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BRIEF REPORT

Innate Lymphoid Cell Phenotypic and Functional Alterations in Patients With Systemic Juvenile Idiopathic Arthritis

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Objective. Systemic juvenile idiopathic arthritis (sJIA) is a chronic childhood disease classically attributed to innate immune cell dysregulation. This study aimed to elucidate the role of innate lymphoid cells (ILCs), including natural killer (NK) cells and helper-ILCs (hILCs), in sJIA during clinically inactive disease (CID) through phenotypic and functional analysis.

Methods. Peripheral ILCs from children with sJIA during CID receiving interleukin-1 (IL-1) inhibitors (n = 40) were analyzed by flow cytometry and compared to 23 healthy children (HC) and 22 patients with unrelated autoinflammatory diseases taking IL-1 inhibitors. Plasma proteomic profiling was also performed.

Results. Patients with sJIA showed a significant reduction in circulating NK cell frequencies compared to HC, with an increased proportion of $CD56^{bright}$ NK cells. Although overall hILC frequencies were comparable to HC, ILC1s were increased, whereas ILC precursors were reduced. ILC1 frequency correlated positively with IL-18 plasma levels, whereas ILC2 frequency correlated negatively. Functional assessments revealed that NK cells from patients with sJIA had variable interferon γ (IFNγ) production upon IL-18/IL-12 stimulation, inversely correlating with IL-18 levels. Additionally, hILCs from these patients showed a specific impairment in IFNγ production despite normal IL-13 production, potentially linked to decreased IL-18 receptor α expression in ILC1s. Proteomic analysis confirmed IL-18 as the most up-regulated cytokine in sJIA plasma.

Conclusion. Patients with sJIA in CID exhibit significant innate immune abnormalities, including altered ILC subset distribution and impaired IFNγ production, strongly associated with IL-18 levels. These findings suggest ongoing immune dysregulation despite clinical remission, underscoring a potential role for ILCs and cytokine interaction in sJIA pathogenesis.

INTRODUCTION

Systemic juvenile idiopathic arthritis (sJIA), or Still disease, is a chronic childhood disorder of unknown cause, characterized by arthritis and systemic inflammatory features, including rash and spiking fever.[1] The pathogenesis of sJIA is complex and dysregulation of innate immune cells is prominent, supporting the classification of sJIA as an autoinflammatory disorder. Approximately 10%

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to 15% of patients develop macrophage activation syndrome (MAS), a potentially fatal hyperinflammatory syndrome classified among the secondary hemophagocytic lymphohistiocytosis.[2] The effect of the innate proinflammatory cytokines interleukin-1β (IL-1β), IL-6, and IL-18 may explain many features of the disease. On the other hand, the contribution of interferon γ (IFNγ) seems to be more relevant for MAS development. Indeed, increased circulating concentrations of IFNγ and IFNγ-induced chemokines CXCL9 and CXCL10 have been found in patients with sJIA with MAS as compared to patients with active sJIA without MAS.[3]

Innate lymphoid cells (ILCs), including natural killer (NK) cells and helper-ILCs (hILCs), are important cellular sources of IFNγ. hILCs serve as the innate counterpart of T helper cells and are specialized in the secretion of cytokines. In peripheral blood (PB), hILCs include ILC1s, ILC2s, and ILC precursors (ILCPs), whereas mature ILC3s are present only in tissues. ILC1s express the transcription factor T bet and produce IFNγ. ILC2 express GATA3 and produce IL-13, whereas ILCPs consist of precursors capable of differentiating into mature ILC subsets in response to specific inflammatory signals. Because of their shared ability to produce IFNγ as a signature cytokine, NK cells and ILC1s were initially collectively referred to as "group 1 ILCs."[4,5]

Patients with active sJIA display an NK cell dysfunction, characterized by decreased number and reduced IFNγ production and cytotoxicity, possibly due to hyporesponsiveness[6,7] to IL-18. Although a growing body of evidence in recent years has implicated ILCs in the pathogenesis of inflammatory arthritis, including JIA, rheumatoid arthritis, and spondyloarthritis,[8,9] the role and phenotype of hILCs in sJIA remain largely unexplored.

The main goal of treatment in sJIA is to rapidly achieve clinically inactive disease (CID).[10] Given the central role of IL-1 and IL-6 in its pathogenesis, inhibitors targeting these cytokines have become standard therapies over the past decades. Nevertheless, despite favorable responses to IL-1 and IL-6 blockade, particularly with early treatments, a subset of patients (15–30%) remains nonresponsive.[11] Moreover, several observations suggest that abnormalities of innate immunity persist in patients with sJIA and CID. These abnormalities involve circulating myeloid cells[12] and elevated IL-18 circulating levels.[7,13] In fact, IL-18 levels often remain high in patients with sJIA and CID, normalizing only after prolonged remission.[12] In the present study, we aimed to investigate the presence and function of ILCs in the PB of patients with sJIA during CID, focusing on their potential role in persistent immune dysregulation and disease pathogenesis.

PATIENTS AND METHODS

Patients and samples. Forty patients with sJIA, as defined by the International League of Associations for Rheumatology criteria,[14] were enrolled in this study (Supplementary Table 1). All patients had CID, as defined according to the Wallace criteria,[10] were off glucocorticoids, and were receiving IL-1 inhibitors (anakinra [n = 31] or canakinumab [n = 9]). Among the 31 patients treated with anakinra, 19 patients were on a daily regimen, whereas 12 patients were on a tapered schedule (decalage), with 9 patients following step 1 (alternate-day regimen) and 3 patients following step 2 (once every three days). Of the nine patients receiving canakinumab, five patients were on full-dose therapy, whereas four patients were on a tapered regimen (three patients receiving doses every 8 weeks and one patient receiving doses every 10 weeks).

Additionally, 22 patients, receiving IL-1 inhibitors (anakinra [n = 11] or canakinumab [n = 11]), with unrelated autoinflammatory diseases (AIDs) were also enrolled (Supplementary Table 1). A control group of 23 healthy children (HC) (12 of 23 were female [53%]; median age 11.0 years, interquartile range [IQR] 5.0–15.0 years) was recruited from the division of Rheumatology at Bambino Gesù Children's Hospital. These children were evaluated for joint pain but were found to have noninflammatory conditions. Biologic samples (plasma and PB mononuclear cells [PBMCs]) were collected. PBMCs were isolated by density gradient centrifugation and cells were used for both phenotypic analysis by flow cytometry and cytokine production assays. Because limited blood volumes were available from pediatric patients, not all analyses were performed on every sample. The study was approved by the Ethics Committee of Bambino Gesù Children's Hospital (1683 OPBG 2018), and written informed consent was obtained from patients and/or their parents.

Cell stimulation, flow cytometry analysis, and cytokine detection. Freshly isolated PBMCs were left unstimulated or stimulated with phorbol 12–myristate 13–acetate (PMA) (Sigma) (10 ng/mL) plus ionomycin (Sigma) (1 μg/mL) in the presence of Golgi Plug (BD Biosciences) for three hours at 37°C. Alternatively, cells were stimulated overnight with IL-12 (Miltenyi) (10 ng/mL) and IL-18 (R&D) (100 ng/mL) at 37°C, and Golgi Plug was added for the last four hours of stimulation. At the end of the stimulation, cells were stained for flow cytometry analysis. PBMCs were analyzed by flow cytometry using directly conjugated monoclonal antibodies. The following antibodies were used for cell surface staining: CD3-FITC, CD19-FITC, CD14-FITC, CD127-APC, CD218 (IL-18 receptor α [IL-18RA])-APC (Miltenyi), CD56-PECy7, CD127-PE, CD45-KrO, CD3-APC-A700, CD14-APC-A700, CD19-APC-A700, CD94-APC, CD117-ECD, CRTH2-FITC (from DURAClone dry preformulated custom antibody panel, Beckman Coulter), and CD7-PECF594 (BD Biosciences). Dead cells were excluded using LIVE/DEAD Fixable Blue Dead Cell Stain Kit (Invitrogen).

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After surface staining, stimulated cells were fixed and permeabilized using Fixation/Permeabilization Solution Kit (BD Biosciences) to detect intracellular cytokines with the following antibodies: IFNγ-APCeFluor780 (eBioscience) and IL-13-PE (Miltenyi). For the analysis of freshly isolated PBMCs, lymphocytes were first gated, then dead cells, doublets, $CD45^-$, and lin(CD3, CD14, CD19)$^+$ cells were excluded. NK cells were gated as $CD94^+$ cells, whereas hILCs were gated as $CD94^-CD127^+$ cells. Among hILCs, ILC1s were gated as $CD117^-CRTH2^-$, ILC2s were gated as $CRTH2^+$, and ILCPs were gated as $CD117^+CRTH2^-$.

For the analysis of stimulated PBMCs, lymphocytes were first gated, then dead cells, doublets, $CD7^-$ and lin(CD3, CD14, CD19)$^+$ cells were excluded. NK cells were gated as $CD56^+$ cells, whereas hILCs were gated as $CD127^+$ cells.

Data were acquired with a Cytoflex LX flow cytometer (Beckman Coulter) and analyzed using FlowJo software version 10.8.1 (BD Biosciences). Plasma levels of IL-18 were measured by enzyme-linked immunosorbent assay using a commercial kit (R&D), according to the manufacturer's instructions.

Ex vivo culture of HC PBMCs and NK cells. NK cells were isolated from HC PBMCs by negative selection using the RosetteSep Human NK Cell Enrichment Cocktail (Stemcell Technologies). Total PBMCs were cultured in 10% fetal bovine serum–supplemented RPMI 1640 medium with the indicated concentrations of IL-18 (R&D) and, in addition to IL-18, NK cells were cultured with IL-2, IL-1β, and IL-6 (R&D, 10 ng/mL each). After six days, total PBMCs or NK cells were stimulated with IL-18/IL-12 as described in the previous paragraph for subsequent cytofluorimetric analysis.

Proteomic assay. Plasma samples were analyzed using the Olink Target 48 cytokine panel based on the highly sensitive and specific proximity extension assay technology as previously described.[15] Briefly, each target protein was recognized by double antibodies linked to complementary DNA tags that hybridize when in proximity, subsequently quantified using a high throughput microfluidic real-time polymerase chain reaction instrument, Olink Signature Q100. Proteins exhibiting values below the limit of detection in over 80% of samples were excluded from the dataset. The data were preprocessed using the NPX Manager Software (version 1.16.0). Data were analyzed with R and Olink Analyze R package (version 1.3.0).

Statistical analysis. Statistical analysis was performed using GraphPad Prism software version 8.0.1. Data are shown as medians and IQR. To compare two groups, the Mann-Whitney U-test was used. To compare two groups in the proteomics analysis we used multiple *t*-tests with Welch correction. To compare more than two groups Kruskal-Wallis test was used, followed by uncorrected Dunn's test. For correlation analyses, the Spearman test was used. *P* values less than 0.05 were considered significant.

RESULTS

Composition of the circulating ILC compartment in patients with sJIA. We analyzed the composition of the circulating ILC compartment in patients with sJIA receiving IL-1 inhibitors during CID. NK cells and hILCs were compared to those observed in HC. Additionally, to evaluate the potential effects of IL-1 inhibitors in modulating the number of ILCs in patients with sJIA, NK cells and hILCs were also assessed in the PB of patients with unrelated AIDs, who were receiving IL-1 inhibitors. NK cells were gated among live lymphocytes as lin-$CD94^+$ cells, whereas hILCs were gated as lin$^-CD94^-CD127^+$ (Supplementary Figure 1A). The frequency of NK cells within lymphocytes and their absolute number were significantly lower in patients with sJIA compared to HC (Figure 1A and B). Among NK cells, the frequency of $CD56^{bright}$ cells was significantly higher in patients with sJIA compared to HC and patients with AID (Figure 1C). The frequency and absolute numbers of hILCs were instead comparable among patients with sJIA, patients with AID, and HC (Figure 1D and E). In patients with sJIA, the frequencies of hILCs, but not of NK cells, correlated with the duration of therapy with IL-1 inhibitors (Supplementary Figure 1B and C). Altogether, our data show that patients with sJIA during CID are characterized by a marked reduction of circulating NK cells, accompanied by an increase in the percentage of $CD56^{bright}$ cells, whereas no significant abnormalities are observed in the percentage and number of hILCs.

Abnormal hILC subset proportions in patients with sJIA and CID correlating with IL-18 plasma concentration. We next investigated the composition of the hILC compartment by measuring the frequency of each subset among lymphocytes in patients with sJIA during CID. ILC1s were identified as $CD117^-CRTH2^-$, ILC2s as $CRTH2^+$ and ILCPs as $CD117^+CRTH2^-$ (Supplementary Figure 1A). Compared to HC, patients with sJIA showed a significant increase in both the frequency and absolute number of ILC1s (Figure 2A and D), and a reduction in both the frequency and absolute number of ILCPs (Figure 2C and F). Although the frequency of ILC2 was comparable between the two groups (Figure 2B), patients with sJIA had significantly reduced absolute numbers of ILC2s (Figure 2E).

Furthermore, we analyzed the proportion of each subset within hILCs. In line with the data on the frequency among total lymphocytes and cell numbers (Figure 2A–F), we found a significantly higher proportion of ILC1s and a lower proportion of ILCPs in patients with sJIA (Figure 2G–I). Of note, these abnormalities were specific to sJIA because they were not observed in patients with AID similarly receiving the same IL-1 inhibitor treatment.

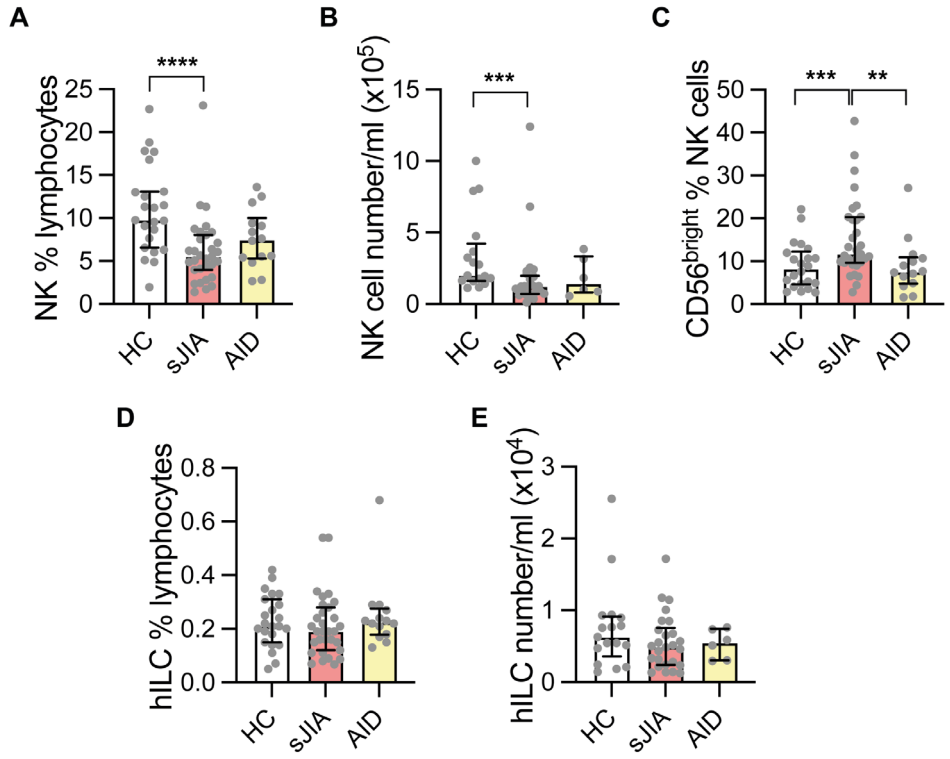

Figure 1. NK cell frequency and number in the peripheral blood of patients with sJIA are decreased compared to HC. (A–C) Scatter dot plots and bars showing the medians and interquartile ranges (IQR) of (A) NK cell frequency within live lymphocytes, (B) their absolute number, (C) and the frequency of CD56^{bright} NK cells among total NK cells. D and E, Scatter dot plots and bars showing the medians and IQR of (D) hILC frequency within live lymphocytes and (E) their absolute number. Symbols represent individual patients. A, C, and D n = 23 HC; n = 31 sJIA; n = 14 AID. B and E n = 17 HC; n =26 sJIA; n =7 AID. Kruskal-Wallis test was used, followed by uncorrected Dunn's test. (**$P < 0.01$; ***$P < 0.005$; ****$P < 0.001$). AID, autoinflammatory disease; HC, healthy children; hILC, helper innate lymphoid cell; NK, natural killer; sJIA, systemic juvenile idiopathic arthritis.

Given the observed changes in NK cell numbers and in hILC subset proportions in patients with sJIA, we next investigated whether these abnormalities were linked to the circulating levels of IL-18, which is the signature inflammatory cytokine in sJIA. We found a significant positive correlation between ILC1 proportion among hILCs and IL-18 plasma concentration (Figure 2L). Consistently, a significant negative correlation between ILC2 proportion and IL-18 levels was also found (Figure 2L). ILC2 (but not ILC1) cell number/mL negatively correlated with IL-18 plasma concentration (r = –0.4017, P = 0.0419). These abnormalities were not linked to surface expression levels of the IL-18 receptor, as we observed that ILC1 from patients with sJIA expressed significantly lower levels of IL-18RA compared to HC and patients with AID (Figure 2M and Supplementary Figure 2A and B). To further explore the role of IL-18, we stimulated HC PBMCs with IL-18, and we found a reduction in ILCP and an increase in ILC1 proportions among hILC, suggesting that IL-18 drives ILCP differentiation toward ILC1 (Supplementary Figure 2C and D). Collectively, our data show that patients with sJIA during CID exhibit an abnormality in the hILC subset proportions, characterized by an increase in the ILC1 subset that is associated with circulating levels of IL-18 and a decrease in the ILCP subset.

Dysfunctional IFNγ production in response to IL-18 stimulation by NK cells and hILCs. To assess the capacity of NK cells and hILCs to produce IFNγ in patients with sJIA as compared to HC, we stimulated PBMCs and measured intracellular IFNγ in these subsets by flow cytometry (Figure 3A–D, Supplementary Figure 3, and Supplementary Figure 4A–D). To evaluate the intrinsic cellular potential to synthesize cytokines, we used PMA/ionomycin as a nonspecific stimulus (Supplementary Figure 3A). Alternatively, we stimulated cells with IL-18 in the presence of IL-12 (Supplementary Figure 3B). We found that in patients with sJIA, the frequency of NK cells capable of producing IFNγ in response to both PMA/ionomycin and IL-18/IL-12 was comparable to that of HC (Figure 3A). However, we found that, on ex vivo cell stimulation with IL-18 and IL-12, patients with sJIA clustered into two distinct subgroups: 5 of 14 patients were hyperresponders (% IFNγ + median = 62.8), whereas the remaining 9 of 14 patients were hypo- or null responders (% IFNγ + median = 3.4) (Figure 3A). The median treatment duration did not differ significantly between hyperresponsive and hyporesponsive patients. Interestingly, we found a negative correlation between the percentage of IFNγ + NK cells and the circulating levels of IL-18 (Figure 3 B). In line with these data, we observed

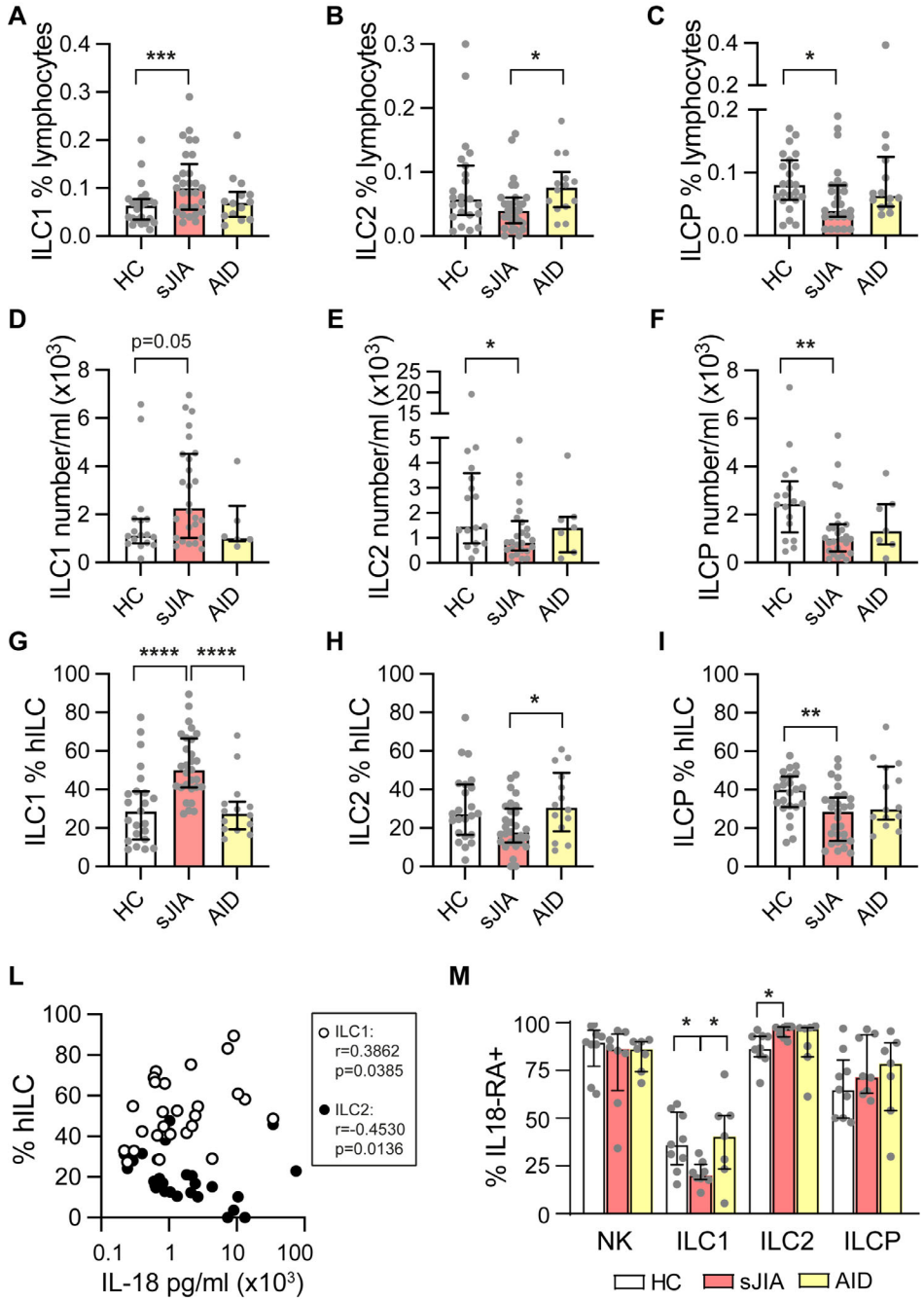

Figure 2. Changes in ILC subset frequencies are associated with IL-18 plasma concentration. (A–I) Scatter dot plots and bars showing for each hILC subsets the medians and interquartile ranges (IQR) of (A–C) cell frequency within live lymphocytes, (D–F) of cell number, and (G–I) cell frequency within total hILCs. A–C and G–I n = 23 HC; n = 29 sJIA, n = 14 AID. D–F n = 17 HC; n = 26 sJIA, n = 7 AID. (L) Scatterplots showing the correlation between IL-18 plasma concentration and ILC1 (empty dots) and ILC2 (black plots) frequency within hILCs. n = 29. r and *P* values were determined by the Spearman correlation test. Symbols represent individual patients. (M) Scatter dot plots and bars showing for each ILC subsets the median and IQR of IL-18RA^{+} cell frequency within each ILC subset. n = 9 HC; n = 8 sJIA; n = 7 AID. In A–I and M, Kruskal-Wallis test was used, followed by uncorrected Dunn's test (*$P < 0.05$; **$P < 0.01$; ***$P < 0.005$; ****$P < 0.001$). AID, autoinflammatory disease; HC, healthy children; hILC, helper innate lymphoid cell; ICL, innate lymphoid cell; IL-18, interleukin 18; IL-18RA, interleukin 18 receptor α; NK, natural killer; sJIA, systemic juvenile idiopathic arthritis.

that preincubating ex vivo NK cells from HC with increasing IL-18 concentrations reduced their subsequent ability to produce IFNγ in response to IL-18/IL-12 stimulation in a dose-dependent manner (Supplementary Figure 4E and F). This desensitization was independent of the presence of IL-1β and IL-6, other cytokines that characterize sJIA milieu (Supplementary Figure 4E and F).

Regarding hILCs, despite a significantly higher proportion of ILC1s in patients with sJIA (Figure 2G), the frequency of IFNγ +

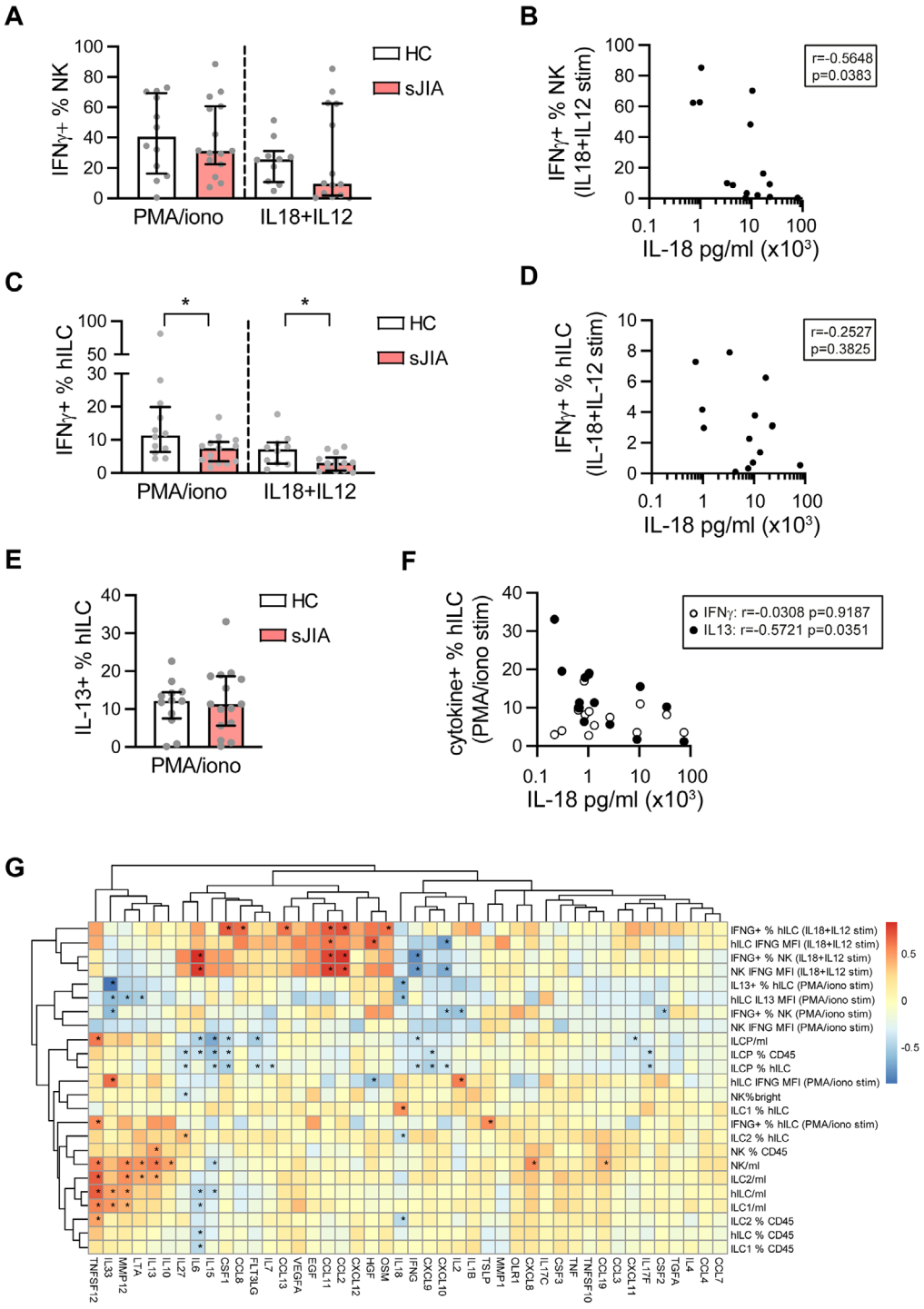

Figure 3. Dysfunctional IFNγ production by ILCs in patient with sJIA. (A, C, and E) Scatter dot plots and bars showing the median and IQR of (A and C) IFNγ$^+$ and (E) IL-13$^+$ cell frequency for cells stimulated as indicated. (B and D) Scatterplots showing the correlation between IL-18 plasma concentration and IFNγ$^+$ cell frequency for (B) NK cells and (D) hILCs stimulated with IL-18 and IL-12. (F) Scatterplot showing the correlation between IL-18 plasma concentration and IFNγ$^+$ (empty dots) or IL-13$^+$ (black dots) cell frequency for hILCs stimulated with PMA/iono. (G) Heatmap of correlations calculated between proteins in sJIA plasma samples and phenotypic/functional ILC data. r values are represented by color, and *P* values <0.05 are represented by an asterisk. Dendrograms represent hierarchical clustering between rows and columns. A, C, and E n = 12 PMA/iono HC; n = 15 PMA/iono sJIA; n = 10 IL-18 and IL-12 HC; n =14 IL-18 and IL-12 sJIA. B, D, and F n = 14. In C, significance was determined by Mann-Whitney U-tests (*$P < 0.05$). In B, D, F, and G, r and *P* values were determined by the Spearman correlation test. Symbols represent individual patients. HC, healthy children; hILC, helper innate lymphoid cell; ILC, helper innate lymphoid cell; IFNγ, interferon γ; IL-18, interleukin 18; iono, ionomycin; IQR, interquartile range; NK, natural killer; PMA, phorbol 12–myristate 13–acetate; sJIA, systemic juvenile idiopathic arthritis.

hILCs following both PMA/ionomycin or IL-18/IL-12 stimulations was significantly lower in patients with sJIA compared to HC (Figure 3C). Differently from NK cells, no significant correlation between the percentage of IFNγ and hILCs, and the circulating levels of IL-18 was found in IL-18/IL-12 stimulation (Figure 3D). As expected, IL-13 was not produced by hILCs in response to IL-18 with IL-12 (data not shown). In PMA/ionomycin stimulation, the frequency of hILCs positive for IL-13, the signature cytokine for ILC2, was comparable between sJIA and HC (Figure 3E). Given the opposite correlation of ILC1 and ILC2 frequencies among hILCs with IL-18 plasma concentration (Figure 2L), we investigated whether there was any association with hILC ability to produce the ILC1 and ILC2 signature cytokines IFNγ and IL-13, respectively. Consistently, IL-18 plasma concentration negatively correlated with the frequency of IL-13 and hILCs, whereas no correlation was observed with the frequency of IFNγ and hILCs (Figure 3F).

Overall, these findings indicate that in patients with sJIA during CID, hILCs display a specific functional impairment in IFNγ production. Conversely, in patients with sJIA, the responsiveness of NK cells to ex vivo stimulation with IL-18 and IL-12, in terms of IFNγ production, is associated with in vivo IL-18 plasma levels.

Proteomic profile of patients with sJIA during CID. To explore the plasma proteomic profile of children with sJIA during CID while receiving IL-1 inhibitors, we quantified the concentrations of 44 proteins in 35 patients and 5 HC. These data confirmed that IL-18 levels were the highest among all the proteins assessed in sJIA samples, and we observed that samples clustered according to IL-18 concentration rather than diagnosis (Supplementary Figure 5A). Indeed, patients with lower plasma levels of IL-18 clustered together with HC and displayed increased expression of tumor necrosis factor superfamily 10 (TNFSF10) and TNFSF12 compared to patients with higher IL-18 levels (Supplementary Figure 5A). Principal component analysis of the protein data set confirmed that patients could not be distinguished from HC (Supplementary Figure 5B), suggesting a similar inflammatory profile between the two groups. Indeed, except for IL-18, which was the top up-regulated cytokine in patients with sJIA, differential analysis between sJIA and HC samples revealed that only IL-15 and colony stimulating factor 3 (CSF3) (also known as G-CSF) were significantly up-regulated in patients with sJIA (Supplementary Figure 5C). Correlation analysis between the expression of pairs of proteins allowed to identify a disease-associated pattern during CID, consisting of 12 proteins including the IFNγ-CXCL9/CXCL10 axis (Supplementary Figure 6). Additionally, we integrated proteomics with the phenotypic and functional ILC analysis data. This comprehensive approach allowed to identify additional correlations and confirmed that ILC phenotypes and functions were primarily linked to IL-18 levels during CID in patients with sJIA (Figure 3G).

DISCUSSION

In this study, we performed immunophenotyping of the ILC compartment in the PB of patients with sJIA during CID receiving IL-1 inhibitors. Similar to what has been shown in active disease,[16] we found a decreased frequency and number of NK cells in patients during CID compared to HC. Notably, we also observed that these patients exhibited an increase in the percentage of NK CD56^{bright} cells compared to HC. This subset of NK cells is typically associated with IFNγ production rather than cytotoxic activity. These data expand on what is already known and suggest that a reduced NK cell frequency may represent a persistent dysfunctional feature of sJIA, even during the inactive phase of the disease.

In contrast to NK cells, we found that in patients with sJIA during CID the frequency and number of hILCs were comparable to HC. Furthermore, in these patients, the hILC frequency positively correlated with the duration of IL-1 inhibitor treatments, suggesting that prolonged control of disease activity may facilitate the recovery of circulating hILCs. Within the hILC compartment, patients with sJIA exhibited a significant increase in ILC1 and a decrease in ILCP frequencies and absolute numbers compared to HC. Importantly, these changes were specific to sJIA and not observed in patients with other autoinflammatory diseases receiving IL-1 inhibitors, suggesting a unique pattern of hILC dysregulation in sJIA. Additionally, we found that both ILC1 and ILC2 proportions among hILCs correlated with plasma IL-18 levels, further supporting the pivotal role of IL-18 in sJIA pathogenesis. The positive correlation between IL-18 levels and the proportion of ILC1s, alongside the negative correlation with the ILC2 proportion, highlights the influence of IL-18 on the hILC landscape. The high IL-18 plasma levels may be responsible for the IL18-driven ILC1 expansion observed, as suggested by the ILCP-ILC1 conversion induced by stimulating HC PBMCs with IL-18 ex vivo. Furthermore, the negative correlation between IL-18 levels and the frequency of IL-13^{+} hILCs underscores the IL-18's role in skewing the balance away from ILC2-driven responses, which could have anti-inflammatory effects. Consistent with our results, hILCs respond to IL-18 during inflammation, with IL-18 driving preferential ILCP differentiation toward ILC1s and production of IFNγ.[17]

Regarding the functional capability of ILCs, we found that both NK cells and hILCs from patients with sJIA exhibited functional abnormalities. Despite the higher frequency of CD56^{bright} cells, NK cells from patients with sJIA displayed a heterogeneous response to IL-18/IL-12 stimulation, with some patients being hyperresponders and others hypo- or nonresponders. This variability in NK cell response was inversely correlated with circulating IL-18 levels, suggesting a potential desensitization mechanism induced by prolonged exposure to high IL-18 concentrations.

Similar findings have been reported in patients with sJIA during active disease and at disease onset.[6,7] This IL-18 desensitization mechanism is also supported by our data on HC NK cells, showing a dose-dependent loss of responsivity to further IL-18 stimulation on preincubation with this cytokine. These findings suggest that prolonged IL-18 exposure may induce a refractory state in NK cells, potentially impairing their ability to mount an appropriate immune response in inflammatory conditions. Reduced IFNγ production by NK cells in response to IL-18 may represent a protective mechanism aimed at preventing excessive IFNγ production and, consequently, the development of MAS during active sJIA. However, in patients with sJIA with low IL-18 plasma levels, despite proteomic data indicating a similarity with HC cytokine expression patterns, NK cells were hyperresponsive to this cytokine in terms of IFNγ production, therefore suggesting that a decrease in IL-18 plasma concentration during CID may not be associated with a recovery of physiologic NK cell function. This hypothesis aligns with data demonstrating that leukocytes from patients with inactive sJIA exhibit underlying alterations in Toll-like receptor signaling and a skewed monocyte phenotype, even in patients who are clinically and transcriptionally comparable to healthy individuals.[12]

Conversely, despite the increased proportion of ILC1s in patients with sJIA and CID, hILCs demonstrated significant functional impairment in their ability to produce IFNγ on stimulation with PMA/ionomycin or IL-18/IL-12, while maintaining normal IL-13 production. This impairment may be due to reduced IL-18RA expression on ILC1s in patients with sJIA and could represent a protective mechanism to avoid excessive IFNγ production.

Proteomic integration analysis confirmed that ILC phenotypes and functions were predominantly associated with IL-18 levels, further emphasizing its role as a key driver of immune dysregulation in sJIA. Additionally, hierarchical clustering revealed distinct groups of highly correlated cytokines, indicating shared biologic pathways. Notably, a very well-defined cluster, including CXCL9, CXCL10, and IFNγ, along with nine other proteins, was identified, suggesting a distinct inflammatory signature driven by IFNγ in patients with sJIA, even during CID.

Because of the low frequency of ILCs in the PB, a limitation of this study is that the number of cells obtained from children's samples was insufficient to perform all analyses in parallel for each patient. To avoid potential alterations in cell subset proportions and function because of cell thawing, we conducted our analyses on freshly isolated PBMCs. This approach limited the number of patients we could enroll, particularly those with treatment-naïve sJIA at disease onset.

Despite these limitations, our study provides the first phenotypic and functional characterization of ILCs in patients with sJIA during CID. Our findings underscore the persistent dysregulation of innate immune cells in patients with sJIA, even in the absence of active disease symptoms, and highlight the potential pathogenic role of specific immune cell subsets and cytokine interactions. We demonstrate that NK cells and hILCs in patients with sJIA during CID exhibit opposite dysfunctional features, with NK cells being more responsive to IL-18 stimulation and hILCs showing an intrinsic impairment in IFNγ production, compared to the respective subsets in HC. These findings further contribute to the definition of recovery from active disease as a state of compensation[2] or adaptation to an altered inflammatory environment, rather than a reversion to an initial normal state.

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AUTHOR CONTRIBUTIONS

All authors contributed to at least one of the following manuscript preparation roles: conceptualization AND/OR methodology, software, investigation, formal analysis, data curation, visualization, and validation AND drafting or reviewing/editing the final draft. As corresponding author, Dr Quatrini confirms that all authors have provided the final approval of the version to be published and takes responsibility for the affirmations regarding article submission (eg, not under consideration by another journal), the integrity of the data presented, and the statements regarding compliance with institutional review board/Declaration of Helsinki requirements.

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