Familial Mediterranean fever (FMF) is a relapsing autoinflammatory disorder, heritable as an autosomal recessive trait, which is caused by various mutations in the gene MEFV. This gene encodes a protein called pyrin, expressed primarily on the innate immune system cells, including neutrophils, and cytokine-activated monocytes (French FMF Consortium, 1997; International FMF Consortium, 1997; Centola et al., 2000). The N-terminal domain of pyrin defines a motif, called the pyrin domain (PyD), which is similar to the structure of death effector domains and caspase recruitment domains (Staub et al., 2001). Through homotypic domain interactions, pyrin binds the common adaptor – apoptosis-associated speck-like protein (ASC) – and participates in at least three important cellular processes: apoptosis, recruitment and activation of procaspase-1 (with associated processing and secretion of IL-1β) and activation of the NF-κB transcription factor (Stehlik & Reed, 2004). Macrophages from mice expressing truncated pyrin, similar to FMF patients, exhibit heightened sensitivity to bacterial lipopolysaccharide, produce more active caspase-1 and IL-1β and show resistance to cytokine- and lipopolysaccharide-induced apoptosis (Chae et al., 2003).

Although the nature of the heightened endotoxin sensitivity state observed in FMF patients at present remains unknown, it was suggested that it may be due to impaired endotoxin tolerance induction. Prior exposure to lipopolysaccharide leads to a transient state of lipopolysaccharide hyporesponsiveness in vivo and in vitro, termed ‘endotoxin tolerance’ (Medvedev et al., 2006). Endotoxin tolerance is thought to limit the inflammatory response induced during infection, and protects the host from developing shock caused by excessive production of inflammatory cytokines by monocytes and macrophages (Varma et al., 2001). Recently we have shown that induction of monocyte
homologous endotoxin tolerance occurs during FMF attack, whereas monocytes from patients in the attack-free period fail to induce lipopolysaccharide tolerance and exhibit heightened sensitivity to bacterial endotoxin (Davtyan et al., 2006a).

Induction and maintenance of endotoxin tolerance is controlled by multiple, sometimes cell- and tissue-specific mechanisms, including modulation of Toll-like receptors (TLR), CD14, CD11a and CD11b surface expression, upregulation of membrane-associated T1/ST-2molecule, as well as increased expression of intracellular mediators involved in negative regulation of the TLR/IL-1-receptor signalling pathway (Liew et al., 2005). CD14 is an important molecule involved in the inflammation induced during sepsis induced by Gram-negative bacteria and endotoxin tolerance (Antal-Szalma, 2000). In addition to CD14, several other molecules, such as CD11b/CD18, facilitate regulation of TLR-mediated signalling receptor and can compensate for the loss of CD14 in response to Gram-negative bacteria (Moore et al., 2000; Perera et al., 2001).

Here we investigated endotoxin-induced intracellular cytokine synthesis by monocytes, the time course of lipopolysaccharide-mediated changes in surface CD14 and CD11b coreceptor expression, and endotoxin-induced apoptosis of neutrophils in FMF patients using a whole blood cell culture technique, which maintains the microenvironment of the blood, and reduces the risk of preactivation or selective depletion or enrichment of cell subsets (Brekke et al., 2007).

Materials and methods

Patients

Peripheral blood samples were obtained from 34 (21 male, 13 female, aged between 18 and 41 years) patients with FMF, diagnosed according to the Tel-Hasomer criteria (Livenh et al., 1997). MEFV mutations were identified in all patients (15 patients were homozygous for the M694V mutation, and the 19 remaining patients were compound heterozygous for the M694V and one of the V724A, M680I, E148Q, R761H and F749L mutations). The following selection criteria were applied to the patients enrolled in the study: (1) age > 16 years; (2) onset of FMF attacks in early childhood; (3) absence of chronic diseases such as renal failure, renal amyloidosis, diabetes mellitus, ischaemic heart disease, malignancy, trauma, infections and rheumatic disease; (4) treatment-naïve and no drug administration within 4 weeks before blood drawing; and (5) analyses were carried out only during attack-free periods. Blood samples from 10 Salmonella enteritidis (SalE)-infected patients at convalescence (10–15 days after a diagnosis of salmonellosis was made), were also analysed during the study. The diagnosis of SalE infection was confirmed based on clinical (diarrhoea, abdominal pain, nausea, vomiting, and fever), epidemiological and bacteriological data. Salmonella enteritidis was isolated from stool samples of all patients and identified by conventional biochemical profiles according to the CLSI (formerly NCCLS) criteria. All patients had given their informed consent to inclusion in this study. Heparinized peripheral blood from 12 sex- and age-matched normal donors (NDs) (eight male, four female) was provided by the Viola Company (Yerevan, Armenia) blood bank. No significant differences existed between FMF and SalE patients and NDs with respect to mean levels of erythrocyte sedimentation rate (ESR) and white blood cell counts.

Endotoxin tolerance

Endotoxin tolerance was monitored by measuring intracellular production of tumour necrosis factor (TNF-α) by whole blood monocytes in response to repeated doses of Escherichia coli O26:B6 lipopolysaccharide (Sigma Chemical, St Louis, MO) as described previously (Davtyan et al., 2006a,b). Briefly, whole blood cells were cultured for 18 h in the presence or absence of 100 ng mL⁻¹ lipopolysaccharide, washed three times with endotoxin-free phosphate-buffered saline, and cultured for an additional 4 h in the presence of 1 μg mL⁻¹ lipopolysaccharide and 10 μg mL⁻¹ brefeldin A (Sigma). After cell harvesting, endotoxin tolerance was assayed by flow cytometry-based determination of intracellular synthesis of TNF-α in activated monocytes.

Intracellular and surface staining of whole blood monocytes

After removal of erythrocytes by lysing, whole blood monocytes were surface stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD11b (clone 44) and phycoerythrin-conjugated anti-human CD14 (Sigma) or IgG1 and IgG2a isotype-matched controls (BD Pharmingen, San Diego, CA). Cells were incubated with 1% FACS permeabilizing solution and stained with phycoerythrin-conjugated antihuman TNF-α (clone MAb11) or matched isotype control, fixed in 1% paraformaldehyde, and subjected to FACS analysis on a FACSCalibur™ instrument using CELLQUEST™ software (Becton Dickinson). Intracellular synthesis of IL-4 and IFN-γ by monocytes was analysed by cell staining with FITC-conjugated anti-human IFN-γ (clone 4S.B3) and phycoerythrin-conjugated anti-human IL-4 (clone 8D4-8) or matched isotype controls (BD Pharmingen).

Measurement of apoptosis

Escherichia coli or S. enteritidis lipopolysaccharide (Sigma) at 1 and 0.1 μg mL⁻¹ concentrations were added to 500 μL of
the whole blood and incubated at 37 °C for 4 h. To detect neutrophil apoptosis at earlier stages, propidium iodide (PI)/Annexin V and TUNEL assays (BD ApoAlert; BD PharMingen) were used according to the manufacturer’s protocol. Apoptosis was monitored by determining the proportion of early apoptotic (Annexin V/FITC- or TUNEL-positive and PI-negative) neutrophils on a FACSCalibur™ instrument using CELQUEST™ software (Becton Dickinson).

Statistical analyses

Dispersion analysis with parametric and nonparametric procedures using the GRAPH PAD PRISM v4.01 software was used. Results of independent experiments were used to calculate mean values ± SEM, and differences were defined as statistically significant by Student’s t-test (P), paired t-test (Pp), Wilcoxon–Mann–Whitney and Welch’s test (PW) at P ≤ 0.05.

Results

Monocytes of FMF patients exhibited lack of endotoxin tolerance induction and higher cytokine production upon stimulation with lipopolysaccharide

We observed that monocytes obtained from NDs with overnight 100 ng mL⁻¹ lipopolysaccharide treatment developed tolerance to the subsequent 1 μg mL⁻¹ lipopolysaccharide challenge by declining intracellular TNF-α synthesis (P = 0.002). Monocytes of FMF patients in the attack-free period failed to induce lipopolysaccharide-homologous tolerance to repeated exposure to lipopolysaccharide (Fig. 1).

Next, we tested if monocytes of FMF patients and NDs produced different levels of cytokines (IL-12, IL-4 and IFN-γ) constitutively or upon lipopolysaccharide activation. Neither IL-4 nor IFN-γ were constitutively produced by freshly isolated whole blood monocytes from NDs, and only a small fraction of monocytes constitutively producing IL-12 was detected in every individual tested (Table 1). In contrast, whole blood monocytes from FMF patients incubated in the medium alone produced detectable IFN-γ and IL-4 and significant levels of IL-12, as compared with NDs. After incubation of monocytes of both NDs and FMF patients for 4 and 18 h with 1 μg mL⁻¹ lipopolysaccharide, we observed an upregulation of cytokine synthesis towards production of IFN-γ over IL-4. However, monocytes from FMF patients produced significantly higher levels of IL-4, IL-12 and IFN-γ upon lipopolysaccharide stimulation, as compared with NDs.

Time course of lipopolysaccharide-induced changes in monocyte surface expression of CD14 and CD11b receptors during FMF

The baseline level of monocytic surface expression of CD14 was higher in NDs than in FMF patients. However, the time course of lipopolysaccharide-induced downmodulation of monocyte surface expression of CD14 in FMF patients and NDs was different and showed delayed turnover in FMF patients (Fig. 2a). The size of the CD14+CD11b+ monocyte population did not differ between analysed groups, but again the time course of lipopolysaccharide-induced changes in the size of the CD14+CD11b+ monocyte population during FMF revealed a pattern similar to that of CD14 surface expression (Fig. 2b). The size of the CD14–CD11b+ monocyte population also did not differ between analysed groups during 4 h of incubation with or without lipopolysaccharide. However, it increased in FMF patients upon 18 h of incubation either in the presence or in the absence of lipopolysaccharide (Fig. 3a). The time course pattern of lipopolysaccharide-induced changes in size of the CD14–CD11b+ monocyte population in both FMF patients and NDs was not similar with that of CD14 surface expression and the CD14+CD11b+ monocyte population; the size of the CD14–CD11b+ monocyte population increased significantly in the presence of lipopolysaccharide during 18 h of incubation. The baseline level of monocytic surface expression of CD11b was found to be higher in NDs than in FMF patients. Lipopolysaccharide stimulation of ND monocytes triggered upregulation of CD11b surface expression, as compared with that of untreated monocytes, but the overall time course revealed that expression of CD11b declined over with incubation time both in the absence and
Results are the means ± SEM of n = 26 FMF patients and n = 12 NDs.

*P<0.05, comparing FMF with ND; †P<0.05 comparing background cytokine synthesis with lipopolysaccharide-induced intracellular cytokine synthesis. Whole blood samples were incubated for 4 and 18 h in the presence or absence of 1 µg mL⁻¹ lipopolysaccharide and intracellular cytokine synthesis was subsequently assayed by flow cytometry.

**CD11b expression in neutrophils and monocytes**

CD11b surface expression was significantly higher in monocytes from FMF patients as compared with untreated monocytes from NDs. CD11b expression increased within the first 4 h of lipopolysaccharide stimulation of both NDs and FMF patient neutrophils (Fig. 2). Although monocyte surface expression of CD11b increased only during the first 4 h of stimulation with lipopolysaccharide in FMF patients as compared with untreated monocytes, the overall time course showed no significant changes in expression of CD11b after 4–18 h in the presence or absence of lipopolysaccharide (Fig. 3). Monocyte surface expression of the CD11b receptor was significantly increased in FMF patients compared with NDs at 18 h of incubation either in the presence or in the absence of lipopolysaccharide. Taken together, these data suggest that the time course of lipopolysaccharide-induced changes in FMF patient monocytes is characterized either by delayed turnover or by increased surface retention of CD14 and CD11b receptors despite their low baseline surface expression levels.

**CD11b surface expression and early apoptosis of neutrophils in FMF patients**

The baseline levels of CD11b neutrophil surface expression did not differ between NDs and FMF patients. Lipopolysaccharide stimulates both NDs and FMF patient neutrophils after 4 and 18 h caused upregulation of CD11b surface expression, compared with untreated neutrophils. CD11b expression declined over the incubation period in the absence of lipopolysaccharide in NDs but not in neutrophils of FMF patients (Fig. 4a). Furthermore, we observed that the percentage of CD11b+ neutrophils was significantly higher in FMF patients compared with NDs; however, 4 h of lipopolysaccharide stimulation of neutrophils from FMF patient did not increase the percentage of CD11b+ neutrophils as much as that for NDs. The percentage of lipopolysaccharide-stimulated and unstimulated CD11b+ neutrophils in both groups decreased after 18 h of incubation, and this was more pronounced in FMF patients than in NDs (Fig. 4b).

Lipopolysaccharide-induced early apoptosis of neutrophils in patients with FMF and NDs was assessed within 4 h of whole blood cultivation during which no significant perturbations in neutrophil death were observed in the presence or absence of lipopolysaccharide (data not shown). The percentage of neutrophils that spontaneously

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**Table 1. Intracellular cytokine synthesis by monocytes of FMF patients**

<table>
<thead>
<tr>
<th></th>
<th>IL-4</th>
<th>IL-12</th>
<th>IFN-γ</th>
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<tbody>
<tr>
<td></td>
<td>Lipopolysaccharide, 4 h</td>
<td>Lipopolysaccharide, 18 h</td>
<td>Lipopolysaccharide, 4 h</td>
</tr>
<tr>
<td>FMF</td>
<td>2.6 ± 0.7</td>
<td>10.5 ± 3.7</td>
<td>7.6 ± 2.4</td>
</tr>
<tr>
<td>ND</td>
<td>2.0 ± 0.2</td>
<td>4.5 ± 0.9</td>
<td>6.1 ± 0.9</td>
</tr>
</tbody>
</table>

†P<0.05 comparing background cytokine synthesis with lipopolysaccharide-induced intracellular cytokine synthesis. Whole blood samples were incubated for 4 and 18 h in the presence or absence of 1 µg mL⁻¹ lipopolysaccharide and intracellular cytokine synthesis was subsequently assayed by flow cytometry.
undergone early apoptosis did not differ significantly between NDs and FMF patients (Fig 5a). In contrast, lipopolysaccharide-induced apoptosis of neutrophils in FMF patients was significantly higher, as compared with NDs, in whom we observed delayed apoptosis of neutrophils in the presence of lipopolysaccharide. Thus, increased lipopolysaccharide-induced apoptosis of neutrophils in FMF patients could indicate a heightened state of endotoxin susceptibility in FMF.

Finally, we compared differences between susceptibility of whole blood neutrophils of NDs and FMF and SalE patients to the apoptotic action of low and high doses of lipopolysaccharide derived from S. enteritidis. The selected NDs (n = 8) and FMF patients (n = 8) with no previous history of salmonellosis were negative for both the conventional Widal agglutination test and S. enteritidis growth in stool samples. Surprisingly, we observed that the percentage of apoptotic neutrophils in FMF patients in the presence of both high and low doses of S. enteritidis lipopolysaccharide was significantly higher than those in NDs and SalE patients (Fig. 5b). In SalE patients, the percentage of apoptotic neutrophils in the presence of high but not low doses of S. enteritidis lipopolysaccharide was decreased compared with NDs. However, unlike NDs, S. enteritidis lipopolysaccharide-induced apoptosis was not dose-dependent in FMF patients and in SalE patients (Fig. 5c). The percentage of
Fig. 5. Apoptosis of granulocytes induced by Escherichia coli lipopolysaccharide (a), Salmonella enteritidis lipopolysaccharide (b) and percentage of persons responding in a dose-dependent manner to S. enteritidis lipopolysaccharide-induced apoptosis (c). Whole blood from FMF patients \( (n=26) \) was treated with 1 \( \mu \text{g mL}^{-1} \) E. coli lipopolysaccharide for 4 h (a). Whole blood from FMF patients \( (n=8) \), NDs \( (n=8) \) with no previous history of salmonellosis and SalE patients \( (n=10) \) was treated either with 0.1 or 1 \( \mu \text{g mL}^{-1} \) lipopolysaccharide from S. enteritidis for 4 h (b). Apoptosis was subsequently assayed by flow cytometry. \( *P \), or \( \text{P}_{0.05} \), comparing unstimulated cells with lipopolysaccharide-stimulated granulocytes (a) or high and low doses of S. enteritidis lipopolysaccharide (b, shown by arrows); \( \text{P}_{0.05} \), comparing FMF, SalE with NDs; \( \text{P}_{0.05} \), comparing FMF with SalE.

NDs whose neutrophils undergo apoptosis in a dose-dependent manner was significantly higher than in FMF and SalE patients. Thus, neutrophils of FMF patients previously unexposed to S. enteritidis exhibited heightened endotoxin susceptibility to the lipopolysaccharide of this pathogen similar to that of SalE patients.

**Discussion**

There is the evidence that, both in human and in primate populations during evolution, pyrin has been subjected to positive Darwinian selection (Schaner et al., 2001). Therefore, it appears that some of the FMF-causing mutations have been selected for, indicating that, under some environmental circumstances, a mutant pyrin allele confers a benefit to the host (Ross, 2007). The failure of monocytes from FMF attack-free patients to induce lipopolysaccharide-homologous tolerance together with a shift in monocyte proinflammatory cytokine synthesis polarization could indicate that the heightened state of inflammatory alert in asymptomatic FMF patients is caused by a lowered threshold of monocyte proinflammatory activation in response to bacterial endotoxins. Similar results were found in a mouse model of FMF, carrying truncated pyrin protein, in which homozygous mice exhibit heightened sensitivity to endotoxin challenge and elevated production of IL-1\( \beta \) (Chae et al., 2003).

Whether the heightened sensitivity to endotoxin during FMF conditioned by impaired endotoxin tolerance induction alone whether there are other occasioning mechanisms remain unknown. Although we have demonstrated that increased endotoxin sensitivity in the attack-free period could be result of a shift in the monocyte activation programme from ‘alternatively’ into ‘classically’ activated (Davtyan et al., 2006a), the precise mechanisms that could explain aberrant endotoxin tolerance during FMF are entirely unknown. One possible scenario connecting pyrin-associated cytoskeleton with endotoxin tolerance is altered surface expression or different recycling rate of CD14 and CD11b receptors. Using similar time course studies as in lipopolysaccharide tolerance experiments, we demonstrated that the rate of lipopolysaccharide-induced CD14 turnover was altered in FMF patients. Although it has been proposed that lipopolysaccharide tolerance is the consequence of a decrease in the number of lipopolysaccharide receptors on the cell surface (Nomura et al., 2000), CD14 expression is not affected or may even be increased in lipopolysaccharide-tolerant cells (Labetz et al., 1993). Nevertheless, in the present study we showed that delayed turnover of CD14 and increased surface retention of CD11b receptors on monocytes is linked to the impairment of endotoxin tolerance induction during FMF. Lipopolysaccharide, as a ligand for CD14, can alter the number of CD14 on monocytes, dependent on cell type, endotoxin concentration and incubation time (Antal-Szalma, 2000). The extent of CD14 expression upon lipopolysaccharide stimulation shows a rapid upregulation (50–100%) in 30–180 min, followed by a decrease (50–75%) after 3–6 h and then again a marked increase (200–300%) after 1–6 days (Landmann et al., 1991). The first rapid increase is the result of intracellular translocation of a CD14 pool to the plasma membrane, while the second upregulation is associated with de novo protein synthesis and might correlate with monocyte differentiation (Landmann et al., 1996). Therefore, it appears that the increased rate of intracellular translocation of a CD14 pool...
to the plasma membrane may contribute to heightened endotoxin susceptibility during FMF.

Another possible contribution to the impaired endotoxin tolerance could be a different cytokine profile during FMF or a C to T polymorphism at position 159 in the promoter of CD14 (C-159T), which changes the affinity of the Sp family of transcription factors, thus modulating the cellular response to endotoxin (LeVan et al., 2001). Recent studies have shown that the CD14 C159T polymorphism in contrast to the Arg753Gln polymorphism of TLR2 is not associated with FMF or development of amyloidosis (Ozen et al., 2006; Keskin et al., 2007). FMF patients both during and between attacks produce increased levels of serum IL-6, IL-8, IL-10, IL-12, IL-18, INF-γ, soluble IL-2 and TNF receptors, relative to controls (Aypar et al., 2003; Baykal et al., 2003; Simsek et al., 2007). Here we demonstrated that proinflammatory cytokine synthesis polarization and lack of tolerance to endotoxin take place during attack-free periods of FMF. This is in agreement with previously reported data that proinflammatory cytokines prevent endotoxin tolerance (Adib-Conquy & Cavalion, 2002) and induce rapid decrease in CD14 expression (Cosentino et al., 1995).

An important cellular participant in FMF attack are neutrophil granulocytes (Schaner & Gumucio, 2005), and one of their main properties, a short lifespan. We therefore studied neutrophil involvement in mechanisms that make for a heightened state of endotoxin susceptibility during FMF. Lipopolysaccharide-induced apoptosis of neutrophils is now recognized as a highly evolutionarily conserved mechanism for resolution of inflammation (Luster et al., 2005; Serhan & Savil, 2005). Extensive in vitro analyses have ascribed various roles to CD11b in cellular processes, many of which are involved in control of bacterial numbers, generation of protective immune response to Gram-negative organisms and in resolution of inflammation (Rijneveld et al., 2005).

In addition, CD11b is required for enhanced neutrophil apoptosis following cellular interactions with pathogens (Pilione et al., 2006). The expression time course of CD11b shows that a decline with incubation time in the absence of lipopolysaccharide in NDs but not in neutrophils of FMF patients. However, the percentage of lipopolysaccharide-stimulated CD11b+ neutrophils declined upon lipopolysaccharide stimulation in FMF patients, which we found to be associated with increasing apoptosis. Previously, we have shown that increased susceptibility of neutrophils to S. enteritidis lipopolysaccharide-induced apoptosis is correlated with the severity of a clinical course of salmonellosis at convalescence (Gyulazyan et al., 2006). Neutrophils from FMF patients unexposed to S. enteritidis exhibited heightened endotoxin susceptibility to low and high doses of lipopolysaccharide of this pathogen similar to that of SaeE patients.

Infection of macrophages with Salmonella spp. leads to caspase-1 activation, release of IL-1β and rapid cell death (Tschopp et al., 2003). Recent studies have established a central role for the ASC and Ipaf inflammasome in caspase-1 activation and IL-1β secretion by Salmonella typhimurium (Lara-Tejero et al., 2006; Lamkanfi et al., 2007). Pyrin regulates caspase-1 activation and consequently IL-1β production through cognate interaction of its N-terminal PyD motif with the ASC adaptor protein (Stehlik & Reed, 2004). However, the preponderance of mutations reside in the C-terminal B30.2 domain of pyrin, which is necessary and sufficient for direct interaction with caspase-1 even in the absence of ASC (Chae et al., 2006). Therefore, a direct, ASC-independent effect of pyrin on IL-1β activation or apoptosis suggests heightened IL-1β responsiveness as one factor selecting for pyrin mutations. The carrier status for MEFV mutations seems to be unique, in that they, as modifiers of a common inflammatory pathway, cause an alteration in the state of ‘health’ (Kalyoncu et al., 2006) and increased incidence of chronic inflammatory diseases (Fidder et al., 2005; Rabinovich et al., 2005; Grimaldi et al., 2006).

In conclusion, MEFV mutation modifiers of a common inflammatory pathway cause an alteration in the state of endotoxin susceptibility, which may be associated with the development of a more severe disease in patients having an inflammatory burden.

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