Original article

The implication of interferon-γ-producing immunocompetent cells for evaluating disease activity and severity in adult-onset Still's disease

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A short running title: IFN-γ related to activity and severity in AOSD

Author's contribution: All authors made the design of this study, developed the structure and argument for this study. Y-Shi, T-I, D-K, K-U recruited blood samples and clinical data. Y-Shi and T-I performed laboratory investigations, and analyzed obtained data. Y-Shi and T-I prepared the draft of this manuscript. Y-Shi and Y-Se contributed to revise the manuscript. All authors revised and approved of the final manuscript.

Acknowledgments

We thank all members of the Department of Medicine (Neurology and Rheumatology), Shinshu University Hospital, for treating the patients. This study was supported by the Shinshu Public Utility Foundation for the Promotion of Medical Science, and a Health and Labour Sciences Research Grant on Rare and Intractable Diseases from the Ministry of Health, Labour and Welfare of Japan.

Conflicts of Interest

The authors declare that they have no financial or personal conflicts of interest.

Abstract

Objective: To investigate the relationship between interferon-γ (IFN-γ), IFN-γ-producing immunocompetent cells, their related cytokines, and the clinical features in adult-onset Still's disease (AOSD).

Methods: Twenty-five patients with AOSD before initiating treatment (acute AOSD), 9 patients after remission (remission AOSD), and 12 healthy controls (HC) were included. Circulating IFN-γ-producing CD4+ and CD8+ cells, natural killer (NK) cells, and IFN-γ production in NK cells were evaluated by flow cytometry. Serum levels of IFN-γ, interleukin (IL)-6, IL-12, IL-15, and IL-18 were also measured. The obtained results were statistically analyzed with clinical findings.

Results: Serum levels of IFN-γ, IL-6, IL-12, IL-18, intracellular expression of IFN-γ in CD4+, CD8+, and NK cells were significantly higher in acute AOSD than in HC. The proportion of NK cells was significantly lower in acute AOSD than in HC. Serum levels of IFN-γ and IFN-γ expression in CD4+ cells were significantly correlated with serum ferritin levels. The proportion of NK cells had a significant inverse correlation with serum IFN-γ levels. A lower proportion of NK cells was significantly noted in patient refractory to initial immunosuppressive treatment. In remission AOSD, serum levels of IL-6, IL-12, and IL-18 were significantly higher than in HC.

Conclusion: Increased serum levels of IFN-γ, increased expression of IFN-γ in CD4+ cells, and decreased NK cell proportion correlate with disease activity in AOSD. Moreover, a lower proportion of NK cells may be useful for predicting a refractory clinical course. Meanwhile, increased serum levels of IL-6, IL-12, and IL-18 may persist after clinical remission.

Keywords: adult-onset Still's disease, interferon-γ, NK cells, CD4+ cells, interleukin-12, interleukin-18

1. Introduction

Adult-onset Still's disease (AOSD) is a systemic inflammatory disorder with characteristic symptoms such as high fever, polyarthritis, evanescent rash, and increases in inflammatory laboratory parameters, including high serum ferritin levels. Considering the clinical course of AOSD, there is a concern that some patients may show resistance to initial immunosuppressive treatment, relapse, or life-threatening involvement. It is still difficult to determine the prognosis of patients with AOSD, although the candidates for prognostic disease factors have been suggested in some previous studies [1, 2]. The pathogenic mechanism underlying AOSD development may be composed of diverse immunological interactions, including enhancing pro-inflammatory cytokine profiles, innate and adaptive immune systems. Notably, macrophage activation contributes to the occurrence of disease as a pathogenic hallmark in AOSD [3]. In the process of activating macrophages, interferon-γ (IFN-γ) is a crucial cytokine that is predominantly produced by natural killer (NK) cells and effector T cells [4, 5]. Increase in serum levels of IFN-γ and frequency of IFN-γ-producing CD4+ cells (type 1 T helper [Th1] cells) were significantly found in AOSD [6-8]. In our recent investigation, increased expression of IFN-γ in NK cells was also significantly demonstrated in the acute phase of AOSD [9], whereas a decreased frequency of NK cells was observed, similar to previous studies [9-11]. Accordingly, it was suggested that secreted IFN-γ and IFN-γ-producing immunocompetent cells might be implicated in developing the disease. On the contrary, it is still uncertain how these key mediators are relevant to clinical features and prognosis in AOSD.

In this study, we investigated how serum levels of IFN-γ and the kinetic profiles of IFN-γ-producing cells, including Th1 cells and NK cells, are related to the clinical features of AOSD. In addition, the participation of IL-

12, IL-15, and IL-18, which play a role in IFN-γ production in both Th1 cells and NK cells [12-15], was also evaluated.

2. Methods

2.1 Patients and samples

Blood samples from 25 patients with AOSD before initiating immunosuppressive treatments (acute AOSD) and 12 healthy controls (HC) were included in this study. The diagnosis of AOSD was definitively made according to the criteria proposed by Yamaguchi *et al* [16] in our hospital. The clinical findings in acute AOSD are shown in **Table 1**, in which the overall disease activity score (Pouchot's score) [17] and the complication of macrophage activation syndrome (MAS) [18, 19] were also evaluated. These findings in Table 1 were obtained and evaluated as the baseline when blood samples were provided. Patients resistant to initial immunosuppressive treatment were defined as having a refractory course, which was determined by requiring additional treatment before tapering initial immunosuppressive agents: 1) increase in corticosteroid, 2) another immunosuppressant, 3) biologics, and/or 4) plasmapheresis. There were no significant differences in age and sex distribution between acute AOSD and HC (median age: 43.5 years [interquartile range (IQR): 40.8–60.0], 6 men and 6 women). Of the 25 patients, blood samples were provided from 9 patients who achieved remission with the Pouchot's score of zero (remission AOSD) to evaluate the remission phase results. Blood samples were obtained at a median period of 18 months (IQR: 10–28) after initiating immunosuppressive therapy. The maintenance therapies at the point of obtaining blood sample were as follows: prednisolone (PSL; n = 7), cyclosporine (n = 3), methotrexate (n = 3), and tocilizumab (n = 2). The local ethics committee in Shinshu University approved this study (the approval number:

601/4294). All participants provided informed consent.

2.2. Flow cytometry

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood samples collected into EDTA-coated tubes by gradient centrifugation with Ficoll-Hypaque PLUS (GE Healthcare, Pittsburgh, PA, USA). CD4+ cells, NK cells, and intracellular IFN-γ expression in CD4+ cells or NK cells were determined by flow cytometric analysis. CD8+ cells and their IFN-γ expression were also evaluated. PBMCs were stimulated with 0.5 μg/mL of ionomycin, 0.04 μg/mL of phorbol myristate acetate (both from Sigma-Aldrich, St.Louis, MO, USA), and 2 μM monensin (BD Bioscience, San Diego, CA, USA) at 37°C for 4 hours. To detect CD4+ cells, CD8+ cells, or NK cells that were phenotypically defined as CD3−CD16+CD56+ cells, stimulated PBMCs were stained with PE/Cy7 anti-CD4 (BioLegend, San Diego, CA, USA), FITC-conjugated anti-CD8 (Beckman Coulter, Brea, CA, USA), or Pacific blue-conjugated anti-CD3 (BioLegend) together with FITC-conjugated anti-CD16 (Beckman Coulter) and PE-conjugated anti-CD56 (Beckman Coulter). PBMCs stained with the above-mentioned cell-surface markers were permeabilized with Cytofix/Cytoperm (BD Bioscience) to determine intracellular IFN-γ expression. Permeabilized cells were subsequently stained with APC-conjugated anti-IFN-γ (BioLegend) in NK cells or CD8+ cells, or FITC-conjugated anti-IFN-γ (Beckman Coulter) in CD4+ cells. Treated cells were acquired on a FACSCanto II flow cytometer (BD Bioscience), and the acquired data were analyzed using FlowJo version 7.6.5 software (Tree Star Inc., Ashland, OR, USA).

2.3. Enzyme-linked immunosorbent assay (ELISA)

Serum samples were stored at −80 °C until ELISA was performed. Serum concentrations of IFN-γ, IL-6, IL-12

(R&D system, Minneapolis, MN, USA), IL-15 (Abcam, San Francisco, CA, USA), and IL-18 (Medical and Biological Laboratories, Nagoya, Japan) were assayed using commercially available ELISA kits.

2.4. Statistical analysis

All data are presented as the median with the interquartile range (IQR). *P-values* of less than 0.05 were defined as statistically significant. The Mann-Whitney U test was used to compare two independent groups. In comparing the results of subsequent patients before and after treatment, the Wilcoxon signed ranked test was employed. A correlation coefficient test was performed to evaluate a significant relationship. All statistical analyses were performed using BellCurve for Excel (SSRI, Tokyo, Japan).

3. Results

Clinical findings in patients with AOSD

The median Pouchot's score before initiating treatment was 6 (IQR: 4−6) in acute AOSD (**Table 1**). Of the 25 patients, 7 (28%) fulfilled the criteria of MAS at baseline, and 18 (72%) were defined as having a refractory course (refractory patients). In the comparison of clinical and laboratory findings between refractory patients and those who did not have a refractory course (non-refractory patients), the number of white blood cells (WBC) was significantly higher in refractory patients than in non-refractory patients ($p = 0.034$), despite no other significant differences (**Table 2**). MAS was ultimately developed in 11 refractory patients until they were defined as having a refractory course, whereas no MAS occurrence was newly shown in non-refractory patients except for 3 presenting with MAS at baseline. In the initial therapy, intravenous infusion of methylprednisolone (1g daily for

3 days) (mPSL) was administered to 11 refractory patients, while non-refractory patients did not entirely require mPSL (p = 0.007). The initial dosage of oral PSL was significantly higher in refractory patients than in non-refractory patients (p = 0.001). Immunosuppressive agents were concomitantly initiated in 6 refractory patients, and not in non-refractory patients, resulting in no significant difference (p = 0.080). Of the 18 refractory patients, mPSL was additionally required in 16 (89%), and increase in the dosage of PSL was given in 7 (39%) (**Supplementary table 1**). Immunosuppressive agents, intravenous immunoglobulin, and plasma exchange were additionally administered for 11 (61%), 4 (22%), and 7 (39%), respectively. In remission AOSD, improved laboratory findings were significantly demonstrated as follows: WBC counts (7120/μL [IQR: 6610–7560], p = 0.005), serum levels of C-reactive protein (0.03 mg/dL [IQR: 0–0.04], $p < 0.0001$), and ferritin (71 ng/mL [IQR: 54–83], $p < 0.0001$).

Expression of IFN-γ-producing cells and serum IFN-γ in AOSD

The proportion of CD4+ cells was not significantly different between patients with AOSD and HC (**Table 3**). The proportion of CD4+IFN-γ+ cells was significantly higher in acute AOSD than in HC (p = 0.0002). In addition, the frequency and median fluorescence intensity (MFI) of IFN-γ in CD4+ cells were significantly higher in acute AOSD than in HC (p = 0.0002 and $p < 0.0001$, respectively) (**Table 3**, **Figure 1(A), 1(B)**). The frequency and MFI of IFN-γ in CD8+ cells, as well as the proportion of CD8+IFN-γ+ cells, were significantly higher in acute AOSD than in HC (p = 0.0002, p = 0.0002, p = 0.041, respectively), whereas the proportion of CD8+ cells was significantly lower in acute AOSD than in HC (p = 0.003) (**Supplementary table 2, Supplementary figure 1**). The proportion of NK cells was significantly lower in acute AOSD than HC (p = 0.0026) (**Table 3**, **Figure 1(C)**),

whereas the frequency and MFI of IFN-γ in NK cells were significantly higher in acute AOSD than in HC (p = 0.0001 and $p < 0.0001$, respectively) (**Table 3**, **Figure 1(D), 1(E)**). Serum levels of IFN-γ were also significantly higher in acute AOSD than in HC ($p < 0.0001$). Meanwhile, there were no significant differences in these results between remission AOSD and HC.

In comparison with consecutive results of 9 patients before and after treatment, decreases in the proportion of CD4+IFN-γ+ cells, frequencies and MFI of IFN-γ expression in CD4+ cells and NK cells, serum levels of IFN-γ, and increase in the proportion of NK cells were significantly demonstrated ($p < 0.05$) (**Figure 2**). The frequency and MFI of IFN-γ in CD8+ cells also significantly decreased after treatment (p = 0.027) (**Supplementary figure 1**). In remission AOSD, 7 refractory patients were included. Of the 7 refractory patients, decrease in the proportion of CD4+IFN-γ+ cells and increase in that of NK cells were shown in 6 after treatment, whereas neither the proportion of CD4+IFN-γ+ cells nor that of NK cells were significantly different from those before treatment (p = 0.062) (**Supplementary figure 2**). MFI of IFN-γ in CD4+ cells and NK cells significantly decreased after treatment (p = 0.018).

Serum IL-6, IL-12, IL-15, and IL-18 expression, and their participation in AOSD

Serum levels of IL-6, IL-12, and IL-18 were significantly higher in acute and remission AOSD than in HC (**Table 3**). In comparisons between acute and remission AOSD, serum levels of IL-6 and IL-18 were significantly decreased (p = 0.008, p = 0.011, respectively), whereas those of IL-12 were not significantly different (p = 0.313). Serum levels of IL-15 were not significantly different in acute AOSD, remission AOSD, and HC. In the regression analyses with serum levels of IFN-γ, intracellular expression of IFN-γ in CD4+ and NK cells, and the proportion

of CD4+IFN-γ+ and NK cells, serum levels of IL-12, IL-15, and IL-18 demonstrated no significant correlations (data not shown). Meanwhile, serum levels of IL-6 had a significant correlation with frequency of IFN-γ in CD4+ cells ($p = 0.009$) (**Supplementary table 3**).

The relationship between IFN-γ-producing cells and clinical findings

We analyzed the statistical relationship between the experimental results and clinical findings shown in Table 1. Serum ferritin levels were significantly correlated with serum levels of IFN-γ, frequency, and MFI of IFN-γ in CD4+ cells ($p = 0.026$, $p = 0.016$, $p = 0.035$, respectively) (**Figure 3(A)**, **3(B)**, **3(C)**). Serum levels of IFN-γ had a significant reverse correlation with NK cells ($p = 0.038$) (**Figure 3(D)**). There were no other significant relationships between the experimental data and clinical findings.

Next, we compared the experimental data obtained between refractory and non-refractory patients. A lower proportion of NK cells was significantly demonstrated in refractory patients than in non-refractory patients (median 5.6% [IQR: 2.4−10.9] vs. 14.1% [10.1−16.2], $p = 0.025$) (**Figure 3(E)**). Meanwhile, there were no significant differences between patients with and without MAS at baseline in the comparison of experimental data (data not shown).

4. Discussion

In this study, we focused on the participation of IFN-γ expression and IFN-γ-producing immunocompetent cells in the clinical features of AOSD. Some investigations previously demonstrated an increase in serum levels of IFN-γ in the acute phase of AOSD [3, 6, 7, 20]. It was also assumed that IFN-γ might be implicated in the pathogenic

mechanism underlying AOSD development as the promoter of macrophage and neutrophil activation [3, 21-23]. However, the role of IFN-γ in evaluating the disease activity and prognosis of AOSD has not been evaluated thus far, whereas the correlation between disease activity markers and IFN-γ-induced chemokines was significantly demonstrated [20]. In our study, serum levels of IFN-γ were significantly correlated with ferritin, suggesting that serum levels of IFN-γ may be regarded as an indicator of disease activity because serum ferritin is a well-known biomarker for estimating disease activity and prognosis [24-26].

Moreover, it has been known that macrophage activation leads to increased serum levels of ferritin in AOSD [1, 2, 27]; namely, IFN-γ expression may participate in disease activity by activating macrophages. Besides, IFN-γ production in CD4+, CD8+, and NK cells was significantly increased in acute AOSD. Although a previous study reported the predominance of Th1 cells in the active phase of AOSD [8], our investigation found that the intracellular expression of IFN-γ in CD4+ cells was significantly correlated with serum ferritin levels. The imbalance or implication of adaptive immunity in the pathogenesis of AOSD has been described to date [2, 3]. The relationship between circulating effector T cells and disease activity has been described in previous investigations of AOSD [8, 28, 29]. The proportion of circulating IL-17-producing CD4+ (Th17) cells was found to be significantly correlated with serum ferritin levels in the acute phase of AOSD [29], whereas our results first demonstrated a significant relationship between Th1 cells and serum ferritin levels, suggesting that the intracellular expression of IFN-γ in CD4+ cells is correlated with disease activity in AOSD. In our recent investigation, the production of IFN-γ, which is usually shown mainly in the $CD56^{bright}$ NK cell population in healthy individuals, was predominant in the $CD56^{dim}$ NK cell population in AOSD [9], ultimately resulting in significantly increased IFN-γ in NK cells

in acute AOSD. The percentage of $CD56^{dim}$ NK cell population is known to be around 90% in total circulating NK cells [14] despite not significantly different between acute AOSD and healthy individual [9], allowing increased expression of IFN-γ in NK cells because a majority of the NK cell population can produce IFN-γ in AOSD. In addition, the proportion of circulating NK cells was significantly decreased in acute AOSD and inversely correlated with serum levels of IFN-γ. However, intracellular IFN-γ in NK cells was not correlated with serum ferritin levels or the proportion of NK cells (data not shown). Accordingly, IFN-γ produced in NK cells may not be a parameter that correlates with disease activity even though NK cells possess the ability to promote IFN-γ production in acute AOSD. Taken together, a decreased proportion of NK cells, increased serum levels of IFN-γ, and intracellular IFN-γ expression in CD4+ cells may be mediators that contribute to disease activity in AOSD.

Serum levels of IL-18 were found to be implicated in the disease activity [2, 3, 30], and those of IL-12 were also increased in AOSD [31]; however, the participation of serum IL-15 has remained obscure in AOSD. Our study ultimately demonstrated that serum levels of IL-12 and IL-18 were significantly higher in AOSD than in HC, but those of IL-15 showed no significant difference. Although serum levels of these cytokines were not correlated with those of IFN-γ, intracellular IFN-γ expression, and the proportion of CD4+IFN-γ+ and NK cells, serum levels of IL-12 and IL-18 were significantly higher in remission AOSD than in HC. Moreover, this suggested that persistent increased serum levels of IL-12 and IL-18 may be the cause of relapse, because IL-18 in combination with IL-12 upregulates IFN-γ-producing signal in CD4+ and NK cells [13, 32-34]. Serum levels of IL-6, which were found to be associated with disease activity of AOSD [1-3, 35], were also significantly higher in acute and remission AOSD than in HC. It was previously shown that exposure to high concentrations of IL-6 might lead to suppressing NK cell

cytotoxicity [36], suggesting that IL-6 may partially affect NK cell activation in the pathogenesis of AOSD.

Refractory patients were classified in this study to determine the predictive factors of severity. The frequency of mPSL administration and PSL dosage was significantly higher in the initial treatment of refractory patients than in non-refractory patients, demonstrating insufficient therapeutic efficacy even in the more potent initial treatment. The previous cohort indicated that a high dose of PSL was usually required to achieve remission in AOSD [37, 38], even though there is a concern that the total dose of corticosteroid was ultimately accumulated in refractory patients [37]. Herein, a lower proportion of circulating NK cells was also significantly indicated in refractory patients. Namely, the frequency of circulating NK cells may be valuable for predicting the severity of AOSD. It has been shown that impaired function of NK cells may result in the inability to regulate the immune system, leading to the activation of lymphocytes and macrophages in systemic juvenile idiopathic arthritis (s-JIA) and AOSD [10, 11, 39-41]. Given our results and these immunological disorders, the induction of NK cells may regulate the pathological signaling underlying the development of AOSD. Besides, the dysfunction of NK cells was found to be implicated in developing MAS, which affects the prognosis of s-JIA and AOSD [40, 42, 43]. The development of MAS was sequentially observed in refractory patients despite no occurrence of that in non-refractory patients after initiating treatment, suggesting that a lower proportion of NK cells at the onset of AOSD may also be predictive of MAS development. However, no significant differences were found between patients with and without MAS at the diagnosis of AOSD in the analyses of experimental results. It has been shown that multiple mediators are implicated in the development of MAS [2, 19, 44], suggesting it may be insufficient to predict the occurrence of MAS only by estimating the expression of NK cells and/or IFN-γ.

In conclusion, serum levels of IFN-γ and intracellular expression of IFN-γ in CD4+, CD8+, and NK cells were significantly increased in acute AOSD. Notably, the expression of IFN-γ in the serum and CD4+ cells was significantly correlated with serum ferritin; meanwhile, a decreased proportion of NK cells was significantly correlated with serum IFN-γ levels, suggesting that IFN-γ and IFN-γ-producing immunocompetent cells are the parameters of disease activity. Furthermore, a lower proportion of NK cells may be a useful indicator for predicting an intractable clinical course. Besides, there is a concern that sustained increases in serum IL-6, IL-12, and IL-18 levels, which were significantly higher in remission AOSD than in HC, may be responsible for relapse. On the other hand, precise machinery for NK cell reduction is still uncertain in AOSD. The generation of NK cell is dependent on response to internal or external pathogens, neoplasm antigen, and immunological signals in the host [45, 46]. Some investigations suggested possible mechanisms leading to NK cell reduction, such as the implication of inhibitory receptors [47], reduced expression of activating receptors [48], or apoptosis mediated by induction of FcγR under IL-2 stimulation [49]. However, this study focused on the limited area of the immune system; thus, further investigations are required to clarify more precise indicators of severity and prognosis in a wide range of immune mechanisms underlying AOSD.

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Figure legends

Figure 1: Comparisons of intracellular IFN-γ expression and NK cells. **(A)** The representative histogram of IFN-γ expression in CD4+ cells. **(B)** Comparison of MFI of IFN-γ in CD4+ cells. **(C)** The representative dot-plots of CD16+CD56+ cells in the population of CD3− cells. **(D)** The representative histogram of IFN-γ expression in NK cells. **(E)** Comparison of MFI of IFN-γ in NK cells. Acute, acute AOSD; Remission, remission AOSD; HC, healthy controls; IFN-γ, interferon-γ; MFI, median fluorescence intensity; NK cells, natural killer cells. *** $p < 0.0001$

Figure 2: Alteration of IFN-γ and IFN-γ-producing cells before and after treatment. Acute, acute AOSD; Remission, remission AOSD; HC, healthy controls; IFN-γ, interferon-γ; MFI, median fluorescence intensity; NK cells, natural killer cells.

Figure 3: Regression analyses related to disease activity, and comparison between refractory and non-refractory patients. IFN-γ, interferon-γ; MFI, median fluorescence intensity; NK cells, natural killer cells; Ref, refractory patients; Non-Ref, non-refractory patients.

Table 1 Clinical characteristics of patients with AOSD (n = 25)

Epidemiological findings	
Age, years, median [IQR]	51 [39–66]
Sex (M/F)	6/19
Physical findings, n (%)	
Fever	25 (100)
Eruption	23 (92)
Sore throat/Pharyngitis	19 (76)
Lymphadenopathy	12 (48)
Arthritis	22 (88)
Myalgia	18 (72)
Pleuritis	5 (20)
Pericarditis	1 (4)
Hepatomegaly	9 (36)
Splenomegaly	13 (52)
Clinical evaluation	
Pouchot's score, median [IQR]	6 [4–6]
Fulfilled MAS criteria, n (%)	7 (28)
Refractory course, n (%)	18 (72)
Laboratory findings, median [IQR]	
White blood cells, /μL	15380 [10600–19080]
Neutrophils, /μL	13396 [7935–17668]
AST, U/L	60 [41–78]
ALT, U/L	51 [29–91]
C-reactive protein, mg/dL	9.66 [3.85–13.1]

ESR, mm/h	48 [31–96]
Ferritin, ng/mL	8191 [2863–11080]

IQR, interquartile range; MAS, macrophage activation syndrome; AST, aspartate transaminase; ALT, alanine transaminase; ESR, erythrocyte sedimentation rate

Table 2 Comparison of clinical findings and initial treatment between refractory and non-refractory patients

	Refractory patients (n = 18)	**Non-refractory patients** (n = 7)	*p* values
Epidemiological findings			
Age, years, median [IQR]	54 [38–75]	43 [42–53]	0.467
Sex (M/F)	3/15	3/4	0.194
Physical findings, n (%)			
Fever	18 (100)	7 (100)	0.623
Eruption	16 (89)	7 (100)	0.510
Sore throat/Pharyngitis	15 (83)	4 (57)	0.194
Lymphadenopathy	10 (56)	2 (29)	0.223
Arthritis	17 (94)	6 (86)	0.820
Myalgia	5 (28)	2 (29)	0.663
Pleuritis	5 (28)	0 (0)	0.161
Pericarditis	1 (6)	0 (0)	0.720
Hepatomegaly	7 (39)	2 (29)	0.501
Splenomegaly	11 (61)	2 (29)	0.155
Clinical evaluation			
Pouchot's score, median [IQR]	5.5 [4–6]	6 [4–7]	0.828
Fulfilled MAS criteria			
at baseline	4 (22)	3 (42)	0.934
throughout the clinical course	11 (61)	3 (42)	0.351
Laboratory findings, median [IQR]			
White blood cells, /μL	17595 [11430–25390]	11520 [8295–13035]	0.034
Neutrophils, /μL	15182 [9433–23078]	10138 [6453–11879]	0.061

AST, U/L	65 [42–88]	54 [41–62]	0.397
ALT, U/L	63 [34–99]	19 [14–57]	0.090
C-reactive protein, mg/dL	9.74 [4.75–14.35]	8.51 [3.58–12.29]	0.468
ESR, mm/h	48 [33–100]	48 [31–80]	0.507
Ferritin, ng/mL	8293 [3846–17757]	2863 [891–8548]	0.090
Initial treatment			
mPSL, n (%)	11 (61)	0	0.007
Oral PSL, mg/kg/day, median [IQR]	1.00 [0.90–1.02]	0.47 [0.38–0.68]	0.001
Immunosuppressive agents, n (%)	6 (33)	0	0.105
CsA	4 (22)	0	0.242
TAC	1 (1.25)	0	0.617
MTX	1 (1.25)	0	0.617

IQR, interquartile range; MAS, macrophage activation syndrome; AST, aspartate transaminase; ALT, alanine transaminase; ESR, erythrocyte sedimentation rate; mPSL, intravenous infusion of methylprednisolone (1g daily for 3 days); PSL, prednisolone; CsA, cyclosporin; TAC, tacrolimus; MTX, methotrexate.

Table 3 Phenotypes of lymphocytes and IFN-γ expression in patients with acute AOSD, remission AOSD, and healthy controls

	acute AOSD	**remission AOSD**	**HC**	*p* value	
	(n = 25)	(n = 9)	(n = 12)	Acute vs. HC	Rem vs. HC
In total lymphocytes					
% CD4+ cells	52.9 [38.9–57.4]	48.7 [36.4–61.5]	53.8 [38.9–58.6]	0.990	0.840
% CD4+IFN-γ+ cells	5.89 [2.63–11.86]	1.26 [0.68–2.28]	1.06 [0.47–1.72]	0.0002	0.693
% CD3- cells	45.0 [35.8–56.1]	39.8 [29.5–55.6]	41.9 [39.9–44.3]	0.807	0.531
% NK cells	8.42 [2.54–12.34]	10.75 [9.13–15.44]	14.28 [11.71–19.05]	0.0026	0.201
In CD4+ cells					
%IFN-γ	5.84 [2.41–9.17]	2.84 [1.95–5.25]	1.75 [0.55–2.68]	0.0002	0.174
In NK cells					
%IFN-γ	41.5 [32.2–73.7]	17.5 [12.2–18.5]	23.1 [18.5–28.8]	0.0001	0.077
In the serum					
IFN-γ (pg/mL)	15.41 [6.81–40.89]	4.16 [1.18–4.82]	1.12 [0.39–3.50]	<0.0001	0.254
IL-6 (pg/mL)	15.84 [7.58–181.3]	3.53 [2.19–12.38]	1.82 [1.29–2.36]	<0.0001	0.024
IL-12 (pg/mL)	1.55 [1.32−1.76]	1.34 [1.21−1.56]	0.59 [0.54−0.67]	<0.0001	0.0001

IL-15 (pg/mL)	6.97 [5.20−9.61]	5.20 [4.32−7.85]	5.20 [4.09−8.06]	0.174	0.971
IL-18 (pg/mL)	2271 [1798−2554]	98.5 [73.7−213.9]	71.6 [70.7−74.1]	<0.0001	0.006

HC, healthy controls; Acute, acute AOSD; Rem, remission AOSD; NK cells, natural killer cells; IFN-γ, interferon-γ; IL, interleukin.

Data are presented as median with interquartile range.

Figure 1

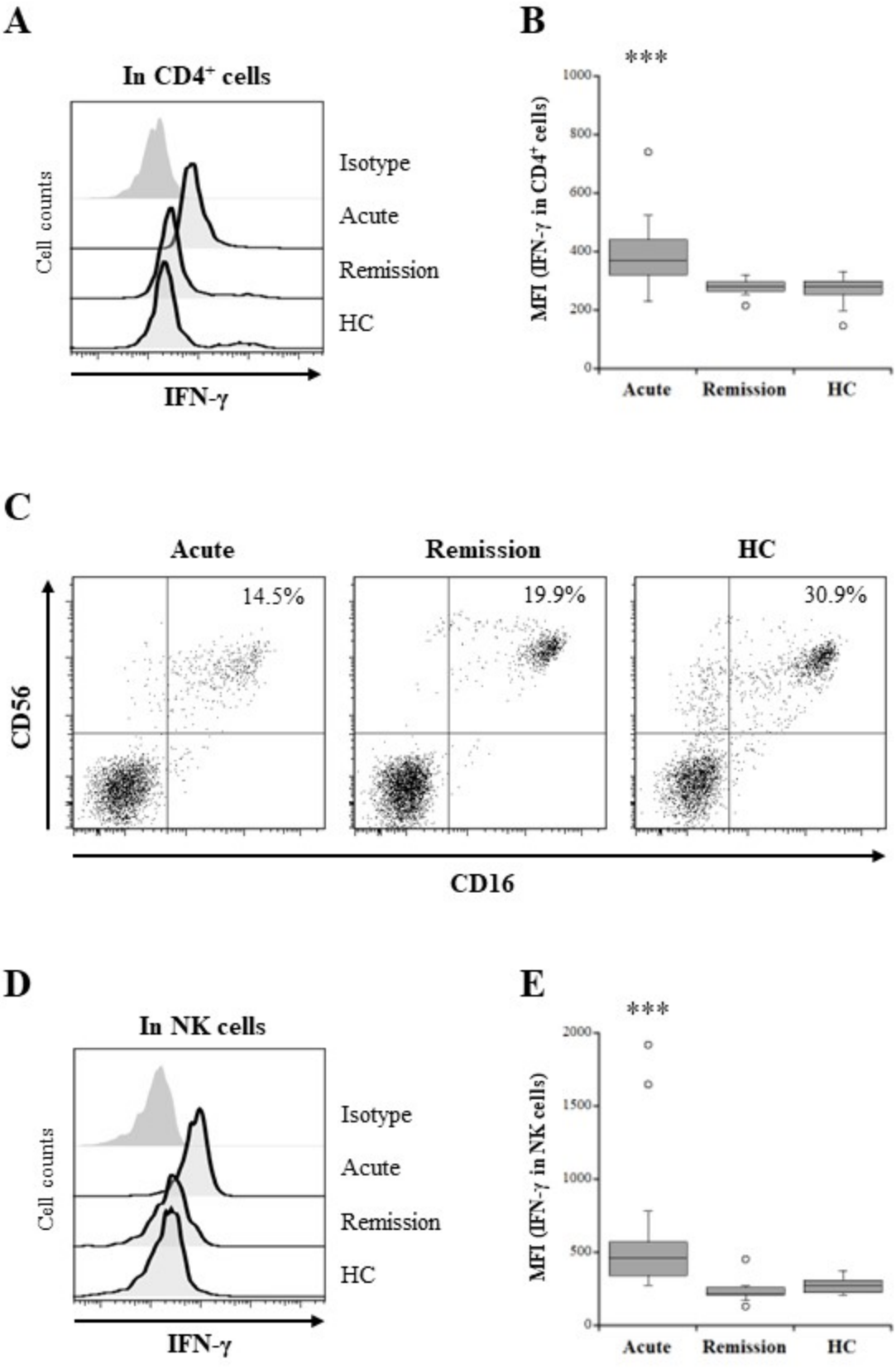

A
In CD4+ cells
Cell counts
Isotype
Acute
Remission
HC
IFN-γ
B
***
MFI (IFN-γ in CD4+ cells)
1000
800
600
400
200
0
Acute
Remission
HC
C
Acute
Remission
HC
14.5%
19.9%
30.9%
CD56
CD16
D
In NK cells
Cell counts
Isotype
Acute
Remission
HC
IFN-γ
E
***
MFI (IFN-γ in NK cells)
2000
1500
1000
500
0
Acute
Remission
HC

Figure 2

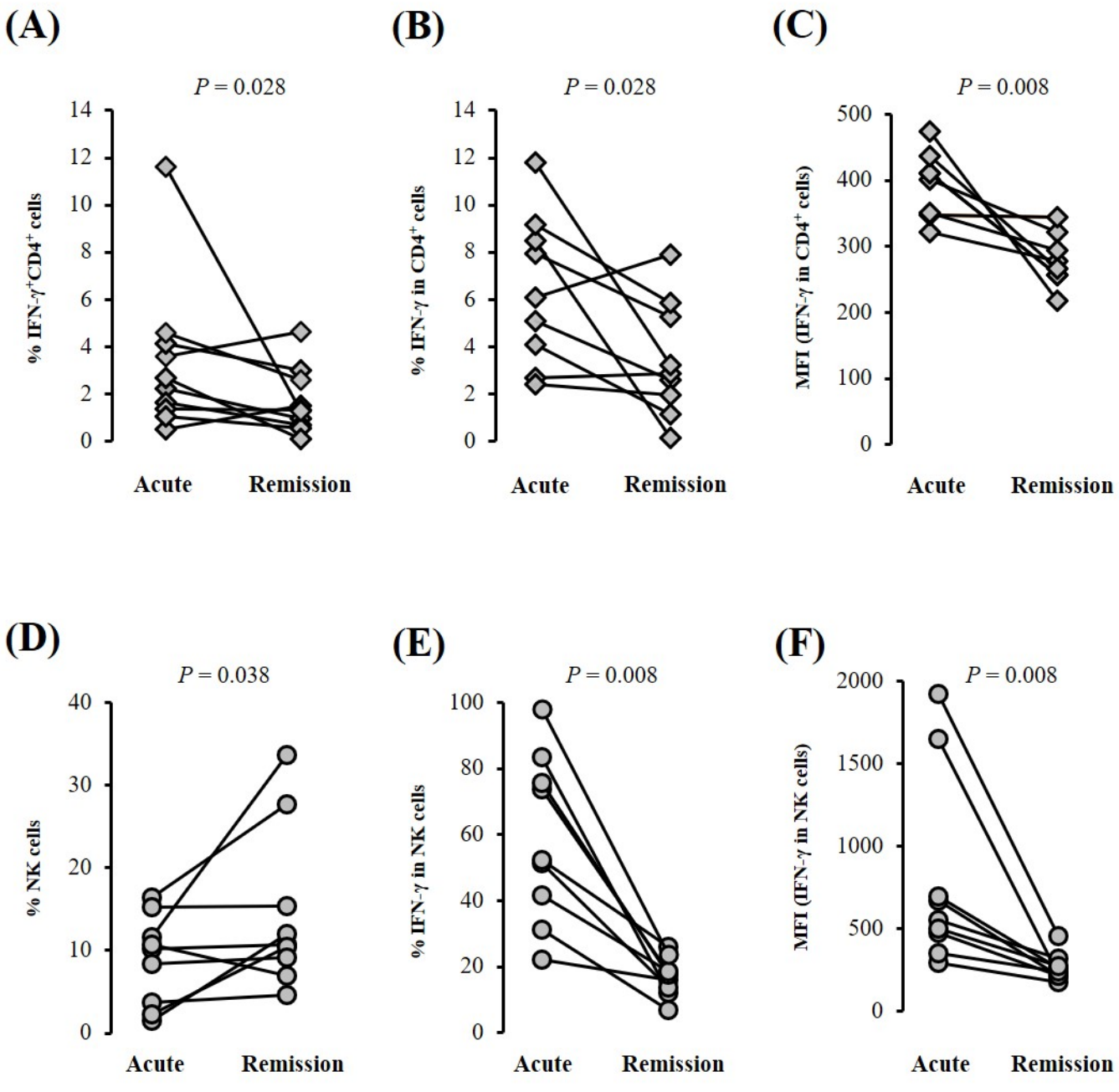
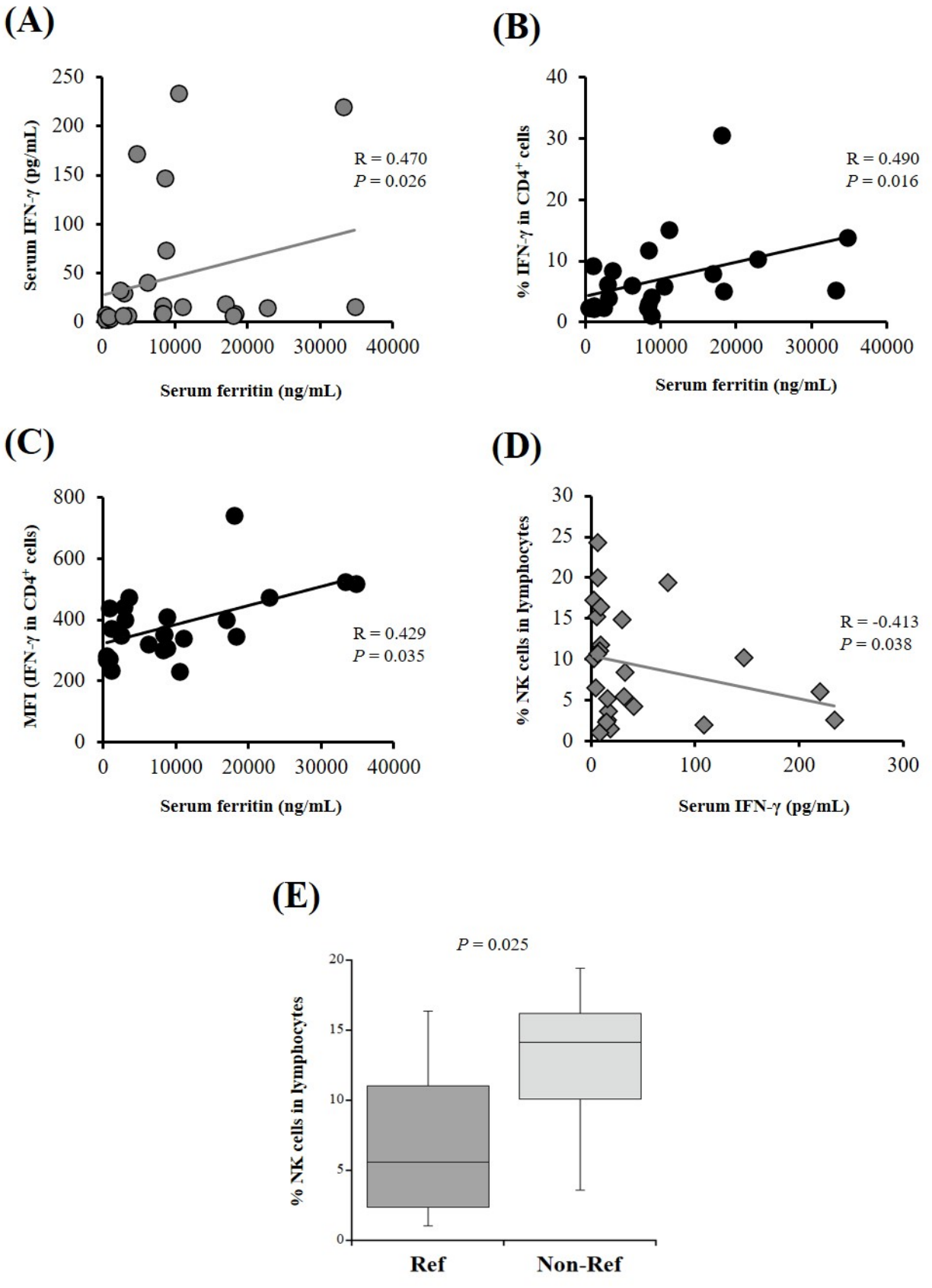
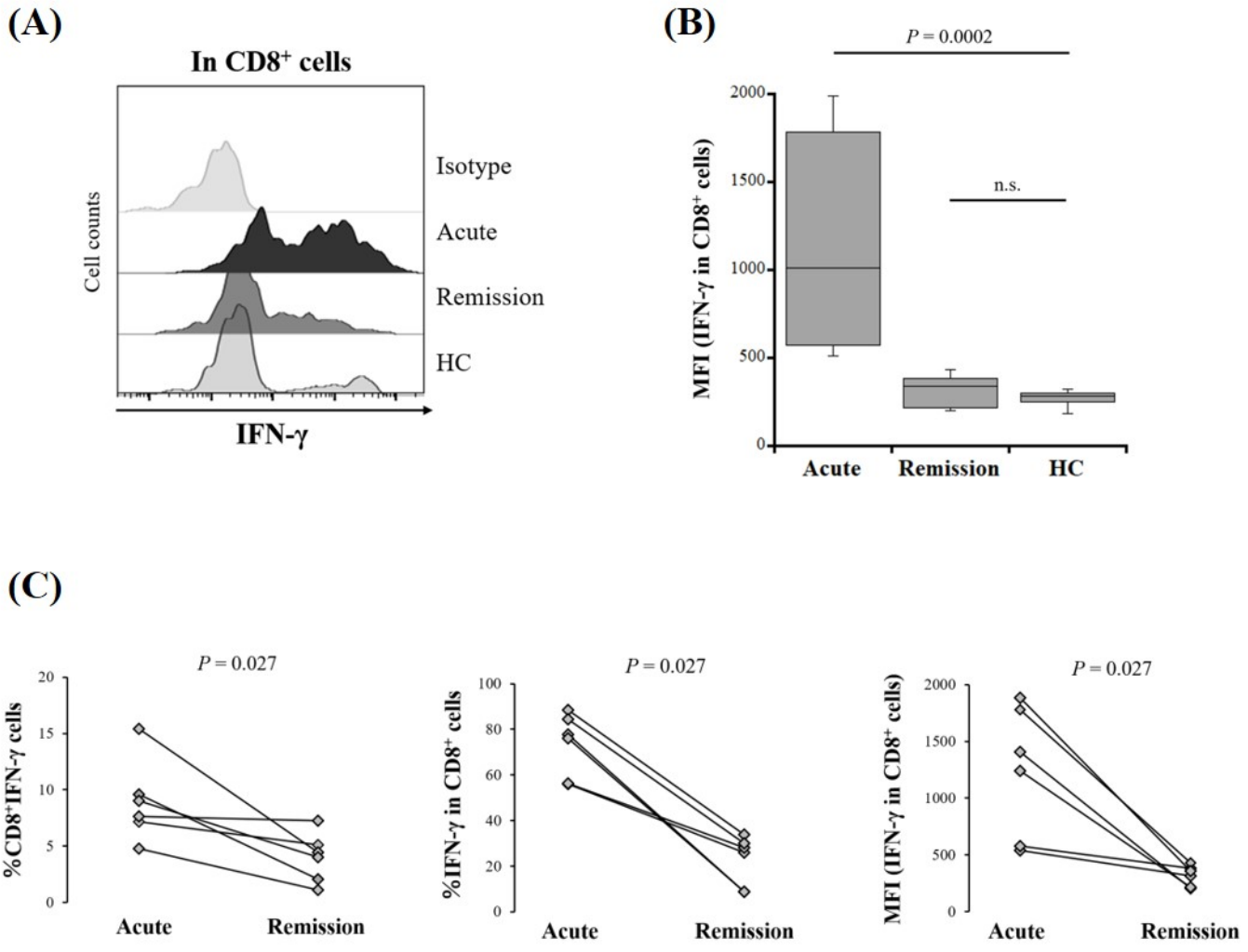

(A)
P = 0.028
% IFN-γ+CD4+ cells
14
12
10
8
6
4
2
0
Acute
Remission
(B)
P = 0.028
% IFN-γ in CD4+ cells
14
12
10
8
6
4
2
0
Acute
Remission
(C)
P = 0.008
MFI (IFN-γ in CD4+ cells)
500
400
300
200
100
0
Acute
Remission
(D)
P = 0.038
% NK cells
40
30
20
10
0
Acute
Remission
(E)
P = 0.008
% IFN-γ in NK cells
100
80
60
40
20
0
Acute
Remission
(F)
P = 0.008
MFI (IFN-γ in NK cells)
2000
1500
1000
500
0
Acute
Remission

Figure 3


(A)
Serum IFN-γ (pg/mL)
250
200
150
100
50
0
0
10000
20000
30000
40000
Serum ferritin (ng/mL)
R = 0.470
P = 0.026
(B)
% IFN-γ in CD4+ cells
40
30
20
10
0
0
10000
20000
30000
40000
Serum ferritin (ng/mL)
R = 0.490
P = 0.016
(C)
MFI (IFN-γ in CD4+ cells)
800
600
400
200
0
0
10000
20000
30000
40000
Serum ferritin (ng/mL)
R = 0.429
P = 0.035
(D)
% NK cells in lymphocytes
30
25
20
15
10
5
0
0
100
200
300
Serum IFN-γ (pg/mL)
R = -0.413
P = 0.038
(E)
P = 0.025
% NK cells in lymphocytes
20
15
10
5
0
Ref
Non-Ref

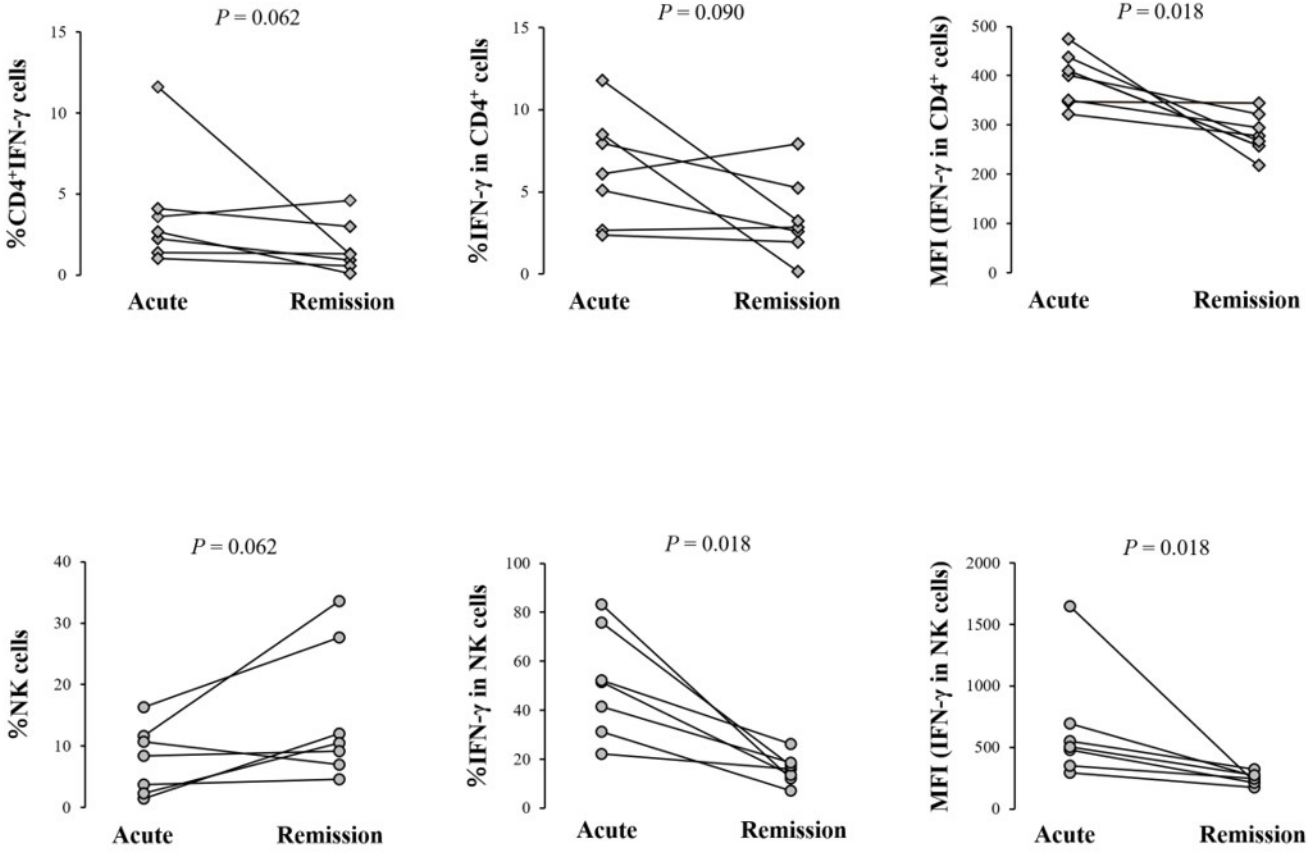
Supplementary Figure 1



Comparisons of intracellular IFN-γ expression in CD8+ cells.

(A) The representative histogram of IFN-γ expression in CD8+ cells. **(B)** Comparison of MFI of IFN-γ in CD8+ cells. **(C)** Alteration of IFN-γ expression in CD8+ cells before and after treatment. Acute, acute AOSD; Remission, remission AOSD; HC, healthy controls; IFN-γ, interferon-γ; MFI, median fluorescence intensity; n.s., not significant.

Supplementary Figure 2



Alteration of IFN-γ-producing cells in refractory patients before and after treatment.

Acute, acute AOSD; Remission, remission AOSD; HC, healthy controls; IFN-γ, interferon-γ; MFI, median fluorescence intensity; NK cells, natural killer cells.