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Lack of HLH in FMF

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Abstract

Background Macrophage activation syndrome (MAS) is a severe complication of systemic juvenile idiopathic arthritis (sJIA), driven by excessive activation of T cells and macrophages, resulting in a cytokine storm. IFN-γ and IL-18 play crucial roles, with monocyte and macrophage hyperresponsiveness to IFN-γ amplifying MAS-related inflammation. Familial Mediterranean Fever (FMF), an autosomal recessive disease, is characterized by recurrent fever episodes due to MEFV gene mutations. Despite intense inflammation in FMF, MAS is rare. This study aimed to compare in vitro responsiveness of peripheral blood mononuclear cells (PBMCs) to IFN-γ between sJIA/MAS and FMF patients.

Methods Five sJIA/MAS and five FMF patients were included. PBMCs were stimulated in vitro with IFN-γ for 45 min. Levels of IFN-γ-induced chemokines CXCL9, CXCL10, and IL-18 in supernatants were measured using cytometric bead arrays before and after stimulation.

Results PBMCs from MAS patients produced higher baseline CXCL9 levels compared to FMF patients in a flare, with differences increasing post-IFN-γ stimulation. IFN-γ stimulation also upregulated IL-18 production in MAS patients but not in FMF patients.

Conclusion Enhanced responsiveness to IFN-γ distinguishes sJIA/MAS from FMF patients, which may explain the lower occurrence of MAS in FMF.

Keywords CXCL-9, CXCL-10, Familial Mediterranean fever, IL-18, Macrophage activation syndrome

Background

Macrophage activation syndrome (MAS) is a severe complication of childhood systemic inflammatory disorders, characterized by fever, hepatosplenomegaly, lymphadenopathy, profound cytopenia, impaired liver function, intravascular coagulation, and central nervous system dysfunction [1].

Familial Mediterranean Fever (FMF) is the most common recurrent fever syndrome. It occurs as a result of autosomal recessive mutations in the MEFV (Mediterranean fever) gene encoding the pyrin protein. Recurrent or self-limiting fever is characterized by serositis and arthritis attacks. Excessive cytokine release, primarily interleukin (IL)-1, secondary to defects in the innate

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immune system, is known to play a role in the pathogenesis of many autoinflammatory diseases, including FMF [2].

Although systemic juvenile idiopathic arthritis (sJIA) is classified under JIA, it is currently thought by many authors to have a predominantly autoinflammatory basis [3]. MAS has been reported in hyperimmunoglobulin D syndrome, TNF **(tumor necrosis factor)** receptor-associated periodic syndrome, and cryopyrin-associated periodic syndromes [4]. However, despite the intense inflammatory response and increased IL-1 levels during the attacks in FMF patients, the risk for MAS in these patients is remarkably low.

Interferon (IFN)-ɣ has emerged as the pivotal cytokine in MAS. A study of longitudinal cytokine changes in **sJIA** patients with MAS demonstrated that IFN-γ itself and IFN-γ-induced chemokines increased markedly with the emergence of clinical features of MAS and returned to normal ranges after MAS resolution [5]. Hemophagocytic macrophages in the bone marrow also showed a proinflammatory expression profile with a strong IFN-induced signature, further supporting the concept that inappropriately increased IFN signaling is the driving force behind macrophage expansion. Additionally, increased responsiveness of monocytes and macrophages to IFN-γ in **sJIA** appears to be another important factor that further amplifies macrophage activation [6, 7].

IL-18 has also emerged as a potential critical link between **sJIA** and MAS. IL-18 is produced by myeloid and epithelial cells and is best known for its ability to amplify, in concert with IL-12, IL-15, or IL-2, lymphocyte production of IFN-γ. Strikingly high serum levels of IL-18 have been observed in patients with sJIA, in sharp contrast to only moderately elevated levels of IL-18 seen in other rheumatic diseases which lack a strong association with MAS. Based on these observations, the current working model is that the combination of IL-18 driven augmented production of IFN-γ by CD8 + cytolytic effector T cells combined with hyper-responsiveness of monocytes and macrophages to IFN-γ may be the key factors responsible for the strong association between MAS and sJIA [8].

In this study, we aimed to assess the reasons for the lack of MAS-like cytokine storm in FMF patients, by comparing serum IL-18 levels and the in vitro responsiveness of peripheral blood mononuclear cells (PBMCs) to IFN-ɣ to patients with sJIA/MAS. Specifically, we sought to identify potential immunological markers or pathways that could explain the differential predisposition to MAS in these two patient groups. By elucidating these mechanisms, our study aims to contribute to a deeper understanding of MAS pathophysiology and to inform potential therapeutic strategies for managing this severe condition.

Methods

Patients and samples

Five sJIA associated MAS patients, 5 FMF patients, and 4 healthy controls (HCs) were enrolled. The diagnosis of MAS was based on the 2016 EULAR/ACR/PRINTO classification criteria [9]. FMF patients met the 2019 Eurofever/PRINTO classification criteria [10]. PBMCs were obtained during both the active and inactive phases of the MAS and FMF.

Venous blood samples were obtained from patients and immediately transferred to EDTA tubes and processed within 30–45 min. Mononuclear cells were separated by Ficoll-based density gradient method and aliquoted as **PBMCs**, and stored at – 80 °C.

Demographic, clinical, and laboratory data were collected retrospectively via medical chart review.

Laboratory tests and cell culture experiments

Patient PBMCs stored at -80 °C were thawed and centrifuged at 400 g for 5 min, and the pellets were resuspended in culture media (RPMI 1640; **10%** Fetal Bovine Serum, **1%** l-glutamine, **1%** penicillin-streptomycin). Cells were seeded into 48-well plates at a density of 3 × 10^4 cells per well and incubated at 37 °C with 5% CO2 for 30 min. For the FMF, MAS, and **HCs**, cells were divided into two aliquots; one was stimulated (with IFN-γ) and the other used as a control sample. **IFN-**γ (1.2 mg/mL) (Biolegend, Cat No: 570206) was added to the relevant wells at a concentration of 50 ng/ml and cells were stimulated for 45 min at 37 °C with 5% CO2. After stimulation, cells were fixed with 2% paraformaldehyde for 10 min at room temperature. Following fixation, cultures were centrifuged at 400 g for 10 min, and supernatants were collected for planned ELISA analysis and stored at -80 °C.

Quantifications of chemokine (C-X-C) motif ligand (CXCL) 9, CXCL10, and IL-18 levels in the supernatants were studied by the cytometric bead-based multiplex assay panel according to the manufacturer's instructions (LEGENDplex HU Proinflammatory Chemokine Panel 1 (13-plex); catalogue number 740985, Biolegend) and analyzed by Novocyte 3005 flow cytometer.

Ethical approval

The study was approved by the local ethical committee of our university (GO20/1154) and all patients/parents provided informed consent.

Statistical analysis

Statistical analysis and graphical representations were performed using the Prism 10 (GraphPad Software, San Diego, CA, USA) and Statistical Package for Social Sciences' (SPSS) 23.0. The normality of the data was tested using the Shapiro-Wilk test. Data were summarized as

Table 1 Comparison of the chemokine levels in the culture supernatants of PBMCs of patients during active period before and after stimulation with IFNɣ

	CXCL9			CXCL10			IL-18		
	Before IFNγ	**After IFNγ**	***p***	**Before IFNγ**	**After IFNγ**	***p***	**Before IFNγ**	**After IFNγ**	***p***
MAS flare	46.3 (0-152.9)	58.9 (0-282.7)	0.06	2.10(0-40.3)	28.8 (0–54)	0.28	0 (0-73.5)	20.1 (0-153)	0.1
FMF flare	0 (0-13.8)	12.2(0-42.9)	0.10	0 (0–20)	0 (0-42.5)	1	73.5 (0-100)	46.8 (0-126.5)	1
HC	0 (0-12.7)	0 (0-28.3)	0.31	0 (0-10.4)	8.4 (0–18)	0.18	73.4 (0-153)	60.5 (0-231)	1

MAS, Macrophage activation syndrome; FMF, Familial Mediterranean Fever, HC, healthy control; IFNγ, interferon-gamma; CXCL, chemokine (C-X-C) motif ligand

$p < 0.05$, statistically significant

Table 2 CXCL9, CXCL10, and IL-18 levels in PBMC Culture supernatants from MAS and FMF patients during active disease

	FMF	**MAS**	***p***
CXCL9 (Before IFNγ,)	0.0 (0-13.8)	46.3 (0-152.9)	**0.03***
CXCL9 (After IFNγ)	12.20 (0-42.9)	58.9 (0-282.7)	0.11
CXCL10 (Before IFNγ,)	0.0 (0–20)	2.10 (0-40.3)	0.73
CXCL10 (After IFNγ)	0.0 (0-42.5)	28.8 (0–54)	0.31
IL-18 (Before IFNγ,)	73.5 (0-100)	0.0 (0-73.5)	0.91
IL-18 (After IFNγ)	46.8 (0-126.5)	20.1 (0-153)	0.18

MAS, Macrophage activation syndrome; FMF, Familial Mediterranean Fever, IFNγ, interferon-gamma; CXCL, chemokine (C-X-C) motif ligand; PBMC: peripheral blood mononuclear cells

*$p < 0.05$, statistically significant

median and range. Parametric tests were used when the data were normally distributed, and non-parametric tests were used when the data were not normally distributed. When the assumptions of parametric tests were met, the Independent Samples t Test was used to compare the difference between the means of two independent groups. Pairwise comparisons were made using the Mann-Whitney test only when the Kruskal-Wallis test was significant after Bonferroni correction. A p-value of < 0.05 was considered statistically significant.

Results

The mean ages of MAS (2 girls, 3 boys) and FMF (**2** girls, 3 boys) patients during the study were 10.4 (± 6.5) and 5.6 (± 3.5) years, respectively. The median disease duration for MAS patients was 0.5 (0.16-6) years, and the mean disease duration for FMF patients was 6.8 (± 2.8) years. The mean age of the HCs (2 boys, 2 girls) was 12.87 (± 3.4) years.

The demographic and clinical features of the patients in each group are summarized in the Supplementary Tables 1 and 2.

For FMF patients during an active attack, the median levels of both CXCL9 and CXCL10 in PBMC culture supernatants (before IFN-γ stimulation) were 0 pg/ml, whereas the median IL-18 level was 73.5 pg/ml (Table 1). In contrast, unstimulated PBMC supernatants from MAS patients with new-onset disease showed higher levels of CXCL9, CXCL10, and IL-18 compared to those of FMF patients ($p = 0.03$ for CXCL9, $p = 0.73$ for CXCL10, and $p = 0.91$ for IL-18) (Table 2). Moreover, that baseline CXCL9 levels were lower in FMF patients compared to MAS patients both before and after stimulation (Fig. 1). Albeit impressive, the lack of statistical significance for the differences between MAS and FMF was attributed to the limited sample sizes.

The IL-18 levels in culture supernatants from FMF patients before and after IFN-γ stimulation were similar to those in healthy controls. In contrast, MAS patients exhibited a moderate increase in IL-18 levels after IFN-γ stimulation, although the difference did not reach statistical significance (Fig. 2). The levels of IL-18 in the PBMC supernatants from both FMF and MAS patients were similar to controls. This was in sharp contrast to the strikingly high levels of total serum IL-18 assessed in 3 patients with FMF and one patient with SJIA/MAS (Table 3).

Discussion

In this study, we aimed to investigate immunologic differences between FMF and sJIA that would explain the lack of MAS in FMF, although it is characterized by intense inflammation. Since abnormal activation of the IL-18-IFN-ɣ axis has been implicated in the pathogenesis of MAS, our study was focused on the responses of PBMCs to IFN-ɣ stimulation in vitro. The main finding in this study is that at baseline, in vitro cultured PBMCs from MAS patients were producing higher levels of CXCL9 compared to the samples from FMF patients obtained at the time of a flare. Since exaggerated IFN responsiveness may be one of the mechanisms driving MAS in SJIA, the absence of such hyper-responsiveness may be one of the reasons for the low risk of MAS in FMF.

MAS is a cytokine storm, where IFNγ and IL-18 often play the leading role [1, 11]. There is increasing evidence for the pivotal role of IFNγ in MAS, and the IFN-induced downstream **CXCL9**, CXCL-10, and CXCL-11 are emerging as MAS biomarkers [5, 12]. In patients with MAS, of the three IFNγ-induced chemokines, CXCL9 was found to have the strongest correlation with IFNγ

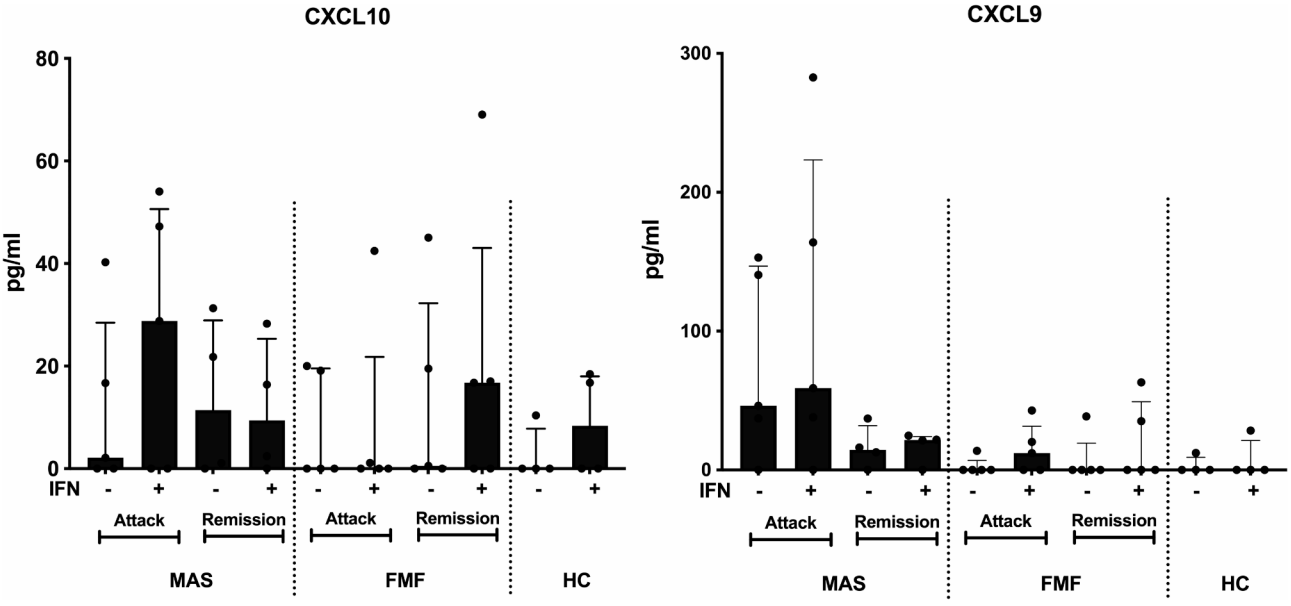

Fig. 1 CXCL10 and 9 responses of the PBMCs from FMF and MAS patients and healthy controls (HC), after stimulation with IFNγ both in attack and remission periods

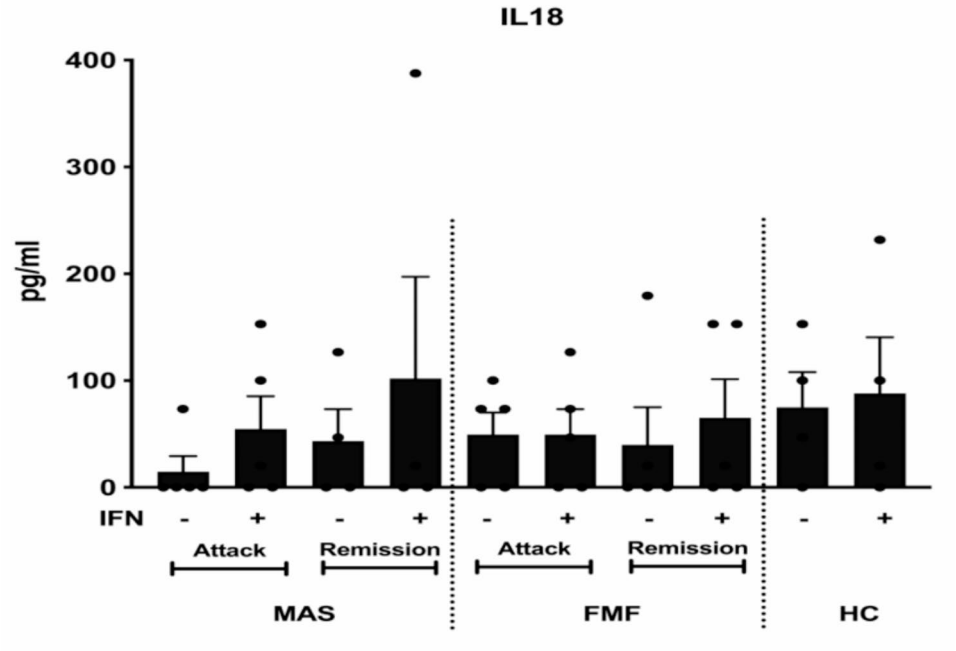

Fig. 2 IL-18 levels of the culture supernatants from PBMCs of FMF and MAS patients and healthy controls (HC), after stimulation with IFNγ both in attack and remission periods

Table 3 Serum Interleukin-18 levels (pg/mL) in selected MAS and FMF patients

FMF patient no 1	3752.03
FMF patient no 2	4519.69
FMF patient no 3	8561.28
MAS patient no 1	> 15,000
MAS patient no 2	1044.20
MAS patient no 3	> 15,000

tissue production and is now increasingly used in clinical practice as a measure of MAS activity [5]. In the present study, we found significantly lower CXCL9 levels (before IFNγ stimulation) in FMF patients when compared to MAS patients (Fig. 1). Therefore, we suggest that this may be one of the reasons for the lower MAS risk in FMF patients.

IL-18, which is an IL-1 family cytokine and previously known as IFNγ-inducing factor, is primarily synthesized by myeloid and epithelial cells and serves as a key stimulant for T cell IFNγ expression. IL-18 is produced by macrophages and some epithelial cells. There is increasing evidence suggesting that IL-18 and maybe tissue IL-18, play a crucial role in s-JIA and may contribute to the development of s-JIA-associated MAS [13–16]. In the MAS and FMF patient samples studied, the IL18 levels were not as high as in the serum samples (Table 3) which supports that the IL18 in MAS is mostly produced in the tissues [13]. On the other hand, although the possible effect of high levels of IL-18 in MAS patients has been shown, another autoinflammatory disease, PAPA (Pyogenic Arthritis, Pyoderma gangrenosum and Acne), which is associated with high levels of IL-18, almost never develops MAS. In a review published by Shimizu et al. [8], the absence of MAS development in PAPA patients has been attributed to the possible presence of excess **IL-18 binding protein (BP)** in the environment, which reduces IL-18 activity, or the distinct inflammatory milieu present in PAPA compared to sJIA. Additionally, there is a possibility that IL-18 originates from different cellular sources in both diseases [8]. This may be one of the factors in the absence of MAS in FMF as well, since IL18 is also elevated, albeit not as significantly as PAPA. In FMF IL-18 activation typically involves proteolytic cleavage, often through inflammasome-mediated activation of caspase 1, and is believed to be released from the cytosol via gasdermin D-mediated pyroptosis. The IL18 produced in FMF is mainly from the monocytes, and this may explain the relatively low levels in this PBMC cell culture.

Our study had some significant limitations. Firstly, the number of patients was low. The small sample size limits the generalizability of the diagnostic findings, especially considering the variability in individual responses to IFN-γ stimulation. Furthermore, the colchicine treatment in FMF patients and steroid/IL-1 blockade therapies in MAS patients may have influenced biomarker levels, potentially affecting diagnostic accuracy. Another important limitation was that we did not assess perforin activity.

On the other hand, our primary objective was original and this is the first study comparing the FMF and MAS patients.

Conclusion

The development of MAS involves upregulation of IFN-induced signaling in the immune cells as evidenced by highly increased levels of the IFN-induced chemokines CXCL9 and CXCL10, as well as the presence of high levels of IL-18 in the peripheral circulation. Based on the differences we demonstrated in our study, the lower risk for MAS development in FMF patients may be attributed to the lack of abnormally high responsiveness of immune cells to IFN-ɣ stimulation. However, it is conceivable that progression to MAS in the context of sJIA may not be restricted only to uncontrolled IL18 and IFNγ activation but might also involve other pathological mechanisms as well. We believe that this study will serve as a step for numerous future investigations and shed light on this topic.

Abbreviations

MAS	Macrophage activation syndrome
FMF	Familial Mediterranean Fever
MEFV	Mediterranean fever
IL	interleukin
sJIA	systemic juvenile idiopathic arthritis
PAPA	Pyogenic Arthritis, Pyoderma gangrenosum and Acne
IFN	Interferon
PBMCS	peripheral blood mononuclear cells
CXCL	chemokine (C-X-C) motif ligand
IL18BP	IL18 binding protein
ELE	Erysipelas-Like Erythema
ESR	erythrocyte sedimentation
CRP	C-reactive protein

Supplementary Information

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Supplementary Material 1

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Author contributions

Study conception and design: OB, ES, BA, NKT, YB, AAG, SO. Statistical analysis: OB, ES. Data acquisition: OB, ES, EAA, EC, SÖ, YB. Data analysis: OB, ES, BA, NKT. Data interpretation: All authors. All authors read, critically revised, and approved the final manuscript.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

The study was approved by the local ethical committee of our university (GO20/1154) and all patients/parents provided informed consent.

Competing interests

The authors declare no competing interests.

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