Analysis of immune reconstitution after autologous CD34+ stem/progenitor cell transplantation for systemic sclerosis: predominant reconstitution of Th1 CD4+ T cells

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Abstract

Objective. The aim of this study is to evaluate the mechanism of long-term effect of autologous haematopoietic stem cell transplantation (ASCT) in treatment of SSc.

Methods. Eleven patients (three males and eight females) with SSc were enrolled. Blood mononuclear cells were harvested after mobilization treatment with CYC and G-CSF. CD34+ haematopoietic stem/progenitor cell fractions were purified and cryopreserved. Patients were transplanted with >2 x 10^6/kg autologous CD34+ cells after high-dose CYC (50 mg/kg for 4 days) conditioning. Immune reconstitution was evaluated serially by analysing lymphocyte subpopulations for 36 months.

Results. Progressive improvement of skin sclerosis has been observed for 3 years in most of the patients. The serum level of anti-Scl-70, an auto-antibody specific to SSc, was progressively decreased after ASCT. Improvement of skin sclerosis was significantly associated with the change in the serum anti-Scl-70 level after ASCT at 36 months. Serum levels of KL-6 and surfactant protein D, indicators for interstitial pneumonia activity, were also significantly decreased. The number of CD8+ T cells immediately recovered within a month after ASCT, while the number of CD4+ T cells remained low for >36 months post-transplant. The majority of CD4+ cells were memory but not naive T cells, and regulatory CD4+ T cells were not recovered. Th1/Th2 ratio was significantly increased after ASCT.

Conclusions. ASCT with purified CD34+ cells was effective in controlling the disease activity of SSc. Th1/Th2 ratio was significantly increased for at least 3 years after ASCT.

Key words: Autoimmune disease, High-dose cyclophosphamide, Transplantation, Immune reconstitution, Th1/Th2 balance.

Introduction

SSc is a heterogeneous autoimmune disease (AD) characterized by predominant T-cell activation, production of auto-antibodies and cytokine release [1, 2]. All of these contribute to diffuse microvascular injury, leading to diffuse sclerosis within the skin and internal organs. DcSSc and internal organ involvement often cause life-threatening status of patients [3].

Autologous haematopoietic stem cell transplantation (ASCT) was introduced for SSc treatment in 1996, and >100 patients with SSc have been treated [4]. Phase I–II studies demonstrated that improvement of skin sclerosis and stabilization of interstitial pneumonia (IP) were achieved [5–10]. We also reported the safety and efficacy of high-dose CYC with ASCT for SSc patients [11]. The complete or partial remission of SSc was maintained for at least 3 years in these reports [6–9].
The working hypotheses of the efficacy of ASCT for SSc might be due to the immune reset that should include: (i) the eradication of auto-reactive lymphocytes by immunosuppressive conditioning; and/or (ii) the correction of dysregulated immune balance by newly developed lymphocytes derived from transplanted hematopoietic stem cells [12]. However, there remains controversy regarding whether such immune reset occurs in patients treated with ASCT. Muraro et al. [13] have reported that naïve CD4+ T cells with diverse T-cell receptor repertoire, which were generated through thymus, developed after ASCT in patients with multiple sclerosis (MS). In contrast, in SSc patients, Farge et al. [14] reported the sustained reduction of CD4+ T cells and B cells after ASCT.

Furthermore, Th1/Th2 balance might also be important in understanding the disease status of SSc. In a tight skin mouse, an animal model for SSc, the stimulation of Th1 immune responses prevents the development of scleroderma-like syndrome [15]. In human SSc, a shift from Th2 to Th1 responses is correlated with improvement of the skin fibrosis by longitudinal analysis of serum cytokine concentrations [16].

These data led us to analyse immune reconstitution in SSc patients treated with ASCT. In this study, we tracked reconstitution of T- and B-cell subpopulations after ASCT with purified CD34+ hematopoietic stem/progenitor cells in SSc patients, and found that despite the resolution of clinical symptoms of SSc, patients did not achieve normalization of lymphocyte compartment, even 3 years after ASCT. All patients showed sustained reduction of CD4+ T cells, but Th1/Th2 ratio was increased during the 3-year observation. Thus, the clinical efficacy of ASCT might be dependent upon the skewed reconstitution of Th1 cells for a long time after ASCT.

**Patients and methods**

The study was approved by the ethics committee of Kyushu University Hospital. Written informed consent was obtained from all patients according to the Declaration of Helsinki.

**Patients and eligibility**

Eligibility of patients with SSc for ASCT was previously described [11]. Briefly, patients with SSc were eligible when they had severe diffuse SSc that had rapidly developed over the previous 4 years. They also had to have at least one of the following organ involvements: (i) pulmonary, (ii) cardiac or (iii) renal involvement. Patients with limited SSc were considered eligible when progressive and life-threatening IP was present. Patients were excluded from the study when they had uncontrolled arrhythmia, severe heart failure, pulmonary hypertension, DLCO <20% of predicted and renal failure as previously described [11]. All patients were followed up for at least 36 months after transplantation for the evaluation of immune reconstitution. Blood was obtained from healthy donors (after informed consent, n = 10) to determine the reference values of lymphocyte subpopulations and Th1/Th2 balance, and from SSc patients (after informed consent, n = 13) for FoxP3 staining.

**Peripheral blood stem cell collection and ASCT**

Peripheral blood stem cells (PBSCs) were mobilized during haematologic recovery after relatively high-dose CYC (2 g/m²) for 2 days followed by administration of recombinant human G-CSF (filgrastim; Kirin Brewery, Tokyo, Japan) as previously described [11]. After collecting PBSCs to obtain 2 × 10⁶ CD34+ cells/kg or more by apheresis, CD34+ cells were positively selected using an immunomagnetic bead with an anti-CD34 mAb (CliniMACS; Miltenyi Biotec, Germany). For pre-transplant conditioning, high-dose CYC (50 mg/kg) was given for 4 days from Day -5 to -2 and frozen-thawed CD34+ cells were transplanted on Day 0. Patients 1, 2, 3, 4 and 6 received G-CSF from Day 1 [11]. Concomitantly administered prednisolone doses were kept unchanged or gradually tapered according to the individual clinical status after ASCT. The concomitantly administered immunosuppressants were stopped before the administration of CYC for mobilization.

**Treatment outcome**

The modified Rodnan skin score (mRSS) was used to evaluate the improvement of skin sclerosis when initial mRSS was ≥15 [11]. Serum levels of anti-ScI 70 (anti-topo I) antibody were measured by an ELISA kit (MBL, Nagoya, Japan). Serum levels of KL-6 and surfactant protein D (SP-D) were measured by ELISA kits (Sanko Junyaku Co., Tokyo and Yamasa Co., Choshi, respectively, Japan). Serum levels of immunoglobulin were measured by laser nephelometry.

**Lymphocyte phenotyping**

Lymphocyte immunophenotyping of samples from whole blood was performed by IF flow cytometry before mobilization, before hematopoietic stem cell transplantation (HSCT) and 1, 3, 6, 12, 18, 24, 36 months after HSCT. The following mAbs and their combinations were used: anti-CD3-FITC; anti-CD19-phycocerythrin (PE); anti-CD20-FITC; anti-CD4-FITC; anti-CD8-PE; anti-CD45RA-PE; anti-CD45RO-PE; anti-CD25-PE; anti-CD16-FITC; anti-CD56-PE; anti-CD69-FITC and anti-CD27-FITC (eBioscience, San Diego, CA, USA). The CD4+FoxP3+ cells were detected by using anti-CD4-FITC, anti-FoxP3-PE and permeabilization buffer (eBioscience) in 9 patients at 37 °C (18.0 months) (one sample for each patient) after ASCT and 13 SSc controls without ASCT. Results were expressed as the absolute number of cells.

**Th1/Th2 balance**

Flow cytometric determination of IFN-γ and IL-4 in the cytoplasm of peripheral CD4+ T cells was performed by a previously described method [17]. Briefly, aliquots (500 µl) of heparinized whole blood were stimulated with a combination of 25 ng/ml phorbol myristate acetate (PMA) and 1 µg/ml ionomycin in the presence of brefeldin A (Sigma, St Louis, MO, USA) and cultured for 4 h at 37 °C

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**Th1 CD4+ T cells after autologous HSCT for SSc**

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in a humidified incubator containing 7% CO₂. Activated cultures were aliquoted and stained with 20 μl of peridinin chlorophyll protein-conjugated CD4-specific mAb (Becton Dickinson, San Jose, CA, USA) for 15 min at room temperature, and then treated with 2 ml of FACS lysing solution (Becton Dickinson). After a short incubation (5 min), the samples were centrifuged and combined with FACS permeabilizing solution (Becton Dickinson) for 10 min at room temperature in the dark. The sample tubes were washed twice and FITC-conjugated IFN-γ-specific mAb and PE-conjugated IL-4-specific mAb (Becton Dickinson) for 30 min at room temperature in the dark. FITC-conjugated mouse IgG2a and PE-conjugated mouse IgG1 were used as controls. After washing again, the cells were resuspended in 1% paraformaldehyde and analysed by flow cytometry. The percentage of IFN-γ or IL-4-positive cells (percentage of IFN-γ or percentage of IL-4) was counted by FACS, and Th1/Th2 balance was evaluated by a ratio of percentage of IFN-γ to percentage of IL-4 (IFN-γ/IL-4).

Cytokine and chemokine levels
Serum levels of TNF-α and TGF-β were measured with sandwich ELISA kits (Quantikine; R&D systems, Minneapolis, MN, USA), according to the manufacturer’s instructions before and after ASCT. Serum levels of IL-6 and soluble receptor for IL-2 (sIL-2R) were measured with CLISA kit (Fujirebio, Tokyo, Japan) and with ELISA kit (Kyowamedix, Tokyo, Japan), respectively.

Statistical analysis
Values were expressed as mean (s.d.). Student’s t-test was used for statistical analysis of the data. The correlations of mRSS with the level of anti-Scl-70 or Th1/Th2 balance were investigated by Pearson’s correlation coefficient test. Difference with P < 0.05 was considered to be statistically significant.

Results
Autologous transplantation with purified CD34+ mobilized blood stem cells was successfully performed without treatment-related mortality
Eleven patients, eight females and three males, were enrolled in this study. Clinical results of Patients 1–6 at 12 months after ASCT were previously reported [11]. The mean (s.d.) of mRSS was 21.5 (9.0), and all patients developed clinical IP with decreased per cent vital capacity (VC) and per cent DLCO [mean (s.d.), 64.9 (14.8) and 45.8 (18.0)%, respectively] (Table 1). The anti-Scl-70 antibody, an auto-antibody specific to diffuse SSc, was detected in 9 out of 11 patients. All patients were resistant to the standard treatment with CS, CYC and/or ciclosporin. Blood mononuclear cells were harvested after mobilization with CYC and G-CSF. CD34+ haematopoietic stem/progenitor cell fractions were purified and cryopreserved. Patients were transplanted with purified autologous CD34+ cells after high-dose CYC (500 mg/kg) conditioning. Consequently, 4.7 × 10⁶/kg CD34+ cells with 1.1 (1.2) × 10⁴/kg CD3+ T-cell contaminants were transplanted. ASCT was successfully performed without transplantation-related mortality in all patients, but Patient 7 died due to the progression of IP at 20 months after ASCT. The clinical status of the remaining 10 patients was followed up for >36 months. There was a variety of post-transplant infections such as adenoviral haemorrhagic cystitis, herpes zoster and cytomegaloviral antigaemia [18].

CD34+ ASCT treatment induced resolution of refractory SSc
The effect of ASCT on skin sclerosis was assessed by the change in mRSS. After ASCT, skin sclerosis was progressively resolved, and had improved by 72.0% at 36 months post-transplant (Fig. 1A). The vital capacity of the lung was increased from 64.9 to 77.8% at 36 months, although
DLCO was unchanged (Fig. 1B). Serum levels of KL-6 and SP-D, indicators of IP activity [19, 20], were significantly decreased (Fig. 1C and D).

Reflecting the resolution of clinical symptoms of SSc, the serum level of anti-ScI-70 progressively decreased after ASCT (Fig. 2A), independent of serum immunoglobulin levels (Fig. 2B). A significant correlation ($r = 0.52$, $P < 0.05$) was observed between the change in mRSS and that in the serum level of anti-ScI-70 at 36 months after ASCT.

Serum levels of pro-inflammatory or pro-fibrotic cytokines and a T-cell activation marker such as TNF-$\alpha$, TGF-$\beta$, IL-6 and soluble IL-2R (sIL-2R), were also decreased after ASCT (Fig. 3A-D). These data show that ASCT with purified CD34$^+$ cells is effective in controlling disease activity of SSc patients refractory to conventional immunosuppressive therapies.

The recovery of CD4$^+$ T cells was particularly retarded after ASCT with purified CD34$^+$ cells in SSc patients

Because the patients were infused with highly purified CD34$^+$ haematopoietic stem/progenitor cells containing very low numbers of lymphocytes, we tracked the immune reconstitution after ASCT by analysing lymphocyte subpopulations for 36 months.

As shown in Fig. 4A, although the absolute number of CD8$^+$ T cells returned to the normal level only a month after ASCT, the recovery of CD4$^+$ T cells was apparently retarded; at 36 months post-transplant, the number of CD4$^+$ cells remained below the normal range, resulting in continuous inversion of the CD4/CD8 ratio. Previous reports have shown that the long-term remission after ASCT in MS and SLE was associated with the increased number of thymus-derived naive CD4$^+$ T cells [13, 21]. Therefore, we analysed functional subsets of CD4$^+$ T cells. As shown in Fig. 4B, the number of memory CD4$^+$CD45RO$^+$ T cells was significantly decreased for 1–3 months after CD34$^+$ ASCT, but returned to the baseline level 24 months after ASCT, although it did not reach the normal range. In contrast, naive CD4$^+$CD45RA$^+$ T cells were significantly decreased after mobilization, and remained at a low level at 36 months post-ASCT. These data show that most recovered CD4$^+$ T cells were memory cells but not naive T cells. The CD4$^+$CD25$^+$ T cell fraction that includes activated and regulatory T cells [22] disappeared after ASCT, and remained low in number even at 36 months post-ASCT (Fig. 4C). The number of CD4$^+$FoxP3$^+$ cells in 9 patients at 37.8 (18.0) months after ASCT was significantly lower than that of 13 SSc controls [8.06 (7.61) vs 27.23 (13.30)/$\mu$l, $P < 0.05$. These results show that although the patients displayed resolution of clinical SSc after ASCT, naive CD45RA$^+$ T cells or regulatory T cells did not reconstitute well after ASCT.

SSc patients have distinct abnormalities of B-cell subpopulations, characterized by expanded naive B cells and activated but diminished memory B cells [23]. The number of CD19$^+$ B cells returned into the normal range 18 months after ASCT.

**Fig. 1** Resolution of SSc after ASCT. (A) mRSS. The proportional change from baseline measurement was calculated for each time point. Patients with an initial mRSS of $\geq 15$ were included. (B) VC and DLoD, (C) KL-6. (D) SP-D. Data are presented as mean (s.d.). The x-axis is not drawn to scale. The data obtained before mobilization and just before transplantation (HSCT) are shown as BM and BT, respectively. *$P < 0.05$ vs BM. (C and D) Dashed line shows upper normal limit.
To study whether correction of abnormalities in functional subsets of B cells was associated with durable remission, we focused on the functional subset of CD19 + B cells. The number of CD19+CD27+ memory B cells was low at baseline, which was characteristic of SSc [23]. An increase in the number of memory B cells, however, was not observed even at 36 months after ASCT, and the vast majority of recovered B cells were naïve CD19+CD27−/CD28/CD45−/CD27 B cells (Fig. 4D).

Th1 CD4+ T cells were predominantly reconstituted after CD34+ ASCT

Previous data showed that Th1/Th2 balance was associated with skin sclerosis in an animal model of SSc [15]. Therefore, we analysed Th1/Th2 balance after ASCT by measuring the ratio of intracellular IFN-γ+/IL-4−/CD4+ to IFN-γ−/IL-4+/CD4+ T cells (Fig. 5). In Fig. 5A, the results of Case 9 before mobilization, 3 and 24 months after HSCT were shown as representative data. IFN-γ+IL-4−/CD4+ and IFN-γ−/IL-4+/CD4+ T cells were considered to be Th1 and Th2 cells, respectively. In this case, elimination of IL-4+/CD4+ (Th2) T cells as well as predominant reconstitution of IFN-γ+/CD4+ (Th1) T cells were observed after ASCT. The ratio of IFN-γ+ to IL-4+ CD4+ T cells was increased from 4.3 before mobilization to 32.8 at 3 months and 197.3 at 24 months after HSCT. When Th1/Th2 balance was analysed in all SSc patients, the ratio of IFN-γ+/ to IL-4+/CD4+ T cells was significantly increased.

Fig. 2 Evaluation of autoantibody and immunoglobulin. (A) Change in the titre of anti-Scl-70. (B) Change in the serum levels of IgG, IgA and IgM. Data are presented as mean (s.d.). The x-axis is not drawn to scale. The data obtained before mobilization and just before transplantation (HSCT) are shown as BM and BT, respectively. *P < 0.05 vs BM.

Fig. 3 Kinetics of immunological markers in SSc patients who received ASCT. Data are mean (s.d.). The x-axis is not drawn to scale. (A) TNF-α, (B) TGF-β, (C) IL-6, (D) sIL-R. The data obtained before mobilization and just before transplantation (HSCT) are shown as BM and BT, respectively. *P < 0.05 vs BM. Dashed line shows upper normal limit.
increased at 1 month and reached a plateau at 6 months after ASCT (Fig. 5B). The skewed reconstitution of Th1 CD4+ T cells was maintained for 36 months after HSCT. There were no significant correlations between the changes in mRSS and those in Th1/Th2 balance.

**Discussion**

In this study, the resolution of disease was progressively obtained in SSc patients for 36 months after ASCT. This durable effect was not due to the reconstitution of naïve CD4+ T cells, regulatory T cells or the correction of B-cell imbalance. On the other hand, the elimination of Th2 cells by high-dose CYC as well as the predominant reconstitution of Th1 cells were observed after ASCT. Reflecting the resolution of clinical symptoms of SSc, serum levels of anti-Scl-70 progressively decreased after ASCT. Serum levels of KL-6 and SP-D, indicators for IP activity, were also significantly decreased.

In patients with SSc, production levels of type 2 cytokines such as IL-4, IL-6 and IL-13 by stimulated peripheral blood mononuclear cells and cultured CD4+ T cells decreased [24, 25]. Our data showed that the ratio of IFN-\(\gamma\)- to IL-4-producing CD4+ T cells was significantly increased in a month and was sustained for 36 months after ASCT. The predominant reconstitution of IFN-\(\gamma\)-producing cells is associated with amelioration of skin sclerosis, probably due to an ability of IFN-\(\gamma\) to reduce excessive collagen synthesis by scleroderma-derived fibroblasts [26]. IL-4 increases collagen production of fibroblasts and induces the production of TGF-\(\beta\) in patients with SSc [27]. Therefore, the elimination of IL-4-producing T cells provides a favourable effect on SSc. The limitation of this study was small sample size and that there were not significant correlations between the changes in mRSS and those in Th1/Th2 balance. It is unclear how predominant reconstitution of Th1 CD4+ T cells after ASCT is induced. Polarization of CD4+ T cells after ASCT may depend on the local levels of cytokines such as IL-12 or IL-4 when naïve CD4+ T cells develop into functional T cells [28]. Predominant reconstitution of Th1 CD4+ T cells after ASCT may also occur in patients with other autoimmune diseases when treated by ASCT. Therefore, it is conceivable that ASCT is potently effective for Th2-related diseases such as SSc and SLE, while its effect on Th1-related diseases such as RA is limited [4]. Macrophage activation syndrome is often observed in patients with JIA after ASCT [29]. It may be associated with a Th1 immune response after ASCT.

Our data showed that despite the resolution of clinical symptoms of SSc, patients did not achieve normalization of lymphocyte compartment, even 3 years after ASCT. The recovery of CD4+ T cell was delayed until 36 months after ASCT. Muraro et al. [13] reported that
naïve CD45RA+ T cells with diverse TCR repertoire and of thymic origin, were increased after ASCT in patients with MS, and that the increase of such cells was associated with long-term suppression of inflammatory activity of MS. In contrast, the present study revealed that the recovery of naïve CD4+CD45RA+ T cells was so severely suppressed for 36 months after HSCT and that most of the recovered CD4+ T cells were memory CD45RO+ T cells (Fig. 4B). This discrepancy of T-cell recovery after ASCT between SSc and MS, may be due to the difference of disease and/or of age at inclusion. In the study of Farge et al. [14], the level of naïve CD4+CD45RA+ T cells was also suppressed for 9 months after ASCT in SSc patients. In the study of Storek et al. [30], naïve and memory CD4+ T cells were equally recovered in 24 months after ASCT in patients with MS or SSc.

CD25+CD4+Foxp3+ regulatory T cells are a major regulator of adaptive immunity [22]. Patients with JIA showed a significant increase in thymus-derived regulatory T cells (CD25+CD4+ Foxp3+) following ASCT [22]. However, in this study, the recovery of CD25+CD4+ T cells was severely delayed compared with that of CD25+CD4+ T cells (Fig. 4C); the number of CD25+CD4+ T cells did not reach the lower limit of normal even at 36 months after ASCT. It is unlikely that the number of regulatory T cells was increased after ASCT, even if we considered that CD25+CD4+ T cells included activated T cells as well as regulatory T cells. When we analysed CD4+Foxp3+ T cells in nine patients with SSc, their recovery after ASCT was retarded. These results show that the expansion of regulatory T cells after ASCT was not the cause of the efficacy of ASCT on SSc.

The number of CD19+CD27+ memory B cells was low in contrast to an increased number of CD19+CD27− naïve B cells at the baseline. Sato et al. [23] reported the B-cell abnormality including the expanded naïve B cells and diminished memory B cells in SSc patients. Unexpectedly, recovery of memory CD19+CD27+ B cells was severely suppressed even at 36 months after ASCT (Fig. 4D). In the study of Storek et al. [30], both naïve and memory B cells recovered to the normal range in 6 months after ASCT. Collectively, the resolution of clinical SSc after ASCT was not due to the reconstitution of naïve CD4+ T cells or to that of regulatory T cells or to the correction of B-cell imbalance.
An anti-Scl-70 antibody, a useful marker in establishing the diagnosis of SSc, predicts diffuse skin involvement and pulmonary fibrosis, and the increased level of this antibody is associated with a poor prognosis. In this study, we, for the first time, showed that the level of an anti-Scl-70 antibody was continuously decreased for 36 months after ASCT, and that the changes in anti-Scl-70 level were correlated significantly with those in mRSS. These results are consistent with a previous report that showed the correlation of serum anti-Scl-70 levels with disease activity in SSc [31], although the role of anti-Scl-70 in the pathogenesis of SSc was not clearly demonstrated in this article. It is of interest that the changes in serum anti-Scl-70 levels were independent of those in serum immunoglobulin levels, which returned to the baseline level at 12 months after ASCT (Fig. 2). In the study of Storek et al. [30], the level of anti-Scl-70 continued to be abnormally high throughout 24 months after ASCT. This difference might come from the difference in transplant conditioning (CYC 200 vs CYC 120 mg/kg + total body irradiation 8 Gy + anti-thymocyte globulin) or in the purity of the CD34+ cells.

Dysregulated cytokine production was reported in SSc patients [32, 33]. In this study, serum levels of TNF-α, TGF-β, IL-6 and sIL-2R increased before mobilization as previously reported [32], but their levels significantly decreased after ASCT (Fig. 3). Serum levels of VEGF and monocyte chemotactic protein 1 (MCP-1), however, did not decrease after ASCT (data not shown). The decreased levels of profibrotic cytokines after ASCT might reflect resolution of the disease.

Patient 7 died due to progressive IP in spite of the improvement of skin sclerosis at 20 months after ASCT. In this patient, IP was already highly advanced (per cent VC 39%) at the time of ASCT. Immune reconstitution after ASCT was similarly obtained in terms of Th1/Th2 balance and serum levels of pro-fibrotic cytokines. Therefore, the disease was fatal because of advanced and refractory IP that did not respond to the resolution of autoimmune reactions. This result suggests that patients with advanced organ involvement need to be excluded in a future study.

In conclusion, ASCT with purified CD34+ cells was effective in controlling the disease activity of SSc. Improvement of skin sclerosis was significantly associated with the change in serum anti-Scl-70 level after ASCT. Th1/Th2 ratio was significantly increased for at least 3 years after ASCT.

**Rheumatology key messages**

- ASCT causes durable remission in patients with SSc.
- Th1/Th2 ratio was significantly increased for at least 3 years after ASCT.

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