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Altered signaling in systemic juvenile idiopathic arthritis monocytes

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Abstract

Systemic juvenile idiopathic arthritis (sJIA) is characterized by systemic inflammation and arthritis. Monocytes are implicated in sJIA pathogenesis, but their role in disease is unclear. The response of sJIA monocytes to IFN may be dysregulated. We examined intracellular signaling in response to IFN type I (IFN α) and type II (IFN γ) in monocytes during sJIA activity and quiescence, in 2 patient groups. Independent of disease activity, monocytes from Group 1 (collected between 2002-2009) showed defective STAT1 phosphorylation downstream of IFNs,

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and expressed higher transcript levels of *SOCS1*, an inhibitor of IFN signaling. In the Group 2 (collected between 2011-2014), monocytes of patients with recent disease onset were IFN γ hyporesponsive, but in treated, quiescent subjects, monocytes were hyperresponsive to IFN γ . Recent changes in medication in sJIA may alter the IFN hyporesponsiveness. Impaired IFN/pSTAT1 signaling is consistent with skewing of sJIA monocytes away from an M1 phenotype and may contribute to disease pathology.

Keywords

Juvenile arthritis; monocytes; IFN signaling; *SOCS1*

1. Introduction

Systemic juvenile idiopathic arthritis (sJIA) is a childhood rheumatic condition that has been classified as one of the subtypes of JIA. sJIA is typically characterized by spiking fever in a quotidian pattern, transient rash, and arthritis. Patients may alternate between periods of disease activity (flare) and inactivity (quiescence). The etiology of sJIA is unknown, but it is hypothesized that infectious agents are triggers of disease in genetically susceptible individuals [1]. The presence of macrophage activation syndrome (MAS) in a subset of sJIA patients and the efficacy of anti-IL-1 and anti-IL-6 therapies highlight a key role for dysfunction of the innate immune system [1, 2]. Absence of typical characteristics of autoimmunity, such as autoantibodies, also implies a less prominent role for altered adaptive immunity, although it may contribute, especially to persistent disease with polyarticular arthritis [3, 4].

Among the cells of the innate immune system, cells of the monocytic lineage are thought to be drivers of sJIA pathology, as these innate cells (monocytes and macrophages) are among the producers of several mediators implicated in sJIA, including the cytokines IL-1, IL-6 and IL-18 and S100 proteins [1]. Monocytes are found in infiltrates of the sJIA rash [5], and monocytosis during sJIA disease activity has been described [6, 7]. Furthermore, gene expression studies of circulating cells show increased levels of transcripts reflective of monocyte/macrophage-associated activation in sJIA [6-8].

Different patterns of monocyte/macrophage activation have been described, with the classical, or M1-type, associated with pro-inflammatory functions, and the alternative, or M2-type (and its subtypes), associated with immunosuppression and tissue repair [9]. Although it is likely that clear polarized profiles may be only observed *in vitro*, the M1/M2 paradigm is a useful framework to examine the roles of monocytes/macrophages in various conditions.

In sJIA, contrary to expectations of a pro-inflammatory monocyte profile, we previously observed reduced proportions of circulating M1-like monocytes and increased mixed M1/M2-like monocytes in active sJIA patients [7]. One possible explanation for these results is that in sJIA, drivers of M1-polarization, such as IFN γ , are decreased. IFN γ has been shown to play an important role in sJIA patient with MAS [1], and levels of plasma IFN γ are comparable or even modestly increased in sJIA in relation to healthy controls during active

disease [10, 11]. Production of IFN γ from sJIA PBMC *in vitro* most often is observed to be similar to controls [[12, 13] and Nguyen, Mellins, unpublished]. Interestingly, independent microarray studies using PBMCs [6, 14] or whole blood [15], have failed to detect an IFN-induced signature in sJIA, with one exception, in which a Japanese population was analyzed [16]. Another IFN, IFN α , has been less studied in sJIA, but as for IFN γ , no evidence of deficient levels has been found [17, 18]. These findings raise the possibility that sJIA cells may not respond normally to M1-driving stimuli and that a defective response of sJIA cells to IFN, rather than a deficit in availability of IFN, may contribute to sJIA pathology.

In this work, we analyze the status of IFN signaling in peripheral monocytes from sJIA patients, using a flow cytometry-based technique, termed “phospho-flow” [19]. We measured the responses of monocytes from patients during sJIA flare or quiescence and compared these responses to those of monocytes from age-matched, immunologically normal children.

2. Patients, materials and methods

2.1 Subject population and clinical data collection

sJIA patients were recruited at the Pediatric Rheumatology Clinics at Lucile Packard Children’s Hospital, UCSF and Seattle Children’s Hospital. The Institutional Review Board of each institution approved this study. sJIA study subjects met the International League of Associations for Rheumatology classification criteria for systemic JIA [20]. Any sJIA patient meeting ILAR criteria could join the study; there were no exclusion criteria. We examined two patient groups, one recruited between 2002-2009 (Group 1), and a smaller group of patients collected between 2011-2014 (Group 2). At each visit, comprehensive clinical information was collected, as described previously [21]. The subject’s clinical status at sample collection was assessed, according to scoring systems that we previously devised and used to grade severity of systemic disease manifestations or of arthritis [4, 7, 21, 22]. Based on these scores, each sample was classified as “flare” or “quiescence”. We obtained control samples from immunologically healthy (based on clinical history) children, who were being seen in the Stanford Endocrinology Clinic, the majority for constitutional growth delay or precocious puberty. Due to limitation in cell number, different samples were used for different experiments. As the demographic and clinical characteristics of the samples in the different experiments were similar, we show the combined data for all the samples in each category (quiescence, flare or control). Demographical and clinical information for patients and controls is shown in Tables 1 and 2.

Venous blood samples from all subjects were treated anonymously throughout the analysis; the samples from both sJIA patients and controls were obtained when there was a clinical need for blood tests. The blood was collected in heparin tubes (BD Vacutainer, BD, Franklin Lakes, NJ); PBMC were isolated by density gradient centrifugation using Ficoll-Hypaque and frozen in 10% DMSO/25% heat inactivated (HI) human AB serum (Mediatech, Manassas, VA).

2.2 Flow cytometry-based detection of cell signaling (phospho-flow)

Phospho-flow [19, 23] was performed as detailed below. PBMC were thawed and rested for 90 min in RPMI with 5% (HI) human AB serum. Cells (5×10^6 /ml) were left unstimulated or stimulated with 10,000 U/ml of IFN α (type I interferon; MBL International, Woburn, MA), 20ng/ml of IFN γ (type II interferon), 10 ng/ml of IL-4, (Peprotech, Rocky Hill, NJ), for 12 min at 37°C, or with 1 μ g of LPS/ml (Escherichia coli, serotype 026:B6, Sigma, St. Louis, MO), for 5 min at 37°C. The stimuli were titrated for maximum signaling activity (not shown). Paraformaldehyde (PFA; Electron Microscopy Services, Fort Washington, PA) was then added to a 2% final concentration for 10 min at room temperature to stop the signaling activity. Cells were permeabilized with 90% ice-cold methanol for 10 min on ice, washed and stained for cell markers and phospho-epitopes. Antibodies to phospho-epitopes (BD Biosciences, San Jose, CA) recognized phospho-STAT1(Y701), phospho-STAT6(Y641), and phospho-p38(T180/Y182). Expression of CD33 was used to identify monocytes; previously published data show that antibodies to CD33 and CD14 recognize overlapping populations of monocytes [23]. In other experiments, CD14+ and CD16+ monocytes were identified using antibodies against HLA-DR, CD14, and CD16 (BD Biosciences). The gating strategy was as follows: cells were gated to exclude events with low size (Forward Scatter, FSC) and high cell granularity (Side Scatter, SSC); HLA-DR+ cells were then selected on SSC $\times$ HLA-DR plot and CD14+ and CD16+ monocytes were defined on a CD14 $\times$ CD16 plot; we did not subset the CD16+ monocytes (representative example in Supplementary Fig. 1A). In these experiments, cells were fixed with BD Phospho Flow Fix Buffer I and permeabilized using BD Phospho Flow PermWash I (BD Biosciences). Data were acquired on a FACSCalibur flow cytometer (BD Biosciences). Phosphorylation ratios were not significantly different between fresh or frozen samples from sJIA or controls (Supplementary Fig. 1B).

2.3 Flow cytometry staining for IFN γ receptor, total STAT1 and SOCS3

Monocytes were identified using HLA-DR, CD14 and CD16, as described above. Cell surface expression of the IFN γ receptor R1 was determined with IFN γ R1 antibody (CD119, R&D Systems, Minneapolis, MN). For intracellular detection of SOCS3, cells were permeabilized with Cytofix/Cytoperm Kit (BD Biosciences), stained for SOCS3 (Abcam, Cambridge, MA), followed by APC-labeled goat anti-rabbit antibody (R&D Systems). For intracellular detection of total STAT1, BD PhosphoFlow Fix Buffer I and Perm/Wash Buffer I were used. Cells were stained for STAT1 (Cell Signaling Technologies, Beverly, MA), followed by FITC-labeled goat anti-mouse antibody (Invitrogen, Carlsbad, CA).

2.4 Gene expression by real time RT-PCR

CD14+ monocytes were positively isolated using CD14+ microbeads (Miltenyi Biotec, San Diego, CA) from previously frozen PBMC. The purity of the isolated cells was determined by flow cytometry and was $92 \pm 6\%$ (average $\pm$ SD); the main contaminating cells were lymphocytes (not shown). The isolated monocytes were lysed in RLT buffer Plus (Qiagen, Valencia, CA), and total RNA was extracted using the RNeasy Plus kit (Qiagen), with gDNA Eliminator spin columns to remove contaminating genomic DNA. RNA quantity and purity were analyzed on a NanoDrop 2000c (Thermo Scientific, Wilmington, DE). cDNA

synthesis was performed using the iScript Reverse Transcription Supermix, and cDNA was amplified with SsoAdvanced™ SYBR® Green Supermix; reactions were performed in a CFX384 real time PCR instrument (Bio-Rad, Hercules, CA). Primers for the IFN γ receptor 2 gene (*IFNGR2*), which encodes the non-ligand-binding beta chain of the IFN γ receptor, and for the housekeeping gene for ribosomal protein L12 (*RPL12*) were from Origene (Rockville, MD). Other primer sequences were obtained from qPrimerDepot (<http://primerdepot.nci.nih.gov/>), synthesized at the Stanford University Protein and Nucleic Acid Facility (PAN) and validated in our laboratory: Suppressor of cytokine signaling 1: *SOCS1* (Right: ATGGTTCCAGGCAAGTA, left: CTACCTGAGCTCCTTCCCCT); inositol polyphosphate-5-phosphatase: *INPP5D* (also known as SH2 domain-containing inositol-5'-phosphatase 1: SHIP1) (Right: GGGTGGAGACACGACACTTT, left: GTGACCCATCTGCAATACCC); protein inhibitor of activated STAT1: *PIAS1* (Right: CGCTGACTGTTGTCTGATGC, left: CAGCTCTTCACCCAGTCCAT); *STAT1* (Right: TGAATATTCCCCGACTGAGC, left: AGGAAGACCCAATCCAGATGT); and the housekeeping genes eukaryotic translation elongation factor 1 alpha1: *EEF1A1* (Right: ACTTGCCCGAATCTACGTGT, left: TTGCCCGCCAGAACACAG), and protein phosphatase 1, catalytic subunit, gamma isoform: *PPP1CC* (Right: GCTGGACATTCTTACCAGGC, left: GCGATGGCGGATTTAGATA). The PCR reactions were tested for assay efficiency, amplification of residual DNA and the profile of the melting curve (not shown). Relative starting amounts of each gene of interest were determined using the standard curve method, and known amounts of cDNA from the Raji B cell line were used to construct the standard curve (not shown). Data from duplicate reactions for each target gene were averaged and normalized based on levels of expression of the housekeeping genes [7, 24].

2.5 Statistical analyses

All statistical procedures were performed with GraphPad Prism Version 6.04 (GraphPad Software, San Diego, CA). Comparisons between more than two groups were made using Kruskal-Wallis test with multiple comparison correction; group to group comparisons used Mann-Whitney test. Paired analyses were performed using Wilcoxon matched-pairs signed rank test. Correlations were tested using Spearman rank correlation. Categorical variables were analyzed using Fisher's exact test. Tests used are also specified in figure legends.

3. Results

3.1 STAT1 phosphorylation is impaired in sJIA monocytes following IFN γ or IFN α stimulation

Phospho-flow data can be expressed as median fluorescence intensity (MFI) for basal (unstimulated) levels and paired stimulated levels for each phospho-protein/stimulus, or as relative levels of phosphorylation (ratio), calculated by dividing MFI after stimulation by MFI from unstimulated cells. This ratio expresses the fold change in signaling response, a more informative value than MFI alone [19]. Information conveyed by fold change (rather than absolute signal) may be the crucial component of the signaling response [25, 26]. Baseline (unstimulated, U) absolute MFI level of the tested phosphorylated proteins analyzed was similar between sJIA and control monocytes in samples collected between

2002-2009 (Group 1) (Fig. 1A). Following IFN γ stimulation, MFI levels showed that CD14 $^{+}$ monocytes from sJIA patients are able to respond to IFN γ stimulation, but the amplitude of the response is lower than in the control group (Fig. 1A and representative example in Supplementary Fig 1C). Ratios of pSTAT1/IFN γ were lower in CD14 $^{+}$ monocytes in sJIA from both flare and quiescent samples compared to controls (Fig. 1B).

There was no correlation between the pSTAT1/IFN γ ratios and clinical or laboratorial parameters in samples from flare patients. For quiescent patients, a negative correlation between prednisone dose and pSTAT1/IFN γ ratios was found ($r=-0.7326$, $p<0.0001$); this negative association is in line with reported effect of steroids inducing expression of the cytokine signaling inhibitor SOCS1 [27]; this correlation was not observed for flare patients, although these patients were on higher doses of prednisone than quiescent patients.

We also analyzed the monocyte response to IFN α ; analysis of MFI levels showed that CD14 $^{+}$ monocytes from many sJIA quiescence samples, and all controls, respond to IFN α stimulation, whereas monocytes from only few flare samples responded (Fig. 1C). pSTAT1 ratios in response to IFN α were significantly lower in CD14 $^{+}$ monocytes during sJIA flare and quiescence compared to controls (Fig. 1D). No correlation with clinical or laboratory variables was found for pSTAT1/IFN α for flare samples. Similar to pSTAT1/IFN γ , there was a negative correlation between prednisone dose and pSTAT1/IFN α ($r=-0.6390$, $p=0.0364$) for quiescence samples.

In contrast to the IFN/pSTAT1 signaling pathways, other pathways analyzed in sJIA monocytes did not differ from controls. pSTAT6 responses to IL-4 stimulation were robust and similar in sJIA and controls (Fig. 2A,B), as were MFI levels and phosphorylated p38 ratios after LPS stimulation of CD14 $^{+}$ monocytes (Fig. 2C, D). No correlations were found with any disease parameter for pSTAT6/IL4 or pp38/LPS for either flare or quiescence.

We have also analyzed signaling responses in CD16 $^{+}$ monocytes; overall, the pattern of response was similar to CD14 $^{+}$ monocytes, but the responses were of lower amplitude for both sJIA and controls (Supplementary Fig 2).

3.2 Higher levels of SOCS1 gene expression in sJIA monocytes

To investigate the basis of lower interferon signaling in sJIA monocytes, we analyzed the expression of the cytokine signaling inhibitor genes, *SOCS1*, *INPP5D* (SHIP1) and *PIAS1*, which regulate the IFN signal transduction pathway at different steps. SOCS1 is an inhibitor of cytoplasmic Janus kinases (Jak) and STAT1 signaling; SHIP1 inhibits PI3-kinase mediated signaling, relocating from the cytoplasm to the plasma membrane, and it may be a nucleocytoplasmic shuttle protein. PIAS1 is a nuclear protein that inhibits gene expression mediated by STAT1. Experiments were performed with isolated CD14 $^{+}$ monocytes; IFN γ /pSTAT1 phosphorylation in monocytes is similar in isolated monocytes and monocytes within PBMC (not shown). We found that baseline levels of *SOCS1* transcripts in isolated CD14 $^{+}$ monocytes were higher in cells from sJIA flare and quiescence samples in comparison to controls (Fig. 3A). Levels of *INPP5D* and *PIAS1* transcripts were not statistically different between sJIA CD14 $^{+}$ monocytes and controls (Fig 3B, C), and levels of SOCS3 protein, which inhibits IL-6 signaling, were comparable in unstimulated CD14 $^{+}$

and CD16+ monocytes from sJIA and controls (Supplementary Fig. 3). For samples with measurements of both cytokine inhibitors and pSTAT1, the correlations between these variables were tested; flare and quiescence samples were analyzed as a single group. A significant negative correlation between SOCS1 levels and pSTAT1/IFN γ fold change was found (Fig. 3D). Together, these data indicate a specific change in SOCS1 levels in sJIA monocytes and suggest that SOCS1 inhibits IFN γ signaling in these cells. Interestingly, *SOCS1* expression in monocytes from patients at flare, but not quiescence, was negatively correlated with disease duration at time of sampling ($r=-0.5579$, $p=0.0161$). No such correlations were found for INPP5D or PIAS1.

We also investigated other components of the IFN γ pathway. The levels of total STAT1 protein in sJIA CD14+ (Fig. 4A) and CD16+ monocytes (Supplementary Fig. 4A) were similar to control cells. Levels of surface staining of IFN γ R2, the signaling chain of the receptor, were very low as measured by flow cytometry, as previously reported [28], and were not different between sJIA and control cells (not shown); by gene expression, levels of IFN γ R2 were similar between sJIA and controls (Fig. 4B). Surface levels of IFN γ R1, the ligand-binding chain of the heterodimeric IFN γ receptor, were also similar in sJIA CD14+ monocytes and controls (Fig. 4C). We noticed that levels of IFN γ R1 were higher in CD14+ compared to CD16+ monocytes, for both sJIA and control monocytes (Supplementary Fig. 4B), a possible explanation for the lower pSTAT1/IFN γ responses observed in CD16+ compared to CD14+ monocytes.

3.3 Monocytes respond to IFN γ in Group 2 sJIA patients

The data above were obtained using samples collected between 2002 and 2009, with >50% of the samples collected before 2007. Samples in Group 2 were collected between 2011 and 2014, and include a group of treatment naïve patients with recent onset and active disease. Similar to samples from Group 1 patients, monocytes from recent onset patients showed lower responses to IFN γ (Fig. 5A) and lower pSTAT1/IFN γ ratios than the control group (Fig. 5B). However, IFN γ responses and ratios of pSTAT1/IFN γ from these samples in Group 2 were comparable or higher than in the control group (Fig. 5A,B), unlike flare and quiescent samples in Group 1 (Fig 5C).

When we compared demographic and clinical characteristics of the samples in Group 1 and 2, the only significant difference was medication usage in the quiescent samples, specifically use of biological therapies (anti-IL-1 or anti-IL6 receptor, Tables 1 and 2), with higher use of biologicals in samples of Group 2.

These findings led us to try to determine if medication could affect components of the IFN signaling pathway. As the medication usage may be a complex factor to dissect, we investigated the expression of SOCS1 and STAT1 in isolated CD14+ monocytes from a group of sJIA patients treated with anti-IL-1 medication (Rilonacept) as part of the RAPPORT trial [29]. The controlled treatment approach in the RAPPORT trial reduces confounding variables and makes data interpretation more straightforward.

We observed that, after a minimum of 10 weeks on therapy, 3/3 responders tested showed reduced SOCS1 expression levels, on average 38 times (range 93-7.5) in relation to baseline

(Fig. 6). In contrast, SOCS1 level increased slightly compared to baseline in the 2/2 non-responders examined (1.3× and 2.2×). STAT1 levels went in the opposite direction: they increased in 3/3 responders (average 4.8× – range 2.2×-7.7×), and decreased (1.7×) or did not change in the non-responders (Fig. 6). These results suggest an association of anti-IL-1 therapy with decreased SOCS1 and increased STAT1 expression, changes that may result in higher pSTAT1 levels following IFN stimulation, especially in patients responsive to therapy.

4. Discussion

In this work, we investigate signaling in sJIA CD14+ monocytes in response to IFN type II and I. In patients with ongoing disease, there is an impaired STAT1 response to IFN in samples collected between 2002-2009 during both flare and quiescence. The hyporesponse to IFN is specific and not part of an overall defect in monocyte signaling in response to other stimuli. In contrast, analysis of samples collected after 2011 show that monocytes from patients have higher ratios of STAT1 phosphorylation in response to IFN γ than control cells. These patients were receiving biological therapies at the time of sampling. Treatment with biologicals may affect expression of components of IFN signaling pathway to favor enhanced pSTAT1/IFN responses in sJIA. A group of treatment naïve patients with recent disease onset collected after 2011 showed impaired STAT1 phosphorylation, similar to samples collected prior to 2011.

Our results suggest that a reduced IFN response may underlie sJIA pathology, a possibility also implied by findings in an animal model, where IFN γ deficiency led to the appearance of a SJIA-like syndrome [30]. The IFN hyporesponsiveness in sJIA monocytes may be associated with their activation state. Human monocytes polarized to an alternatively activated, regulatory M2 phenotype through endotoxin tolerance induction display impaired STAT1 phosphorylation in response to IFN γ [31]. Inhibition of the IFN γ /pSTAT1 response also is observed during infection of murine macrophages and human monocytes with *Francisella tularensis* [32], an intracellular pathogen that reprograms infected macrophages to express an alternatively activated, M2 phenotype [33]. Of note, in the endotoxin tolerance model, the cells maintain normal responsiveness to IL-4. Similarly, we saw normal levels of pSTAT6 after IL-4 stimulation in sJIA monocytes. An M2-like profile in a subset of sJIA blood monocytes during flare and quiescence [7] indicates that these monocytes may have an anti-inflammatory role.

The defective phosphorylation of STAT1 in response to IFN in sJIA monocytes may be due to higher expression of SOCS1, one of the most important signaling inhibitors of STAT1 [34]. Increased SOCS1 is another feature of M2 polarization [35]. High levels of SOCS1 in circulating monocytes may be especially important close to disease onset, as SOCS1 expression appears negatively correlated with disease duration in active patients. Previous studies have shown that induction of SOCS1 occurs during M2-type polarization, whereas SOCS3 increases during M1 polarization [36, 37]. Furthermore, SOCS1 has a functional role in maintaining M2 polarization [36]. In our analysis of sJIA monocytes, SOCS1 gene expression is increased and expression of SOCS3 protein is not altered. Similarly, in an animal model of SLE, SOCS1 increases and IFN γ signaling is attenuated with disease

development, whereas SOCS3 does not increase [38]. Preliminary experiments show that plasma from sJIA patients induces gene expression of SOCS1, but not of SOCS3, in normal monocytes (not shown). An anti-apoptosis role for SOCS1 also has been demonstrated in immune cells [39], and, interestingly, we have found that sJIA monocytes are more resistant to apoptosis induction *in vitro* [40].

SOCS1 can be induced by several cytokines, including IFN γ as a part of a negative feedback loop. However, IFN γ might not be the main factor driving increased SOCS1 expression in sJIA monocytes. SOCS1 can also be induced by IL-21 [41], one product of Th17 cells [42]. A higher proportion of Th17 cells among circulating CD4 $^{+}$ and CD4 $^{-}$ T cells has been reported in sJIA patients, independent of disease status, in comparison to age-matched controls [13]. This result would be in line with known effects of IL-1 and IL-6 on Th17 differentiation [43]. Elevated levels of circulating IL-17 in sJIA compared to healthy controls have also been described [11], and we observed that CD4 $^{+}$ T cells isolated from sJIA patients and cultured in Th17-inducing conditions release more IL-17 than cells from control subjects (Wong, Macaubas and Mellins, unpublished observations). SOCS1 is important for Th17 differentiation, as suppressor of IFN γ signaling [44]. In sJIA, higher SOCS1 levels may promote Th17 development, leading in turn to production of IL-21 and further induction of SOCS1.

The use of biologicals, specifically anti-cytokine therapy, may alter initially defective STAT1 phosphorylation, especially in patients who respond to the therapy. Our findings are similar to a study by Sikora et al [45], who showed that fresh, positively (CD14 $^{+}$) isolated, sJIA CD14 $^{+}$ monocytes from 4 active and 3 inactive sJIA patients did not exhibit hyporesponsiveness to IFN γ , as assessed by levels of pSTAT1. All the inactive sJIA patients were on IL-1 blockade and showed a higher fold increase in IFN γ -induced pSTAT1 than active patients or controls [45]. Interestingly, defective phosphorylation in response to IL-18 in sJIA NK cells was also restored following successful treatment with an IL-1 inhibitor [46].

The changes in IFN signaling following anti-cytokine therapy might be a direct effect of the blockade of IL-1 or IL-6 in sJIA. It has been shown that both IL-1 β and IL-6 can induce SOCS1 expression [47, 48], and the blockade of these cytokines could affect SOCS1 levels and increase IFN responsiveness. In fact, blockade of IL-1 reveals an IFN signature in whole blood from some sJIA patients [15]. We found that in CD14 $^{+}$ monocytes from patients responsive to anti-IL1 therapy, SOCS1 levels went down and STAT1 levels went up, while in non-responders, the opposite pattern was observed, suggesting that successful response to anti-cytokine therapy is associated with effects on IFN signaling pathway that may result in a higher pSTAT1/IFN response.

IFNs are important immune regulators, and an impaired IFN response may contribute to sJIA pathology by dysregulating responses to infectious agents. It is unknown if an impaired IFN response is primarily or secondarily associated with sJIA. Decreased responses to IFN γ and IFN α could contribute to exuberant IL-1 β activity in sJIA, as both type I IFN α and IFN γ have been shown to be able to suppress production of IL-1 by different mechanisms [49, 50]. Also, IFN γ and IFN α inhibit IL-1 β effects, including induction of pro-inflammatory

mediators, synthesis of matrix metalloproteinases (MMPs) and cartilage degradation [51, 52]. Alternatively, excess IL-1 and IL-6 may result in blockade of the IFN pathway [15], for example, by inducing SOCS1 production.

Further studies are needed to elucidate the specific mechanisms regulating the IFN response in sJIA, and to determine the association between medication profile, medication response and the IFN response. The elucidation of the molecular and cellular basis of the interplay between IFNs and IL-1 β in sJIA is an important scientific goal.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- sJIA CD14 monocytes show specific, defective STAT1 phosphorylation downstream of IFNs
- sJIA monocytes express higher transcript levels of the IFN signaling inhibitor *SOCS1*
- Recent changes in medication use in sJIA may alter the IFN hyporesponsiveness
- Impaired IFN/pSTAT1 signaling consistent with monocyte skewing away from M1 phenotype
- Impaired IFN/pSTAT1 signaling may contribute to disease pathology in sJIA

Figure 1.

Defective response to IFN γ and IFN α in sJIA monocytes in samples collected between 2002-2009 (Group 1). **A.** Median Fluorescence Intensity (MFI) of phosphorylated STAT1 (pSTAT1) in unstimulated (U) and IFN γ -stimulated CD14+ monocytes in sJIA and controls. PBMCs were stimulated *ex vivo* with cytokine, fixed, permeabilized, and stained for surface molecules and intracellular phospho-protein. **B.** IFN γ -stimulated pSTAT1 ratios in CD14+ monocytes from sJIA and controls, calculated by dividing the MFI from IFN γ -stimulated by the MFI from unstimulated monocytes, expressed in a log₂ scale. **C.** MFI of pSTAT1 in unstimulated (U) and IFN α -stimulated CD14+ monocytes in sJIA and controls. **D.** IFN α -stimulated pSTAT1 ratios in CD14+ monocytes from sJIA and controls. Q: quiescence; F: flare. Statistical analysis used Kruskal-Wallis with Dunn's Multiple Comparison test (represented by *), or Mann-Whitney test (#) for group to group comparisons. One symbol, $p < 0.05$; two symbols, $p < 0.01$.

Figure 2.

Other signaling pathways are preserved in sJIA monocytes. **A.** MFI of pSTAT6 in unstimulated (U) and IL4-stimulated CD14+ monocytes in sJIA and controls. **B.** IL4-stimulated pSTAT6 ratios in CD14+ monocytes from sJIA and controls. **C.** MFI of pp38 in unstimulated (U) and LPS-stimulated CD14+ monocytes in sJIA and controls. **D.** LPS-stimulated pp38 ratios in CD14+ monocytes from sJIA and controls. PBMCs were processed and ratios calculated as described in Figure 1. Q: quiescence; F: flare. Statistical analysis used Kruskal-Wallis with Dunn's Multiple Comparison test or Mann-Whitney test for group to group comparisons. All comparisons were deemed non-significant ($p > 0.05$).

Figure 3.

Higher expression of *SOCS1* in sJIA monocytes. **A.** Expression of *SOCS1*, *INPP5D* (SHIP1) (**B**) and *PIAS1* (**C**) in sJIA and control CD14+ monocytes. Monocytes were positively selected with CD14 microbeads, total RNA was extracted and RT-PCR was performed. Results expressed as relative levels in relation to transcript levels from housekeeping genes. Detection of SOCS-1 protein requires higher cell numbers than were available in our pediatric cohorts (not shown). Statistical analysis used Kruskal-Wallis with Dunn's Multiple Comparison test (*) or Mann-Whitney test for group to group comparisons; *, $p < 0.05$; **, $p < 0.01$. **D.** Correlation between expression level of *SOCS1*, *INPP5D* (SHIP1) (**E**) and *PIAS1* (**F**) and ratios of IFN γ /pSTAT1 in CD14+ monocytes, analyzed using Spearman rank correlation. Q: Quiescence; F: Flare.

Figure 4.

Total STAT1 and IFN γ receptor levels are similar between sJIA and control monocytes. **A.** Total STAT1 in CD14+ monocytes from sJIA and control subjects. Total STAT1 levels were assessed by intracellular staining of permeabilized cells and flow cytometry. Data are expressed as MFI. **B.** Expression of *IFNGR2*. Total RNA was extracted from PBMC from sJIA patients and controls, and RT-PCR was performed. Results expressed as relative levels in relation to transcript levels from housekeeping gene. **C.** Surface expression of IFN γ R1 in CD14+ monocytes from sJIA and control subjects, assessed by flow cytometry. Data are expressed as MFI. Q: quiescence; F: flare. Statistical analysis used Kruskal-Wallis with Dunn's Multiple Comparison test or Mann-Whitney test. All comparisons were deemed non-significant ($p > 0.05$).

Figure 5.

Response to IFN γ in sJIA patients collected between 2011-2014 (Group 2). **A.** MFI of pSTAT1 in unstimulated (U) and IFN γ -stimulated CD14+ monocytes in sJIA and controls. **B.** IFN γ -stimulated pSTAT1 ratios in CD14+ monocytes from sJIA and controls. **C.** IFN γ -stimulated pSTAT1 ratios from sJIA samples and controls from both groups. PBMCs were processed as described in Figure 1. Q: quiescence; F: flare. G1: Group 1 (samples collected between 2002-2009); G2: Group 2 (samples collected between 2011-2014). Statistical analysis used Mann Whitney test for group to group comparisons. # $p < 0.05$; ## $p < 0.01$.

Figure 6.

Expression level of *SOCS1* and *STAT1* in unstimulated CD14+ monocytes isolated from 5 sJIA patients from the RAPPORT trial (Patients A-E) at different time points. Monocytes were processed as in Figure 3. Responders (A-C) and non-responders (D, E) were defined based on American College of Rheumatology (ACR) Pediatric 30 criteria. W: week.

Table 1

Subjects demographic and clinical characteristics – Group 1

	sJIA Quiescence	sJIA Flare	Control
N (subjects) ¹	35	37	46
Female/Male	19/16	18/19	22/24
Median age (yr) at disease onset (range) (all)	5.4(0.5-16)		NA ⁸
Median age (yr) at sample (range) ²	13.5 (1.2-20.1)	9.9 (1-18.9)	12.3 (5-23.8)
Fever/total no. samples (%) ³	0/58 (0%)	9/63 (14%)	NA
Rash/total no. samples (%) ⁴	0/58 (0%)	14/63 (22%)	NA
Median joint count (range) ⁵	0 (0-4)	3 (0-28)	NA
Median WBC (×10 ³ /ul) (range) ⁵	8.1 (3.4-15.3)	12.5 (5.8-50)	NA
Median platelets (×10 ³ /ul) (range) ⁵	294.5 (201-496)	467 (198-1103)	NA
Median ESR (mm/h) (range) ⁵	7 (0-38)	36 (3-125)	NA
Median prednisone dose, mg/kg/day (range) ⁶	0 (0-1.04)	0.15 (0-3.16)	NA
NSAIDs/total no. samples (%) ⁴	38/58 (66%)	53/63 (84%)	NA
Methotrexate/total no. samples (%)	23/58 (40%)	27/63 (43%)	NA
Anti-TNF /total no. samples (%)	24/58 (41%)	25/63 (40%)	NA
Biologicals /total no. samples (%)	11/58 (19%)	13/63 (21%)	NA

Other medications: Cyclosporine A, thalidomide (one each flare and quiescence), and 1 flare patient with abatacept.

¹ Twenty-four patients contributed samples during flare and quiescence

² sJIA flare samples are younger than sJIA quiescence and controls (p<0.05); Kruskal Wallis with Dunn's Multiple Comparison test

³ higher in sJIA flare than sJIA quiescence (p<0.003); Fisher's exact test

⁴ higher in sJIA flare than sJIA quiescence (p<0.0001); Fisher's exact test

⁵ higher in sJIA flare than sJIA quiescence (p<0.0001); Mann-Whitney test

⁶ higher in sJIA flare than sJIA quiescence (p=0.0002); Mann-Whitney test

⁷ anti-IL1 or anti-IL6R medication

⁸ not applicable

Table 2

Subjects demographic and clinical characteristics – Group 2

	sJIA Quiescence	sJIA Flare	Recent Onset	Control
N (subjects)	4	5	4	8
Female/Male	4/0	3/2	2/2	4/4
Median age (yr) at disease onset (range)	7.2 (1.5-11.6)	9 (1.5-13.3)	5.4 (2.9-7.3)	NA ⁸
Median age (yr) at sample (range)	10.9 (2.4-17.2)	10.3 (3.1-15.3)	5.5 (2.9-7.9)	9.4 (4.7-13.7)
Fever/total no. samples (%)	0/4 (0%)	4/5(80%)	4/4 (100%)	NA
Rash/total no. samples (%)	0/4 (0%)	1/5 (20%) ¹	4/4 (100%)	NA
Median joint count (range)	0 (0-1) ²	7 (1-14)	14.5 (0-28)	NA
Median WBC (×10 ³ /ul) (range)	7.6 (4.2-9.2)	17.2 (3.6-19.4)	21.1 (13.5-22.4)	NA
Median platelets (×10 ³ /ul) (range)	301 (207-435) ³	425 (162-470)	526 (480-625)	NA
Median ESR (mm/h) (range)	8 (3-19) ⁴	78 (42-80)	74 (66-99)	NA
Median prednisone dose, mg/kg/day (range)	0 (0)	0.13 (0-2) ¹	0	NA
NSAIDs/total no. samples (%)	2/4 (50%)	2/5 (40%)	4/4 (100%)	NA
Methotrexate/total no. samples (%)	2/4 (50%)	0/5 (0%)	0/4 (0%)	NA
Anti-TNF /total no. samples (%)	0/4 (0%)	0/5 (0%)	0/4 (0%)	NA
Biologicals /total no. samples (%) ⁷	4/4 (100%)	4/5 (80%) ⁵	0/4 (0%) ⁶	NA

¹ higher in sJIA new diagnosis than sJIA flare (p<0.05); Mann-Whitney test² higher in sJIA flare than sJIA quiescence (p<0.05); Mann-Whitney test³ higher in sJIA new diagnosis than sJIA quiescence (p<0.05); Mann-Whitney test⁴ higher in sJIA new diagnosis than sJIA quiescence (p<0.05); Mann-Whitney test⁵ 5th patient had a steroid pulse (no biologicals)⁶ higher in sJIA flare and in sJIA quiescence than sJIA new diagnosis (p<0.05); Mann-Whitney test⁷ anti-IL1 or anti-IL6R medication⁸ not applicable