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Alternative activation in systemic juvenile idiopathic arthritis monocytes

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Abstract

Systemic juvenile idiopathic arthritis (SJIA) is a chronic autoinflammatory condition. The association with macrophage activation syndrome, and the therapeutic efficacy of inhibiting monocyte-derived cytokines, has implicated these cells in SJIA pathogenesis. To characterize the activation state (classical/M1 versus alternative/M2) of SJIA monocytes, we immunophenotyped monocytes using several approaches. Monocyte transcripts were analyzed by microarray and quantitative PCR. Surface proteins were measured at the single cell level using flow cytometry. Cytokine production was evaluated by intracellular staining and ELISA. CD14⁺⁺CD16[–] and CD14⁺CD16⁺ monocyte subsets are activated in SJIA. A mixed M1/M2 activation phenotype is apparent at the single cell level, especially during flare. Consistent with an M2 phenotype, SJIA monocytes produce IL-1 β after LPS exposure, but do not secrete it. Despite the inflammatory nature of active SJIA, circulating monocytes demonstrate significant anti-inflammatory features. The persistence of some of these phenotypes during clinically inactive disease argues that this state reflects compensated inflammation.

Keywords

Juvenile arthritis; monocytes; activation phenotype; cytokines

1. Introduction

Systemic juvenile idiopathic arthritis (SJIA) is a rheumatic condition characterized by remitting fever, transient rash, and relapsing arthritis. Although SJIA has been considered an autoimmune disease, the paucity of specific autoantibodies or predisposing major histocompatibility complex (MHC) alleles suggest an auto-inflammatory nature, in contrast to the non-systemic subtypes of JIA [1–2]. Consistent with this, SJIA clinical features

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include thrombocytosis, granulocytosis, and the up-regulation of acute-phase proteins [3]. The fact that macrophage activation syndrome (MAS) and amyloidosis are complications of SJIA further supports the involvement of the innate immune system and monocyte/macrophages in particular [4].

Mechanistic studies of SJIA also point to activation of the monocyte lineage. Transcriptome analyses of PBMC reflect likely stimulation of innate immune pathways in monocytes at flare [5–8]. Pro-inflammatory cytokines, including interleukin-1 β (IL-1 β), IL-6, IL-18, and TNF α , are elevated in the serum and/or synovial fluid of SJIA patients with active disease [9–10]; activated monocytes are potential sources of these cytokines. Importantly, biologic therapies targeting IL-1 β and IL-6 ameliorate disease, at least in subsets of patients [6, 11–14]. High serum concentrations of calcium-binding proteins S100A8, S100A9, and S100A12 correlate with disease activity; these proteins also indicate the activation of monocytes and/or granulocytes [3]. Thus, numerous lines of evidence suggest that activation of monocytes may play an important role in SJIA pathophysiology.

Monocytes can be induced into specific activation phenotypes, depending on the microenvironment. Activation by IFN- γ and LPS results in “classical” or M1 monocytes/macrophages and is strongly linked with T helper 1 (Th1) polarization, whereas activation by type 2 cytokines IL-4 and IL-13 results in “alternative” or M2 monocyte/macrophages, which are associated with Th2 polarization [15]. M1 and M2 phenotypes likely represent the extremes of a continuum of monocyte activation states. M1 polarization confers a pro-inflammatory phenotype, associated with elevated production of IL-1 β , TNF α , and IL-6 [16] and enhanced killing of intracellular pathogens [17]. M2 polarization is associated with regulatory and inhibitory functions, counterbalancing proinflammatory mechanisms, and M2 monocytes provide a niche for chronic infection by some pathogens, such as parasites and certain bacteria [17–18]. Although much remains to be clarified regarding the alternative activation of monocytes/macrophages [19], the M1/M2 paradigm already has been used to characterize monocyte/macrophage activation in situations other than Th1/Th2 dominance. Other M2-associated conditions include endotoxin tolerance [20], obesity and insulin resistance, atherogenesis and tumor-associated macrophages [21]. Notably, cells with a mixed M1/M2 phenotype, such as monocytic myeloid-derived suppressor cells (MDSC) have been described in cancer, infectious diseases, and autoimmune diseases, and are potent inhibitors of immune responses, both adaptive as well as innate responses [22–23].

Despite the pro-inflammatory nature of the SJIA cytokine environment, some markers associated with an M2 profile are highly expressed in SJIA. These include IL-1Ra [24], IL-10 [7], MS4A4A [8], surface [25] and soluble CD163 [26] and serum heme oxygenase-1 [27–28]. These results suggest that monocytes may play both pro- and anti-inflammatory roles in SJIA.

Human monocytes also can be divided into two major subsets, based on cell surface expression of CD14 and CD16 [29–30]. The CD14⁺⁺CD16[–] subset (CD14⁺ subset, hereafter) is predominant (~85% of monocytes) in the absence of infection. This subset expresses higher levels of molecules with potential antimicrobial function [31] and of receptors involved in endocytosis [32]. The CD14⁺CD16⁺ subset (CD16⁺ subset) is expanded in a range of inflammatory conditions, including Crohn’s disease, rheumatoid arthritis, asthma, sarcoidosis [33], Kawasaki disease [34] and hemophagocytic syndrome [35]. This subset is associated with production of pro-inflammatory cytokines and has elevated Fc-mediated phagocytosis [33]. The CD14⁺ and CD16⁺ subsets appear to have a common myeloid progenitor [32], and may even have a precursor (CD14⁺)/product (CD16⁺) relationship [30]. However, the circulating subsets express a distinct profile of

adhesion molecules, chemokine receptors and cell activation markers, with CD16+ expressing more macrophage- and dendritic cell-related markers [32].

Previous work from our group demonstrated expansion of the monocyte lineage in active SJIA [36]. Both CD14+ and CD16+ subsets contribute to this increase and proportions observed in normal individuals are maintained. Further, both at flare and quiescence, we observed increased expression of the CD14 and CD16 surface markers in their respective subsets, a sign of monocyte activation [36]. In addition, increased CD14 expression may be linked to increased SJIA monocyte resistance to apoptosis [37], another phenotype we have described [38].

To further explore the role of monocytes in SJIA pathogenesis, we sought to determine the activation profile of circulating CD14+ and CD16+ monocyte subsets in SJIA at different disease stages. We analyzed gene expression, surface phenotype and cytokine production. Our results suggest a novel activation state of circulating SJIA monocytes that may shed light on the role that these cells play in disease.

2. Patients and methods

2.1 Subject population and clinical data collection

The Institutional Review Board of Stanford University approved this study. SJIA patients were followed at the Pediatric Rheumatology Clinic at Lucile Packard Children's Hospital, and were enrolled after consent. All SJIA study subjects met the International League of Associations for Rheumatology criteria for JIA [39]. Comprehensive clinical information was collected at each SJIA patient visit [36]. Clinical status was assigned according to our scoring system to grade severity of systemic disease manifestations or arthritis ([36, 40–41] and Supplemental Tables 1 and 2). Each sample was classified as “flare” (systemic score of ≥ 1) or “quiescence” (systemic score=0, arthritis score= A or B). Control samples were from age-matched immunologically healthy children (based on clinical history) from the Stanford Endocrinology Clinic. The majority of these children were seen for growth delay (mostly constitutional delay) or precocious puberty. The demographics of patient and control groups were similar for all assays shown, with the exception of SJIA samples analyzed by RT-qPCR, where the paired samples from quiescence were from a later time (older age) than the samples from flare. Venous blood samples from all subjects were treated anonymously throughout the analysis; these samples were obtained only when there was a clinical need for blood tests [36].

2.2 RNA preparation, reverse transcription (RT)-qPCR and microarray analysis of SJIA PBMCs

Purified PBMCs were lysed in RLT reagent (QIAGEN, Valencia, CA) and total RNA was isolated (RNeasy mini kit, QIAGEN), including on-column DNase I treatment, per manufacturer's instructions. The RNA concentration and purity were measured using either the Ribogreen assay (Molecular Probes, USA) or by spectrophotometry. The integrity of the RNA samples was also checked by either agarose gel electrophoresis or using the Agilent 2100 Bioanalyzer (Agilent Technologies, USA). The RT-qPCR assay was performed as described [42]. Briefly, all reactions were performed in duplicate as single-step RT-PCR reactions, using total PBMC RNA and SYBR green chemistry. Data from duplicate reactions for each gene were averaged and normalized based on the average of the expression levels of 5 housekeeping genes: EEF1A1, PPP1CA, PPP1CC, RPL12, RPL41.

PBMC RNA samples for microarray analysis were processed as described [38, 43] and analyzed using Lymphochip cDNA microarrays [44]. Hierarchical clustering was performed with the Cluster program and visualized using TreeView [45]. Differentially expressed

genes (flare vs. quiescence) were identified by Significance Analysis of Microarrays (SAM) [46].

2.3 Monocyte phenotyping by flow cytometry

Monocytes were identified using HLA-DR, CD14 and CD16 (BD Biosciences, San Jose, CA); other surface markers were: CD40, CD80, CD86, CD163, CD206 (BD Biosciences) and CCR2 (R&D Systems, Minneapolis, MN). For intracellular cytokines, antibodies against IL-1 β , IL-6, TNF and IL-10 were used (BD Biosciences). Antibody fluorochromes were chosen as appropriate for acquisition on a FACSCalibur flow cytometer (BD Biosciences). Data were analyzed with FlowJo (Treestar, Ashland, OR).

2.4 *In vitro* stimulation of PBMC and cytokine detection

Previously frozen, thawed and rested PBMC [1×10^6 cells/ml in RPMI with 10% heat-inactivated (HI) FCS] were stimulated with LPS (from *Escherichia coli*, serotype 026:B6, Sigma, St. Louis, MO) plus Brefeldin A (Sigma) at 10 μ g/ml. Control samples, containing only Brefeldin A, were processed in parallel. Samples for analysis of IL-1 β , IL-6 and TNF were incubated at 37°C for 4 h with 20 ng/ml of LPS and for IL-10 with 1 μ g/ml of LPS for 24h. After the stimulation, cells were stained for monocyte markers, fixed and permeabilized with Cytofix/Cytoperm solution (BD Biosciences) and stained for cytokines. Secreted IL-1 β was detected in cell-free supernatants from PBMC or monocytes (enriched by adherence to plastic for 2h followed by washes to remove non-adherent cells) stimulated for 4h or 24h with 20 ng/ml or 1 μ g/ml of LPS; the human IL-1 β Cytometric Bead Array (CBA, BD Biosciences) was used. Comparison of fresh and frozen cells showed that secretion of IL-1 β was significantly increased in supernatants of previously frozen PBMC in comparison to fresh PBMC. Importantly, this pattern was observed for both control and SJIA samples (Supplementary Fig. 1A). It appears that previously frozen cells, both SJIA and controls, secrete larger amounts of cytokine, as levels of secreted IL-8 from frozen cells also tend to increase, in both SJIA and controls samples (Suppl. Fig. 1B). Intracellular levels of TNF with or without LPS stimulation, were similar between fresh and frozen samples, especially at a higher LPS dose (Supplementary Fig 1C). Levels of intracellular IL-1 β following LPS stimulation tend to be lower in frozen monocytes compared to fresh monocytes. This pattern was similar for both SJIA and control samples (Suppl. Fig. 1D).

2.5 Statistical analysis

Multiple group comparisons used ANOVA with Bonferroni correction for multiple comparisons. Group to group comparisons were made using Student's t-test or a nonparametric test. All tests were performed with GraphPad Prism (GraphPad Software, San Diego, CA).

3. Results

3.1 Activation phenotype of SJIA PBMC by gene expression

In a microarray screen of paired flare/quiescence PBMC samples from 14 SJIA subjects, we, like others [5–8], found that gene expression patterns correlate with disease activity (Fig. 1A). In the “flare signature,” we observed mRNAs linked to M1 and M2 phenotypes [15, 20, 47–49], indicating that the PBMCs either included a mixture of M1 and M2 cells or included cells with a mixed M1/M2 phenotype. Follow-up analysis by RT-qPCR showed that, for ~40% of M1- and M2-associated genes, the expression levels were ≥ 2 times higher than the fold increase in monocyte number at flare (Fig. 1B), arguing that monocytes are activated, not just expanded in number. The differences in flare versus quiescence profiles could not be attributed to differences in medication use in the sample groups (not shown).

3.2 Surface phenotype of CD14 and CD16 monocyte subsets in SJIA

In the microarray and in the RT-qPCR we also observed co-expression of CD14 and CD16-associated genes in PBMCs (Supplementary Fig 1A, B). Using cell surface phenotyping (Fig. 2A), we found the distribution of the CD14⁺ and CD16⁺ subsets was not altered in SJIA cells in comparison to control subjects (Fig. 2B), in line with our previous results [36]. We then determined the expression of several M1- and M2-associated surface markers in the CD14 and CD16 monocyte subsets during flare and quiescence in comparison to controls. More robust changes in markers were observed during flare. Increases in M1-associated markers CCR2, [MCP-1 receptor involved in monocyte infiltration into tissue sites [47]], and co-stimulatory molecules, CD40 [50–51] and CD80 [17], were found on SJIA CD14⁺ monocytes at flare compared to quiescence or controls (Fig. 3A). However, CD14⁺ cells did not show increased CD86 or HLA-DR, proteins that are enhanced by the M1-polarizing cytokine, IFN γ [17, 52] (Fig. 3A). Higher levels of the M2 markers CD163 (scavenger receptor), CD206 (mannose receptor) and CD14 [15] were also expressed on CD14⁺ monocytes during flare (Fig. 3B). CD163 expression is particularly associated with IL-10-induced alternative activation of macrophages [50]. During quiescence, levels of CD14 were higher on CD14⁺ monocytes than in controls, although lower than on CD14⁺ cells from flare samples. Other M2 markers were expressed during quiescence at levels that trend toward higher than controls (Fig. 3B).

CD16⁺ monocytes showed increased expression of the M1 markers CD16 [47] and CD40 at flare (Fig. 3A), without statistically significant changes in other M1 or M2 markers (Fig 3A, B). During quiescence, levels of CD16 (M1 marker) were higher on CD16⁺ monocytes in comparison to controls (Fig. 3A).

To distinguish between a mixture of M1 and M2 cells and cells co-expressing M1 and M2 markers, we tested 6 additional SJIA flare samples. In all SJIA samples, we found that >90% of CD14⁺ monocytes co-express the M1 marker CD40 and the M2 marker CD163, and the CD40 and the M2 marker CD206; in samples from 4 controls, an average of 43% of CD14⁺ monocytes co-express these markers; a representative example is shown in Figure 3C.

Our results argue that CD14⁺ SJIA monocytes express a mixed M1/M2-type phenotype at flare and exhibit a trend toward a more M2-like picture at quiescence. By marker profile alone, CD16⁺ monocytes appear more M1-like at flare and quiescence (but see below). Higher levels of CD14 and CD16 during quiescence indicate that monocyte phenotypes are not equivalent to control monocytes, even when SJIA is controlled or inactive.

3.3 Cytokine profiles of SJIA monocytes

We next determined the cytokine profile of SJIA CD14⁺ and CD16⁺ monocytes, as M1 and M2 activation states are associated with production of different cytokines [17]. We did not observe differences in ‘spontaneous’ cytokine production [i.e., in the presence of brefeldin (B) only] between SJIA and control monocytes, in either subset (Fig. 4). In monocytes from both SJIA and control subjects, LPS stimulation induced robust IL-1 β , TNF α , and detectable IL-6 production in both monocyte subsets, with the exception of IL-6 in CD16⁺ monocytes at flare (Table 1). LPS stimulated IL-10 in CD14⁺, but not CD16⁺ cells (Table 1), as previously reported [53]. SJIA monocytes from both flare and quiescent samples responded with higher levels of IL-1 β production than controls, whereas IL-6, TNF α and IL-10 responses were similar to controls (Fig. 4). We next tested whether higher intracellular levels of IL-1 β corresponded to higher secreted levels. Strikingly, secretion of IL-1 β was lower in culture supernatants from SJIA compared to control PBMC after LPS (Fig. 5A), regardless of prednisone treatment (Fig. 5B); similar results were obtained with shorter

stimulation period (4 h, Fig. 5C) and higher dose of LPS (1 μ g/ml, Fig. 5D). Monocytes that were enriched by plastic adherence also gave comparable results (not shown). Both previously frozen and fresh cells showed lower IL-1 β secretion in response to LPS in SJIA compared to normal subjects (Suppl. Fig. 1A). As IL-1 β secretion appears to be directly linked to processing of pro-IL-1 β to mature IL-1 β and secretion may even be enhanced by brefeldin [54–55], the pattern of high intracellular IL-1 β and low secreted IL-1 β suggests decreased pro-IL-1 β cleavage. This pattern is observed in both monocyte subsets, regardless of disease activity, and is similar to a previously described M2 pattern [48].

4. Discussion

Multiple lines of evidence implicate monocytes in SJIA pathogenesis. However, the specific role of monocytes in SJIA is still unknown, and relatively few studies have examined monocyte phenotype in depth. Using the framework of M1 and M2 phenotypes, we analyzed the activation state of SJIA monocyte subsets during flare and quiescence. Our analysis highlights a mixed M1/M2-type activation program, especially during disease flare. Our results showing expression of M2-related genes and surface expression of CD163 corroborate and expand other indications of some degree of alternative activation in SJIA during flare [7–8, 24–27]. However, our study represents, to the best of our knowledge, the first in-depth analysis of protein expression at the single cell level in SJIA and reveals that a mixed M1/M2 phenotype is present at the level of individual cells. This activation profile is similar to the previously described MSDC, detected in cancer and infectious diseases [23]. Cells that express a mixed phenotype have been also observed in other conditions, such as circulating monocytes in sepsis [56–57], and adipose tissue macrophages (ATM) in obesity [50]. This mixed M1/M2 phenotype has been mostly associated with suppression or regulation of immune responses. These results indicate that circulating monocytes in SJIA may have a suppressive/regulatory role.

During quiescence, evidence of M1 activation is largely absent, but expression of M2-related markers is observed. Although the changes during quiescence are subtle compared to changes during flare, comparisons to cells from control individuals show that SJIA quiescence monocytes have not reverted to normal.

As we have reported before [36], the distribution of the CD14⁺ and CD16⁺ subsets is normal in SJIA. In the current study, we did not find evidence for preferential activation of a particular monocyte subset during active SJIA. For CD14⁺ monocytes, we observed co-expression of M1/M2 surface markers. For CD16⁺ monocytes, surface markers at flare were more M1-like, but cytokine production indicates M2-like features. Thus, both monocyte subsets show a M1/M2 profile during flare and a more M2-like activation program when disease is controlled or in remission.

As noted, monocyte activation phenotype is also defined by the cytokine profile. We did not find evidence for higher expression of IL-1 β , IL-6, IL-10 or TNF in unstimulated SJIA monocytes, tested after freeze/thaw/rest protocols. Following LPS stimulation, SJIA monocytes increased intracellular production of IL-6, IL-10 and TNF, cytokine networks that are implicated in SJIA [10]. However, the increases were comparable to those seen in stimulated control monocytes, even when SJIA monocytes from disease flare were assayed. Interestingly, transcript levels for these cytokines were noted to be elevated during SJIA flare compared to quiescence, in this and other studies [7], suggesting posttranscriptional control of levels of these cytokines. For IL-10, this finding might be evidence of M2-type polarization, as polarization of cells towards M2 phenotype by induction of LPS tolerance is associated with elevated IL-10 gene expression, but normal IL-10 protein production [20].

In contrast to the other cytokines tested, intracellular levels of LPS-induced IL-1 β were higher in SJIA monocytes from both flare and quiescence than in control monocytes. Strikingly, however, the levels of secreted IL-1 β in response to LPS were lower in SJIA samples than in controls. IL-1 β is synthesized as pro-IL-1 β and requires inflammasome assembly and activation of caspase 1 for processing to mature IL-1 β , which is then secreted [55]. Scotton et al. [48] demonstrated that stimulation of human monocytes with IL-13 (M2 polarization) is associated with downregulation of caspase-1 expression and a reduction in LPS-induced caspase 1 activity, with resulting reduced cleavage of pro-IL-1 β and diminished secretion of LPS-induced IL-1 β . Gattorno et al reported that LPS-induced IL-1 β secretion was low in fresh SJIA monocytes and that caspase-1 activation was undetectable: these phenotypes persisted even after activation with ATP, a known enhancer of caspase 1 activation [11]. In the Gattorno study, low IL-1 β secretion after LPS was associated with steroid treatment (differing from our results), but LPS+ATP induced IL-1 β secretion was low, regardless of steroid treatment. The high intracellular IL-1 β /low IL-1 β secretion observed in SJIA resembles an intermediate M2 phenotype as described in a study of murine macrophages [58]. Specifically, cells partially polarized towards an M2 phenotype show high intracellular IL-1 β , whereas fully polarized M1 and M2 cells show high or very low intracellular IL-1 β , respectively. Interestingly, the small number of patients in our study who received newer biologicals (the anti-IL6R, Actemra and the IL-1 trap, Riloncept) showed a phenotype further skewed towards M2 (low intracellular and secreted IL-1 β , Suppl. Fig. 1). Further investigation will be needed to determine whether the lack of IL-1 β secretion by SJIA monocytes reflects a defect in inflammasome assembly and/or activation or results from changes in the IL-1 β /IL-18 secretion pathway in monocytes, which remains poorly defined [55].

There is an apparent discrepancy between the reduced IL-1 β secretion by circulating SJIA monocytes and the fact that a significant proportion of SJIA patients improve clinically with IL-1 inhibition [6, 11, 13]. One possible explanation is that IL-1 β may be secreted from other cellular sources, such as neutrophils [59], B cells [60] or endothelial cells [61] in SJIA. Indeed, the M1/M2 monocyte phenotype we observe may be part of a negative feedback loop associated with a high IL-1 β environment. Interestingly, anakinra treatment in SJIA leads to increased expression of the ATP receptor, P2RX7, in PBMC [13]. The P2RX7 receptor mediates autocrine ATP stimulation of caspase-1 activation in monocytes [62]. The anakinra-mediated increase in P2RX7 implies that IL-1 suppresses this receptor. In fact, Allantaz et al [63] found that one of 12 genes that could accurately classify SJIA patients was chloride intracellular channel 2 (CLIC-2), and chloride has an inhibitory effect on IL-1 β secretion through effects on P2RX7 [64]. It will be of interest to assess cell surface expression of this receptor on SJIA monocytes. Another plausible scenario that would rationalize our monocyte results with the therapeutic efficacy of IL-1 inhibition is that the SJIA monocytes containing pro-IL-1 β may be recruited to sites of inflammation and proceed to die by proinflammatory-mediated cell death, such as pyroptosis, and release pro-IL-1 β , which can be cleaved by caspase-1-independent mechanisms [65]. It also is possible that the conditions in our assays lead to low secretion of LPS-induced IL-1 β in SJIA monocytes which differs from *in vivo* monocyte behavior. Of note, however, lower IL-1 β secretion by fresh SJIA monocytes has been noted by others [11], and is not restricted to LPS stimulation, as described by Gattorno et al [11]. Additional work is needed to elucidate the regulation of the IL-1 network in SJIA patients.

The specific triggers for the development of a mixed M1/M2-like phenotype in SJIA monocytes during active disease remain to be determined. We found higher levels of IL-13 transcripts in PBMCs during flare, but plasma levels were not altered, making a contribution of IL-13 less likely (not shown). Another possibility is that M2-like responses are favored in SJIA by defective NK cell cytotoxic activity. In an animal model, the absence of NK cells

facilitates expansion of M2-type monocytes [66]. NK cell and CD8⁺ T cell cytotoxic functions have been noted to be defective in SJIA [67], and polymorphisms in genes associated with cytolytic function may contribute to this phenotype in SJIA [68–70].

During disease quiescence, prolonged M2-type polarization may reflect a successful compensatory mechanism for on-going (subclinical) inflammation. Indeed, levels of serum amyloid A in quiescent SJIA subjects imply persistent disease activity [71]. Our results suggest that circulating monocytes may contribute to anti-inflammatory mechanisms in both on-going and inactive SJIA.

Highlights

- Monocytes are implicated in SJIA pathogenesis based on clinical and mechanistic data
- We characterized the activation phenotype of SJIA monocytes during flare and quiescence
- SJIA monocytes, especially during flare, show a mixed M1/M2-like activation phenotype
- During quiescence SJIA monocytes tend to exhibit a M2-like phenotype
- Circulating monocytes may play a pro- but also an anti-inflammatory role in SJIA

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

A. Hierarchical clustering analysis of genes differentially expressed in PBMC at SJIA flare and quiescence in 14 SJIA patients. The list of differentially expressed genes was generated using a paired SAM analysis (see Methods). Genes and samples were clustered. Each column represents a separate sample; each row represents a separate gene. Samples from SJIA patients in quiescence, (Q) are shown as green branches in the dendrogram; samples from SJIA patients in flare (F) are red. Asterisks indicate two samples from the same individual from distinct periods of flare or quiescence. In the heatmap, black indicates the median level of expression; red indicates greater expression than the median, green less expression and gray missing data. M1-associated genes are labeled in red, and M2-associated genes are in black. **B.** RT-PCR was performed with total RNA from PBMC from

15 paired SJIA samples (flare and quiescence). Data are expressed as a ratio of mean normalized gene expression in flare samples/ mean normalized gene expression in quiescence samples. Dotted line shows 2× the mean increase in monocyte numbers (assessed by CBC) during SJIA flare.

Figure 2.

A. Representative example of monocyte subset gating. CD14⁺⁺/CD16⁻ and CD14⁺/CD16⁺ monocyte subsets are gated from live (based on FSC × SSC plot), HLA-DR⁺ cells. **B.** CD14⁺⁺/CD16⁻ and CD14⁺/CD16⁺ monocyte subsets distribution in SJIA and controls. Percentage of monocyte subsets presented as % of total monocytes. Monocyte subsets were identified by HLA-DR, CD14 and CD16 surface staining. SJIA Q (quiescence) n=10 subjects; SJIA F (flare) n=12 subjects. Control n=10 subjects. Statistical analysis used ANOVA with multiple test correction. All comparisons were deemed non-significant (p ≥0.05).

Figure 3.

Expression of M1- and M2-related surface markers in CD14⁺/CD16⁻ and CD14⁺/CD16⁺ monocyte subsets in SJIA and controls. Monocyte subsets were identified by HLA-DR, CD14 and CD16 surface staining. Surface levels are expressed as median fluorescent intensity (MFI). SJIA Q (quiescence) n=10 subjects; SJIA F (flare) n=12 subjects; Control n=10 subjects. Statistical analysis used ANOVA with Bonferroni correction. * p<0.05; ** p≤0.01; *** p≤0.001. **A:** M1 markers. **B:** M2 markers. Medication usage did not differ between SJIA flare and quiescence groups (not shown). **C:** representative example of a co-staining experiment for CD40 and CD163 in CD14⁺ monocytes; CD40 is M1-associated and CD163 is M2-associated markers. CD14⁺ monocytes were gated from live, CD14⁺ cells. Backgating analysis showed that the cells correspond to the monocyte population on FSC × SSC plot (not shown).

Figure 4.

A. Intracellular cytokine production by CD14⁺⁺/CD16⁻ and CD14⁺/CD16⁺ monocytes subsets from SJIA and control subjects. PBMC were cultured in brefeldin (B) only or with LPS+brefeldin (L) for 4 hours for IL-1 β , IL-6 and TNF, and for 24h for IL-10. Monocyte subsets were identified by HLA-DR, CD14 and CD16 surface staining. Data are expressed as MFI. SQ: SJIA quiescence, n=10; SF: SJIA flare, n=10; HC: Healthy control, n=14. Star symbol represents a sample from a patient in remission for B and LPS values. Statistical analysis used ANOVA with Bonferroni correction. ** $p \leq 0.01$; *** $p \leq 0.001$.

Figure 5.

A. Levels of secreted IL-1 β in supernatants of PBMC cultured without (M=media) or with LPS (20 ng/ml) for 24 h, according to clinical status. **B.** Same data as in **A** (for LPS) according to use of prednisone (PO): PO low <0.16 mg/kg/day; PO high $\geq$ 0.16 mg/kg/day. Star symbols represent samples from 2 patients in remission. **C.** Levels of secreted IL-1 β in supernatants of PBMC cultured without (M=media) or with LPS (20 ng/ml) for 4 h, according to clinical status. **D:** Levels of secreted IL-1 β in supernatants of PBMC cultured with LPS (1 μ g/ml) for 24 h. SQ: SJIA quiescence (8–13 subjects); SF: SJIA flare (4–10 subjects); HC: Healthy control (10–21 subjects). Due to limitation in cell number not all samples were used in all conditions. Statistical analysis used ANOVA Bonferroni correction. * $p < 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

Table 1

p values for paired statistical analysis between Brefeldin (B) and LPS stimulated (LPS) in monocyte subsets.

CD14++CD16– monocytes					
Group	Comparisons	IL-1 β	TNF	IL-6	IL-10
Control	B $\times$ LPS	0.0017	0.0003	<0.0001	0.022
SJIA Q	B $\times$ LPS	0.0072	0.0134	0.0039	0.014
SJIA F	B $\times$ LPS	<0.0001	0.002	0.0488	0.0273
CD14+CD16+ monocytes					
Group	Comparisons	IL-1 β	TNF	IL-6	IL-10
Control	B $\times$ LPS	0.0015	0.003	0.0157	0.4557
SJIA Q	B $\times$ LPS	0.0045	0.0012	0.0217	0.1948
SJIA F	B $\times$ LPS	<0.0001	0.0017	0.303	0.3051