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ORIGINAL ARTICLE

Circulating regulatory T cells in adult-onset Still's disease: Focusing on their plasticity and stability

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Abstract

We investigated the characteristics of regulatory T cells in adult-onset Still's disease (AOSD) with a focus on their plasticity, stability and relationship to disease severity. The proportion of circulating $CD4^+CD25^+$forkhead box protein 3 ($FoxP3^+$) cells (T_{regs}) and intracellular expression of effector cytokines, including interferon (IFN)-γ, interleukin (IL)-17 and IL-4, was analysed in 27 untreated patients with AOSD (acute AOSD), 11 of the 27 patients after remission and 16 healthy controls (HC) using flow cytometry. The suppressive ability of T_{regs} was also evaluated. Regression analyses of the results were performed. The proportion of T_{regs} was significantly lower in patients with acute AOSD than in the HC. The expression levels of IFN-γ, IL-17 and IL-4 in T_{regs} were significantly increased in patients with acute AOSD. IFN-γ and IL-4 expression levels were inversely correlated with the proportion of T_{regs} and positively correlated with serum ferritin levels. Decreased expression of FoxP3 in $CD4^+CD25^+$ cells, which was correlated with increased expression of IL-17, and impaired suppressive function were observed in T_{regs} in acute AOSD. However, these aberrant findings in T_{regs}, including the reduced circulating proportion and functional ability and altered intracellular expression levels of cytokines and FoxP3, were significantly improved after remission. In acute AOSD, T_{regs} show plastic changes, including effector cytokine production and reductions in their proportion and functional activity. IFN-γ and IL-4 expression levels in T_{regs} may be associated with disease severity. Also, down-regulation of FoxP3 may be related to IL-17 expression in T_{regs}. Importantly, the stability of T_{regs} can be restored in remission.

KEYWORDS

adult-onset Still's disease, effector cytokines, helper T cell-related cytokines, plasticity, regulatory T cells

INTRODUCTION

Adult-onset Still's disease (AOSD) is an autoinflammatory disease that is clinically characterized by spike fever, skin rash, sore throat and arthritis with elevated neutrophil counts and serum ferritin levels. Severe complications, such as macrophage activation syndrome (MAS), thrombotic thrombocytopenic purpura or other visceral impairments, sometimes develop [1–3]. In the disease mechanism of AOSD, activation of the innate immune system, including macrophages and neutrophils, is the hallmark of pathogenesis [3]. In the acute phase of AOSD, expression of proinflammatory cytokines is increased, and the relationship between serum proinflammatory cytokine levels and disease activity has been demonstrated [1,2]. Among the proinflammatory cytokines in AOSD, interleukin (IL)-18 was found to be a potential biomarker [4–6], and interferon (IFN)-γ may be associated with the

induction of macrophages and neutrophils during the development of AOSD [7–9]. Our recent study revealed the characteristics of the natural killer cells that produce IFN-γ in AOSD [10]. Polarization of helper T (Th) type 1 cells, which also produce IFN-γ, has also been demonstrated in AOSD [11], implicating acquired immune responses in the development of AOSD. Increased frequency of Th17 cells was found to be associated with disease activity in AOSD [12]. In the acquired immune system, regulatory T cells (T_{regs}), which express CD4, CD25 and the transcription factor forkhead box P3 (FoxP3), play a crucial role in mediating immune tolerance and regulating immune responses [13,14]. To the best of our knowledge, only one study has examined the role of T_{regs} in AOSD, which showed a reduced frequency of $CD4^+CD25^{high}$ cells [15]. However, it has been reported that the plasticity of T_{regs}, i.e. a shift to Th-like cells by effector cytokines, may be promoted in the inflammatory environments of autoimmune disease [16,17]. Accordingly, it is necessary to assess the plasticity and stability of T_{regs} in AOSD, a representative systemic inflammatory disease.

In this study, we investigated the characteristics of circulating T_{regs}, including their proportion, intracellular expression of effector cytokines related to Th cells and suppressive ability in AOSD. In addition, the relationships between T_{regs} and clinical findings were also evaluated.

MATERIAL AND METHODS

Patients

Twenty-seven patients with AOSD who had not yet received immunosuppressive therapy (acute AOSD), with a mean age [standard deviation (SD)] of 53.5 (16.9) years, were enrolled into this study. All patients fulfilled the diagnostic criteria for AOSD proposed by Yamaguchi *et al*. [18]. For the control group, 16 age-matched healthy controls (HC), with a mean age (SD) of 51.5 (11.6) years (seven men and nine women), were included for comparison. Whole blood samples were obtained from all study participants. The clinical findings of patients with acute AOSD (Table 1) were recorded when whole blood samples were provided. The comprehensive disease score (Pouchot score) [19] and the development of macrophage activation syndrome (MAS) [20,21] were also included in the analysis. Eleven of the 27 patients whose Pouchot score was zero after remission were also evaluated as the remission AOSD group. Whole blood samples were collected at a mean (SD) of 33.1 (34.3) months after initiating immunosuppressive therapy. Of the 11 patients in the remission AOSD group, nine were on maintenance therapy, including prednisolone [mean (SD) = 4.4 (4.3) mg daily) (n = 8)], methotrexate (n = 3), cyclosporin (n = 4) and/or tocilizumab (n = 2). The local ethics committee of Shinshu University approved this study (approval no.: 601/4294). All participants provided written informed consent.

Flow cytometry

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood samples, which were collected into ethylene diamine tetraacetic acid (EDTA)-coated tubes by gradient centrifugation with Ficoll-Hypaque PLUS (GE Healthcare, Pittsburgh, Pennsylvania, USA). Surface and intracellular markers in PBMCs were detected by flow cytometric analysis. The population of $CD4^+CD25^+FoxP3^+$ cells was defined as T_{regs}. PBMCs were stimulated with 0.5 μg/ml ionomycin, 0.04 μg/ml phorbol myristate acetate (both from Sigma-Aldrich, St Louis, Missouri, USA) and 2 μM monensin (BD Biosciences, San Diego, California, USA) at 37°C for 4 h. Stimulated PBMCs were stained with phycoerythrin/cyanin 7 (PE/Cy7)-conjugated anti-CD4 (BioLegend, San Diego, California, USA) and PC5-conjugated anti-CD25 (Beckman Coulter, Brea, California, USA) with or without PE-conjugated anti-CD152 [cytotoxic T lymphocyte antigen 4 (CTLA-4] (Beckman Coulter) antibodies. For detecting the population of $CD4^+CD25^+CD127^{-/low}CD45RA^+FoxP3^+$ cells, stimulated PBMCs were alternatively stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD4 (Beckman Coulter), allophycocyanin (APC)-conjugated anti-CD25 (BioLegend), PE/Cy7-conjugated anti-CD127 (BD Biosciences) and PE/Cy5-conjugated anti-CD45RA (BioLegend) antibodies. The stained PBMCs were permeabilized with Cytofix/Cytoperm (BD Biosciences) and then stained with FITC-conjugated anti-IFN-γ (Beckman Coulter), PE-conjugated anti-IL-17 (BD Biosciences), PE-conjugated anti-IL-4 (Beckman Coulter), FITC-conjugated transforming growth factor (TGF)-β1 (BioLegend) or PE-conjugated IL-10 (BioLegend) antibodies, as well as PE-conjugated (BD Biosciences), FITC-conjugated or Pacific blue-conjugated anti-FoxP3 (both BioLegend). The permeabilized PBMCs, which were stained with anti-CD4, anti-CD25, anti-CD127 and anti-CD45RA antibodies, were alternatively stained with PE-conjugated anti-T-bet, anti-RAR-related orphan receptor gamma (RORγt) or anti-GATA binding protein 3 (GATA3) antibody (all from BD Biosciences), as well as Pacific blue-conjugated anti-FoxP3 antibody. To assess the suppressive ability of T_{regs}, suppression assays were performed and evaluated as previously described [22,23]. The $CD4^+CD25^+$ regulatory T cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) was used to isolate

TABLE 1 Epidemiological and clinical findings of patients with AOSD

		Acute AOSD	Remission AOSD	HC	*p* value*
	Number	27	11	16	
	Mean age, years	53.5	—	51.5	0.889
	Sex (M/F)	7/20	—	7/9	0.091
Physical findings, *n* (%)					
	Spike fever (more than 38°C)	27 (100)	—	—	
	Skin eruption	25 (93)	—	—	
	Sore throat	20 (74)	—	—	
	Lymphadenopathy	14 (52)	—	—	
	Arthritis	24 (89)	—	—	
	Myalgia	9 (33)	—	—	
	Pleuritis	5 (19)	—	—	
	Lung involvement	3 (11)	—	—	
	Pericarditis	1 (4)	—	—	
	Hepatomegaly	10 (37)	—	—	
	Splenomegaly	13 (48)	—	—	
	Abdominal pain	2 (7)	—	—	
Laboratory data, mean ± SD					***p*** value**
	White blood cells/μl	17 205 ± 11352	7345 ± 1787	—	0.001
	Neutrophils/μl	13 878 ± 10527	4988 ± 1547	—	<0.0001
	Lymphocytes/μl	971 ± 509	1706 ± 596	—	0.001
	C-reactive protein, mg/dl	11.23 ± 8.16	0.04 ± 0.05	—	<0.0001
	ESR, mm/h	66 ± 36	5.1 ± 2.9	—	0.010
	Ferritin, ng/ml	9618 ± 9545	70 ± 48	—	<0.0001
	AST, U/l	82 ± 87	20 ± 6	—	<0.0001
	ALT, U/l	86 ± 103	20 ± 8	—	0.001
	LDH, U/l	622 ± 435	215 ± 43	—	<0.0001
Activity evaluation					
	Pouchot score, mean ± SD	5.5 ± 1.7	0	—	
	Fulfilled MAS criteria, *n* (%)	8 (30)	—	—	

Abbreviations: ALT, alanine transaminase; AST, aspartate transaminase; ESR, erythrocyte sedimentation rate; HC, healthy controls; MAS, macrophage activation syndrome; SD, standard deviation.

*Comparisons between 27 acute adult-onset Still's disease (AOSD) patients and 16 HC using the Mann–Whitney *U*-test for age and Fisher's exact probability test for sex.; **Comparisons between 27 acute and 16 remission AOSD patients using the Mann–Whitney *U*-test. A *p* value less than 0.05 was considered statistically significant.

conventional T (con-T) cells ($CD4^+CD25^-$ cells) and T_{regs} from unstimulated PBMCs. Allogenic con-T cells labeled with carboxyfluorescein succinimidyl ester (CFSE, 2 μM; Invitrogen, Carlsbad, California, USA) and T_{regs} were co-cultured with anti-CD3/CD28 microbeads (at 1:1:1) at 37°C for 4 days. The proliferation of con-T cells was analysed by the CSFE dilution method, both with and without T_{regs}. Treated cells were acquired on a FACSCanto II flow cytometer (BD Bioscience), and the data were analysed using FlowJo software version 7.6.5 (Tree Star Inc., Ashland, Oregon, USA).

Enzyme-linked immunosorbent assay (ELISA)

ELISA kits were used to measure serum levels of IL-4, IL-6, IL-12, IL-17, IL-21, IFN-γ (R&D Systems, Minneapolis,

MN, USA) and IL-18 (Medical and Biological Laboratories, Nagoya, Japan).

Statistical analysis

All data are presented as mean ± standard deviation (SD). The Mann–Whitney *U*-test and Fisher's exact probability test were used to compare two independent groups. Patient characteristics before and after treatment were compared by Wilcoxon's signed-rank test. The Kruskal–Wallis test was performed for comparisons between three independent groups and the Steel–Dwass test was used for multiple comparisons. A correlation coefficient test was performed to evaluate the significance of relationships. A p value less than 0.05 was defined statistically significant. All statistical analyses were performed using BellCurve for Excel (SSRI, Tokyo, Japan) and JMP (SAS Institute, Cary, North Carolina, USA).

RESULTS

Clinical characteristics of patients with AOSD

Of the 27 AOSD study patients, all (100%) had spike fever, 25 (93%) indicated skin eruption and eight (30%) fulfilled the criteria for MAS (Table 1). The mean Pouchot score was 5.5 ± 1.7 in the acute AOSD group. In the comparisons between three different clinical courses, which are classified into monocyclic, polycyclic and chronic pattern [1,2], serum levels of C-reactive protein at AOSD diagnosis were significantly higher in the polycyclic pattern than the chronic pattern (Supporting information, Table S1). Laboratory findings were significantly improved in the remission AOSD group and were within the normal ranges (Table 1).

Proportion of T_{regs} and intracellular expression of effector cytokines in acute AOSD

The proportion of circulating T_{regs} was significantly lower in the acute AOSD patients than in the HC (5.06 ± 3.92 versus 7.33 ± 3.47, $p = 0.016$) (Figure 1a). This value was significantly increased in the remission AOSD patients (8.90 ± 4.40, $p = 0.004$) (Figure 1b). Intracellular expression of IFN-γ, IL-17 and IL-4 in T_{regs} was significantly higher in the acute AOSD patients than in the HC ($p < 0.0001$) (Figure 2a, Table 2). Expression levels of the aforementioned cytokines were significantly lower in the remission AOSD patients [median fluorescence index (MFI): $p = 0.003$, $p = 0.003$, $p = 0.004$, respectively (Figure 2b) (frequency: $p = 0.004$, $p = 0.003$, $p = 0.003$, respectively] (Table 2). Meanwhile, the proportion of circulating $CD4^+CD25^+CD127^{-/low}CD45RA^+FoxP3^+$ cells was also significantly lower in the acute AOSD patients than in the HC (1.50 ± 0.51 versus 7.13 ± 3.27, $p = 0.0001$), and was significantly increased after remission (9.20 ± 2.50, $p = 0.027$) (Supporting information, Figure S1). Intracellular expression of IFN-γ, IL-17, and IL-4 in $CD4^+CD25^+CD127^{-/low}CD45RA^+FoxP3^+$ cells was also significantly higher in the acute AOSD patients than in the HC ($p = 0.0006$, $p = 0.006$, $p = 0.0006$, respectively), and their expression was significantly decreased after remission ($p = 0.027$) (Supporting information, Figure S2). In addition, intracellular expression of T-bet,

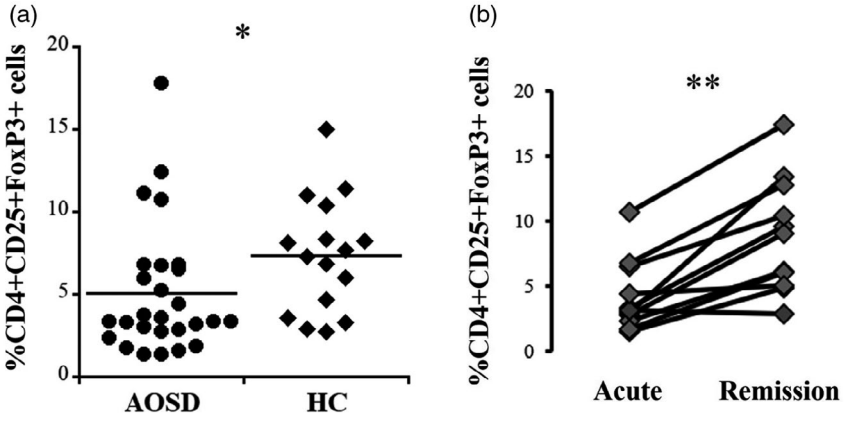

FIGURE 1 Proportions of circulating $CD4^+CD25^+$forkhead box protein 3 ($FoxP3^+$) cells [regulatory T cells (T_{regs})]. (a) Comparison of T_{regs} between patients with acute adult-onset Still's disease (AOSD) and healthy controls (HC). (b) Differences in the proportions of T_{regs} in 11 patients between the acute and remission phases of AOSD. The Mann–Whitney *U*-test was used for comparison between the acute AOSD patients and HC. Wilcoxon's signed-rank test was used for comparison between acute and remission phases in 11 AOSD patients. *$p < 0.05$, **$p < 0.005$

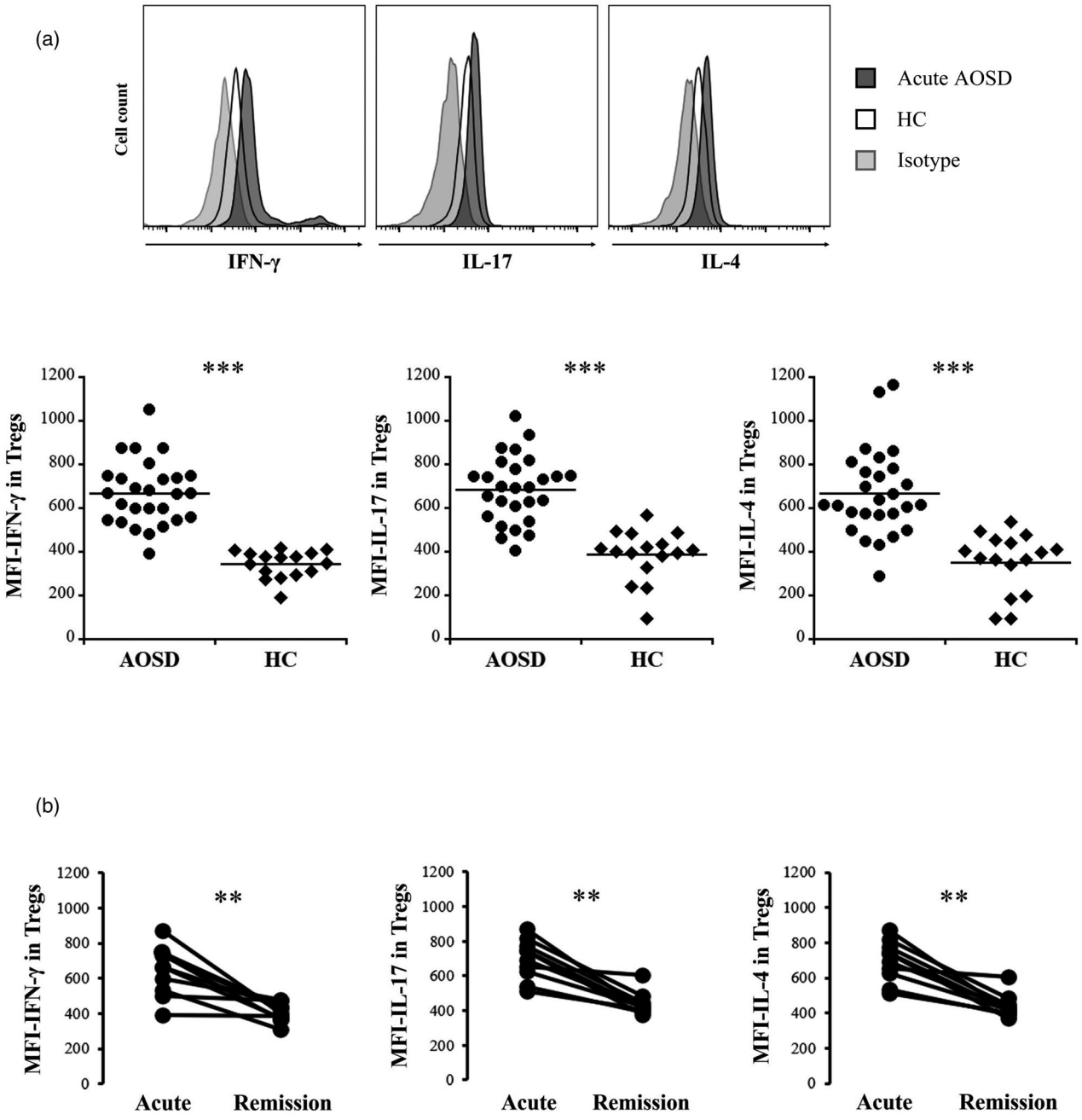

FIGURE 2 Intracellular expression levels of effector cytokines in $CD4^+CD25^+$forkhead box protein 3 ($FoxP3^+$) cells [regulatory T cells (T_{regs})]. (a): (Upper panels) Representative histograms of interferon (IFN)-γ, interleukin (IL)-17 and IL-4 expression in T_{regs} (lower bar graphs). Comparisons of the median fluorescence index (MFI) of IFN-γ, IL-17 and IL-4 in T_{regs} between patients with acute adult-onset Still's disease (AOSD) and HC. (b) Differences in the MFI of IFN-γ, IL-17 and IL-4 in T_{regs} from 11 patients between acute and remission phases. The Mann–Whitney *U*-test was used for comparisons between the acute AOSD patients and the HC. The Wilcoxon signed-rank test was used for comparisons between acute and remission phases in 11 AOSD patients. $^{**}p < 0.005$, $^{***}p < 0.0001$

RORγt and GATA3, which are T helper type (Th)1, Th17 and Th2-related transcription factors, respectively, in $CD4^+CD25^+CD127^{-/low}CD45RA^+FoxP3^+$ cells, were also significantly higher in the acute AOSD patients than in the HC ($p = 0.0001$, $p = 0.0001$, $p = 0.0006$, respectively), and their expression was significantly decreased after remission ($p = 0.027$) (Supporting information, Figure S3).

Serum levels of Th cell-related cytokines and their relationships with T_{reg} plasticity in AOSD

Serum levels of IL-4, IL-6, IL-12, IL-17, IL-18, IL-21 and IFN-γ were significantly higher in the acute AOSD patients than in the HC (Table 3). Comparisons of the acute

TABLE 2 Frequencies of intracellular cytokines in T_{regs} in patients with acute AOSD, remission AOSD and healthy controls

		Acute AOSD ($n = 27$)	Remission AOSD ($n = 11$)	HC ($n = 16$)	*p* value */**
In $CD4^+CD25^+FoxP3^+$ cells					
	%IFN-γ	24.18 ± 14.92	5.20 ± 9.61	4.33 ± 3.64	0.004/<0.0001
	%IL-17	23.58 ± 21.15	1.20 ± 1.36	1.84 ± 2.19	0.003/<0.0001
	%IL-4	26.97 ± 23.27	0.78 ± 1.41	2.04 ± 2.15	0.003/<0.0001

Note: Data are presented as mean ± standard deviation (SD).

Abbreviations: FoxP3, forkhead box protein 3; HC, healthy controls; IFN-γ, interferon-γ; IL, interleukin; T_{regs}, regulatory T cells.

*Comparisons between acute and remission phases in 11 acute adult-onset Still's disease (AOSD) patients using Wilcoxon's signed-rank test.; **Comparisons between 27 acute AOSD patients and 16 HC using the Mann–Whitney *U*-test. A *p* value less than 0.05 was considered statistically significant.

TABLE 3 Serum cytokine levels in patients with acute and remission AOSD and healthy controls

Cytokines	Acute AOSD ($n = 27$)	Remission AOSD ($n = 11$)	HC ($n = 16$)	*p* value */**
IL-4 (pg/ml)	13.1 ± 6.1	8.7 ± 2.1	8.5 ± 2.0	0.102/0.033
IL-6 (pg/ml)	109.7 ± 139.4	11.7 ± 20.3	1.7 ± 0.6	0.005/<0.0001
IL-12 (pg/ml)	1.5 ± 0.5	1.8 ± 1.0	0.7 ± 0.2	0.726/<0.0001
IL-17 (pg/ml)	32.3 ± 17.6	23.3 ± 2.8	21.5 ± 4.1	0.368/0.025
IL-18 (pg/ml)	2039.4 ± 779.6	351.6 ± 286.6	72.0 ± 11.1	<0.0001/<0.0001
IL-21 (pg/ml)	26.6 ± 41.2	8.4 ± 1.5	9.2 ± 1.8	<0.0001/<0.0001
IFN-γ (pg/ml)	75.3 ± 167.3	3.3 ± 2.0	2.3 ± 3.2	<0.0001/<0.0001

Note: Data are presented as the mean ± standard deviation (SD).

Abbreviations: HC, healthy controls; IL, interleukin; IFN-γ, interferon-γ.

*Comparisons between acute and remission phases in 11 acute adult-onset Still's disease (AOSD) patients using Wilcoxon's signed-rank test.; **Comparisons between 27 acute AOSD patients and 16 HC using the Mann–Whitney *U*-test. A *p* value less than 0.05 was considered statistically significant.

and remission AOSD patients showed no significant differences in serum levels of IL-4, IL-12 or IL-17, despite significant reductions in the levels of the other evaluated cytokines. However, serum levels of these cytokines were not significantly correlated with the proportion of circulating T_{regs} or the intracellular expression levels of IFN-γ, IL-17 and IL-4 in T_{regs} (data not shown).

Correlations among circulating T_{regs}, intracellular effector cytokine expression and disease severity in acute AOSD

Correlation analyses of circulating T_{regs} showed that the proportion of T_{regs} was inversely correlated with the MFI of IFN-γ and IL-4 in T_{regs} in the acute AOSD patients, but was not correlated with that of IL-17 (Table 4). Frequencies of IFN-γ, IL-17 and IL-4 were inversely correlated with the proportion of T_{regs}. Moreover, the MFI and frequencies of IFN-γ and IL-4 in T_{regs} were correlated with serum ferritin levels in the acute AOSD patients, whereas those of IL-17 showed no correlation. There was no significant correlation between the proportion of T_{regs} and serum ferritin levels (data not shown).

Suppressive ability of T_{regs} in AOSD

To evaluate the suppressive ability of T_{regs}, the proliferation of con-T target cells was evaluated with and without T_{regs} (Figure 3a,b). The proliferation of con-T cells was significantly lower in the presence of T_{regs} from HC than in the absence of T_{regs} ($p = 0.001$). The proliferation of con-T cells did not differ significantly in the presence of T_{regs} from acute AOSD patients or in the absence of T_{regs} ($p = 0.193$); however, proliferation was significantly higher in the presence of T_{regs} from acute AOSD patients

TABLE 4 Correlations between intracellular cytokine levels and the proportions of T_{regs} or serum ferritin in patients with acute AOSD

		Versus %$CD4^+CD25^+FoxP3^+$ cells		Versus serum ferritin levels	
		Coefficient	*p* value	Coefficient	*p* value
MFI in $CD4^+CD25^+FoxP3^+$ cells					
	MFI^-IFN-γ	−0.421	0.029	0.433	0.027
	MFI^-IL-17	−0.324	0.099	0.156	0.425
	MFI^-IL-4	−0.449	0.019	0.413	0.035
% Frequency in $CD4^+CD25^+FoxP3^+$ cells					
	% IFN-γ	−0.499	0.008	0.390	0.047
	% IL-17	−0.547	0.003	0.184	0.349
	% IL-4	−0.680	0.0005	0.435	0.030

Note: A *p* value less than 0.05 was considered statistically significant.

Abbreviations: AOSD, acute adult-onset Still's disease; Coefficient, correlation coefficient; FoxP3, forkhead box protein 3; IL, interleukin; IFN-γ, interferon-γ; MFI, median fluorescence index.

than in the presence of T_{regs} from HC ($p = 0.003$), indicating abrogated suppressive function of T_{regs} in acute AOSD. The suppressive ability of T_{regs} from remission AOSD patients was significantly improved ($p = 0.043$) (Figure 3c). The expression levels of representative suppressive mediators, including TGF-β1, IL-10 and CTLA-4 in T_{regs} from acute AOSD patients were not significantly different than those in T_{regs} from HC ($p = 0.845$, $p = 0.220$, $p = 0.259$, respectively) (Figure 4a). In contrast, the MFI and frequencies of FoxP3 in $CD4^+CD25^+$ cells were significantly lower in the acute AOSD patients than in the HC ($p < 0.0001$) (Figure 4b, Table 5) and were significantly increased after remission ($p = 0.004$, $p = 0.013$, respectively) (Figure 4c, Table 5). In $CD4^+CD25^+CD127^{-/low}CD45RA^+$ cells, the MFI and frequency of FoxP3 were also significantly lower in the acute AOSD patients than in the HC ($p = 0.0002$, $p = 0.0001$, respectively), and were significantly increased after remission ($p = 0.027$) (Supporting information, Figure S4). The MFI of FoxP3 was inversely correlated with the MFI and frequency of IL-17 in T_{regs} (Figure 4d), but was not correlated with those of either IFN-γ or IL-4 (data not shown).

The relationship with clinical findings and clinical course

We investigated the relationship between the obtained results and clinical findings shown in Table 1. The expression of IL-17 in T_{regs} was significantly higher in patients with sore throat than in those without ($p = 0.015$) (Supporting information, Figure S5). The expression of IL-4 in T_{regs} was significantly higher in patients with pleuritis than in those without ($p = 0.005$). In accordance with previous investigation in which a cut-off at 7.0 of the Pouchot score has an impact on the prognosis [24], we additionally compared the experimental results between patients showing more and less than this score, resulting in no significant differences (data not shown). Any experimental results had no significant differences between monocyclic, polycyclic and chronic pattern (data not shown).

DISCUSSION

In our first attempt at studying AOSD, we investigated the characteristics of circulating T_{regs} in AOSD, with a focus on their plasticity and stability. Previous investigations of T_{regs} in autoimmune diseases, such as rheumatoid arthritis, systemic lupus erythematous, systemic vasculitis and multiple sclerosis, inconsistently showed decreased and increased expression; however, defective T_{reg} function was largely a consistent result [17,22,25,26]. Our study demonstrated a decreased proportion of T_{regs} and dysfunction in acute AOSD patients as significant results. However, an increase in the proportion of T_{regs} and improved suppressive activity were both observed in remission AOSD patients, suggesting that the T_{reg} disorder in AOSD may be reversible.

Intracellular expression of the effector cytokines IFN-γ, IL-17 and IL-4 in the population of $CD4^+CD25^+FoxP3^+$ cells was significantly higher in patients with acute AOSD. T_{regs} may include memory/effector phenotypes in the environment of chronic inflammation and/or in the process of immune-aging [27,28]. Meanwhile, subsets of $CD127^{-/low}$ and $CD45RA^+$ are found to be phenotypical markers for

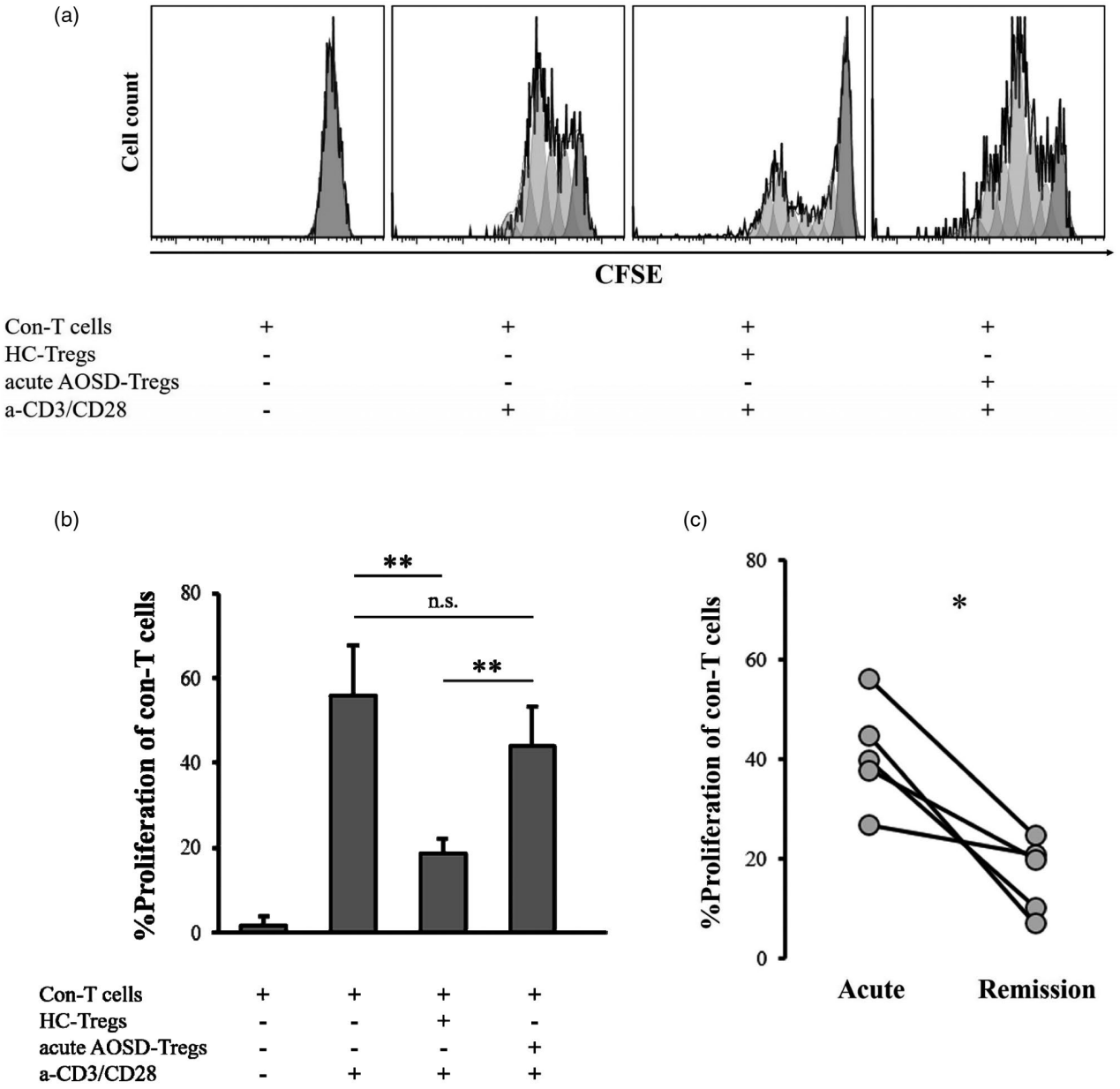

FIGURE 3 Suppression assay of regulatory T cells [regulatory T cells (T_{regs})]. (a) Representative histograms showing the proliferation of conventional T cells (con-T cells) with and without T_{regs}. (b) Comparisons of proliferative frequency of con-T cells without T_{regs} ($n = 9$) and with T_{regs} from either acute adult-onset Still's disease (AOSD) patients ($n = 8$) or healthy controls (HC; $n = 9$). (c) Differences in the proliferative frequency of con-T cells in the presence of T_{regs} from five patients collected in the acute and remission phases ($n = 5$). CFSE = carboxyfluorescein diacetate succinimidyl ester; HC-T_{regs} = T_{regs} from HC; acute AOSD-T_{regs} = T_{regs} from patients with acute AOSD; a-CD3/CD28 = anti-CD3/CD28 microbeads; NS = not significant. The Kruskal–Wallis and Steel–Dwass tests were used for comparisons of proliferative frequency of con-T cells between three independent groups. Wilcoxon's signed-rank test was used for comparisons between acute and remission phases in five AOSD patients. *$p < 0.05$, **$p < 0.005$

certifying the homeostasis of T_{regs} in healthy individuals [29,30]. The proportion of $CD4^+CD25^+CD127^{-/low}CD45RA^+FoxP3^+$ cells was significantly lower than that of $CD4^+CD25^+FoxP3^+$ cells in the acute AOSD patients, suggesting that conversion to effector phenotypes might be shown even in the population expressing FoxP3. However, expression of IFN-γ, IL-17, IL-4 and their transcription factors in $CD4^+CD25^+CD127^{-/low}CD45RA^+FoxP3^+$ cells was significantly higher in the acute AOSD patients than the HC, demonstrating that the phenotypical changes may be the pivotal features of T_{regs} in the acute phase of AOSD. The plasticity of T_{regs} includes Th-like changes, characterized by the expression of effector cytokines in the conventional phenotype of T_{regs} that express FoxP3 [16,17,31,32], ultimately resulting in an imbalance in immune tolerance due to insufficient intervention by T_{regs}

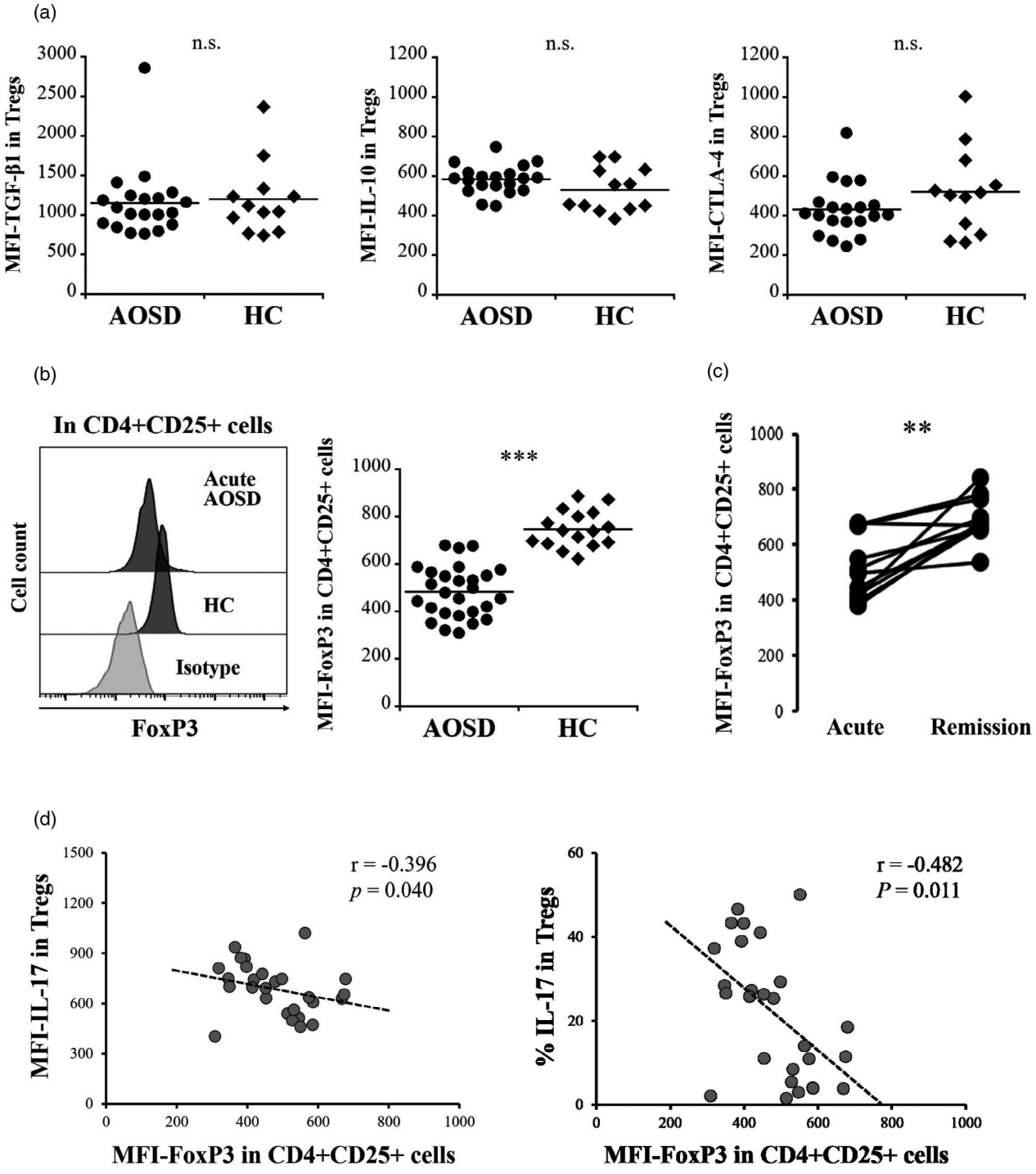

FIGURE 4 Expression of functional mediators in regulatory T cells (T_{regs}). (a) Comparisons of median fluorescence index (MFI) of transforming growth factor (TGF)-β, interleukin (IL)-10, and cytotoxic T lymphocyte antigen (CTLA)-4 in $CD4^+CD25^+$forkhead box protein 3 ($FoxP3^+$) cells collected from patients with acute acute adult-onset Still's disease (AOSD) and healthy controls (HC). (b) (Left panel) Representative histograms of FoxP3 expression in $CD4^+CD25^+$ cells from patients with AOSD and HC (right bar graph). Comparison of the MFI for FoxP3 in $CD4^+CD25^+$ cells from patients with acute AOSD and HC. (c) Differences in the MFI of FoxP3 in $CD4^+CD25^+$ cells from 11 patients with AOSD collected in the acute and remission phases. (d) Regression analyses of MFI and frequency of IL-17 in MFI^-FoxP3 in $CD4^+CD25^+$ cells from patients with acute AOSD. The Mann–Whitney *U*-test was used for comparisons between the acute AOSD patients and the HC. Wilcoxon's signed-rank test was used for comparisons between acute and remission phases in 11 AOSD patients. $^{**}p < 0.005$, $^{***}p < 0.0001$; NS = not significant

TABLE 5 Frequency of FoxP3 in $CD4^+CD25^+$ cells in patients with acute AOSD, remission AOSD and healthy controls

		Acute AOSD ($n = 27$)	Remission AOSD ($n = 11$)	HC ($n = 16$)	*p* value */**
In total lymphocytes					
	% $CD4^+CD25^+$ cells	45.75 ± 15.48	43.40 ± 10.89	40.14 ± 18.02	0.374/0.218
In $CD4^+CD25^+$ cells					
	% FoxP3	8.43 ± 7.83	18.73 ± 8.48	25.24 ± 9.62	0.013/<0.0001

Note: Data are presented as the mean ± standard deviation (SD).

Abbreviations: HC, healthy controls; FoxP3, forkhead box protein 3.

*Comparisons between acute and remission phases in 11 acute adult-onset Still's disease (AOSD) patients by using Wilcoxon's signed-rank test.;

**Comparisons between 27 acute AOSD patients and 16 HC using the Mann–Whitney *U*-test. A *p* value less than 0.05 was considered statistically significant.

[16,33]. We have two hypotheses to explain the impaired homeostasis of T_{regs} in AOSD. First, the decrease in the proportion of circulating T_{regs} might be attributed to intracellular induction of effector cytokines. Notably, IFN-γ and IL-4 expression levels in T_{regs} were associated with the reduction in circulating T_{regs}. Although no significant correlation was found between the MFI of IL-17 in T_{regs} and the proportion of T_{regs} in this study, plastic alteration of T_{regs} into Th17-like cells has also been shown in some autoimmune diseases [34,35]. Secondly, the increased intracellular expression of IFN-γ, IL-17 and IL-4 might impair the suppressive function of T_{regs}. In fact, both proportion and functional capacity of T_{regs} recovered after remission with a significant reduction in intracellular cytokine expression. However, in this study there were no significant deficiencies in TGF-β1, IL-10 or CTLA-4, which are mediators of the suppressive activity of T_{regs} [14,25]. Our result demonstrated significantly decreased FoxP3 expression in $CD4^+CD25^+$ cells in patients with acute AOSD. Moreover, this was related to increased intracellular IL-17 expression in T_{regs}. Conventional T_{regs} express FoxP3 as a pivotal regulator preventing extreme immune responses [36,37]; T_{regs} down-regulate FoxP3 expression in response to inflammatory signals [38–40]. It has been shown that intracellular induction of IL-17 in T_{regs} leads to the down-regulation of FoxP3 expression [39,41]. Therefore, we assumed that the T_{regs} in patients with acute AOSD could be converted into not only Th-like T_{regs} that retain FoxP3 expression but also Th phenotypes that lose FoxP3 expression, resulting in a decreased proportion of circulating T_{regs}. In addition, intracellular expression of IL-17 might contribute to the loss of FoxP3 in T_{regs}, leading to impaired function. In contrast, it has also been reported that Th-like T_{regs} which retain FoxP3 have less suppressive activity than conventional T_{regs}, although their suppressive capacity is not abolished [16,31]. Under our experimental conditions, $CD4^+CD25^+$ cells, which were isolated using a commercially available magnetic isolation kit, were defined as T_{regs} and their suppressive activity was evaluated. Therefore, the isolated T_{regs} might include cell populations with down-regulated FoxP3 levels and Th-like $FoxP3^+$ populations, suggesting that both populations have defective suppressive activity.

Some studies have shown elevated serum levels of IFN-γ or IL-17 as well as an increase in the proportion of circulating Th1 or Th17 cells in AOSD [8,11,12,42]. Increased serum IL-4 levels were also shown to be significantly correlated with disease activity [43,44]. Accordingly, Th1, Th2 and Th17-related cytokines may be strongly implicated in the pathogenesis of AOSD. Based on the results of previous investigations, plastic alteration of T_{regs} is promoted by exposure to Th cell-related inflammatory cytokines [16,17,32,45–48]. However, our results showed that serum levels of these cytokines were not significantly correlated with the intracellular expression levels of IFN-γ, IL-17 or IL-4 in T_{regs}. In contrast, IFN-γ and IL-4 expression levels in T_{regs} were associated with serum levels of ferritin, which is a biomarker commonly used for evaluating disease severity in AOSD [2,3,49,50]. Hence, it was suggested that the plastic change of T_{regs} especially expressing IFN-γ or IL-4 may be significantly associated with disease severity in AOSD.

There are some limitations in this study. Our results statistically demonstrated low correlation coefficients in the regression analyses of intracellular cytokines in T_{regs}, suggesting that their correlations with FoxP3 expression, the proportion of T_{regs} and serum ferritin levels are very weak. Therefore, Th-like changes in T_{regs} may have limited implications for the variation of T_{regs} and disease severity in AOSD. Also, it has been shown that ethnic and genetic variations may affect the immunity of AOSD [1–3] as well as the development of T_{regs} [51,52], although a small number of Japanese patients were employed in this study.

In conclusion, in acute AOSD, circulating T_{regs}, which showed increases in the intracellular expression levels of IFN-γ, IL-17 and IL-4, were significantly reduced in both proportion and suppressive activity. The expression

levels of IFN-γ and IL-4 in T_{regs} might be implicated in a decreased proportion of T_{regs} and elevated serum levels of ferritin. In addition, decreased expression of FoxP3 in $CD4^+CD25^+$ cells was significantly shown in acute AOSD, and might be associated with IL-17 expression in T_{regs}. These findings were significantly improved in patients with remission AOSD, suggesting that the Th-like shift and functional impairment of T_{regs} are reversible. Disease activity may affect the stability of T_{regs} in AOSD. However, numerous immunopathogenic mechanisms are involved in the development of AOSD, and the immune response of T_{regs} can be affected by broad immune system interactions. Thus, further investigations on the immune signals affecting the plasticity of T_{regs} in AOSD are required.

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CONFLICTS OF INTEREST

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

AUTHOR CONTRIBUTIONS

All authors designed this study, developed the structure and argument for this study. Y.-S., T.-I., D.-K. and R.-T. recruited blood samples and clinical data. Y.-S. performed laboratory investigations and analyzed obtained data and prepared the draft of this manuscript. Y.-S. and Y.-S. contributed to revision of the manuscript. All authors revised and approved the final manuscript.

DATA AVAILABILITY STATEMENT

The data for the analyses in this study are available from the corresponding author upon reasonable request.

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REFERENCES

1. Giacomelli R, Ruscitti P, Shoenfeld Y. A comprehensive review on adult onset Still's disease. J Autoimmun. 2018;93:24–36.
2. Feist E, Mitrovic S, Fautrel B. Mechanisms, biomarkers and targets for adult-onset Still's disease. Nat Rev Rheumatol. 2018;14:603–18.
3. Gerfaud-Valentin M, Jamilloux Y, Iwaz J, Seve P. Adult-onset Still's disease. Autoimmun Rev. 2014;13:708–22.
4. 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.
5. Priori R, Colafrancesco S, Alessandri C, Minniti A, Perricone C, Iaiani G, et al. Interleukin 18: a biomarker for differential diagnosis between adult-onset Still's disease and sepsis. J Rheumatol. 2014;41:1118–23.
6. 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.
7. Billiau A, Matthys P. Interferon-gamma: a historical perspective. Cytokine Growth Factor Rev. 2009;20:97–113.
8. Han JH, Suh CH, Jung JY, Ahn MH, Han MH, Kwon JE, et al. Elevated circulating levels of the interferon-γ-induced chemokines are associated with disease activity and cutaneous manifestations in adult-onset Still's disease. Sci Rep. 2017;7:46652.
9. 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.
10. Shimojima Y, Kishida D, Ueno KI, Ushiyama S, Ichikawa T, Sekijima Y. Characteristics of circulating natural killer cells and their interferon-γ production in active adult-onset Still disease. J Rheumatol. 2019;46:1268–76.
11. Chen DY, Lan JL, Lin FJ, Hsieh TY, Wen MC. Predominance of Th1 cytokine in peripheral blood and pathological tissues of patients with active untreated adult onset Still's disease. Ann Rheum Dis. 2004;63:1300–6.
12. Chen DY, Chen YM, Lan JL, Lin CC, Chen HH, Hsieh CW. Potential role of Th17 cells in the pathogenesis of adult-onset Still's disease. Rheumatology. 2010;49:2305–12.
13. Lan Q, Fan H, Quesniaux V, Ryffel B, Liu Z, Zheng SG. Induced Foxp3(+) regulatory T cells: a potential new weapon to treat autoimmune and inflammatory diseases? J Mol Cell Biol. 2012;4:22–8.
14. Sakaguchi S, Yamaguchi T, Nomura T, Ono M. Regulatory T cells and immune tolerance. Cell. 2008;133:775–87.
15. Chen DY, Chen YM, Chen HH, Hsieh CW, Lin CC, Lan JL. The associations of circulating $CD4^+$CD25high regulatory T cells and TGF-β with disease activity and clinical course in patients with adult-onset Still's disease. Connect Tissue Res. 2010;51:370–7.
16. Qiu R, Zhou L, Ma Y, Zhou L, Liang T, Shi L, et al. Regulatory T cell plasticity and stability and autoimmune diseases. Clin Rev Allergy Immunol. 2020;58:52–70.
17. Zhang X, Olsen N, Zheng SG. The progress and prospect of regulatory T cells in autoimmune diseases. J Autoimmun. 2020;111:102461.
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. 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.
20. Ahn SS, Yoo BW, Jung SM, Lee SW, Park YB, Song JJ. Application of the 2016 EULAR/ACR/PRINTO classification criteria for macrophage activation syndrome in patients with adult-onset Still disease. J Rheumatol. 2017;44:996–1003.
21. 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.
22. Shimojima Y, Ishii W, Kishida D, Fukushima K, Ikeda SI. Imbalanced expression of dysfunctional regulatory T cells and T-helper cells relates to immunopathogenesis in polyarteritis nodosa. Mod Rheumatol. 2017;27:102–9.
23. Wen Z, Shimojima Y, Shirai T, Li Y, Ju J, Yang Z, et al. NADPH oxidase deficiency underlies dysfunction of aged $CD8^+$ Tregs. J Clin Invest. 2016;126:1953–67.
24. Ruscitti P, Cipriani P, Masedu F, Iacono D, Ciccia F, Liakouli V, et al. Adult-onset Still's disease: evaluation of prognostic tools and validation of the systemic score by analysis of 100 cases from three centers. BMC Med. 2016;14:194.
25. Miyara M, Gorochov G, Ehrenstein M, Musset L, Sakaguchi S, Amoura Z. Human $FoxP3^+$ regulatory T cells in systemic autoimmune diseases. Autoimmun Rev. 2011;10:744–55.
26. Morgan MD, Day CJ, Piper KP, Khan N, Harper L, Moss PA, et al. Patients with Wegener's granulomatosis demonstrate a relative deficiency and functional impairment of T-regulatory cells. Immunology. 2010;130:64–73.
27. Jagger A, Shimojima Y, Goronzy JJ, Weyand CM. Regulatory T cells and the immune aging process: a mini-review. Gerontology. 2014;60:130–7.
28. Campbell DJ, Koch MA. Phenotypical and functional specialization of $FOXP3^+$ regulatory T cells. Nat Rev Immunol. 2011;11:119–30.
29. Schmidt A, Oberle N, Krammer PH. Molecular mechanisms of Treg-mediated T cell suppression. Front Immunol. 2012;3:51.
30. Rodríguez-Perea AL, Arcia ED, Rueda CM, Velilla PA. Phenotypical characterization of regulatory T cells in humans and rodents. Clin Exp Immunol. 2016;185:281–91.
31. Hori S. Lineage stability and phenotypic plasticity of $Foxp3^+$ regulatory T cells. Immunol Rev. 2014;259:159–72.
32. Brown CY, Sadlon T, Hope CM, Wong YY, Wong S, Liu N, et al. Molecular insights into regulatory T-cell adaptation to self, environment, and host tissues: plasticity or loss of function in autoimmune disease. Front Immunol. 2020;11:1269.
33. Frangogiannis NG. Protean functions and phenotypic plasticity of regulatory T cells in chronic ischemic heart failure. Circulation. 2019;139:222–5.
34. Noack M, Miossec P. Th17 and regulatory T cell balance in autoimmune and inflammatory diseases. Autoimmun Rev. 2014;13:668–77.
35. Komatsu N, Okamoto K, Sawa S, Nakashima T, Oh-hora M, Kodama T, et al. Pathogenic conversion of $Foxp3^+$ T cells into TH17 cells in autoimmune arthritis. Nat Med. 2014;20:62–8.
36. Wan YY, Flavell RA. Regulatory T-cell functions are subverted and converted owing to attenuated Foxp3 expression. Nature. 2007;445:766–70.
37. Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of $CD4^+CD25^+$ regulatory T cells. Nat Immunol. 2003;4:330–6.
38. Duarte JH, Zelenay S, Bergman ML, Martins AC, Demengeot J. Natural Treg cells spontaneously differentiate into pathogenic helper cells in lymphopenic conditions. Eur J Immunol. 2009;39:948–55.
39. Yurchenko E, Shio MT, Huang TC, Da Silva MM, Szyf M, Levings MK, et al. Inflammation-driven reprogramming of $CD4^+$ $Foxp3^+$ regulatory T cells into pathogenic Th1/Th17 T effectors is abrogated by mTOR inhibition *in vivo*. PLOS ONE. 2012;7:e35572.
40. Hoffmann P, Boeld TJ, Eder R, Huehn J, Floess S, Wieczorek G, et al. Loss of FOXP3 expression in natural human $CD4^+CD25^+$ regulatory T cells upon repetitive in vitro stimulation. Eur J Immunol. 2009;39:1088–97.
41. Yang XO, Nurieva R, Martinez GJ, Kang HS, Chung Y, Pappu BP, et al. Molecular antagonism and plasticity of regulatory and inflammatory T cell programs. Immunity. 2008;29:44–56.
42. 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.
43. Saiki O, Uda H, Nishimoto N, Miwa T, Mima T, Ogawara T, et al. Adult Still's disease reflects a Th2 rather than a Th1 cytokine profile. Clin Immunol. 2004;112:120–5.
44. Fujii T, Nojima T, Yasuoka H, Satoh S, Nakamura K, Kuwana M, et al. Cytokine and immunogenetic profiles in Japanese patients with adult Still's disease. Association with chronic articular disease. Rheumatology. 2001;40:1398–404.
45. Dominguez-Villar M, Baecher-Allan CM, Hafler DA. Identification of T helper type 1-like, $Foxp3^+$ regulatory T cells in human autoimmune disease. Nat Med. 2011;17:673–5.
46. Noval Rivas M, Burton OT, Wise P, Charbonnier LM, Georgiev P, Oettgen HC, et al. Regulatory T cell reprogramming toward a Th2-cell-like lineage impairs oral tolerance and promotes food allergy. Immunity. 2015;42:512–23.
47. Massoud AH, Charbonnier LM, Lopez D, Pellegrini M, Phipatanakul W, Chatila TA. An asthma-associated IL4R variant exacerbates airway inflammation by promoting conversion of regulatory T cells to TH17-like cells. Nat Med. 2016;22:1013–22.
48. Koenen HJ, Smeets RL, Vink PM, van Rijssen E, Boots AM, Joosten I. Human CD25highFoxp3pos regulatory T cells differentiate into IL-17-producing cells. Blood. 2008;112:2340–52.
49. 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.
50. 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.
51. Arvey A, van der Veeken J, Plitas G, Rich SS, Concannon P, Rudensky AY. Genetic and epigenetic variation in the lineage specification of regulatory T cells. eLife. 2015;4:e07571.
52. Huehn J, Beyer M. Epigenetic and transcriptional control of $Foxp3^+$ regulatory T cells. Semin Immunol. 2015;27:10–8.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

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