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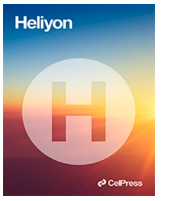

Research article

Altered subgroups of regulatory T cells in patients with primary Sjögren's syndrome

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ABSTRACT

Primary Sjögren syndrome (pSS) is a systemic autoimmune inflammatory disease. Up to now, the role of regulatory T cells (Tregs) and their subgroups in pSS is still in controversial. In this study we tried to elucidate the roles of Tregs and its subgroups in pSS. Total 43 pSS patients and 23 health persons as control were enrolled in this study. We grouped the pSS patients according to the anti-SSa/SSb and the EULAR Sjögren's syndrome disease activity index (ESSDAI). Among the 43 pSS patients, 14 patients were followed after treatment. The percentage of rTregs (resting Treg cells) among Tregs was increased in the pSS group, and decreased after treatment. In the high disease activity subpopulation (ESSDAI $\geq$ 5), the percentage of rTregs among Tregs decreased after treatment. On the contrary, the percentage of aTregs (activated Treg cells) increased after treatment. It was in an inverse correlation between the percentage of aTreg and rTreg in pSS patients. The Tregs are co-cultured with responder T cells. Tregs from pSS patients showed poorer proliferation inhibitory function. Our results show that the percentages of Tregs and their subgroups altered in pSS patients. The percentage of aTreg and the percentage of rTreg have an inverse correlation in pSS patients. Compared to the control group, the percentage of rTregs among Tregs was increased in the pSS patients and decreased after the treatment. Our study also showed that The Tregs from pSS patients may have poorer inhibitory functions.

1. Introduction

Primary Sjögren syndrome (pSS) is a multi-genic and multi-factorial systemic autoimmune disease with presentation of xerostomia and xerophthalmia. Until now, the pathogenesis of pSS remains obscure in spite of extensive studies. It's difficult to evaluate the patients' systemic inflammation and disease progression for clinician. Up to date, the most useful clinical tool in determining pSS disease activity is ESSDAI and scores less than 5 is considered to be in low disease activity [1]. Several studies show that an imbalance among regulatory T cells (Tregs), T helper cells type I (Th1), T helper cells type II (Th2), T helper cells type 17 (Th17) and the related cytokines in peripheral blood and from the minor salivary gland biopsy in pSS patients [2–5]. Some reports mentioned the population

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of Tregs was decreased in pSS patients' blood and salivary glands [6,7], but another study doesn't demonstrate the same phenomenon [8]. Recently, more and more reports have been focusing on the role which the subgroups of Tregs plays in the autoimmune diseases. As preview studies reported, the majority Tregs can be divided into two groups, the resting Tregs and the memory/active Tregs [9,10]. The resting Tregs are those cells below the threshold for full activation, and the memory/active Tregs are those cells received strong antigen stimulation. In some cases, the resting Tregs can turn into the active Tregs enhancing the suppressive function and migrating into inflamed tissue to conduct its function of modulate the immune system [9,11,12]. Sakaguchi et al. reported Tregs can be divided into three subpopulations: $Foxp3^{high}CD45RA^{-}$ activated Tregs (aTreg), $CD45RA^{+}$ $FoxP3^{lo}$ Tregs (rTreg) and non-suppressive $CD45RA^{-}FoxP3^{lo}$ T cells (nTreg) [10]. In disease situation, the population of rTregs increases in patients with systemic lupus erythromatosus and is responsible for the decreased suppression ability of Tregs in these patients [10,13,14]. However, the subgroups of Tregs in patients with pSS have not been explored yet. Therefore, the purpose of this study was to elucidate the roles of Tregs and its subgroups play in pSS patients during before and after treatment, and the correlation with disease activity.

2. Results

2.1. Demographic, clinical and laboratory data in 44 patients with pSS

There were totally 43 pSS patients and 23 age and gender matched healthy volunteers enrolled into the study (Table 1). There was no significant difference between the health people and pSS patients in terms of age (60.0 ± 15.1 versus 61.3 ± 15.4 years) and gender. Most patient received hydroxychloroquine (47%), azathioprine (28%), sulfasalazine (21%) and small amount of them received steroid therapy (12%). Among all patients, 26 (61%) patients were seropositive (either anti-SSA or anti-SSB was positive), and 17 (25%) patients were ESSDAI ≥ 5. There was no significant difference between seropositive and seronegative pSS group in terms of age (61.6 ± 17.6 versus 60.8 ± 11.6 years), disease duration (10.8 ± 14.6 versus 27.9 ± 29.2 months, $p < 0.05$), IgG level, C3, C4 and ESSDAI scores. We found that more elevated ESR (15.8 ± 8.7 versus 29.0 ± 21.1 mm/h) but not CRP can be observed in seropositive group. Further, there were no difference in terms of age between low and high disease activity groups (59.5 ± 16.2 versus 63.1 ± 14.6 years), and disease duration (21.0 ± 23.3 versus 13.9 ± 22.3months). IgG, C3, C4 showed no difference between low and high disease activity groups, (1.9 ± 1.2 versus 7.1 ± 2.5, $p < 0.05$).

Table 1
Clinical and immunological features in health control and pSS patients.

Variables	Control	All pSS patients	p	Serology		p	Disease activity		p
	(n = 23)	(n = 43)		Seronegative (n = 17)	Seropositive (n = 26)		ESSDAI<5 (n = 26)	ESSDAI ≥ 5 (n = 17)	
Demographic									
Sex (female, n (%))	19 (83%)	40 (93%)		2 (12%)	25 (96%)		24 (92%)	16 (94%)	
Age (years)	60.0 ± 15.1	61.3 ± 15.4C	0.760	60.8 ± 11.6	61.6 ± 17.6	0.871	59.3 ± 15.5	64.2 ± 15.2	0.309
Disease duration (months)	–	17.6 ± 22.9		27.9 ± 29.2	10.8 ± 14.6[b]	0.036	24.5 ± 26.2	6.9 ± 10.1[b]	0.004
Seropositive	–	26 (61%)		–	–		13 (50%)	13 (76%)	
Disease activity (ESSDAI ≥ 5)	–	17 (25%)		4 (22%)	13 (50%)		–	–	
ESSSDAI	–	4.0 ± 3.1		3.0 ± 2.1	4.6 ± 3.5	0.094	1.9 ± 1.2	7.1 ± 2.5[b]	<0.001
IgG abnormal (>1600 mg/dL)		8 (19%)		1 (6%)	7 (27%)		5 (19%)	3 (18%)	
C3 abnormal (<80 mg/dL)		4 (9%)		2 (12%)	2 (8%)		2 (8%)	2 (12%)	
C4 abnormal (<16 mg/dL)		3 (7%)		2 (12%)	1 (4%)		2 (8%)	1 (6%)	
Prednisolone		5 (12%)		1 (6%)	4 (15%)		2 (8%)	3 (18%)	
HCQ		20 (47%)		7 (41%)	13 (50%)		9 (35%)	11 (65%)[b]	
AZA		12 (28%)		5 (29%)	7 (27%)		7 (27%)	5 (29%)	
SSZ		9 (21%)		4 (24%)	5 (19%)		5 (19%)	4 (24%)	
Laboratory Data									
ESR (mm/hr)	–	23.6 ± 18.2		15.8 ± 8.7	29.0 ± 21.1[b]	0.020	20.3 ± 13.9	29.0 ± 23.1	0.133
CRP (mg/L)	–	5.2 ± 15.6		1.4 ± 0.8	7.9 ± 20.2	0.197	1.8 ± 1.2	10.4 ± 24.6	0.186
Among $CD4^{+}$ cells									
Treg cells (%)	8.8 ± 2.6	8.2 ± 3.2	0.466	7.7 ± 3.0	8.5 ± 3.3	0.458	7.9 ± 2.6	8.6 ± 3.9	0.541
Among $CD4^{+}FoxP3^{+}$ T cells									
aTreg cells (%)	16.7 ± 5.5	18.3 ± 10.2	0.428	16.9 ± 11.3	19.2 ± 9.4	0.484	18.5 ± 10.1	17.9 ± 10.5	0.852
rTreg cells (%)	8.6 ± 5.1	12.7 ± 7.8[a]	0.014	12.8 ± 6.2[a]	12.6 ± 8.8[a]	0.961	13.6 ± 7.9	11.3 ± 7.7	0.358

Legend: "a" denotes statistical significance with control group ($p < 0.05$); "b" denotes statistical significance between groups ($p < 0.05$); "aTreg" denotes $Foxp3^{high}CD45RA^{-}$activated Tregs; "rTreg" denotes $CD45RA^{+}$ $FoxP3^{lo}$Tregs;.

2.2. The population of Tregs and its subgroups between health controls and patients with pSS

The percentages of Tregs were analyzed with flow cytometer in the patients and healthy controls (Fig. 1). We were first gated on a forward scatter (FSC) and side scatter (SSC) plot to identify of Foxp3+CD4$^+$ T cells including PBMCs and tissue-infiltrating lymphocytes were isolated (Fig. 1A) and gated on the CD4$^+$ population (Fig. 1B). Then we further gated for the subsets of Foxp3^{high}CD45RA$^-$ activated Tregs (aTreg) and Foxp3^{high}CD45RA$^+$ resting Tregs (rTreg) (Fig. 1C and D). However, there was no significant difference in the percentage of Tregs in CD4$^+$ T cells between healthy controls and pSS patients (8.8 ± 2.6 versus 8.2 ± 3.2) (Table 1).

As shown in the previous studies, the increased rTreg change the component of Tregs and decrease the suppression ability of Tregs in patients with SLE [14]. We evaluated the population of aTregs and rTregs in Treg cells between the healthy and pSS patients. The percentage of rTreg cells significantly increased in the group of pSS patients than the healthy controls (12.7 ± 7.8versus 8.6 ± 5.2, $p < 0.05$) (Table 1). However, the population of aTregs in the Tregs cells was similar between the two groups.

Further, we studied Tregs and its subgroups to clarify the association with serology or disease activity. There is no significant difference in the percentage of Tregs and its subgroups between the seropositive and seronegative groups of the patients (Table 1). Patients with high disease activity (ESSDAI ≥ 5) had the significantly shorter disease durations. However, there is no significant difference in terms of the percentage of Tregs and its subgroups.

Furthermore, we analyzed the correlation between Tregs subgroups and ESSDAI scores in pSS patients (Fig. 2). Tregs, aTregs and rTregs percentages have no significant correlation with ESSDAI score (Fig. .2A–C). Intriguingly, in pSS patients, we observed the rTregs percentage was negatively correlated with aTregs percentage (Fig. 2D) but not in health controls (data not shown).

2.3. ESSDAI statistically significance decreased after therapy in clinical high disease activity group

Among the 43 pSS patients, we followed 14 patients after therapy. Demographic, clinical and laboratory data of patients are summarized (Table 2). They were all females whose mean age was 65.1 ± 11.6 years old, with 21.8 ± 29.0 months as mean disease

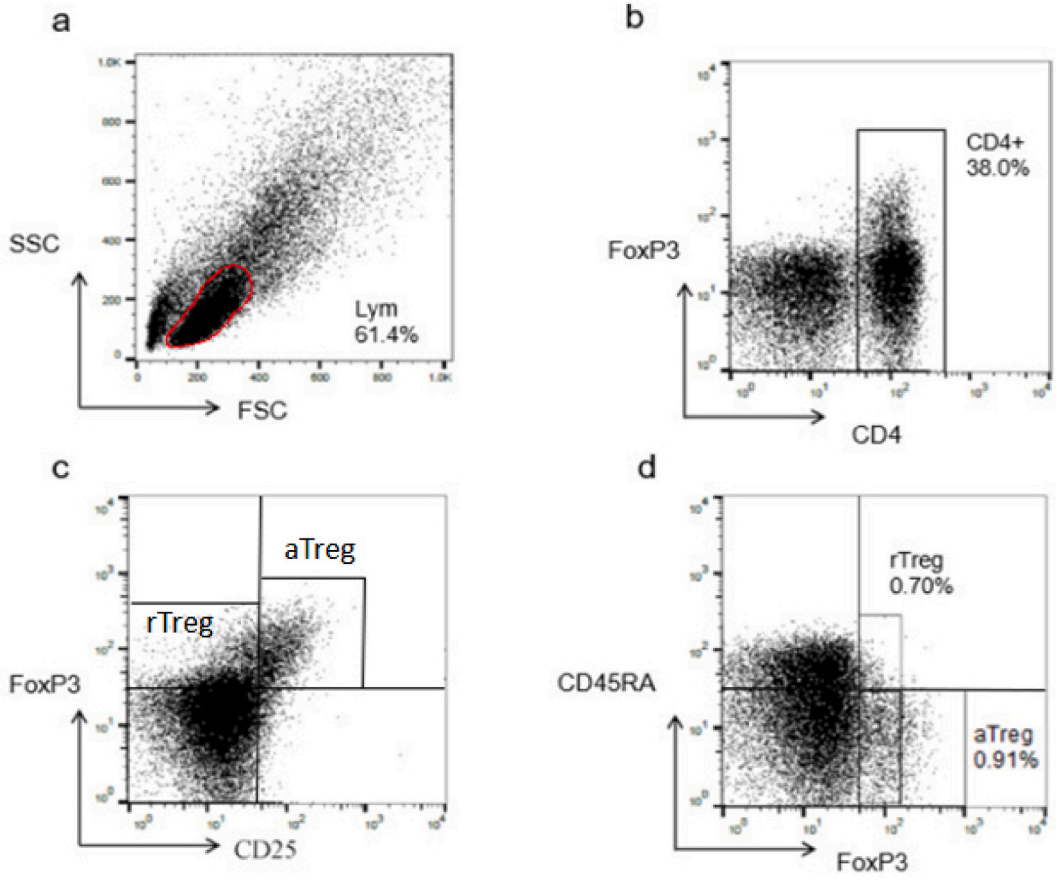

Fig. 1. Treg and subgroups gating in flowcytometry Legend: a to d shows the gating line, lymphocytes legend a were first gated on a forward scatter (FSC) and side scatter (SSC) plot to identify of Foxp3+CD4$^+$ T cells including PBMCs and tissue-infiltrating lymphocytes were isolated and legend b gated on the CD4$^+$ population. Legend c and d were then further gated for the subsets of Foxp3^{high}CD45RA$^-$ activated Tregs (aTreg) and Foxp3^{high}CD45RA$^+$ resting Tregs (rTreg).

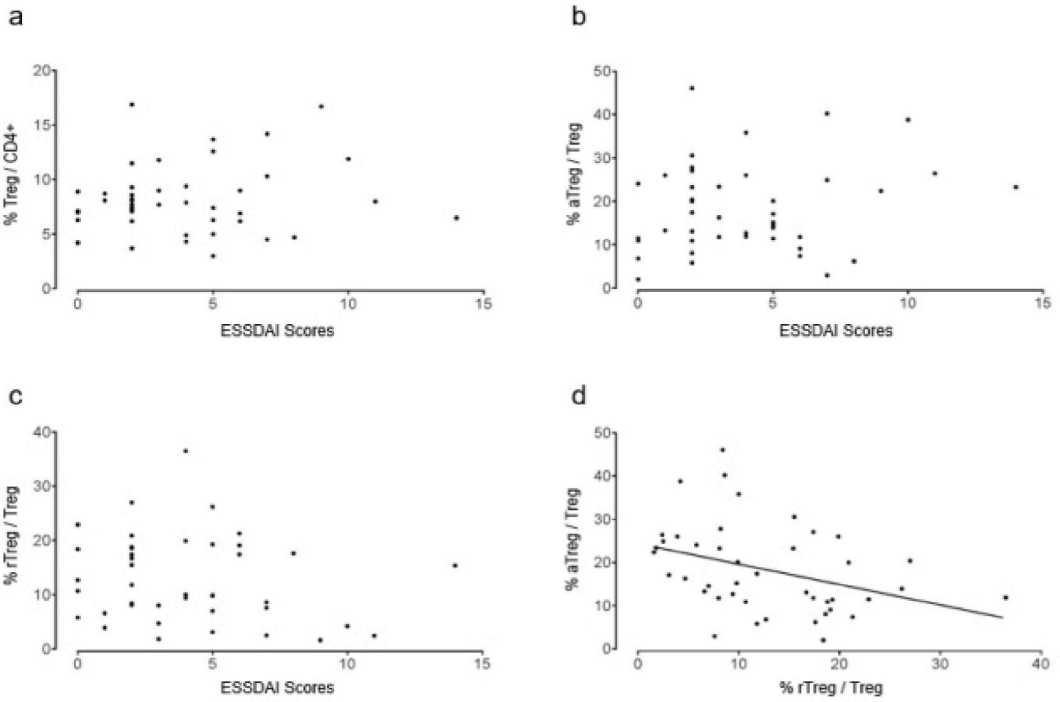

Fig. 2. The correlation of Tregs subgroups with ESSDAI. Legend a the correlation between Treg percentage and ESSDAI in pSS patients, Spearman r = −0.241 and $p = 0.118$; legend b the correlation between aTreg percentage and ESSDAI in pSS patients, Spearman r = 0.120, $p = 0.441$; legend c the correlation between rTreg percentage and ESSDAI in pSS patients, Spearman r = −0.175, $p = 0.261$; legend d the correlation between aTreg percentage and rTreg in pSS patients, Spearman r = −0.413 and $p < 0.01$.

Table 2
Clinical and immunological features of pSS patients before and after treatment.

Variables	pSS patients			Disease activity					
	(n = 14)			ESSDAI<5 (n = 8)			ESSDAI ≥ 5 (n = 6)		
	Before	After	*p*	Before	After	*p*	Before	After	*p*
Demographic									
Sex (female, n (%))	14 (100%)		–		–				
Age (years)	65.1 ± 11.6		63.7 ± 8.8		67.0 ± 15.2				
Disease duration (months)	21.8 ± 29.0		2.7 ± 2.7		0.6 ± 1.0				
Seropositive	7 (50%)		4 (50%)		3 (50%)				
Disease activity (ESSDAI ≥ 5)	6 (43%)		–		–				
ESSSDAI	4.2 ± 3.5	2.7 ± 2.9	0.328	2.0 ± 1.5	4.0 ± 3.2	0.193	7.7 ± 3.4	1.1 ± 1.3[a]	0.002
IgG abnormal (>1600 mg/dL)	3 (21%)		2 (25%)		1 (17%)				
C3 abnormal (<80 mg/dL)	1 (7%)		0 (0)		1 (17%)				
C4 abnormal (<16 mg/dL)	1 (7%)		1 (13%)		0 (0)				
prednisolone	1 (7%)		0 (0)		1 (17%)				
HCQ	7 (50%)		2 (25%)		5 (83%)				
AZA	7 (50%)		5 (63%)		2 (33%)				
SSZ	4 (29%)		2 (25%)		2 (33%)				
Laboratory Data									
ESR (mm/hr)	22.2 ± 11.3	17.5 ± 7.3	0.297	20.7 ± 9.1	17.7 ± 5.8	0.191	24.3 ± 14.3	17.2 ± 10.0	0.744
CRP (mg/L)	3.4 ± 5.9	3.2 ± 4.6	0.982	1.9 ± 1.2	3.4 ± 5.3	0.490	5.3 ± 9.0	3.0 ± 3.9	0.451
Among $CD4^+$ cells									
Treg cells (%)	7.4 ± 2.5	8.1 ± 2.9	0.208	7.1 ± 2.1	7.4 ± 1.8	0.682	7.7 ± 3.2	9.1 ± 3.9	0.241
Among $CD4^+FoxP3^+$ T cells									
aTreg cells (%)	18.5 ± 11.8	20.5 ± 6.7	0.686	23.5 ± 12.7	18.1 ± 7.1	0.423	11.9 ± 6.9	23.6 ± 4.8[a]	0.047
rTreg cells (%)	14.6 ± 8.1	8.4 ± 4.9[a]	0.040	14.2 ± 10.0	9.6 ± 5.3	0.323	15.1 ± 5.3	6.7 ± 4.2[a]	0.034

Legend: The "a" denotes statistical significance ($p < 0.05$); "aTreg" denotes $Foxp3^{high}CD45RA^-$ activated Tregs; "rTreg" denotes $CD45RA^+ FoxP3^{lo}$ resting Tregs. ESR and CRP mildly declined after treatment but there is no statistical significance found.

duration and 2.2 ± 1.4 as the mean ESSDAI scores. Among them, seven patients (50%) were seropositive and eight patients (57%) were clinically active pSS (ESSDAI ≥ 5). After treatment, the ESSDAI score showed no significant difference in the low disease activity group (ESSDAI<5). Most of them received hydroxychloroquine therapy (50%) and only one person received steroid therapy. As we expected, decreased ESSDAI with statistically significance could be observed in the group after therapy especially in clinical high disease activity group.

2.4. The population of Tregs and its subgroups changed in patients with pSS after therapy

After treatment, the percentage of Tregs in $CD4^+$ cells showed no statistical significance in the 14 patients (Table 2). The percentages of rTregs were significantly decreased after treatment (14.6 ± 8.1% versus 8.4 ± 4.9%, $p < 0.05$, Table 2, Fig. 4D). Similarly, in patients with high disease activity, the percentages of rTregs were significantly decreased after treatment (Fig. 3D). However, in the low activity subgroups, there was no significance (Fig. 3B). On the other hand, the percentage of aTreg cells increased significantly after treatment in patients with high disease activity groups (11.9 ± 6.9% versus 23.6 ± 4.8%, $p < 0.05$, Fig.s. 3C and 4A) though not in patients with low disease activity (Fig. 3A). The percentage of $FoxP3^{lo}$ $CD25^+CD45RA^+$ Treg (non-suppressive Treg) decreased in all groups (Fig. 4C).

"All" denotes all 14 pSS patients; "Sero(−)" denotes seronegative group; "Sero(+)" denotes seropositive group.

2.5. Proliferation assay of Treg, aTreg, and rTreg between pSS patients and health controls

To further investigate the imbalance of Treg subsets in primary sjögren syndrome patients, we assessed CFSE expression in responder T cells co-cultured with Treg subpopulations, including Tregs, rTregs and aTregs from pSS patients after treatment and health controls. We found out that the function of proliferation between pSS and control were significantly different in rTregs ($p = 0.03$). In spite of increased aTreg and decreased rTreg in pSS patients after treatment (Fig. 3), Treg and subgroups showed Responder T cell, rTreg, aTreg, Treg cells were gated in flowcytometry, we use different colors to show different cells (Fig. 5A) and the proliferation of responder T cells co-cultured with rTregs was significant increased in another 16 pSS patients (Fig. 5B). It showed proliferation of responder T cells cultured with CD3/CD28 beads and subgroups of Treg, rTreg, and aTreg cells between health controls and pSS patients after treatment. ($^*p < 0.05$) (Fig. 5C).

3. Discussion

The major findings of this study could be explained in several aspects. In respect of the Tregs population in peripheral blood, there

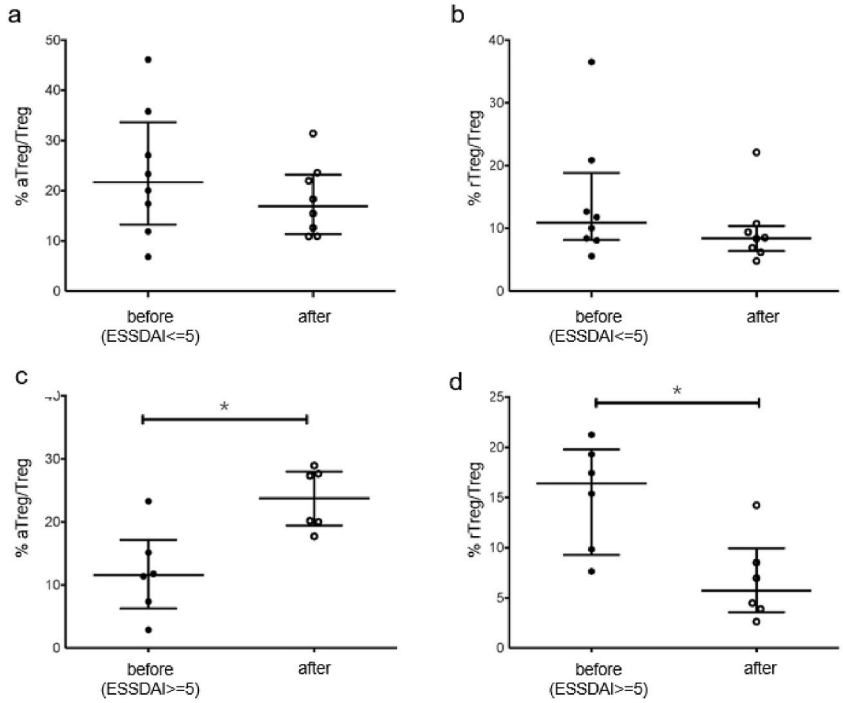

Fig. 3. The change of aTreg and rTreg after treatment in Sjogren patients. Legend a and b represent the difference of patients and the $Foxp3^{high}CD45RA^-$ activated Tregs (aTreg) percentage in low disease activity, $p = 0.422$, and $CD45RA^+$ $FoxP3^{lo}$ Tregs (rTreg) percentage, $p = 0.322$. Legend c and d represent the difference of aTreg and rTreg percentage in high disease activity patients. $p = 0.047$, $p = 0.033$ ($^*p < 0.05$).

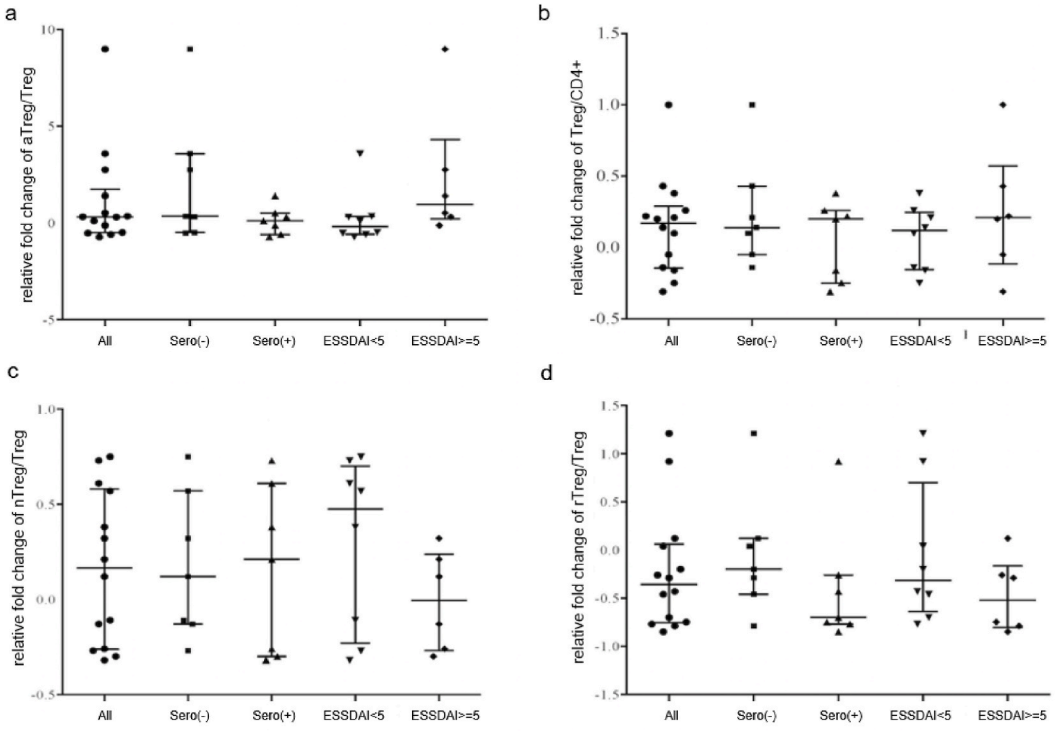

Fig. 4. Relative fold changes of Treg subgroups after treatment. Legend a Foxp3^{high}CD45RA^{-} activated Tregs (aTreg) percentage increased in all groups after treatment. Legend b CD4^{+}CD25^{+} FoxP3^{+} Lymphocytes (Treg) percentage decreased in all groups after treatment. Legend c represents relative fold change of FoxP3^{lo} CD25^{+}CD45RA $^{+}$ Treg (non-suppressive Treg) in each group. Legend d represent rTreg percentage change in all groups.

was no significant difference between the healthy and the pSS or before and after treatment in the pSS. As for the Treg subgroups considered, rTregs was significantly higher in pSS patients (Table 1). The rTregs percentage declined after treatment in our pSS patients, especially in the high disease activity group (ESSDAI $\geq$ 5) (Fig.s. 3D and 4B). The population of aTregs was significantly increased after treatment in the high disease activity group (ESSDAI $\geq$ 5) (Fig.s. 3C and 4A). Furthermore, the populations of rTregs and aTregs in Tregs had an inverse correlation in pSS patients (Fig. 2D) but not in the healthy. Our findings indicated that it was the subgroup of Tregs rather than the total Tregs is associated with the Primary Sjögren syndrome.

Primary Sjögren syndrome is an inflammatory disease characterized by autoantibody anti-SSa and anti-SSb. There are still controversial about the phenotype of seropositive and seronegative groups in Sjögren's syndrome [15,16]. Our study indicated that the seropositive pSS had elevated ESR, but they shared the same immunological features with the seronegative group. The response to the treatment was different between these two groups. After treatment, the seropositive pSS got more improvement in ESSDAI, but seronegative group did not. In such a context, the findings of our study provided more confusing data for the present debate, and the conclusion and difference about seropositive and seronegative pSS still need more studies to elucidate.

Tregs exerts inhibitory activity toward auto-reactive lymphocytes via cell-cell contact or cytokines releasing such as IL-10 and transforming growth factor-β (TGF-β) [17]. Altered peripheral percentage of Tregs has been observed in pSS in a variety of studies, but remains no consistent conclusion [6–8,18–21]. In this study, we showed that Treg cells percentage in CD4^{+} T cells in the pSS patients was similar to the control group. Further, there was no difference when comparing between seropositive and seronegative group or high and low disease activity in cross-sectional observation.

As for Tregs subgroups were considered, the population of rTregs in pSS patient is larger than health controls. Furthermore, the formerly increased rTregs would decrease after treatment. In addition, rTregs and aTregs showed inverse correlation only in the pSS patients but not in the healthy control. It had already been reported that the aTregs but not rTregs is the Treg cells could migrate into inflamed tissue to mitigate tissue damage during the heightened responses of pro-inflammatory memory cells [9,10]. When suppressor potential is concerned, the rTregs are lower than the aTregs [10]. Therefore, we propose that the change of Treg cells component by increased rTreg cells is the reason aggravating/inducing the Primary Sjögren syndrome. Similar to our observation, it had been demonstrated that the rTregs increase in patients with systemic lupus erythromatosus [10], increased other Treg subgroups is also described in Chen's report [21]. When the function of Tregs is concerned, our study shows the trend of poor responder T proliferation inhibitory function of Treg compared with health control (Fig. 5B).

There are several possible explanations for our findings. First, increased aTreg number may be only an epiphenomenon of successful treatment. Second, the number and perhaps, functions, of aTregs may be more important in high disease activity patients. We surmise that in pSS, more aTregs are needed to inhibit the inflammation, and the condition can be correct by regular treatment of pSS.

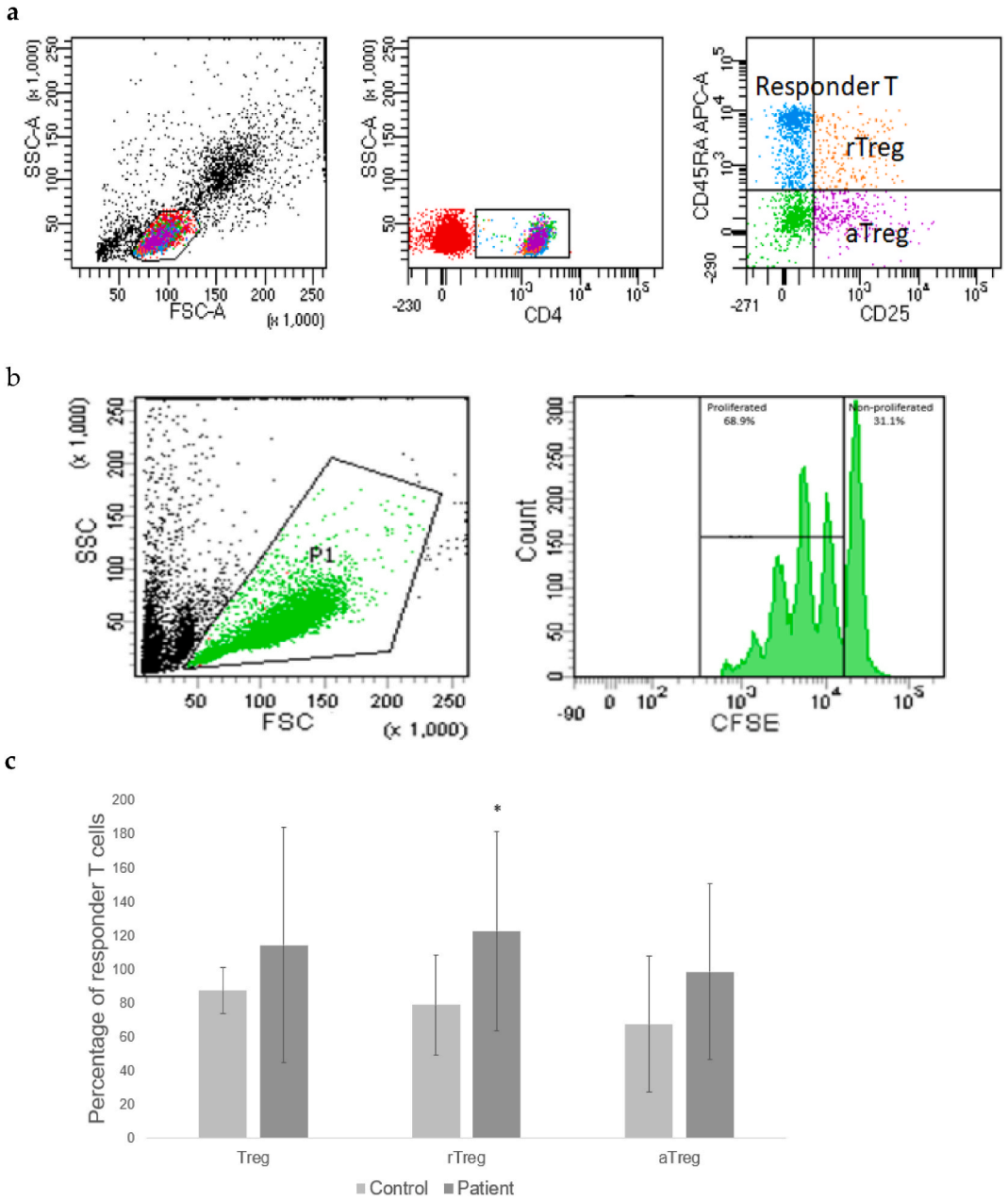

Fig. 5. Responder T cells inhibition by Treg. Legend a Treg and subgroups showed Responder T cell, rTreg, aTreg, Treg cells were gated in flowcytometry, and the suppression assay of Responder T cell were cultured with Treg, aTreg and rTreg cells after 96 h. Legend b showed the gating line of responder T cells stain in CFSE (P1), and the proliferation of responder T cells cultured with CD3/CD28 beads for 96 h (right). Legend c showed proliferation of responder T cells cultured with CD3/CD28 beads and subgroups of Treg cells between health controls and pSS patients after treatment. (*$p < 0.05$).

Third, aTregs are considered to migrate to peripheral inflamed tissue for exerting inhibitory function [9]. The decreased aTregs numbers in blood may represent their migration to peripheral tissue. After treatment, when the peripheral tissue inflammation improved, the aTreg in blood then increased in number and percentage. The condition can be observed especially in high ESSDAI patients may be due to their prominent generalized inflammation. The second hypothesis about the phenomena we found is that the differentiation of naïve T to resting Treg and active Tregs are disturbed in pSS patients. The rTreg and non-suppressive Tregs increased in treatment naïve pSS patients and decreased after medication treatment (Fig. 4C). The study also showed that all Tregs from pSS patients including aTreg, rTreg and even $CD4^{+}CD45^{-}CD25^{hi}$ aTregs had lower ability to inhibit the proliferation of co-cultured responder T cells (Fig. 5B). And the pSS patients failed to yield more Treg numbers for compensating Tregs function defect. Although the resting Tregs exert less inhibitory function than active Tregs, they exert inhibitory functions when an inflammatory

condition occurs. The rTregs may still play a role in inhibiting pSS inflammation.

Monitoring disease activity of pSS with ESSDAI is quite time consuming for clinical rheumatologists and the most relevant lab data with the pSS inflammation are elevating IgG, lower C3 and C4. In our study showed that the rTreg and aTreg are more relevant with ESSDAI than IgG and complements. The finding might worth more investigation in future works.

There are still some limitations about our study. First, the annual incidence in Taiwan is around 6/100000 [22] and the population of the county nearby is about 300000. During our 3-year study the new case of pSS is estimated to be 18 (and we got 14). A small number of our pSS patients may affect the statistics. Second, ESSDAI reflect systemic organ damage more than current inflammation condition, but the serum Tregs and Tregs subgroups are more likely to demonstrate current inflammation status. Third, the responder T proliferation inhibition study cannot represent all inhibitory function of Tregs.Previous study also demonstrated that Treg dysfunction and decreased numbers in pSS patients. But the report uses the GITR + Tregs with low CD25 as target study group [23]. Our result showed that not only GITR + CD25 low Tregs but also CD25 high Tregs dysfunctioned in pSS patients, Future works include long-term follow up of the clinical course and complications of pSS. For verifying our assumptions, our team will exam immunohistochemical stain on patients' salivary gland specimen and do more functional studies of the Treg subgroups when available.

4. Conclusion

We found the composition of Tregs altered in pSS patients. The percentage of rTregs in Tregs increased in patients with Sjögren's syndrome and decreased after treatment. The percentage of aTregs and rTregs in Tregs have an inverse correlation in the pSS patients but not in the healthy control. The Treg inhibitory function are damped in pSS patients. These results indicated that rTreg, the subgroups of Tregs, rather than Tregs is the associated with the autoimmune disease pSS.

5. Methods

5.1. Subjects and study design

Patients with pSS who investigated in this study were recruited from the Department of Rheumatology, Chang Gung Memorial Hospital, Chiayi, Taiwan. The diagnosis of pSS was based on the 2002 revised classification criteria proposed by American-European Consensus Group [24]. Ethical approval was obtained from the Institutional Review Board of Chang Gung Memorial Hospital (IRB 100–3498B) prior to the implementation of the study and the study was carried out in accordance with relevant guidelines. Informed consents were obtained from all patients who participated in this study. Forty-three patients with pSS (40 females; mean age: 61.3 ± 15.4yr) and 23 age- and sex-matched healthy volunteers (19 females; mean age: 60.0 ± 15.1yr) were enrolled as control. 14 newly diagnosed patients among them were checked twice for following the effect of treatment on Tregs and ESSDAI [1]. pSS is categorized as catastrophic disease in National Health Insurance Bureau in Taiwan and the diagnosis date of each patient is recorded. We also used the diagnosis date to calculate the disease duration.

We compared healthy control and all pSS patients in the first, and further sub-grouped the patients with disease activity and the presence of autoantibody. Patients who had either Anti-SSA or anti-SSB were classified as "seropositive", in contrast, as "seronegative". In addition, we sub-grouped the patients according to the disease activity scores (ESSDAI) of pSS, low disease activity (ESSDAI$<$5), and high disease activity (ESSDAI $\geq$ 5).

5.2. Clinical and immunological data

All patients underwent extensive medical examinations and serological evaluations, such as saliva production, erythrocyte sedimentation rate (ESR), Hemoglobin (Hgb), C-reactive protein (CRP), neutrophil segment, white blood cells (WBCs) and platelet (PLT), IgG, C3, C4, along with anti-SSA and anti-SSB were measured. The ESSDAI disease activity of patients were measured [1] and recorded by a rheumatologist, the ESSDAI scores equal or greater than 5 are considered to be with clinically active disease based on suggestions [1].

5.3. Surface and intracellular staining and flow cytometry

The blood samples were collected into collection tubes, which are coated with EDTA-K2 (BD Biosciences), and processed on the day of collection. Peripheral blood mononuclear cells (PBMCs) were prepared over Ficoll-Paque Plus gradients (GE Healthcare). For the detection of Treg cells, PBMCs were analyzed by 3- or 4-color flow cytometry according to the manufacturer's protocol. The following antibodies were used: anti-human CD4 FITC, anti-human CD45RA PE-Cy™7, anti-human CD25 APC (all BD Bioscience) and anti-human FoxP3 PE (eBioscience). Intracellular staining of FoxP3 was performed after fixation and permeabilization according to the manufacturer's (eBioscience) protocol. After staining, cells were washed twice and resuspended in fluorescence-activated cell sorting solution (phosphate buffered saline [25], with 1% Fetal Bovine Serum. Flow cytometry was performed on an FACS Canto flow cytometer (BD Biosciences), and followed by analysis using FlowJo software (Tree Star).

5.4. Cell sorting and proliferation assay

For functional assays, CD4+T cells were firstly isolated by positive selection of PBMCs (obtained from 30 ml whole blood) were

collected into collection tubes, which are coated with EDTA-K2 (BD Biosciences), and processed on the day of collection. Peripheral blood mononuclear cells (PBMCs) were prepared over Ficoll-Paque Plus gradients (GE Healthcare). Purified $CD4^+$ cells were stained with CD4, CD25, and CD45RA antibodies, and then sorted into $CD4^+CD25{+}{+}CD45RA+$ cells (rTregs), $CD4^+CD25+CD45RA-$cells (aTregs), and $CD4^+CD25^-CD45RA+$ cells (responder T cells) using a BD FACSAria Fusion flow cytometer from health controls and patients were labeled with 1 μ M CFSE (Biolegend) and were then cocultured with (2×10^4) unlabeled, sorted rTreg, aTreg, and Treg at a 1:1 Treg subpopulations/$CD4^+CD25^-CD45RA+$ responder cell ratio in anti-CD3/CD28 beads (gibco, Dynabeads) for 96 h at 37 °C and 5% CO_2 in complete medium (RPMI 1640 with 10% fetal calf serum). CFSE-labeled cells was assessed by flow cytometry. Proliferation assays were performed, suppression was measured using the formula:

100−[(%Proliferation in presence of Treg/% Proliferation in absence of Treg) × 100].
100−[(%Proliferation in presence of aTreg/% Proliferation in absence of aTreg) × 100].
100−[(%Proliferation in presence of rTreg/% Proliferation in absence of rTreg) × 100].

5.5. Statistical analysis

SPSS version 15.0 package was used for statistical analysis. The Kolmogorov-Smirnov test was used to evaluate the distribution of each parameter. When data were not normally distributed, the Mann-Whitney test was used to compare differences between two groups, and Kruskal-Wallis test with Dunn post-hoc test was performed if there were three groups. Spearman's rank correlation coefficient was used to assess the correlation. The number that follows the ± sign is a standard deviation (s.d.). The p values were considered statistical significance at $p < 0.05$.

Author contribution statement

Jing-Chi Lin, M.D.: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Kuo-Li Pan; Kam-Fai Lee; Ko-Ming Lin; Chun-Yen Lin: Conceived and designed the experiments.

Cheng-Feng Li; Kuan-Yu Lin: Performed the experiments; Analyzed and interpreted the data.

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Data availability statement

Data included in article/supp. material/referenced in article.

Declaration of interest's statement

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e15565.

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