Diminished IL-10 production is Associated with Impaired Versatility of Monocytes in Familial Mediterranean Fever

Tigran K. Davtyan¹*, Gagik S. Hakobyan²*, Samvel A. Avetisyan³, Anna G. Sukiasyan⁴ and Yuri T. Aleksanyan⁴

¹Laboratory of Immunology and Virology, "Armenicum" Research Centre, CJSC Armenicum, 37 Nabbandyan St., Yerevan, Republic of Armenia
²Department of Internal Medicine, Yerevan State Medical University, Koryun 2 St., Yerevan, Republic of Armenia
³Department of Pathophysiology, Yerevan State Medical University, Koryun 2 St., Yerevan, Republic of Armenia
⁴Laboratory of Epidemiology and Immunology, Institute of Epidemiology, Virology and Medical Parasitology, Ministry of Health RA, Yerevan, Armenia

Corresponding author: Dr. Tigran K. Davtyan, PhD, ScD, Analytical Laboratory Branch, Scientific Centre of Drug and Medical Technology Expertise JSC, Armenia, Tel: +374 10 23-72-61; Fax: +374 10 28-07-33; E-mail: tigdav@excite.com

Received date: Jan 15, 2014, Accepted date: Mar 10, 2014, Published date: Mar 17, 2014

Abstract

Purpose: The nature of the heightened endotoxin sensitivity state observed in Familial Mediterranean Fever (FMF) at present remains unknown. To assess the possibility that IL-10 plays a role in setting the inflammatory threshold, we studied IL-10 production by monocytes and dendritic cells as well as endotoxin tolerance induction in FMF patients.

Methods: 46 attack-free FMF patients were included in this study. The production of IL-10 by NLR- or TLR-agonist-stimulated monocytes and dendritic cells were assayed either by conventional ELISA or flow cytometry. The versatility of monocytes was studied by measuring the production of IL-10 and IL-1β after stimulation by pro- and anti-inflammatory agents, and after stimulation arrest or a further counter stimulation. Monocyte endotoxin tolerance and cross-tolerance induction were assayed by measuring the production of IL-1β, IL-10, TNF-α and IFN-γ after pre-stimulation by NLR- or TLR-ligands and after re-stimulation with LPS.

Results: In FMF patients, we observed down-regulation of circulating CD36⁺ peripheral blood lymphoid cells but not monocytes, constitutively producing IL-10. The production of IL-10 by TLR- and NLR-agonist-stimulated monocytes and dendritic cells declines in FMF patients. Monocytes isolated from FMF patients failed to switch from a pro-inflammatory activated state to anti-inflammatory phenotype and still produce IL-1β but not IL-10, which cause impaired endotoxin tolerance and cross-tolerance induction. The IL-10 production and endotoxin tolerance induction by monocytes and dendritic cells were restored by NOD2- ligand MDP and colchicine treatment.

Conclusion: The reduced IL-10 production was associated with the impaired setting of feedback inhibition of inflammatory response and caused impaired resolution of inflammation and endotoxin tolerance induction.

Keywords: Familial Mediterranean fever; IL-10; Endotoxin tolerance; Monocyte; Dendritic cells; IL-1β

Introduction

Familial Mediterranean fever (FMF) is a systemic relapsing auto-inflammatory 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 [1-3]. 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 the activation of pro-caspase-1 (with associated processing and secretion of IL-1β) and activation of the NF-κβ transcription factor [4]. Macrophages from mice expressing truncated pyrin similar to FMF patients - exhibit heightened sensitivity to bacterial lipopolysaccharide (LPS), produce more active caspase-1 and IL-1β and show resistance to cytokine- and LPS-induced apoptosis [5,6]. Although the nature of heightened endotoxin sensitivity state observed in FMF at present remains unknown, it was suggested that it may be due to impaired endotoxin tolerance induction. Prior exposure to LPS leads to a transient state of LPS hypersensitivity in vivo and in vitro, termed ‘endotoxin tolerance’ [7]. Endotoxin tolerance is thought to limit the inflammatory response induced during infection, and protects the host from developing shock caused by the excessive production of inflammatory cytokines by monocytes and macrophages [8]. Recently, we have shown that induction of monocyte homologous endotoxin tolerance occurs during an FMF attack, whereas monocytes from patients in the attack-free period fail to induce LPS tolerance and exhibit heightened sensitivity to bacterial endotoxin [9,10]. Impaired LPS tolerance induction in attack-free FMF patients correlates with both the increased LPS-induced pro-inflammatory cytokine synthesis polarization and the different time course pattern of LPS-induced changes on monocyctic surface expression of CD14, CD11a/CD18 and CD11b co-receptors. In addition, enhancement of LPS-induced apoptosis of neutrophils is observed in FMF patients, which is further confirmed by the fact that neutrophils from FMF patients previously unexposed to Salmonella enteritidis exhibited heightened
susceptibility to the LPS of this pathogen similar with that of *Salmonella enteritidis* infected patients [11].

Although dominance of anti-inflammatory cytokines such as IL-10 is associated with reduced immune responsiveness and susceptibility to persistent infection, conditions such as chronic inflammation are linked to lower IL-10 levels [12]. An appropriate threshold for immune activation is critical for optimal protection from infection and conversely, from short- and long-term side-effects of immune effector mechanisms. In the absence of appropriate feedback control, inflammatory responses can lead to vast insalubrity and death. Due to its primary ability to restrain inflammation, IL-10 has been a topic of long-standing interest [13]. Numerous clinical observations have validated mouse studies by linking IL-10 levels with disease outcomes [14,15]. Likewise, disease association studies identifying correlations between IL-10 levels and disease susceptibility have bolstered the belief that appropriate regulation of IL-10 expression is fundamental to governing host inflammatory responses [16,17]. To assess the possibility that IL-10 plays a role in setting inflammatory threshold and immune silence in auto-inflammatory disorders, we characterized the IL-10 production by monocytes and dendritic cells and endotoxin tolerance induction in patients with FMF.

Material and Methods

Patient population

Peripheral blood samples were obtained from 46 attack-free FMF patients with family history (26 male, 20 female, aged between 18-41 years), diagnosed according to the Tel-Hasomer criteria [18]. MEFV mutations in exon 10 were identified in all patients (patients were compound heterozygous for the M694V and one of the V724A, M680T, E148Q, R761H and F749L mutations). The following selection criteria were applied to the patients enrolled in the study: 1) age>16 years, 2) absence of chronic diseases such as chronic renal failure, renal amyloidosis, diabetes mellitus, ischemic heart disease, malignancy, trauma, infections and rheumatic disease, 3) treatment-naive and no drug administration within 4 weeks before blood drawing. All patients had given their informed consent to the inclusion in this study. Heparinized peripheral blood was obtained from 43 ND and 46 FMF patients and samples were stored at -80°C until solid phase enzyme-immune assay. IL-10 and IL-1β concentration in serum samples was determined by ELISA. Peripheral blood monocytes were isolated by cell adherence of PBMC to 25 cm² plastic flasks during 45 min incubation at 37°C in an atmosphere containing 6% CO₂ [19]. Monocytes (95% CD14 positive cells) were then washed three times with endotoxin-free PBS and cultured at 5 ×10⁶ cells/ml density in RPMI-1640 containing 10% FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin and 100 μg/ml streptomycin. Either of 2.5 μg/ml peptidoglycan (PGN) from *E. coli* 0111:B4 (an NOD1 and NOD2 ligand) and synthetic Pam3CSK4 bacterial lipoprotein (an TLR2-TLR1 ligand) or 5 μg/ml muramyl dipeptide (MDP, an NOD2 ligand) were included in the culture media from day 0 to day 3. All reagents were purchased from InvivoGen (San Diego, USA). To assess the versatility of monocyte activation, 5 ×10⁵ cells in 1 ml of complete RPMI-1640 medium were incubated with recombinant IL-4 (10 ng/ml, eBioscience) or 100 ng/ml LPS from *E. coli* 026:B6 (Sigma Chemical Co., St. Louis, MO) from day 0 to day 3. One day 3, the cells washed and fresh medium added or stimulated with opposite pro-inflammatory (LPS) or anti-inflammatory (IL-4) molecules for an additional 3 days [19]. Cell-free supernatants were decanted at days 3 and 6, then stored at -80°C and IL-10 and IL-1β concentration in samples determined by ELISA.

Generation of monocyte-derived dendritic cells (DCs)

For the generation of immature DCs (iDCs), plastic adherence monocytes were cultured for 6 days with GM-CSF (10 ng/ml) and IL-4 (10 ng/ml) as originally described by Morse et al. [20]. Mature DCs (mDCs) were generated *in vitro* by monocytes with incubation with GM-CSF (10 ng/ml), IL-4 (10 ng/ml) and pro-inflammatory TNF-α (10 ng/ml) and cultured for 6 days [20]. On day 6, the cells were washed and fresh medium was added or stimulated with 100 ng/ml LPS or 2 μg/ml colchicine for an additional 24 h. Cell-free supernatants were decanted and stored at -80°C and IL-10 concentration in samples determined by ELISA.

Flow cytometry

PBMC were surface-stained by 0.3 μg fluorescein isothiocyanate (FITC)-conjugated anti-CD36 (Serotec) or IgM-FITC isotype control and fixed in 1% paraformaldehyde (Becton Dickinson). Stained cells were incubated with 1% FACS permeabilizing solution (Becton Dickinson) and incubated with phycoerythrin (PE)-conjugated anti-CD36 (Serotec) or IgM-FITC isotype control. DC surface staining was done as described in Electronic Supplementary Material —Supplementary methods Table S1).

Serum cytokine profiling

Serum samples were collected from 43 ND and 46 FMF patients and samples were stored at -80°C until solid phase enzyme-immune assay. IL-10 and IL-1β concentration in serum samples was determined by conventional ELISA using human IL-10 and IL-1β Ready-SET-Go test kit (eBioscience), with a detection limit of 2 pg/ml, according to the manufacturer’s recommendations.

Monocytes isolation and activation

Heparinized peripheral blood samples were obtained from 15 (9 male, 6 female) out of a randomly selected 46 FMF patients, and from 7 out of 43 sex- and age-matched (3 male and 4 female) ND. Peripheral blood mononuclear cells (PBMC) were separated from heparinized whole blood by Ficoll-Hypaque (histopaque) (Sigma Chemical Co., St. Louis, MO) density gradient centrifugation. Peripheral blood monocytes were isolated by cell adherence of PBMC to 25 cm² plastic flasks during 45 min incubation at 37°C in an atmosphere containing 6% CO₂ [19]. Monocytes (95% CD14 positive cells) were then washed three times with endotoxin-free PBS and cultured at 5 ×10⁶ cells/ml density in RPMI-1640 containing 10% FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin and 100 μg/ml streptomycin. Either of 2.5 μg/ml peptidoglycan (PGN) from *E. coli* 0111:B4 (an NOD1 and NOD2 ligand) and synthetic Pam3CSK4 bacterial lipoprotein (an TLR2-TLR1 ligand) or 5 μg/ml muramyl dipeptide (MDP, an NOD2 ligand) were included in the culture media from day 0 to day 3. All reagents were purchased from InvivoGen (San Diego, USA). To assess the versatility of monocyte activation, 5 ×10⁵ cells in 1 ml of complete RPMI-1640 medium were incubated with recombinant IL-4 (10 ng/ml, eBioscience) or 100 ng/ml LPS from *E. coli* 026:B6 (Sigma Chemical Co., St. Louis, MO) from day 0 to day 3. One day 3, the cells washed and fresh medium added or stimulated with opposite pro-inflammatory (LPS) or anti-inflammatory (IL-4) molecules for an additional 3 days [19]. Cell-free supernatants were decanted at days 3 and 6, then stored at -80°C and IL-10 and IL-1β concentration in samples determined by ELISA.

Endotoxin tolerance

Endotoxin tolerance was monitored by measuring the production of IL-1β, IL-10, FN-γ and TNF-α by monocytes in response to LPS after preincubation in the presence or absence of two doses of LPS [9]. Monocytes at 5 ×10⁶ cells/ml density were cultured for 18 h in the presence or absence of 100 ng/ml LPS or either with 2 μg/ml colchicine or 2.5 μg/ml PGN or 5 μg/ml MDP or 10 ng/ml recombinant IL-4. After the first 18 h of culture, samples were washed three times with endotoxin-free PBS, and cultured for an additional 4 h in the presence of 1 μg/ml LPS. At the end of the culture period, cell-free supernatants were harvested, and endotoxin tolerance assayed by...
conventional ELISA determination of human IL-1β, IL-10, IFN-γ and TNF-α using Ready-SET-Go test kit (eBioscience) according to the manufacturer’s recommendations.

Statistics

The method of dispersion analysis with parametric and non-parametric procedures was used in this study. Results of independent experiments were used to calculate mean values ± SD, and differences were defined as statistically significant by Student’s t-test (P<0.05), paired t-test (P<0.01), Wilcoxon-Mann-Whitney, and Welch’s test (PW<0.05) at P ≤ 0.05.

Results

IL-10 production in FMF patients

IL-10 and IL-1β concentration in serum samples was measured in 43 ND and 46 FMF patients. The serum IL-10 was detectable only in 7 (16.3%) ND and in only 2 (4.2%, P<0.04 compared with the ND) FMF patients (described in Electronic Supplementary Material—Supplementary results, Figure S1). We found that the serum IL-1β is not detectable either in ND or in FMF.

Next, we studied how cellular production of IL-10 is different between ND and FMF patients. Previously it had been shown that circulating CD36+ peripheral blood cells constitutively produce IL-10 in healthy individuals and play a potential role in homeostatic innate immune suppression [21]. In PBMC isolated from ND and FMF patients, the number of CD36+ cells intracellularly synthesizing IL-10 was determined by flow cytometry (described in Electronic Supplementary Material—Supplementary results, Figure S2). The percentage of CD36+ monocytes (Figure 1A) was found to be significantly higher than CD36+ lymphocytes both in FMF and ND (P<0.0001 and PW<0.0008 respectively). Interestingly, the percentage of CD36+ monocytes was found to be significantly higher (P<0.05) in FMF patients compared with ND (Figure 1A). In contrast to this, the number of CD36+ lymphocytes is higher in ND (9.7 ± 3.7%) compared with FMF patients (6.9 ± 0.8%), however, this difference did not reach a statistically significant level (the variances were found to be higher in ND, P<0.0003).

Figure 1: Flow cytometry analysis of CD36+ monocytes and lymphocytes (A) and CD36+ cells, intracellularly synthesizing IL-10 (B) in ND and FMF patients. PBMC were surface stained by anti-CD36, CD3 or CD14 and fixed and permeabilized cells incubated with anti-human IL-10 or matched isotype control and subjected to FACS analysis. All data represent means ± SEM (error bars) and are significantly different comparing FMF with ND (* PW<0.05) or comparing monocytes with lymphocytes (# PW<0.001).

Results of independent experiments were used to calculate mean values ± SD, and differences were defined as statistically significant by Student’s t-test (P<0.05), paired t-test (P<0.01), Wilcoxon-Mann-Whitney, and Welch’s test (PW<0.05) at P ≤ 0.05.

Results of independent experiments were used to calculate mean values ± SD, and differences were defined as statistically significant by Student’s t-test (P<0.05), paired t-test (P<0.01), Wilcoxon-Mann-Whitney, and Welch’s test (PW<0.05) at P ≤ 0.05.

Results of independent experiments were used to calculate mean values ± SD, and differences were defined as statistically significant by Student’s t-test (P<0.05), paired t-test (P<0.01), Wilcoxon-Mann-Whitney, and Welch’s test (PW<0.05) at P ≤ 0.05.

Results of independent experiments were used to calculate mean values ± SD, and differences were defined as statistically significant by Student’s t-test (P<0.05), paired t-test (P<0.01), Wilcoxon-Mann-Whitney, and Welch’s test (PW<0.05) at P ≤ 0.05.

Results of independent experiments were used to calculate mean values ± SD, and differences were defined as statistically significant by Student’s t-test (P<0.05), paired t-test (P<0.01), Wilcoxon-Mann-Whitney, and Welch’s test (PW<0.05) at P ≤ 0.05.

Results of independent experiments were used to calculate mean values ± SD, and differences were defined as statistically significant by Student’s t-test (P<0.05), paired t-test (P<0.01), Wilcoxon-Mann-Whitney, and Welch’s test (PW<0.05) at P ≤ 0.05.

Production of IL-10 by monocytes and monocyte-derived dendritic cells in FMF patients

Furthermore, we studied whether the production of IL-10 by isolated monocytes and monocyte-derived dendritic cells is different between ND and FMF patients when the cells are stimulated with pro-inflammatory and anti-inflammatory molecules. LPS, colchicine and IL-4 were included in the culture medium of isolated monocytes for 3 days and IL-10 production by monocytes was assayed by ELISA (Figure 2A). We found that IL-10 production by untreated as well as IL-4-treated monocytes in ND does not significantly differ from the IL-10 production by the monocytes isolated from FMF patients. Surprisingly, we found that LPS-stimulation significantly enhanced IL-10 production (Pt<0.04) by monocytes isolated from ND in comparison with the LPS-stimulated monocytes isolated from FMF patients. As shown in Figure 2A, colchicine induced a weak increase of IL-10 production by monocytes in FMF, while in ND it induced a statistically significant increase in the production of IL-10 (P<0.02).
up-regulation of IL-10 production by mDCs generated from ND monocytes (Figure 2C). In contrast, mDCs from FMF patients produced an elevated level of IL-10 after treatment with both LPS and colchicine ($P_{p}<0.05$). Thus, we saw that, during FMF, both iDCs and mDCs produced a decreased level of anti-inflammatory cytokine IL-10. However, IL-10 production by DCS was restored after LPS stimulation or colchicine treatment which indicates that they induced tolerogenic DCS in FMF patients but not in ND.

To confirm this, we evaluated the expression of CD86, CD206, CD14 and Annexin V in unstimulated, LPS- and colchicine-treated DCS, generated from FMF patients and ND. The results suggested that the surface expression of CD86, which is the marker for pro-inflammatory activated DCS, is up-regulated in FMF patients and LPS stimulation, in contrast to colchicine, caused further enhancement of this activation pattern (described in Electronic Supplementary Material - Figure S3). In addition, neither any analyzed surface marker nor apoptosis induction were found in parallel with the pattern of IL-10 production by differentially activated DCS in FMF patients (discussed in Electronic Supplementary Material—Supplementary results). Therefore, we postulated that the cellular pro- and anti-inflammatory activation plasticity is altered in FMF, and furthermore we studied the versatility of monocytes activation type switching.

**Versatility of monocyte pro-inflammatory activation switching in FMF patients**

Next, we studied how reduced LPS-induced IL-10 production by monocytes, isolated from FMF patients, is associated with the impaired versatility of monocyte activation switching. Monocytes were incubated with or without recombinant IL-4 or LPS for 3 days and on day 3, cells were washed and a fresh medium was added or stimulated with opposite pro- or anti-inflammatory molecules for an additional 3 days. Monocyte CD206 mannose receptor surface expression was analyzed by flow cytometry and IL-10 production by monocytes assayed by ELISA.

We found that CD206 surface expression, as a marker of alternatively activated monocytes [19], in differentially activated monocytes during stimulation arrest or counter stimulation, did not significantly differ between ND and FMF patients (described in Electronic Supplementary Material - Figure S4), which may suggest that the activation of monocytes during FMF is not associated with the impaired versatility of cell activation switching. To confirm these results, we next studied the pattern of IL-10 production by differentially activated monocytes. We found that the pattern of IL-10 production by activated and, through future stimulation, arrested or counter-stimulated monocytes is quite different in ND and FMF patients. IL-10 production by untreated and future IL-4- and LPS-stimulated monocytes was significantly enhanced ($P_{t}<0.04$ and $P_{t}<0.02$, accordingly) in ND, compared with monocytes isolated from FMF patients (Figure 3A). IL-10 production by IL-4-pretreated and future counter-stimulated by LPS monocytes was significantly enhanced ($P_{t}<0.03$) in ND, compared with FMF patients (Figure 3B). IL-10 production by LPS-pretreated (but those not future stimulation-arrested or counter-stimulated by IL-4) monocytes were found to be enhanced in ND ($P_{t}<0.04$) compared to FMF (Figure 3C). Thus, in contrast to ND, monocytes from FMF patients failed to switch from a pro-inflammatory activated state to anti-inflammatory phenotype, which may cause the impaired resolution of inflammation.

![Figure 2](image-url)
Figure 3: Versatility of monocytes pro-inflammatory activation switching in FMF patients. IL-10 production by monocytes isolated from ND and FMF patients after stimulation by pro- and anti-inflammatory agents and after stimulation arrest or a further counter stimulation (A, B and C). Monocytes were unstimulated or stimulated by recombinant IL-4 or LPS for 3 days and on day 3, cells washed and fresh medium added or stimulated with opposite pro- or anti-inflammatory molecules for additional 3 days. The IL-10 production by monocytes was assayed by ELISA. All data represent means ± SEM (error bars) and are significantly different comparing FMF with ND (* P<0.05).

Influence of TLR-, NOD-agonsitcs and colchicine on monocyte IL-10 and IL-1β production in FMF patients

We studied whether the production of IL-10 and IL-1β by isolated monocytes is different between ND and FMF patients, when the cells are stimulated with NOD1 and NOD2 ligand PGN or TLR2-TLR1 ligand Pam3CSK4 or NOD2 ligand MDP for 3 days. We found that PGN, MDP and Pam3CSK4 significantly enhanced IL-10 production (P<0.04, Pr<0.05 and Pw<0.02, respectively) by monocytes isolated from ND in comparison with stimulated monocytes isolated from FMF patients (Figure 4A).
In contrast to IL-10, the production of IL-1β by monocytes stimulated with PGN or Pam3CSK4 or MDP did not show any significant differences between ND and FMF patients. MDP induced a weak increase of IL-1β production by monocytes in ND, compared with FMF (Figure 4B). Interestingly, colchicine induced a statistically significant increase of IL-1β production by monocytes in both FMF ($P_{<0.001}$) and ND ($P_{<0.02}$). However, the production of IL-1β by colchicine-treated monocytes in FMF was significantly higher compared to ND ($P_{<0.02}$).

Next, we study the production of IL-10 and IL-1β by PGN or Pam3CSK4 or MDP pre-stimulated monocytes for 3 days and counter-stimulated with recombinant IL-4 for an additional 3 days. As shown in Figure 4C, the IL-1β production by IL-4-induced PGN- and Pam3CSK4-pre-stimulated monocytes is significantly enhanced in FMF patients but not in ND ($P_{<0.02}$). In contrast, the IL-10 production by unstimulated monocytes and future stimulated by IL-4 is significantly enhanced in ND ($P_{<0.04}$) compared to FMF patients. The IL-10 production by IL-4-treated and Pam3CSK4- or MDP-pre-stimulated monocytes is significantly enhanced in FMF patients, compared with ND (Figure 4D). Thus, in contrast to ND, monocytes from FMF patients failed to switch from a pro-inflammatory activated state to an anti-inflammatory phenotype, and still produced more IL-1β. Colchicine treatment could not restore the increased production of IL-1β in FMF, and only a combination of IL-4 and NOD2 ligand MDP could partially restore anti-inflammatory cytokine IL-10 production and versatitity of monocyte activation type switching.

**Endotoxin tolerance induction by monocytes isolated from FMF patients**

We studied how impaired versatility of pro-inflammatory activated monocytes in FMF patients caused disruptions in endotoxin tolerance induction. Monocytes, isolated from ND and FMF patients, were pretreated with LPS for 18 h or other TLR- and NOD-agonists, colchicine or IL-4 and were washed and subjected to another dose of LPS for 4 h and the production of IL-1β, IL-10, TNF-α and IFN-γ was subsequently assayed by ELISA.

We found that during the first 18 h of incubation, the production of IL-1β by TLR- and NOD- agonists- as well as IL-4- stimulated monocytes in FMF patients (Figure 5), was significantly higher than in ND ($P_{<0.03}$ for PGN, $P_{<0.03}$ for MDP, $P_{<0.002}$ for LPS and $P_{<0.03}$ for IL-4 respectively). We observed that overnight LPS-treated monocytes, isolated from ND, develop tolerance to subsequent LPS challenges by declining the production of IL-1β (Figure 5B). In contrast, monocytes from FMF patients failed to induce LPS-homologous tolerance to the repeated action of LPS and produced equal amounts of IL-1β during repetitive LPS stimulation (Figure 5B). Colchicine pretreatment did not induce a hyporesponsive state of monocytes in FMF patients to LPS challenge. Similarly, PGN pretreatment failed to induce monocyte endotoxin cross tolerance after LPS re-stimulation in FMF patients (Figure 5A). However, in ND both PGN and colchicine pretreatment induced monocyte endotoxin tolerance by subsequent decreasing of IL-1β production by LPS re-stimulated monocytes ($P_{<0.04}$, Figure 5A). Interestingly, MDP was found to be unable to induce IL-1β production by monocytes both in FMF patients and ND and induce endotoxin tolerance in FMF patients as well (Figure 5). Similarly, IL-4 pretreatment caused endotoxin tolerance induction in FMF patients and ND monocytes to subsequent LPS restimulation, suggesting that monocytes alternative activation caused endotoxin-tolerance induction both in FMF and normal donors.

**Figure 5: Influence of TLR- and NOD-agonists, colchicine and IL-4 on monocyte IL-1β production and endotoxin tolerance induction in FMF patients (A) and ND (B).** Monocytes were pretreated with 100 ng/ml LPS or either with 2 μg/ml colchicine or 2.5 μg/ml PGN or 5 μg/ml MDP or 10 ng/ml recombinant IL-4 for 18 h, washed with LPS-free PBS and cultured for an additional 4 h in the presence of 1 μg/ml LPS. The production of IL-1β monocytes was subsequently assayed by ELISA. All data represent means ± SEM (error bars) and are significantly different comparing FMF with ND ($^*P_{<0.05}$ or $^*P_{<0.002}$) or comparing pretreatment with LPS re-stimulation pairs ($^#P_{<0.05}$).

Next, we studied how monocytes, isolated from FMF patients and ND, responded to repeated action of LPS challenge by changing the production of IL-10 (Figure 6A). First, we compared the production of IL-10 by untreated and LPS-treated monocytes between ND and FMF patients at 18 h incubation. As it is shown in Figure 6A, IL-10 production by untreated monocytes in ND was significantly higher than in FMF patients ($P_{<0.01}$). Surprisingly, LPS-induced production of IL-10 by monocytes at 18 h incubation were found to be significantly higher in FMF patients ($P_{<0.04}$ and $P_{<0.05}$, respectively) compared with ND. However, LPS-pretreated monocytes from FMF patients failed to produce a detectable level of IL-10 after 4 h LPS re-stimulation, while monocytes isolated from ND produced a detectable level of IL-10 during repeated action of LPS (comparing NT +LPS with the LPS+LPS pairs, Figure 6A). We do not find statistically significant differences in IL-10 production by TLR4 and NOD2 agonists (LPS, PGN and MDP) - as well as IL-4- or colchicine- pretreated and LPS re-stimulated monocytes in FMF patients (data not shown).
LPS significantly increased overnight production of TNF-α in FMF patients (P<0.05) compared with ND (comparing NT+NT with NT+LPS parries accordingly, Figure 6B). We observed that monocytes, isolated from FMF patients and ND develop homologous tolerance to LPS challenge by declining production of TNF-α after overnight LPS treatment (comparing NT+LPS with the LPS+LPS parries, Figure 6B). However, 4 h LPS re-stimulation of monocytes, isolated from FMF patients caused only 3.0 fold reduction of TNF-α production, while in ND it caused up to 25.0 fold reduction of TNF-α production (comparing NT+LPS with the LPS+LPS parries, Figure 6C). Thus, the observed differences in monocyte cytokine production in response to repeated action of LPS could suggest that endotoxin tolerance induction is impaired in FMF patients due to down-regulation of IL-10 and IFN-γ production and up-regulation of IL-1β and TNF-α production by monocytes.

Figure 6: LPS homologous tolerance induction in patients with FMF and ND. Monocytes were pretreated with 100 ng/ml LPS for 18 h, washed with LPS-free PBS and cultured for an additional 4 h in the presence of 1 μg/ml LPS. The production of TNF-α (A), IL-10 (B) and IFN-γ (C) was subsequently assayed by ELISA. All data represent means ± SEM (error bars) and are significantly different comparing FMF with ND (*P<0.05, **P<0.002) or comparing pretreatment with LPS re-stimulation pairs (#P<0.05).
IL-10 secretion rate in CD36⁺ lymphocytes is higher than in CD36⁻ monocytes, as it was originally shown using ELISPOT techniques [21].

Innate immune compartment is another potentially important source of IL-10 given that various cell types can be divided into distinct subpopulations based on IL-10 expression patterns. Most notably, monocytes, alternatively activated and type I macrophages, and DCs, including myeloid (but not plasmacytoid) DCs provide sources of IL-10 from the myeloid lineage [25,26]. Here we focused mainly on studying the production of IL-10 by monocytes and DCs in FMF patients, but not lymphocytes, based on the two reasons. First, it is well known that the MEFV-encoded pyrin is selectively expressed in monocytes and neutrophils, and participates in inflammatory activation, apoptosis, and secretion of IL-1β [5,6,27]. Second, the immunoregulatory function of myeloid- but not T-cell-derived IL-10 in many animal and human studies is well characterized during sepsis and the anti-inflammatory response to LPS [28-30]. The importance of IL-10 in such responses is particularly evident in the gut, where despite the large burden of commensal bacteria, there is a delicate balance of pro-inflammatory and anti-inflammatory cytokines which act in concert to maintain a steady state [31-33]. Here we demonstrated that the monocyte IL-10 production induced by LPS and other TLR and NLR-agonists, including PGN, Pam3CSK4 and MDP is declined in FMF patients. Furthermore, the production of IL-10 was found to be diminished in both types of iDCs and mDCs, generated from FMF patients. Moreover, we found that DCs surface expression of co-stimulatory molecule CD86 is up-regulated in FMF, further suggesting that IL-10 antagonizes the expression of CD86 [34].

Do these data indicate that FMF is an IL-10-deficiency associated disease? Two data sets, obtained in this study suggested against this notion. First, the LPS-induced production of IL-10 by monocytes at 18 h incubation was found to be significantly higher in FMF patients, and second, the IL-10 production by IL-4-treated and Pam3CSK4- or MDP-stimulated monocytes was significantly enhanced in FMF patients, compared with ND. Other studies also demonstrated that the serum IL-10 levels were not different in FMF patients with attacks and attack-free, and healthy controls [35]. In contrast, a significantly higher level of IL-10 in attack vs. control was confirmed by two independent groups of investigators [36,37]. In addition, a potentially important consideration in the analysis of human IL-10 expression is the well-described inter-individual variation in IL-10 production which is associated with single nucleotide polymorphisms in the IL-10 promoter [38,39]. However, there is no data available for FMF studies. These data may suggest that the diminished IL-10 production by monocytes is indirectly associated with mutation in MEFV and rather reflected the regulatory role of pyrin in IL-1β processing and release. Indeed, pyrin levels directly correlate with IL-1β processing in monocytes and macrophages in the context of endotoxin-induced activation and pyrin protein levels are down-regulated in monocytes as they mature into monocyte-derived macrophages [40]. These changes occur at the same time that IL-1β processing and release is also down-regulated. These data are in agreement with our observation that spontaneous and inducible production of IL-1β by monocytes at 18 h incubation were found to be significantly higher in FMF patients, while there is no significant differences observed between ND and FMF patients during the 3-day incubation period. Thus, pyrin may function to augment caspase-1 activation and IL-1β processing events in the early stages of innate immune response settings including IL-10 dependent regulatory pathways against some pathogen (damage)-associated molecular patterns.

TLR- or NLR-mediated recognition of pathogen (damage)- associated molecular patterns initiates MyD88- or inflammasome-dependent signaling pathways correspondingly that orchestrates the production of pro-inflammatory cytokines by innate immune cells [41]. Even though these cytokines are crucial for host defense, excess pro-inflammatory cytokines give rise to systemic metabolic and hemodynamic disturbances that are harmful to the host. To avert these deleterious effects, IL-10 is also produced by stimulated monocytes or DCs [42], leading to Stat3 phosphorylation that has been correlated with the dampening of inflammatory response [43]. Upon encounter of monocytes or immature DCs with microbial, pro-inflammatory, or T cell-derived stimuli, characteristic phenotypic and functional changes are induced and cells recruit to sites of inflammation, a process referred to as pro-inflammatory (classical) activation of monocytes or maturation of DCs [44]. Mature DCs and activated monocytes exhibit reduced phagocytic activity and increased expression of co-stimulatory molecules, secreting large amounts of immunostimulatory cytokines [45]. However, some types of DCs or the newly activated monocytes in the gut and maintain immunosuppression in vivo. Tolerogenic DCs, induced by various anti-inflammatory and immunosuppressive agents, are maturation-resistant or alternatively activated DCs that express low ratio of co-stimulatory surface molecules and are able to synthesize anti-inflammatory cytokines, including IL-10 and TGF-β1 [46]. The fact that LPS treatment of mature DCs in FMF patients, in contrast to ND, leads to the increased production of IL-10 indicated the high turnover potential for switching to the anti-inflammatory phenotype of mature DCs during the encounter to pro-inflammatory factors and may be associated with the characteristic self-limited inflammatory attack observed in FMF. In addition we observed that IL-10 production by DCs was restored after colchicine treatment which may indicate that tolerogenic DCs are induced in FMF patients but not in ND. However, we found that the surface expression of co-stimulatory molecules are not paralleled with the pattern of IL-10 production by differentially activated DCs and monocytes in FMF patients, further suggesting that cellular pro-inflammatory activation plasticity is altered in FMF and reduced the IL-10 production associated with impaired setting of feedback inhibition of the pro-inflammatory response. It has been shown that the increase of IL-10 and TGF-β1 in attack may play a compensatory and regulatory role with respect to proinflammatory cytokines and may contribute to the self-resolving nature of inflammatory attacks in FMF [36].

To address this hypothesis, we investigate here the impaired versatility of monocytes, obtained from FMF patients, by measuring the production of IL-10 and IL-1β after stimulation by pro- and anti-inflammatory agents, and after stimulation arrest or a further counter stimulation. In contrast to ND, monocytes from FMF patients failed to switch from a pro-inflammatory activated state to an anti-inflammatory phenotype and still produced more pro-inflammatory IL-1β and less IL-10, which may cause the impaired resolution of inflammation. Furthermore, this conclusion was confirmed by the observed impaired endotoxin tolerance and cross-tolerance induction in monocytes, obtained from FMF patients by measuring the production of IL-1β, IL-10, TNF-α and IFN-γ after pre-stimulation by NLR- or TLR-ligands and after re-stimulation with LPS. Again, in contrast to ND, monocytes from FMF patients failed to switch from a pro-inflammatory activated state to an anti-inflammatory phenotype in response to repeated action of LPS, suggesting that endotoxin tolerance induction is impaired in FMF patients due to down-regulation of IL-10 and IFN-γ production by monocytes. These data
agree with the findings that IL-10 not only antagonizes the expression of co-stimulatory molecules and the pro-inflammatory cytokines IL-1β, IL-6, IL-8, and TNF-α, but also induces endotoxin tolerance production of IFN-γ [16,17,47].

Previously, we had found that colchicine is able to restore impaired LPS-homologous tolerance induction (assayed by flow cytometry of monocytes, intracellularly synthesizing TNF-α), in FMF patients upon increasing the intracellular IL-4 synthesis and monocyte “alternative” activation [9]. In this study we observed that colchicine increased both IL-10 and IL-1β production by monocytes obtained from ND and FMF patients. However, the production of IL-1β by colchicine-treated monocytes in FMF was found to be higher and, vice versa, the production of IL-10 was significantly lower comparing with ND. In addition, colchicine failed to induce tolerance of monocytes to the repeated action of LPS (assayed by ELISA of monocytes IL-1β and IL-10 production), suggesting that colchicine alone is not sufficient to restore impaired endotoxin tolerance during FMF. It is known that taxol and colchicine, two drugs that affect microtubule structure and function, increase LPS-induced IL-1β by an increase in the production of the pro- IL-1β precursor molecule [48]. Suppressing this, we found that colchicine could not restore the polarization of IL-1β and IL-10 production in FMF, and only a combination of IL-4 and noninflammasome NLR (NOD2)- ligand MDP could partially restore the anti-inflammatory cytokine production and versatility of monocyte activation switching. Moreover, MDP was found to be unable to induce IL-1β production by monocytes and IL-4, MDP-pretreatment were able to induce endotoxin tolerance in FMF patients. These results are in agreement with recent studies illustrating a role for chronic stimulation of NOD2 in mediating tolerance to bacterial products [49]. It is possible that pyrin represents a positive regulator in the context of LPS but a negative regulator of inflammation in the context of other types of intracellular infectious challenges, including MDP. Our future studies are directed at this question to understand further details of the regulatory role of noninflammasome NLR NOD2 in mechanisms required for inflammation resolution, which may underpin the development of new therapeutic approaches that can resolve FMF inflammation in directed and controlled ways. The finding that (NOD2)- ligand MDP differentially affects the monocyte activation program shift and endotoxin tolerance induction may have important implications for the treatment of FMF.

In conclusion, reduced IL-10 production by monocytes and dendritic cells is associated with the impaired setting of feedback inhibition of the pro-inflammatory response and caused impaired resolution of inflammation and endotoxin tolerance induction in response to repeated action of endotoxine. The IL-10 production by monocytes and dendritic cells and endotoxin tolerance induction are restored by NOD2- ligand MDP and partially by colchicine which may underpin the development of new therapeutic approaches that can resolve FMF inflammation in directed and controlled ways.

Acknowledgements

The International Science and Technology Center (ISTC Grant A-1580), and “Armenicum+” CJSC, Yerevan, Armenia, supported this study. We thank Professor Tamara Sarkisian PhD, Dr.Sc, Center of Medical Genetics and Primary Health, for her critical reading of the manuscript, Dr. Nazareth Seferian MD, for language revision and Honorary Doctor of NAS RA Levon A. Gevorkyan for his support of this study in the Armenian Research Centre.
21. Barrett L, Dai C, Gamberg J, Gallant M, Grant M (2007) Circulating CD14-CD36+ peripheral blood mononuclear cells constitutively produce interleukin-10. J Leukoc Biol 82: 133-141.

22. Berg DJ, Kühn R, Rajewsky K, Müller W, Menon S, et al. (1995) Denning TL, Wang YC, Patel SR, Williams IR, Pulendran B (2007) Regulatory T cells in the control of host-microorganism interactions (*). Annu Rev Immunol 27: 551-589.

23. Belkaid Y, Tarbell K (2009) Interleukin-10 production in response to MyD88- and TRIF-dependent TLR signals, and TLR-independent signals. J Immunol 177: 7551-7558.

24. Boonstra A, Rijsbah R, Holman M, Marques R, Asselin-Paturel C, et al. (2006) Macrophages and myeloid dendritic cells, but not plasmacytoid dendritic cells, produce IL-10 in response to MyD88- and TRIF-dependent TLR signals, and TLR-independent signals. J Immunol 177: 7551-7558.

25. Chae JJ, Cho YH, Lee GS, Cheng J, Liu PP, et al. (2011) Gain-of-function Pyrin mutations induce NLRP3 protein-independent interleukin-1β activation and severe autoinflammation in mice. Immunity 34: 755-768.

26. Mosser DM, Edwards JP (2008) Exploring the full spectrum of macrophage activation. Nat Rev Immunol 8: 958-969.

27. Ciaj J, Cho YH, Lee GS, Cheng J, Liu PP, et al. (2011) Gain-of-function Pyrin mutations induce NLRP3 protein-independent interleukin-1β activation and severe autoinflammation in mice. Immunity 34: 755-768.

28. Kwan WH, Boix C, Gougelet N, Fridman WH, Mueller CG (2007) Interleukin-10 derived from macrophages and/or neutrophils regulates the inflammatory response to LPS but not the response to CpG DNA. Eur J Immunol 36: 3248-3255.

29. Auffray C, Sieweke MH, Geissmann F (2009) Blood monocytes: development, heterogeneity, and relationship with dendritic cells. Annu Rev Immunol 27: 669-692.

30. Berci AP, Trinchieri G (2004) Interleukin-10 in viral diseases and cancer: exiting the labyrinth? Immunol Rev 202: 223-236.

31. Chaudhuri S, Duncan MD, Hart JM, Gavrillon MA, Wewers MD (2007) Pyrin levels in human monocytes and monocyte-derived macrophages regulate IL-1β processing and release. J Immunol 179: 1274-1281.

32. Beutler B (2009) Microbe sensing, positive feedback loops, and the pathogenesis of inflammatory diseases. Immunol Rev 227: 248-263.

33. Manukyan GP, Ghazaryan KA, Ktsoyan ZhA, Tatyan MV, Khachatryan ZA, et al. (2008) Cytokine profile of Armenian patients with Familial Mediterranean fever. Clin Exp Immunol 153: 256-253.

34. Lazaras R, Vercelli D, Palmer LJ, Klimecki WJ, Silverman EK, et al. (2002) Single nucleotide polymorphisms in innate immunity genes: abundant variation and potential role in complex human disease. Immunol Rev 190: 9-25.

35. Vicari AP, Trinchieri G (2004) Interleukin-10 in viral diseases and cancer: exiting the labyrinth? Immunol Rev 202: 223-236.

36. Seshadri S, Duncan MD, Hart JM, Gavrillon MA, Wewers MD (2007) Pyrin levels in human monocytes and monocyte-derived macrophages regulate IL-1β processing and release. J Immunol 179: 1274-1281.

37. Beutler B (2009) Microbe sensing, positive feedback loops, and the pathogenesis of inflammatory diseases. Immunol Rev 227: 248-263.

38. Samarasinghe R, Tailor P, Tamura T, Kiskir T, Akira S, et al. (2006) Induction of an anti-inflammatory cytokine, IL-10, in dendritic cells after toll-like receptor signaling. J Interferon Cytokine Res 26: 893-900.

39. Melillo JA, Song L, Bhagat G, Blazquez AB, Plumlee CR, et al. (2010) Dendritic cell (DC)-specific targeting reveals Stat3 as a negative regulator of DC function. J Immunol 184: 2638-2645.

40. Ueno H, Kleeckevi E, Morita R, Aspod C, Cao T, et al. (2007) Dendritic cell subsets in health and disease. Immunol Rev 219: 118-142.

41. Fiorentino DF, Zlotnik A, Mosmann TR, Howard M, O’Garra A (1991) Interleukin-10, a new member of the cytokine family. Annu Rev Immunol 9: 459-482.

42. Morelli AE1, Thomson AW (2007) Tolerogenic dendritic cells and the quest for transplant tolerance. Nat Rev Immunol 7: 610-621.

43. Marchant A, Bruyns C, Vandenebree P, Ducarme M, Gérard C, et al. (1994) Interleukin-10 controls interferon-gamma and tumor necrosis factor production during experimental endotoxemia. Eur J Immunol 24: 1167-1171.

44. Denning TL, Wang YC, Patel SR, Williams IR, Pulendran B (2007) Lamina propria macrophages and dendritic cells differentially regulate interleukin 17-producing T cell responses. Nat Immunol 8: 1086-1094.

45. Murai M, Turovskaya O, Kim G, Madan R, Karp CL, et al. (2009) Interleukin 10 acts on regulatory T cells to maintain expression of the transcription factor Foxp3 and suppressive function in mice with colitis. Nat Immunol 10: 1178-1184.