Published in final edited form as:

Clin Immunol. 2010 August ; 136(2): 257–268. doi:10.1016/j.clim.2010.04.003.

Monocytes are resistant to apoptosis in systemic juvenile idiopathic arthritis

Shivani Srivastava¹, Claudia Macaubas², Chetan Deshpande², Heather C. Alexander³, Sheng-Yung Chang³, Yue Sun², Jane L. Park^{2,4}, Tzielan Lee⁴, Ann Begovich³, and Elizabeth D. Mellins²

¹Department of Biology, Stanford University

²Department of Pediatrics, Program in Immunology, Stanford University

³Celera, Alameda, CA

⁴Division of Pediatric Rheumatology, Department of Pediatrics, Stanford University

Abstract

We investigated whether circulating monocytes from patients with systemic juvenile idiopathic arthritis (SJIA) are resistant to apoptosis and which apoptotic pathway(s) may mediate this resistance. A microarray analysis of peripheral blood mononuclear cells (PBMC) of SJIA samples and RT-PCR analysis of isolated monocytes showed that monocytes from active SJIA patients express transcripts that imply resistance to apoptosis. SJIA monocytes incubated in low-serum show reduced annexin binding and diminished FasL up-regulation compared to controls. SJIA monocytes are less susceptible to anti-Fas-induced apoptosis and, upon activation of the mitochondrial pathway with staurosporine, show diminished Bid cleavage and Bcl-w down-regulation compared to controls. Exposure to SJIA plasma reduces responses to apoptotic triggers in normal monocytes. Thus, SJIA monocytes are resistant to apoptosis due to alterations in both the extrinsic and intrinsic apoptosis pathways, and circulating factors associated with active SJIA may confer this phenotype.

Keywords

Systemic juvenile arthritis; monocytes; apoptosis

INTRODUCTION

Systemic juvenile idiopathic arthritis (SJIA) is a chronic inflammatory disease of childhood characterized by a combination of systemic features (fever, rash, adenopathy, serositis) and arthritis. Although SJIA represents only 10-20% of all JIA, it accounts for more than 2/3 of JIA mortality [1]. Approximately 10% of SJIA patients develop a potentially fatal complication known as “macrophage activation syndrome” (MAS). MAS is characterized by uncontrolled

© 2010 Elsevier Inc. All rights reserved.

Address correspondence and reprint requests to Dr. Elizabeth D. Mellins, MD, Department of Pediatrics, Stanford University School of Medicine, CCSR 2115c, 300 Pasteur Drive, Stanford, CA 94305. mellins@stanford.edu. Phone: 650-498-7350. Fax: 650-736-0194..

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

activation of macrophages and Tlymphocytes, resulting in fever, hepatic dysfunction, severe cytopenia, disseminated intravascular coagulation, and neurological involvement [2].

Monocytes show a tendency for expansion and activation in SJIA even in the absence of clinically diagnosed MAS [3;4]. Compared to normal monocytes, SJIA monocytes produce more pro-inflammatory cytokines, including IL-1 β and IL-6 [5;6]. They also show enhanced proteolytic activity, degrading more bone *in vitro* than normal monocytes [7]. Additionally, SJIA serum contains significantly elevated levels of S100A8, S100A9, and S100A12, proteins that are secreted during activation of neutrophils and monocytes [8]. Levels of macrophage migration inhibitory factor (MIF), which up-regulates phagocytic function and secretion of pro-inflammatory cytokines by macrophages, are also significantly elevated in SJIA serum and synovial fluid [9] and may drive monocyte activation in SJIA, at least in part. Activated monocytes are found in inflamed joints of SJIA patients [10], and circulating levels of chemotactic factors for activated monocytes are found during periods of active disease (6).

Uncontrolled monocyte activation may result from a killing defect in NK cells. NK cytolytic activity and perforin expression are reported to be lower in at least a subgroup of SJIA patients (even in the absence of MAS) compared to other JIA subtypes and controls [11;12;13]. As NK cells have been shown to regulate macrophage activity by directly killing activated macrophages [14], dysfunctional NK cytolytic activity could contribute to uncontrolled activation of monocytes in SJIA.

There is some evidence that activation of normal monocytes is associated with resistance to apoptosis [15;16]. We hypothesized that monocyte activation in SJIA may be associated with dysregulation of apoptosis, either as a cause or effect. Apoptosis is the primary form of programmed cell death and is crucial to balancing cell activation/proliferation with cell death [17]. Apoptosis results in a chromatin condensation and characteristic cell morphology, involving cell shrinkage, blebbing, and breakage into smaller apoptotic bodies. Under normal conditions, monocytes develop in the bone marrow daily and survive for 24-48 hours in the circulation before undergoing spontaneous apoptosis [18].

Apoptosis is mediated by the extrinsic (death receptor) or the intrinsic (mitochondrial) pathways [19]. In the extrinsic pathway, a death-inducing ligand (e.g. Fas ligand) binds to its receptor (e.g.Fas) on target cells, initiating signal transduction and the formation of the death-inducing signaling complex (DISC). The adapter molecule Fas-associated death domain protein (FADD) is recruited to DISC and then recruits pro-caspase 8, which undergoes autocatalytic cleavage/activation. Active caspase 8 is released from DISC and initiates the caspase cascade by cleaving caspase 3. Caspase 3 activation results in DNA fragmentation, degradation of key cellular proteins and cell death. FLIP proteins act as dominant negative regulators of this pathway by interfering with Fas-induced DISC formation.

The intrinsic pathway of apoptosis is initiated by DNA damage, hypoxia or other severe cell stress. These stimuli influence the Bcl-2 protein family, which may be induced, repressed or post-translationally modified to regulate activity. Cell stress can result in p53 stabilization and accumulation in the nucleus, where it enhances the expression of a number of pro-apoptotic genes involved in the intrinsic pathway, including *BAX*, *NOXA*, *PUMA*. In addition, p53 can exert pro-apoptotic effects by direct binding of anti-apoptotic Bcl-2 proteins or by activating pro-apoptotic Bcl-2 proteins [20]. The balance between pro-apoptotic Bcl-2 family members (e.g., Bad, PUMA) and anti-apoptotic members (e.g., Bcl-2, Bcl-w) controls the intrinsic pathway. Pro-apoptotic members induce loss of integrity of the outer mitochondrial membrane, but can be inhibited by anti-apoptotic members. Upon mitochondrial membrane permeabilization, cytochrome c and the SMAC/DIABLO protein translocate into the cytosol. SMAC/DIABLO promotes apoptosis by blocking inhibitors of apoptosis proteins (IAPs),

which inactivate caspases. Cytochrome c binds Apaf-1 (apoptotic protease activating factor-1), forming the apoptosome. Procaspase-9 is recruited into the apoptosome, where it is allosterically activated. Activated caspase 9 in turn activates the downstream effector caspases 3, 6, and/or 7.

Fas pathway signaling through DISC assembly and caspase-8 activation is insufficient to induce apoptosis in certain cell types, such as hepatocytes and pancreatic β -cells [21;22;23;24]. These cells, termed “type II” cells, depend upon the mitochondrial apoptosis pathway to amplify the initial death receptor signal [25]. Specifically, activated caspase-8 cleaves the pro-apoptotic Bcl-2 family member Bid, which translocates to the mitochondrial membrane, induces loss of membrane potential and release of cytochrome c, activating the caspase cascade. Bcl2a1 and Bcl-w proteins directly inhibit Bid cleavage.

Apoptotic death of activated monocytes could be a mechanism for controlling inflammation. Dysregulated apoptosis has been postulated to play a role in several autoimmune diseases [26;27;28]. The few studies investigating apoptosis in SJIA focus on peripheral blood lymphocytes and describe increased susceptibility to apoptosis [29;30]. To date, no study has investigated monocyte apoptosis in SJIA. We hypothesized that SJIA monocytes are resistant to apoptosis, enabling these cells to persist in and enhance pro-inflammatory microenvironments.

MATERIALS AND METHODS

Study population and blood collection

SJIA patients were recruited through the Stanford Pediatric Rheumatology Clinic. All SJIA study subjects met American College of Rheumatology criteria for the diagnosis of SJIA [31] and were enrolled after informed consent. The study was approved by the Stanford Institutional Review Board. Clinical data were collected at each visit when blood samples were drawn; clinical data included history, physical exam, and clinical laboratory values (including CBC, ESR). A disease activity scoring system was developed to facilitate correlation of clinical data with cellular data (Supplementary Tables S1, S2). For controls, blood samples were obtained from immunologically healthy children, primarily through the Stanford Pediatric Endocrinology Clinic. The mean age of subjects in the SJIA and control groups was comparable (mean $\pm$ SD: SJIA 11.8 ± 5.1 , controls 12.6 ± 4.6 , in years). Venous blood samples were processed within two hours of collection. After centrifugation for separation of plasma, PBMCs were isolated by Ficoll-Hypaque gradient (Sigma-Aldrich, St. Louis MO). PBMCs were frozen in liquid nitrogen until analysis. Plasma was centrifuged twice at 1000xg for removal of platelets and stored (-80°C) until analysis. To analyze the effect of SJIA and control plasma on normal monocytes, we used cells from buffy coats from normal adult donors, obtained through the Stanford Blood Bank.

RNA preparation

For some RNA studies, PBMCs were isolated in cell preparation tubes, per manufacturer's directions (Becton Dickinson, USA). RNA was isolated from PBMC or MACS-purified monocytes (MACS Monocyte Separation Kit, Miltenyi Biotech), using the RNeasy mini kit (QIAGEN, USA), per the manufacturer's protocol. Samples received an additional on-column DNase I treatment (QIAGEN GmbH, Hilden, Germany). RNA purity was assessed using Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA) and reverse transcription (RT)-PCR amplification of a test gene. RNA concentration was measured by RiboGreen assay (Molecular Probes, USA) or spectrophotometry.

Microarray analysis of SJIA PBMC

PBMC RNA samples for microarray analysis were processed essentially as described [32]. Briefly, patient PBMC RNA samples and Universal Human Reference RNA (Stratagene, USA) were linearly amplified (Ambion Inc, USA) and aRNA was reverse transcribed with CyDye™-labeled dNTPs (Amersham Biosciences, UK) to generate labeled cDNA. The labeled cDNAs were hybridized to Lymphochip cDNA microarrays (37,632 array elements:~18,000 genes) [33]. The reference RNA (Cy3-labeled) was mixed with the Cy5-labeled experimental sample before hybridization as a common internal reference for comparison of relative gene expression levels across arrays [32]. Hybridized cDNA arrays were scanned using a GenePix 4000B scanner (Axon Instruments, USA) and images were analyzed using GenePix Pro 5.0 (Axon Instruments, USA). A total intensity normalization method was applied to data from each array. Array elements that were not well measured on > 80% of the samples were excluded. Remaining data were log-transformed and centered by subtracting the mean observed value. Hierarchical clustering was performed using Cluster and visualized using TreeView [34]. Cluster analysis was “unsupervised”, meaning that gene expression data alone drove clustering, without prior classification by clinical status. Differentially expressed genes (flare vs. quiescence) were identified by Significance Analysis of Microarrays (SAM) [35].

qRT-PCR analysis of SJIA PBMC

The kinetic RT-PCR assay was performed as described [36]. Briefly, all reactions were carried out 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*. The normalized expression level, housekeeping normalized units, of each gene was used to determine the fold change among samples.

Apoptosis-related gene expression in SJIA monocytes

cDNA was synthesized from monocyte RNA, according to the protocol from the RT² PCR Array First Strand Kit (C-02) (Superarray, Bioscience Corp.). For real-time PCR, the SuperArray PCR master mix was added to the first strand cDNA synthesis reaction and aliquoted into the PCR Array plate. Reactions were run in the RT-PCR machine (7900HT Applied Biosystems) under a two-step cycling program (1 cycle of 10 min at 95°C; 40 cycles of 15 min at 95°C and 1 min at 60°C). Threshold cycles were calculated for each gene; threshold cycles > 40 were considered as not detected.

Low serum induction of apoptosis

PBMCs were thawed in RPMI + 10% human AB serum (HAB) and monocytes isolated by adherence and then incubated (37°C; 24h) in either low-serum (RPMI + 0.2% HAB) or normal serum (RPMI + 20% HAB). Cells were harvested by washing wells with cold PBS, incubating cells with 1ml Cell Dissociation Buffer (Invitrogen™) on ice, and washing again. After harvesting, cells were resuspended in binding buffer (BD Biosciences), stained with annexin V-FITC and propidium iodide (PI), and analyzed on the FACSCalibur (BD Biosciences). FACS data were analyzed using FlowJo (Tree Star, Inc.) and Prism (GraphPad Software, Inc.).

Cell surface staining for Fas and FasL

PBMC (SJIA n=17, control n=13) were suspended in FACS Wash Buffer with 4% fetal calf serum (FCS) immediately after thawing. Another set (SJIA n=13, control n=10) was incubated (37°C; 24h) in RPMI + 0.2% HAB, followed by harvesting with cold PBS and Cell Dissociation Buffer (Invitrogen™) and resuspension in FACS Wash Buffer with 4% FCS. Six samples (SJIA n=3, control n=3) were assessed under both conditions. Cells were stained with CD14-FITC (BD Biosciences), FasAPC and FasL-PE antibodies (BioLegend), using APC (BD

Biosciences) and IgG1-PE (BD Biosciences) isotype controls. Samples were resuspended in FACS Buffer + 1% paraformaldehyde and analyzed by flow cytometry.

Induction of monocyte apoptosis with anti-Fas antibody

Wells of a 24-well plate were incubated (37°C; 2h) with 0, 1, 5, or 15 ug/ml of anti-Fas antibody (IgM, clone 7C11; Beckman Coulter). PBMC (SJIA n=10, control n=10) were suspended in X-Vivo (Gibco). 1×10^6 cells/coated well were incubated with 1 ng/ml LPS (per manufacturer's instructions) at 37°C for 3h, washed briefly with X-Vivo, and then incubated at 37°C for an additional 16-18h. Cells were harvested and analyzed by annexin V/PI staining.

Intracellular staining of mitochondrial proteins

PBMC were thawed in X Vivo (Gibco) and incubated (37°C; 1hr) in a 24-well plate (2×10^6 cells/well) for monocyte enrichment by adherence. Wells were washed and media replaced with X Vivo with or without 1 μ M staurosporine (Sigma) and incubated (37°C; 3 hrs). A similar dose of staurosporine was previously used in monocyte studies [16]. Cells were harvested, fixed in PBS + 4% paraformaldehyde. After resuspension in 90% cold methanol (30 min; on ice), cells ($0.5-1 \times 10^6$) were resuspended in P-FACS buffer (4% FCS, 0.1% NaN₃) and stained with CD4-PE, CD33-PerCP-Cy5.5, and CD3-APC (BD Biosciences). CD33 (Siglec-3 [37]) was used to identify monocytes due to the stability of the epitope recognized by the CD33 antibody after methanol fixation in comparison to anti-CD14 antibodies; it has been shown that CD14 and CD33 recognize similar populations within PBMC [38]. Cells were incubated with rabbit antibodies against uncleaved Bid (Abcam,) (SJIA n=9, control n=10), Bcl-w (Cell Signaling) (SJIA n=11, control n=12), Bcl2a1 (Novus) (SJIA n=4, control n=4) or no antibody as a control. Cells were washed, stained with goat anti-rabbit IgG secondary antibody (Invitrogen) (1:1000 in P-FACS buffer) and analyzed by flow cytometry.

Effects of SJIA plasma on monocyte apoptosis

Previously frozen PBMC isolated from buffy coats of normal adults (one donor/experiment) were thawed in RPMI + 5% FCS and incubated (37°C; 1h) in RPMI + 5% HAB to enrich for monocytes by adherence. Wells were washed briefly with RPMI (no HAB) and incubated (37°C; 24h) in RPMI (no HAB) + 1%, 5%, 10%, or 20% SJIA (n=6) or control plasma (n=5). Cells were harvested and analyzed using annexin V/PI staining.

RESULTS

In active SJIA, PBMC express altered levels of genes regulating apoptosis

In an initial screen for gene expression changes associated with active SJIA disease, we classified samples into two disease categories: active (systemic score ≥ 2 and/or arthritis score $\geq B$) and inactive (systemic score ≤ 1 and arthritis score=A) (see Supplementary Table S1, S2). We compared PBMC transcript profiles in paired samples (active/ inactive disease) from 14 children with SJIA. Unsupervised clustering of gene expression data revealed distinct patterns of gene expression associated with active versus inactive disease (Fig 1). We used Significance Analysis of Microarrays to identify genes that were differentially expressed at a statistically significant level (false discovery rate $< 1\%$) between the two disease states and found some related to apoptosis (partial gene list on Fig. 1). Interestingly, apoptosis-related genes up-regulated during active disease included both pro-apoptotic (e.g. *GADD45B*, *PPM1A*, *TNFRSF25*) and anti-apoptotic genes (e.g., *GPX4*, *BCL2L1*, *PIMI*, *MCL1*, *BCL2A1*).

Genes associated with inhibition of apoptosis are expressed in SJIA monocytes

We next sought to confirm and extend these results by measuring changes in transcript levels more quantitatively in 11 PBMC sample pairs (22 samples of which 6, including 2 pairs, overlap

with samples in Fig. 1). Using kPCR, we analyzed expression of 8 apoptosis-related genes, including genes related to both the extrinsic and intrinsic pathways. We found that 5 of 8 genes tested were differentially expressed in PBMC from active and inactive SJIA, 4 of 5 being related to the intrinsic pathway (Table 1). Consistent with the microarray results, the anti-apoptotic genes *BCL2A1* and *BCL2L1* were expressed at significantly higher levels in active SJIA PBMC ($p=0.0024$ and $p=0.008$, respectively); the pro-apoptotic *BNIP3* was also increased in PBMC from active disease ($p=0.0035$). Based on prior reports of increased sensitivity to apoptosis in SJIA lymphocytes [29;30], we hypothesized that this mixed genetic picture reflected opposing trends in lymphocytes and monocytes. In line with this hypothesis, we found that the anti-apoptotic genes *BCL2A1* and *FLIP* were expressed at higher levels in purified monocytes from individuals with active SJIA compared to controls, whereas the pro-apoptotic genes *TP53*, *BNIP3* (in contrast to expression in PBMC) and *FASLG* transcripts were down-regulated in SJIA monocytes (Table 1). Taken together, these data revealed altered expression of genes from the extrinsic and intrinsic apoptosis pathways, suggesting resistance to apoptosis in monocytes during active SJIA.

SJIA monocytes are resistant to apoptosis induced by low serum

We tested the prediction that SJIA monocytes are more resistant to apoptosis, using *in vitro* stimuli. Using monocytes purified by adherence, we induced apoptosis by 24-hour incubation in low-serum media and stained with annexin V /propidium iodide (PI) to measure apoptosis. Both control and SJIA monocytes cultured overnight in control conditions (10% HAB) showed a low proportion of apoptotic cells (Fig. 2, left panels). In contrast, the percentage of annexin + PI- and annexin+PI+ monocytes was significantly lower in SJIA monocytes than in controls after incubation in low-serum (Fig. 2, right panels). We observed this phenotype in monocytes from SJIA subjects even when disease was controlled by medication (not shown).

SJIA monocytes fail to up-regulate FasL normally

Some evidence suggests that monocyte apoptosis induced by low-serum is mediated by the extrinsic Fas/FasL pathway [39]. Therefore, we measured the surface expression of Fas and FasL in SJIA and control monocytes immediately after thawing and after 24-hour incubation in low-serum. Levels of FasL were comparable in SJIA and control monocytes immediately after thawing (Fig. 3A, left), but were significantly lower in SJIA monocytes after 24-hour induction of apoptosis ($p=0.03$) (Fig. 3A right). As we observed with the low serum induction of apoptosis, the failure to upregulate FasL was similar in cells from both active and inactive SJIA patients, although statistical significance was reached only with cells from active patients (Supp. Fig. S1). In an intra-group comparison of FasL levels at time 0 and after 24 h induction of apoptosis, FasL levels in SJIA monocytes before and after apoptosis induction were roughly the same (Fig. 3B, left); by contrast, in control monocytes, FasL levels were up-regulated when apoptosis was induced ($p=0.04$), (Fig. 3B, right). Together with the data showing lower levels of *FASLG* transcripts in monocytes from active SJIA (Table 1), it appears likely that SJIA monocytes fail to up-regulate FasL, although we have not ruled out increased secretion/shedding of FasL by SJIA cells [40].

Fas levels did not significantly differ between SJIA and control monocytes immediately after thawing (Fig 3C, left), or after 24h induction of apoptosis (Fig 3C, right). In an intra-group comparison, after 24-hour induction of apoptosis Fas levels were down-regulated to approximately the same extent after induction of apoptosis in SJIA (Fig. 3D, left), and controls (Fig. 3D, right). We have not determined the basis of this down-regulation. However, the resistance to apoptosis in SJIA monocytes cannot be ascribed to reduced levels of surface Fas compared to normal monocytes.

Treatment with anti-Fas agonist antibodies does not restore apoptosis in SJIA monocytes

We next investigated whether steps downstream in the Fas pathway were defective in SJIA monocytes. Using samples from active SJIA and controls, we stimulated apoptosis with plate-bound anti-Fas antibody over a range of concentrations. We initially incubated SJIA or control PBMC with stimulatory anti-Fas antibody and 1 ng/ml LPS to activate cells (per manufacturer's instructions) for three hours. The cultures were then washed to reduce LPS and enrich for monocytes via adherence, and the remaining adherent cells were incubated with anti-Fas antibody overnight. We then measured apoptosis using annexin V/PI staining. To control for differences in initial cell viability, we analyzed the data by fold change, comparing percentage of apoptotic cells after anti-Fas treatment to percentage of apoptotic cells in mock-treated cultures. Treatment with anti-Fas resulted in significantly reduced early (annexin+ PI-) and late (annexin+ PI+) apoptotic cells in SJIA monocytes compared to control monocytes [Fig. 4A and 4B (representative data)]. Thus, antibody-mediated stimulation of the Fas receptor did not appear to restore normal levels of apoptosis in SJIA monocytes, arguing that steps in the downstream Fas pathway are also defective in SJIA monocytes.

Expression of mitochondrial apoptosis-related proteins

Our gene expression data suggested the mitochondrial apoptosis pathway was also affected in SJIA monocytes. To confirm and extend these results, we measured the levels of selected proteins in this pathway in SJIA and control monocytes. As *BCL2A1* transcripts were more abundant in active SJIA monocytes compared to controls (Table 1), we tested Bcl2a1 as an initial candidate and also measured expression of Bcl-w, which (like Bcl2a1) directly inhibits Bid cleavage. We also measured levels of the pro-apoptotic protein Bid as this protein is the primary link between the Fas and mitochondrial pathways.

We did not detect a difference in expression of Bcl2a1, Bcl-w or uncleaved Bid in SJIA compared to control monocytes immediately after thawing, nor were there differences observed after induction of apoptosis by 24-hour incubation in low serum (data not shown). As serum-withdrawal is thought to induce apoptosis by a Fas-mediated pathway in cultured monocytes [41;42], the mitochondrial pathway may not have been sufficiently stimulated by serum starvation. We therefore measured levels of the same Bcl-2 family proteins after induction of apoptosis by staurosporine, a compound known to stimulate the mitochondrial apoptosis pathway. We determined the dose of staurosporine required to induce apoptosis (annexin-positivity) in normal monocytes following 3-hour stimulation (Supp. Fig. S2); this dose was the same as that used by other investigators studying the mitochondrial pathway in monocytes [16]. Under these conditions, levels of uncleaved Bid were higher in SJIA monocytes compared to controls ($p=0.038$; Fig. 5A). Levels of uncleaved Bid decreased in control monocytes ($p=0.0026$), whereas they did not change significantly in SJIA monocytes (Fig. 5B). Levels of Bcl-w were down-regulated in control monocytes ($p=0.0488$), but not in SJIA monocytes following staurosporine-induced apoptosis (Fig. 5C). Bcl2a1 levels were down-regulated to roughly the same extent in both SJIA and control monocytes upon stimulation with staurosporine (Fig. 5D). Together these data support a model in which SJIA monocytes fail to down-regulate Bcl-w when induced to undergo apoptosis by staurosporine and consequently show reduced Bid cleavage.

SJIA plasma has a protective effect on monocyte viability

To determine whether resistance to apoptosis can be conferred on normal monocytes by soluble mediators in SJIA plasma, we used buffy coats from normal adult donors to isolate PBMCs and then purified monocytes by adherence. We incubated these cells with increasing amounts (1%, 5%, 10%, 20%) of either SJIA or control plasma. The baseline level of early apoptosis (annexin+/PI-) in monocytes derived from normal buffy coats is typically 25-45%, immediately after thawing and after 24-hour incubation in media with 1-20% normal human

plasma (not shown); this suggests that these cells receive an apoptotic stimulus during buffy coat preparation. Increasing the amount of control plasma to 20% did not reduce the proportion of apoptotic cells; in contrast, increasing amounts of SJIA plasma caused a dose-dependent reduction in apoptosis (Fig. 6). These data suggest that SJIA plasma has a protective effect on monocyte viability and is capable of rescuing normal monocytes that have received an apoptotic stimulus.

DISCUSSION

In this study, we find that SJIA monocytes are resistant to apoptosis induced by several stimuli. Activation of normal monocytes is characterized in part by resistance to apoptosis [43;44]. Activation confers survival signals, enabling monocytes to persist and function in sites of inflammation containing cytotoxic inflammatory mediators [16]. As such, the anti-apoptotic phenotype of SJIA monocytes may be a consequence of activation, arising from similar mechanisms that operate in normal monocytes. Alternatively, the SJIA phenotype may derive from mechanisms particular to SJIA.

In studies of apoptosis resistance, it has been observed that after activation, normal monocytes resist apoptosis induced by Fas cross-linking or by DNA damage from gamma irradiation [16;39]. In one potential mechanism, pro-inflammatory cytokines like TNF α have been shown to protect monocytes from death receptor-mediated apoptosis through up-regulation of FLIP, an enzymatically inactive homologue of pro-caspase-8 that blocks death receptor signaling by preventing caspase-8 activation [45;46]. Apoptosis of normal monocytes triggered by serum withdrawal is mediated primarily by the Fas pathway [41;42]. Thus, activated normal monocytes would be predicted to acquire resistance to serum withdrawal-induced apoptosis due to changes downstream of Fas, including increased expression of FLIP. Consistent with a contribution of activation state to apoptosis resistance in SJIA monocytes, treatment with an anti-Fas agonist does not result in normal levels of apoptosis (Fig. 4) and *FLIP* expression is up-regulated (3.1-fold) in active SJIA versus control monocytes (Table 1). The reduced response to anti-Fas in SJIA monocytes is not likely due to their resistance to the effects of short exposure to LPS that enhance responsiveness to anti-Fas in our assay (see methods), as SJIA monocytes from subjects with inactive or active disease have normal or even enhanced responses to LPS as measured by induction of IL-1 β and other cytokines (not shown). Additional studies will be needed to determine the extent of FLIP contribution to the SJIA phenotype, including determining whether cleaved FLIP is recruited to the DISC complex [47].

Our investigations also show that SJIA monocytes fail to up-regulate FasL upon incubation in low serum. Measurements of *FASLG* gene expression (Table 1) and cell surface FasL expression (Fig. 3) suggest defective FasL induction. This phenotype may also be related to monocyte activation by pro-inflammatory cytokines in SJIA. Exposure of normal monocytes to TNF α has been associated with a decrease in FasL expression [39;44;48]. Shedding of soluble FasL may also contribute to apoptosis resistance. We did not measure levels of sFasL in the monocyte culture supernatants, and to our knowledge there are no data on levels of circulating sFasL in SJIA. A study of a small number of patients found that 3/10 JIA patients (combined polyarticular and SJIA) had elevated levels of soluble Fas in comparison to a normal control group [49]. However, in our studies, Fas expression was not significantly different between SJIA and control monocytes after thawing or after 24-hour induction of apoptosis (Fig. 3C and D). FasL expression is more tightly controlled than Fas expression [50], such that even small differences in FasL expression can produce large differences in apoptosis induction.

As regards the Fas pathway of apoptosis, the relationship between the SJIA monocyte phenotype and the phenotype of activated monocytes is not simple. LPS-induced activation of

normal monocytes causes a dramatic up-regulation of *BCL2A1* mRNA along with a rapid down-regulation of *CASP8* mRNA (encoding caspase-8) [16]. Although *BCL2A1* transcripts were more abundant in active SJIA monocytes compared to controls, we did not observe reduced *CASP8* mRNA in monocytes from active SJIA compared to inactive disease or compared to normal monocytes (not shown),

In addition to effects on signaling through the Fas pathway, dysregulation of components of the mitochondrial apoptosis pathway could also explain why SJIA monocytes are resistant to Fas-induced apoptosis. Weglarczyk *et al.* have provided evidence that monocytes are “type II” cells, depending upon the mitochondrial apoptosis pathway to amplify the initial death receptor signal to induce apoptosis [51]. We found that active SJIA monocytes up-regulate anti-apoptotic *BCL2A1* and down-regulate proapoptotic genes of the mitochondrial pathway (*TP53*, *BNIP3*) relative to control monocytes. When the mitochondrial apoptosis pathway is activated by staurosporine, SJIA monocytes fail to down-regulate Bcl-w and show reduced Bid cleavage. *BID* mRNA expression is not increased in active SJIA compared to control monocytes (not shown), arguing that SJIA monocytes show reduced Bid cleavage and not higher Bid expression. Because Bcl-w is known to specifically inhibit Bid cleavage, failure to down-regulate Bcl-w in SJIA monocytes likely results in continued inhibition of Bid cleavage, preventing activation of the mitochondrial apoptosis pathway. Dysregulation of Bcl-w and Bid may thus explain why treatment with staurosporine does not stimulate normal levels of apoptosis in SJIA and, further, may constitute “downstream defects” in the Fas-mediated death pathway in these type II cells.

Notably, in contrast to our data with SJIA monocytes, activation of normal monocytes with LPS does not induce resistance to staurosporine-mediated (mitochondrial pathway) apoptosis [16]. A number of stimuli (serotonin, reactive oxygen species, and histamine) have been shown to up-regulate Bcl-2 and/or Mcl-1 in normal monocytes [52;53;54], but these changes were not consistently observed in SJIA monocytes. Conversely, the persistence of Bcl-w observed in SJIA monocytes has not been reported after activation of normal monocytes, although human alveolar macrophages infected with *Mycobacterium tuberculosis* up-regulate Bcl-w [55]. Thus, it will be of interest to further investigate whether altered regulation of Bcl-w is a primary defect in SJIA monocytes that is separable from the exposure of these cells to the inflammatory environment during active disease.

At least some of the cellular changes that lead to monocyte resistance to apoptotic stimuli may be induced by soluble mediators in SJIA plasma, as exposure to SJIA plasma reduces apoptosis in normal monocytes that receive a death-inducing stimulus. Further studies will be necessary to determine the similarities and differences between the phenotype induced by SJIA plasma and the phenotype of SJIA monocytes. Potential inducers of resistance to monocyte apoptosis in SJIA plasma include pro-inflammatory cytokines such as IL-1 β and TNF α . Another candidate is MIF, which promotes monocyte/macrophage survival by binding to p53 and suppressing its function in activation of downstream target genes, thereby reducing apoptosis through the mitochondrial pathway [56;57]. All three cytokines are significantly elevated in SJIA serum [9;58]. Importantly, a polymorphism in the *MIF* gene is associated with SJIA susceptibility [59] and with higher serum and synovial MIF levels, and poorer outcome and responses to treatment in SJIA [60]. Enhanced MIF activity may thus link resistance to monocyte apoptosis in SJIA with disease pathogenesis. Another potential inducer of resistance to apoptosis in SJIA monocytes is S100A12. A microarray analysis of S100A12-induced gene expression in monocytes showed induction of genes associated with activation, chemotaxis and repression of apoptosis [61]. Elevated levels of circulating S100A12 have been described in SJIA [62;63].

Resistance to apoptosis also is a characteristic of macrophages [64]. In a microarray study of gene expression in peripheral blood mononuclear cells from children with new onset SJIA, Fall et al observed increased expression of genes associated with monocyte differentiation into macrophages [3]. Thus, the anti-apoptotic phenotype may be evidence of monocyte differentiation towards macrophages in SJIA.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank the patients, their families and the medical staff of Pediatric Rheumatology and Pediatric Endocrinology Clinics at Lucile Packard Hospital for Children. This work was supported by The Wasie Foundation, the Dana Foundation, the Child Health Research Program of Stanford University and the National Institutes of Health. J.L. Park is supported by the American College of Rheumatology Research and Education Foundation Physician Scientist Development Award and the Ernest and Amelia Gallo Endowed Postdoctoral Fellowship Fund. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of Arthritis and Musculoskeletal and Skin Diseases or the National Institutes of Health.

REFERENCES

1. Schneider R, Laxer RM. Systemic onset juvenile rheumatoid arthritis. *Baillieres Clin Rheumatol* 1998;12:245–71. [PubMed: 9890097]
2. Ravelli A. Macrophage activation syndrome. *Curr Opin Rheumatol* 2002;14:548–52. [PubMed: 12192253]
3. Fall N, Barnes M, Thornton S, Luyrink L, Olson J, Ilowite NT, Gottlieb BS, Griffin T, Sherry DD, Thompson S, Glass DN, Colbert RA, Grom AA. Gene expression profiling of peripheral blood from patients with untreated new-onset systemic juvenile idiopathic arthritis reveals molecular heterogeneity that may predict macrophage activation syndrome. *Arthritis Rheum* 2007;56:3793–804. [PubMed: 17968951]
4. Macaubas C, Nguyen K, Deshpande C, Phillips C, Peck A, Lee T, Park J, Sandborg C, Mellins E. Distribution of circulating cells in systemic juvenile idiopathic arthritis across disease activity states. *Clinical immunology*. 2009
5. Martini A, Ravelli A, Notarangelo LD, Maccario R, Lanfranchi A, Rondena D, Ugazio AG, Burgio R. Enhanced interleukin 1 and depressed interleukin 2 production in juvenile arthritis. *J Rheumatol* 1986;13:598–603. [PubMed: 3090248]
6. Pascual V, Allantaz F, Arce E, Punaro M, Banchereau J. Role of interleukin-1 (IL-1) in the pathogenesis of systemic onset juvenile idiopathic arthritis and clinical response to IL-1 blockade. *J Exp Med* 2005;201:1479–86. [PubMed: 15851489]
7. Key LL Jr. Hoch S, Cairns L, Carnes D, Beyer E, Anast CS. Monocyte bone degradation: in vitro analysis of monocyte activity in patients with juvenile rheumatoid arthritis. *J Pediatr* 1986;108:405–9. [PubMed: 3081699]
8. Foell D, Wittkowski H, Hammerschmidt I, Wulffraat N, Schmeling H, Frosch M, Horneff G, Kuis W, Sorg C, Roth J. Monitoring neutrophil activation in juvenile rheumatoid arthritis by S100A12 serum concentrations. *Arthritis Rheum* 2004;50:1286–95. [PubMed: 15077313]
9. Meazza C, Travaglini P, Pignatti P, Magni-Manzoni S, Ravelli A, Martini A, De Benedetti F. Macrophage migration inhibitory factor in patients with juvenile idiopathic arthritis. *Arthritis Rheum* 2002;46:232–7. [PubMed: 11817596]
10. Frosch M, Metze D, Foell D, Vogl T, Sorg C, Sunderkotter C, Roth J. Early activation of cutaneous vessels and epithelial cells is characteristic of acute systemic onset juvenile idiopathic arthritis. *Exp Dermatol* 2005;14:259–65. [PubMed: 15810883]
11. Wulffraat NM, Rijkers GT, Elst E, Brooimans R, Kuis W. Reduced perforin expression in systemic juvenile idiopathic arthritis is restored by autologous stem-cell transplantation. *Rheumatology (Oxford)* 2003;42:375–9. [PubMed: 12595640]

12. Villanueva J, Lee S, Giannini EH, Graham TB, Passo MH, Filipovich A, Grom AA. Natural killer cell dysfunction is a distinguishing feature of systemic onset juvenile rheumatoid arthritis and macrophage activation syndrome. *Arthritis Res Ther* 2005;7:R30–7. [PubMed: 15642140]
13. Bleesing J, Prada A, Siegel DM, Villanueva J, Olson J, Ilowite NT, Brunner HI, Griffin T, Graham TB, Sherry DD, Passo MH, Ramanan AV, Filipovich A, Grom AA. The diagnostic significance of soluble CD163 and soluble interleukin-2 receptor alpha-chain in macrophage activation syndrome and untreated new-onset systemic juvenile idiopathic arthritis. *Arthritis Rheum* 2007;56:965–71. [PubMed: 17328073]
14. Nedvetzki S, Sowinski S, Eagle RA, Harris J, Vely F, Pende D, Trowsdale J, Vivier E, Gordon S, Davis DM. Reciprocal regulation of human natural killer cells and macrophages associated with distinct immune synapses. *Blood* 2007;109:3776–85. [PubMed: 17218381]
15. Um HD, Orenstein JM, Wahl SM. Fas mediates apoptosis in human monocytes by a reactive oxygen intermediate dependent pathway. *J Immunol* 1996;156:3469–77. [PubMed: 8617975]
16. Perera LP, Waldmann TA. Activation of human monocytes induces differential resistance to apoptosis with rapid down regulation of caspase-8/FLICE. *Proc Natl Acad Sci U S A* 1998;95:14308–13. [PubMed: 9826696]
17. Platt N, da Silva RP, Gordon S. Recognizing death: the phagocytosis of apoptotic cells. *Trends Cell Biol* 1998;8:365–72. [PubMed: 9728398]
18. Fahy RJ, Doseff AI, Wewers MD. Spontaneous human monocyte apoptosis utilizes a caspase-3-dependent pathway that is blocked by endotoxin and is independent of caspase-1. *J Immunol* 1999;163:1755–62. [PubMed: 10438906]
19. Gupta S. Molecular signaling in death receptor and mitochondrial pathways of apoptosis (Review). *Int J Oncol* 2003;22:15–20. [PubMed: 12469180]
20. Chipuk JE, Green DR. Dissecting p53-dependent apoptosis. *Cell Death Differ* 2006;13:994–1002. [PubMed: 16543937]
21. Scaffidi C, Fulda S, Srinivasan A, Friesen C, Li F, Tomaselli KJ, Debatin KM, Krammer PH, Peter ME. Two CD95 (APO-1/Fas) signaling pathways. *EMBO J* 1998;17:1675–87. [PubMed: 9501089]
22. Yin XM, Wang K, Gross A, Zhao Y, Zinkel S, Klocke B, Roth KA, Korsmeyer SJ. Bid-deficient mice are resistant to Fas-induced hepatocellular apoptosis. *Nature* 1999;400:886–91. [PubMed: 10476969]
23. Kaufmann T, Tai L, Ekert PG, Huang DC, Norris F, Lindemann RK, Johnstone RW, Dixit VM, Strasser A. The BH3-only protein bid is dispensable for DNA damage- and replicative stress-induced apoptosis or cell-cycle arrest. *Cell* 2007;129:423–33. [PubMed: 17448999]
24. McKenzie MD, Carrington EM, Kaufmann T, Strasser A, Huang DC, Kay TW, Allison J, Thomas HE. Proapoptotic BH3-only protein Bid is essential for death receptor-induced apoptosis of pancreatic beta-cells. *Diabetes* 2008;57:1284–92. [PubMed: 18252892]
25. Certo M, Del Gaizo Moore V, Nishino M, Wei G, Korsmeyer S, Armstrong SA, Letai A. Mitochondria primed by death signals determine cellular addiction to antiapoptotic BCL-2 family members. *Cancer Cell* 2006;9:351–65. [PubMed: 16697956]
26. Korb A, Pavenstadt H, Pap T. Cell death in rheumatoid arthritis. *Apoptosis* 2009;14:447–54. [PubMed: 19199037]
27. Lee SC, Pervaiz S. Apoptosis in the pathophysiology of diabetes mellitus. *Int J Biochem Cell Biol* 2007;39:497–504. [PubMed: 17074529]
28. Munoz LE, van Bavel C, Franz S, Berden J, Herrmann M, van der Vlag J. Apoptosis in the pathogenesis of systemic lupus erythematosus. *Lupus* 2008;17:371–5. [PubMed: 18490410]
29. Smolewska E, Brozik H, Smolewski P, Biernacka-Zielinska M, Darzynkiewicz Z, Stanczyk J. Apoptosis of peripheral blood lymphocytes in patients with juvenile idiopathic arthritis. *Ann Rheum Dis* 2003;62:761–3. [PubMed: 12860732]
30. Smolewska E, Brozik H, Smolewski P, Darzynkiewicz Z, Stanczyk J. Regulation of peripheral blood and synovial fluid lymphocyte apoptosis in juvenile idiopathic arthritis. *Scand J Rheumatol* 2004;33:7–12. [PubMed: 15124936]
31. Cassidy JT, Levinson JE, Bass JC, Baum J, Brewer EJ Jr, Fink CW, Hanson V, Jacobs JC, Masi AT, Schaller JG, et al. A study of classification criteria for a diagnosis of juvenile rheumatoid arthritis. *Arthritis Rheum* 1986;29:274–81. [PubMed: 3485433]

32. Alizadeh AA, Eisen MB, Davis RE, Ma C, Lossos IS, Rosenwald A, Boldrick JC, Sabet H, Tran T, Yu X, Powell JI, Yang L, Marti GE, Moore T, Hudson J Jr, Lu L, Lewis DB, Tibshirani R, Sherlock G, Chan WC, Greiner TC, Weisenburger DD, Armitage JO, Warnke R, Levy R, Wilson W, Grever MR, Byrd JC, Botstein D, Brown PO, Staudt LM. Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. *Nature* 2000;403:503–11. [PubMed: 10676951]
33. Alizadeh A, Eisen M, Davis RE, Ma C, Sabet H, Tran T, Powell JI, Yang L, Marti GE, Moore DT, Hudson JR Jr, Chan WC, Greiner T, Weisenburger D, Armitage JO, Lossos I, Levy R, Botstein D, Brown PO, Staudt LM. The lymphochip: a specialized cDNA microarray for the genomic-scale analysis of gene expression in normal and malignant lymphocytes. *Cold Spring Harb Symp Quant Biol* 1999;64:71–8. [PubMed: 11232339]
34. Eisen MB, Spellman PT, Brown PO, Botstein D. Cluster analysis and display of genome-wide expression patterns. *Proc Natl Acad Sci U S A* 1998;95:14863–8. [PubMed: 9843981]
35. Tusher VG, Tibshirani R, Chu G. Significance analysis of microarrays applied to the ionizing radiation response. *Proc Natl Acad Sci U S A* 2001;98:5116–21. [PubMed: 11309499]
36. Baechler EC, Batliwalla FM, Karypis G, Gaffney PM, Moser K, Ortmann WA, Espe KJ, Balasubramanian S, Hughes KM, Chan JP, Begovich A, Chang SY, Gregersen PK, Behrens TW. Expression levels for many genes in human peripheral blood cells are highly sensitive to ex vivo incubation. *Genes Immun* 2004;5:347–53. [PubMed: 15175644]
37. Crocker P, Paulson J, Varki A. Siglecs and their roles in the immune system. *Nature Reviews. Immunology* 2007;7:255–266.
38. Lee AW, Sharp ER, O'Mahony A, Rosenberg MG, Israelski DM, Nolan GP, Nixon DF. Single-cell, phosphopeptide-specific analysis demonstrates cell type- and pathway-specific dysregulation of Jak/STAT and MAPK signaling associated with in vivo human immunodeficiency virus type 1 infection. *J Virol* 2008;82:3702–3712. [PubMed: 18216116]
39. Kiener PA, Davis PM, Starling GC, Mehlin C, Klebanoff SJ, Ledbetter JA, Liles WC. Differential induction of apoptosis by Fas-Fas ligand interactions in human monocytes and macrophages. *J Exp Med* 1997;185:1511–6. [PubMed: 9126933]
40. Mizuta M, Nakajima H, Mizuta N, Kitamura Y, Nakajima Y, Hashimoto S, Matsuyama H, Shime N, Amaya F, Koh H, Ishizaka A, Magae J, Tanuma SI. Fas ligand released by activated monocytes causes apoptosis of lung epithelial cells in human acute lung injury model in vitro. *Biol Pharm Bull* 2008;31:386–90. [PubMed: 18310897]
41. Hu CL, Cowan RG, Harman RM, Porter DA, Quirk SM. Apoptosis of bovine granulosa cells after serum withdrawal is mediated by Fas antigen (CD95) and Fas ligand. *Biol Reprod* 2001;64:518–26. [PubMed: 11159354]
42. Caricchio R, D'Adamio L, Cohen PL. Fas, ceramide and serum withdrawal induce apoptosis via a common pathway in a type II Jurkat cell line. *Cell Death Differ* 2002;9:574–80. [PubMed: 11973615]
43. Mangan DF, Wahl SM. Differential regulation of human monocyte programmed cell death (apoptosis) by chemotactic factors and pro-inflammatory cytokines. *J Immunol* 1991;147:3408–12. [PubMed: 1940344]
44. Mangan DF, Welch GR, Wahl SM. Lipopolysaccharide, tumor necrosis factor-alpha, and IL-1 beta prevent programmed cell death (apoptosis) in human peripheral blood monocytes. *J Immunol* 1991;146:1541–6. [PubMed: 1993844]
45. Perlman H, Pagliari LJ, Nguyen N, Bradley K, Liu H, Pope RM. The Fas-FasL death receptor and PI3K pathways independently regulate monocyte homeostasis. *Eur J Immunol* 2001;31:2421–30. [PubMed: 11500826]
46. Krueger A, Schmitz I, Baumann S, Krammer PH, Kirchhoff S. Cellular FLICE-inhibitory protein splice variants inhibit different steps of caspase-8 activation at the CD95 death-inducing signaling complex. *J Biol Chem* 2001;276:20633–40. [PubMed: 11279218]
47. Snow AL, Chen LJ, Nepomuceno RR, Krams SM, Esquivel CO, Martinez OM. Resistance to Fas-mediated apoptosis in EBV-infected B cell lymphomas is due to defects in the proximal Fas signaling pathway. *J Immunol* 2001;167:5404–5411. [PubMed: 11673559]
48. Flad HD, Grage-Griebenow E, Petersen F, Scheuerer B, Brandt E, Baran J, Pryjma J, Ernst M. The role of cytokines in monocyte apoptosis. *Pathobiology* 1999;67:291–3. [PubMed: 10725805]

49. Knipping E, Krammer PH, Onel KB, Lehman TJ, Mysler E, Elkon KB. Levels of soluble Fas/APO-1/CD95 in systemic lupus erythematosus and juvenile rheumatoid arthritis. *Arthritis and rheumatism* 1995;38:1735–1737. [PubMed: 8849344]
50. Suzuki I, Fink PJ. The dual functions of fas ligand in the regulation of peripheral CD8+ and CD4+ T cells. *Proc Natl Acad Sci U S A* 2000;97:1707–12. [PubMed: 10677522]
51. Weglarczyk K, Baran J, Zembala M, Pryjma J. Caspase-8 activation precedes alterations of mitochondrial membrane potential during monocyte apoptosis induced by phagocytosis and killing of *Staphylococcus aureus*. *Infect Immun* 2004;72:2590–7. [PubMed: 15102767]
52. Soga F, Katoh N, Inoue T, Kishimoto S. Serotonin activates human monocytes and prevents apoptosis. *J Invest Dermatol* 2007;127:1947–55. [PubMed: 17429435]
53. Cristofanon S, Nuccitelli S, D'Alessio M, Dicato M, Diederich M, Ghibelli L. Oxidation-dependent maturation and survival of explanted blood monocytes via Bcl-2 up-regulation. *Biochem Pharmacol* 2008;76:1533–43. [PubMed: 18765235]
54. Soga F, Katoh N, Kishimoto S. Histamine prevents apoptosis in human monocytes. *Clin Exp Allergy* 2007;37:323–30. [PubMed: 17359382]
55. Spira A, Carroll JD, Liu G, Aziz Z, Shah V, Kornfeld H, Keane J. Apoptosis genes in human alveolar macrophages infected with virulent or attenuated *Mycobacterium tuberculosis*: a pivotal role for tumor necrosis factor. *Am J Respir Cell Mol Biol* 2003;29:545–551. [PubMed: 12748057]
56. Mitchell RA, Liao H, Chesney J, Fingerle-Rowson G, Baugh J, David J, Bucala R. Macrophage migration inhibitory factor (MIF) sustains macrophage proinflammatory function by inhibiting p53: regulatory role in the innate immune response. *Proc Natl Acad Sci U S A* 2002;99:345–50. [PubMed: 11756671]
57. Jung H, Seong HA, Ha H. Critical role of cysteine residue 81 of macrophage migration inhibitory factor (MIF) in MIF-induced inhibition of p53 activity. *J Biol Chem* 2008;283:20383–96. [PubMed: 18502749]
58. Mangge H, Kenzian H, Gallistl S, Neuwirth G, Liebmann P, Kaulfersch W, Beaufort F, Muntean W, Schauenstein K. Serum cytokines in juvenile rheumatoid arthritis. Correlation with conventional inflammation parameters and clinical subtypes. *Arthritis Rheum* 1995;38:211–20. [PubMed: 7848311]
59. Donn RP, Shelley E, Ollier WE, Thomson W. A novel 5'-flanking region polymorphism of macrophage migration inhibitory factor is associated with systemic-onset juvenile idiopathic arthritis. *Arthritis Rheum* 2001;44:1782–5. [PubMed: 11508429]
60. De Benedetti F, Meazza C, Vivarelli M, Rossi F, Pistorio A, Lamb R, Lunt M, Thomson W, Ravelli A, Donn R, Martini A. Functional and prognostic relevance of the -173 polymorphism of the macrophage migration inhibitory factor gene in systemic-onset juvenile idiopathic arthritis. *Arthritis Rheum* 2003;48:1398–407. [PubMed: 12746913]
61. Wittkowski H, Viemann D, Lueken A, Barczyk K, Vogl T, Roth J, Foell D. The Damage Associated Molecular Pattern (DAMP) molecule S100A12 induces pro-inflammatory responses in monocytes via innate immunity signalling pathways. *Pediatric Rheumatology* 2008;6:P2.
62. Foell D, Roth J. Proinflammatory S100 proteins in arthritis and autoimmune disease. *Arthritis and rheumatism* 2004;50:3762–71. [PubMed: 15593206]
63. Wittkowski H, Frosch M, Wulffraat N, Goldbach-Mansky R, Kallinich T, Kuemmerle-Deschner J, Frhwald M, Dassmann S, Pham T-H, Roth J, Foell D. S100A12 is a novel molecular marker differentiating systemic-onset juvenile idiopathic arthritis from other causes of fever of unknown origin. *Arthritis and rheumatism* 2008;58:3924–31. [PubMed: 19035478]
64. Murphy J, Summer R, Wilson AA, Kotton DN, Fine A. The prolonged life-span of alveolar macrophages. *Am J Respir Cell Mol Biol* 2008;38:380–385. [PubMed: 18192503]
65. Lotito AP, Campa A, Silva CA, Kiss MH, Mello SB. Interleukin 18 as a marker of disease activity and severity in patients with juvenile idiopathic arthritis. *J Rheumatol* 2007;34:823–30. [PubMed: 17343316]

Figure 1. Hierarchical clustering analysis of genes differentially expressed at flare and quiescence in 14 SJIA patients

The gene list was generated using a paired SAM analysis (see Methods). The genes and samples were then organized using hierarchical clustering. Each column represents a separate sample; each row represents a separate gene. Samples from patients with inactive SJIA are shown as green branches in the dendrogram; samples from patients with active SJIA are red. Asterisks indicate two samples from the same individual from distinct periods of active or inactive disease. Apoptosis-related genes are identified close to their position in the gene dendrogram. In the heatmap, black indicates the median level of expression, red indicates greater expression than the median, green less expression and gray missing data.

Figure 2. Apoptosis in SJIA and control monocytes after 24h incubation in normal or low serum media

Percentage of apoptotic SJIA (open circles) and control monocytes (filled circles) after 24-hour incubation in normal serum (left) or low-serum (right) media. Monocytes were identified as CD33+CD3-. Apoptotic cells were identified as annexin (Ann)+ PI- and annexin (Ann)+ PI+. Individual values are plotted, with horizontal bars indicating the mean for each group. P-values determined by unpaired t-test.

Figure 3. Fas/FasL expression in SJIA and control monocytes after 0h and 24h incubation in low serum media

Levels of FasL (A, B) and Fas (C, D) in SJIA (open symbols) and control monocytes (filled symbols). A: Inter-group comparison of FasL levels immediately after thawing (left) and after 24h incubation in low-serum media (right). B: Intra-group comparison of FasL level immediately after thawing and after 24h incubation in low serum media in SJIA (left) and control monocytes (right). C: Inter-group comparison of Fas levels immediately after thawing (left) and after 24h incubation in low-serum media (right). D: Intra-group comparison of Fas level immediately after thawing and after 24h incubation in low serum media in SJIA (left) and control monocytes (right). Monocytes were identified as CD14+. Data are expressed as ratio of MFI of antibody against Fas or FasL relative to MFI of isotype control. P-values determined by unpaired t-test. Individual values are plotted, with horizontal bars indicating the mean for each group. Connecting lines indicate paired samples.

Figure 4. Induction of apoptosis in SJIA and control monocytes after treatment with anti-Fas antibody

A: White boxes represent SJIA monocytes; shaded boxes represent control monocytes. Fold increase of early apoptotic cells (identified as annexin (Ann)+PI-) is shown (left); fold increase of late apoptotic cells (identified as annexin (Ann)+PI+) is shown (right). Data are fold increase of percentage of apoptotic cells after treatment with anti-Fas to percentage of apoptotic cells when mock-treated. Boxes indicate median and quartiles. Whiskers indicate range. P-values determined by non-parametric t-test. B: Sample FACS plots from a SJIA sample (top panels) and an healthy control sample (bottom panels) are shown after treatment with 0 ng/ml (left panels) and 5 ng/ml of anti-Fas agonist (right panels).

Figure 5. Expression of mitochondrial proteins Bid, Bcl-w, and BCL2A1 in SJIA and control monocytes before and after stimulation with 1 μ M staurosporine
Monocytes were identified as CD33+ CD3-. Data are ratio of MFI of antibody against Bid (A and B), Bcl-w (C), or BCL2A1 (D) relative to the MFI of negative control. Representative FACS histograms are on the right of each column. A: Inter-group comparison of levels of uncleaved Bid in SJIA (open circles) and control monocytes (filled circles) after incubation with 0 μ M (left) or 1 μ M (right) staurosporine. Individual values are plotted, with horizontal bars indicating the mean and quartiles for each group. P-values determined by non-parametric t-test. B, C, and D: Levels of uncleaved Bid (B), Bcl-w (C), and Bcl2a1 (D) after stimulation with 0 μ M and 1 μ M staurosporine in SJIA (left panels, hatched bars) and control monocytes (right panels, solid bars). Bars indicate group mean + standard error of the mean (SEM). P-values determined by non-parametric paired t-test.

Figure 6. Effect of SJIA plasma on normal monocyte apoptosis

Percentage of apoptotic control monocytes after 24-hour incubation in decreasing concentrations of normal serum (filled symbols) or SJIA serum (open symbols) in media. Left panel represents percentage of annexin+PI- cells, and right panel, percentage of annexin+PI+ cells. Statistical analysis used ANOVA with Bonferroni correction. * $p<0.05$; **: $p\leq0.01$.

Table 1

Genes showing differential regulation in active SJIA

Gene ^a	Expression in Active vs Inactive SJIA PBMC		Function	Pathway Involvement ^b	
				Extrinsic	Intrinsic
BCL2A1	Higher	p=0.0024 ^c	Anti-apoptosis		X
BNIP3	Higher	p=0.0035	Pro-apoptosis		X
BCL2L1	Higher	p=0.0080	Anti-apoptosis		X
FASLG	Lower	p=0.0279	Pro-apoptosis	X	
TP53	Lower	p=0.0481	Pro-apoptosis		X
Gene	Expression in Active SJIA / Control Monocytes ^d		Function	Pathway Involvement ^b	
				Extrinsic	Intrinsic
CASP1 ^e		++	Part of inflammasome; activates IL-1, IL-18		
BCL2A1		+	Anti-apoptosis		X
FLIP		+	Anti-apoptosis	X	
TP53		-	Pro-apoptosis		X
MCL1		-	Anti-apoptosis		X
BNIP3		--	Pro-apoptosis		X
FASLG		-	Pro-apoptosis	X	

^aTRADD, TRAF2, and CASP10 expression were not significantly different between active and inactive SJIA PBMC (not shown). These genes are involved in the extrinsic pathway.

^bRefers to whether the encoded protein is involved in the extrinsic or intrinsic apoptosis pathway.

^cP-values determined by paired t-test.

^d+, >3 fold; ++, >10 fold up-regulation; -, >3 fold; --- >10 fold down-regulation in active SJIA relative to control monocytes. Active SJIA (n=3); Control (n=3).

^eBoth IL-1 and IL-18 have been implicated in SJIA [6:65]