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ORIGINAL ARTICLE

Decrease in $CD4^+CD25^+$ and $CD8^+CD28^+$ T cells in interstitial pneumonitis associated with rheumatic disease

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Abstract The expression of CD25 or CD28 on T cells was examined in patients with rheumatic diseases associated with interstitial pneumonitis (IP), in order to investigate the conditions of $CD4^+CD25^+$ regulatory T cells and $CD8^+CD28^-$ suppressor T cells. Fifty-five patients with various rheumatic diseases and 23 normal controls were enrolled. $CD4^+CD25^+$ T cells of patients with IP were significantly decreased in comparison with non-IP patients, and the ratio of $CD8^+CD28^-$ T cells in patients with IP was significantly higher than that in non-IP patients or normal controls. These results for $CD8^+CD28^-$ T cells were in accord with the decrease in $CD8^+CD28^+$ T cells, and may be related to activation-induced $CD8^+CD28^+$ T-cell death. Thus, the abnormality of $CD4^+CD25^+$ regulatory T cells may be related to the pathogenesis of IP, and the survival and activation of $CD8^+$ T cells.

Keywords $CD4^+CD25^+$ regulatory T cell · $CD8^+CD28^-$ suppressor T cell · Interstitial pneumonitis · Rheumatic diseases · CD62L

Introduction

Interstitial pneumonitis (IP) is frequently associated with various rheumatic diseases, and influences their prognosis. Although the pathogenesis of IP is still unclear, previous studies have demonstrated that activated T cells, including $CD8^+$ T cells, are involved to varying degrees [1]. The activation of T cells is regulated both positively and negatively through the influence of both enhancing and inhibitory factors. Generally, it is postulated that an imbalance of these factors contributes to the onset of autoimmune diseases. Enhancing factors include various cytokines or co-stimulatory molecules. In IP, inflammatory cytokines such as TNF-α [2–4], IFN-γ [5], IL-1 [4, 6], TGF-β [7] and IL-13 [8, 9], and chemokines such as MIP-1α [10] and IL-8 are important inflammatory promoters, and it is speculated that TGF-β promotes fibrogenesis during the period of fibrotic transformation [7]. With regard to co-stimulatory molecules, increased expression of CD80 on lung macrophages or CD154 on T cells has been demonstrated in IP [11]. Thus, enhancing factors greatly contribute to the development of autoimmune diseases, including IP.

On the other hand, various inhibitory factors, such as inhibitory cytokines, inhibitory molecules and regulatory cells, are also involved in the immune system, and suppress excessive immune responses. It can be assumed that a decrease in these inhibitory factors induces autoimmune diseases. Although TGF-β and IL-16 are reported to be inhibitory cytokines, no studies have demonstrated that their levels are decreased in antoimmune disease [12]. Inhibitory molecules such as CD22 on B cells and CD152 (CTLA-4) on T cells have been reported, and abnormal expression of these molecules in autoimmune diseases has been demonstrated [13–16]. Among regulatory cells, suppressor T cells and $CD25^+$ regulatory T cells have been reported. The former include $CD28^-$ T cells, which have been shown to be decreased in patients with systemic lupus erythematosus (SLE) [17], although the relationship between this population and others ($CD28\alpha\alpha$, $CD8^+CD25^+$,

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$CD8^+CD45RClow$, $CD8^+CD75^+$, $CD8^+CD122^+$) is still unclear. A decrease in the latter has been demonstrated to induce organ-specific autoimmunity [18, 19], although no previous reports have focused on systemic autoimmunity or the involvement or function of any regulatory cells in IP. However, it is can be speculated that an abnormality of regulatory cells contributes to the onset of IP.

In the present study, we determined the expression of CD25 and CD28 on T cells of patients with IP, in order to investigate the role of $CD4^+CD25^+$ regulatory T cells and $CD8^+CD28^-$ suppressor T cells in this disease.

Materials and methods

Patients

For this study, we enrolled 55 patients (11 men and 44 women; mean age 56.6 years; age range 22–88 years) with various rheumatic diseases [14 with rheumatoid arthritis (RA), 5 with Sjögren's syndrome (SjS), 10 with systemic sclerosis (SSc), 14 with polymyositis/dermatomyositis (PM/DM), 11 with mixed connective tissue disease (MCTD), and one with microscopic polyangitis (MPA)]; 33 of these patients had associated IP and 21 did not. We also enrolled 23 normal controls (9 men and 14 women) (Table 1). Patients with SLE were excluded, since there were no such patients with IP. SSc and RA were diagnosed according to the American College of Rheumatology criteria [20, 21]. MCTD was diagnosed according to Kasukawa's criteria [22] and PM/DM was diagnosed according to Bohan's criteria [23]. Pneumonitis was generally diagnosed by chest X-ray, chest CT, and pulmonary function tests. The diagnosis was confirmed by plain CT in 9 cases, high-resolution CT in 20 cases, TBLB in 4 cases, and BAL in one case. The disease duration was 5.6 years (range 0.2–30 years) for patients with IP, and 7.9 years (range 0.4–30 years) for those without IP. Although only one patient with RA received methotrexate, this patient did not have associated IP. Patients with drug-induced interstitial pneumonitis were excluded. Eight patients with IP and seven without IP had a history of smoking. Each rheumatic disease was of similar severity. Although some patients received low-dose steroid (predonisolone, maximum 20 mg/day), the samples were collected from these patients before any increase in the therapeutic dose.

Informed consent to participate in the study was obtained from all patients. All of the normal controls matched the patients in age and sex [8 men and 15 women; mean age 28.9 years (range 23–35 years)].

Table 1 Demographic features of the patients included in this study

	IP+ ($n = 34$)	IP− ($n = 21$)	NC ($n = 23$)
Males/females	9/25	2/19	9/14
Age (mean)	60.4 (26–81)	52.8 (22–88)	28.8 (23–35)
Disease duration (mean)	5.6 (0.2–30)	7.9 (0.2–30)	
Groups (n)			
RA	8	6	
SjS	4	1	
SSc	7	3	
PM/DM	8	6	
MCTD	6	5	
MPA	1	0	

IP interstitial pneumonitis, *NC* normal contro, *RA* rheumatoid arthritis, *SjS* Sjögren's disease, *SSc* systemic sclerosis, *PM/DM* polymyositis/dermatomyositis, *MCTD* mixed connective tissue disease, *MPA* mycroscopic polyangitis

Cell preparation

Peripheral venous blood samples from the 55 patients and 23 normal controls were diluted 1:2 with phosphate-buffered saline (PBS) (Dulbecco, Nissui, Japan), and the peripheral blood mononuclear cells (PBMCs), including lymphocytes, were isolated by density gradient centrifugation with lymphocyte separation (Lymphoprep, Nycomed Pharma AS, Oslo, Norway). After two washings with PBS, the number of PBMCs was determined with a blood cell counter, and the cell suspension was adjusted to a concentration of 1×10^6 cells/ml in PBS.

Staining and flow-cytometric analysis

Ten microliters of anti-human CD4, CD8, CD25, CD28, CD62L or CD95 (Fas) antibody (PharMingen, San Diego, CA, USA) conjugated with fluorescein isothiocyanate (FITC), 10 μl of anti-human CD8, CD25, CD28, CD62L (PharMingen) or IL-2Rγ antibody (Biosource International, Inc.) conjugated with phycoerythrin (PE), and 10 μl of anti-human CD4 or CD8 antibody (PharMingen) conjugated with APC were added and incubated for 30 min. After two additional washes, the samples were fixed with 0.5% paraformaldehyde in PBS. Then two- or three-color analysis was performed using a FACScalibur (Becton Dickinson, San Jose, CA, USA) and the data were processed with the CellQuest program (Becton Dickinson).

Statistical analysis

The data were analyzed with the Stat View 5.0 statistical software package (SAS Institute Inc., Cary, NC, USA). The Kruskal–Wallis test and Fisher's protected least significant difference (Fisher's PLSD) test were used to determine the statistical significance of the data. Differences at $P < 0.05$ were considered significant.

Results

Expression of CD25 on $CD4^+$ T cells

First we investigated the ratio of $CD4^+CD25^+$ regulatory T cells in lymphocytes from 34 patients with IP, 21 patients without IP and 23 normal controls. The ratio of $CD4^+$ T cells expressing CD25 in patients with IP was significantly lower than that in non-IP patients ($P < 0.05$), as shown in Fig. 1a. However, since this ratio was higher than that in the controls ($P < 0.05$) (Fig. 1a), it is possible that this increase in CD25 was related to the activation of T cells. On the other hand, it was shown that the bright CD25-positive population corresponded to regulatory T cells. Therefore, we examined this population, and found that it was also significantly decreased in patients with IP ($P < 0.05$) (Fig. 1b). On the other hand, with regard to absolute cell number, $CD4^+CD25^+$ T cells tended to be decreased in patients both with and without IP, and there was no significant difference in this respect between the two patient groups (data not shown). Furthermore, it has recently been reported that the expression of CD62L on $CD4^+CD25^+$ T cells plays a major regulatory role. Therefore, we also investigated the ratio of $CD4^+$ $CD25^+$ $CD62L^+$ T cells among lymphocytes, and found that these cells were significantly decreased in patients with IP in comparison with non-IP patients ($P < 0.01$) (Fig. 1c).

Expression of CD28 on $CD8^+$ T cells

Next, we investigated the expression of CD28 on $CD8^+$ T cells. The ratio of $CD8^+CD28^-$ T cells in patients with IP was significantly higher than that in non-IP patients and normal controls ($P < 0.01$ and $P < 0.0001$, respectively) (Fig. 2a). Also, in terms of absolute cell number, similar results were obtained (data not shown). This result was in accord with the finding that $CD8^+CD28^-$ T cells were significantly increased in patients with IP compared with non-IP patients ($P < 0.05$) (Fig. 2b), and that $CD8^+CD28^+$ T cells from patients with IP were decreased in comparison

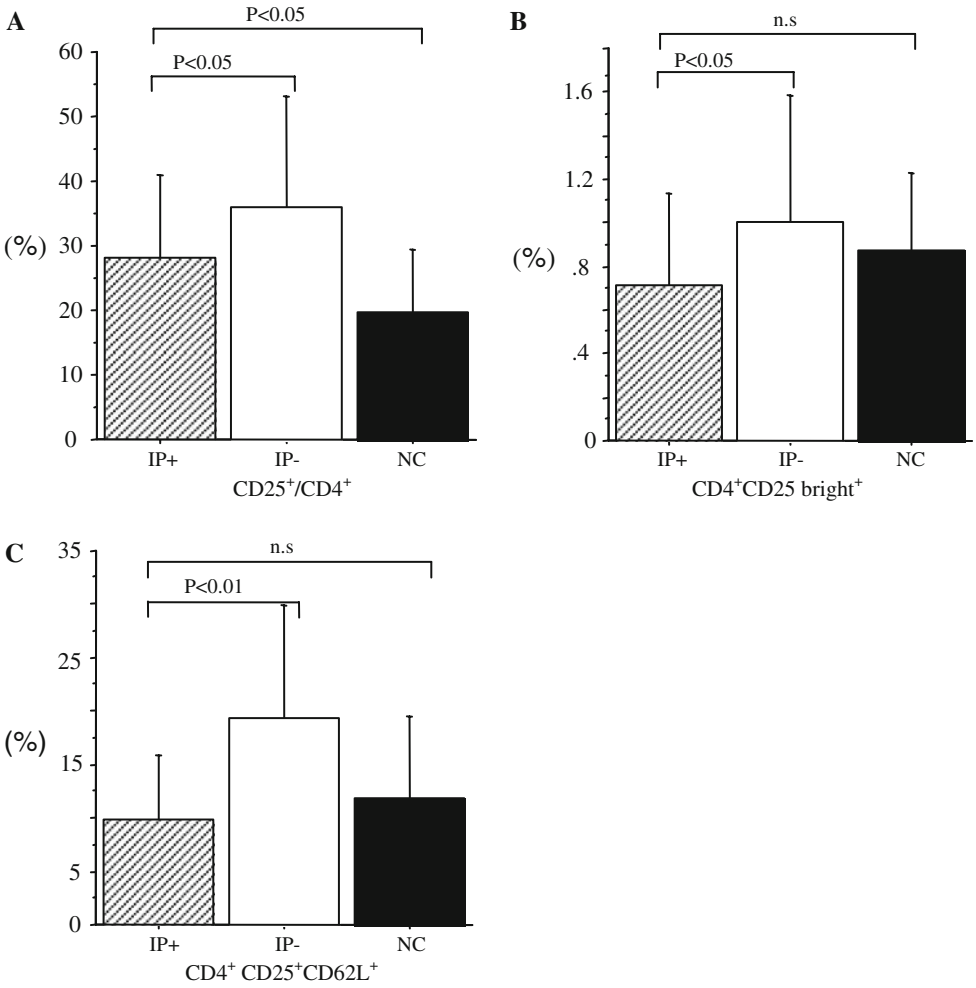

Fig. 1 The ratio of $CD4^+CD25^+$ T cells among $CD4^+$ T cells (**a**), the ratio of $CD4^+$ bright CD25-positive T cells in the lymphocyte population (**b**), and the ratio of $CD4^+CD25^+CD62L^+$ T cells in the lymphocyte population (**c**) in patients with IP (IP+ *hatched bar*), patients without IP (IP− *clear bar*) and normal controls (NC *black bar*). The ratio of $CD4^+CD25^+$ T cells in patients with IP was significantly lower than that in non-IP patients ($P < 0.05$), but higher than in normal controls ($P < 0.05$) (**a**). The ratio of $CD4^+$ bright CD25-positive T cells in patients with IP was significantly lower than in non-IP patients ($P < 0.05$) (**b**). The ratio of $CD4^+$ $CD25^+CD62L^+$ T cells in patients with IP was significantly lower than in non-IP patients ($P < 0.01$) (**c**)

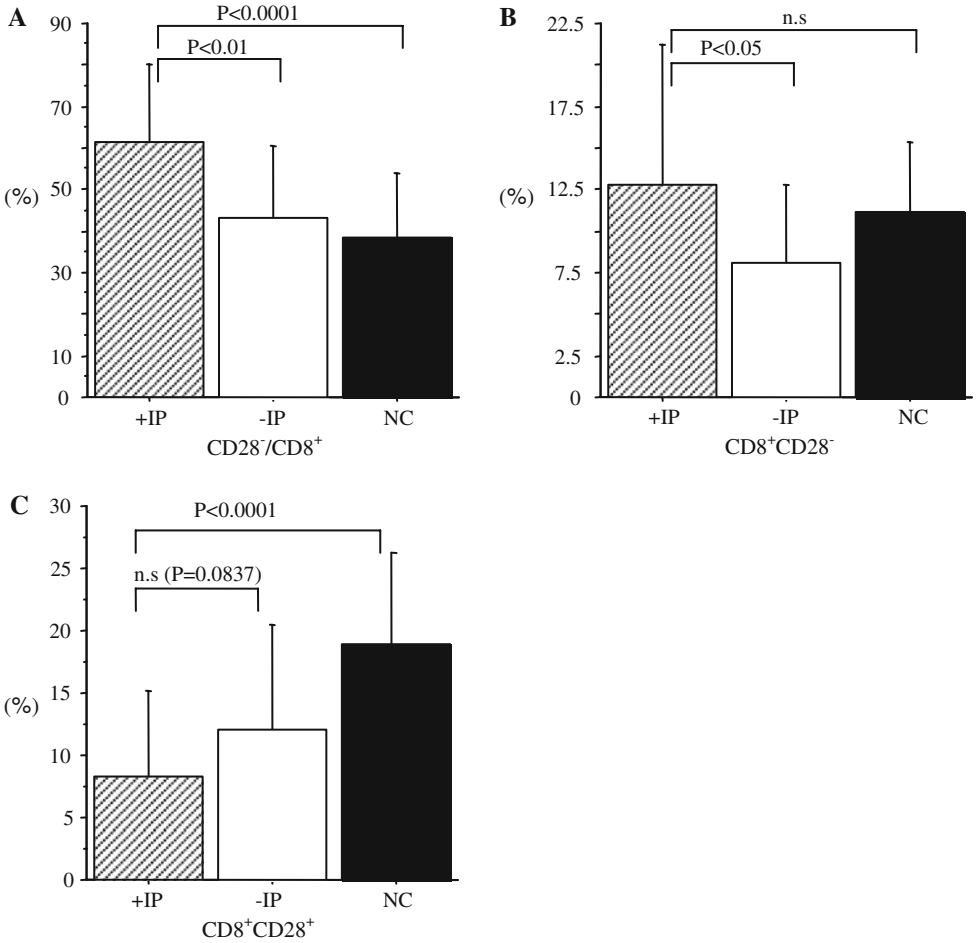

Fig. 2 The ratio of $CD8^+CD28^-$ T cells among $CD8^+$ T cells (**a**), the ratio of $CD8^+CD28^-$ T cells in the lymphocyte population (**b**), and the ratio of $CD8^+CD28^+$ T cells in the lymphocyte population (**c**) in patients with IP (IP+ *hatched bar*), patients without IP (IP− *clear bar*) and normal controls (NC *black bar*). The ratio of $CD8^+CD28^-$ T cells in patients with IP was significantly higher than in non-IP patients and normal controls ($P < 0.05$ and $P < 0.01$, respectively) (**a**). $CD8^+CD28^-$ T cells in patients with IP were significantly increased as compared with non-IP patients ($P < 0.05$) (**b**), and $CD8^+CD28^+$ T cells in patients with IP were decreased in comparison with normal controls ($P < 0.0001$) (**c**)

with normal controls ($P < 0.0001$) (Fig. 2c). The decrease of $CD8^+CD28^+$ T cells in patients with IP may be related to the translocation of these cells into the lung tissue, or to activation-induced cell death (AICD). Therefore, we investigated the expression of CD62L on $CD8^+CD28^+$ T cells, since the lymphocytes may translocate into inflammatory tissue, resulting in low expression of CD62L [24, 25]. However, there was no significant difference in CD62L expression between patients with IP and those without (Fig. 3a). Next, in order to determine whether there is a relationship to AICD, we investigated the expression of CD95 (Fas) on $CD8^+CD28^+$ T cells. The ratio of $CD8^+CD28^+$ T cells expressing CD95 in both patients with and without IP was significantly higher than in normal controls ($P < 0.01$), although there was no significant difference between IP and non-IP patients (Fig. 3b). Furthermore, we investigated the expression of IL-2Rγ on $CD8^+CD28^+$ T cells because IL-15 inhibits AICD by binding to this receptor, and it increased in IP as previously reported [26]. The ratio of IL-2Rγ expression on $CD8^+CD28^+$ T cells was increased in both patients with and and those without IP as compared with normal controls ($P < 0.05$), although there was no significant difference between IP and non-IP patients (Fig. 3c).

Relationship to clinical parameters and serial changes

Finally, we examined clinical findings. The ratio of $CD4^+CD25^+$ T cells or $CD8^+CD28^-$ T cells was not significantly different among the various rheumatic diseases. We investigated the relationship between $CD4^+CD25^+$ or $CD8^+CD28^-$ T cells and clinical parameters such as KL-6, lactate dehydrogenase (LDH) and C-reactive protein (CRP). However, no significant relationship was demonstrated (data not shown). Furthermore, we investigated serial changes in $CD4^+CD25^+$ or $CD8^+CD28^-$ T cells in four patients before and after treatment (Fig. 4). The ratio of $CD4^+CD25^+$ T cells tended to increase after treatment, and two patients with PM or MPA revealed increases in $CD8^+CD28^\pm$ T cells after treatment, while two patients with SSc showed no such change. Thus, with regard to post-therapeutic changes in $CD8^+CD28^\pm$ T cells, there was a difference among the various rheumatic diseases.

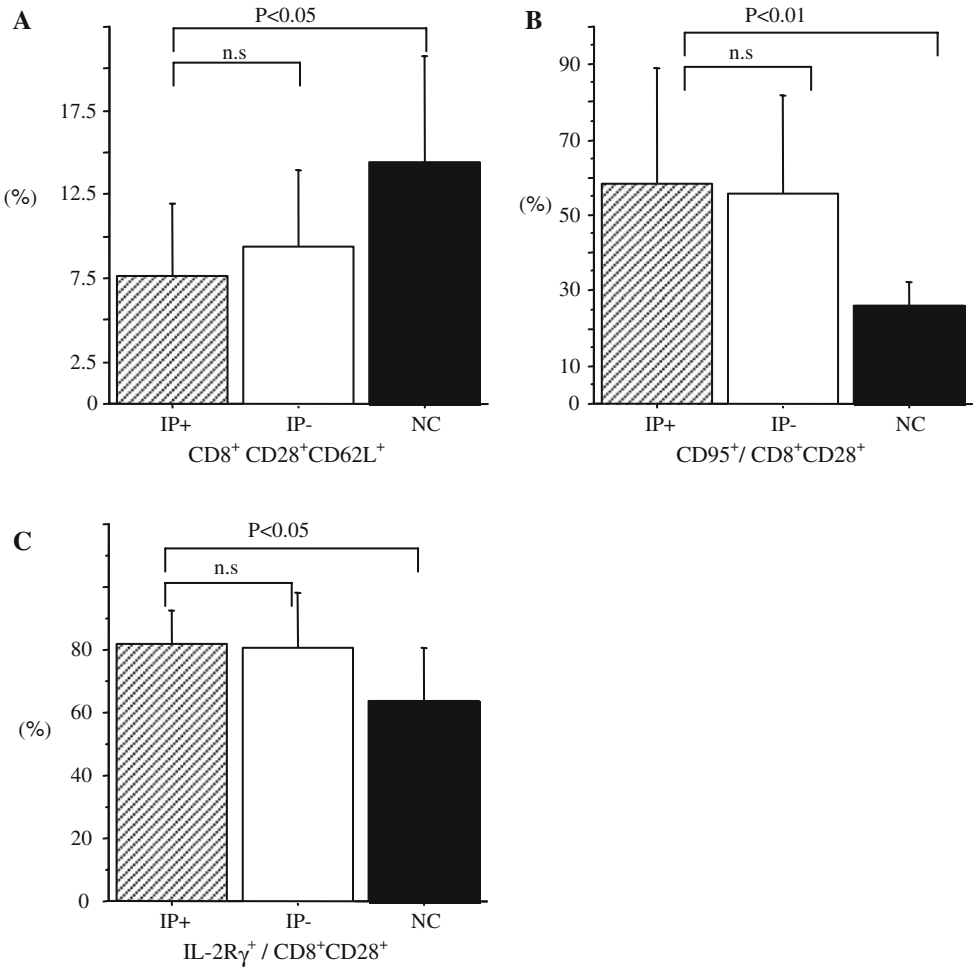

Fig. 3 The ratio of $CD8^+CD28^+CD62L^+$ T cells in the lymphocyte population (**a**), the ratio of CD95 $(Fas)^+$ cells among $CD8^+CD28^+$ T cells (**b**), and the ratio of IL-$2R\gamma^+$ cells among $CD8^+CD28^+$ T cells (**c**) in patients with IP (IP+ *hatched bar*), patients without IP (IP− *clear bar*) and normal controls (NC *black bar*). There was no significant difference in CD62L expression between patients with and without IP (**a**). The ratio of $CD8^+CD28^+$ T cells expressing CD95 and IL-$2R\gamma$ in patients with and without IP was increased as compared with normal controls ($P < 0.05$), but there was no significant difference between patients with and without IP (**b**, **c**)

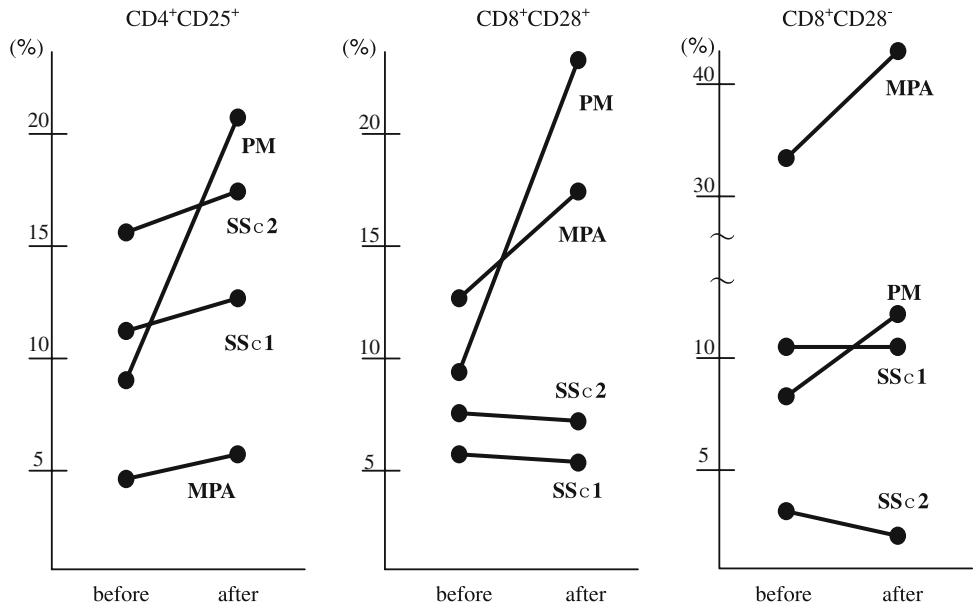

Fig. 4 Serial changes in $CD4^+CD25^+$ or $CD8^+CD28^\pm$ T cells in four patients before and after treatment. The ratio of $CD4^+CD25^+$ T cells tended to increase after treatment. With regard to $CD8^+CD28^\pm$ T cells, those of two patients with PM or MPA increased after treatment, whereas no differences in the T cells were evident in two patients with SSc

Discussion

In this study, we investigated the expression of CD25 on $CD4^+$ T cells and CD28 on $CD8^+$ T cells, and related regulatory T cells. We found that $CD25^+CD4^+$ T cells of patients with IP were significantly decreased in comparison with those of non-IP patients. However, these cells in patients with IP were significantly more numerous than in the controls, possibly because $CD4^+CD25^+$ T cells also include activated T cells. Although the difference between regulatory T cells and activated T cells among $CD4^+CD25^+$ T cells has been difficult to determine, a recent study has demonstrated that the bright positive population of $CD4^+CD25^+$ cells includes a high ratio of regulatory T cells. This difference can be determined by examining the CD62L molecule, and this has demonstrated that $CD4^+CD25^+CD62L^+$ T cells have a major regulatory function [27]. Therefore, we analyzed this cell population, and were able to demonstrate and confirm this difference. Although this result was similar to that for $CD4^+CD25^+$ T cells, it was of interest that $CD4^+$ bright CD25-positive T cells or $CD4^+CD25^+CD62L^+$ T cells of patients with IP were decreased in comparison with those of non-IP patients, and that these cells in patients with non-IP were increased in comparison with the controls. Although some previous studies [28, 29] have demonstrated no difference or decrease in $CD4^+CD25^+$ T cells in RA, the patients were not divided according to the presence of IP. Indeed, in the present study, there was no difference in this population between RA patients as a whole and normal controls. Probably, in patients without IP, regulatory $CD4^+CD25^+$ T cells may increase through a feedback mechanism, while this mechanism may be inactivated in patients with IP, and it is possible that this dysfunction of regulatory T cells may be related to the pathogenesis of IP. Although it could not be proven that this decrease was related to clinical markers of IP activity, the decrease was ameliorated by treatment, and therefore this suppression certainly contributes to development of the disease. However, since the data for absolute cell number demonstrated no clear difference, and previous studies [28, 29] suggested the possibility of transfer into joint, it seems unlikely that dysfunction of the feedback mechanism alone contributes to the pathogenesis of IP. The relationship between $CD4^+CD25^+$ regulatory T cells and autoimmunity has only been demonstrated in mice with organ-specific autoimmune disease [18, 19]. Although we previously investigated $CD4^+CD25^+$ T cells in various rheumatic diseases, no abnormality was revealed (data not shown), and the present study is the first to demonstrate such an abnormality. This feature of IP suggests that abnormality of $CD4^+CD25^+$ T cells contributes to local, rather than systemic autoimmunity. However, since we were unable to examine any function such as the production of IL-10 or TGF-β, any relationship to pathogenesis is still unclear. Although patients in this study had various rheumatic diseases, this decrease in $CD4^+CD25^+$ T cells has been commonly recognized in patients with IP, and therefore we speculate that it commonly contributes to the pathogenesis of IP in various rheumatic diseases.

Our next focus of interest was the reason for the decreases in $CD4^+CD25^+$ T cells, as the mechanism responsible for the regulation of these cells is still unclear. Recently, a study has demonstrated that IL-12 suppresses $CD4^+CD25^+$ T cells, and therefore it is possible that IL-12 produced by macrophages, especially those localized in the lung, act in this way. Therefore, we investigated the level of IL-12 in our study patients, and found that it was increased in some patients with IP (data not shown). Although there was no significant correlation between the level of IL-12 and the ratio of $CD4^+CD25^+$ T cells, the latter result cannot be ignored. Lung macrophages are activated in IP, and react with T cells in the lung (unpublished data). This interaction is closely related to development of the disease, and it is possible that a high level of IL-12 is produced via this pathway. Although it is possible that IL-12 induces a Th1 response in the lung, and activates $CD8^+$ killer T cells, which are closely related to the pathogenesis of IP, we speculate that IL-12 also suppresses regulatory T cells and contributes to development of the disease. Previous studies have demonstrated that $CD4^+CD25^+$ regulatory T cells are translocated into inflammatory tissue [28, 29]. Since we were unable to examine local tissue in the lung, we could not rule out the possibility that $CD4^+CD25^+$ cells have been translocated into the lung.

On the other hand, our results for CD28 were unexpected, as the ratio of $CD8^+CD28^+$ T cells, including suppressor T cells, was increased, reflecting the decrease of $CD8^+CD28^-$ T cells. Thus, we were unable to demonstrate a decrease of suppressor T cells. However, these results are very significant in view of the characteristics of a population of $CD8^+$ T cells. It has been reported that these cell populations translocate into inflammatory tissue including the lung, and cells that have infiltrated the lung need to be studied. However, we were unable to obtain any samples of lung tissue, since it was difficult to obtain informed consent, or the volume of samples obtained was very small. Therefore, we examined the cells in terms of their CD62L expression, and found no significant difference in this respect, thus suggesting a high possibility of AICD. This decrease in $CD8^+CD28^+$ T cells was not related to CD95 expression, suggesting independent CD95 AICD, as revealed in our previous study of patients with SLE [30]. The present study also yielded another interesting finding: the $CD8^+$ T cells that survived commonly expressed CD95 and IL-2Rγ chains on both $CD28^+$ and $CD28^-$ cells. Since

our previous study demonstrated that patients with IP concomitant with rheumatic diseases had high serum levels of IL-15 [26], it is possible that IL-15 binding to IL-2Rγ is the one of the factors related to resistance to CD95-dependent AICD. This finding is of considerable interest, since no previous studies have investigated the IL-2Rγ chain in patients with rheumatic disease. Therefore, we speculate that these activated $CD8^+$ cells with AICD resistance participate in the pathogenesis of IP in lung.

The results for $CD4^+CD25^+$ cells differed from those for $CD8^+CD28^+$ cells. However, if it can be considered that $CD8^+$ T cells are mainly related to the pathogenesis of IP, then the possibility that dysfunction of regulatory $CD4^+CD25^+$ T cells induces the activation and survival of $CD8^+$ cells cannot be discounted. It is very important to understand how T cells contribute to IP, and the results of the present study should provide clues that can aid the clarification of this issue.

Conflict of interest All of the authors confirm that there is no conflict of interest with regard to this work.

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