Short title: IFN-γ in sJIA and MAS

Cytokines in systemic juvenile idiopathic arthritis and macrophage activation syndrome: tipping the balance between interleukin-18 and interferon-gamma

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Abstract

Objectives. To study the role of interferon-gamma (IFN-γ) in the pathogenesis of systemic juvenile idiopathic arthritis (sJIA) and macrophage activation syndrome (MAS) by searching for an IFN-γ profile and assess its relation with other cytokines.

Methods. Patients with inactive (n=10) and active sJIA (n=10), MAS (n=5) and healthy controls (n=16) were enrolled in the study. Cytokines and IFN-γ-induced proteins were determined in plasma by ELISA and HPLC-MS, in patient peripheral blood mononuclear cells (PBMCs) (qPCR, flow cytometry, western blot and ELISA) and in lymph node biopsies of one patient during both sJIA and MAS episodes (immunohistochemistry). IFN-γ responses were investigated in healthy donor PBMCs, primary fibroblasts and endothelial cells.

Results. Plasma IFN-γ, IL-6 and IL-18 were elevated in active sJIA and MAS. Levels of IFN-γ and IFN-γ-induced proteins (IP-10/CXCL-10, IL-18BP and IDO) in MAS were highly surpassing levels in active sJIA. Free IL-18 and ratios of IL-18/IFN-γ were higher in active sJIA versus MAS. MAS PBMCs showed a hyporesponsiveness to IFN-γ *in vitro*. Endothelial cells and fibroblasts expressed IFN-γ-induced proteins *in situ* in lymph node stainings of a MAS patient and *in vitro* upon stimulation with IFN-γ.

Conclusions. Patients with active sJIA and MAS show distinct cytokine profiles with highly elevated plasma levels of IFN-γ and induced proteins typically found in MAS. In addition to PBMCs, histiocytes, endothelial cells and fibroblasts may contribute to an IFN-γ profile in plasma. Increasing levels of IFN-γ compared to IL-18 may raise suspicion for development of MAS in sJIA.

Key words: systemic juvenile idiopathic arthritis, macrophage activation syndrome, interferon-gamma, interleukin-18, cytokine, plasma, peripheral blood mononuclear cells

INTRODUCTION

Systemic juvenile idiopathic arthritis (sJIA) is a pediatric immune-inflammatory disorder, characterized by arthritis and systemic features including fever, rash and lymphadenopathy [1-3]. A striking aspect of sJIA is its strong association with macrophage activation syndrome (MAS), a condition caused by excessive activation of T cells and macrophages leading to hemophagocytic activity and massive inflammatory responses [4,5]. Whereas secondary MAS can be a complication of infections, malignancies and childhood systemic inflammatory disorders -especially sJIA-, primary MAS occurs in the context of hereditary hemophagocytic lymphohistiocytosis (HLH) and has a genetic cause related to defective cytotoxic activity [6].

Several findings support the concept that sJIA is an auto-inflammatory disease driven by pro-inflammatory cytokines such as IL-1β, IL-6 and IL-18 [7]. These findings include detection of elevated innate cytokines in plasma of patients [8-16], induction of IL-1β-related genes in peripheral blood mononuclear cells (PBMCs) from healthy controls by sera of sJIA patients [17] and the successful treatment of sJIA with IL-1 and IL-6 antagonists [18-21].

Despite the high inflammatory status of both sJIA and MAS, the role of interferon-gamma (IFN-γ), a key cytokine in inflammation and macrophage activation, is incompletely understood [22]. Gene expression studies performed on PBMCs from sJIA patients revealed an absence of IFN-γ-upregulated genes [23]. Sikora *et al.* reported an intact transcriptional response of sJIA PBMCs to IFN-γ, suggesting that the absent IFN-γ gene signature might be the result of a low *in vivo* exposure to IFN-γ [24]. Conversely, a major role of IFN-γ in the pathogenesis of MAS is presumed from our observations demonstrating *in situ* IFN-γ in liver biopsies in a heterogeneous group of MAS patients [25], from elevated IFN-γ plasma levels in HLH [26-28] and from animal models for HLH [29,30], in which the symptoms were inhibited by anti-IFN-γ antibody treatment. We recently described a new mouse model showing typical clinical and pathological features reminiscent of sJIA [31]. Intriguingly, sJIA-

like features, provoked by challenging mice with Freund's adjuvant, were more evident in IFN-γ-deficient mice than in wild-type counterparts, suggesting a protective role of IFN-γ in this model. Indeed, in addition to its pro-inflammatory activities, IFN-γ also possesses profound anti-inflammatory effects such as promoting regulatory T cell activity, inhibiting development and activity of T helper 17 cells and suppressing IL-1β-signaling (i.e. IL-8) [32,33].

In this study, we investigated the role of IFN-γ in a comprehensive way in patients with sJIA and MAS. Levels of IFN-γ, IFN-γ-induced proteins (IP-10/CXCL-10, IL-18BP and IDO) and pro-inflammatory cytokines (IL-1β, IL-6, IL-18) were measured in plasma and patient PBMCs. IFN-γ-induced gene and/or protein expression was also determined upon IFN-γ stimulation of patient PBMCs and *in situ* on lymph nodes of a patient both during sJIA and MAS episodes. We further compared IFN-γ stimulation of normal PBMCs, primary human fibroblasts and endothelial cells and investigated the potential regulatory effect of IFN-γ on IL-1β signaling in PBMCs.

PATIENTS AND METHODS

Patients

Patients and healthy controls were recruited from the University Hospital of Leuven and Hôpital Necker, Enfants Malades, Paris, after giving informed consent. The study was approved by the Institutional Review Boards. Patient samples were collected at sJIA disease flare or at the occurrence of MAS before additional therapeutic intervention, and were compared to samples of inactive sJIA patients and age-matched controls. 5 patients in MAS episode, 10 inactive and 10 active sJIA patients and 16 age-matched healthy controls were enrolled. All sJIA patients met the criteria of the International League of Associations for Rheumatology [3] and were grouped according to their disease state into active or inactive sJIA. Inactive sJIA was defined by absence of fever, rash, arthritis and inflammatory parameters [34]. Table I summarizes demographic data, diagnosis, treatment, clinical characteristics and laboratory values at the time of sampling. Diagnosis of MAS was based on the diagnostic guidelines set by Ravelli *et al.* and criteria set in the HLH-2004 protocol [35,36]. All MAS patients underwent genetic testing for HLH-associated genes. This analysis revealed a heterozygous mutation in Munc13/4 (p.Arg83X; p.Ala1018Asp) in one patient presenting with MAS (MAS3). In an EBV-associated MAS patient (MAS4), a combination of two heterozygous unclassified variants were found in both the perforin and the Munc13/4 gene.

Plasma isolation and cell cultures

Within two hours after withdrawal of EDTA-anticoagulated blood, plasma was separated by centrifugation (300g and 2000g) and stored at -80°C. Lymphoprep purified patient PBMCs (Axis-Shield PoC AS, Oslo, Norway) were cultured in RMPI 1640 medium containing 10% fetal bovine serum (FBS) (Lonza BioWhittaker, Walkersville, MD, USA) and stimulated with human recombinant IFN-γ (PeproTech, London, UK). For the experiments described in

Figure 4 and 5, healthy adult PBMCs were obtained by the same procedure out of buffy coats from the Red Cross of Flanders. PBMCs were frozen in liquid nitrogen and thawed at the time of stimulation with human recombinant IFN-γ and/or IL-1β (PeproTech). Primary human retinal microvascular endothelial (HRMVE) cells were cultured in endothelial basal medium-2, supplemented with the endothelial growth medium-2MV Bullet kit (Lonza BioWhittaker). Primary human diploid skin/muscle-derived fibroblasts (E1SM) were grown in Eagle's minimal essential medium (EMEM; Lonza BioWhittaker) containing 10% FBS. Monolayers were grown to confluency and stimulated with IFN-γ. After 24 hours, cells were harvested for analysis of gene expression and induced proteins were detected in supernatant.

RNA extraction, qPCR and ELISA

RNA was purified using the RNeasy Micro kit (Qiagen, Hilden, Germany) for patient material or the PureLink RNA mini kit (Invitrogen, Carlsbad, CA, USA). cDNA synthesis was performed using Super Script II Reverse Transcriptase (Invitrogen), according to manufacturer's protocols. mRNA levels were analyzed in duplicate by qPCR using TaqMan Gene Expression Assays [Applied Biosystems, Foster City, CA, USA; Assay IDs: Hs00171042_m1 (IP-10/CXCL10), Hs00158032_m1 (IDO), Hs00271720_m1 (IL-18BP), Hs01555410_m1 (IL-1β), Hs00174103_m1 (IL-8/CXCL8)] and normalized to housekeeping gene GAPDH. Cytokine levels in plasma were measured by sandwich ELISA according to the manufacturer's protocols (IFN-γ, IL-6, IL-1β High Sensitivity ELISA, eBioscience, San Diego, CA, USA; Human IL-18 ELISA kit MBL International, Woburn, MA, USA; Human IL-18BPa Duoset, R&D Systems, Minneapolis, MN, USA). IP-10, MIG and IL-8 levels were quantified by sandwich ELISA as described [37,38]. Levels of free IL-18 were calculated as described [39].

Flow cytometry, western blot, immunohistochemistry and HLPC-MS

PBMCs were stained with anti-CD11c-PE (eBioscience). Intracellular IDO staining was performed using the Cytofix/Cytoperm kit (BD Biosciences) and the anti-IDO-alexa633 mouse monoclonal antibody 4.16H1, developed and validated at the Ludwig Institute, Brussels [40]. For western blot analysis, PBMCs were lysed in RIPA buffer containing protease inhibitors. Lysates were run on SDS/PAGE and blotted on a PVDF membrane. Immunoblots were performed using anti-IDO (clone10.1), anti-GAPDH (clone6C5) (EMD Millipore Corporation, Billerica, MA, USA) and secondary horseradish peroxidase conjugates followed by enhanced chemiluminescence detection. Parrafin-embedded lymph node sections were stained with monoclonal mouse anti-IDO (EMD Millipore Corporation) and polyclonal rabbit anti-IP-10 antibodies (Abcam, Cambridge, UK) according to manufacturers' instructions. Tryptophan (Trp) and kynurenine were quantified in plasma by tandem LC-MS^n. Detailed methods can be found in supplementary file S1.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 software. Wilcoxon signed rank test was used for induction experiments, Mann-Whitney U test for comparison of two groups not showing Gaussian distribution, and for multiple comparisons Kruskal-Wallis tests were performed with Dunn's post test. P values $\leq .05$ were considered indicative of statistical significant differences.

RESULTS

Increased cytokine levels in active sJIA and MAS patients

IFN-γ levels in plasma were quantified and compared with other cytokines (Figure 1A, Supplementary table S2). Concentrations of IFN-γ were approximately 5 times higher in plasma of active sJIA patients than in inactive sJIA patients and healthy controls (Figure 1A). In MAS patients, IFN-γ levels were more than 100 times higher than in controls ($p<0.001$) and were increased compared to inactive ($p<0.01$) and active (not significant (ns)) sJIA patients.

IL-6 plasma levels were elevated in active sJIA patients as compared to healthy controls ($p<0.001$) (Figure 1A). Also in inactive sJIA the levels of IL-6 were higher than in healthy controls (ns). Median IL-6 levels in MAS patients were even higher compared to active sJIA and were elevated compared to healthy controls ($p<0.001$). A 10 to 100-fold difference in IL-6 levels was noted between the three patients with MAS complicating sJIA, and those of two other patients, one with primary MAS and one with EBV-associated MAS (open squares in Figure 1A).

As reported, IL-1β levels are difficult to detect in plasma of sJIA patients [8,15,17]. Likewise, we could not detect IL-1β in the plasma of active sJIA patients. Yet, IL-1β levels were elevated in all three patients with MAS complicating sJIA (Figure 1A).

Levels of IL-18, a member of the IL-1β-family, were increased in active sJIA patients in comparison to inactive sJIA patients ($p<0.05$) and healthy controls ($p<0.001$) (Figure 1A). IL-18 in MAS patients was significantly higher than in controls ($p<0.05$).

Together, these data clearly show a cytokine storm in both active sJIA and MAS patients, with raised levels of IFN-γ, IL-6 and IL-18.

Elevation of IP-10, IL-18BP and IDO activity in plasma of active sJIA and MAS patients

We next quantified IFN-γ-induced proteins. Interferon inducible protein-10 (IP-10/CXCL10) [41], was detected in 4 of 7 active sJIA patients and was elevated in MAS patients compared to inactive sJIA ($p<0.001$) and to healthy controls ($p<0.001$) (Figure 1B).

IL-18 binding protein (IL-18BP), an antagonist of IL-18 that is known to be induced by IFN-γ [42], was higher in active sJIA patients compared to healthy controls ($p<0.05$) (Figure 1B). IL-18BP levels were elevated more than 5-fold in patients with MAS compared to active sJIA, resulting in significant differences with inactive sJIA patients ($p<0.01$) and healthy controls ($p<0.001$).

Indoleamine 2,3-dioxygenase (IDO), an intracellular enzyme catalyzing the degradation of the essential amino acid tryptophan (Trp) to kynurenine, is induced by IFN-γ [43]. IDO activity in plasma was measured by detecting the ratio of kynurenine to Trp by HPLC. An image similar to IP-10 and IL-18BP was found for IDO activity with moderately raised IDO activity in active sJIA and highly elevated ratio of kynurenine/Trp in MAS patients compared to inactive sJIA patients ($p<0.05$) and healthy controls ($p<0.01$) (Figure 1C).

Based on the above plasma levels, we conclude that MAS patients have a distinct IFN-γ plasma profile with high levels of both IFN-γ and its induced proteins, while active sJIA patients show a moderate IFN-γ profile.

High IL-18 plasma levels in active sJIA

IL-18 was originally identified as an IFN-γ-inducing factor [44]. In view of the high IL-18 levels found in active sJIA, the only moderately elevated levels of IFN-γ and related proteins in these patients were surprising. Accordingly, the median ratio of IL-18 to IFN-γ was more than 200 times higher in active sJIA plasma compared to MAS (Figure 2A, $p<0.01$). Based on plasma levels of both IL-18 and its inhibitor IL-18BP, calculated free IL-18 levels were found

to be significantly higher in plasma of active sJIA but not in MAS patients (Figure 2B, $p<0.01$). These data show a different balance of IL-18 and IFN-γ in sJIA versus MAS with an important role of IL-18 in sJIA and an IFN-γ-tipped balance in MAS.

Defective induction of IDO, IP-10 and MIG upon *in vitro* stimulation of MAS PBMCs with IFN-γ

In addition to plasma protein levels, we analyzed the corresponding mRNA levels of IFN-γ-induced proteins in freshly isolated PBMCs of all patients, and confirmed the reported lack of an IFN-γ gene signature in sJIA [23]. In PBMCs of patients with MAS, there was a trend towards higher IFN-γ, IP-10 and IDO mRNA (data not shown). The elevated levels of IFN-γ in plasma of active sJIA and especially of MAS patients seem counterintuitive with the absence of a clear-cut IFN-γ signature in their freshly isolated PBMCs. Therefore, we investigated the response of patient PBMCs to IFN-γ stimulation *in vitro*. Figure 3A shows a significant increase of IDO and IP-10 mRNA in response to IFN-γ in healthy controls ($p<0.001$) as well as in inactive ($p<0.05$) and active ($p<0.05$) sJIA patients. In MAS patients, IP-10 and IDO mRNA were both induced, however not significantly and to a lesser extent than in the other groups. To confirm these data at the protein level, the induction of IDO was analyzed by intracellular flow cytometry. IFN-γ induced IDO in PBMCs from healthy controls, inactive and active sJIA patients. IDO levels were significantly lower in MAS patients compared to healthy controls ($p<0.05$) and inactive sJIA patients ($p<0.05$) (Figure 3B). In addition, lower levels of IFN-γ-induced IDO were found in two active sJIA patients. Western blot analysis confirmed flow cytometric results, with little or no IDO expression in PBMCs of two active sJIA and one MAS patient, after stimulation with a concentration of IFN-γ as low as 1.5 ng/ml (Figure 3C). In order to determine induction of other IFN-γ-induced

proteins, IP-10/CXCL10 and MIG/CXCL9 protein levels were measured in the supernatant of IFN-γ-stimulated PBMC cultures. Both IP-10 and MIG were induced in healthy controls, inactive and active sJIA patients (Figure 3D). In contrast, in PBMCs of MAS patients, IFN-γ failed to induce IP-10 and MIG proteins (Figure 3D). Taken together, freshly isolated PBMCs of healthy controls, inactive sJIA and active sJIA patients responded to an *in vitro* stimulation with IFN-γ. In contrast, PBMCs of MAS patients demonstrated a decreased response to IFN-γ.

Endothelial cells and fibroblasts as alternative sources of plasma cytokines

The IFN-γ profile seen in the plasma of sJIA and MAS patients (Figure 1) is not in line with gene expression findings of freshly isolated PBMCs of the patients ([23,45] and data not shown). We hypothesized that cells other than PBMCs might be a source of IFN-γ-induced proteins. Therefore, IFN-γ responses were measured in primary fibroblast and endothelial cells and compared with adult donor PBMCs. Induction of IP-10, IL-18BP and IDO mRNA by IFN-γ was 10 to 100 times higher in fibroblasts and endothelial cells compared to PBMCs (Figure 4A). The inductions were confirmed at the protein level for IP-10 and IL-18BP (Figure 4B). However, PBMCs did not produce IL-18BP in response to IFN-γ. We conclude that in addition to PBMCs, fibroblasts and endothelial cells respond to IFN-γ and might contribute to an IFN-γ protein profile in plasma.

Production of IP-10 and IDO by histiocytes, endothelial cells and fibroblasts in a MAS lymph node

Two years before development of MAS, during active sJIA, patient ‘MAS2’ (Table I) presented with persistently enlarged lymph nodes; a lymph node biopsy was performed to exclude lymphoproliferative disorder. A second lymph node biopsy was taken at the time of

overt MAS. Immunohistochemical staining showed a limited number of IP-10 and IDO positive, mainly monocytic cells, during the stage of active sJIA (Figure 4C, left panels). During the MAS episode, a prominent increase of both IP-10 and IDO staining was observed, corresponding to the IFN-γ profile in plasma (Figure 4C, right panels). Next to histiocytes (upper right panel), both endothelial cells and fibroblasts in the lymph node sections stained for IP-10 and IDO (arrows, lower right panels), endorsing the importance of alternative sources of IFN-γ-associated proteins.

Anti-inflammatory role of IFN-γ through inhibition of IL-1β signaling in PBMCs

IFN-γ exerts both pro- and anti-inflammatory functions. One of its anti-inflammatory properties is regulation of pro-inflammatory cytokines, including IL-1β signaling [33]. As shown in Figure 5A+B, in healthy adult PBMCs, IFN-γ inhibited IL-1β-induced production of IL-1β as well as the production of IL-8/CXCL8, an important chemokine for attraction and activation of polymorphonuclear neutrophils [46]. Hence, elevated IFN-γ levels observed in the plasma of MAS patients might have an influence on the IL-1β levels in patient PBMCs. To test this assumption, IL-1β and IL-8 mRNA levels were quantified in freshly isolated sJIA and MAS patient PBMCs. PBMCs of MAS patients showed lower mRNA levels of both IL-1β and IL-8 as compared to other patients and controls, with a significant decrease compared to inactive and active sJIA patients (Figure 5C). The decreased IL-1β and IL-8 mRNA in MAS PBMCs may be a consequence of the highly elevated IFN-γ plasma levels.

DISCUSSION

As previously described, both active sJIA and MAS patients experience a cytokine storm with raised levels of IL-6 and IL-18 [8-14,16], and these findings are confirmed in our study. Though IFN-γ is reported to be elevated in primary MAS [26-28] and MAS secondary to

infection or malignancy [13], we are the first to report high levels of IFN-γ and its induced proteins in plasma of patients with MAS complicating sJIA. Since IFN-γ inhibits IL-1β-signaling in PBMCs ([33] and our data in Figure 5A), decreased IL-1β and IL-8 expression in MAS PBMCs might be linked to high IFN-γ levels in these patients.

Reports concerning IFN-γ plasma levels in sJIA are scarce and not always in agreement. While Gattorno *et al.* reported significantly elevated levels of IFN-γ in the serum of active sJIA patients compared to healthy controls [15], de Jager *et al.* did not observe raised IFN-γ [8]. We did observe moderately - though not significantly- elevated IFN-γ in the plasma of active sJIA patients. However, we found a marked difference in the balance of IFN-γ and the other cytokines. IFN-γ is more than 20 times higher in MAS patients as compared to active sJIA patients, while IL-6 and IL-18 are not significantly different between both entities. Conversely, active sJIA patients showed a highly elevated ratio of IL-18 to IFN-γ and high free IL-18 levels as compared to MAS. Since IL-18 is an important inducer of IFN-γ in NK cells [44], the low IFN-γ levels in active sJIA patients were counterintuitive. In this context, de Jager *et al.* reported a defective phosphorylation of the IL-18 receptor β-chain in NK cells of sJIA patients leading to defective IL-18-induced IFN-γ production *in vitro* [47]. Intriguingly, a defective IFN-γ production may be in line with our recently developed mouse model reminiscent of sJIA, in which the clinical, biological and pathological features of sJIA were more prominent in IFN-γ deficient mice [31]. These data suggest that IFN-γ is not required for development of sJIA and may exert protective activity in the pathogenesis of sJIA.

The role of IFN-γ in MAS seems different. Indeed, we observed a substantial increase of IFN-γ-induced proteins IP-10 and IDO in a lymph node biopsy from a patient during MAS episode as opposed to its active sJIA phase. IFN-γ plays an essential role in the primary HLH-

like syndrome elicited in LCMV-infected mice with a genetic defect in cytoxicity [29,30]. In contrast, Canna *et al.* ascribed only a minor role to IFN-γ in a murine model of TLR-9-induced fulminant MAS where IFN-γ mediates anemia but is dispensable for the other manifestations [48]. Despite the high levels of IFN-γ and its downstream proteins in MAS, its exact role remains to be elucidated.

Looking at PBMCs *in vitro*, we confirmed the gene expression results of Fall *et al.* and Sumegi *et al.*, who reported the absence of an IFN-γ gene signature in PBMCs of active sJIA and primary MAS patients, respectively [23,45]. In PBMCs of MAS patients we observed a trend towards an IFN-γ gene signature - albeit not in accordance to the highly elevated IFN-γ levels found in the plasma of these patients. As mRNA expression data in freshly isolated PBMCs did not correspond to the levels of IFN-γ in the plasma of patients, we investigated IFN-γ responses in PBMCs. We report an intact transcriptional response in sJIA patients, as was shown previously by Sikora *et al.* [24]. In addition, we found that IFN-γ induced IDO, IP-10 and MIG proteins in PBMCs of both controls and sJIA patients. PBMCs of MAS patients showed a hyporesponsiveness to IFN-γ, which is unexpected in view of the extremely high levels of IFN-γ, IDO and IP-10 in the plasma. We hypothesize that the defect observed in the *in vitro* culture might be explained by a functional exhaustion where PBMCs, that have been producing massive amounts of IDO and IP-10 in plasma, fail to continue this production upon *in vitro* restimulation. In systemic diseases like sJIA and MAS, PBMCs are the most suitable cells for research, reflected by their use in many reports. We found a robust IFN-γ responsiveness in primary fibroblasts and endothelial cells, in addition to PBMCs. The production of IFN-γ-induced IP-10 and IDO by these cells was shown *in situ* in a lymph node biopsy of a MAS patient. We propose that in the interpretation of studies in PBMCs, other cell types should also be taken into account.

We recognize that the number of MAS patients tested in this study is limited, which is related to the rarity of the condition. However, since our findings were not different between three patients under treatment, compared to two patients tested before receiving any immunosuppressive therapy, we believe that our findings are of relevance. One out of five patients presenting with full-blown MAS was later diagnosed as primary MAS. Although it is well known that primary and secondary MAS have different genetic backgrounds, they both share a final common pathway with a similar clinical and pathological picture [6]. Therefore, for the purpose of our study, all MAS patients were analyzed as one group.

In conclusion, sJIA and MAS patients both show a prominent inflammatory cytokine response, though with a distinct profile. MAS patients show a clear-cut IFN-γ profile, in contrast to active sJIA in which IL-18 levels greatly outweigh the relatively low IFN-γ levels. IFN-γ responsiveness of PBMC is equally different between both entities, with low responsiveness of MAS PBMC. In addition to PBMCs, histiocytes, endothelial cells and fibroblasts may contribute to an IFN-γ profile, and therefore these cells should be taken into account for research into the pathogenesis of sJIA and MAS. A decreased IL-18/IFN-γ ratio might be an additional tool heightening the suspicion of a MAS episode in sJIA.

Key messages

- IFN-γ and its downstream proteins distinguish MAS from sJIA
- High free IL-18 accompanied by low IFN-γ is typically seen in sJIA
- Endothelial cells and fibroblasts, besides PBMCs can contribute to the MAS IFN-γ profile

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Table I: Patient Characteristics

Patient	Age(yr) /Gender	Treatment	AR	FEVER	RASH	HSM	LA	CNS	WBC 10^9/l	RBC 10^{12}/l	Hb g/dl	PLT 10^9/l	CRP mg/dl	AST/ALT U/ml	LDH U/ml	FTN
sJIA1	19/M	Cn							4.58	4.8	15.4	227	3	22/18	152	29.8
sJIA2	11/M	Tc CS MTX							8.08	4.6	13.3	319	3	27/20	187	24
sJIA3	10/F	Cn MTX							9.59	5	10.8	400	6	22/9	177	19.9
sJIA4	12/F	Tc MTX CS							4.15	4	11.9	276	4	24/15	200	35
sJIA5	9/F	Tc MTX							6.02	4.7	13.4	289	2	31/17	272	5
sJIA6	15/F	MTX							5.1	4.75	14.4	274	1	27/15	419	33
sJIA7	2/F	Tc							10.2	5.49	10.8	366	9	ND	ND	ND
sJIA8	17/F	Tc CS							7	4.55	11.6	231	9	21/15	ND	15
sJIA9	7/M	Tc CS							5.5	4.79	13.2	211	6	24/11	ND	41
sJIA10	16/M	Cn NSAID MTX CS							4.7	4.8	12.7	192	6	22/19	117	4
sJIA11*	10/M	CS	+	+					32.65	5.3	12.2	582	195	55/77	290	ND
sJIA12*	15/F	NSAID		+					10	4.07	10.6	338	41	28/15	ND	236
sJIA13*	3/F	NSAID	+	+	+				15.2	4.74	10.5	498	112	33/21	483	428
sJIA14*	11/F	CS MTX NSAID	+	+	+				23.9	4.7	10.8	366	118	22/17	723	787
sJIA15*	3/F	-	+	+	+				29	2.86	7.8	595	177	38/16	417	4469
sJIA16*	1/F	NSAID Cefotaxime	+	+	+	+			12.5	3.53	9.3	414	60	38/25	370	7397
sJIA17*	8/M	NSAID	+	+	+		+		16.7	4.03	10.4	327	48	22/9	289	2549
sJIA18*	3/F	NSAID	+	+	+				16.2	4.54	12	591	66.1	24/9	201	226.1
sJIA19*	1/F	-		+	+	±	±		13	3.54	8.6	200	78	800/675	1613	3393
sJIA20*	4/M	-	+	+	+		+		40.2	4.18	9.6	656	112	31/23	523	171
MAS1#	28/F	Tc CS MTX NSAID		+	+	+	+		5.6	2.69	6.9	77	28	976/361	8228	53947
MAS2#	19/M	Tc NSAID		+	+	+	+		3.4	2.62	6.7	48	102	122/57	2172	10991
MAS3$	1/M	-		+		+	+	+	5.3	3.49	8.3	84	34	94/74	705	542
MAS4£	4/F	-		+		+	+		0.6	2.78	6.8	17	29	339/84	3700	31085
MAS5#	21/M	Tc NSAID		+		+			4.1	3.14	9.1	63	1	491/551	1531	6325

Treatment: Cn, Canakinumab (anti-IL-1β); Tc, Tocilizumab (anti-IL-6 receptor); CS, corticosteroids; NSAID, non-steroidal anti-inflammatory drugs; MTX, metothrexate.

AR, active arthritis; HSM, hepatosplenomegaly; LA, lymphoadenopathy; CNS, central nervous system dysfunction; WBC, white blood cell count; RBC, red blood cell count; Hb, hemoglobin; CRP, C-reactive protein; AST/ALT, aspartate/alanine transaminase; LDH, lactate dehydrogenase; FTN, ferritin; ND, not determined. * sJIA patients in an active disease state; # Patients with MAS complicating sJIA; $ Primary MAS patient with a *Munc13/4* mutation; £ EBV-associated MAS

Figures

Figure 1

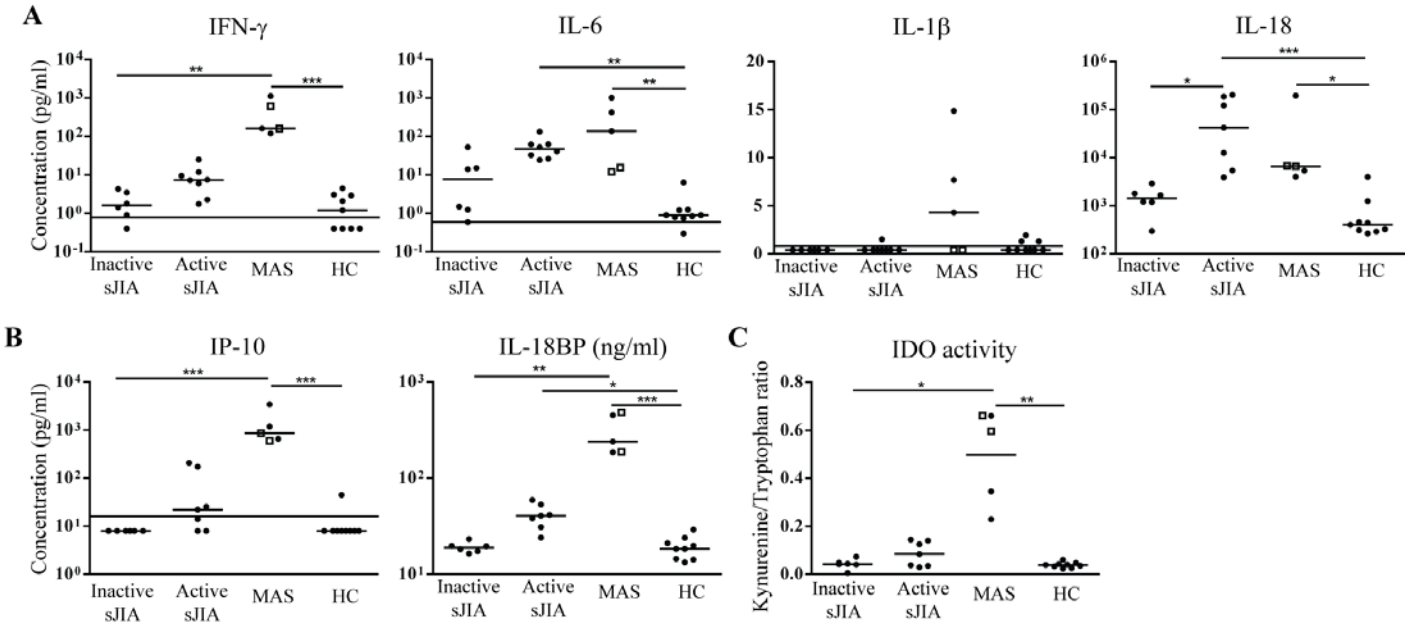

Figure 2

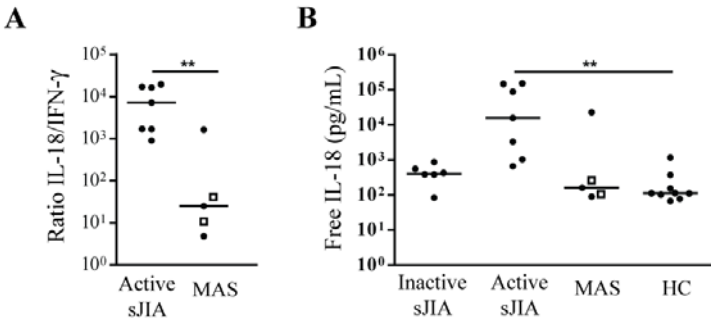

Figure 3

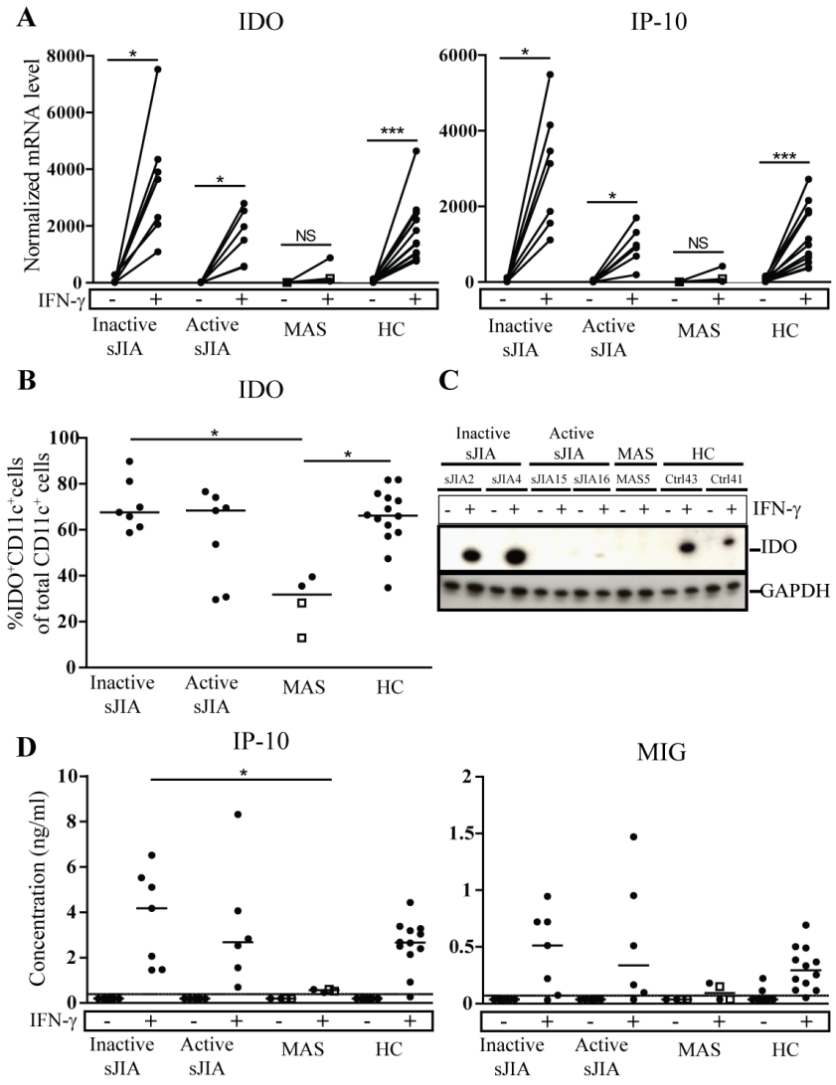
A
IDO
IP-10
Normalized mRNA level
8000
6000
4000
2000
0
6000
4000
2000
0
*
*
NS
***
IFN-γ
- + - + - + - +
Inactive sJIA
Active sJIA
MAS
HC
B
IDO
%IDO+CD11c+cells of total CD11c+ cells
100
80
60
40
20
0
C
Inactive sJIA
Active sJIA
MAS
HC
sJIA2
sJIA4
sJIA15
sJIA16
MAS5
Ctrl43
Ctrl41
IFN-γ
IDO
GAPDH
D
IP-10
MIG
Concentration (ng/ml)
10
8
6
4
2
0
2
1.5
1
0.5
0

Figure 4

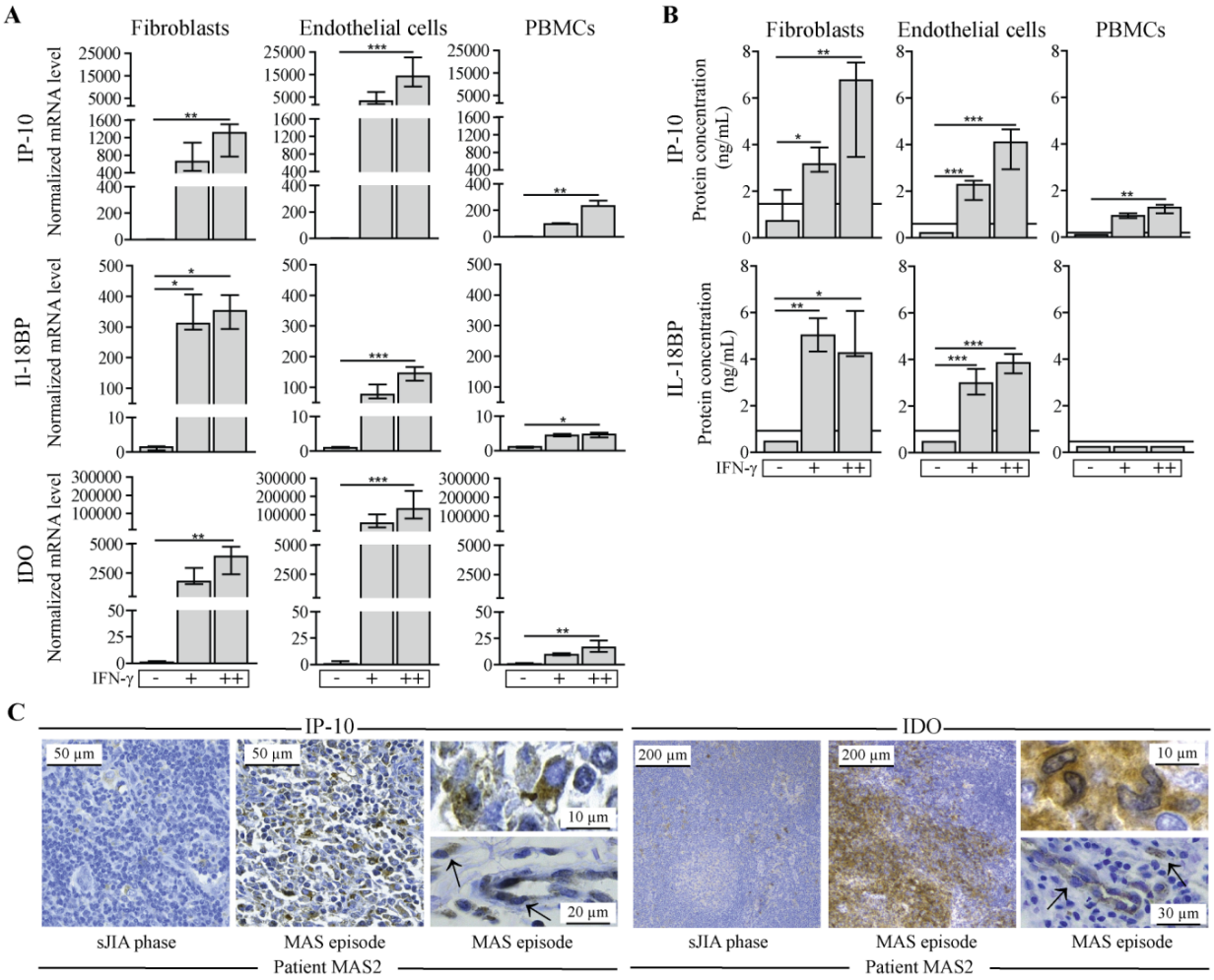
A
Fibroblasts
Endothelial cells
PBMCs
IP-10
Il-18BP
IDO
Normalized mRNA level
IFN-γ
B
IP-10
IL-18BP
Protein concentration (ng/mL)
C
IP-10
IDO
50 µm
10 µm
20 µm
200 µm
30 µm
sJIA phase
MAS episode
Patient MAS2

Figure 5

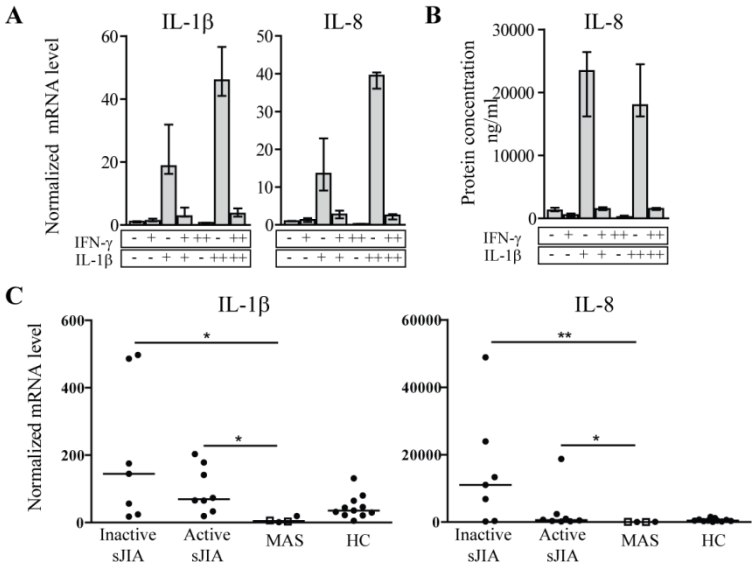
A
IL-1β
IL-8
Normalized mRNA level
IFN-γ
IL-1β
B
IL-8
Protein concentration ng/ml
C
IL-1β
IL-8
Normalized mRNA level
Inactive sJIA
Active sJIA
MAS
HC

Figure legends

Fig. 1 Elevated cytokine levels in plasma of active sJIA and MAS patients. Levels of cytokines and IFN-γ-induced proteins were determined by ELISA in the plasma of inactive and active sJIA patients, MAS patients and healthy controls. **A.** Concentrations (pg/ml) of IFN-γ, IL-6 and IL-18 are depicted in a logarithmic scale, IL-1β in a linear scale. **B.** IFN-induced proteins IP-10 (pg/ml) and IL-18BP (ng/ml) were analyzed in plasma of patients and both displayed in a logarithmic scale. **C.** Plasma concentrations of tryptophan and kynurenine were determined by HPLC. The ratio of kynurenine to tryptophan is depicted, and reflects a measure of IDO activity in the patients. Dots represent single patients, with open squares representing a patient with primary MAS and one with EBV-associated MAS; horizontal bars represent the median. Horizontal line depicts the lower detection limit of the ELISAs. *p<0.05; **p<0.01; ***p<0.001, Kruskal-Wallis with Dunn's post test.

Fig. 2 Distinct levels of IL-18 in active sJIA versus MAS plasma. A. Ratio of IL-18 to IFN-γ plasma levels in active sJIA and MAS patients. **B.** Levels of free IL-18 in plasma of inactive and active sJIA patients, MAS patients and healthy controls. Dots represent single patients, with open squares representing a patient with primary MAS and one with EBV-associated MAS; horizontal bars represent the median. **p<0.01, Mann-Whitney U test (A); Kruskal-Wallis with Dunn's post test (B).

Fig. 3 IFN-γ responses in PBMCs of sJIA and MAS patients. **A.** Induction of IDO and IP-10 mRNA levels in PBMCs stimulated with IFN-γ (15 ng/ml; 16h); dots and connecting lines represent individual patients with (+) or without (-) IFN-γ; NS: not significant; *p<0.05; ***p<0.001, Wilcoxon signed rank test. **B.** Flow cytometric analysis showing IDO^+CD11c^+ cells as a percentage of total $CD11c^+$ cells after treatment of PBMCs with IFN-γ (15 ng/ml,

16h). **C.** Western blots for IDO in cell lysates of IFN-γ [(+) 1,5 ng/ml, 16h] stimulated PBMCs. GAPDH is included as a loading control. **D.** IP-10 and MIG protein in supernatant of medium (-) and IFN-γ-stimulated [(+) 15 ng/ml; 16h] PBMCs. Each dot represents one patient, open squares represent a patient with primary MAS and one with EBV-associated MAS; horizontal bars are medians. *p<0.05, Kruskal-Wallis with Dunn's post test.

Fig. 4 IFN-γ-induced genes/proteins in fibroblasts, endothelial cells and PBMCs. A+B. The induction of IP-10, IDO and IL-18BP by IFN-γ was investigated in fibroblasts, endothelial cells and adult donor PBMCs. Cell cultures were incubated without (-) or with IFN-γ [1.5 (+) or 15 (++) ng/ml] for 24 hours. (**A**) Normalized mRNA expression relative to the medium condition and (**B)** protein production in supernatant were determined. Bars represent medians with interquartile range (fibroblasts, n=4; endothelial cells, n=6; PBMCs, n=4). Results are representatives of at least two independent experiments. *p<0.05; **p<0.01, ***p<0.001, Kruskal-Wallis with Dunn's post test. **C.** Immunohistochemical stainings for IP-10 (left) and IDO (right) on lymph node sections of patient MAS2, during the stage of active sJIA, and during a MAS episode. On the right side of each panel, enlargements of the MAS phase lymph node section show IP-10/IDO positive histiocytes (above) and endothelial cells and fibroblasts (below, arrows). Object lenses used were 10x and 40x.

Fig. 5 IFN-γ inhibits IL-1β-induced gene and protein expression in PBMCs. A+B. The regulation of IL-1β and IL-8 by IL-1β and/or IFN-γ was investigated in healthy adult donor PBMCs. Cells were incubated for 24 hours with IFN-γ [1.5 ng/ml (+) or 15 ng/ml (++)] or IL-1β [1 ng/ml (+) or 10 ng/ml (++)] and combination of both cytokines. **A.** mRNA levels of IL-1β and IL-8 are depicted normalized to GAPDH and relative to the untreated medium condition. **B.** IL-8 protein levels were detected in the culture supernatant by ELISA. Bars

represent medians with interquartile range (n=3). **C.** mRNA expression of IL-1β and IL-8 was analyzed by qPCR in PBMCs of inactive and active sJIA patients, MAS patients and healthy controls. mRNA expression was normalized to the housekeeping gene GAPDH. Dots represent single patients, with open squares representing a patient with primary MAS and one with EBV-associated MAS; horizontal bars represent the median. *$p<0.05$; **$p<0.01$, Kruskal-Wallis with Dunns' post test.