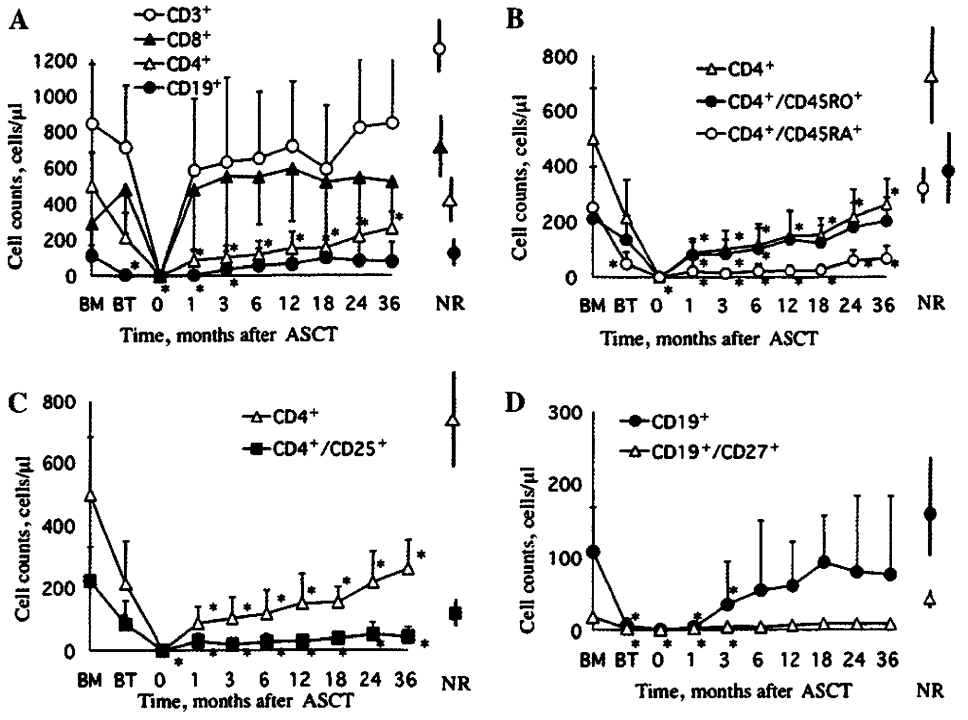
FIG. 4 Immune reconstitution after ASCT. Change in absolute cell counts of immune cells. (**A**) $CD3^+$, $CD4^+$, $CD8^+$, $CD19^+$ cells. (**B**) $CD4^+$, $CD4^+CD45RO^+$, $CD4^+CD45RA^+$ cells. (**C**) $CD4^+$, $CD4+CD25^+$cells. (**D**) $CD19^+$, $CD19^+CD27^+$ cells. Data are presented as mean (S.D.). 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. Normal ranges (NR: 95% CI) are shown as right-sided vertical bars.



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.

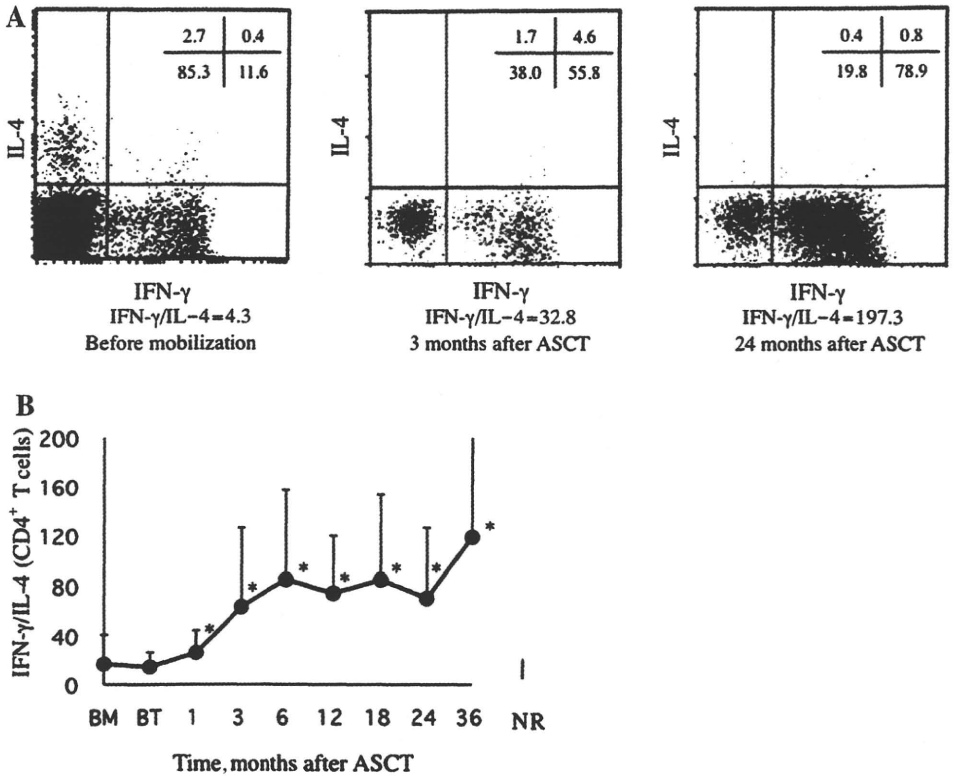
Discussion

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-γ- 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-γ-producing cells is associated with amelioration of skin sclerosis, probably due to an ability of IFN-γ to reduce excessive collagen synthesis by scleroderma-derived fibroblasts [26]. IL-4 increases collagen production of fibroblasts and induces the production of TGF-β 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

Fig. 5 Evaluation of Th1/Th2 balance after ASCT. (**A**) Representative expression of IFN-γ and IL-4 in the cytoplasm of $CD4^+$ T cells (Case 9) before mobilization, 3 months and 24 months after ASCT. IFN-γ /IL-4 was defined as the ratio of IFN-γ^+/IL-4^- to IFN-γ^-/IL-4^+. Number of analysed cells was decreased after ASCT since ratio of $CD4^+$ T cells in gated cells was decreased after ASCT. (**B**) Change in the ratio of intracellular IFN-γ^+ to IL-4^+ $CD4^+$ T cells after ASCT in patients with SSc. 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. Normal range (NR: 95% CI) is shown as right-sided vertical bar.



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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References

1 LeRoy EC, Black CM, Fleischmajer R *et al.* Scleroderma (systemic sclerosis): classification, subsets and pathogenesis. J Rheumatol 1998;15:202–5.

2 Yurovsky VV. The repertoire of T-cell receptors in systemic sclerosis. Crit Rev Immunol 1995;15:155–65.

3 Steen VD, Medsger TA Jr. Severe organ involvement in systemic sclerosis with diffuse scleroderma. Arthritis Rheum 2000;43:2437–44.

4 Hough RE, Snowden JA, Wulffraat NM. Haemopoietic stem cell transplantation in autoimmune diseases: a European perspective. Br J Haematol 2005;128:432–59.

5 McSweeney PA, Nash RA, Sullivan KM *et al.* High-dose immunosuppressive therapy for severe systemic sclerosis: initial outcomes. Blood 2002;100:1602–10.

6 Nash RA, McSweeney PA, Crofford LJ *et al.* High-dose immunosuppressive therapy and autologous hematopoietic cell transplantation for severe systemic sclerosis: long-term follow-up of the US multicenter pilot study. Blood 2007;110:1388–96.

7 Farge D, Passweg J, van Laar JM *et al.* Autologous stem cell transplantation in the treatment of systemic sclerosis: report from the EBMT/EULAR Registry. Ann Rheum Dis 2004;63:974–81.

8 Vonk MC, Marjanovic Z, van den Hoogen FH *et al.* Long-term follow-up results after autologous haematopoietic stem cell transplantation for severe systemic sclerosis. Ann Rheum Dis 2008;67:98–104.

9 Bohgaki T, Atsumi T, Bohgaki M *et al.* Immunological reconstitution after autologous hematopoietic stem cell transplantation in patients with systemic sclerosis: relationship between clinical benefits and intensity of immunosuppression. J Rheumatol 2009;36:1240–8.

10 Oyama Y, Barr WG, Statkute L *et al.* Autologous non-myeloablative hematopoietic stem cell transplantation in patients with systemic sclerosis. Bone Marrow Transpl 2007;40:549–55.

11 Tsukamoto H, Nagafuji K, Horiuchi T *et al.* Phase I-II trial of autologous peripheral blood stem cell transplantation in the treatment of refractory autoimmune disease. Ann Rheum Dis 2006;65:508–14.

12 Burt RK, Marmont A, Oyama Y *et al.* Randomized controlled trials of autologous hematopoietic stem cell transplantation for autoimmune diseases: the evolution from myeloablative to lymphoablative transplant regimens. Arthritis Rheum 2006;54:3750–60.

13 Muraro PA, Douek DC, Packer A *et al.* Thymic output generates a new and diverse TCR repertoire after autologous stem cell transplantation in multiple sclerosis patients. J Exp Med 2005;201:805–16.

14 Farge D, Henegar C, Carmagnat M *et al.* Analysis of immune reconstitution after autologous bone marrow transplantation in systemic sclerosis. Arthritis Rheum 2005;52:1555–63.

15 Shen Y, Ichino M, Nakazawa M, Minami M. CpG oligodeoxynucleotides prevent the development of scleroderma-like syndrome in tight-skin mice by stimulating a Th1 immune response. J Invest Dermatol 2005; 124:1141–8.

16 Matsushita T, Hasegawa M, Hamaguchi Y, Takehara K, Sato S. Longitudinal analysis of serum cytokine concentrations in systemic sclerosis: association of interleukin 12 elevation with spontaneous regression of skin sclerosis. J Rheumatol 2006;33:275–84.

17 Akahoshi M, Nakashima H, Tanaka Y *et al.* Th1/Th2 balance of peripheral T helper cells in systemic lupus erythematosus. Arthritis Rheum 1999;42:1644–8.

18 Kohno K, Nagafuji K, Tsukamoto H *et al.* Infectious complications in patients receiving autologous CD34-selected hematopoietic stem cell transplantation for severe autoimmune diseases. Transpl Infect Dis 2009;11:318–23.

19 Ohnishi H, Yokoyama A, Kondo K *et al.* Comparative study of KL-6, surfactant protein-A, surfactant protein-D, and monocyte chemoattractant protein-1 as serum markers for interstitial lung diseases. Am J Respir Crit Care Med 2002;165:378–81.

20 Kuroki Y, Takahashi H, Chiba H, Akino T. Surfactant proteins A and D: disease markers. Biochim Biophys Acta 1998;1408:334–45.

21 Alexander T, Thiel A, Rosen O *et al.* Depletion of autoreactive immunologic memory followed by autologous hematopoietic stem cell transplantation in patients with refractory SLE induces long-term remission through de novo generation of a juvenile and tolerant immune system. Blood 2009;113:214–23.

22 de Kleer I, Vastert B, Klein M *et al.* Autologous stem cell transplantation for autoimmunity induces immunologic self-tolerance by reprogramming autoreactive T cells and restoring the $CD4^+CD25^+$ immune regulatory network. Blood 2006;107:1696–702.

23 Sato S, Fujimoto M, Hasegawa M, Takehara K. Altered blood B lymphocyte homeostasis in systemic sclerosis: expanded naive B cells and diminished but activated memory B cells. Arthritis Rheum 2004;50: 1918–27.

24 Needleman BW, Wigley FM, Stair RW. Interleukin-1, interleukin-2, interleukin-4, interleukin-6, tumor necrosis factor alpha, and interferon-gamma levels in sera from patients with scleroderma. Arthritis Rheum 1992;35: 67–72.

25 Mavalia C, Scaletti C, Romagnani P *et al.* Type 2 helper T-cell predominance and high CD30 expression in systemic sclerosis. Am J Pathol 1997;151:1751–8.

26 Serpier H, Gillery P, Salmon-Ehr V *et al.* Antagonistic effects of interferon-gamma and interleukin-4 on fibroblast cultures. J Invest Dermatol 1997;109:158–62.

27 Mueller R, Krahl T, Sarvetnick N. Tissue-specific expression of interleukin-4 induces extracellular matrix accumulation and extravasation of B cells. Lab Invest 1997;76: 117–28.

28 Vieira PL, de Jong EC, Wierenga EA, Kapsenberg ML, Kaliński P. Development of Th1-inducing capacity in myeloid dendritic cells requires environmental instruction. J Immunol 2000;164:4507–12.

29 Brinkman DM, de Kleer IM, ten Cate R *et al.* Autologous stem cell transplantation in children with severe progressive systemic or polyarticular juvenile idiopathic arthritis: long-term follow-up of a prospective clinical trial. Arthritis Rheum 2007;56:2410–21.

30 Storek J, Zhao Z, Lin E *et al.* Recovery from and consequences of severe iatrogenic lymphopenia (induced to treat autoimmune diseases). Clin Immunol 2004;113: 285–98.

31 Hu PQ, Fertig N, Medsger TA Jr, Wright TM. Correlation of serum anti-DNA topoisomerase I antibody levels with disease severity and activity in systemic sclerosis. Arthritis Rheum 2003;48:1363–73.

32 Sakkas LI. New developments in the pathogenesis of systemic sclerosis. Autoimmunity 2005;38:113–6.

33 Scala E, Pallotta S, Frezzolini A *et al.* Cytokine and chemokine levels in systemic sclerosis: relationship with cutaneous and internal organ involvement. Clin Exp Immunol 2004;138:540–6.