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Absolute reduction of regulatory T cells and regulatory effect of short-term and low-dose IL-2 in polymyositis or dermatomyositis

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ABSTRACT

Aim: The study aimed to investigate the changes in peripheral lymphocyte and $CD4^+$T subsets and to observe the regulatory effect of low-dose interleukin-2 (ld-IL2) on these cells in polymyositis or dermatomyositis (PM/DM).

Methods: Lymphocyte subsets ($CD3^+$T, $CD4^+$T, $CD8^+$T, B and natural killer (NK) cells), $CD4^+$T subsets (Th1, Th2, Th17 and regulatory T (Treg) cells) and multiple cytokines of 71 patients after admission and treatment were measured by flow cytometry, as well as these indicators in 30 healthy controls (HCs). In DM, 35 cases were administrated with ld-IL2 combined with conventional therapy, the remaining 26 patients received conventional therapy only.

Results: The numbers of $CD3^+$T and $CD4^+$T cells in PM/DM were markedly decreased. Meanwhile, the absolute number and percentage of peripheral Treg cells in PM/DM, as well as Th1 cells in DM, were significantly lower than those in HCs ($P < 0.05$), but Th2 and Th17 cells had no significant difference. The ratio of Th17/Treg in PM ($P = 0.031$) and in DM ($P = 0.003$) were obviously higher than that in HCs. The deficiency of Treg cells was associated with the occurrence of interstitial lung disease (ILD) in myositis patients. Meanwhile, reduced production of IL-2 was also observed in PM/DM ($P < 0.001$). ld-IL2 combination therapy could significantly increase the numbers of $CD4^+$T subsets in DM, especially Treg cells (expanded 2.5 times).

Conclusions: The decline of peripheral Treg cells and serum IL-2 were found in PM/DM. ld-IL2 combination therapy could significantly increase the number of Treg cells.

1. Introduction

Dermatomyositis (DM) and polymyositis (PM) are chronic autoimmune disorders primarily involving skeletal muscle, which is infiltrated by mononuclear cells ($CD4^+$T, $CD8^+$T cells and macrophages) [1]. Other organs like the skin, heart and lungs are also often involved. Although the pathogenesis of PM/DM is not well understood at present, immunological imbalance is considered to be central to the disease progress [2].

Naive $CD4^+$ T cells could differentiate into effector T helpers including Th1, Th2, Th9, Th17 and anti-inflammatory regulatory T (Treg) cells [3]. Excessive immune responses exerted by these T helper cells could cause autoimmune and inflammatory diseases. Treg cells are critical for the suppression of excessive immune and inflammatory response and can be served as a hallmark of immune tolerance [4]. Conventional view was that the pathogenesis of autoimmune disease was caused by over-activation of effector T cells, so corticosteroids and immunosuppressive agents were adopted as the main treatment option. Recently, immune intolerance caused by quantitative and/or qualitative deficiencies of Treg cells is regarded as the pivotal origin of autoimmune diseases. Cytokines are also thought to be involved in the onset of PM/DM [5–9]. Despite all of the above, studies recording the role of peripheral lymphocyte and $CD4^+$T subsets, especially Th17 and Treg cells, and cytokines in PM/DM are rare.

Several studies have demonstrated that low-dose IL-2 (ld-IL2) administration is safe and effective in maintaining the abundance and

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function of Treg cells in patients with graft-versus-host disease and autoimmune diseases [10–13]. So immunoregulation executed by ld-IL2 represents a potential therapy to maintain self-tolerance in autoimmune diseases [11]. However, the treatment effect of ld-IL2 in PM/DM has not been reported. The present work aimed to investigate the changes in peripheral lymphocyte and $CD4^+$T subsets and to observe the regulatory effect of ld-IL2 on these cells in PM/DM.

2. Materials and methods

2.1. Patients

A total of 71 patients with myositis (10 PM and 61 DM patients admitted to our inpatient department between January 2016 and February 2019) and 30 age- and gender-matched healthy controls (HCs) were recruited into the study. All the patients were diagnosed based on the Bohan and Peter diagnostic criteria [14]. Patients older than 80 and/or are unable to exercise were excluded. Seven PM and 35 DM patients were administrated with short-term and low-dose rhIL-2 (recombinant human interleukin-2, Beijing, China) and conventional therapy, while 3 PM and 26 DM only received conventional therapy. The rhIL-2, which was produced by *Escherichia coli* and has similar bioactivity with Proleukin (aldesleukin), passed the review by the State Food and Drug Administration, China. The rhIL-2 was administered subcutaneously at a dose of 0.5 million IU per day for 5 days. Venous blood samples were drawn on the second day after admission and one week after treatment to detect various indicators. The diagnosis of interstitial lung disease (ILD) was based on the respiratory symptoms, HRCT scan findings and clinical presentations [15]. The study was approved by the Ethics Committee of the Second Hospital of Shanxi Medical University (2016KY007) and informed consent was obtained from all patients and controls.

2.2. Assessment of disease activity of PM/DM patients

The disease activity of PM/DM was evaluated by two experienced physicians, using the Myositis Disease Activity Assessment Visual Analogue Scales (MYOACT), which was established by the International Myositis Assessment and Clinical Studies (IMACS) group [16], including constitutional, cutaneous, skeletal, gastrointestinal, pulmonary, cardiovascular, muscle, extra-muscular and a global score.

2.3. Analysis of the numbers of lymphocytes

The absolute numbers of peripheral lymphocyte subgroups ($CD3^+$T/$CD4^+$T/$CD8^+$T/B/NK cells) were analyzed by our modified one-platform flow cytometry method, which is simpler and more accurate than previous two-platform methods. First, we added 50 μL EDTA-anticoagulated venous blood by reverse pipetting into Trucount tube (Becton-Dickinson, USA) A and B respectively, which itself could eliminate the variation produced by adding reference beads manually. Next, anti-CD3/CD4/CD8/CD45 antibodies and anti-CD3/CD16/CD56/CD45/CD19 antibodies were added into tube A and tube B, respectively. Finally, cells were collected by flow cytometry (BD FACSCalibur) and detected by MultiSET software. For $CD4^+$T subsets (Th1/Th2/Th17/Treg cells), we, first, added Ionomycin, PMA and GolgiStop into heparin-anticoagulated venous blood to stimulate Th1/Th2/Th17 cells. Then, cells were labeled by anti-CD4 antibody, followed by 1 mL fresh prepared Fixation/Permeabilization. Finally, we stained cells using anti-IFN-γ/IL-4/IL-17 antibodies to identify Th1/Th2/Th17 cells. Treg cells in 80 μL heparin-anticoagulated venous blood were labeled by anti-CD4 and anti-CD25 antibodies, followed by 1 mL fresh prepared Fixation/Permeabilization, and then were stained by anti-FoxP3 antibody. We detected $CD4^+$T subsets by flow cytometry (BD FACSCalibur) and analyzed them by MultiSET software. The calculation formula is that the number of $CD4^+$T subsets = the number of $CD4^+$T cells * the percentage of $CD4^+$T subsets.

2.4. Analysis of cytokines

The levels of serum cytokines [interleukin-2 (IL-2), IL-4, IL-6, IL-10, tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ) and IL-17] in PM/DM (serums were kept at −80 °C until analysis) were detected by magnetic bead-based multiplex immunoassay using Human Th1/Th2/Th17 subgroup test kit (JIANGXI CELLGENE BIOTECH CO., LTD) according to the manufacturer's protocol. The Bio-Plex 200 reader was used to acquire the data of cytokines, which was output as Median Fluorescence Intensity (MFI) and concentration (pg/mL) using the Bio-Plex Manager software.

2.5. Statistical analysis

All data were analyzed by SPSS22.0 and/or Graphpad Prism 6. Continuous data were expressed as Mean ± standard deviation (M ± SD) or Median (Q_{25}, Q_{75}). Categorical variables were reported as numbers. Results in three groups were compared by one-way analysis of variance (ANOVA) or the Independent-samples Kruskal-Wallis test one-way ANOVA. Paired-samples *t*-test or paired-samples Wilcoxon test was used for comparison of changes before and after treatment. Independent-samples *t*-test or Mann-Whitney *U* test was used to compare the differences between two groups. Spearman's rank correlation test was used to evaluate the correlation between MYOACT and other indicators. The correlation coefficient of 0.1 to 0.3 was weak correlation, 0.3 to 0.5 was moderate correlation, and 0.5 to 1.0 was strong correlation. Binary logistic regression was conducted to determine which cell was an independent risk factor for ILD in PM/DM. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Characteristics and demographic data of patients and HCs

Table 1 summarized the PM/DM patients' baseline characteristics and demographic data. Proximal muscle weakness was present in 88.73%, rash in 81.69%, arthritis in 45.07%, dysphagia in 32.39% and myositis-associated ILD in 26.76% of all myositis patients. Mechanic's hands and Raynaud's phenomenon were only observed in DM and accounted for 3.28% and 4.92% of DM, respectively.

3.2. Manifestations of various indicators in PM and DM

The numbers of $CD3^+$T ($P = 0.015$, $P = 0.002$) and $CD4^+$T ($P = 0.038$, $P = 0.018$) were markedly decreased in PM/DM. While $CD8^+$T ($P = 0.004$) and NK ($P < 0.001$) cells were only significantly reduced in DM, and B cells ($P = 0.012$) were only obviously lower in PM than that in HCs (Additional file 1). Furthermore, the number and percentage of Treg cells in PM/DM, and those of Th1 cells in DM also showed a significant decline compared with HCs ($P < 0.05$), but Th2 and Th17 cells had no significant changes (Fig. 1). Intriguingly, the ratio of Th17/Treg in PM/DM ($P = 0.031$, $P = 0.003$) was significantly higher than that in HCs (Additional file 1). No significant difference of the above cells was observed between PM and DM patients ($P > 0.05$). Notably, the concentration of soluble interleukin-2 receptor α (sIL-2Rα) in PM/DM ($P = 0.001$, $P < 0.001$) was greatly higher than that in HCs. Corresponding to this, the level of IL-2 in patients with PM/DM was significantly lower than that in HCs ($P < 0.001$, $P < 0.001$). Other cytokines, however, including IL-4 ($P = 0.001$), IL-6 ($P = 0.035$, $P < 0.001$), IL-10 ($P = 0.027$, $P < 0.001$), TNF-α ($P < 0.001$), IFN-γ ($P < 0.001$, $P < 0.001$) and IL-17 ($P = 0.004$, $P < 0.001$), in PM/DM were obviously higher than HCs, except for IL-4 ($P = 0.093$) and TNF-α ($P = 0.487$) in PM (Additional file 2).

Table 1
Laboratory data and clinical manifestations of PM, DM and HCs.

	PM (n = 10)	DM (n = 61)	HCs (n = 30)	*P*		
				PM vs. HCs	DM vs. HCs	PM vs. DM
Age (years)[a]	48.00 (34.00,59.25)	56.00 (47.00,63.50)	53.00 (48.00,60.25)		0.225	
Female/male[b]	5/5	42/19	20/10		0.521	
WBC ($*10^9$/L)[a]	7.25 (5.42, 8.20)	7.49 (5.50, 9.34)	5.83 (4.73, 6.72)	0.283	0.004	1.000
NEUT ($*10^9$/L)[a]	5.48 (3.58, 6.74)	5.58 (3.49, 7.04)	3.28 (2.57, 3.96)	0.013	< 0.001	1.000
LYMP ($*10^9$/L)[a]	0.85 (0.45, 1.93)	1.15 (0.83, 1.76)	1.91 (1.70, 2.27)	0.002	< 0.001	1.000
PLT ($*10^9$/L)[a]	282.00 (182.25, 314.75)	240.00 (174.00, 306.00)	245.50 (218.25, 270.25)		0.791	
RDW (fL)[a]	46.20 (43.15, 52.10)	44.70 (41.88, 48.43)	41.30 (40.48, 43.38)	0.010	0.001	1.000
ESR (mm/h)[a]	73.00 (26.25, 120.00)	40.00 (23.25, 71.50)	12.50 (6.00, 15.00)	< 0.001	< 0.001	0.943
CRP (mg/L)[a]	18.95 (9.31, 74.63)	12.85 (5.08, 49.55)	2.73 (1.88, 3.91)	< 0.001	< 0.001	0.984
ALT (U/L)[c]	50.10 (25.70, 63.50)	34.75 (16.35, 65.95)	–	–	–	0.412
AST (U/L)[c]	35.25 (26.35, 62.58)	36.30 (25.78, 72.50)	–	–	–	0.880
CK (U/L)[c]	536.50 (38.00, 2291.50)	56.00 (35.77, 354.00)	–	–	–	0.111
LDH (U/L)[c]	406.50 (324.00, 632.63)	338.00 (262.00, 476.00)	–	–	–	0.168
HBDH (U/L)[c]	286.50 (223.00, 527.48)	247.00 (195.50, 328.50)	–	–	–	0.116
IgG (g/L)[c]	15.70 (6.57, 17.85)	13.50 (9.65, 16.05)	–	–	–	0.765
IgA (g/L)[c]	2.48 (1.15, 4.86)	2.39 (1.72, 3.44)	–	–	–	0.911
IgM (g/L)[c]	1.02 (0.48, 1.37)	1.08 (0.83, 1.72)	–	–	–	0.217
sIL-2Rα (pg/mL)[a]	330.05 (262.73, 584.35)	497.85 (293.98, 703.53)	119.50 (95.17, 145.05)	0.001	< 0.001	1.000
Muscle weakness[b]	8	55	–	–	–	0.687
Rash[b]	3	55	–	–	–	< 0001
Mechanic's hands[b]	0	2	–	–	–	1.000
Raynaud's phenomenon[b]	0	3	–	–	–	1.000
Arthritis[b]	3	29	–	–	–	0.490
Interstitial lung disease[b]	2	17	–	–	–	0.892
Dysphagia[b]	3	20	–	–	–	1.000

Data were reported as number or mean ± SD or median (IQR).
Abbreviations: WBC: white blood cell; NEUT: neutrophile granulocyte; LYMP: lymphocyte; PLT: platelet; RDW: red cell distribution width; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; ALT: alanine aminotransferase; AST: aspartate aminotransferase; CK: creatine kinase; LDH: lactate dehydrogenase; HBDH: hydroxybutyrate dehydrogenase; sIL-2Rα: soluble interleukin-2 receptor α.
[a] Intergroup and within-group data were compared by the Independent-Samples Kruskal-Wallis test one-way ANOVA.
[b] Differences of categorical variables were measured by the Chi-square test.
[c] Data between both groups were compared using the Mann-Whitney *U* test.

3.3. Comparison of conventional treatment and short-term and low-dose IL-2 treatment in DM

Since only 10 PM patients were recruited in the study, we only discussed the changes of various indicators in DM before and after treatment. Twenty-six DM patients received only conventional treatment while 35 DM were given ld-IL2 combined with conventional drugs. After conventional treatment, the numbers of lymphocyte subsets (except for B cells) showed downward trends, as well as the numbers of Th1, Th2 and Th17 cells, but no significant difference existed. The number of Treg cells remained the same as before treatment (Additional file 3). As a result, the numbers of Th1, Th2 and Treg cells after conventional treatment were significantly lowered than HCs ($P = 0.016$, $P = 0.033$, $P = 0.038$, respectively) (Fig. 2). Contrary to conventional treatment, the lymphocyte subsets (except for NK cells) of DM receiving ld-IL2 combination therapy have sharply proliferated and the numbers of $CD3^+T$, $CD4^+T$ and B cells returned to normal levels. The numbers of $CD4^+T$ subsets were also significantly increased (Fig. 2), as well as the percentage of Treg cells ($P = 0.048$) (Additional file 4). The levels of CRP ($P < 0.001$, $P = 0.028$) and ESR ($P < 0.001$, $P = 0.001$) were down-regulated obviously in both conventional therapy and ld-IL2 combination therapy group, and the former returned to normal level but the latter was still higher than HCs. It is worth noting that the serum concentration of CK ($P < 0.001$, $P < 0.001$), which reflects the disease activity of myopathy, as well as LDH ($P = 0.002$, $P = 0.001$) and HBDH ($P = 0.006$, $P = 0.025$), belonging to muscle enzymes, significantly dropped in DM who received conventional therapy and ld-IL2 combination therapy. Moreover, the level of sIL-2Rα reduced significantly from 558.89 ± 250.15 (pg/mL) to 475.54 ± 167.13 (pg/mL) in DM who were administrated with ld-IL2 combination therapy ($P = 0.022$). In terms of cytokines, the concentration of IL-2 was significantly up-regulated ($P = 0.002$) to normal level in DM after ld-IL2 combination therapy ($P = 0.659$). The levels of IL-4 and IL-10, in DM, also sharply increased ($P = 0.009$, $P = 0.048$), but no significant changes in IL-6, TNF-α, IFN-γ and IL-17 were found (Fig. 3).

In our study, DM patients treated by ld-IL2 were divided into 18 new-onset cases and 17 treated patients based on disease history. The changes of multiple indicators in new and treated DM patients before and after ld-IL2 combination therapy were also investigated. After ld-IL2, the numbers of $CD3^+T$ ($P = 0.019$), $CD4^+T$ ($P < 0.001$) and Treg cells ($P = 0.015$) increased to normal level in new DM patients. In addition to B cells, however, no significant change about lymphocyte and $CD4^+T$ subsets was found in treated DM patients.

3.4. Association between various indicators and myositis disease activity

We analyzed the disease activity of myositis patients (Additional file 5). The levels of lymphocyte subgroups, $CD4^+T$ subsets, multiple muscle enzymes, et al. were found to be associated with the disease activity of DM patients. The results showed that constitutional disease activity score was negatively correlated with the numbers of $CD3^+T$ ($r = -0.398$, $P = 0.001$), $CD4^+T$ ($r = -0.400$, $P = 0.001$), $CD8^+T$ ($r = -0.298$, $P = 0.020$), NK ($r = -0.347$, $P = 0.006$), Th1 ($r = -0.337$, $P = 0.008$), Th2 cells ($r = -0.303$, $P = 0.018$), but it was positively associated with the percentage of B cells ($r = 0.314$, $P = 0.014$), and the levels of ALT, AST, LDH and HBDH ($r = 0.282$, $P = 0.029$; $r = 0.359$, $P = 0.005$; $r = 0.317$, $P = 0.013$; $r = 0.341$, $P = 0.007$, respectively). With regard to cutaneous disease activity score, negative correlations were found with the numbers of $CD3^+T$ ($r = -0.336$, $P = 0.008$), $CD4^+T$ ($r = -0.314$, $P = 0.014$), $CD8^+T$ ($r = -0.325$, $P = 0.011$), Th1 ($r = -0.267$, $P = 0.037$), Th2

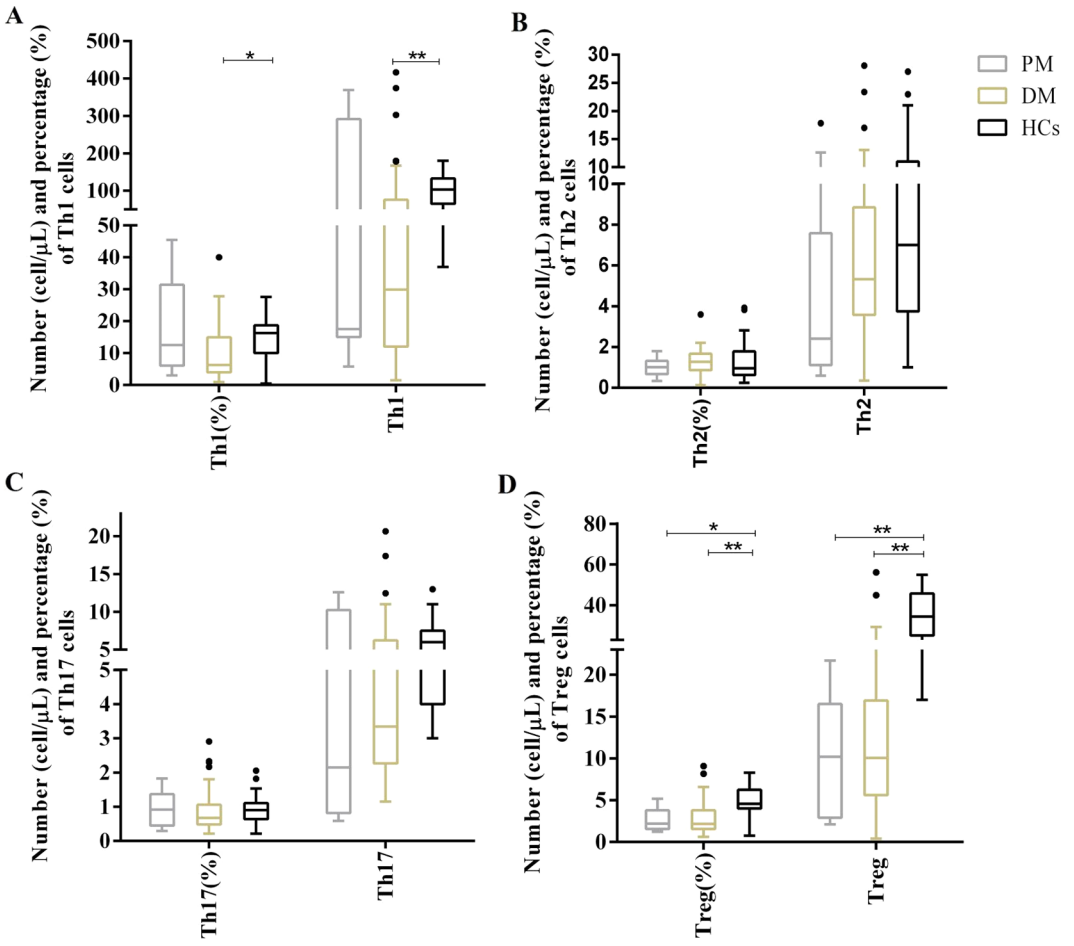

Fig. 1. Differences of the number and percentage of CD4$^+$T subsets among PM (n = 10), DM (n = 61), and healthy controls (n = 30). Data were presented as Median (IQR) and were compared by the Independent-Samples Kruskal-Wallis test one-way ANOVA. *P < 0.05, **P < 0.001. P < 0.05 was considered statistically significant.

(r = −0.296, P = 0.021) and Treg cells (r = −0.315, P = 0.013), and positive correlation was found with the percentage of B cells (r = 0.426, P = 0.001). As for skeletal disease activity score, no correlation was found with lymphocyte and CD4$^+$T subsets, but it was positively correlated with the levels of ESR, CRP, LDH and HBDH (r = 0.392, P = 0.002; r = 0.342, P = 0.010; r = 0.276, P = 0.031; r = 0.265, P = 0.039, respectively). Furthermore, gastrointestinal disease activity score was inversely correlated with the numbers of CD8$^+$T (r = −0.313, P = 0.014), NK (r = −0.272, P = 0.034) and Th1 cells (r = −0.329, P = 0.010), but it was associated with the percentage of B cells (r = 0.333, P = 0.009) positively. In addition, negative correlations were found between pulmonary disease activity score and the numbers of CD3$^+$T, CD4$^+$T, CD8$^+$T, Th1, Th17, Treg cells (r = −0.352, P = 0.005; r = −0.334, P = 0.009; r = −0.311, P = 0.015; r = −0.375, P = 0.003; r = −0.328, P = 0.017; r = −0.284, P = 0.027, respectively). No correlation was found in

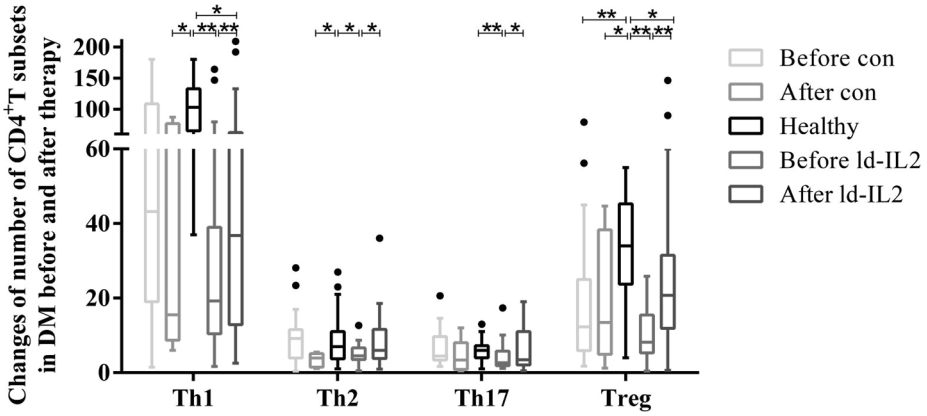

Fig. 2. Comparison of CD4$^+$T subsets in healthy controls (HCs) and DM patients before and after conventional and low-dose IL-2 (ld-IL2) treatment. Data were expressed as Median (Q_{25}, Q_{75}) and were analyzed by the Independent-Samples Kruskal-Wallis test one-way ANOVA. *P < 0.05;**P < 0.001. P < 0.05 was considered statistically significant.

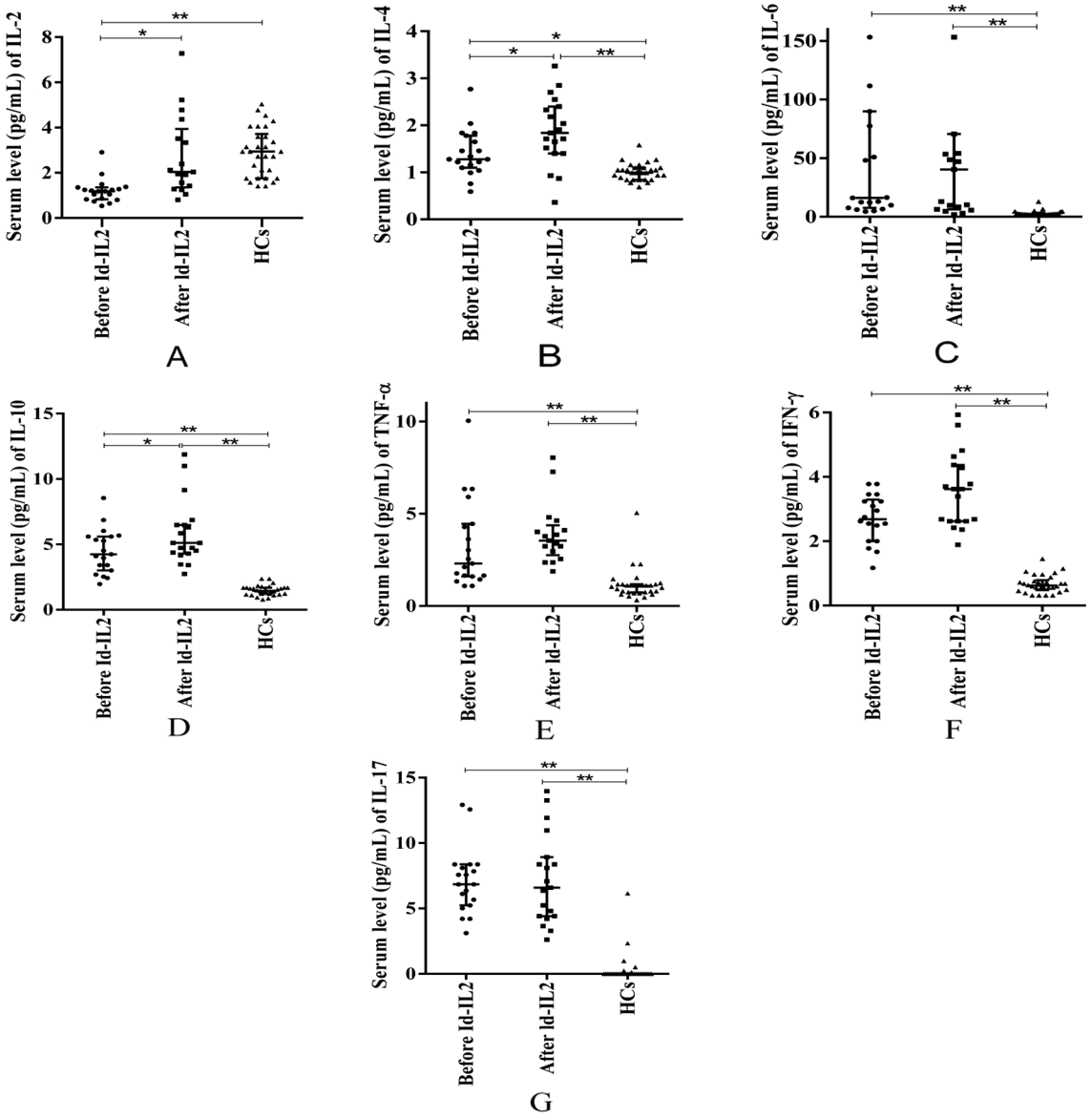

Fig. 3. Comparison of serum concentrations of cytokine (including IL-2, IL-4, IL-6, IL-10, TNF-α, IFN-γ and IL-17) between healthy controls (HCs) and DM patients before and after low-dose IL-2 (ld-IL2) combination therapy. Paired sample *T* test and Wilcoxon symbolic rank test were used to compare the levels of serum cytokines between DM patients before and after ld-IL2 injection. Mann-Whitney *U* test was used to compare the levels of serum cytokines between HCs and DM patients after ld-IL2 combination treatment. *$P < 0.05$; **$P < 0.001$. $P < 0.05$ was considered statistically significant.

cardiovascular disease activity with other indicators. Intriguingly, serum concentration of IL-2 was strongly correlated with muscle disease activity score ($r = -0.503$, $P = 0.024$) (Fig. 4A). Our results also showed negative correlations between extra-muscular score and the numbers of $CD3^+T$, $CD4^+T$, $CD8^+T$, Th1, Th2 and Th17 cells ($r = -0.384$, $P = 0.002$; $r = -0.363$, $P = 0.004$; $r = -0.353$, $P = 0.005$; $r = -0.360$, $P = 0.004$; $r = -0.290$, $P = 0.024$; $r = -0.277$, $P = 0.046$, respectively), but the former was positively associated with the percentage of B cells ($r = 0.255$, $P = 0.047$) and the level of AST ($r = 0.291$, $P = 0.024$). Global score was associated with the numbers of $CD3^+T$ ($r = -0.388$, $P = 0.002$), $CD4^+T$ ($r = -0.360$, $P = 0.004$), $CD8^+T$ ($r = -0.364$, $P = 0.004$), Th1 ($r = -0.386$, $P = 0.002$), Th2 ($r = -0.282$, $P = 0.028$), Treg cells ($r = -0.262$, $P = 0.041$) negatively, but correlated with AST positively ($r = 0.289$, $P = 0.025$) (Fig. 4B).

3.5. Analysis of the role of lymphocyte subgroups and $CD4^+T$ subsets in contributing to ILD in myositis patients

To reveal the role of lymphocyte and $CD4^+T$ subsets in contributing to ILD in myositis patients, logistic regression analysis was performed. The numbers of $CD3^+T$, $CD8^+T$ and Treg cells were significantly lower in ILD subsets by univariate analysis. Next, multivariate analysis was conducted. The above-described meaningful cell subsets were included as independent variables, and the presence or absence of ILD as the dependent variable. The results showed only Treg cells were most significantly associated with the occurrence of ILD in PM/DM [$\beta = -0.129$, $P = 0.009$, odds ratio (OR) 0.879, 95% confidence interval (CI) 0.798–0.968]. Based on this, a receiver operating characteristic (ROC) curves was drawn to assess the accuracy of Treg cells in predicting whether or not patients with PM/DM have ILD, and the area under the ROC curve (AUC) was 0.720, sensitivity and specificity were 89.47%, 53,85%, respectively (Fig. 5).

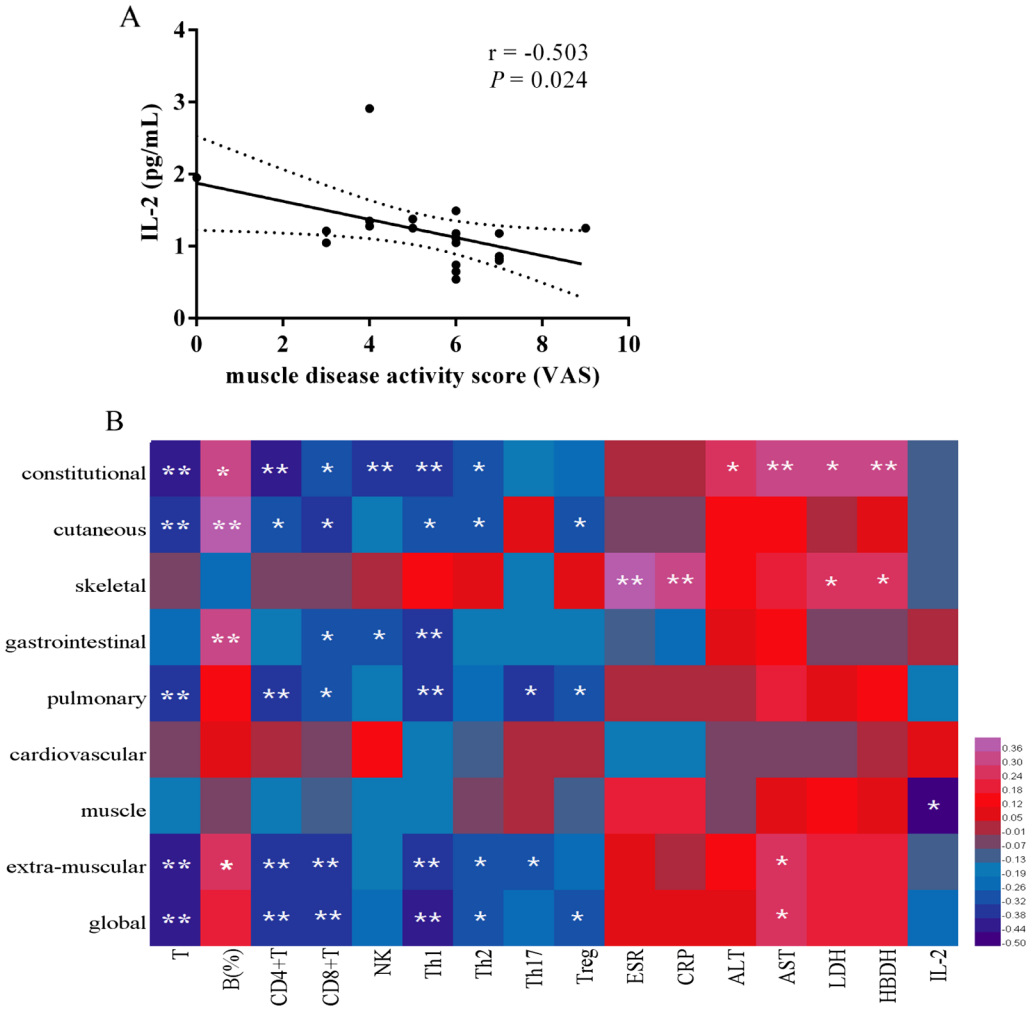

Fig. 4. (A) The correlation between serum IL-2 and muscle disease activity score (VAS). Shown are linear regression lines with interpolated 95% confidence interval curves (broken lines). (B) Correlations between multiple indicators and MYOACT. Heatmap representation of correlation of MYOACT (including constitutional, cutaneous, skeletal, gastrointestinal, pulmonary, cardiovascular, muscle, extra-muscular and a global MYOACT score) with lymphocyte subpopulation (including $CD3^+$T, B, $CD4^+$T, $CD8^+$T and NK cells), $CD4^+$T subsets (including Th1, Th2, Th17 and Treg cells), Erythrocyte Sedimentation Rate (ESR), C-Reactive Protein (CRP), Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST), Lactate Dehydrogenase (LDH) and Hydrox ybutyrate Dehydrogenase (HBDH) in DM patients (n = 61). $^*P < 0.05$, $^{**}P < 0.01$ by Spearman correlation test.

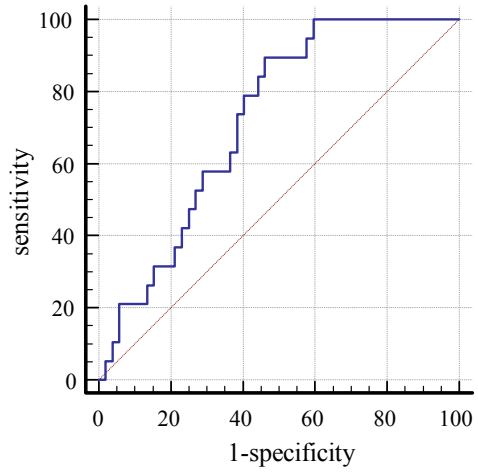

Fig. 5. Receiver operating characteristic (ROC) curve of Treg cells for predicting the occurrence of interstitial lung disease (ILD) in myositis patients. The area under the ROC curve (AUC) was 0.720, sensitivity and specificity were 89.47%, 53.85%, respectively.

3.6. Comparison of the number of Treg cells labeled by different forms

Treg cells were defined by different forms in several studies, including $CD4^+CD25^+$, $CD4^+FoxP3^+$, $CD4^+CD25^+FoxP3^+$, etc. In our study, Treg cells were determined by $CD4^+CD25^+FoxP3^+$T cells. Different definitions of Treg cells may contribute to controversial results. We compared the number of Treg cells labeled by $CD4^+CD25^+$ and $CD4^+CD25^+FoxP3^+$ in all myositis patients. The results showed that the number of Treg cells labeled by $CD4^+CD25^+FoxP3^+$ was markedly lower than that marked by $CD4^+CD25^+$ [(9.09 (5.08,16.40) vs. 12.64 (6.62,22.31), $P = 0.048$].

4. Discussion

The results of our study demonstrated 1) the number of Treg cells in PM/DM patients was only about one-third that of HCs, while no obvious change was found in Th17 cells; 2) Opposite to sIL-2Rα, the serum concentration of IL-2 in PM/DM was significantly lower than that in HCs; 3) ld-IL-2 could up-regulate lymphocyte cells, especially Treg cells (expanded 2.5 times); 4) In general, patients' disease activity was negatively correlated with T cell subsets, IL-2, ESR and CRP, but it was positively associated with the percentage of B cells and the levels of muscle enzymes; 5) the deficiency of Treg cells was an independent risk factor for the occurrence of ILD in myositis patients, and Treg cells could be used to assist in the diagnosis of ILD in myositis patients.

Although the pathogenesis of PM/DM is not well clear thus far, there is a body of evidence that the onset of PM/DM is due to the disruption of immune balance [17–19]. Both the over-activation of T helpers and the reduction of Treg cells could lead to immune imbalance, but the exact cause remains to be determined. Limited number of samples made our results cannot represent the truth of pathogenesis of PM/DM, but can reflect certain trends to some extent. Consistent with our findings, a body of studies have confirmed that

lymphocytopenia (low levels of $CD3^+T$, $CD4^+T$, $CD8^+T$, B and NK cells) was common in DM[2,20], indicating that immune hyperfunction is not the main reason of PM/DM. We found, interestingly, the absolute number of Treg cells was fivefold more than Th17 cells among the $CD4^+T$ subsets in HCs, implying that Treg cells play a more important role in maintaining self-immune-balance rather than Th17 cells. Furthermore, the number and percentage of Treg cells in PM/DM were significantly lower than those in HCs, but no obvious change was found in Th17 cells. As a result, the ratio of Th17/Treg in PM/DM was markedly increased compared than HCs, further implying that immune imbalance may be caused by Treg cells reduction rather than the overactivation of T helpers in PM/DM and providing a theoretical basis for the idea that we should use immunoregulation to induce immune tolerance rather than immunosuppression therapy. In addition, the suppressive function of Treg cells need to be further verified. The concentration of sIL-2Rα, which could compete with membrane surface IL-2Rα to bind to IL-2, has been observed to be up-regulated in many autoimmune disease [21,22], and sIL-2Rα could be used to assess myositis activity [21]. Jing He and Masayuki Mizui observed the production of IL-2 was reduced in patients with SLE [23,24]. Similarly, we also found elevated serum sIL-2Rα and decreased IL-2 in myositis patients. In autoimmune hepatitis (AIH) patients, IL-2 deficiency has been reported to trigger apoptosis of Treg cells [25]. Thus, we hypothesize that the decline of Treg cells in PM/DM may be partly caused by the increased apoptosis of Treg cells, which is arisen by IL-2 deficiency. Furthermore, many researches have also found serum concentrations of IL-4, IL-6, IL-10, TNF-α and IFN-γ were over-expressed in many autoimmune diseases, such as multiple sclerosis (MS), rheumatoid arthritis (RA), systemic lupus erythematosus (SLE) and idiopathic inflammatory myopathies (IIM) [5–9]. IL-6, a cytokine with pro-inflammatory and anti-inflammatory role, was confirmed as a contributor to muscle cell reduction and could be served as a biomarker to predict disease activity of adult and juvenile DM [26,27].

Conventional treatment for PM/DM relies upon the empirical application of corticosteroids and immunosuppressive agents, accompanying with substantial side-effects, such as infection and cancer [28]. Moreover, corticosteroid itself is related with the occurrence of myopathy, including muscle weakness [28,29], and quite a few people are refractory to conventional therapy [30]. In recent years, IL-2 was confirmed that it has dual and opposing role in immunity. High-level IL-2 could promote the development of effector cells whereas low-dose IL-2 was believed to be safe in vivo and to play a critical role in increasing immune tolerance by enhancing Treg cells differentiation, function and survival [23,31]. Owing to the deficiency of Treg cells and reduced production of serum IL-2 in PM/DM, it was reasonable that therapeutic strategies of PM/DM turn to using ld-IL2. The application of ld-IL2, however, for the management of PM/DM are still unreported, the study conducted by us was the first to investigate the regulatory effect of ld-IL2 on lymphocyte and $CD4^+T$ subsets in PM/DM. Peripheral lymphocyte subgroups (except for NK cells) and $CD4^+T$ subsets were significantly increased in DM who received ld-IL2 combination therapy, and Treg cells' amplification was most pronounced, approximately 2.5 times of that before treatment, indicating that Treg cells were highly sensitive to IL-2. Jing He et al. also demonstrated that not only could ld-IL2 enhance the abundance and the suppressive function of Treg cells, but it markedly alleviate the disease activity of SLE patients [24]. The application of ld-IL2 not only enhance immune tolerance by increasing the number of Treg cells, but also reduce the risk of infection by suitably increasing the number of effector T cells, which was an indirect evidence that immunoregulation therapy using ld-IL2 was more superior than immunosuppressive therapy for autoimmune diseases. Furthermore, the level of sIL-2Rα decreased greatly in DM after ld-IL2 administration, which was also a good sign for patients. The serum level of IL-2 was elevated to normal level after the administration of ld-IL2, which may be due to the injection of rhIL-2 and/or the proliferation of Th1 cells after ld-IL2 combination therapy. The concentration of IL-4 and IL-10 were also obviously elevated after the injection of ld-IL2 in DM. We hypothesized that this might be due to the increased number of Th2 cells after ld-IL2 administration. Although there was no significant difference, the lymphocyte and $CD4^+T$ subsets, on the contrary, showed a downward trend in DM who only received conventional drugs. That is to say, the problem of immune intolerance still exists. We hypothesized that the lack of statistical differences in the data may be attributed to the small sample size of this group. Moreover, the regulatory effects of ld-IL2 on lymphocyte and $CD4^+T$ subsets in new and treated DM patients were different. Treg cells remarkedly proliferated in new DM patients, but not in treated DM. The reason, we hypothesized, might be that treated DM patients used to be administrated with immunosuppressive agents, leading to the decrease of pre-Treg cells. We also found the proliferation of Treg cells was the major part of $CD4^+T$ cell expansion for new DM patients. Thus, we guess ld-IL2 was more efficient for patients without history of treatment.

In current study, multiple indicators were found to be associated with MYOACT in DM, implying the engagement of these indicators in the occurrence of DM. Our results demonstrated that, in brief, the deficiency of lymphocytes other than B cells, which could speed up the progression of autoimmune diseases by producing auto-antibodies, mainly increase constitutional, cutaneous, gastrointestinal, pulmonary disease activity. Consistent with our results, other studies also found associations between increased disease activity and decreased numbers of $CD3^+T$, $CD4^+T$ and $CD8^+T$ cells, which suggested that occurrence of PM/DM may not be due to the activation of effector cells [2]. Furthermore, our results demonstrated that the levels of ESR, CRP, LDH and HBDH could reflect joint involvement in some degree. Reduction of IL-2, notably, was a strong risk factor for muscle damage, which strengthened our belief that the development of DM (especially muscle damage) was closely related to the lack of IL-2, and gave us more confidence to treat DM with ld-IL2. Evaluation of these markers, which could reflect the disease activity of DM to some extent, is very useful in assisting clinicians in making clinical decisions.

As we all know, PM and DM are usually complicated by ILD [32], which lead to increased morbidity and mortality of patients with myositis patients [33]. Thus, we also investigated that whether the occurrence of ILD was associated with the reduction of lymphocyte and $CD4^+T$ subsets. Our results showed that Treg cells [$\beta = -0.129$, $P = 0.009$, OR 0.879, 95% CI 0.798–0.968] were an independent protective factor against ILD in patients with myositis. In other words, the reduction of Treg cells was a risk factor for the happening of ILD in myositis patients. In addition, a study conducted by Kotsianidis et al. has confirmed that patients with idiopathic pulmonary fibrosis (IPF) had impaired abundance and function of Treg cells, which were closely related to the degree of pulmonary fibrosis [34], suggesting the important role of Treg cells in inhibiting pulmonary fibrosis.

Another advantage of our study was that Treg cells were labeled by anti-CD4/CD25 /FoxP3 antibodies. FoxP3, regarded as the most specific marker of Treg cells, was truly important for the suppressive function of Treg cells [35,36]. Our results showed that the number of Treg cells defined as $CD4^+CD25^+FoxP3^+$ was obviously lower than those labeled by $CD4^+CD25^+$, suggesting that $CD4^+CD25^+T$ cells could not exhibit the true level of Treg cells. Moreover, we observed, in our study, the changes of the proportion and absolute number of cells were sometimes not very consistent, and the change of the percentage of one subset is not only absolutely due to changes in its cell number, but the changes in the number of other cells. Thus, proportion of cells should not completely replace the absolute number of cells to represent the cellular level, and we should pay more attention to the absolute number of cells.

Despite the remarkable and clinically relevant findings in this study, there are some drawbacks to our study. First, the number of patients, especially PM, was not large. We further need to expand the sample size to study the pathogenesis of PM/DM. Secondly, patients were administrated with ld-IL2 therapy for a short time, and the long-term effects of

ld-IL2 combination therapy require further observation.

In conclusion, the size of peripheral Treg cells pool was contracted while Th17 cells had no significant difference in PM/DM. ld-IL2 combination treatment could up-regulate Treg cells to restore immune balance and decrease serum enzymes concentration related to DM disease activity. The reduction of Treg cells was the independent risk factor for the happening of ILD in myositis patients.

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Declaration of Competing Interest

None.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2019.105912.

References

[1] F. Suzuki, T. Kubota, Y. Miyazaki, K. Ishikawa, M. Ebisawa, S. Hirohata, et al., Serum level of soluble CX3CL1/fractalkine is elevated in patients with polymyositis and dermatomyositis, which is correlated with disease activity, Arthritis. Res. Ther. 14 (2) (2012) R48.
[2] D.X. Wang, X. Lu, N. Zu, B. Lin, L.Y. Wang, X.M. Shu, et al., Clinical significance of peripheral blood lymphocyte subsets in patients with polymyositis and dermatomyositis, Clin. Rheumatol. 31 (12) (2012) 1691–1697.
[3] S.G. Zheng, Regulatory T cells vs Th17: differentiation of Th17 versus Treg, are the mutually exclusive? Am. J. Clin. Exp. Immunol. 2 (1) (2013) 94–106.
[4] O. Boyman, J. Sprent, The role of interleukin-2 during homeostasis and activation of the immune system, Nat. Rev. Immunol. 12 (3) (2012) 180–190.
[5] Y. Shimojima, W. Ishii, M. Matsuda, S. Ikeda, Phenotypes of peripheral Blood Lymphocytes and cytokine expression in polymyositis and Dermatomyositis before Treatment and after clinical Remission, Clin. Med. Insights. Arthritis. Musculoskelet Disord. 5 (2012) 77–87.
[6] M. Yang, X. Cen, Q. Xie, C. Zuo, G. Shi, G. Yin, Serum Interleukin-6 Expression Level and Its Clinical Significance in Patients with Dermatomyositis, Clin. Dev. Immunol. 2013 (2013) 717808.
[7] A. Sabry, H. Sheashaa, A. El-Husseini, K. Mahmoud, K.F. Eldahshan, S.K. George, et al., Proinflammatory cytokines (TNF-and IL-6) in Egyptian patients with SLE: its correlation with disease activity, Cytokine 35 (3–4) (2006) 148–153.
[8] A. Notarnicola, G. Lapadula, D. Natuzzi, I.E. Lundberg, F. Iannone, Correlation between serum levels of IL-15 and IL-17 in patients with idiopathic inflammatory myopathies, Scand. J. Rheumatol. 44 (3) (2015) 224–228.
[9] M. Tucci, C. Quatraro, F. Dammacco, F. Silvestris, Interleukin-18 overexpression as a hallmark of the activity of autoimmune inflammatory myopathies, Clin. Exp. Immunol. 146 (1) (2006) 21–31.
[10] A. Ballesteros-Tato, Beyond regulatory T cells: the potential role for IL-2 to deplete Tfollicular helper cells and treat autoimmune diseases, Immunotherapy 6 (11) (2014) 1207–1220.
[11] D. Klatzmann, A.K. Abbas, The promise of low-dose interleukin-2 therapy for autoimmune and inflammatory diseases, Nat. Rev. Immunol. 15 (5) (2015) 283–294.
[12] M. Miao, Z. Hao, Y. Guo, X. Zhang, S. Zhang, J. Luo, et al., Short-term and low-dose IL-2 therapy restores the Th17/Treg balance in the peripheral blood of patients with primary Sjögren's syndrome, Ann. Rheum. Dis. 77 (12) (2018) 1838–1840.
[13] M. Shao, X.L. Sun, H. Sun, J. He, R.J. Zhang, X. Zhang, et al., Clinical Relevance of Autoantibodies against Interleukin-2 in Patients with Systemic Lupus Erythematosus, Chin. Med. J. (Engl) 131 (13) (2018) 1520–1526.
[14] M.Z. Gao, Y.L. Huang, X.D. Wu, Q.W. Xu, R. Ji, B. Gu, et al., Red blood cell distribution width and neutrophil to lymphocyte ratio are correlated with disease activity of dermatomyositis and polymyositis, J. Clin. Lab. Anal. 32 (1) (2018).
[15] M. Chen, C. Quan, L. Diao, F. Xue, K. Xue, B. Wang, et al., Measurement of cytokines and chemokines and association with clinical severity of dermatomyositis and clinically amyopathic dermatomyositis, Br. J. Dermatol. 179 (6) (2018) 1334–1341.
[16] D.A. Isenberg, E. Allen, V. Farewell, M.R. Ehrenstein, M.G. Hanna, I.E. Lundberg, et al., International consensus outcome measures for patients with idiopathic inflammatory myopathies development and initial validation of myositis activity and damage indices in patients with adult onset disease, Rheumatology (Oxford) 43 (1) (2004) 49–54.
[17] A.M. Brunasso, W. Aberer, C. Massone, New onset of dermatomyositis/polymyositis during anti-TNF-α therapies: a systematic literature review, Sci. World J. 179180 (2014).
[18] M.J. Bendewald, D.A. Wetter, X. Li, M.D. Davis, Incidence of dermatomyositis and clinically amyopathic dermatomyositis: a population-based study in Olmsted County, Minnesota, Arch. Dermatol. 146 (1) (2010) 26–30.
[19] N. Martin, C.K. Li, L.R. Wedderburn, Juvenile dermatomyositis: new insights and new treatment strategies, Ther. Adv. Musculoskelet. Dis. 4 (1) (2012) 41–50.
[20] M. Viguier, S. Fouéré, P. de. la Salmonière, C. Rabian, C. Lebbe, L. Dubertret, et al., Peripheral blood lymphocyte subset counts in patients with dermatomyositis: clinical correlations and changes following therapy, Medicine (Baltimore) 82 (2) (2003) 82–86.
[21] A. Tournadre, J.J. Dubost, M. Soubrier, M. Ruivard, P. Souteyrand, J. Schmidt, et al., Soluble IL-2 receptor: a biomarker for assessing myositis activity, Dis. Markers (2014) 472624.
[22] L.M. Maier, D.E. Anderson, C.A. Severson, C. Baecher-Allan, B. Healy, D.V. Liu, et al., Soluble IL-2RA levels in multiple sclerosis subjects and the effect of soluble IL-2RA on immune responses, J. Immunol. 182 (3) (2009) 1541–1547.
[23] M. Mizui, G.C. Tsokos, Low-dose IL-2 in the treatment of lupus, Curr. Rheumatol. Rep. 18 (11) (2016) 68.
[24] J. He, X. Zhang, Y. Wei, X. Sun, Y. Chen, J. Deng, et al., Low-dose interleukin-2 treatment selectively modulates CD4(+) T cell subsets in patients with systemic lupus erythematosus, Nat. Med. 22 (9) (2016) 991–993.
[25] K. John, M. Hardtke-Wolenski, E. Jaeckel, M.P. Manns, K. Schulze-Osthoff, H. Bantel, Increased apoptosis of regulatory T cells in patients with active autoimmune hepatitis, Cell. Death. Dis. 8 (12) (2017) 3219.
[26] R.C. Langen, A.M. Schols, M.C. Kelders, J.L. Van. Der. Velden, E.F. Wouters, Y.M. Janssen-Heininger, Tumor necrosis factor-inhibits myogenesis through redoxdependent and -independent pathways, Am. J. Physiol. Cell. Physiol. 283 (3) (2002) C714–21.
[27] H. Bilgic, S.R. Ytterberg, S. Amin, K.T. McNallan, J.C. Wilson, T. Koeuth, et al., Interleukin-6 and type I interferon-regulated genes and chemokines mark disease activity in dermatomyositis, Arthritis. Rheum. 60 (11) (2009) 3436–3446.
[28] A.R. Findlay, N.A. Goyal, T. Mozaffar, An overview of polymyositis and dermatomyositis, Muscle. Nerve. 51 (5) (2015) 638–656.
[29] S.B. Helmers, M. Bruton, I. Loell, A.K. Ulfgren, A.J. Gracie, I.B. McInnes, et al., Expression of interleukin-18 in muscle tissue of patients with polymyositis or dermatomyositis and effects of conventional immunosuppressive treatment, Rheumatology (Oxford) 57 (12) (2018) 2149–2157.
[30] S.K. Shinjo, F.H. de Souza, J.C. de Moraes, Dermatomyositis and polymyositis: from immunopathology to immunotherapy (immunobiologics), Rev. Bras. Reumatol. 53 (1) (2013) 101–110.
[31] A.K. Abbas, E. Trotta, D.R. Simeonov, A. Marson, J.A. Bluestone, Revisiting IL- 2: Biology and therapeutic prospects, Sci. Immunol. 3 (25) (2018).
[32] T. Gono, H. Kaneko, Y. Kawaguchi, M. Hanaoka, S. Kataoka, M. Kuwana, et al., Cytokine profiles in polymyositis and dermatomyositis complicated by rapidly progressive or chronic interstitial lung disease, Rheumatology (Oxford) 53 (12) (2014) 2196–2203.
[33] Li Zhang, Guoqin Wu, Di Gao, Guijian Liu, Lin Pan, Liyan Ni, Zheng Li, Qiang Wang, Toby M Maher, Factors associated with interstitial lung disease in patients with polymyositis and dermatomyositis: a systematic review and meta-analysis, PLoS ONE 11 (5) (2016) e0155381, https://doi.org/10.1371/journal.pone.0155381.10.1371/journal.pone.0155381.g00110.1371/journal.pone.0155381.g00210.1371/journal.pone.0155381.g00310.1371/journal.pone.0155381.g00410.1371/journal.pone.0155381.g00510.1371/journal.pone.0155381.t00110.1371/journal.pone.0155381.t00210.1371/journal.pone.0155381.s00110.1371/journal.pone.0155381.s00210.1371/journal.pone.0155381.s00310.1371/journal.pone.0155381.s004.
[34] I. Kotsianidis, E. Nakou, I. Bouchliou, A. Tzouvelekis, E. Spanoudakis, P. Steiropoulos, et al., Global impairment of CD4+CD25+FOXP3+ regulatory T cells in idiopathic pulmonary fibrosis, Am. J. Respir. Crit. Care. Med. 179 (12) (2009) 1121–1130.
[35] L. Passerini, F.R. de. Sio Santoni, M.G. Roncarolo, R. Bacchetta, Forkhead box P3: the peacekeeper of the immune system, Int. Rev. Immunol. 33 (2) (2014) 129–145.
[36] F. Ramsdell, S.F. Ziegler, FOXP3 and scurfy: how it all began, Nat. Rev. Immunol. 14 (5) (2014) 343–349.