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Mutations in *MEFV* cause alterations in neutrophil F-actin and phagocytosis dynamics

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Aim. *To examine neutrophil F-actin, phagocytosis and macropinocytosis dymanics in patients with Familial Mediterranean Fever (FMF), in an effort to understand the mechanisms that regulate switch of neutrophil activation program.* ***Methods***. *Whole blood neutrophils obtained from 37 attack-free FMF patients and 20 normal donors (ND) were activated with N-formyl-Met-Leu-Phe (fMLP), phorbolmyristate acetate (PMA) or lipopolysaccharide (LPS) and cellular F-actin content, phagocytosis and macropinocytosis determined by flow cytometry. F-actin oscillation amplitude and period were calculated from the curves generated by mathematical simulation giving the assumption that in neutrophil F-actin oscillates about a fixed point in a harmonic motion.* ***Results***. *Unstimulated neutrophil F-actin content was markedly increased in FMF patients. fMLP- but not PMA- or LPS-stimulated and Col-pretreated neutrophils where characterized by different pattern of F-actin dynamics and delayed time period of F-actin oscillation during FMF. Neutrophils from FMF patients failed to induce chemoattractant receptor desensitization during repeated action of fMLP, while in ND it occurred with significant reduction of F-actin oscillation amplitude and period. In FMF patients we observed significant enhancement of phagocytosis but not macropinocytosis amplitude and frequency.* ***Conclusions***. *Impaired neutrophil F-actin, phagocytosis and macropinocytosis oscillations amplitude and frequency that tightly regulate switch of neutrophil activation program during its encounter with increasing concentration of chemoattractants may be a potential pathogenic mechanism causing aberrant resolution of inflammation during FMF.*

Keywords: familial Mediterranean fever, neutrophil, F-actin, phagocytosis, macropinocytosis, N-formyl-Met-Leu-Phe, phorbolmyristate acetate.

Introduction. The discovery of mutations in the *MEFV* (for MEditerranean FeVer) locus has changed considerably the understanding of the most common auto-inflammatory disease Familial Mediterranean Fever (FMF). This gene encodes a protein called pyrin, expressed primarily on the innate immune system cells, including neutrophils, and cytokine-activated monocytes [1]. While it is known that pyrin interacts with five cellular proteins: PSTPIP1 [2], 14-3-3 [3], Caspase-1 [4], ASC [5], and Siva [6], the exact function of pyrin in cell and the mechanism underlying the pathological effect of pyrin mutations are yet to be revealed. In transfected cells pyrin was observed to co-localize with actin in lamellar structures and in supranuclear ruffles, but not with stress fibers [7]. Since, actin plays a central role in biological motility as an essential constituent of cytoskeleton and a partner of intracellular signaling pathways associated with chemoattractant-receptor activation, here we investigated neutrophil F-actin, phagocytosis and macropinocytosis dynamics during neutrophil chemoattractant-dependent activation in FMF patients carrying homozygous or compound heterozygous mutations in the *MEFV* locus.

Materials and methods. Peripheral blood samples were obtained from 37 attack-free FMF patients, diag-

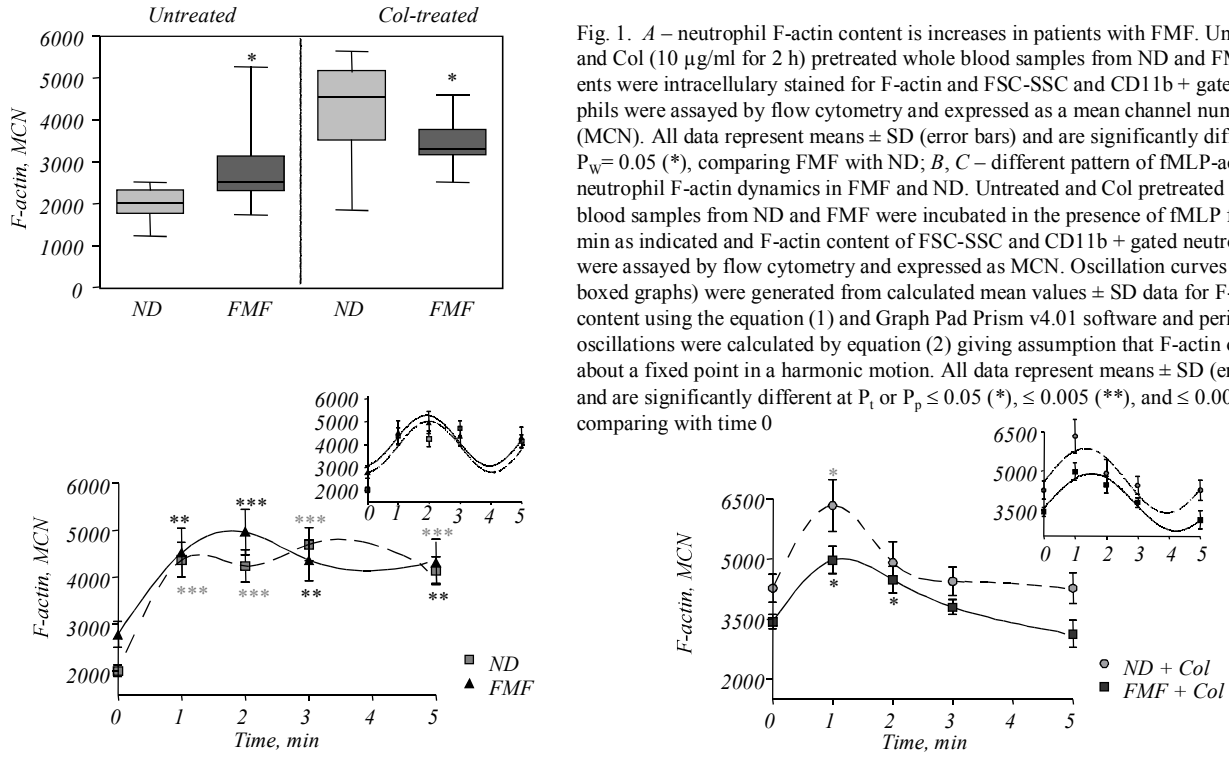

Fig. 1. *A* – neutrophil F-actin content is increases in patients with FMF. Untreated and Col (10 μg/ml for 2 h) pretreated whole blood samples from ND and FMF patients were intracellulary stained for F-actin and FSC-SSC and CD11b + gated neutrophils were assayed by flow cytometry and expressed as a mean channel number (MCN). All data represent means ± SD (error bars) and are significantly different at P_W= 0.05 (*), comparing FMF with ND; *B*, *C* – different pattern of fMLP-activated neutrophil F-actin dynamics in FMF and ND. Untreated and Col pretreated whole blood samples from ND and FMF were incubated in the presence of fMLP for 1–10 min as indicated and F-actin content of FSC-SSC and CD11b + gated neutrophils were assayed by flow cytometry and expressed as MCN. Oscillation curves (upper boxed graphs) were generated from calculated mean values ± SD data for F-actin content using the equation (1) and Graph Pad Prism v4.01 software and periods of oscillations were calculated by equation (2) giving assumption that F-actin oscillates about a fixed point in a harmonic motion. All data represent means ± SD (error bars) and are significantly different at P_t or $P_p \leq 0.05$ (*), ≤ 0.005 (**), and ≤ 0.0005 (***), comparing with time 0

nosed according to the Tel-Hasomer criteria [8] and 20 sex- and age-matched normal donors (ND). MEFV mutations in exon 10 were identified in all patients (18 patients were homozygous for the *M694V* mutation, 19 remaining patients were compound heterozygous for the *M694V* and one of the *V726A*, *M680I*, *E148Q*, *R761H* and *F479L* mutations). The quantitative flow cytometry determination of intracellular F-actin content was performed [9]. Whole blood samples were incubated with 20 ng/ml phorbol-12-myristate-13-acetate (PMA) or 10^{-7} M N-formyl-Met-Leu-Phe (fMLP) or 1 μg/ml lipopolysaccharide (LPS) from *Escherichia coli* 026:B6 or 0.1–10 μg/ml colchicine (Col) for 1–10 min. Phagocytosis assay was performed using latex beads and dextran-FITC. Oscillation time-response curves were generated from calculated mean values ± SD data for F-actin content using the equation (1):

$$Y = B + A \cdot \sin(F \cdot t + P), \quad (1)$$

where *Y* represents a relative F-actin content, expressed as a mean channel number – MCN; *B* is a base line; *A* is an amplitude; *F* is a frequency and *P* is a phase shift of oscillation and *t* is time in min. *A* and *F* values were calculated using Graph Pad Prism v4.01 software and periods of oscillations (*T*) were calculated by equation (2) giving assumption that F-actin oscillates about a fixed point in a harmonic motion [10]:

$$T = 2\pi/F. \quad (2)$$

Results and discussion. Unstimulated neutrophils F-actin content in FMF patients was significantly higher ($P_W < 0.01$) than in ND (Fig. 1, *A*). Pretreatment of whole blood with 10 μg/ml Col for 2 h increased neutrophil F-actin content in both FMF ($P_W < 0.007$) and ND ($P_t < 0.0001$). However, Col-pretreated neutrophils F-actin content in FMF patients was found to be significantly lower ($P_W \pm 0.02$) than in ND (Fig. 1, *A*). The existence of several shifted maximums in F-actin dynamics of ND and FMF patient's activated neutrophils led us to test if this is due to different pattern of actin cytoskeleton polymerization and depolymerization regular cycles. fMLP-induced neutrophil F-actin oscillation showed differences in baseline, but not in amplitude and frequency of F-sactin oscillations (Fig. 1, *B*, *C*). The period of fMLP-induced neutrophil F-actin oscillation, for FMF patients was 4.08 ± 0.46 min and

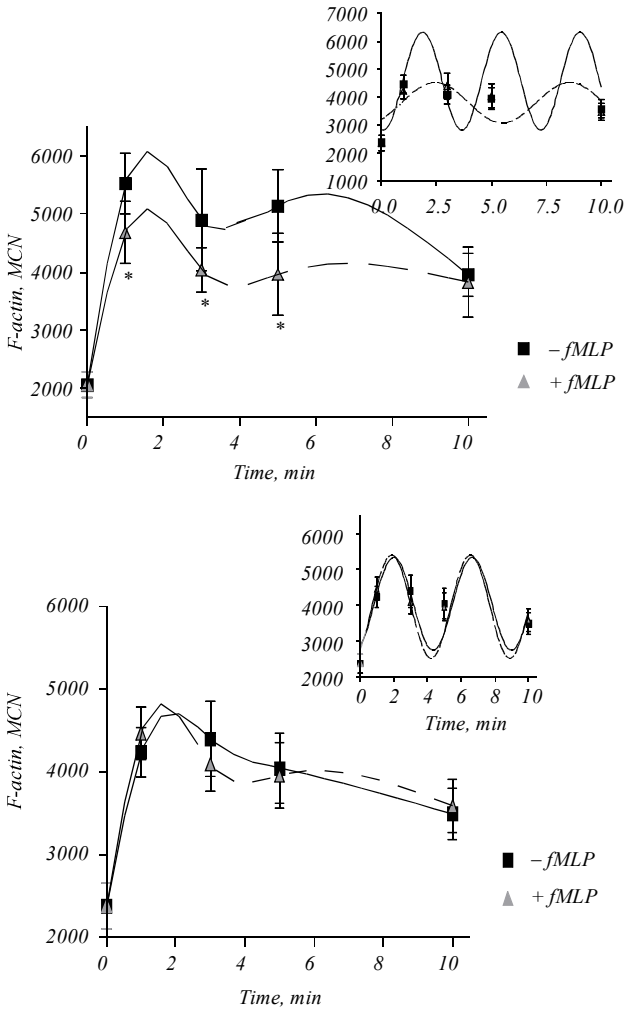

Fig. 2. Neutrophil F-actin dynamics in the presence of double doses of chemoattractant. Neutrophils from ND (*A*) and FMF (*B*) in 100 μl of whole blood were incubated for 10 min with (+) or without (–) fMLP and then equal concentration of these chemoattractant added to blood samples and incubated for additional 1–10 min. The reaction is stopped at the appropriate time and the intracellular F-actin content is measured as described in Materials and methods. Oscillation curves (upper boxed graphs) were generated using the equations (1) and (2). All data represent means ± SD (error bars) and are significantly different at P_t or $P_p \leq 0.05$ (*), comparing with single and double fMLP-treated neutrophils

4.09 ± 0.18 min for ND. As we expected, the period of F-actin oscillation in Col-pretreated neutrophil, activated by fMLP increased to 1.31 min in FMF patients (5.39 ± 0.13) and to 1.1 min in ND (5.19 ± 0.21). Thus, fMLP-stimulated and Col-pretreated neutrophils were characterized by different pattern of F-actin dynamics and delayed shift of maximums during FMF.

We found that fMLP-, LPS- or PMA-stimulated neutrophil F-actin dynamics in FMF patients is characterized by oscillations with different amplitude and periods, whereas in ND's activated neutrophil F-actin dynamics has an undulating shape in the presence of fMLP only. Next, we analyzed how delayed F-actin oscillation period in FMF could contribute to altered F-actin dynamics during repeated fMLP action. We incubated ND's and FMF patient's whole blood neutrophils with fMLP for 10 min, then added equal amount of fMLP for 1–10 min and assayed the cellular F-actin content. We observed that repeated action of fMLP in ND (Fig. 2, *A*) induced significant reduction in both overall F-actin content and cellular F-actin oscillation amplitude (1751 ± 166 in single dose fMLP treated cells versus 725 ± 120 MCN in double fMLP treated cells, P_t = = 0.03). In contrast to ND, FMF patients' neutrophils failed to induce reduced F-actin dynamics during repeated action of fMLP (Fig. 2, *B*). Indeed, neither F-actin oscillation amplitude (1298 ± 104 in single dose fMLP treated cells versus 1442 ± 93 MCN in double fMLP treated cells) nor oscillation period (4.64 ± 0.11 versus 4.66 ± 0.09 min, respectively) were changed in FMF patient's neutrophils in the presence of single or double doses of fMLP.

The period of unstimulated neutrophil phagocytosis oscillation for FMF patients was 3.65 ± 0.03 min and 6.06 ± 0.12 min for ND (P_t = 0.02) with enhanced amplitude (274.9 ± 37 MCN versus 68.7 ± 19 MCN in ND, P_t = 0.04) and frequency of phagocytic uptake of latex beads. However, fMLP-stimulated neutrophil phagocytosis dynamics revealed oscillation behavior in FMF patients with two-fold increase in the period of latex beads phagocytic uptake (6.03 ± 0.05 min) and linear time-dependence in ND's. Unstimulated neutrophil macropinocytosis dynamics revealed oscillation behavior in ND's with the period of FITC-dextran uptake 7.21 ± 0.16 min, whereas the linear time-dependence was observed for FMF patients. The period of fMLP-stimulated neutrophil macropinocytosis oscillation for FMF patients was found to be 4.78 ± 0.03 min and 6.4 ± 0.01 min for ND (P_t = 0.04). Thus, the period of fMLP-stimulated neutrophil macropinocytosis oscillation in FMF patients was found to be much closer to the period of fMLP-stimulated F-actin oscillation (4.73 ± 0.13 min for F-actin and 4.78 ± 0.03 min for FITC-dextran uptake) compared with two-fold increase in the period of FITC-dextran uptake in ND (3.58 ±

± 0.17 min for F-actin and 6.49 ± 0.01 min for FITC-dextran uptake).

There are a number of unexpected findings described in this study. The first unexpected finding is the increasing of overall F-actin content in unstimulated neutrophils in FMF patients. Second, we found that repeated action of fMLP in ND induced significant reduction of both overall F-actin content and cellular F-actin oscillation amplitude, which paralleled with double increasing of neutrophil F-actin oscillation period. The resolution of inflammation can now be regarded as an integral component of the neutrophil activation negative regulation programs [11]. The best known example is the phenomenon, known as neutrophil chemoattractant receptors desensitization. When neutrophils encounter increasing concentration of chemoattractant, they gradually become nonresponsive to further stimulation by the same agent. In contrast to ND, FMF patients' neutrophils failed to induce reduced F-actin dynamics during repeated action of fMLP, suggesting that neutrophils from FMF patients fail to induce chemoattractant receptor desensitization. In conclusion we suggested that mutant *MEFV*-encoded pyrin may contribute to the decreasing of plasticity of cellular cytoskeleton and neutrophils failed to induce reduced F-actin or phagocytosis dynamics during repeated action of different chemoattractant or particles.

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Мутації в гені *MEFV* спричиняють порушення у вмісті F-актину нейтрофілів та динаміці фагоцитозу

Резюме

Мета. *Вивчення динаміки внутрішньоклітинного вмісту F-актину, фагоцитозу і макропіноцитозу нейтрофілів у хворих на сімейну середземноморську лихоманку (ССЛ) для визначення молекулярно-клітинних механизмів періодичної активації.* ***Методи***. *Нейтрофіли цільної крові 37 хворих на ССЛ поза нападом і 20 нормальних донорів (НД) активували хемоатрактантним пептидом N-форміл-Мет-Лей-Фен (fMLP), форболмірістатацетатом (PMA) або ліполісахаридом (LPS). Вміст клітинного F-актину, фагоцитоз і макропіноцитоз визначали методом проточної цитофлуориметрії. Амплітуду і період осциляції F-актину вираховували з кривих, отриманих методом математичної обробки даних, із допущенням, що F-актин осцилює гармонійними коливаннями навколо фіксованої точки.* ***Результати***. *Вміст F-актину у нестимульованих нейтрофілах виявився достовірно вищим у хворих на ССЛ. fMLP-, але не PMA- або LPS-стимульовані і попередньо оброблені колхіцином нейтрофіли характеризувалися різними динамічними змінами у вмісті F-актину з подовженим періодом його осциляції при ССЛ. За повторної дії fMLP нейтрофіли хворих на ССЛ не індукували десенсибілізацію хемоатрактантних рецепторів, однак у НД десенсибілізація розвивалася із значним зменшенням амплітуди і періоду осциляції клітинного F-актину. У хворих на ССЛ виявлено істотне зростання амплітуди і періоду осциляції фагоцитарної активності, але не макропіноцитозу нейтрофілів.* ***Висновки***. *Порушення динамічних змін фагоцитозу, макропіноцитозу і цитоскелета, які регулюють програми активації і дезактивації нейтрофілів за умов безперервної дії хемоатрактантів, може бути потенційним патогенетичним механізмом аберантної резолюції запалення при ССЛ.*

Ключові слова: сімейна середземноморська лихоманка, нейтрофіл, F-актин, фагоцитоз, макропіноцитоз, N-форміл-Мет-Лей-Фен, форболмірістатацетат.

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Мутации в гене *MEFV* приводят к нарушению содержания F-актина нейтрофилов и динамики фагоцитоза

Резюме

Цель. *Изучение динамики внутриклеточного содержания F-актина, фагоцитоза и макропиноцитоза нейтрофилов у больных семейной средиземноморской лихорадкой (ССЛ) для выяснения молекулярно-клеточных механизмов периодической активации.* ***Методы***. *Нейтрофилы цельной крови 37 больных ССЛ вне приступа и 20 нормальных доноров (НД) активировали хемоаттрактантным пептидом N-формил-Мет-Лей-Фен (fMLP), форболмиристатацетатом (PMA) или липолисахаридом (LPS). Содержание клеточного F-актина, фагоцитоз и макропиноцитоз определяли методом проточной цитофлуориметрии. Амплитуду и период осцилляции F-актина вычисляли из кривых, полученных методом математической обработки данных, с допущением, что F-актин осциллирует гармоничными колебаниями вокруг фиксированной точки.* ***Результаты***. *Содержание F-актина в нестимулированных нейтрофилах оказалось достоверно выше у больных ССЛ. fMLP-, но не PMA- или LPS-стимулированные и предварительно обработанные колхицином нейтрофилы характеризовались разными динамическими изменениями в содержании F-актина с удлиненным периодом его осцилляции при ССЛ. При повторном воздействии fMLP нейтрофилы больных ССЛ не индуцировали десенсибилизацию хемоаттрактантных рецепторов, однако у НД десенсибилизация развивалась со значительным уменьшением амплитуды и периода осцилляции клеточного F-актина. У больных ССЛ выявлено существенное увеличение амплитуды и периода осцилляции фагоцитарной активности, но не макропиноцитоза нейтрофилов.* ***Выводы***. *Нарушение динамических изменений фагоцитоза, макропиноцитоза и цитоскелета, регулирующих программы активации и дезактивации нейтрофилов в условиях непрерывного воздействия хемоаттрактантов, может быть потенциальным патогенетическим механизмом аберрантной резолюции воспаления при ССЛ.*

Ключевые слова: семейная средизимноморская лихорадка, нейтрофил, F-актин, фагоцитоз, макропиноцитоз, N-формил-Мет-Лей-Фен, форболмиристатацетат.

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