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Inflammatory gene expression profile and defective IFN-γ and granzyme K in natural killer cells of systemic juvenile idiopathic arthritis patients

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Abstract

Objective. Systemic juvenile idiopathic arthritis (sJIA) is an immune-inflammatory disease characterized by arthritis and systemic features. The role of natural killer (NK) cells in sJIA pathogenesis remains unclear. Therefore, we performed a comprehensive analysis of NK cell phenotype and functionality in sJIA patients.

Methods. NK cell-specific transcriptional alterations were investigated by RNA-sequencing of highly purified NK cells from six active sJIA patients and six healthy controls. NK cell-stimulating and other cytokines were quantified in plasma (n=18). NK cell phenotype and cytotoxic activity against tumor cells were determined (n=10), next to their IFN-γ-producing function (n=8).

Results. NK cells of sJIA patients showed an altered gene expression profile compared to controls, with enrichment of immune-inflammatory pathways, increased expression of innate genes including *TLR4* and *S100A9* and decreased expression of immune-regulating genes like *IL10RA* and *GZMK*. In plasma of sJIA patients, IL-18 was increased and a decreased IFN-γ/IL-18 ratio was observed. sJIA NK cells exhibited specific alterations in the balance of inhibitory and activating receptors with decreased KLRG1 and increased NKp44 expression. Although sJIA NK cells showed increased granzyme B expression, in line with intact cytotoxicity and degranulation against a tumor cell line, we demonstrated decreased granzyme K expression in $CD56^{bright}$ NK cells and defective IL-18-induced IFN-γ production and signaling.

Conclusion. NK cells are active players in the inflammatory environment typical of sJIA. Although their cytotoxic function is globally intact, subtle defects in NK-related pathways such as granzyme K expression and IL-18-driven IFN-γ production may contribute to the immune-inflammatory dysregulation in sJIA.

Systemic juvenile idiopathic arthritis (sJIA) is a chronic immune-inflammatory childhood disorder of unknown etiology, which 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 sJIA patients develop macrophage activation syndrome (MAS), a potentially fatal hyper-inflammatory syndrome classified as secondary hemophagocytic lymphohistiocytosis (HLH) (3), and a further 50% of sJIA 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 the 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 (i.e. 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 IL-2, IL-12, IL-15 and IL-18 (6). Although some studies reported normal NK cell numbers in sJIA 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 sJIA and may account for its association with MAS (8;11).

sJIA differs from other JIA subtypes as it lacks involvement of autoreactive T cells or autoantibodies. Together with a prominent innate immune activation, this has led to the classification of sJIA 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 pro-inflammatory environment, levels of IFN-γ are barely increased (8;15), which is intriguing given the high levels of IL-18, a strong IFN-γ-

inducing factor in NK cells (18). Of note, low expression of IFN-γ is in accordance with our recently published sJIA-like mouse model, in which IFN-γ-deficient mice challenged with complete Freund's adjuvant (CFA) developed clinical and pathological features of sJIA. In addition, these sJIA-like mice demonstrated defective NK cytotoxicity (19).

Driven by the hypothesis that dysfunctional NK cells may underlie the pathogenesis of sJIA, we here performed an in-depth analysis of NK cells from active sJIA patients. We performed RNA-sequencing (RNA-seq) on highly purified NK cells from sJIA patients and healthy controls. Furthermore, we report on NK–activating cytokines in plasma, on the expression of activating and inhibitory receptors and cytotoxic molecules by NK cells, on their cytolytic activity against a tumor cell line and on their capacity to produce IFN-γ in response to IL-18. We found that sJIA NK cells display a characteristic inflammatory gene expression, but an overall normal phenotype and globally intact cytotoxic function. However, sJIA NK cells manifested decreased granzyme K expression and defective IL-18-driven IFN-γ production, which may contribute to the pathogenesis of sJIA.

Patients and Methods

Patients and sampling

19 sJIA patients and 20 age-matched healthy controls were recruited from the University Hospital of Leuven, after giving informed consent, according to the declaration of Helsinki. The study was approved by the Institutional Review Board. All sJIA patients met the criteria of the International League of Associations for Rheumatology (20) and all patients had active disease at the time of sampling. Supplementary Table S1 summarizes demographic data, clinical characteristics, laboratory values and treatment at time of sampling. Four patients were under corticosteroid treatment with a median dosage of 0.6 mg/kg/day (range 0.3 to 2

mg/kg/day). One patient was sampled prior to a new infusion with IL-1β antagonist (canakinumab). We examined the data of treated patients in detail and found no diverging results when compared to patients without concurrent treatments. Two sJIA patients presented with the first symptoms of an emerging MAS episode and are indicated with open squares in Figures 2-4. Plasma was separated from EDTA-anti-coagulated blood samples (10 mL) and stored at -80°C. Lymphoprep (Axis-Shield PoC AS)-isolated peripheral blood mononuclear cells (PBMCs, 15-30x10^6) were frozen in liquid nitrogen (10% DMSO (Sigma Aldrich), 90% fetal bovine serum (FBS, Lonza BioWhittaker)) and thawed for analysis.

PBMC cultures

PBMCs (1.5x10^6/mL) were cultured in RPMI-1640 medium containing 10% FBS and stimulated with human recombinant IL-18 (100 ng/mL, MBL International) with or without human recombinant 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 min. Intracellular IFN-γ was detected after 18 h, with GolgiStop (BD Biosciences) added for the last 4 h. IFN-γ production in cell culture supernatant was measured after 48 h.

Flow cytometry

PBMCs (0.3x10^6) were incubated with Fc-receptor blocking reagent (Miltenyi Biotec) followed by staining for surface markers (Supplementary Table S2). Intracellular staining was performed using the Cytofix/Cytoperm kit (BD Biosciences). Phosphorylation staining (2x10^6 PBMCs) was performed using the Phosflow protocol (BD Biosciences). Cell sorting was performed on a FACSAria III instrument. Representative gating strategy is depicted in Supplementary Figure S3.

RNA sequencing (RNA-seq)

The number and purity of NK cells (median purity 99%) used in the RNA-seq is enlisted in Supplementary Table S4. RNA was extracted from purified NK cells (Qiagen RNeasy Micro Kit) and RNA integrity and quantity were checked with the Bioanalyzer 6000 pico chip (Agilent). Whole-transcriptome analysis with next-generation sequencing (RNA-seq), preparative techniques and statistical comparison (21) were performed by the VIB Nucleomics Core (KU Leuven, www.nucleomics.be). Detailed methods can be found in Supplementary File S5.

Cytokine measurement

Meso Scale Discovery's multiplex assay was used to detect following cytokines in plasma: IFN-γ, IL-1β, IL-2, IL-6, IL-10, IL-12p70, TNF-α (Proinflammatory Panel 1); IL-15 (Cytokine Panel 1) and IL-18. In culture supernatants, IFN-γ was quantified by Duoset ELISA (R&D Systems).

NK cell assays

NK cytotoxicity was measured by ^{51}Cr release assay. PBMCs were thawed and cultured with the NK-sensitive human K562 leukemia cell line (ATCC) that lacks expression of MHC-I. ^{51}Cr–labeled K562 cells were cultured with PBMCs ($2.5x10^6$/mL) at a 50:1 effector:target (E:T) ratio for 4 h and release of ^{51}Cr was measured. Spontaneous and maximal release was determined by incubation of labeled target cells with medium or saponin (Merck), respectively. The specific lysis was calculated as [(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 2 h incubation of PBMCs ($1x10^6$/mL) with K562 cells (E:T 1:1). Perforin and granzyme expression was analyzed *ex vivo* by intracellular flow cytometry. For intracellular expression of IFN-γ in NK cells by flow cytometry, PBMCs ($1x10^6$/mL) were cultured in the presence of K562 cells for

6 h (E:T, 1:1), with addition of BD GolgiStop (1:150) and GolgiPlug (1:100) (BD Biosciences) for the final 5 h.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 6 software. Mann-Whitney U test was used for comparison of two groups, and for multiple comparisons Kruskal-Wallis tests were performed with Dunn's post test. p values < 0.05 were considered statistically different.

Results

The inflammatory environment influences gene expression in sJIA NK cells

To dissect the involvement of NK cells in sJIA pathogenesis, an in-depth analysis of NK gene expression by RNA-seq was performed on purified NK cells from 6 active sJIA patients and 6 age-matched healthy controls. The purity of the NK cell transcriptome was confirmed using Cell Type enrichment (CTen) software (22), which demonstrated highest enrichment of NK cell-specific genes (Supplementary Figure S6A). Upon principal component analysis, NK cell data from 5 out of 6 sJIA patients clustered together (Figure 1A). Comparable symptoms and treatment were noted in the outlier patient as compared to the other patients, therefore all patients were included in further analyses. Statistical analysis of the transcriptome revealed 107 differentially expressed genes of which 71 genes were up-regulated and 36 were down-regulated in sJIA NK cells. A comprehensive list of differentially expressed genes can be found in Supplementary Table S7.

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, hematological system development and function, immune cell trafficking and inflammatory response, compatible with modification of key immunological pathways

(Supplementary Table S8). Among key immune-inflammatory genes, sJIA NK cells demonstrated increased gene 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 S7). Analysis of transcriptional pathways identified components within the inflammatory environment, among which IL-10, IL-1β, IFN-γ, TNF-α and IL-6, as potential upstream mediators (z-score >|1.5|, $p<10^{-5}$) of the observed changes in NK transcriptional profile (Figure 1B). Of note, NK-activating IL-18 was not identified as major upstream regulator in sJIA NK gene signature (z-score=-0.80, p=1.7 $x10^{-3}$).

In contrast to what we expected from highly purified NK cells, we found evidence for genes normally expressed by erythrocytes (e.g. *HBB,* Supplementary Table S7). Spearman correlation with highly expressed cell-specific erythrocyte genes revealed clustering of sJIA patients, indicating a potential erythrocyte contamination (Supplementary Figure S6B). A similar enrichment of erythrocyte-specific genes was detected in previous gene expression studies on total PBMCs, which Hinze et al. attributed to the presence of erythrocyte precursors in sJIA PBMCs (23-26). Although we used highly purified NK cells (median post sort purity of 99%, Supplementary Table S4 and Figure S6C), contamination of a minimal amount of erythrocyte precursors in sJIA samples might influence the results.

Therefore, in order to extract biologically relevant data, an NK cell-specific gene list was generated by filtering the list of significantly differentially expressed genes with IPA software (Supplementary File S5), which revealed increased *S100A9* and *TLR4* and decreased *IL10RA* and *GZMK*, among others (Figure 1C). When performing clustering analysis of the 23 NK cell-specific genes, all sJIA 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 immunological disease genes (data not shown). Analysis of the functional association of key cytokines in sJIA disease with differentially expressed NK cell genes demonstrated that IL-1β and IL-6 are linked to multiple differentially expressed NK cell genes (Supplementary Figure S9), underscoring the inflammatory response in sJIA NK cells.

Together, these results demonstrate that NK cells from sJIA patients display an altered gene expression profile as compared to healthy control NK cells with a manifest enrichment of inflammatory response-related genes, including pro-inflammatory IL-1β- and IL-6-signaling pathways, suggesting their biological behavior has changed secondary to the inflammatory environment characteristic of sJIA.

Increased IL-18, but not IL-2 or IL-12 in plasma of sJIA patients

Although transcriptome analysis identified pro-inflammatory cytokines, i.e. IL-1β and IL-6, as up-stream regulators of sJIA NK cell gene expression, it did not provide data concerning cytokines on 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 plasma of 18 sJIA patients and 11 age-matched healthy controls. While IL-2 and IL-12 plasma levels did not differ between patients and controls, IL-15 ($p<0.01$) and IL-18 ($p<0.0001$) levels were moderately and highly increased in active sJIA plasma, respectively (Figure 2A). Of note, IL-15 levels were measured irrespective of IL-15Ra levels, thus representing both active and inactive forms of IL-15. IL-1β and IFN-γ tended to be higher in sJIA patients, although not significantly (Figure 2B). Two patients with sJIA and emerging MAS symptoms had highly elevated IFN-γ plasma levels (Figure 2B, open squares). In all sJIA patients, a large increase of IL-6 ($p<0.001$) was observed, while TNF-α ($p<0.05$) and IL-10 ($p<0.01$) were moderately raised. The ratio of IFN-γ to IL-18 levels was

significantly decreased in sJIA as compared to controls ($p<0.0001$) (Figure 2C), confirming our previous findings in a different cohort of patients (15). In conclusion, sJIA 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, TNF-α) and on the other hand includes moderate to high increases in IL-15 and IL-18, but not IL-2 or IL-12, which are known to stimulate NK cells.

Subtle change of inhibitory and activating receptors in sJIA NK cells

To decipher the impact of the altered cytokine environment and gene expression on NK cell phenotypes, we performed NK cell phenotyping on PBMCs of 10 active sJIA patients and 10 controls, amongst which the individuals included for RNA-seq analysis. NK cells can be subdivided into two main subsets: $CD56^{bright}CD16^{-/dim}$ NK cells, that have an important cytokine-producing function, and $CD56^{dim}CD16^{+}$ NK cells, which are believed to be mainly cytotoxic (27). Percentages and absolute numbers of total NK cells, and of $CD56^{bright}$ and $CD56^{dim}$ subsets were not significantly different in sJIA compared to control PBMCs (Figure 3A). We observed no difference in expression of CD16, killer cell immunoglobulin-like receptors (KIR), NKG2D, NKG2A, CD94, NKp30 and NKp46 in total NK cells. However, NK cells of active sJIA patients showed decreased expression of the inhibitory receptor KLRG1 ($p<0.01$, Figure 3B), and increased expression of the activating NKp44 receptor ($p<0.01$, Figure 3B). Nonetheless, based on their globally normal phenotype, NK cells from sJIA patients showed no evidence for altered functionality.

sJIA patients manifest intact NK cytotoxicity but decreased expression of granzyme K

NK cytotoxicity against tumor target cells was measured by ^{51}Cr-release and CD107a degranulation assays. PBMCs of sJIA 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, co-culturing PBMCs with K562 tumor cells significantly increased both the percentage of CD107a$^+$ NK cells (HC $p<0.01$, sJIA $p<0.001$) and the expression intensity of CD107a per cell (HC and sJIA $p<0.01$), with similar increases in sJIA patients compared to healthy controls (Figure 4B). Subsequently, we measured the expression of perforin and granzymes in NK cells. sJIA patients showed similar percentages of perforin$^+$ and granzyme A$^+$ NK cells and elevated percentages of granzyme B$^+$ NK cells ($p<0.05$) as compared to healthy controls (Figure 4C). The expression of granzyme K has been described to be characteristic for CD56^{bright} NK cells (28). Interestingly, the percentage of granzyme K$^+$ CD56^{bright} NK cells was decreased in sJIA patients compared to controls ($p<0.05$, Figure 4D), in agreement with RNA-seq findings. Taken together, NK cells of sJIA patients in this cohort have a globally intact cytotoxic profile and function upon *in vitro* stimulation with tumor cells, including increased granzyme B levels, but show decreased granzyme K-expressing CD56^{bright} NK cells.

sJIA NK cells display defective IL-18-induced IFN-γ production and signaling

To measure the cytokine production capacity of NK cells, we focused on the most appreciated NK cell-derived cytokine, IFN-γ. IL-18 is commonly recognized as the main IFN-γ inducer in NK cells. Intriguingly, despite highly elevated IL-18 plasma levels in sJIA patients, levels of IFN-γ remain low, resulting in a decreased IFN-γ/IL-18 ratio (Figure 2). To analyze sJIA NK cell responses to IL-18, PBMCs of 9 active sJIA patients and 8 healthy controls were stimulated *in vitro* with IL-18. IL-18 induced IFN-γ in a small percentage of healthy control NK cells, which was significantly lower in sJIA patients ($p<0.001$, Figure 5A, left panel). Since IL-18 synergizes with IL-12 to induce IFN-γ (29), a condition with IL-18+IL-12 was

included, which resulted in highly increased numbers of IFN-γ^+ NK cells in both controls and patients (Figure 5A, central panel). However, the expression intensity of IFN-γ in IFN-γ^+ NK cells was lower in sJIA ($p<0.05$, Figure 5A, right panel), again indicating defective IL-18 (±IL-12)-induced IFN-γ production. This was further confirmed by the observation of lower secreted IFN-γ levels in supernatant of sJIA PBMCs cultured with IL-18+IL-12 ($p<0.05$) compared to controls (Figure 5B). Production of IFN-γ after stimulation with PMA/ionomycin or after co-culturing PBMCs with K562 cells was not altered in sJIA patients, demonstrating no overall defect in IFN-γ production (Figure 5B+C). De Jager et al. showed a defective IL-18-induced phosphorylation of IL-18 receptor β in sJIA NK cells (9). Therefore, we analyzed the IL-18-driven phosphorylation of ERK1/2 and p38 MAP kinase, both involved in signaling downstream of the IL-18 receptor. The upregulation of phosphorylated ERK1/2 and p38 MAP kinase after IL-18±IL-12 stimulation was less pronounced in sJIA NK cells compared to healthy control NK cells (n=4, Figure 5D). Expression of IL-18 receptor α and β was unchanged in sJIA and healthy control NK cells (data not shown). Together, NK cells of sJIA patients show a specific defect in IL-18-induced IFN-γ 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 sJIA and its association with MAS has received increased attention in recent years (7;8;10;11;30). Here, we performed a comprehensive analysis of NK cell characteristics in sJIA patients during active disease.

To our knowledge, this study is the first to analyze gene expression in highly purified NK cells of sJIA patients. The NK cell transcriptome of active sJIA patients demonstrated

enrichment of inflammation-related genes in comparison to controls. sJIA NK cells displayed increased expression of innate pathways such as TLR4 and S100 proteins, and are under the influence of pro-inflammatory cytokines, i.e. IL-1β and IL-6. In addition, we reported decreased gene expression of immune-regulating granzyme K and IL-10 receptor together with the absence of increased IFN-γ expression, next to absence of IL-18 among significant upstream regulators, which is consistent with the concept of a defective immune regulation in sJIA (1;31). Although NK cells only comprise 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 sJIA-related innate inflammatory pathways and decrease of specific immune-regulating genes in sJIA NK cells, suggests a secondary effect on NK cell biological behavior by the inflammatory environment characteristic in sJIA, identifying NK cells as important targets and essential players in sJIA development and pathogenesis.

In this study, we found equal numbers of NK cells in patient versus control PBMCs, which is in line with earlier reports (7;33;34), but in contrast with studies demonstrating decreased numbers of total (9;10;23;35), $CD56^{dim}$ (23), or $CD56^{bright}$ NK cells (7;9). NK cells from our sJIA patient cohort displayed few alterations in the balance of activating and inhibitory receptors, with significantly increased expression levels of activating receptor NKp44 and decreased levels of the inhibitory receptor KLRG1. Thus, NK cells from sJIA patients do not show any defects in receptors that are necessary for activation.

Much of what is known concerning the pathogenesis of sJIA and associated MAS has been deducted from observations in primary HLH in which mutations leading to a defective cytotoxicity of NK cells and $CD8^{+}$ cytotoxic T cells underlie disease. Although different studies reported polymorphisms in cytotoxicity-linked genes in patients with sJIA/MAS (36-

40), Donn et al. found no association of sJIA (without MAS) with single-nucleotide polymorphisms in gene loci for *PRF1*, *GZMB*, *UNC13D* and *RAB27A* (41). To clarify the present controversy about NK cells in sJIA, we performed an extensive analysis of sJIA NK cell function. In line with the study of Donn et al. (41), we found no intrinsic defect in NK cytotoxicity against a tumor cell target in sJIA patients. However, a large variability between patients was observed, with some demonstrating decreased and others increased cytotoxic capacity. Importantly, the high patient variability in NK cytotoxicity as observed in our study dissolved 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 expression of cytotoxic granule components, we observed normal to increased levels of perforin and granzyme A and B in NK cells of sJIA patients, endorsing our findings of no defective NK cytotoxicity against a tumor cell target in sJIA patients. These findings are in contrast to a study of Wulffraat et al., who observed decreased perforin expression in NK cells of sJIA patients (42), albeit with a high variability. As $CD56^{dim}$ NK cells are generally regarded as the predominant cytotoxic subset, most attention has been given to this subset and its products granzyme A and B. However, during the last decade, $CD56^{bright}$ NK cells received more attention, especially since this population has been found to kill autologous activated T cells in multiple sclerosis patients via granzyme K, a protease predominantly expressed by $CD56^{bright}$ NK cells (28;43). In this 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 of sJIA patients. So far, NK cell cytotoxic capacity has been largely investigated using tumor target cells. However, as decreased granzyme K expression in sJIA patients might influence the killing of activated

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

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

Decreased NK cell IFN-γ production, together with relatively low IFN-γ plasma levels and the absence of an IFN-γ gene signature in PBMCs (23), is in accordance with our sJIA mouse model in which sJIA-like disease develops in the absence of IFN-γ (19). In addition, in this model, NK cells are the major source of IFN-γ, suggesting a regulatory role in disease pathogenesis (unpublished observations). We therefore hypothesize that IFN-γ production by NK cells may constitute a regulatory mechanism in sJIA. Of note, this possibly protective role of IFN-γ in sJIA is opposite to its probable disease-promoting role in MAS and HLH (44). Indeed, highly elevated IFN-γ plasma levels are found in MAS and HLH patients (15), IFN-γ 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 reported anti-IFN-γ as a safe and effective treatment option in refractory primary HLH patients (46). In view of the pathogenic role for IFN-γ in the development of MAS and HLH, an alternative hypothesis might be that IL-18 hyporesponsiveness and decreased IFN-γ production by NK cells may protect sJIA from developing MAS.

Our data endorse the concept that the altered NK cell function observed in sJIA patients (i.e. decreased production of IFN-γ and granzyme K) is triggered by the pro-inflammatory environment typical of sJIA. 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 healthy control NK cells *in vitro* and proposed a similar effect of high IL-6 levels in sJIA, as tocilizumab-treated patients showed an increased expression of perforin and granzyme B (47). Although high IL-6 was detected in our patients, this was not associated with decreased perforin or granzyme B expression compared to controls, nor with decreased NK cytotoxicity. However, we did find decreased granzyme K expression, as well as IL-6-related gene expression alterations in purified sJIA NK cells, indicating 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 sJIA, as shown in this study and described in detail in a review by Avau et al. (8), may underlie the subtle but probably substantial alterations in sJIA NK cell function we observed here. Acquired impaired NK cell function may be part of the immune dysregulation seen in sJIA and may as well constitute the link between sJIA and MAS.

In conclusion, NK cells from active sJIA patients are targeted by the inflammatory environment as evident from their *ex vivo* transcriptome. In our cohort of active sJIA patients, 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 IFN-γ production and granzyme K expression might contribute to sJIA pathogenesis. Together, our data identify NK cells as important target cells in disease, endorse the concept that altered NK cell function is part of the immune-inflammatory dysregulation seen in sJIA, and pave the way for further studies of their potential regulatory activity in this disease.

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Figure Legends

Figure 1. NK-specific altered gene expression in sJIA patients. Gene expression was analyzed by RNA-seq in FACS-sorted NK cells of 6 active sJIA patients (P1-6) and 6 age-matched healthy controls (C1-6). **A.** Principal component analysis of RNA-seq data of sJIA (red) and control (blue) NK cells. **B.** Potential upstream regulators of NK gene expression as determined by Ingenuity Pathway Analysis selected on activation z-scores >|1.5| and overlap p-values <1E-05. **C.** Log 2 relative fragments per kilobase million (FPKM) of each differentially expressed NK cell-specific gene in each examined sample versus their mean FPKM in the controls. Genes and samples are clustered using a complete linkage algorithm, using (1- spearman correlation)/2 as the measure of dissimilarity.

Figure 2. Cytokine levels in plasma of sJIA patients. A+B. Plasma levels of cytokines (pg/mL) in healthy controls (HC, n=11) and active sJIA patients (n=18). Gray bars depict the detection limit. **C.** Ratio of IFN-γ to IL-18 in plasma of controls and active sJIA patients. Symbols represent individual patients with median and interquartile range. Open squares represent 2 sJIA patients with emerging MAS symptoms. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001, Mann-Whitney U test.

Figure 3. Subtle changes in phenotype of sJIA NK cells. A+B. NK cell markers were analyzed on PBMCs from sJIA patients (n=10) and healthy controls (HC, n=10) by flow cytometry. **A.** Percentage of $CD3^-CD56^+$ NK cells of live singlets, absolute numbers of $CD3^-CD56^+$ NK cells per mL of whole blood and percentages of $CD56^{bright}$ and $CD56^{dim}$ cells of total NK cells. Symbols represent individual patients with median and interquartile range. Open squares represent 2 sJIA patients with emerging MAS symptoms. **B.** Expression of different activating and inhibitory receptors is depicted as a percentage of total $CD3^-CD56^+$ NK cells in healthy controls (light gray bars) and active sJIA patients (dark gray bars). **p<0.01, Mann-Whitney U test.

Figure 4. sJIA NK cells display an intact cytotoxic profile with the exception of granzyme K expression in $CD56^{bright}$ NK cells. A+B. PBMCs of controls (HC) and active sJIA patients were co-cultured with K562 cells. **A.** Percentage of specific lysis of ^{51}Cr-labeled K562 cells co-cultured with PBMCs for 4 h as such (left panel), and corrected for the number of NK cells as defined by immune phenotyping (right panel), at an E:T ratio of 50:1. **B.** Percentage of $CD107a^+$ cells of total $CD3^-CD56^+$ NK cells and the median fluorescence intensity (MFI) of $CD107a^+$ NK cells analyzed 2 h after incubation of PBMCs with K562 cells. **C+D.** Intracellular flow cytometric staining of perforin, granzyme A, B and K in control and sJIA NK cells depicted as the percentage of positive cells of total $CD3^-CD56^+$ NK cells

(C) or of CD56^{bright} NK cells (D). Symbols represent individual patients with median and interquartile range. Open squares represent 2 sJIA patients with emerging MAS symptoms. *p<0.05; **p<0.01; ***p<0.001, Mann-Whitney U test.

Figure 5. NK cells of sJIA patients display defective IL-18±IL-12-induced IFN-γ production and phosphorylation of ERK1/2 and p38 MAP kinase. PBMCs of active sJIA patients (red triangles) and age-matched healthy controls (HC, blue circles) were cultured in the presence of IL-18, IL-18+IL-12, PMA+ionomycin (P/I) or K562 cells for 18 h (A), 48 h (B), 6 h (C) or 15 min (D). IFN-γ was measured by intracellular flow cytometry (A+C) or ELISA (B). Phosphorylation of ERK1/2 and p38 MAP kinase was measured by flow cytometry (D). **A.** Percentage of IFN-γ$^+$ cells out of total CD3$^-$CD56$^+$ NK cells after IL-18 (left panel) or IL-18+IL-12 (central panel) stimulation. Median fluorescence intensity (MFI) of IFN-γ in IFN-γ$^+$ NK cells (right panel). **B.** IFN-γ concentration in supernatant. **C.** Percentage of IFN-γ$^+$ cells of total CD3$^-$CD56$^+$ NK cells after co-culturing with K562 cells. **D.** Percentage of phosphorylated (p)-ERK1/2$^+$ (left panel) and p-p38 MAP kinase$^+$ (right panel) cells out of total CD3$^-$CD56$^+$ NK cells. Symbols represent individual patients, with median and interquartile range. *p<0.05; ***p<0.001; Mann-Whitney U test (A-C) for comparison between HC and sJIA patients; Kruskal-Wallis test (D) for comparison within groups.

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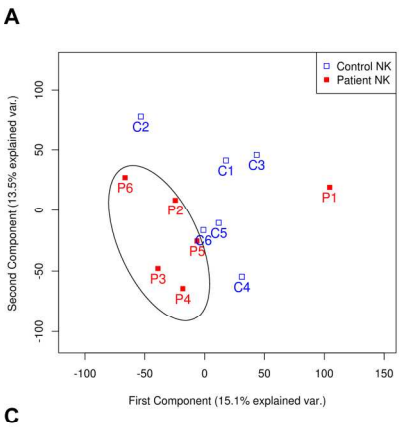

B

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

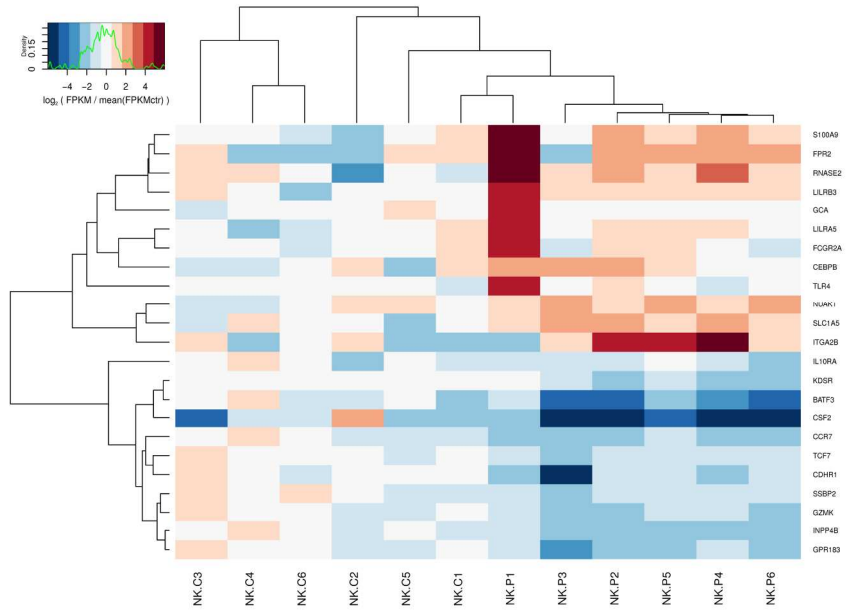

Figure 1. NK-specific altered gene expression in sJIA patients.

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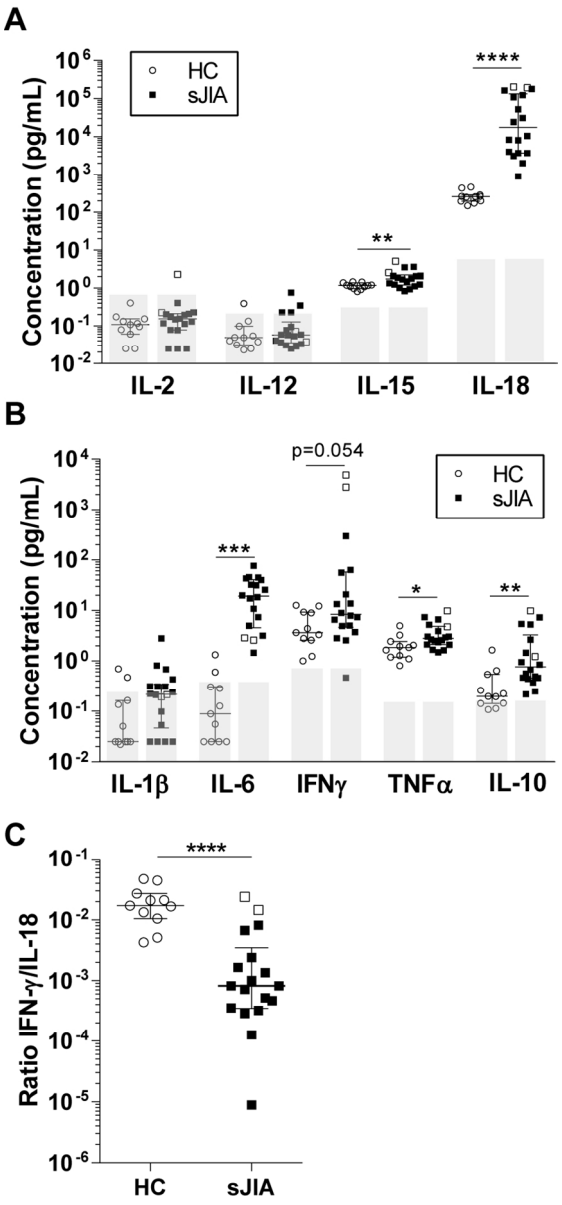

Figure 2. Cytokine levels in plasma of sJIA patients

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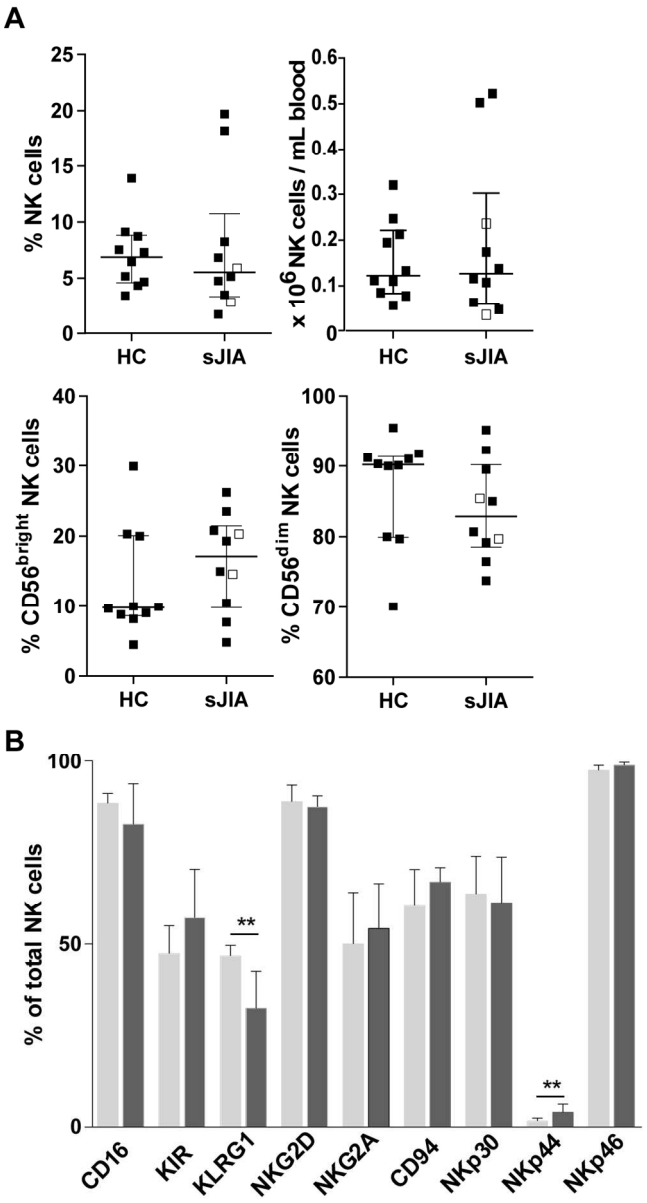

Figure 3. Subtle changes in phenotype of sJIA NK cells.

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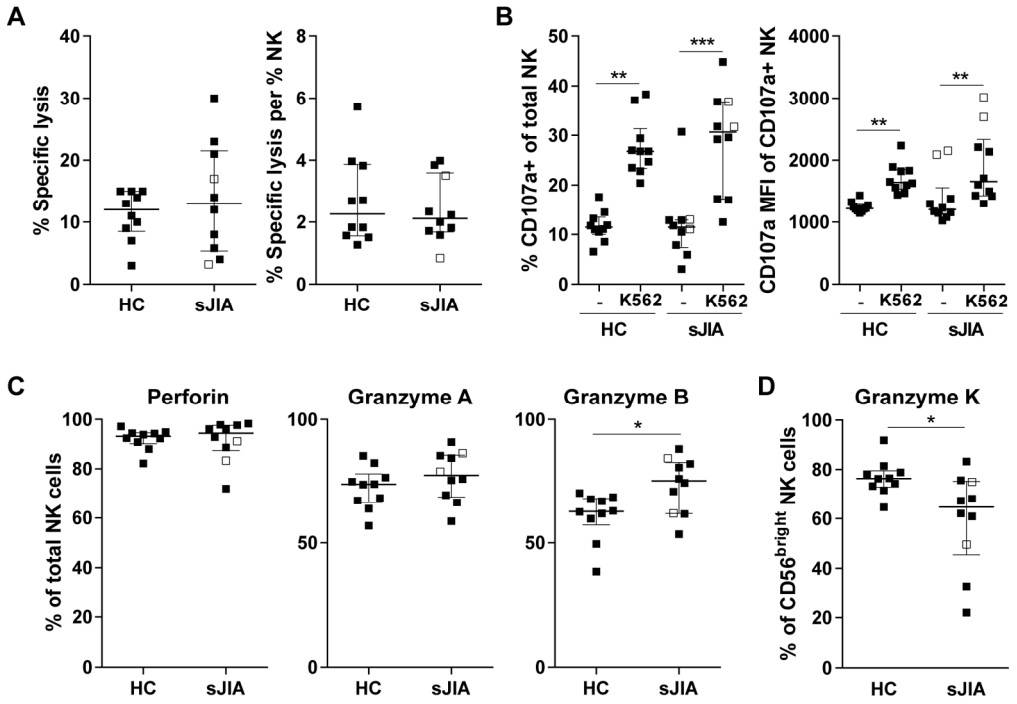

Figure 4. sJIA NK cells display an intact cytotoxic profile with the exception of granzyme K expression in CD56^{bright} NK cells.

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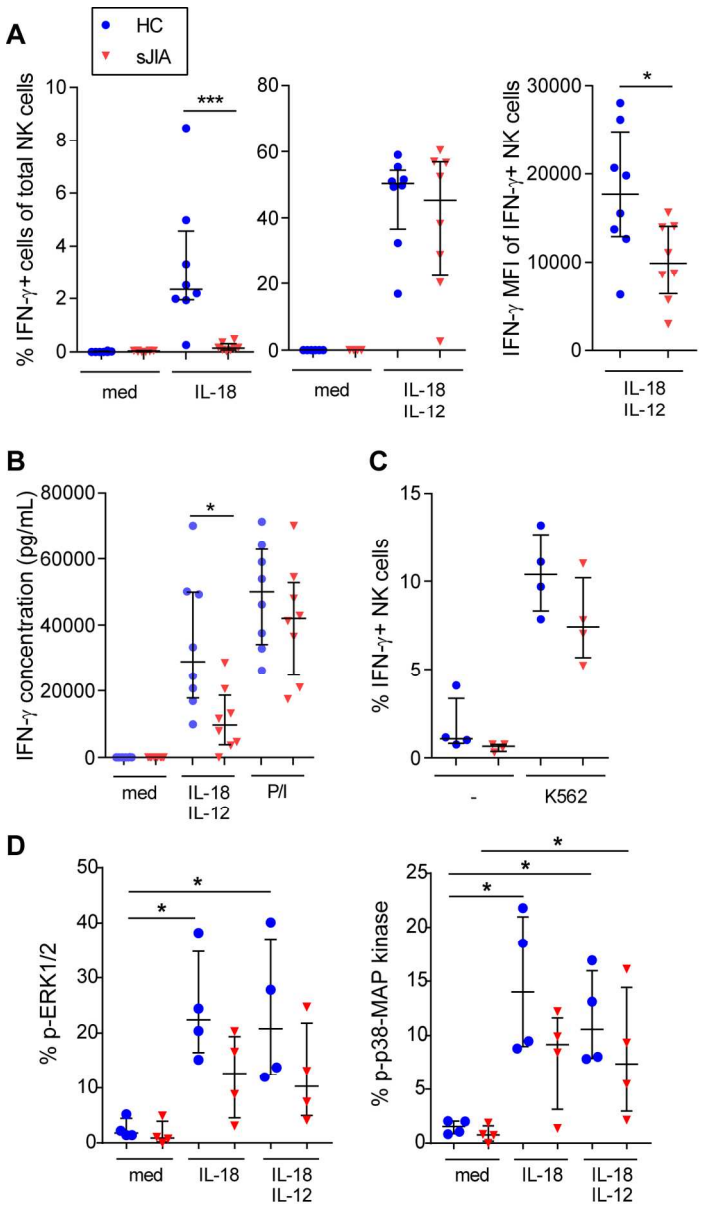

Figure 5. NK cells of sJIA patients display defective IL-18±IL-12-induced IFN-γ production and phosphorylation of ERK1/2 and p38 MAP kinase.

119x184mm (300 x 300 DPI)