the activation of neutrophils and displays potent chemotactic activity [3, 4]. On the other hand, the vast majority of C5L2 exists intracellularly in endosomal compartments and is translocated to the cell surface after cell activation [3]. C5L2 was initially characterized as a default or scavenger receptor [5]. However, recent data suggest that C5L2 plays a key role in biological activities of C5a [6–8]. The balance between C5aR1 and C5L2 expression is proposed to determine the inflammatory response [3, 4]. Based on the aforementioned reports, we propose that the downregulated *C5L2* expression in neutrophils from FMF patients may represent a dysregulation of this balance. On the other hand, a persistent subclinical inflammation is observed in attack-free patients with FMF, as shown by high levels of acute phase reactants and inflammatory cytokines [22]. This chronic inflammatory environment may result among others in the aforementioned downregulation of *C5L2* expression levels. However, further studies are required to reveal a functional role for this disequilibrium. Riedemann et al. have demonstrated in a mouse model of sepsis, that the expression

Table 3 The presence of C5R1 *N279 K* polymorphism in association with *MEFV* gene mutations and clinical features of FMF

MEFV mutations	Number of patients	Number of C5R1 *N279 K* carriers
M680I/M680I	10	1
M694 V/M694 V	19	2
V726A/V726A	2	
M694/0	17	2
M694 V/M680I	23	3
M694I/M680I	2	
M680I/V726A	9	
M694 V/M694I	5	1
M694 V/V726A	10	1
M694/E148Q	4	
Total	105	11

Clinical features	Number of patients	Number of C5R1 *N279 K* carriers
Fever	98 (93.3%)	10
Abdominal pain	96 (91.4%)	11
Monoarthritis	24 (22.8%)	3
Thoracic pain	12 (11.4%)	
Serositis	8 (7.6%)	1
Myalgia	6 (6.6%)	
Henoch-Schönlein purpura	1 (0.95%)	
Amyloidodis	1 (0.95%)	

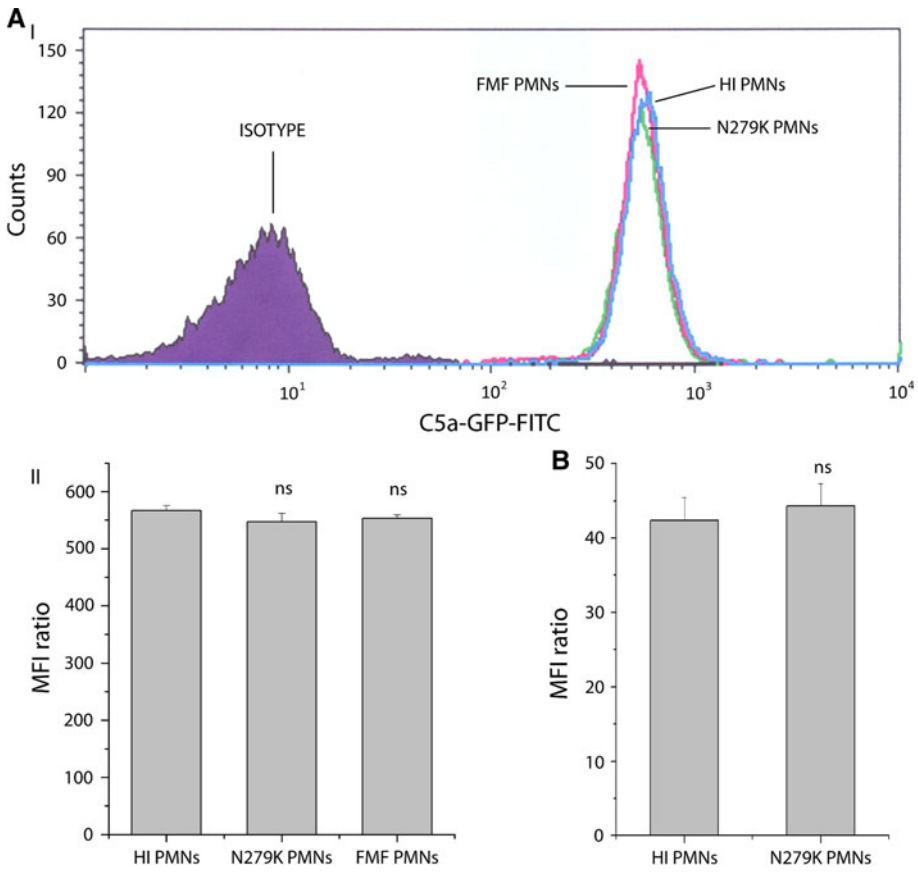

Fig. 3 Assessment of C5a binding capacity and ROS production in neutrophils from FMF patients and neutrophils with the N279 K variant of C5aR1. **a** GFP-rhC5a binding capacity to C5aR1 as observed by flow cytometry (I) and MFI ratio representation (II). Representative data of PMNs derived from control subjects ($n = 8$), a subject with the N279 K variant of C5aR1 ($n = 8$) and a subject with FMF without the polymorphism ($n = 8$), as determined by forward- and side-scatter characteristics. Isotype negative control is shown as filled histogram. **b** ROS production, after C5a stimulation, represented as MFI (bar 1: Healthy individual, bar 2: PMNs with the N279 K variant, $n = 8$ for each group)

levels of C5aR1 are increased in solid organs [23]. On the other hand, recent data demonstrated the decrease of C5aR1 levels in neutrophils from mice in early sepsis, which influences the chemotactic activity of neutrophils [9]. The expression of C5L2 is also downregulated in severe sepsis, which was attributed to systemic generation of C5a [10]. Moreover, a recent study demonstrated that Toll-like receptor signaling inhibited C5L2 expression and activity upon C5a stimulation, resulting in hypersensitivity to C5a [24].

FMF is an autoinflammatory syndrome. This group of diseases is associated with the dysregulation of innate immune responses in the absence of autoantibodies or antigen specific T cells [25]. Based on the critical role of the neutrophil chemotaxis at the sites of inflammation during FMF crisis, several studies attempted to shed light on the involvement of complement system in the pathogenesis of the disease. It has been reported that complement system is activated in FMF patients not receiving colchicine compared to colchicine receivers and healthy individuals [26]. However, another study did not demonstrate increased C5a levels in FMF patients compared to healthy individuals [27]. Recently, an effort was made to correlate a polymorphism in *C5aR1* gene with the clinical features of FMF [28]. Although there was no difference in the frequency of the polymorphism in patients and in healthy population, this receptor genotype tended to associate with the subgroup of FMF patients suffering from Henoch-Schönlein purpura. In the present study, we investigated the frequency of a polymorphism in *C5aR1* gene. The frequency of this polymorphism has not been previously associated with inflammatory disorders. However, N279 K polymorphic variant of *C5aR1* gene was not correlated with the disease.

In conclusion, neutrophils from attack free FMF patients indicated similar binding capacity to C5a and subsequent ROS production compared to control neutrophils, despite the downregulation of *C5L2* gene expression. Moreover, genetic analysis did not reveal novel mutations in *C5aR1* and *C5L2* genes, while N279 K polymorphism of *C5aR1* gene was equally distributed between investigated populations. Even though genetic analysis of C5a receptors was negative and C5a binding and subsequent ROS generation was similar in FMF patients and controls, further studies are needed to elucidate the possible role of other members of complement system in FMF pathogenesis.

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