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Inflammatory Gene Expression Profile and Defective Interferon-γ and Granzyme K in Natural Killer Cells From Systemic Juvenile Idiopathic Arthritis Patients

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Objective. **Systemic juvenile idiopathic arthritis (JIA) is an immunoinflammatory disease characterized by arthritis and systemic manifestations. The role of natural killer (NK) cells in the pathogenesis of systemic JIA remains unclear. The purpose of this study was to perform a comprehensive analysis of NK cell phenotype and functionality in patients with systemic JIA.**

Methods. **Transcriptional alterations specific to NK cells were investigated by RNA sequencing of highly purified NK cells from 6 patients with active systemic JIA and 6 age-matched healthy controls. Cytokines (NK cell–stimulating and others) were quantified in plasma samples (n = 18). NK cell phenotype and cytotoxic activity against tumor cells were determined (n = 10), together with their interferon-γ (IFNγ)–producing function (n = 8).**

Results. **NK cells from the systemic JIA patients showed an altered gene expression profile compared to cells from the healthy controls, with enrichment of immunoinflammatory pathways, increased expression of innate genes including *TLR4* and *S100A9*, and decreased expression of immune-regulating genes such as *IL10RA* and *GZMK*. In the patients' plasma, interleukin-18 (IL-18) levels were increased, and a decreased ratio of IFNγ to IL-18 was observed. NK cells from the patients exhibited specific alterations in the balance of inhibitory and activating receptors, with decreased killer cell lectin-like receptor G1 and increased NKp44 expression. Although NK cells from the patients showed increased granzyme B expression, consistent with intact cytotoxicity and degranulation against a tumor cell line, decreased granzyme K expression in CD56^{bright} NK cells and defective IL-18–induced IFNγ production and signaling were demonstrated.**

Conclusion. **NK cells are active players in the inflammatory environment typical of systemic JIA. Although their cytotoxic function is globally intact, subtle defects in NK-related pathways, such as granzyme K expression and IL-18–driven IFNγ production, may contribute to the immunoinflammatory dysregulation in this disease.**

Systemic juvenile idiopathic arthritis (JIA) is a chronic immunoinflammatory childhood disorder of unknown etiology that is characterized by arthritis and systemic features such as quotidian fever, rash, lymphadenopathy, and serositis (1,2). An interplay of environmental factors and genetic predisposition is considered to underlie the pathogenesis (1). About 10% of systemic JIA patients develop

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macrophage activation syndrome (MAS), a potentially fatal hyperinflammatory syndrome classified as secondary hemophagocytic lymphohistiocytosis (HLH) (3), and a further 50% of systemic JIA patients present with a subclinical form of MAS (4). MAS is characterized by excessive activation of T cells and macrophages, which may be linked to defective cytotoxicity of CD8+ T cells and natural killer (NK) cells (5). NK cells kill infected, transformed, or stressed cells by the release of cytotoxic molecules such as perforin and granzymes. In addition, NK cells produce cytokines, thereby modulating other cells of the immune system. The activity of NK cells is defined by a balance of signals from activating and inhibitory receptors and by activating cytokines such as interleukin-2 (IL-2), IL-12, IL-15, and IL-18 (6). Although some studies found normal NK cell numbers in systemic JIA patients, others demonstrated decreased NK numbers or defective NK function (7–10), supporting the hypothesis that dysfunctional NK cells are involved in the pathogenesis of systemic JIA and may account for its association with MAS (8,11).

Systemic JIA differs from other JIA subtypes in that it lacks involvement of autoreactive T cells or autoantibodies. Together with a prominent innate immune activation, this has led to the classification of systemic JIA as an autoinflammatory disease (1). The contribution of the innate immune system is further exemplified by the role of innate cytokines, including IL-1β, IL-6, and IL-18 (12–17). In contrast with the proinflammatory environment, levels of interferon-γ (IFNγ) are barely increased (8,15), which is intriguing given the high levels of IL-18, a strong IFNγ-inducing factor in NK cells (18). Of note, low expression of IFNγ is consistent with our recently described mouse model of systemic JIA, in which IFNγ-deficient mice challenged with Freund's complete adjuvant developed clinical and pathologic features of systemic JIA. In addition, these mice had defective NK cytotoxicity (19).

Driven by the hypothesis that dysfunctional NK cells may underlie the pathogenesis of systemic JIA, we performed an in-depth analysis of NK cells from patients with active systemic JIA. We also performed RNA sequencing of highly purified NK cells from systemic JIA patients and age-matched healthy controls. Furthermore, we examined NK-activating cytokines in plasma, the expression of activating and inhibitory receptors and cytotoxic molecules by NK cells, their cytolytic activity against a tumor cell line, and their capacity to produce IFNγ in response to IL-18. We found that NK cells from the systemic JIA patients displayed a characteristic inflammatory gene expression, but an overall normal phenotype and globally intact cytotoxic function. However, these NK cells manifested decreased granzyme K expression and defective IL-18–driven IFNγ production, which may contribute to the pathogenesis of the disease.

PATIENTS AND METHODS

Patients and sampling. A total of 19 patients with systemic JIA and 20 age-matched healthy control subjects were recruited from the University Hospitals Leuven. All subjects gave their informed consent, and the study was conducted in accordance with the Declaration of Helsinki. The study was approved by the Institutional Review Board of the University Hospitals Leuven. All patients met the International League of Associations for Rheumatology criteria for the classification of JIA (20), and all patients had active disease at the time of sampling. Supplementary Table 1 (available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39933/abstract) summarizes the demographic data, clinical characteristics, laboratory values, and treatment at the time of sampling. Four patients were receiving corticosteroid treatment, with a median dosage of 0.6 mg/kg/day (range 0.3–2 mg/kg/day). One patient was sampled prior to starting a new course of IL-1β antagonist (canakinumab). We examined the data from the treated patients in detail and found no diverging results when compared to patients without concurrent treatments. Two of the study patients presented with the first symptoms of an emerging MAS episode.

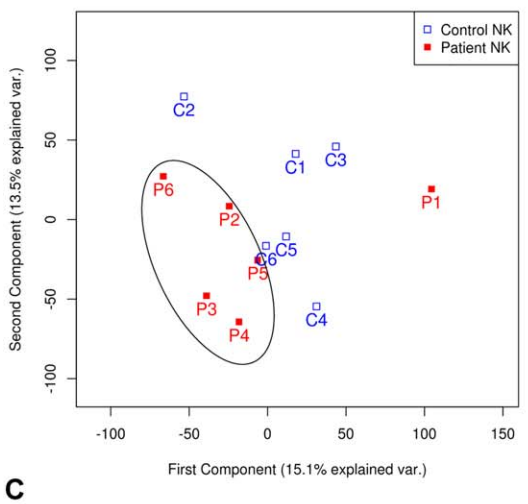
Plasma was separated from EDTA-anticoagulated blood samples (10 ml) and stored at −80°C. Peripheral blood mononuclear cells (PBMCs; 15–30 $\times$ 10^6) were isolated using Lymphoprep (Axis-Shield), frozen in liquid nitrogen using a freezing medium of 10% DMSO (Sigma-Aldrich) and 90% fetal bovine serum (FBS; Lonza BioWhittaker), and thawed prior to analysis.

PBMC cultures. We cultured PBMCs (1.5 $\times$ 10^6) in RPMI 1640 medium containing 10% FBS and stimulated them with recombinant human IL-18 (100 ng/ml; MBL International) in the presence or absence of recombinant human IL-12 (2 ng/ml; PeproTech) or with phorbol 12-myristate 13-acetate (PMA; 50 ng/ml) and ionomycin (500 ng/ml) (both Sigma-Aldrich). Phosphorylation staining was performed after 15 minutes. Intracellular IFNγ was detected after 18 hours, with GolgiStop (BD Biosciences) added for the last 4 hours. IFNγ production in cell culture supernatants was measured after 48 hours.

Flow cytometry. PBMCs (0.3 $\times$ 10^6) were incubated with Fc receptor–blocking reagent (Miltenyi Biotec) followed by staining for surface markers (Supplementary Table 2, available at http://onlinelibrary.wiley.com/doi/10.1002/art.39933/abstract). Intracellular staining was performed using a Cytofix/Cytoperm kit (BD Biosciences). Phosphorylation staining (2 $\times$ 10^6 PBMCs) was performed according to the Phosflow protocol (BD Biosciences). Cell sorting was performed on a FACSAria III instrument. A representative gating strategy is depicted in Supplementary Figure 1 (available at http://onlinelibrary.wiley.com/doi/10.1002/art.39933/abstract).

RNA sequencing. The number and purity of NK cells (median purity 99%) used in the RNA sequencing are shown in Supplementary Table 3 (available at http://onlinelibrary.wiley.com/doi/10.1002/art.39933/abstract). RNA was extracted from purified NK cells using a Qiagen RNeasy Micro kit, and RNA integrity and quantity were checked with an Agilent Bioanalyzer

A



B

Upstream Regulator	Molecule Type	Activation z-score	p-value of overlap	# target molecules
GATA1	transcription regulator	2.938	3.90E-11	12
E. coli B4 LPS	chemical toxicant	2.356	1.89E-06	9
TGM2	enzyme	2.345	3.48E-07	9
tretinoin	chemical	2.312	7.83E-07	20
IL10	cytokine	2.164	1.82E-07	11
IL1B	cytokine	2.083	8.16E-06	14
EPO	cytokine	2.082	2.05E-08	11
LY-2510924	biologic drug	2.000	1.22E-07	4
fluticasone	chemical drug	1.914	7.24E-06	7
TNF	cytokine	1.783	1.19E-07	23
trichostatin A	chemical drug	1.765	5.45E-07	13
LPS	chemical drug	1.718	2.03E-10	27
IL6	cytokine	1.620	4.65E-06	13
E. coli LPS	chemical	1.549	8.34E-06	5
IFNG	cytokine	1.546	1.39E-07	20
Immunoglobulin	complex	-4.054	7.80E-17	17

C

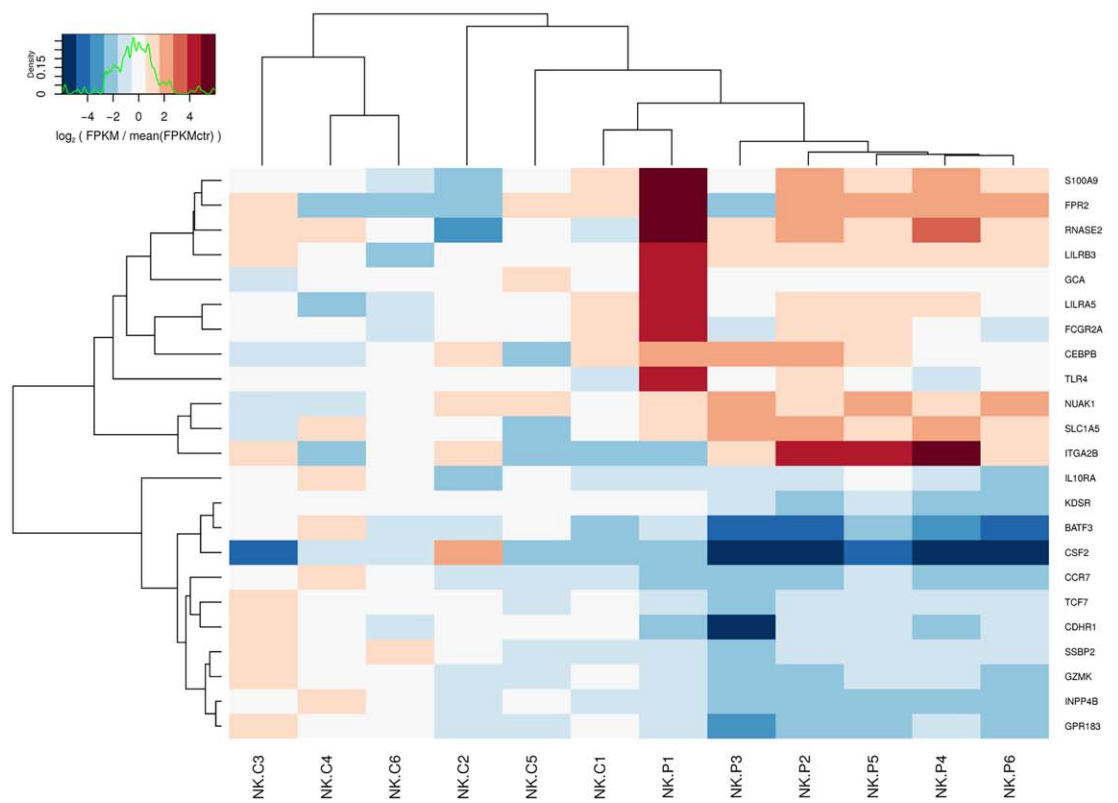


Figure 1. Natural killer (NK) cell–specific alteration of gene expression in patients with systemic juvenile idiopathic arthritis (JIA). Gene expression was analyzed by RNA sequencing of NK cells from 6 patients with active systemic JIA (P1–P6) and 6 age-matched healthy control subjects (C1–C6) isolated by fluorescence-activated cell sorting. **A,** Principal components analysis of RNA sequencing data from systemic JIA and control NK cells. **B,** Potential upstream regulators of NK gene expression, as determined using IPA software, with activation Z scores of $>|1.5|$ and overlap P values of $<10^{-5}$. **C,** Heatmap showing the $\log_2$ relative fragments per kilobase million (FPKM) of each differentially expressed NK cell–specific gene in each sample versus the mean FPKM in the controls (FPKMctr). Genes and samples were clustered using a complete linkage algorithm, with (1 – Spearman's correlation)/2 as the measure of dissimilarity. var. = variance.

with an RNA 6000 Pico LabChip kit. Whole-transcriptome analysis with next-generation RNA sequencing, preparative techniques, and statistical comparison (21) were performed by the VIB Nucleomics Core at KU Leuven (www.nucleomics.be). Detailed methods are given in the Supplementary Methods (available at http://onlinelibrary.wiley.com/doi/10.1002/art.39933/abstract).

Cytokine measurements. A multiplex assay from Meso Scale Discovery was used to detect the following cytokines in plasma: IFNγ, IL-1β, IL-2, IL-6, IL-10, IL-12p70, tumor necrosis factor (TNF) (Proinflammatory Panel 1), IL-15 (Cytokine Panel 1), and IL-18. IFNγ was quantified in culture supernatants using a DuoSet enzyme-linked immunosorbent assay (R&D Systems).

NK cell assays. NK cytotoxicity was measured in a ^{51}Cr release assay. PBMCs were thawed and cultured with the NK-sensitive human K562 leukemia cell line (ATCC) that lacks expression of major histocompatibility complex class I. ^{51}Cr-labeled K562 cells were cultured for 4 hours with PBMCs (2.5×10^6/ml) at a 50:1 effector:target cell (E:T) ratio, and the release of ^{51}Cr was measured. Spontaneous and maximal release was determined by incubation of labeled target cells with medium or saponin (Merck), respectively. Specific lysis was calculated as follows: (experimental release – spontaneous release)/(maximal release – spontaneous release) ×100. The degranulation capacity of NK cells was measured by the induction of CD107a surface expression after a 2-hour incubation of PBMCs (1×10^6/ml) with K562 cells (E:T ratio 1:1). Perforin and granzyme expression was analyzed ex vivo by intracellular flow cytometry. For intracellular expression of IFNγ in NK cells by flow cytometry, PBMCs (1×10^6/ml) were cultured for 6 hours in the presence of K562 cells (E:T ratio 1:1), with the addition of BD GolgiStop (1:150 dilution) and GolgiPlug (1:100 dilution) (both from BD Biosciences) for the final 5 hours.

Statistical analysis. Statistical analysis was performed using GraphPad Prism 6 software. Mann-Whitney U test was used for comparisons of 2 groups, and for multiple comparisons, Kruskal-Wallis test was performed with Dunn's post hoc test. *P* values less than 0.05 were considered statistically significant.

RESULTS

Influence of the inflammatory environment on gene expression in NK cells from systemic JIA patients. To dissect the involvement of NK cells in the pathogenesis of systemic JIA, an in-depth analysis of NK gene expression by RNA sequencing was performed on purified NK cells from 6 patients with active systemic JIA and 6 age-matched healthy controls. The purity of the NK cell transcriptome was confirmed using Cell Type Enrichment software (22), which demonstrated highest enrichment of NK cell–specific genes (Supplementary Figure 2A, available at http://onlinelibrary.wiley.com/doi/10.1002/art.39933/abstract). Using principal components analysis, NK cell data from 5 of the 6 systemic JIA patients clustered together (Figure 1A). Comparable symptoms and treatment were noted in the outlier patient, and therefore, all patients were included in further analyses. Statistical analysis of the transcriptome revealed 107 differentially expressed genes; 71 of them were up-regulated and 36 were down-regulated in NK cells from the patients. A comprehensive list of differentially expressed genes is shown in Supplementary Table 4 (available at http://onlinelibrary.wiley.com/doi/10.1002/art.39933/abstract).

In order to make an unbiased analysis of the differentially expressed genes, we performed Ingenuity Pathway Analysis (IPA). Categories with the highest enrichment comprised genes related to cell-to-cell signaling and interaction, cellular function and maintenance, cellular movement, hematologic system development and function, immune cell trafficking, and inflammatory response, compatible with modification of key immunologic pathways (Supplementary Table 5, available at http://onlinelibrary.wiley.com/doi/10.1002/art.39933/abstract). Among key immunoinflammatory genes, NK cells from systemic JIA patients demonstrated increased expression of *S100A8*, *S100A9*, and *S100A12*, different leukocyte immunoglobulin-like receptors (*LILRA3*, *LILRA5*, and *LILRB3*), formyl peptide receptors (*FPR1* and *FPR2*), *IFNGR2*, *TLR4*, *NLRP12*, and displayed decreased expression levels of *CSF2*, *CCR7*, *IL10RA*, and *GZMK* (Supplementary Table 4). Analysis of transcription pathways identified components within the inflammatory environment, such as IL-10, IL-1β, IFNγ, TNF, and IL-6, as potential upstream mediators of the observed changes in the NK cell transcription profile (Z scores $>|1.5|$, $P < 10^{-5}$) (Figure 1B). Of note, NK-activating IL-18 was not identified as a major upstream regulator in the NK gene signature of patients with systemic JIA (Z score $= -0.80$, $P = 1.7 \times 10^{-3}$).

In contrast to what we expected to see in highly purified NK cells, we found evidence of genes that are normally expressed by erythrocytes (e.g., *HBB* [see Supplementary Table 4]). Spearman's correlation analysis of highly expressed cell-specific erythrocyte genes revealed clustering of systemic JIA patients, indicating a potential erythrocyte contamination (Supplementary Figure 2B). A similar enrichment of erythrocyte-specific genes was detected in previous gene expression studies of total PBMCs, which has been attributed to the presence of erythrocyte precursors in systemic JIA PBMCs (23–26). Although we used highly purified NK cells (median postsort purity of 99%; Supplementary Table 3 and Supplementary Figure 2C), contamination of a minimal amount of erythrocyte precursors in systemic JIA samples might influence the results.

Therefore, in order to extract biologically relevant data, an NK cell–specific list of genes was generated by filtering the list of significantly differentially expressed genes using IPA software (Supplementary Methods), which revealed increased *S100A9* and *TLR4* and decreased *IL10RA* and *GZMK*, among other genes (Figure 1C). When performing clustering analysis of the 23 NK cell–specific genes, all systemic JIA patients clustered together, confirming the clinical relevance of our NK cell–specific gene list (Figure 1C). Further network analysis of NK cell–specific genes confirmed the involvement of inflammatory response and immunologic disease genes (data not

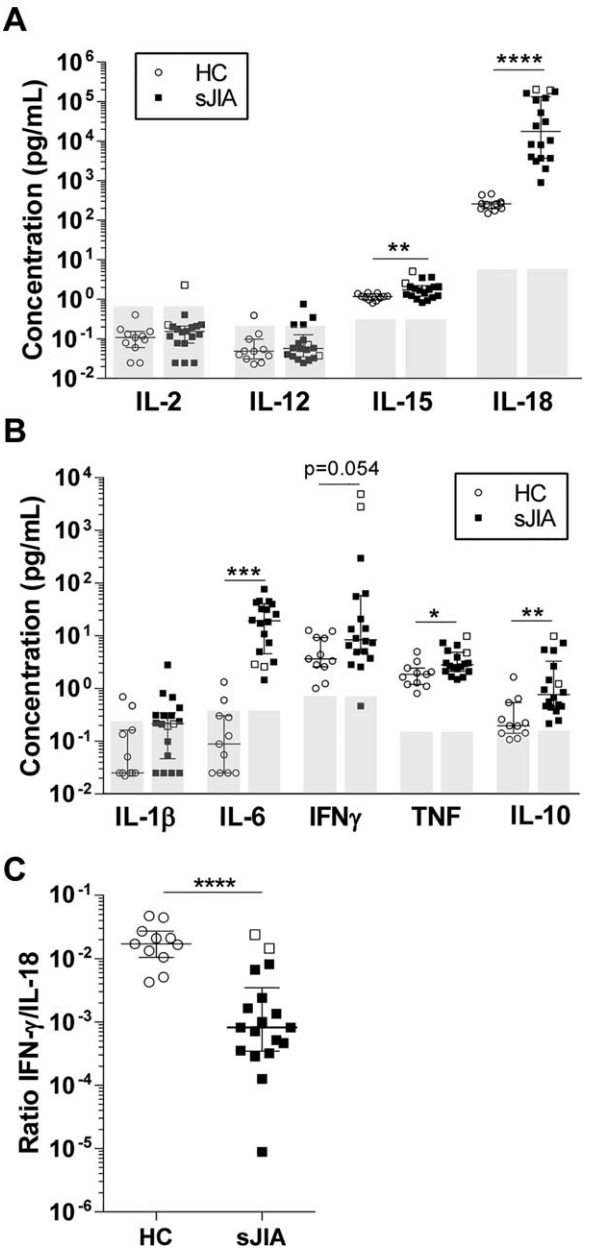


Figure 2. Cytokine levels in the plasma of patients with systemic juvenile idiopathic arthritis (JIA). **A** and **B,** Plasma levels of the cytokines in age-matched healthy controls (HC; n = 11) and patients with active systemic JIA (sJIA; n = 18). Shaded bars indicate the detection limits. **C,** Ratio of interferon-γ (IFNγ) to interleukin-18 (IL-18) in the plasma of controls and patients with active systemic JIA. Open squares represent 2 systemic JIA patients with emerging symptoms of macrophage activation syndrome. Each symbol represents an individual subject; horizontal lines with bars show the median and interquartile range. $* = P < 0.05$; $** = P < 0.01$; $*** = P < 0.001$; $**** = P < 0.0001$ by Mann-Whitney U test. TNF = tumor necrosis factor.

shown). Analysis of the functional association of key cytokines in systemic JIA with differentially expressed NK cell genes demonstrated that IL-1β and IL-6 are linked to multiple differentially expressed NK cell genes (Supplementary Figure 3, available at http://onlinelibrary.wiley.com/doi/10.1002/art.39933/abstract), underscoring the inflammatory response in systemic JIA NK cells.

Together, these results demonstrate that NK cells from systemic JIA patients display an altered gene expression profile as compared to healthy control NK cells, with a manifest enrichment of inflammatory response–related genes, including proinflammatory IL-1β and IL-6 signaling pathways. This finding suggests that their biologic behavior has changed secondary to the inflammatory environment that is characteristic of systemic JIA.

Increased plasma levels of IL-18, but not IL-2 or IL-12, in systemic JIA patients. Although transcriptome analysis identified proinflammatory cytokines (IL-1β and IL-6) as being upstream regulators of NK cell gene expression in systemic JIA patients, it did not provide data concerning the cytokines upon which NK cells rely for survival and activation. We therefore investigated the presence of NK cell–stimulating cytokines IL-2, IL-12, IL-15, and IL-18 together with other inflammatory cytokines in the plasma of 18 systemic JIA patients and 11 age-matched healthy controls. While IL-2 and IL-12 plasma levels did not significantly differ between patients and controls, IL-15 ($P < 0.01$) and IL-18 ($P < 0.0001$) levels were moderately and highly increased in plasma from the patients, respectively (Figure 2A). Of note, IL-15 levels were measured irrespective of the levels of IL-15 receptor antagonist, thus representing both active and inactive forms of IL-15.

Levels of IL-1β and IFNγ tended to be higher in systemic JIA patients, although not significantly so (Figure 2B). Two patients with systemic JIA and emerging MAS symptoms had highly elevated IFNγ plasma levels (Figure 2B). In all systemic JIA patients, a large increase in IL-6 levels was observed ($P < 0.001$), while TNF ($P < 0.05$) and IL-10 ($P < 0.01$) levels were moderately raised. The ratio of IFNγ to IL-18 levels was significantly decreased in systemic JIA patients as compared to controls ($P < 0.0001$) (Figure 2C), confirming our previous findings in a different cohort of patients (15).

In conclusion, systemic JIA patients present with a complex change in plasma cytokine levels, which on the one hand parallels the inflammation-related differential gene expression in NK cells (e.g., IL-1β, IL-6, IL-10, and TNF) and on the other hand includes moderate to high increases in the levels of IL-15 and IL-18, but not IL-2 or IL-12, which are known to stimulate NK cells.

Subtle change in inhibitory and activating receptors in NK cells from systemic JIA patients. To decipher the impact of the altered cytokine environment and gene expression on NK cell phenotypes, we performed NK cell phenotyping on PBMCs from 10 patients with active systemic JIA and 10 healthy controls, among which were

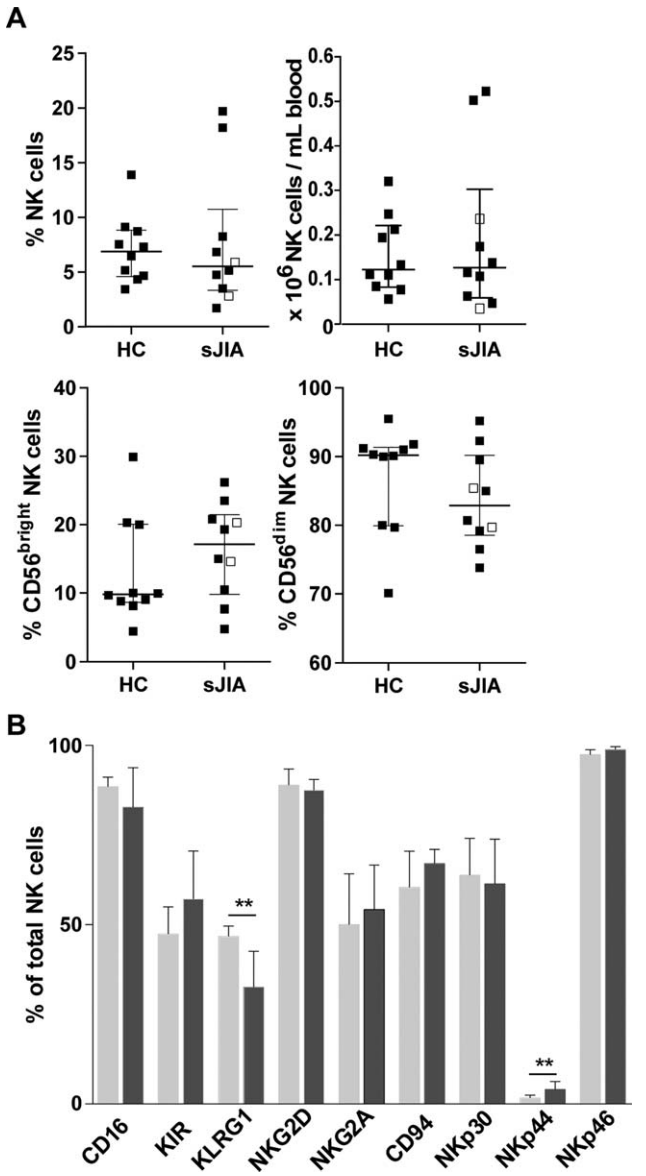


Figure 3. Subtle changes in phenotype of natural killer (NK) cells from patients with systemic juvenile idiopathic arthritis (JIA). NK cell markers on peripheral blood mononuclear cells from age-matched healthy controls (HC; n = 10) and patients with systemic JIA (sJIA; n = 10) were determined by flow cytometry. **A,** Percentage of CD3–CD56+ NK cells among live singlets, absolute numbers of CD3–CD56+ NK cells per milliliter of whole blood, and percentages of $CD56^{bright}$ and $CD56^{dim}$ cells among total NK cells. Open squares represent 2 systemic JIA patients with emerging symptoms of macrophage activation syndrome. Each symbol represents an individual subject; horizontal lines with bars show the median and interquartile range. **B,** Expression of the indicated activating and inhibitory receptors as a percentage of total CD3–CD56+ NK cells in healthy controls (lightly shaded bars) and patients with active systemic JIA (darkly shaded bars). ** = $P < 0.01$ by Mann-Whitney U test.

the individuals included in the RNA sequencing analysis. NK cells can be subdivided into 2 main subsets: $CD56^{bright}CD16^{-/dim}$ NK cells, which have an important cytokine-producing function, and $CD56^{dim}$CD16+ NK cells, which are believed to be mainly cytotoxic (27). The percentages and absolute numbers of total NK cells and $CD56^{bright}$ and $CD56^{dim}$ subsets were not significantly different in PBMCs from the patients as compared to the controls (Figure 3A). We observed no differences in the expression of CD16, killer cell immunoglobulin-like receptors, killer cell lectin-like receptor (KLR-2D) and KLR-2A (NKG2-D and NKG2-A), CD94, NKp30, and NKp46 in total NK cells. However, NK cells from the patients showed decreased expression of the inhibitory receptor killer cell lectin-like receptor G1 (KLR-G1) ($P < 0.01$) and increased expression of the activating NKp44 receptor ($P < 0.01$) (Figure 3B). Nonetheless, based on their globally normal phenotype, NK cells from systemic JIA patients showed no evidence of altered functionality.

Intact NK cell cytotoxicity, but decreased granzyme K expression, in systemic JIA patients. NK cell cytotoxicity against tumor target cells was measured by ^{51}Cr-release and CD107a degranulation assays. PBMCs from systemic JIA patients and healthy controls showed a similar cytolytic capacity against ^{51}Cr-labeled K562 cells, albeit with a large variation (Figure 4A). Correction for the percentage of NK cells normalized the results, indicating an intact cytotoxic function. In the degranulation assay, coculturing PBMCs with K562 tumor cells significantly increased both the percentage of CD107a+ NK cells ($P < 0.01$ for the controls and $P < 0.001$ for the patients) and the expression intensity of CD107a per cell ($P < 0.01$ for the comparisons in each study group), with similar increases in patients as compared to the controls (Figure 4B). Subsequently, we measured the expression of perforin and granzymes in NK cells. Systemic JIA patients had similar percentages of perforin-positive and granzyme A–positive NK cells and elevated percentages of granzyme B–positive NK cells ($P < 0.05$) as compared to healthy controls (Figure 4C). The expression of granzyme K has been described as being characteristic of $CD56^{bright}$ NK cells (28). Interestingly, the percentage of granzyme K–positive $CD56^{bright}$ NK cells was decreased in patients as compared to controls ($P < 0.05$) (Figure 4D), which is consistent with the RNA sequencing findings. Taken together, our findings showed that NK cells from this cohort of systemic JIA patients had a globally intact cytotoxic profile and function upon in vitro stimulation with tumor cells, including increased granzyme B levels, but had decreased granzyme K–expressing $CD56^{bright}$ NK cells.

Defective IL-18–induced IFNγ production and signaling in NK cells from systemic JIA patients. To measure the cytokine-producing capacity of NK cells, we focused on the best-characterized NK cell–derived cytokine, IFNγ. IL-18 is commonly recognized as the main

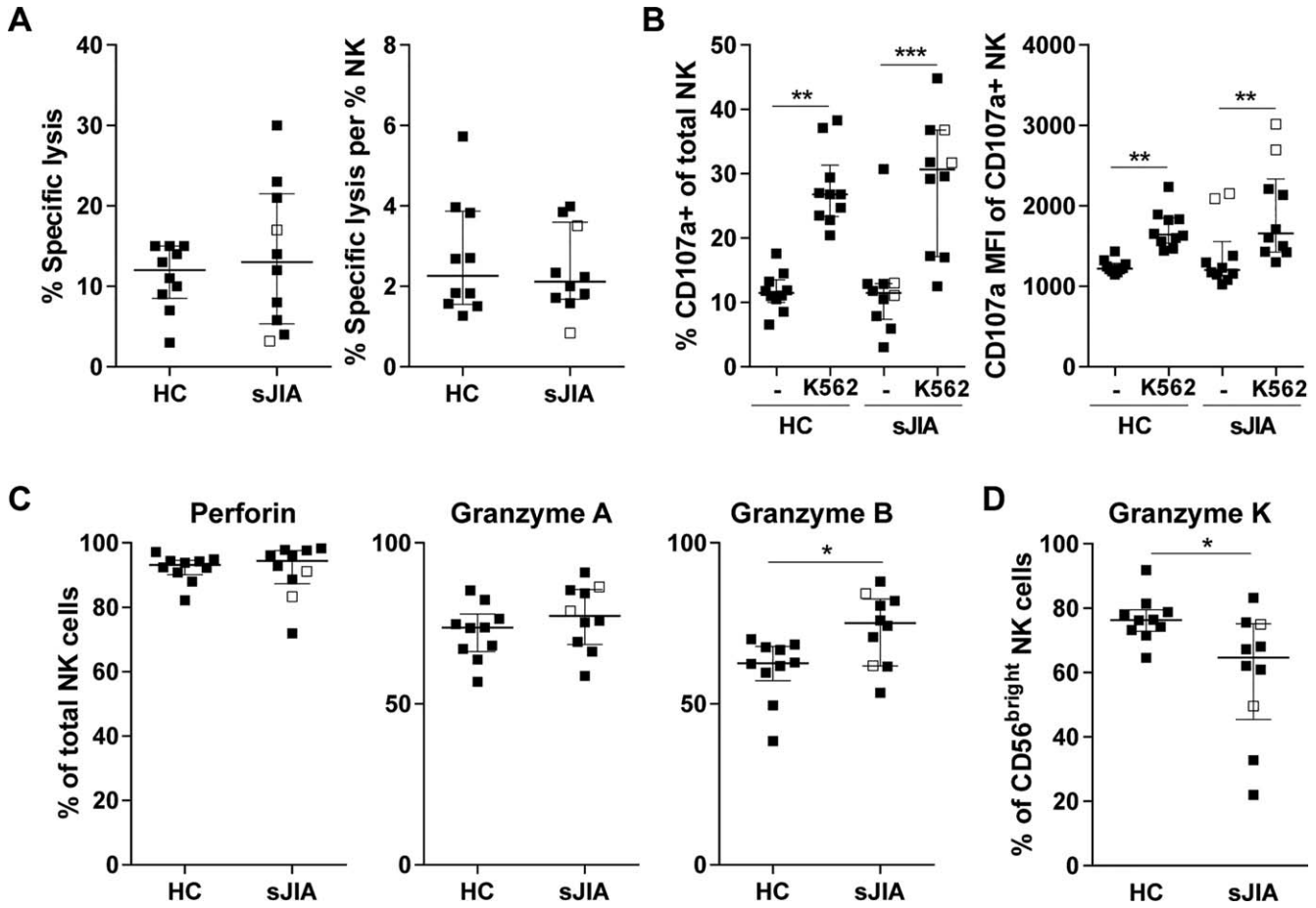


Figure 4. Intact cytotoxicity profile of natural killer (NK) cells from patients with systemic juvenile idiopathic arthritis (JIA), except for granzyme K expression in CD56^{bright} NK cells. **A,** Percentage of specific lysis of ^{51}Cr-labeled K562 cells cultured for 4 hours with peripheral blood mononuclear cells (PBMCs) from age-matched healthy controls (HC) and patients with active systemic JIA (sJIA) (left), with correction for the number of NK cells as defined by immune phenotyping (right), at an effector-to-target ratio of 50:1. **B,** Percentage of CD107a+ cells among total CD3–CD56+ NK cells (left) and the median fluorescence intensity (MFI) of CD107a+ NK cells (right), as determined 2 hours after incubation of control and patient PBMCs either alone or with K562 cells. **C** and **D,** Intracellular flow cytometry of perforin, granzyme A, and granzyme B (**C**), as well as granzyme K (**D**), in NK cells from controls and systemic JIA patients. Results are given as the percentage of positive cells among total CD3–CD56+ NK cells (**C**) or as the percentage of CD56^{bright} NK cells (**D**). Open squares represent 2 systemic JIA patients with emerging symptoms of macrophage activation syndrome. Each symbol represents an individual subject; horizontal lines with bars show the median and interquartile range. $* = P < 0.05$; $** = P < 0.01$; $*** = P < 0.001$ by Mann-Whitney U test.

IFNγ inducer in NK cells. Intriguingly, despite highly elevated plasma levels of IL-18 in systemic JIA patients, the levels of IFNγ remained low, resulting in a decreased ratio of IFNγ to IL-18 (Figure 2). To analyze NK cell responses to IL-18, PBMCs from 9 patients with active systemic JIA and 8 healthy controls were stimulated in vitro with IL-18. We found that IL-18 induced IFNγ in a small percentage of control NK cells, with significantly lower expression in the patients ($P < 0.001$) (Figure 5A).

Since IL-18 synergizes with IL-12 to induce IFNγ (29), we stimulated cells with IL-18 plus IL-12. This resulted in highly increased numbers of IFNγ+ NK cells in both patients and controls (Figure 5A). However, the expression intensity of IFNγ in IFNγ+ NK cells was lower in the patients ($P < 0.05$) (Figure 5A), again indicating defective IL-18–induced IFNγ production in the presence or absence of IL-12. This was further confirmed by the observation of lower levels of secreted IFNγ in the supernatants of IL-18 plus IL-12–stimulated PBMCs from the patients ($P < 0.05$) as compared to the controls (Figure 5B). Production of IFNγ after stimulating PBMCs with PMA/ionomycin or after coculturing PBMCs with K562 cells was not altered in the systemic JIA patients, demonstrating no overall defect in IFNγ production (Figures 5B and C).

De Jager et al (9) showed defective IL-18–induced phosphorylation of IL-18 receptor β in NK cells from systemic JIA patients. We therefore analyzed the

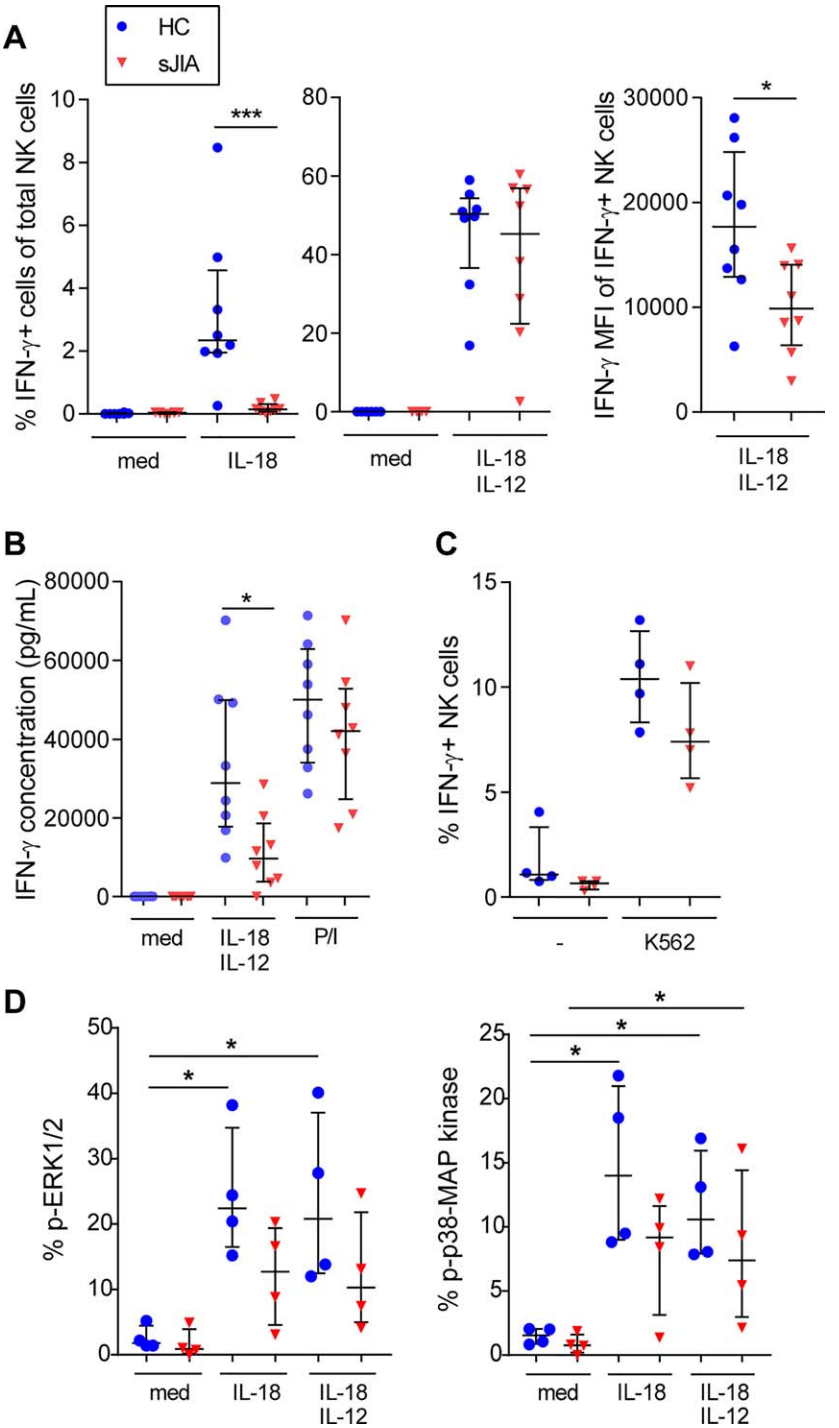


Figure 5. Defective production of interferon-γ (IFNγ) and phosphorylation of ERK-1/2 and p38 MAP kinase in natural killer (NK) cells from patients with systemic juvenile idiopathic arthritis (JIA) following stimulation with interleukin-18 (IL-18) in the presence or absence of IL-12. Peripheral blood mononuclear cells from age-matched healthy controls (HC) and patients with active systemic JIA (sJIA) were cultured in medium (med) alone or in the presence of IL-18, IL-18 plus IL-12, phorbol myristate acetate (PMA) plus ionomycin (P/I), or K562 cells for 18 hours (**A**), 48 hours (**B**), 6 hours (**C**), or 15 minutes (**D**). IFNγ was measured by intracellular flow cytometry (**A** and **C**) or enzyme-linked immunosorbent assay (**B**). Phosphorylation of ERK-1/2 and p38 MAP kinase was measured by flow cytometry (**D**). **A,** Percentage of IFNγ+ cells among total CD3–CD56+ NK cells after the indicated stimulations (left), and the median fluorescence intensity (MFI) of IFNγ in IFNγ+ NK cells (right). **B,** IFNγ concentrations in supernatants. **C,** Percentage of IFNγ+ cells among total CD3–CD56+ NK cells after coculture with K562 cells. **D,** Percentage of phosphorylated ERK-1/2–positive cells (left) and p38 MAP kinase–positive cells (right) among total CD3–CD56+ NK cells. Each symbol represents an individual subject; horizontal lines with bars show the median and interquartile range. * = $P < 0.05$; *** = $P < 0.001$ by Mann-Whitney U test in **A–C** and by Kruskal-Wallis test in **D**.

IL-18–driven phosphorylation of ERK-1/2 and p38 MAP kinase, both of which are involved in signaling downstream of the IL-18 receptor. The up-regulation of phosphorylated ERK-1/2 and p38 MAP kinase after stimulation with IL-18 in the presence or absence of IL-12 was less pronounced in NK cells from the patients as compared to the controls (n = 4) (Figure 5D). Expression of IL-18 receptor α and β was unchanged in NK cells from the patients and controls (data not shown). Taken together, these results show that NK cells from systemic JIA patients have a specific defect in IL-18–induced IFNγ production, which at least partially results from a dysfunctional signaling pathway downstream of the IL-18 receptor.

DISCUSSION

The potential role of NK cells in the pathogenesis of systemic JIA and the association of the disease with MAS has received increased attention in recent years (7,8,10,11,30). In the present study, we performed a comprehensive analysis of NK cell characteristics in systemic JIA patients during active disease. To our knowledge, this study is the first to analyze gene expression in highly purified NK cells from systemic JIA patients.

The NK cell transcriptome in these patients demonstrated enrichment of inflammation-related genes as compared to controls. NK cells from the patients displayed increased expression of innate pathways, such as Toll-like receptor 4 (TLR-4) and S100 proteins, and were influenced by proinflammatory cytokines, such as IL-1β and IL-6. In addition, we found decreased gene expression of immune-regulating granzyme K and IL-10 receptor together with an absence of increased IFNγ expression, as well as an absence of IL-18 among the significant upstream regulators, all of which are consistent with the concept of defective immune regulation in systemic JIA (1,31). Although NK cells comprise only 5–15% of PBMCs, our data on purified NK cells showed activation of pathways comparable to those in total PBMCs (23,25,32). The expression of typical systemic JIA–related innate inflammatory pathways and the decrease in specific immune-regulating genes in the patients' NK cells suggest a secondary effect of the inflammatory environment characteristic of systemic JIA on the biologic behavior of NK cells, identifying NK cells as important targets and essential players in the development and pathogenesis of the disease.

In this study, we found equal numbers of NK cells in patient and control PBMCs, which is consistent with some previous studies (7,33,34) but contrasts with other studies demonstrating decreased numbers of total (9,10,23,35), $CD56^{dim}$ (23), or $CD56^{bright}$ (7,9) NK cells. NK cells from our systemic JIA patient cohort displayed few alterations in the balance of activating and inhibitory receptors, with significantly increased expression of activating receptor NKp44 and decreased expression of the inhibitory receptor KLR-G1. Thus, NK cells from systemic JIA patients do not show defects in any receptors that are necessary for activation.

Much of what is known about the pathogenesis of systemic JIA and associated MAS has been deduced from observations in primary HLH, in which mutations leading to defective cytotoxicity of NK cells and CD8+ cytotoxic T cells underlie disease. Although different studies identified polymorphisms in cytotoxicity-linked genes in patients with systemic JIA/MAS (36–40), Donn et al found no association of systemic JIA (without MAS) with single-nucleotide polymorphisms in gene loci for *PRF1*, *GZMB*, *UNC13D*, or *RAB27A* (41). To investigate the present controversy about NK cells in systemic JIA, we performed an extensive analysis of NK cell function in our patient cohort. Consistent with the study by Donn et al (41), we found no intrinsic defect in NK cytotoxicity against a tumor cell target in our systemic JIA patients. However, a large variability between patients was observed, with some demonstrating decreased, and others increased, cytotoxic capacity. Importantly, the high level of variability in NK cytotoxicity we observed disappeared when we corrected for the number of NK cells in total PBMCs. Previous studies did not correct for NK numbers (7,9,10), making it difficult to draw conclusions about the intrinsic cytotoxic potential.

When further analyzing the expression of cytotoxic granule components, we observed normal-to-increased levels of perforin and granzyme A and B in NK cells of systemic JIA patients, supporting our findings of no defective NK cytotoxicity against a tumor cell target in systemic JIA patients. These findings are in contrast to those of a study by Wulffraat et al, who observed decreased perforin expression in NK cells from systemic JIA patients (42), albeit with a high variability. As $CD56^{dim}$ NK cells are generally regarded as the predominant cytotoxic subset, most of the attention has been paid to this subset and its products granzyme A and granzyme B. However, during the last decade, $CD56^{bright}$ NK cells have received more attention, especially since this population has been found to kill autologous activated T cells in patients with multiple sclerosis via granzyme K, a protease predominantly expressed by $CD56^{bright}$ NK cells (28,43). In the present study, we found decreased granzyme K protein expression in $CD56^{bright}$ NK cells, as well as decreased mRNA expression of granzyme K in purified (total) NK cells from systemic JIA patients. So far, NK cell cytotoxic capacity has been largely investigated using tumor target cells. However, as decreased granzyme K expression in systemic JIA patients might influence the killing of activated autologous cells, we suggest

that the use of autologous cells as target may potentially be more relevant when studying cytotoxicity in this disease.

In addition to their cytotoxic function, NK cells are important sources of IFNγ (6). We demonstrated decreased IL-18–induced IFNγ production in NK cells from systemic JIA patients. This was consistent with the ex vivo data, as we observed no increased IFNγ gene expression in systemic JIA NK cells and only moderately increased plasma IFNγ, even though the patients presented with highly elevated IL-18 plasma levels. Remarkably, IL-18 was not identified as an upstream regulator of NK gene expression in the patients. These data are consistent with those reported by de Jager et al, who similarly found that the decreased NK cell responses to IL-18 in systemic JIA patients was associated with decreased phosphorylation of IL-18 receptor β (9). The latter finding is consistent with our data on a reduced induction of phosphorylation of the downstream signaling pathway of the IL-18 receptor, which may explain why, in our study, the defective IFNγ production was restricted to IL-18 stimulation and not to other triggers, such as PMA or tumor cells.

Decreased NK cell IFNγ production, together with relatively low plasma levels of IFNγ and the absence of an IFNγ gene signature in PBMCs (23), is consistent with our systemic JIA mouse model, in which a systemic JIA–like disease develops in the absence of IFNγ (19). In addition, NK cells are the major source of IFNγ in this model, suggesting a regulatory role in disease pathogenesis (Avau A, et al: unpublished observations). We therefore hypothesize that IFNγ production by NK cells may constitute a regulatory mechanism in systemic JIA. Of note, this possibly protective role of IFNγ in systemic JIA is the opposite of its probable disease-promoting role in MAS and HLH (44). Indeed, highly elevated levels of IFNγ in plasma are found in MAS and HLH patients (15), IFNγ is the major pathogenic factor in most mouse models of HLH (45), and a recent phase II clinical trial in 13 patients with (suspected) primary HLH showed anti-IFNγ to be a safe and effective treatment option in primary HLH patients with refractory disease (46). In view of the pathogenic role of IFNγ in the development of MAS and HLH, an alternative hypothesis might be that IL-18 hyporesponsiveness and decreased IFNγ production by NK cells may protect systemic JIA patients from developing MAS.

Our data support the concept that the altered NK cell function observed in systemic JIA patients (i.e., decreased production of IFNγ and granzyme K) is triggered by the proinflammatory environment that is typical of systemic JIA. Indeed, both IL-6 and IL-18 have been associated with impaired NK cell activity. Cifaldi et al demonstrated that IL-6 exposure reduced perforin and granzyme B expression in NK cells from healthy control subjects in vitro, and they proposed a similar effect of high levels of IL-6 in systemic JIA, as tocilizumab-treated patients showed an increased expression of perforin and granzyme B (47). Although high levels of IL-6 were detected in our patients, this was not associated with decreased perforin or granzyme B expression as compared to that in the healthy controls, nor was it associated with decreased NK cytotoxicity. However, we did find decreased granzyme K expression, as well as alterations in IL-6–related gene expression in purified NK cells from the patients, indicating that NK cells are indeed affected by IL-6. As to IL-18, chronically high levels of this NK-activating cytokine (48) have been shown to impair NK cell function (49,50). We therefore hypothesize that the altered cytokine environment in systemic JIA, as shown in this study and as described in detail in a review by Avau et al (8), may underlie the subtle but probably substantial alterations in NK cell function in systemic JIA patients observed in the present study. Acquired impairment of NK cell function may be part of the immune dysregulation seen in systemic JIA and may also constitute the link between systemic JIA and MAS.

In conclusion, NK cells from patients with active systemic JIA are targeted by the inflammatory environment, as evidenced by their ex vivo transcriptome. In our cohort of patients with active systemic JIA, NK cells displayed only minor alterations in phenotype and a globally intact cytotoxic profile against a tumor cell line. Nevertheless, defects in NK immune-regulating mechanisms, such as IL-18–induced IFNγ production and granzyme K expression, might contribute to the pathogenesis of systemic JIA. Taken together, our data identify NK cells as important target cells in disease, support the concept that altered NK cell function is part of the immunoinflammatory dysregulation seen in systemic JIA, and pave the way for further studies of their potential regulatory activity in this disease.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Drs. Matthys and Wouters had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Put, Vandenhaute, Avau, Brisse, Toelen, Waer, Leclercq, Goris, Liston, De Somer, Wouters, Matthys.

Acquisition of data. Put, Vandenhaute, van Nieuwenhuijze, Rutgeerts, Garcia-Perez, Wouters, Matthys.

Analysis and interpretation of data. Put, Vandenhaute, Avau, van Nieuwenhuijze, Brisse, Dierckx, Waer, Leclercq, Goris, Van Weyenbergh, Liston, De Somer, Wouters, Matthys.

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