Characteristics of Circulating Natural Killer Cells and Their Interferon-γ Production in Active Adult-onset Still Disease

Yasuhiro Shimojima, Dai Kishida, Ken-ichi Ueno, Satoru Ushiyama, Takanori Ichikawa, and Yoshiki Sekijima

ABSTRACT. ***Objective.*** To investigate the characteristics of circulating natural killer (NK) cells and their interferon (IFN)-γ–producing ability in adult-onset Still disease (AOSD).

Methods. Peripheral blood mononuclear cells were obtained from 22 patients in the acute phase of AOSD (acute AOSD); 7 of the 22 patients after treatment (remission AOSD), and 11 healthy controls (HC). NK cells and their IFN-γ expression levels were analyzed by flow cytometry. Additionally, the cytokine receptors of interleukin (IL)-12, IL-15, and IL-18 on NK cells were also evaluated.

Results. The frequency of NK cells was significantly lower in acute AOSD than in HC. NK cell counts significantly increased in remission AOSD. Expression of IL-12 and IL-15 receptors on NK cells was significantly increased in acute AOSD, whereas that of IL-18 receptor indicated no significant difference among 3 groups. IFN-γ expression in NK cells was significantly higher in acute AOSD than in HC, and significantly decreased in remission AOSD. The absolute number of NK cells and IFN-γ–expressing NK cells revealed an inverse correlation with serum ferritin levels in acute AOSD. In 2 distinct subsets of NK cells, $CD56^{dim}$ NK cells significantly exhibited higher IFN-γ expression than $CD56^{bright}$ NK cells in acute AOSD.

Conclusion. In acute AOSD, NK cells displayed lower proportion, whereas they had higher ability for IFN-γ production than in HC; moreover, upregulation of IL-12 and IL-15 receptors on NK cells may promote IFN-γ production. In addition, disease activity may be implicated in regulating the number of NK cells and IFN-γ–expressing NK cells in AOSD. (J Rheumatol First Release June 15 2019; doi:10.3899/jrheum.181192)

Key Indexing Terms:
ADULT-ONSET STILL DISEASE
INTERFERON-γ
NATURAL KILLER CELL
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Adult-onset Still disease (AOSD) is a systemic autoinflammatory disease characterized by daily spike fevers, polyarthritis, evanescent rash, pharyngitis, lymphadenopathy, and hepatosplenomegaly. Further, AOSD sometimes indicates life-threatening conditions such as aseptic meningitis, thrombotic thrombocytopenic purpura, disseminated intravascular coagulation, and macrophage activation syndrome (MAS) as a reactive hemophagocytic lymphohistiocytosis. The hallmarks of AOSD are hyperferritinemia, increased levels of inflammatory mediators including C-reactive protein (CRP), and increased white blood cell counts[1,2]. In addition, elevated levels of proinflammatory cytokines, including interleukin (IL)-1β, IL-6, IL-8, IL-12, IL-18, interferon (IFN)-γ, and tumor necrosis factor-α are found in the acute phase of AOSD[3,4,5,6]. These cytokines activate macrophages and neutrophils, which play a pivotal role in the pathogenesis[2,7,8], suggesting that innate immunity contributes to the development of AOSD. Immune impairment of natural killer (NK) cells is also associated with the pathogenesis of AOSD or systemic juvenile idiopathic arthritis (sJIA), which have been regarded as the adult or juvenile spectrum of same disease, respectively[9]. Moreover, lower expression and defective cytotoxicity of NK cells were demonstrated in an active phase of disease[10,11,12,13].

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NK cells are identified as cytotoxic cells in the category of innate lymphoid cells capable of immune response without antigen-specific cross-linking. To defend the host against invasive and neoplastic pathogens, they stimulate macrophages, upregulate MHC class I on antigen-presenting cells, and promote effector function in T cell lineage by IFN-γ release; alternatively, they perform direct cytotoxicity[14,15]. In addition, a functional response of NK cells can be promoted by cytokine combination signals with IL-12, IL-15, and IL-18; notably, their relevant cytokine receptors are found on NK cells[14,15,16,17]. Accordingly, a specific immune response of NK cells in AOSD is supposed to be affected by the exposure of proinflammatory cytokines related to the disease. On the other hand, the IFN-γ–producing ability of NK cells remains unclear in AOSD.

We investigated the characteristics of circulating NK cells including their IFN-γ–producing ability as well as the relevant cytokine receptor expression in patients with AOSD.

MATERIALS AND METHODS

Patients and samples. Twenty-two patients with AOSD took part in this study [mean age: 51 ± 16 yrs (range 25–80 yrs), 5 men and 17 women]. They were definitively diagnosed according to the criteria proposed by Yamaguchi, *et al*[18] in Shinshu University hospital. The clinical characteristics of the diagnosis and other features related to the disease are shown in Table 1. The complication of MAS and the activity score were also investigated according to the proposal diagnostic criteria[19] and the systemic score proposed by Pouchot, *et al*[20], respectively. Blood samples were obtained prior to the immunosuppressive treatments. As for the healthy controls (HC), blood samples from 11 individuals (mean age: 47 ± 12 yrs, 6 men and 5 women) were also provided. No significant differences in the mean age and distribution of sex were shown between patients with AOSD and HC.

To evaluate the results in the remission phase of AOSD, blood samples were taken from 7 of 22 patients at the mean period of 39 ± 43 months after starting immunosuppressive therapy. Remission was defined by fulfilling both assignments as follows: the Pouchot's score was achieved in 0, and no physical findings concerning disease activity shown in Table 1 were found. When the blood samples were taken, 5 patients had received maintenance therapy including prednisolone (n = 4), cyclosporine (n = 1), methotrexate (n = 2), golimumab (n = 1), and tocilizumab (n = 2). Their laboratory findings were also significantly improved as follows (Table 1): the number of white blood cells (p = 0.032), neutrophils (p = 0.014), serum levels of aspartate transaminase (p = 0.0004), alanine transaminase (p = 0.022), lactate dehydrogenase (p = 0.0003), CRP (p < 0.0001), erythrocyte sedimentation (p = 0.0004), and ferritin (p < 0.0001).

This study was approved by the Local Ethics Committee at Shinshu University (approval number: 601). All individual participants provided informed consent.

Sample preparation and flow cytometry. Whole blood samples were collected into EDTA-coated tubes. Peripheral blood mononuclear cells (PBMC) from whole blood samples were isolated by gradient centrifugation with Ficoll-Hypaque PLUS (GE Healthcare). To define NK cells in flow cytometric analysis, unstimulated PBMC were stained with Pacific blue–conjugated anti-CD3 (BioLegend), FITC-conjugated anti-CD16, and phycoerythrin-conjugated anti-CD56 (both from Beckman Coulter). NK cells were phenotypically defined as CD3–CD16+CD56+ cells in the population gated on total lymphocytes. NK cells were additionally stained with allophycocyanin (APC)-conjugated anti-CD212 (IL-12Rβ1) (Miltenyi Biotec), alternatively, with APC-conjugated anti-CD215 (IL-15Rα; BioLegend), or APC-conjugated anti-CD218 (IL-18Rα; Miltenyi Biotec). To examine intracellular IFN-γ expression in NK cells, PBMC were stimulated with 0.5 μg/ml of ionomycin, 0.04 μg/ml of phorbol myristate acetate (both from Sigma-Aldrich), and 2 μm monensin (BD Bioscience) at 37°C for 4 h. Stimulated PBMC were permeabilized with Cytofix/Cytoperm (BD Bioscience) after being stained with above-described cell-surface makers including CD3, CD16, and CD56. Permeabilized cells were subsequently stained with APC-conjugated anti-IFN-γ (BioLegend). IFN-γ expression was detected in the population gated on NK cells. Stained cells were acquired on a FACSCanto II flow cytometer (BD Bioscience), and the acquired data were analyzed by FlowJo version 7.6.5 software (Tree Star Inc.).

Serum IL-18 measurement. Serum samples were stored at –80°C until use with ELISA. The serum concentration of IL-18 was measured using commercially available ELISA kit (Medical and Biological Laboratories). The minimal detectable concentration of IL-18 was 12.5 pg/ml.

Statistical analysis. The clinical findings shown in Table 1 were represented as the median (interquartile range). The analyzed results were shown as the mean ± SD. Statistical significance was defined as 2-sided p values < 0.05. To compare the analyzed findings between patients with AOSD and HC, the Mann-Whitney U test was used. The Wilcoxon signed-rank test was performed to compare data before and after treatment in patients with AOSD. Correlation coefficient test was used for evaluating a significant relationship between the analyzed data and clinical findings.

RESULTS

Circulating NK cell proportion and counts in AOSD. To determine the proportion of NK cells in the peripheral blood, we compared the frequency of NK cells in the population of peripheral lymphocytes between 22 patients with AOSD prior to the treatment (acute AOSD), 7 of those in the remission phase of AOSD (remission AOSD), and HC. The proportion of NK cells was significantly lower in acute AOSD than in HC (mean 8.2% vs 19.2%; p = 0.002; Figure 1A). Meanwhile, no significant difference was demonstrated between remission AOSD and HC (mean 12.6%, p = 0.063). Of 7 patients in remission AOSD, 6 indicated increased counts of NK cells, ultimately demonstrating statistical significance (p = 0.042; Figure 1B).

Expression of cytokine receptors on NK cells in AOSD. Since IL-12, IL-15, and IL-18 are pivotal cytokines promoting NK cell lineage, we investigated the expression of cytokine receptors responsive to IL-12, IL-15, and IL-18 (IL-12Rβ1, IL-15Rα, and IL-18Rα, respectively) on NK cells (Supplementary Figure 1A, available with the online version of this article). IL-12Rβ1 expression was significantly higher in acute AOSD than in HC [mean 94.0% vs 87.2%, p = 0.004; median fluorescence intensity (MFI), p = 0.002; Figure 2A, 2B]. No significant difference was shown between remission AOSD and HC (mean 90.2%, p = 0.717; MFI, p = 0.497; Supplementary Figure 2A, 2B). A significant decrease of IL-12Rβ1-MFI was demonstrated in a remission phase (p = 0.017; Figure 3A), whereas IL-12Rβ1+ NK cell counts were not significantly different between an acute and remission phase (p = 0.062; Figure 3B). IL-15Rα proportion was significantly higher in acute and remission AOSD than in HC (mean 21.4%, 9.4%, 4.6%, respectively; acute AOSD vs HC, p < 0.0001; remission AOSD vs HC, p = 0.016; Figure 2C and Supplementary Figure 2C), and IL-15Rα-MFI was significantly higher in acute AOSD than in HC

Table 1. Clinical characteristics of patients with adult-onset Still disease in the acute and remission phases.

	Acute (total), n = 22	Consecutive Patients (n = 7)		HC, n = 11	p	
		Acute*	Rem			
Patients					vs HC	
					Acute (total)	Acute*
Age, yrs (range)	51 (25–80)	49 (34–74)		47 (36–69)	n.s.	n.s.
Sex, M/F	5/17	2/5		6/5	n.s.	n.s.
Physical findings, n (%)					Consecutive Patients Acute* vs Rem	
Fever	22 (100)	7 (100)	0		0.0003	
Rash	21 (95)	7 (100)	0		0.0003	
Sore throat/pharyngitis	16 (73)	5 (71)	0		0.010	
Lymphadenopathy	11 (50)	4 (57)	0		0.035	
Arthritis	18 (82)	5 (71)	0		0.010	
Myalgia	6 (27)	1 (14)	0		n.s.	
Pleuritis	5 (23)	1 (14)	0		n.s.	
Pericarditis	2 (9)	0	0		n.s.	
Hepatomegaly	9 (41)	4 (57)	0		0.035	
Splenomegaly	11 (50)	4 (57)	0		0.035	
MAS criteria	7 (32)	2 (29)	0		n.s.	
Pouchot's score, median (IQR)	6 (4.25–6)	6 (5–7.5)	0		0.018	
Laboratory findings, median (IQR)					vs Rem	
					Acute (total)	Acute*
White blood cells, /μl	14770 (9633–20820)	10400 (9485–22735)	7120 (6940–8670)		0.032	n.s.
Neutrophils, /μl	12721 (7640–18397)	9412 (7627–20834)	1582 (1169–2443)		0.014	n.s.
Platelet, 10^4/μl	29.2 (19.5–34.5)	26.5 (20.1–32.2)	24.0 (18.7–26.3)		n.s.	n.s.
AST, u/l	63 (40–109)	49 (40–64)	22 (16–26)		0.0004	0.018
ALT, u/l	55 (30–138)	34 (31–48)	20 (16–21)		0.022	0.034
LDH, u/l	516 (175–906)	449 (375–651)	219 (182–223)		0.0003	0.018
CRP, mg/dl	9.0 (3.8–12.9)	11.8 (9.6–19.5)	0.02 (0–0.04)		< 0.0001	0.018
ESR, mm/h	42.0 (21.0–91.0)	65.0 (34.0–94.0)	6.0 (3.0–15.0)		0.0004	0.028
Ferritin, ng/ml	8392 (1462–15456)	8706 (2995–17627)	24 (16–79)		< 0.0001	0.018

* Seven patients in the acute phase of disease who were consecutively followed. Rem: remission; HC: healthy controls; MAS: macrophage activation syndrome; IQR: interquartile range; AST: aspartate transaminase; ALT: alanine transaminase; LDH: lactate dehydrogenase; ESR: erythrocyte sedimentation rate; n.s.: not significant; CRP: C-reactive protein.

($p < 0.0001$) while being not significantly different between remission AOSD and HC ($p = 0.205$; Figure 2D and Supplementary Figure 2D). In comparison between an acute and remission phase, a decrease in IL-15Rα-MFI was significant ($p = 0.028$) despite no statistical significance in IL-15Rα+ NK cell counts ($p = 0.225$; Figure 3C, 3D). Percent frequency of IL-18Rα was around 80% in acute, remission AOSD, and HC (mean 80.4%, 77.7%, and 82.7%, respectively), showing no significant difference (acute vs HC, $p = 0.516$; remission vs HC, $p = 0.441$; Figure 2E and Supplementary Figure 2E). No statistical significance was indicated in the comparison of IL-18Rα-MFI (acute vs HC, $p = 0.169$; remission vs HC, $p = 0.556$; acute vs remission, $p = 0.612$) or that of IL-18Rα+ NK cell counts between an acute and remission phase ($p = 0.063$; Figure 2F, 3E, 3F, and Supplementary Figure 2F).

Kinetic evaluation of IFN-γ–expressing NK cells in AOSD. IFN-γ production is a crucial function in NK cell machinery. Therefore, we additionally investigated IFN-γ expression in NK cells. In acute AOSD, IFN-γ expression in NK cells was significantly higher than in HC (mean 53.2% vs 24.1%; $p = 0.0001$; MFI, $p = 0.0001$; Figure 4A, 4B, and Supplementary Figure 1B, available with the online version of this article). In remission AOSD, IFN-γ expression in NK cells was significantly lower than in HC (mean 13.1%; $p = 0.008$; MFI, $p = 0.040$; Figure 4C, 4D). IFN-γ-MFI

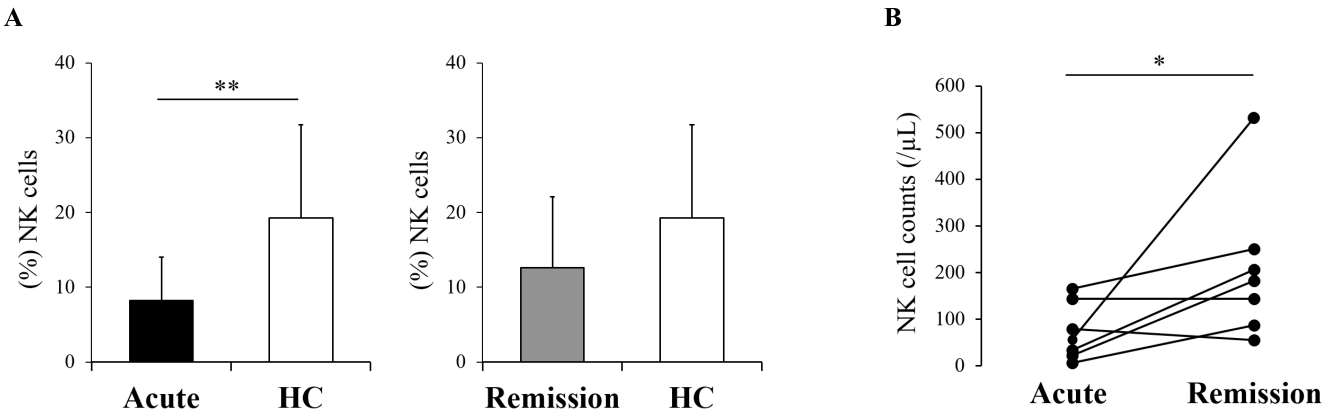

Figure 1. NK cell counts in patients with AOSD. Frequencies of NK cells in peripheral blood lymphocytes were compared between 22 patients with acute AOSD, 7 with remission AOSD, and 11 healthy controls (A). NK cell counts were sequentially evaluated in 7 patients between acute and remission phase (B). Values are shown as the mean ± SD. Statistically significant difference was evaluated by the Mann-Whitney U test (A) or Wilcoxon signed-rank test (B), indicating as follows: *p < 0.05 and **p < 0.005. NK: natural killer; AOSD: adult-onset Still disease; HC: healthy controls.

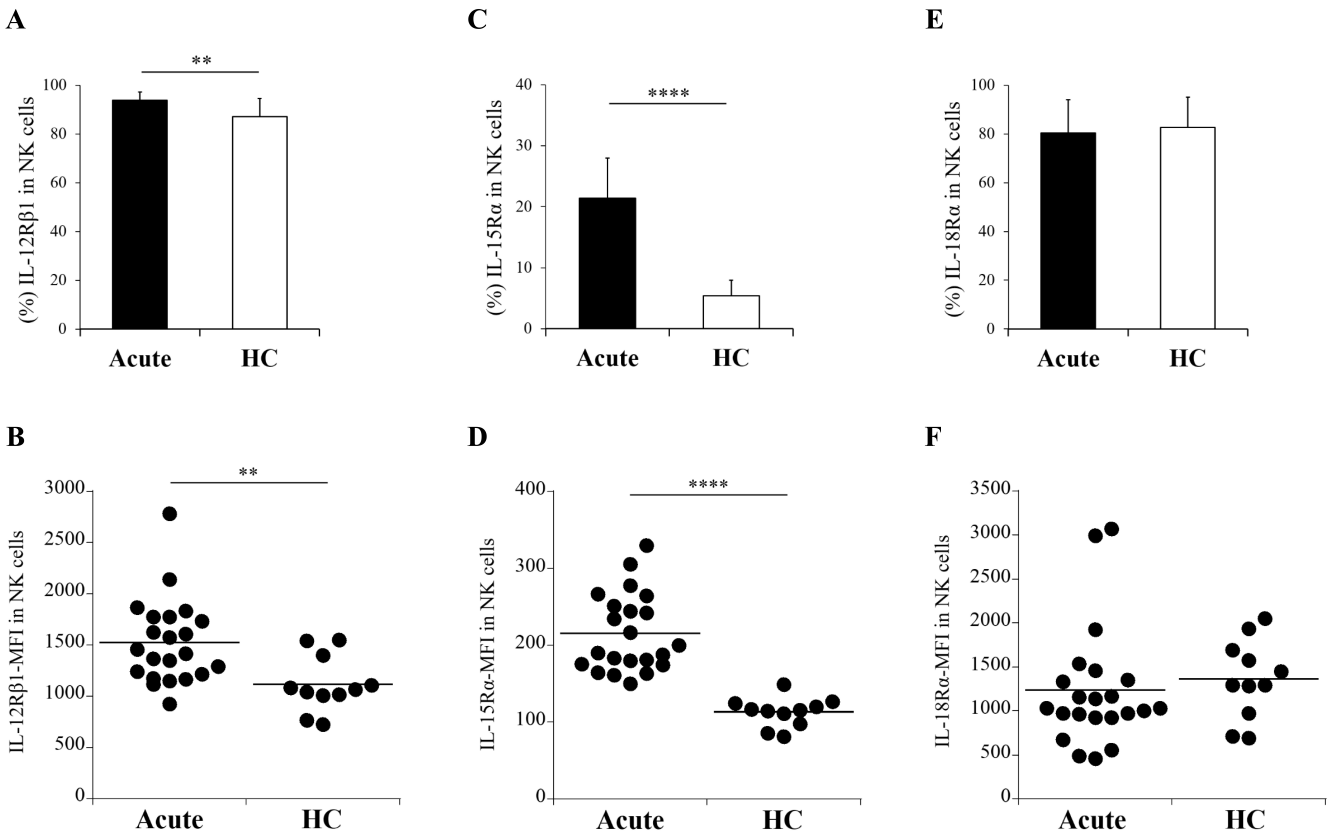

Figure 2. The comparison of interleukin (IL)-12Rβ1, IL-15Rα, and IL-18Rα expression on NK cells between acute AOSD and HC. Percentage of IL-12Rβ1, IL-15Rα, or IL-18Rα (A, C, E) and median fluorescence intensity (MFI) of them (B, D, F) in NK cells were compared between patients with acute AOSD and HC. Values are shown as the mean ± SD. The Mann-Whitney U test was used in the comparison. Statistically significant differences are indicated as follows: **p < 0.005 and ****p < 0.0001. NK: natural killer; AOSD: adult-onset Still disease; HC: healthy controls.

decreased in 7 patients at a remission phase, showing statistical significance in remission AOSD (p = 0.018; Figure 4E). Meanwhile, no significant difference was shown in the comparison of IFN-γ–expressing NK cell counts (p = 0.398; Figure 4F). Even among 3 patients who had been treated with biologics, only 1 patient revealed a decrease in IFN-γ–expressing NK cell counts (data not shown).

Relationship between IFN-γ–expressing NK cells and serum

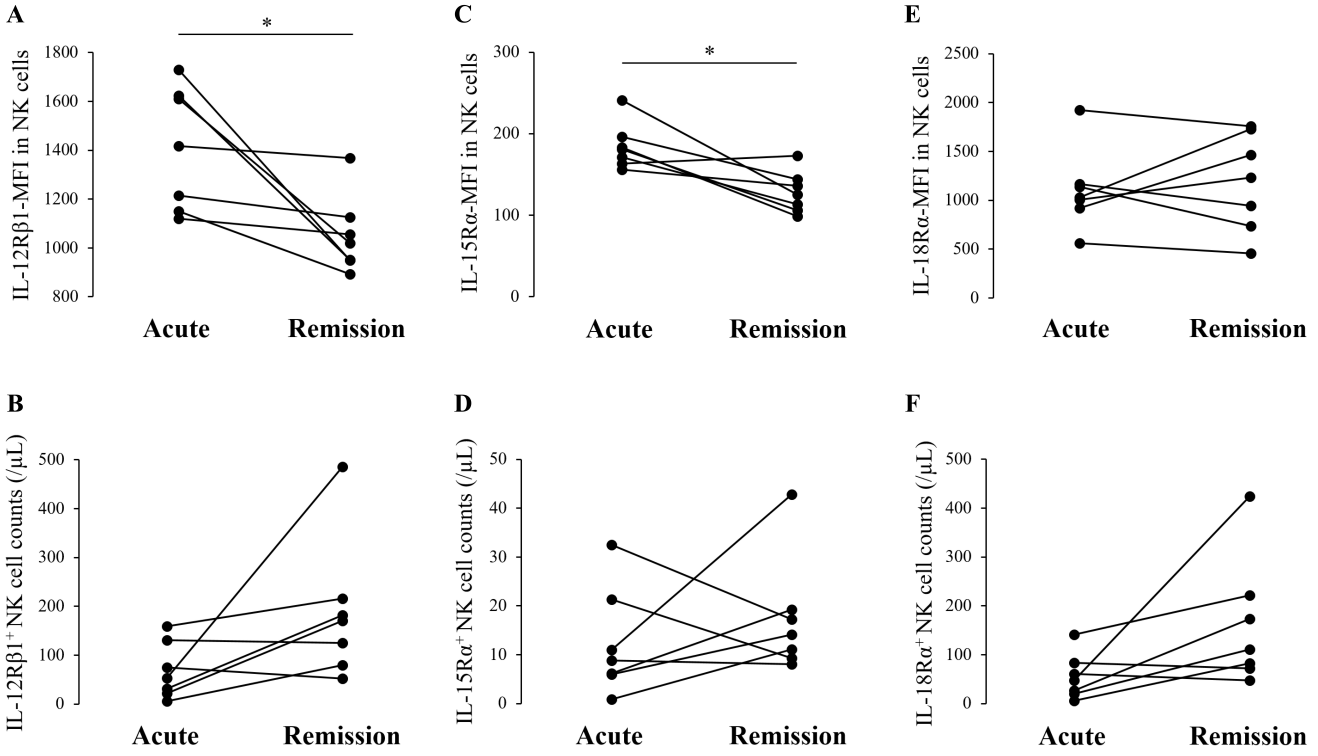

Figure 3. The evaluation of interleukin (IL)-12Rβ1, IL-15Rα, and IL-18Rα expression on NK cells in a remission phase. IL-12Rβ1, IL-15Rα, or IL-18Rα-MFI in NK cells (A, C, E) and their expressing NK cell counts (B, D, F) were sequentially evaluated in 7 patients between acute and remission phase. The Wilcoxon signed-rank test was used in the comparison. Statistically significant difference is indicated as *p < 0.05. NK: natural killer; MFI: median fluorescence intensity.

ferritin levels in AOSD. We analyzed the relationship between NK cells and clinical findings. It has been found that serum IL-18 was strongly associated with the disease activity and/or the clinical features in AOSD[2,3,4,5,21,22]. Therefore, serum IL-18 levels were additionally measured. Serum levels of IL-18 were higher in acute AOSD than in HC (mean 2212.4 pg/ml vs 71.6 pg/ml, p < 0.0001; Supplementary Figure 3A, available with the online version of this article). They were still higher in remission AOSD than in HC (mean 109.2 pg/ml, p = 0.008), while a significant decrease was demonstrated in a remission phase (p = 0.018; Supplementary Figure 3B). However, serum IL-18 levels had significant correlations with neither the clinical findings described in Table 1, including MAS complication and the Pouchot's score, nor any data analyzed by flow cytometry (data not shown). MAS complication or the Pouchot's score had no significant correlation with any data analyzed by flow cytometry (data not shown). On the other hand, serum ferritin levels significantly demonstrated inverse correlations with the absolute number of NK cells and IFN-γ–expressing NK cells in acute AOSD (p = 0.017 and p = 0.003, respectively) despite no correlation with IFN-γ–MFI in NK cells (p = 0.202; Figure 5A, 5B, 5C).

Next, we re-analyzed the property of NK cells in relation to IFN-γ production by subdividing the CD3−CD16+CD56+ population into high- and low-density expression of CD56 ($CD56^{bright}$ and $CD56^{dim}$, respectively; Supplementary Figure 4A, available with the online version of this article). The proportion of $CD56^{bright}$ in NK cells was almost equal between acute AOSD and HC (mean 6.7% vs 7.4%, p = 0.268) while being lower in remission AOSD than in HC (mean 3.9%, p = 0.042; Figure 5D and Supplementary Figure 4B). IFN-γ-MFI in $CD56^{bright}$ was significantly lower in acute AOSD than in HC (p = 0.014), whereas that in $CD56^{dim}$ was significantly higher in acute AOSD than in HC (p = 0.012; Figure 5E and Supplementary Figure 5A). In acute AOSD, IFN-γ-MFI was significantly lower in $CD56^{bright}$ than in $CD56^{dim}$ (p = 0.0003). IFN-γ-MFI in $CD56^{dim}$ significantly decreased in a remission phase (p = 0.018; Figure 5F). Although IFN-γ-MFI in $CD56^{bright}$ showed no significant difference in a remission phase (p = 0.310), increased expression of that was shown in 3 patients who had been treated with biologics (Supplementary Figure 5C).

DISCUSSION

The proportion of NK cells was found to be significantly lower in acute AOSD than in HC, supporting previous studies, which demonstrated impairment of NK cells based on disease activity of AOSD[10,11]. MAS may be attributed to

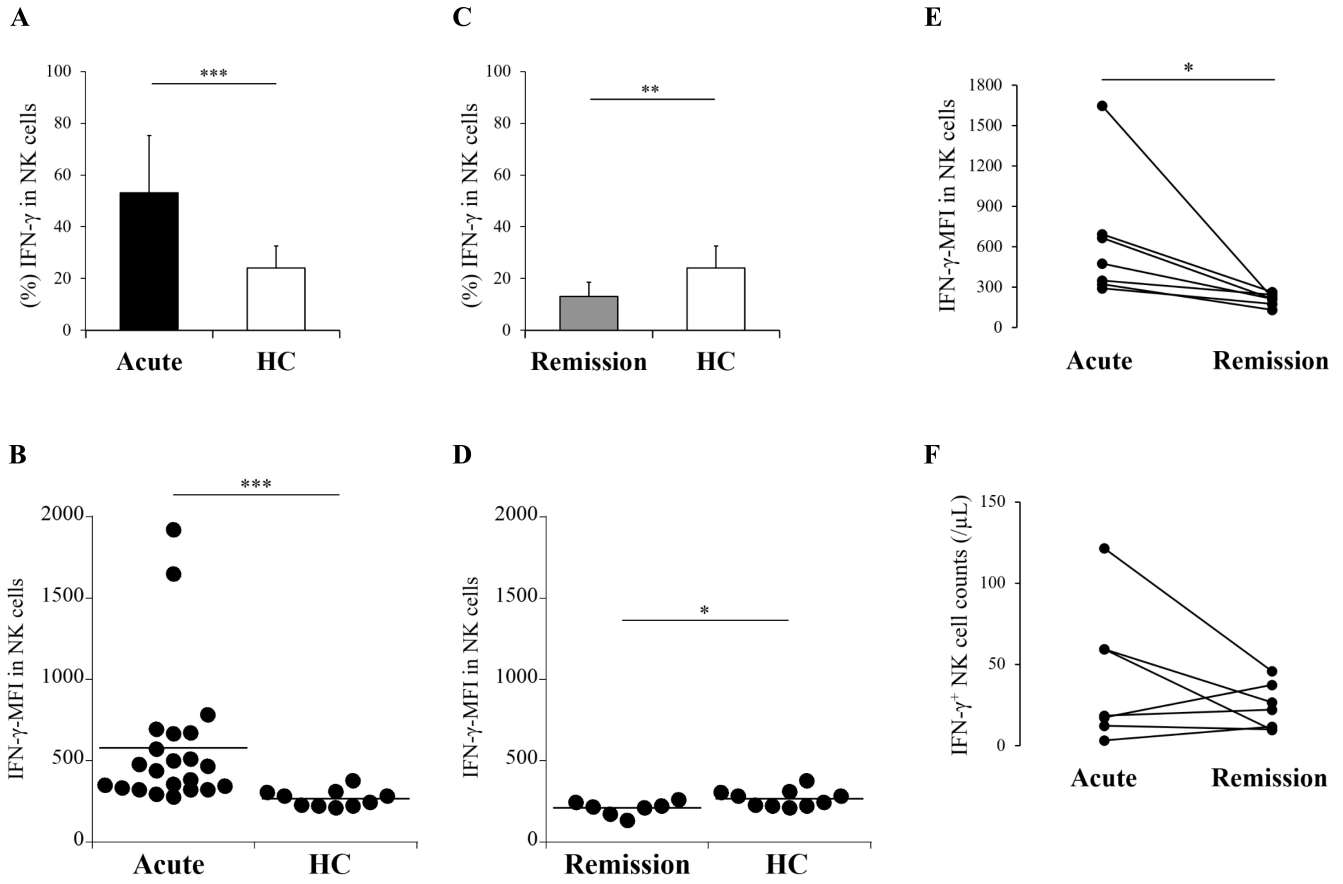

Figure 4. IFN-γ production in NK cells from patients with AOSD. Percentage and MFI of IFN-γ in NK cells were compared between patients with acute AOSD and HC (A, B), or between those with remission AOSD and HC (C, D). IFN-γ-MFI in NK cells (E) and IFN-γ–expressing NK cell counts (F) were sequentially evaluated in 7 patients between acute and remission phase. Values are shown as the mean ± SD. The Mann-Whitney U test was used in the comparison between 3 groups. The Wilcoxon signed-rank test was used in comparison between an acute and remission phase. Statistically significant differences are indicated as follows: *p < 0.05, **p < 0.01, and ***p = 0.0001. NK: natural killer; AOSD: adult-onset Still disease; HC: healthy controls; IFN: interferon; MFI: median fluorescence intensity.

defective cytotoxic function of NK cells as a hallmark of AOSD pathogenesis[7,13]. A high amount of serum IL-18, which is strongly associated with the disease activity in AOSD[2,3,4,5,21,22], reduces functional NK cells, and is implicated in MAS induction[21,23], suggesting that the dysfunction of NK cells is a fundamental immune disorder in AOSD development. Although it was insufficient to indicate direct relationship between NK cell reduction and MAS or serum IL-18 levels in our study, NK cell reduction was significantly related to high levels of serum ferritin. Meanwhile, high elevation of serum ferritin is found to be associated with MAS and/or serum IL-18 elevation in AOSD[5,24]; moreover, serum ferritin is a valuable serological marker of disease activity[2,25,26]. Accordingly, this result may suggest that the higher AOSD disease activity develops, the more the reduction of NK cells may be substantially promoted. In fact, NK cell counts significantly increased in remission AOSD.

IFN-γ, which is known to activate macrophages, is primarily produced by activated NK cells and effector T cells[27]. Previous studies have shown that serum levels of IFN-γ significantly increase in the acute phase of AOSD[4,5,6]. Indeed, our study demonstrated that IFN-γ expression in NK cells was significantly higher in acute AOSD than HC. In the activating cascade of NK cells, IL-12, IL-15, and IL-18 are potential inducers of IFN-γ secretion[15,28]. Therefore, upregulation of proinflammatory cytokines including IL-18 and IL-12, which are shown in an acute phase of AOSD[4], is supposed to enhance NK cell activation. Elevated expression of serum IL-15 was also demonstrated in patients with sJIA[29].

In a series of our study, we also focused on the relevant cytokine receptors on NK cells. IL-18 receptor expression on NK cells in acute AOSD was not significantly different from that in both remission AOSD and HC. In NK cells, IFN-γ can be predominantly produced based on IL-18 participation in the presence of IL-12 or IL-15 under physiological conditions[28,30]. When extracellular IL-18 binds to IL-18 receptor α (IL-18Rα), IL-18 receptor β (IL-18Rβ) is recruited as the co-receptor to form a high-affinity receptor complex in the

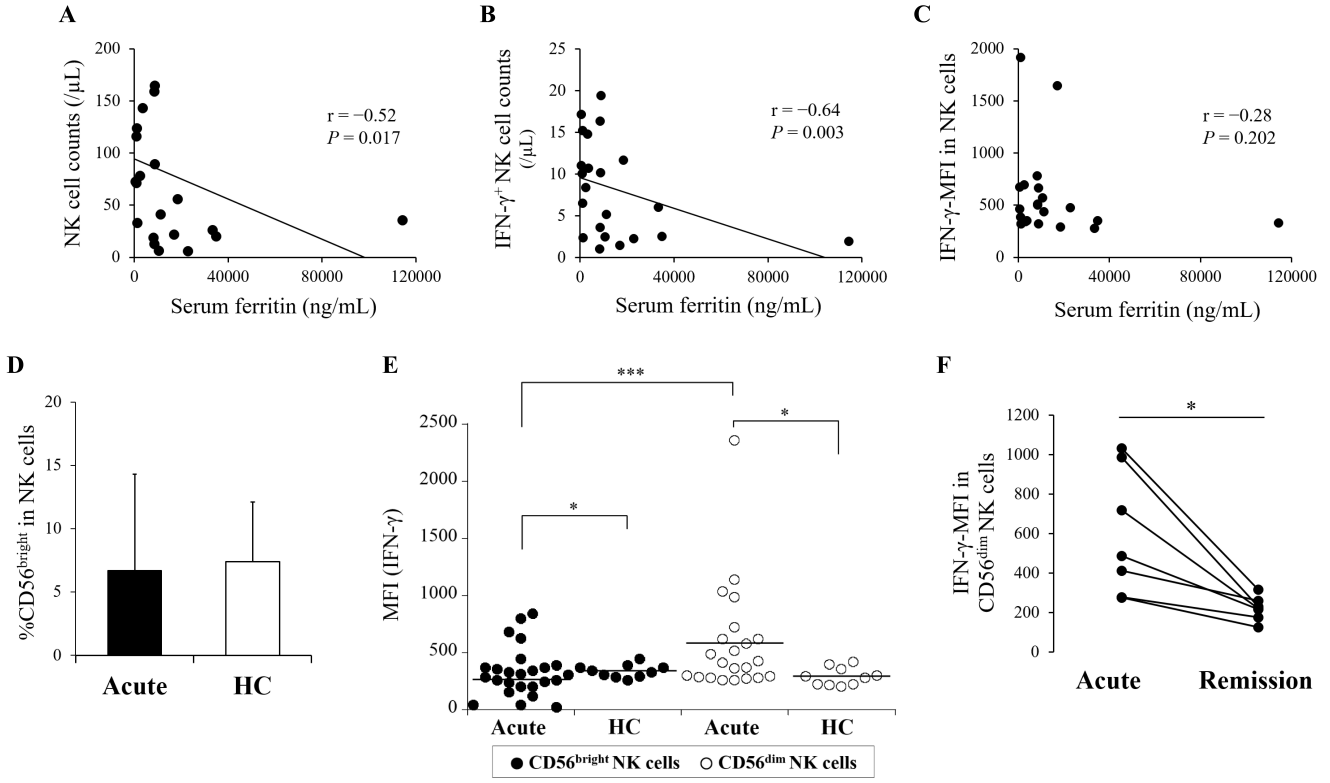

Figure 5. Inverse correlation with serum ferritin levels and IFN-γ-producing ability in 2 distinct NK cell subsets. Regarding the absolute number of NK cells, IFN-γ-expressing NK cells, and IFN-γ-MFI in NK cells, the correlations with serum ferritin levels were evaluated in patients with acute AOSD by the correlation coefficient test (A, B, C). In the population of NK cells (CD3−CD16+CD56+ cells), $CD56^{bright}$ and $CD56^{dim}$ subsets were divided. The proportion of $CD56^{bright}$ in NK cells was compared between patients with acute AOSD and HC (D). In the population of $CD56^{bright}$ or $CD56^{dim}$ NK cells, IFN-γ–producing ability was evaluated. IFN-γ-MFI in each subset was compared between patients with acute AOSD and HC (E). In addition, IFN-γ–MFI in $CD56^{dim}$ NK cell population was compared between an acute and remission phase (F). Values are shown as the mean ± SD. The Mann-Whitney U test was used in the comparison between patients with acute AOSD and HC. The Wilcoxon signed-rank test was used for the comparison between $CD56^{bright}$ and $CD56^{dim}$ in acute AOSD, or for that between an acute and remission phase. Statistically significant differences are indicated as follows: *p < 0.05 and ***p < 0.0005. NK: natural killer; AOSD: adult-onset Still disease; HC: healthy controls; IFN: interferon; MFI: median fluorescence intensity.

process of intracellular IL-18 signal transduction[28,31,32,33]. However, defective phosphorylation of IL-18Rβ impaired NK cell function and reduced IFN-γ secretion even after IL-18 stimulation in sJIA[32]. Accordingly, IL-18 signaling within NK cells from patients with AOSD may be impaired even though IL-18Rα expression remains. This theory may explain the causal attribution of our result that no significant correlation was shown between serum IL-18 levels and IFN-γ–expressing NK cells in acute AOSD. On the other hand, expression of IL-12 and IL-15 receptors on NK cells was significantly higher in acute AOSD than in both remission AOSD and HC. The ability to drive IFN-γ secretion by IL-12 or IL-15 seems to be restricted under deficient IL-18 signaling[15,30]. However, NK cells may exert the compensatory mechanism mediating IFN-γ production by inducing intracellular IL-12 and IL-15 signals by upregulating their affiliate receptors in AOSD.

In acute AOSD, IFN-γ–expressing NK cell counts was inversely correlated with serum ferritin levels. Given the evaluation of NK cell features divided into 2 subsets based on CD56 antigen in this study, IFN-γ expression in $CD56^{bright}$ was significantly lower in acute AOSD than in HC; and conversely, increased IFN-γ expression in $CD56^{dim}$ was significantly demonstrated. This result is considered paradoxical as the physiological phenomenon because $CD56^{bright}$ NK cells are recognized as the main producer of IFN-γ[16,34,35]. However, $CD56^{dim}$ NK cells can more prominently produce proinflammatory cytokine after K562 cell interaction as a target cell than $CD56^{bright}$ NK cells[36]. In fact, K562 cell interaction was found to reduce the proportion and/or function of NK cells in sJIA and AOSD[10,13]. Besides, cell-cell interaction with dendritic cells may also affect NK cell function in which IFN-γ production can be induced[15]. Therefore, $CD56^{dim}$ NK cells may develop their IFN-γ–producing ability through crosstalk with immunopathogenic cells related to AOSD development. Further, it is hypothesized that the absolute number of IFN-γ–expressing NK cells may be changed during IFN-γ–producing dominancy being shifted from $CD56^{bright}$ to $CD56^{dim}$ in acute AOSD, allowing that the inverse correlation with serum ferritin levels might be ultimately determined as the result of reducing the absolute number of IFN-γ–expressing NK cells depending on high disease activity.

Meanwhile, IFN-γ expression in NK cells significantly decreased in remission AOSD compared with that in HC. Previous studies demonstrated that NK cell function including IFN-γ production is suppressed by the treatment with immunosuppressive agents[37,38]. Given the above-mentioned results as well as the relevant citations, it should be considered that defective NK cell function may contribute to not only onset of AOSD, but also insufficient host immunity against infectious microbes during the treatment. However, only 7 patients in remission AOSD could be sequentially analyzed in our study, thus the number of samples might be insufficient for obtaining a complete determination.

The proportion of NK cells was significantly lower in acute AOSD than in HC, whereas NK cells revealed higher expression of IFN-γ as well as IL-12 and IL-15 receptors in acute AOSD than in HC. It was suggested that upregulation of IL-12 and IL-15 receptors may be implicated in compensating increased intracellular IFN-γ production in NK cells from patients with acute AOSD despite no significant expression of IL-18 receptor. Meanwhile, the numbers of NK cells and IFN-γ–expressing NK cells were correlatively reduced in accordance with elevated serum levels of ferritin. In addition, $CD56^{dim}$ NK cells prominently produced IFN-γ compared with $CD56^{bright}$ NK cells in acute AOSD. It was assumed that the ability of IFN-γ production in NK cells may be affected depending on a disease activity in AOSD. On the other hand, NK cells are also induced in response to certain viruses and haptens through a different mechanism[14,17,39]. Some inhibitory receptors binding to host-MHC class I regulate NK cell activity including cytotoxicity and cytokine production[15,40]. Moreover, the experimental system with multiple-proinflammatory cytokines affecting AOSD development may be needed for clarifying more precise IFN-γ–producing machinery in NK cells, because the activation with phorbol myristate acetate/ionomycin was used solely in our study. Therefore, further investigation requires evaluating a wide variety of immune reactions in NK cell lineage together with recruiting more patients.

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ONLINE SUPPLEMENT

Supplementary material accompanies the online version of this article.

REFERENCES

1. Efthimiou P, Paik PK, Bielory L. Diagnosis and management of adult onset Still's disease. Ann Rheum Dis 2006;65:564-72.
2. Gerfaud-Valentin M, Jamilloux Y, Iwaz J, Seve P. Adult-onset Still's disease. Autoimmun Rev 2014;13:708-22.
3. Chen DY, Lan JL, Lin FJ, Hsieh TY. Proinflammatory cytokine profiles in sera and pathological tissues of patients with active untreated adult onset Still's disease. J Rheumatol 2004;31:2189-98.
4. Rau M, Schiller M, Krienke S, Heyder P, Lorenz H, Blank N. Clinical manifestations but not cytokine profiles differentiate adult-onset Still's disease and sepsis. J Rheumatol 2010;37:2369-76.
5. Choi JH, Suh CH, Lee YM, Suh YJ, Lee SK, Kim SS, et al. Serum cytokine profiles in patients with adult onset Still's disease. J Rheumatol 2003;30:2422-7.
6. Hoshino T, Ohta A, Yang D, Kawamoto M, Kikuchi M, Inoue Y, et al. Elevated serum interleukin 6, interferon-gamma, and tumor necrosis factor-alpha levels in patients with adult Still's disease. J Rheumatol 1998;25:396-8.
7. Bae CB, Jung JY, Kim HA, Suh CH. Reactive hemophagocytic syndrome in adult-onset Still disease: clinical features, predictive factors, and prognosis in 21 patients. Medicine 2015;94:e451.
8. Komiya A, Matsui T, Nogi S, Iwata K, Futami H, Takaoka H, et al. Neutrophil CD64 is upregulated in patients with active adult-onset Still's disease. Scand J Rheumatol 2012;41:156-8.
9. Pay S, Turkcapar N, Kalyoncu M, Simsek I, Beyan E, Ertenli I, et al. A multicenter study of patients with adult-onset Still's disease compared with systemic juvenile idiopathic arthritis. Clin Rheumatol 2006;25:639-44.
10. Lee SJ, Cho YN, Kim TJ, Park SC, Park DJ, Jin HM, et al. Natural killer T cell deficiency in active adult-onset Still's Disease: correlation of deficiency of natural killer T cells with dysfunction of natural killer cells. Arthritis Rheum 2012;64:2868-77.
11. Park JH, Kim HS, Lee JS, Kim JJ, Jung KH, Park YW, et al. Natural killer cell cytolytic function in Korean patients with adult-onset Still's disease. J Rheumatol 2012;39:2000-7.
12. Grom AA. Natural killer cell dysfunction: A common pathway in systemic-onset juvenile rheumatoid arthritis, macrophage activation syndrome, and hemophagocytic lymphohistiocytosis? Arthritis Rheum 2004;50:689-98.
13. Villanueva J, Lee S, Giannini EH, Graham TB, Passo MH, Filipovich A, et al. Natural killer cell dysfunction is a distinguishing feature of systemic onset juvenile rheumatoid arthritis and macrophage activation syndrome. Arthritis Res Ther 2005;7:R30-7.
14. Berrien-Elliott MM, Wagner JA, Fehniger TA. Human cytokine-induced memory-like natural killer cells. J Innate Immun 2015;7:563-71.
15. Cooper MA, Yokoyama WM. Memory-like responses of natural killer cells. Immunol Rev 2010;235:297-305.
16. Cooper MA, Fehniger TA, Caligiuri MA. The biology of human natural killer-cell subsets. Trends Immunol 2001;22:633-40.
17. Min-Oo G, Kamimura Y, Hendricks DW, Nabekura T, Lanier LL. Natural killer cells: walking three paths down memory lane. Trends Immunol 2013;34:251-8.
18. Yamaguchi M, Ohta A, Tsunematsu T, Kasukawa R, Mizushima Y, Kashiwagi H, et al. Preliminary criteria for classification of adult Still's disease. J Rheumatol 1992;19:424-30.
19. Ravelli A, Minoia F, Davi S, Horne A, Bovis F, Pistorio A, et al. 2016 classification criteria for macrophage activation syndrome complicating systemic juvenile idiopathic arthritis: a European League Against Rheumatism/American College of Rheumatology/Paediatric Rheumatology International Trials Organisation Collaborative Initiative. Arthritis Rheumatol 2016;68:566-76.
20. Pouchot J, Sampalis JS, Beaudet F, Carette S, Decary F, Salusinsky-Sternbach M, et al. Adult Still's disease: manifestations, disease course, and outcome in 62 patients. Medicine 1991; 70:118-36.
21. Inoue N, Shimizu M, Tsunoda S, Kawano M, Matsumura M, Yachie A. Cytokine profile in adult-onset Still's disease: comparison with systemic juvenile idiopathic arthritis. Clin Immunol 2016;169:8-13.
22. Girard C, Rech J, Brown M, Allali D, Roux-Lombard P, Spertini F, et al. Elevated serum levels of free interleukin-18 in adult-onset Still's disease. Rheumatology 2016;55:2237-47.
23. Shimizu M, Nakagishi Y, Inoue N, Mizuta M, Ko G, Saikawa Y, et al. Interleukin-18 for predicting the development of macrophage

activation syndrome in systemic juvenile idiopathic arthritis. Clin Immunol 2015;160:277-81.
24. Ruscitti P, Iacono D, Ciccia F, Emmi G, Cipriani P, Grembiale RD, et al. Macrophage activation syndrome in patients affected by adult-onset Still disease: analysis of survival rates and predictive factors in the Gruppo Italiano di Ricerca in Reumatologia Clinica e Sperimentale cohort. J Rheumatol 2018;45:864-72.
25. Lee SW, Park YB, Song JS, Lee SK. The mid-range of the adjusted level of ferritin can predict the chronic course in patients with adult onset Still's disease. J Rheumatol 2009;36:156-62.
26. Kong XD, Xu D, Zhang W, Zhao Y, Zeng X, Zhang F. Clinical features and prognosis in adult-onset Still's disease: a study of 104 cases. Clin Rheumatol 2010;29:1015-9.
27. Billiau A, Matthys P. Interferon-gamma: a historical perspective. Cytokine Growth Factor Rev 2009;20:97-113.
28. Dinarello CA, Novick D, Kim S, Kaplanski G. Interleukin-18 and IL-18 binding protein. Front Immunol 2013;4:289.
29. Gaspari S, Marcovecchio ML, Breda L, Chiarelli F. Growth in juvenile idiopathic arthritis: the role of inflammation. Clin Exp Rheumatol 2011;29:104-10.
30. Chaix J, Tessmer MS, Hoebe K, Fuseri N, Ryffel B, Dalod M, et al. Cutting edge: priming of NK cells by IL-18. J Immunol 2008;181:1627-31.
31. Kim SH, Reznikov LL, Stuyt RJ, Selzman CH, Fantuzzi G, Hoshino T, et al. Functional reconstitution and regulation of IL-18 activity by the IL-18R beta chain. J Immunol 2001;166:148-54.
32. de Jager W, Vastert SJ, Beekman JM, Wulffraat NM, Kuis W, Coffer PJ, et al. Defective phosphorylation of interleukin-18 receptor beta causes impaired natural killer cell function in systemic-onset juvenile idiopathic arthritis. Arthritis Rheum 2009;60:2782-93.
33. Kato Z, Jee J, Shikano H, Mishima M, Ohki I, Ohnishi H, et al. The structure and binding mode of interleukin-18. Nat Struct Biol 2003;10:966-71.
34. Cooper MA, Fehniger TA, Turner SC, Chen KS, Ghaheri BA, Ghayur T, et al. Human natural killer cells: a unique innate immunoregulatory role for the CD56(bright) subset. Blood 2001;97:3146-51.
35. Fehniger TA, Shah MH, Turner MJ, VanDeusen JB, Whitman SP, Cooper MA, et al. Differential cytokine and chemokine gene expression by human NK cells following activation with IL-18 or IL-15 in combination with IL-12: implications for the innate immune response. J Immunol 1999;162:4511-20.
36. Fauriat C, Long EO, Ljunggren HG, Bryceson YT. Regulation of human NK-cell cytokine and chemokine production by target cell recognition. Blood 2010;115:2167-76.
37. Ohira M, Nishida S, Tryphonopoulos P, Ruiz P, Ohdan H, Tzakis AG. Impact of steroids on natural killer cells against cytotoxicity and hepatitis C virus replication. Transplant Proc 2017;49:1160-4.
38. Morteau O, Blundell S, Chakera A, Bennett S, Christou CM, Mason PD, et al. Renal transplant immunosuppression impairs natural killer cell function in vitro and in vivo. PLoS One 2010;5:e13294.
39. O'Sullivan TE, Sun JC, Lanier LL. Natural killer cell memory. Immunity 2015;43:634-45.
40. Lanier LL. Up on the tightrope: natural killer cell activation and inhibition. Nat Immunol 2008;9:495-502.