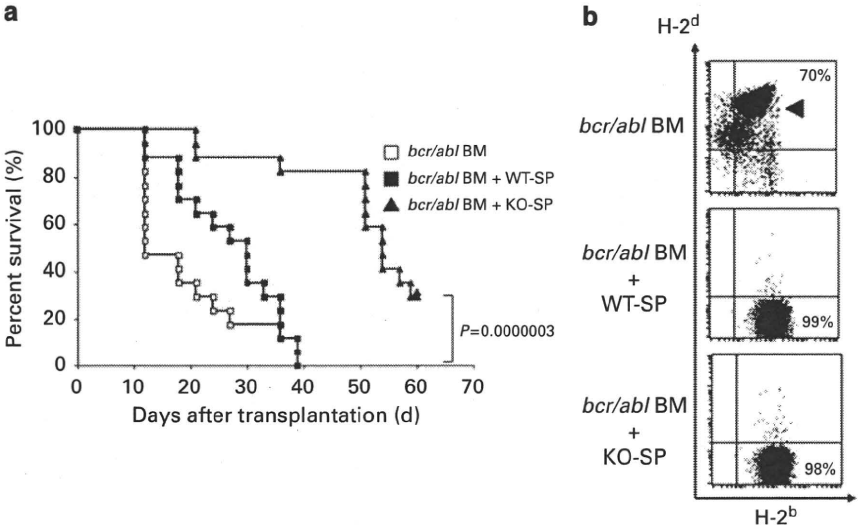

Figure 3 Survival curve with *bcr-abl*-transduced BM as leukemic cells. (**a**) C57BL/6-DBA2 F1 recipients were transplanted with *bcr-abl*-transduced recipient BM (H-2^d/H-2^b) (starting dose: 5×10^5), 5×10^6 TCD-BM (H-2^b) and 2×10^7 WT-SP or KO-SP (H-2^b). Open squares, filled squares and filled triangles indicate transplantation without splenocytes ($n = 17$), with WT-SP ($n = 17$) and with KO-SP ($n = 17$), respectively. The combined results of three independent experiments are shown. *P*-value was calculated by the Log-rank method. (**b**) Peripheral blood cell phenotypes 2 weeks after transplantation. H-2^b single-positive phenotype depicts donor-derived cells. Double-positive population indicates recipient-derived *bcr-abl*-transduced leukemic cells (arrowhead).

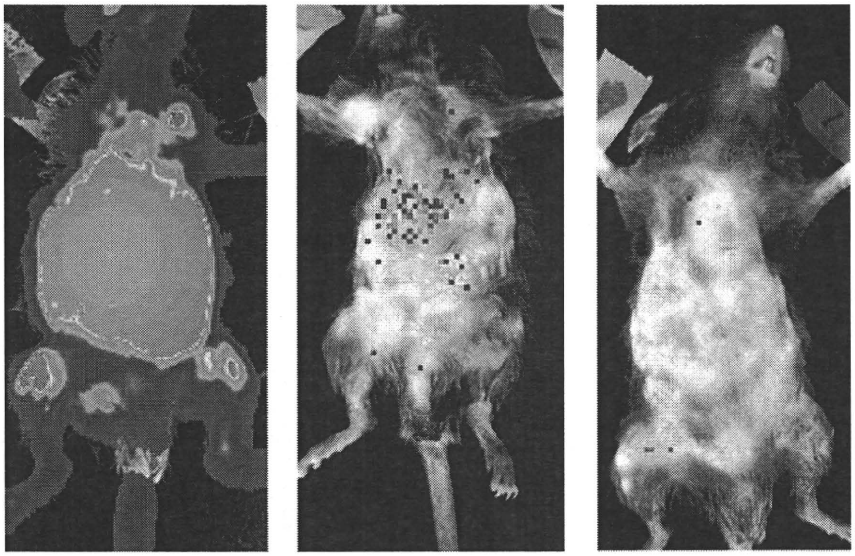

Figure 4 GVL effect *in vivo*. A difference in GVL effect is evident between transplanted mice without splenocytes (control) vs those receiving either wild-type or IL-21R$^{-/-}$ splenocytes (WT-SP or KO-SP) at day 28 after transplantation. C57BL/6-DBA2 F1 recipients (H-2^d/H-2^b) were transplanted with 2×10^4 P815 leukemic cells (H-2^d), 5×10^6 TCD-BM (H-2^b) and 5×10^6 WT-SP or KO-SP (H-2^b).

from early deaths of highly positive mice until the indicated days (Figure 5, third and bottom right panels). Taken together, the threshold for P815 cells in our experimental conditions was between 5×10^5 and 5×10^6, regardless of genotype of IL-21R.

CD8-depleted grafts showed attenuated GVL effect

Our previous experiments demonstrated that IL-21R$^{-/-}$ CD4$^+$ T cells are defective in GVH reactivity after transplantation.[32] It was therefore of great interest to determine whether IL-21R$^{-/-}$ CD4$^+$ T cells are also defective in GVL effect. To evaluate the contribution of CD4$^+$ T cells to GVL effect, we performed transplantation with CD8-depleted splenocytes with dose reduction as above. As CD8 T cells compose only ~10% of splenocyte, we chose CD8-depletion rather than CD4 purification, so we could use the same dose of CD8-depleted splenocytes as in the case of bulk splenocytes. As shown in Figure 6, CD8-depleted KO-SP at the dose of 5×10^7 demonstrated attenuated GVHD and prolonged survival (top left panel), consistent with our previous experiments above[29] (Figure 1).

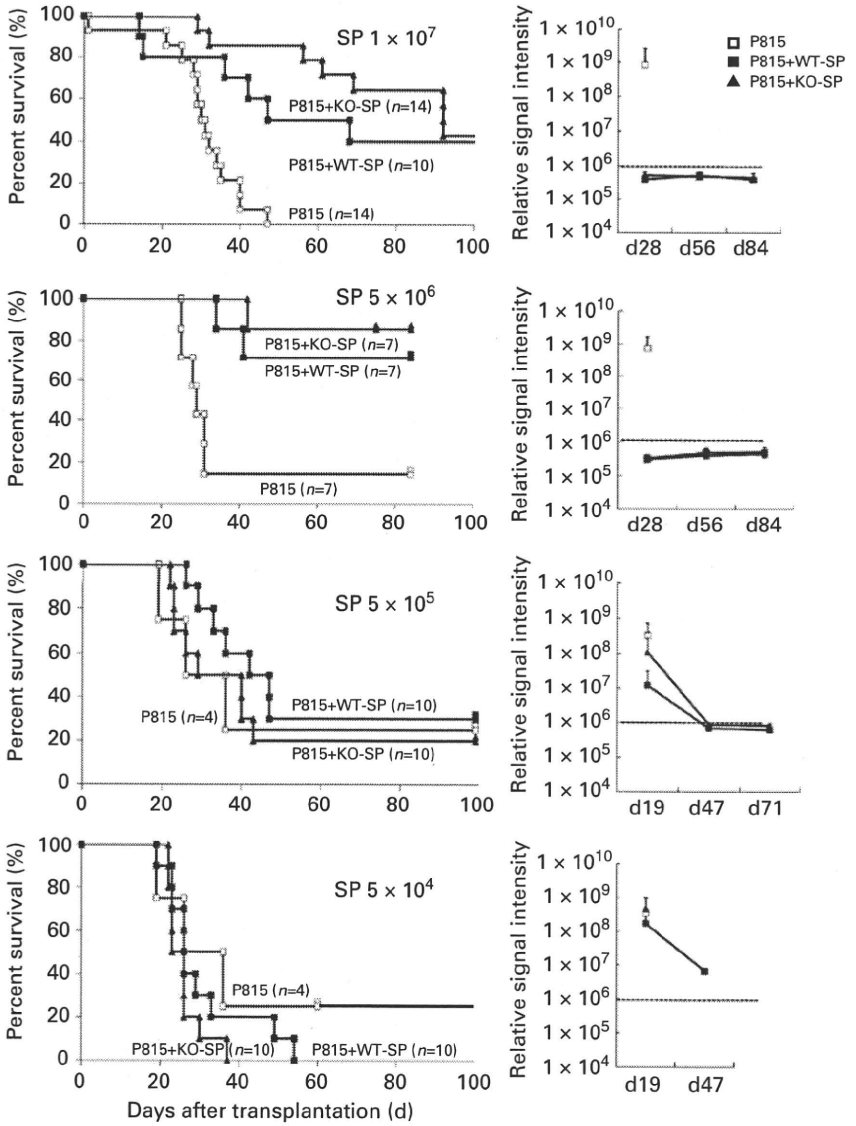

Figure 5 Survival curve and leukemic-tumor-burden in dose-reduction transplantation with bulk splenocytes. C57BL/6-DBA2 F1 recipients were transplanted with 2×10^4 P815 leukemic cells expressing luciferase (H-2^d), 5×10^6 TCD-BM (H-2^b) and 1×10^7 WT-SP or KO-SP (H-2^b). Then, the dose of bulk splenocytes was reduced to 5×10^6, 5×10^5 and 5×10^4 cells. Open squares, filled squares and filled triangles indicate transplantations without splenocytes, with WT-SP and with KO-SP, respectively. Right panels indicate the average of signal intensity of luminescence in survivors at indicated days after transplantation, which correlates with leukemic-tumor-burden. Empirically determined positive–negative threshold indicated in broken line was 10^6 relative signal intensity per mouse.

No increase of luciferase activity indicated that all deaths were primarily due to GVHD (top right panel). However, interestingly, CD8-depleted KO-SP at the dose of 5×10^6 demonstrated diminished GVL effect. Higher luciferase activity (middle right panel) and more deaths (middle left panel) in the KO-SP group indicated leukemic deaths. According to luciferase activity, with CD8-depleted KO-SP at the dose of 5×10^6 cells, 10 out of 21 recipients showed leukemic growth at day 21, whereas for CD8-depleted WT-SP, only 3 out of 21 mice showed leukemic growth at day 21 (middle right panel); only 7 out of 21 mice survived in recipients of KO-SP, but 13 out of 21 mice survived in recipients of WT-SP at day 100 after transplantation (middle left panel), indicating that IL-21R$^{-/-}$ CD4 cells have lower GVL effect than wild-type CD4 cells.

CD8-purified grafts did not show significant reduction in GVL effect

To further evaluate the contribution of CD8$^+$ T cells to GVL effect, we performed transplantation with CD8-purified splenocytes with dose reduction as above. As shown in Figure 7a, CD8-purified KO-SP at the dose of 5×10^5 did not demonstrate a significant increase in the incidence of leukemic cell death ($P = 0.1$), but if anything, CD8-purified KO-SP might have a slight decrease in GVL effect (lower left panel). Consistent with the survival, both CD8-purified KO-SP and WT-SP showed increased luciferase activity at day 21 (lower right panel). To confirm these results, we performed standard cytotoxic T cell assay using ^{51}Cr. Splenocytes from recipients of C57BL/6-DBA-2-F1 mice demonstrated similar and comparable killing activity

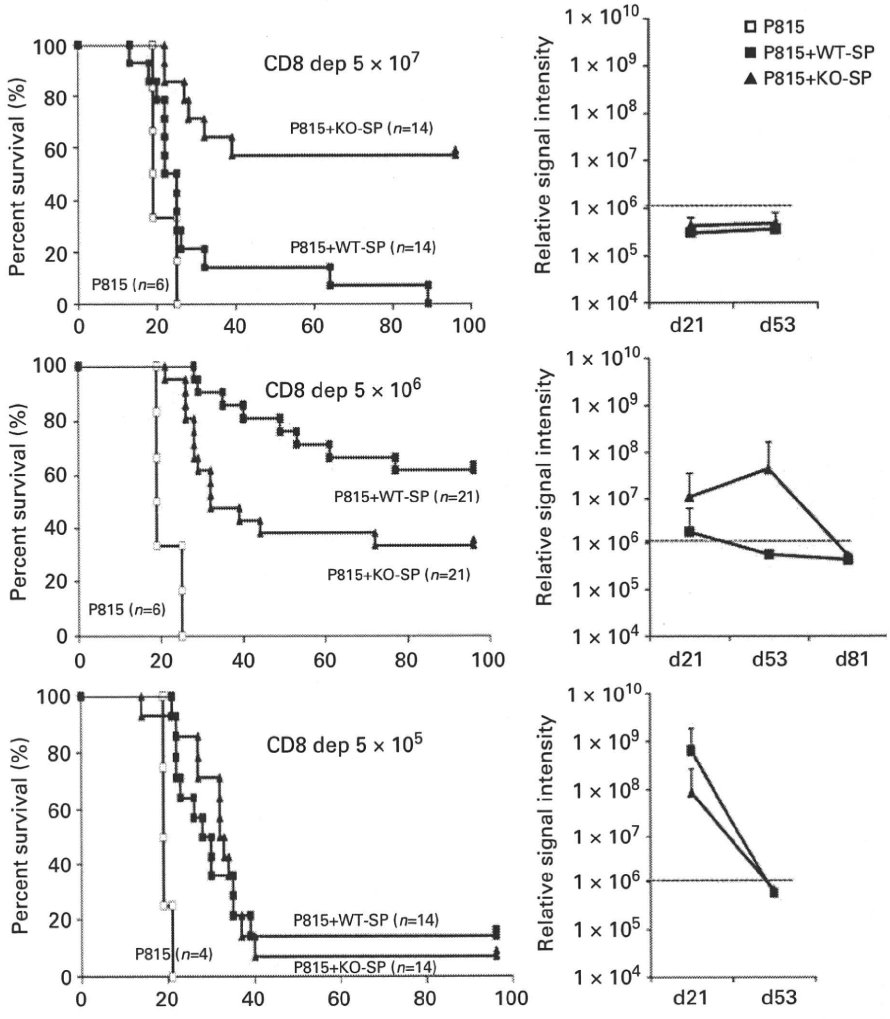

Figure 6 Survival curve and leukemic-tumor-burden in dose-reduction experiments with CD8-depleted splenocytes. Experiments were the same as in Figure 5 but the donor splenocyte was CD8 depleted. Like the bulk splenocyte experiment (Figure 5), the starting dose was 5×10^7 WT-SP or KO-SP (H-2^b). Then, the dose of CD8-depleted splenocytes was reduced to 5×10^6 and 5×10^5, gradually. Open squares, filled squares and filled triangles indicate transplantations without splenocytes, with WT-SP and KO-SP, respectively. Right panels indicate the average of signal intensity of luminescence in survivors at indicated days after transplantation, which correlates with leukemic-tumor-burden. Empirically determined positive–negative threshold indicated in broken line was 10^6 relative signal intensity per mouse.

against allogeneic cells but not syngeneic cells (Figure 7b). Cytokine production, at least, interferon-γ from T cell and $CD8^+$ T cell in MLR was comparable between KO and WT (Supplementary Figure S1).

Discussion

Here, we demonstrated that KO-SP, which ameliorate GVHD, do not attenuate graft-versus-leukemia (GVL) effect even in the splenocyte-dose titration. In the further detailed analysis with CD8-depleted and CD8-purified splenocytes, GVL effect with IL-21$R^{-/-}$ CD8-depleted splenocytes but not CD8-purified splenocytes was significantly diminished compared with wild type, suggesting that IL-21$R^{-/-}$ CD4 cells have lower GVL activity than wild-type cells but IL-21$R^{-/-}$ CD8 cells have no or only a tiny decrease in GVL effect. These results suggested that no reduction in the GVL effect with bulk splenocytes was because of a compensatory effect of IL-21$R^{-/-}$ CD8 cells on GVL effect. This conclusion is reasonable in view of the fact that IL-21$R^{-/-}$ CD4 cells or splenocytes induce less severe GVHD.[29,32] As no immunosuppressive reagent specific for GVHD but not GVL effect is clinically available, the specific suppression of GVHD in the presence of both CD4 and CD8 cells is of interest and could contribute to the development of GVHD treatment.

During this manuscript preparation, Bucher *et al.* reported similar results[30] with our present and published data.[29,32] The authors concluded that the GVL effect was maintained even after IL-21 neutralization, however, we believe that titration experiments are required in order to determine if GVL strength is the same between normal and IL-21 neutralized conditions, because the threshold of the required number of T-cells for GVL is much less than for GVHD, as we have shown in this study. One must titrate the dose of splenocytes to compare the GVL strength between the two groups, but this was not done by Bucher *et al.*[30]

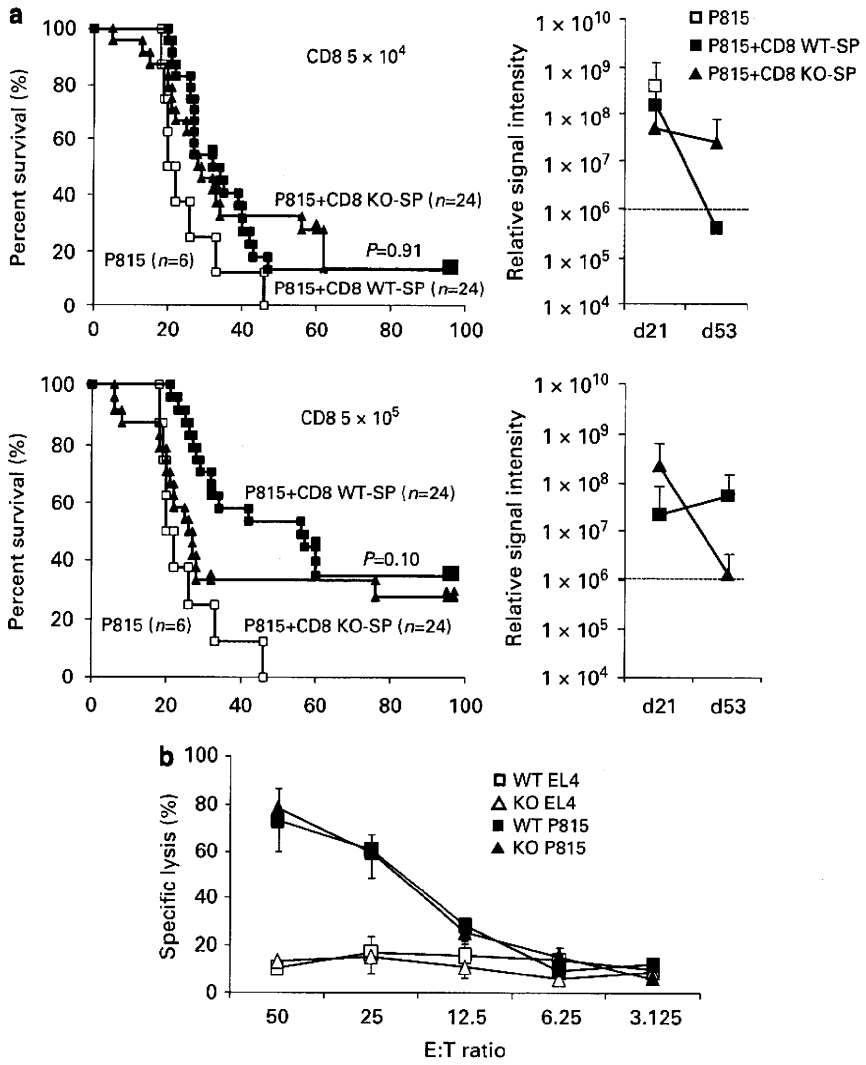

Figure 7 Survival curve in dose-reduction experiments with CD8-purified splenocytes and cytotoxic T-cell assay. (**a**) Experiments were the same as in Figure 5, but the donor cells were CD8-purified splenocytes. The doses of purified CD8 T cells were 5×10^4 and 5×10^5. Open squares, filled squares and filled triangles indicate transplantations without splenocytes, with CD8-purified WT-SP and with CD8-purified KO-SP, respectively. Right panels indicate the average of signal intensity of luminescence in survivors at indicated days after transplantation. (**b**) ^{51}Cr-release assay. At 2 weeks after cotransplantation with splenocytes from either KO or WT mice, splenocytes from recipient C57BL/6-DBA-2 F1 mice were incubated with ^{51}Cr-preloaded allogeneic (P815, H-2^d) and syngeneic (EL4, H-2^b) target cells. Specific lysis was calculated as follows: (sample count–background)/(maximum count–background) (%). Error bars are ± s.e.m.

We found that there were different thresholds and time frames between GVHD and GVL. Under our experimental conditions, 5×10^6 bulk splenocytes did not induce death from GVHD but the dose can completely eliminate P815 leukemic cells. Thus, the required number of splenocytes was apparently less for the GVL effect than for GVHD. As we have shown, timings of GVHD and GVL effects appeared to be different. At 2 weeks after transplantation, leukemic cells in peripheral blood had been already eradicated (Figure 3) but symptoms of GVHD only became clinically obvious 3–4 weeks after transplantation. Our previous study demonstrated that CD4 dysfunction occurs only after transplantation,[32] suggesting the possibility that in the very early phase of GVHD, T cells still have normal GVL capacity to eliminate leukemic cells but GVL capacity gradually diminishes later on. However, our present study revealed that the incidence of leukemia was higher in the KO group than in the WT group at day 21 at the dose of 5×10^6 CD8-depleted splenocytes. This experiment was performed with the same dose and analyzed at the same time point, suggesting that the cause of the diminished GVL effect may be CD4 dysfunction itself rather than differences in dose and timing.

In summary, our results revealed the attenuated GVL effect of IL-21R$^{-/-}$ splenocytes in the absence of CD8 T cells, whereas bulk splenocytes and CD8-purified splenocytes did not demonstrate such a defect, together indicating the importance of CD8 T cells for the effect. Our detailed analysis provides new insights regarding the role for IL-21 in GVL effect.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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Mesenchymal stromal cells inhibit Th17 but not regulatory T-cell differentiation

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Abstract

Background aims. A previous study has demonstrated that mouse mesenchymal stromal cells (MSC) produce nitric oxide (NO), which suppresses signal transducer and activator of transcription (STAT) 5 phosphorylation and T-cell proliferation under neutral and T helper 1 cells (Th1) conditions. We aimed to determine the effects of MSC on T helper 17 cells (Th17) and regulatory T-cell (T-reg) differentiation. *Methods.* CD4 T cells obtained from mouse spleen were cultured in conditions for Th17 or Treg differentiation with or without mouse MSC. Th17 and Treg differentiation was assessed by flow cytometry using antibodies against interleukin (IL)-17 and forkhead box P3 (Foxp3), a master regulator of Treg cells. *Results.* MSC inhibited Th17 but not Treg differentiation. Under Th17 conditions, MSC did not produce NO, and inhibitors of indoleamine-2,3-dioxygenase (IDO) and prostaglandin E_2 (PGE2) both restored MSC suppression of differentiation, suggesting that MSC suppress Th17 differentiation at least in part through PGE2 and IDO. *Conclusions.* Our results suggest that MSC regulate CD4 differentiation through different mechanisms depending on the culture conditions.

Key Words: *indoleamine-2,3-dioxygenase, interleukin-17, mesenchymal stromal cells, prostaglandin E2, regulatory T cells, Th17*

Introduction

Mesenchymal stromal cells (MSC), also called mesenchymal stem cells (1,2), have been shown to have immunomodulatory functions (1–5). Although cell–cell contact may be required for full immunomodulation (6,7), we and many other groups have suggested that soluble factors are important for this function (7–9). Such soluble factors include transforming growth factor (TGF-β), hepatocyto growth factor (HGF), indoleamine-2,3-dioxygenase (IDO) and prostaglandin E_2 (PGE2) (8,10,11). MSC have been reported to be the source of IDO and PGE2 (10,11). We have previously reported that nitric oxide (NO), which is produced by MSC and suppresses T-cell proliferation, is a novel regulator of immunomodulation by MSC (7). We have also demonstrated that a combination of interferon (IFN)-γ and lipopolysaccharide (LPS) or tumor necrosis factor (TNF)-α enables MSC to produce NO (12). Moreover, we have demonstrated that IFN-γ is crucial for NO production (12).

Cell therapy with MSC has been proposed as a salvage therapy for steroid-refractory severe, acute graft-versus-host disease (GvHD) (13), which is a major complication following hematopoietic stem cell transplantation (14,15), sometimes with a fatal outcome. Indeed, MSC therapy induced a high response rate in a phase II study conducted by the European Group for Blood and Marrow Transplantation (EBMT) (16). However, the molecular mechanisms underlying the MSC effect are largely unknown (3,17). GvHD may involve T helper 17 cells (Th17) and regulatory T cells (Treg), which are subtypes of CD4 T cells that have been identified fairly recently (18–24) and which can be induced in the presence of TGF-β and interleukin (IL)-6 (25–27) or TGF-β and IL-2 (28–30), respectively. Treg have been reported to ameliorate mouse GvHD (31), and the induction of Treg following MSC treatment has been reported (11,32–34). A role for IL-17 (also referred to as IL-17A) in GvHD has been controversial (35–37). Yi *et al.* (35) reported that the lack of IL-17 promotes GvHD, suggesting a suppressive effect of IL-17. However, Kappel *et al.* (36) reported that IL-17$^{-/-}$ CD4 T cells ameliorate very early GvHD, suggesting a promoting effect of IL-17. Carlson *et al.* (37) reported that *ex vivo* differentiated Th17 cells induce

Author contributions: RT performed all of the experiments with assistance from IO, KH, AM, HM and KS; KO (Ozaki) designed the study and wrote the manuscript; KO (Ozawa) directed and supervised the entire study. KO (Ozawa) provided financial and administrative support.

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organ-specific, skin and lung GvHD. As a reciprocal relationship is known to exist between Th17 and Treg differentiation (27), it is of great interest, in terms of future GvHD treatment, to determine the effect of MSC on Th17 and Treg differentiation and to understand the molecular mechanisms of MSC immunomodulation.

Methods

Preparation of MSC and CD4 T cells

C57BL/6 mice were purchased from Clea Japan (Tokyo, Japan). All mice were housed in our mouse facility (Jichi Medical University, Tochigi, Japan), which is regulated by an intramural small animal committee, and were treated in accordance with the Jichi Medical University guidelines. MSC were harvested from mouse bone marrow as described previously (7). Briefly, femurs and tibias were excised and bone marrow cells were flushed out with a small amount of phosphate-buffered saline (PBS) using a syringe and a needle. The capacity of the cells to differentiate into adipocytes and osteocytes was determined using Oil Red O and alkaline phosphatase staining, respectively. Cell-surface expression of stem cell antigen (Sca-1) and CD44, and the lack of expression of CD45 and CD11b, which is typical of MSC, was confirmed by flow cytometric analysis. CD4 T cells were isolated by positive selection from a single cell spleen suspen-

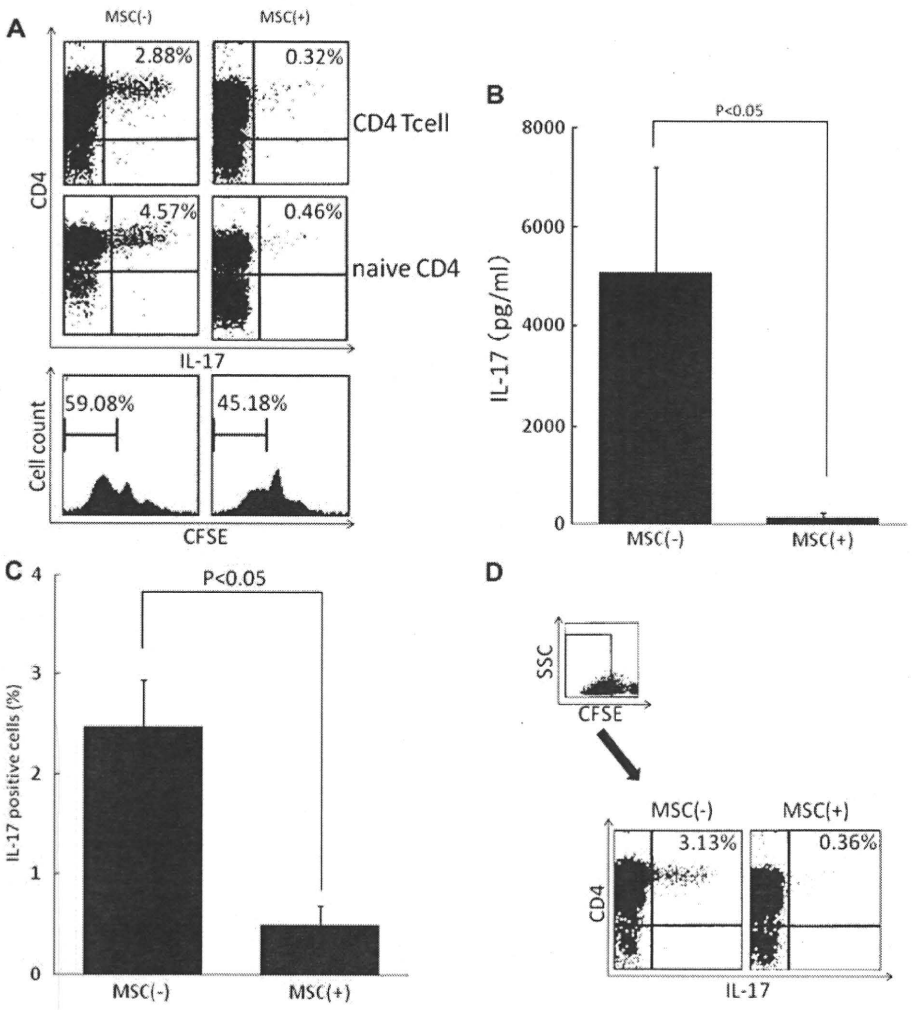

Figure 1. Effect of MSC on Th17 differentiation. (A) Th17 differentiation in the absence or presence of MSC. Purified CD4 T cells (upper panels) or $CD4^+$ $CD25^-$ $CD44^{low}$ $CD62L^{high}$ cells (middle panels) used as naive CD4 T cells (5×10^4/well in a 24-well plate) were cultured for 4 days in the absence or presence of MSC (5×10^3/well in a 24-well plate) and stimulated with anti-CD3/CD28 beads in the presence of hTGF-β and mIL-6. At the beginning of the culture, the CD4 T cells were stained with CFSE, and cell-division was monitored at the end of the culture by flow cytometric analysis (lower panels). (B) The IL-17 concentration in the supernatants, after culture under the Th17 conditions in (A) in the absence or presence of MSC, was assayed using an ELISA. (C) The average percentage of IL-17-positive CD4 T cells was calculated following analysis by intracellular staining and flow cytometry. The number of experiments was six in total. (D) Similar analysis as in (A) with gating on CFSE-low CD4 T cells that had already divided, to exclude non-dividing cells.

sion that was subjected to purification by AutoMACS (Miltenyi Biotec KK, Tokyo, Japan), following which the purity was >90%, or by sorting against $CD4^+$ $CD25^-$ $CD62L^{high}$ $CD44^{low}$ cells using a cell sorter (FACSAria; BD Bioscience, San Jose, CA, USA), following which the purity was >98%.

Th17 and Treg differentiation

Culture medium was RPMI-1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Sigma, St Louis, MO, USA), 50 μM 2-mercaptoethanol (Sigma), 2 mM L-glutamine (Invitrogen), 0.1 mg/mL streptomycin and 100 U/mL penicillin G (Invitrogen). CD4 T cells (5×10^4/well in a 24-well plate) were cultured for 4 days in the absence or presence of MSC (5×10^3/well in a 24-well plate) and stimulated with anti-CD3/CD28 beads (Dynal Biotech ASA, Oslo, Norway). The conditions for Th17 differentiation were human(h) TGF-β (3 ng/mL) (Peprotech, Rocky Hill, NJ, USA) and mouse (m)IL-6 (20 ng/mL) (Peprotech) in addition to T-cell stimulation. The conditions for Treg differentiation were hTGF-β (2.5 ng/mL) and hIL-2 (50 U/mL) (provided by Shionogi & Co., Osaka, Japan).

Flow cytometric analysis

Fragment crystallizable (Fc)-block® solution (BD Biosciences-Pharmingen, San Diego, CA, USA) was used to prevent non-specific antibody binding to Fc receptors. Anti-CD4, -CD25, -CD62L, -CD44, -CD11b, -Sca-1, -CD45, -Foxp3 and -IL-17A antibodies used for flow cytometry were purchased from BD Biosciences-Pharmingen or eBioscience (San Diego, CA, USA). Cells were stained in fluorescence-activated cell sorting (FACS) buffer (PBS supplemented with 10% FBS) with antibodies for 30 min on ice, and washed with FACS buffer. For intracellular staining, Cytofix/Cytoperm® (BD Biosciences) was used for fixation/permeabilization. Then cells were stained with anti-Foxp3 or anti-IL-17 antibody for 30 min on ice and washed. An LSR flow cytometer (BD Biosciences-Immunocytometry Systems, San Jose, CA, USA) was used for data collection, and data were analyzed using CellQuest software (BD Biosciences-Immunocytometry Systems).

Carboxy-fluorescein diacetate succinimydyl ester staining

Carboxy-fluorescein diacetate succinimydyl ester (CFSE) cell staining was performed according to the manufacturer's instructions (Molecular Probes-Invitrogen, Carlsbad, CA, USA). Briefly, the cells were incubated in PBS in the presence of CFSE (10 μM) for 10 min and washed twice with medium containing 10% serum.

Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) kits for the quantification of IL-17, IL-21, IL-23 and PGE2 in supernatants after Th17 and Treg differentiation were purchased from R&D Systems (IL-17 and IL-21; Minneapolis, MN, USA), eBioscience (IL-23) and Cayman Chemical Company (PGE2; Ann Arbor, MI, USA). The cytokine concentrations were determined according to the manufacturer's instructions.

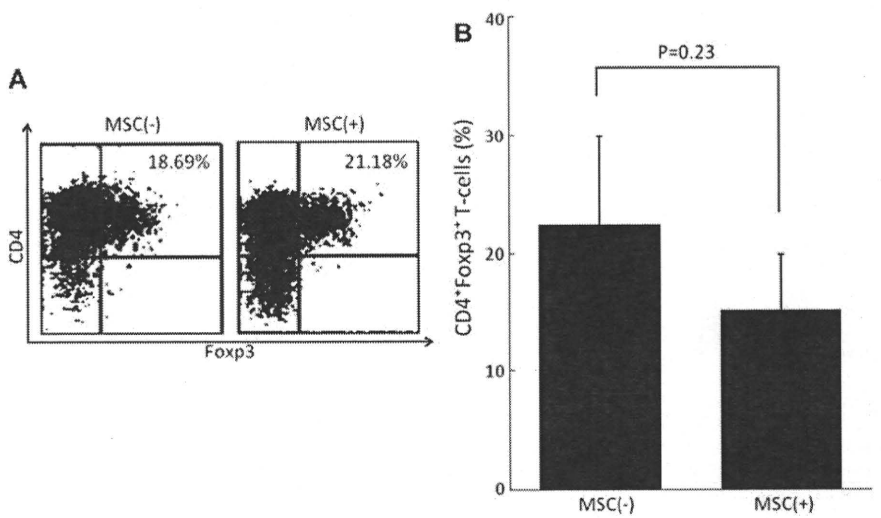

Figure 2. Effect of MSC on Treg differentiation. (A) Purified CD4 T cells were cultured for 4 days in the presence or absence of MSC under Treg conditions (hTGF-β and hIL-2). The cells were then stained with anti-CD4 and Foxp3 antibodies and analyzed using flow cytometry. (B) The average percentage of $CD4^+Foxp3^+$ cells.

Measurement of NO production

The concentrations of NO in cell culture supernatants were determined by using a Griess reagent kit (Wako Pure Chemical Industries, Osaka, Japan) according to the manufacturer's instructions.

Statistical analysis

The Student's *t*-test was used for all statistical analyses, and all of the error bars are SD, unless otherwise specified.

Results

MSC inhibit Th17 but not Treg differentiation

We first assayed the effect of MSC on the differentiation of Th17 and Treg cells. MSC were prepared from mouse bone marrow, cultured *in vitro*, and characterized as described previously (7). In the absence of MSC, and in the presence of TGF-β and IL-6, CD4 T cells purified from mouse spleen (Figure 1A, upper left panel) as well as naive $CD4^+$ $CD25^-$ $CD62L^{high}$ $CD44^{low}$ T cells (Figure 1A, middle left panel) differentiated into Th17 cells, which are CD4 cells that produce IL-17 (also referred to as IL-17A). The addition of MSC suppressed Th17 differentiation of both cell populations (Figure 1A, upper and middle right panels, respectively). For this assay, we used a relatively low concentration of MSC so that the presence of MSC showed a tiny or slight decrease in T-cell proliferation (Figure 1A, lower panels). The suppression of IL-17 production by the MSC was confirmed using an ELISA (Figure 1B) as well as by intracellular staining (Figure 1C). As T-cell differentiation requires T-cell proliferation, we also analyzed the effect of MSC on a gated CFSE-low population of cells, which are cells that have already divided (Figure 1D, left panel). This population of cells gave similar results to the non-gated total cell population (Figure 1D, right panel), suggesting that the suppression of Th17 was independent of inhibition of cell proliferation. We used this method hereafter except for an analysis of Treg.

In contrast to their effect on Th17 differentiation, MSC did not inhibit Treg differentiation (Figure 2A). Thus, in either the absence or presence of MSC, in the presence of TGF-β and IL-2 a similar percentage of naive CD4 T cells differentiated into Treg, which are CD4 T cells that express Foxp3. A representative result of flow cytometry (Figure 2A) and the average of $CD4^+$ $Foxp3^+$ Treg cells (Figure 2B) are shown. Based on these data we focused our study on the mechanisms by which MSC suppress Th17 differentiation.

NO, chemokine (C-C motif) ligand 2, IL-21 and IL-23 do not mediate Th17 suppression by MSC

To address the molecular mechanism by which MSC inhibit Th17 differentiation, we first assessed whether MSC produce NO. We have previously shown that NO is produced by MSC under neutral and Th1 conditions (7,12) and NO is also known to be a strong T-cell suppressor. In contrast to the neutral condition as a control (Figure 3Ai, left bar), we found that MSC did not produce NO under conditions that induce Th17 differentiation (Figure 3Ai, right bar). Consistent with this finding, the addition of a well-known specific inhibitor of NO synthase, L-NAME, had no effect on MSC suppression of Th17 differentiation (Figure 3Aii,iii). Second, we determined whether neutralization of the chemokine (C-C motif) ligand 2 (CCL-2) using a specific antibody might abrogate MSC inhibition of Th17 differentiation, as Rafei *et al.* (38) have reported that MSC can inhibit Th17 differentiation through the secretion of CCL-2. However, anti-CCL-2 did not have any effect on MSC suppression of Th17 differentiation (Figure 3B). Third, we determined whether MSC suppression might involve secretion of the cytokines IL-21 or IL-23 (25,39–42). However, there was no significant difference in the level of these cytokines in the supernatant of the CD4 T cells with or without MSC (Figure 3C). Finally, we investigated whether MSC suppression was the result of secretion of a soluble factor by assay of Th17 differentiation in the presence of MSC using a Transwell culture dish. MSC inhibited Th17 differentiation even when direct cell–cell contact was inhibited by the Transwell dish (Figure 4), suggesting that MSC primarily inhibit Th17 differentiation by means of a soluble factor.

PGE2 partly mediates Th17 suppression by MSC

One potential candidate for this soluble factor was PGE2, which has been reported to mediate MSC suppression of T-cell proliferation (11) that we have confirmed under neutral conditions in a previous study (7). We therefore investigated whether indomethacin, a well-known inhibitor of prostaglandin production, might inhibit MSC suppression of Th17 differentiation. PGE2 was detected in the supernatant of co-cultured MSC and CD4 T cells at a concentration of approximately 1–2 ng/mL (varying from one experiment to another), by using an ELISA, and PGE2 was detected in neither the absence of MSC nor presence of indomethacin (Figure 5A). Indomethacin partially restored Th17 differentiation (Figure 5B), suggesting that PGE2, at least in part, mediates

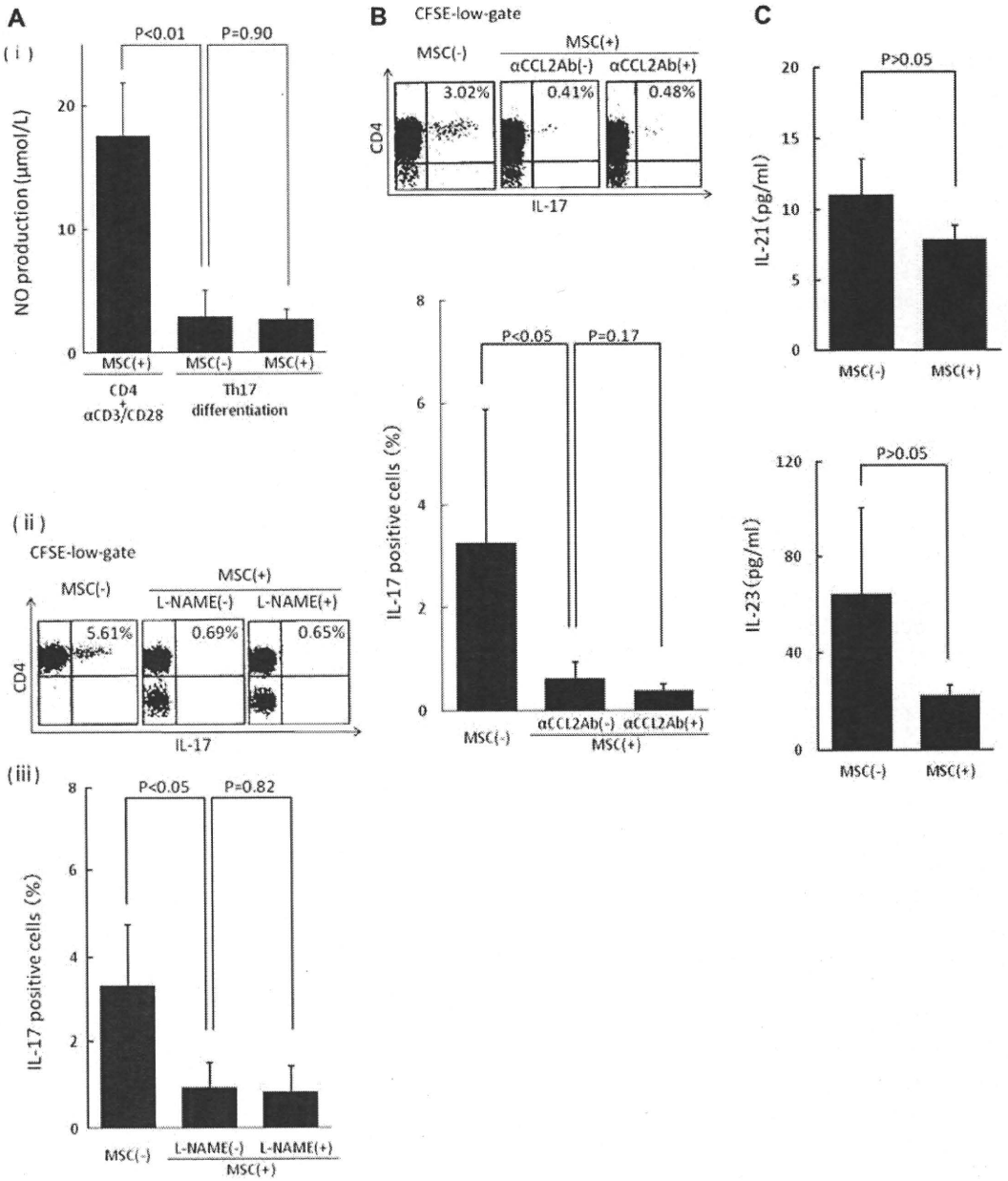

Figure 3. Analysis of the molecular mechanism of Th17 suppression by MSC. (A) (i) NO production by MSC under Th17 conditions was assayed using a Griess method. Left bar denotes a positive control of CD4 T-cell stimulation in neutral conditions. (ii, iii) The effect of a specific inhibitor of NO synthase, L-NAME (1 mM), on MSC-induced Th17 differentiation was assayed using flow cytometry. A representative dot-plot and the average of three independent experiments are shown. (B) The effect of the CCL-2 neutralizing antibody (15 μg/mL) on Th17 differentiation. A representative dot-plot (upper panel) and the average of three independent experiments are shown (lower panel). (C) Purified CD4 T cells were cultured with or without MSC for 4 days under Th17 conditions, following which the concentrations of IL-21 (upper panel) and IL-23 (lower panel) in the culture supernatants were determined by ELISA.

the MSC blockade of Th17 differentiation. Finally, direct addition of 2 ng/mL PGE2 into the conditioned medium of the Th17 differentiation assay in the absence of MSC resulted in inhibition of Th17 differentiation compared with the control without PGE2 (Figure 5Ci). Moreover, a titration of PGE2 demonstrated a gradual dose-dependent inhibition of Th17 differentiation (Figure 5Cii). As shown above (Figure 5A), the level of PGE2 produced by MSC in our conditions was ~2ng/mL and PGE2 at that concentration inhibited Th17 differentiation, but the inhibition appeared to be not as strong as MSC (Figure 5Ci, middle versus right bar, Figure 5Cii, upper versus lower panel). These results suggested a contribution of other factors in addition to PGE2. As is known, PGE2 per se blocks T-cell proliferation in a dose-dependent manner (Figure 5Ciii).

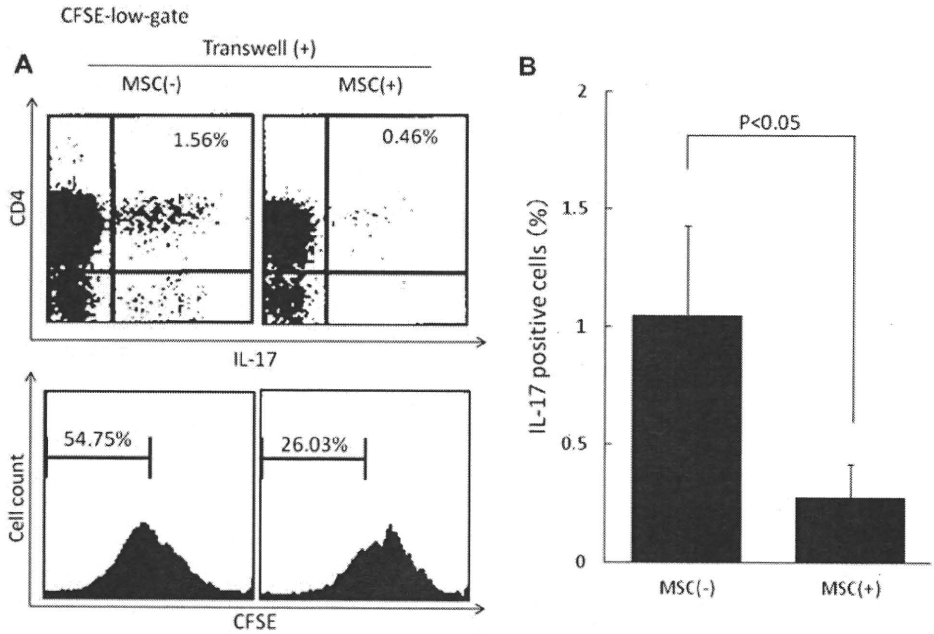

Figure 4. MSC–Th17 suppression is mediated by a soluble factor. (A) CD4 T cells (3×10^4/well) were stimulated in the upper chamber of a Transwell apparatus under Th17 conditions in the presence or absence of MSC (5×10^3/well) in the lower chamber. IL-17-positive CD4 T cells and cell proliferation were assayed by flow cytometry. Representative dot-plots of three experiments are shown. (B) The average percentage of IL-17 positive CD4 T cells was calculated from three independent experiments.

IDO partly mediates Th17 suppression by MSC

As PGE2 does not completely revert MSC suppression, we searched for other factors that might contribute to this suppression. IDO has also been reported to mediate the suppression of T-cell proliferation by MSC (10), although we were unable to confirm an involvement of IDO under neutral conditions using mouse MSC in a previous study (7). To investigate whether IDO mediates the suppression of Th17 differentiation by MSC, we assayed the effect of the addition of a well-known inhibitor of IDO, 1-methyl-DL-tryptophan (1-MT). The addition of 1-MT inhibited MSC suppression of Th17 differentiation (Figure 6A, upper dot-plot and lower panel) without affecting T-cell proliferation (Figure 6A, middle histogram), suggesting that IDO, at least in part, mediates MSC inhibition of Th17 differentiation. To determine whether the effect of indomethacin and 1-MT is additive or not, we repeated these experiments in the presence of each inhibitor alone or in combination. The combination of both inhibitors showed additive restoration (Figure 6B, upper dot-plot and lower panel), suggesting that PGE2 and IDO play independent roles in the molecular mechanism of Th17 suppressionby MSC.

Discussion

In this report we provide evidence indicating that MSC inhibit Th17, but not Treg, differentiation. We also show that, although NO is an important mediator of MSC effects under neutral and Th1 conditions (7,12), NO is not produced by MSC under Th17 conditions. Our results demonstrated that PGE2 and IDO, but not NO, may play a role in the inhibition of Th17 differentiation by MSC.

It is difficult to dissociate the effect of MSC on differentiation from its effects on proliferation, because T-cell differentiation requires T-cell proliferation. In the case of Th17/Treg as well as Th1/Th2 differentiation, MSC primarily block proliferation. This fact makes it difficult to distinguish whether MSC inhibition of differentiation is actually the result of inhibition of differentiation itself or is secondary to MSC inhibition of proliferation. In this study, in order to resolve this issue, we finely adjusted the number of MSC in MSC–T-cell co-cultures so that proliferation would not be apparently blocked. Furthermore, we also analyzed CFSE-low cells alone that had already divided. Thus our data suggest that MSC can block not only T-cell proliferation but also Th17 differentiation *per se*.

Our data suggesting PGE2 partly mediates MSC suppression of Th17 differentiation are in apparent contrast to two previous reports that PGE2 promotes Th17 differentiation (43,44). However, this discrepancy may be explained by the fact that the culture conditions and timing of PGE2 addition were quite different in the previous studies from those of our study. The two previous studies investigated the

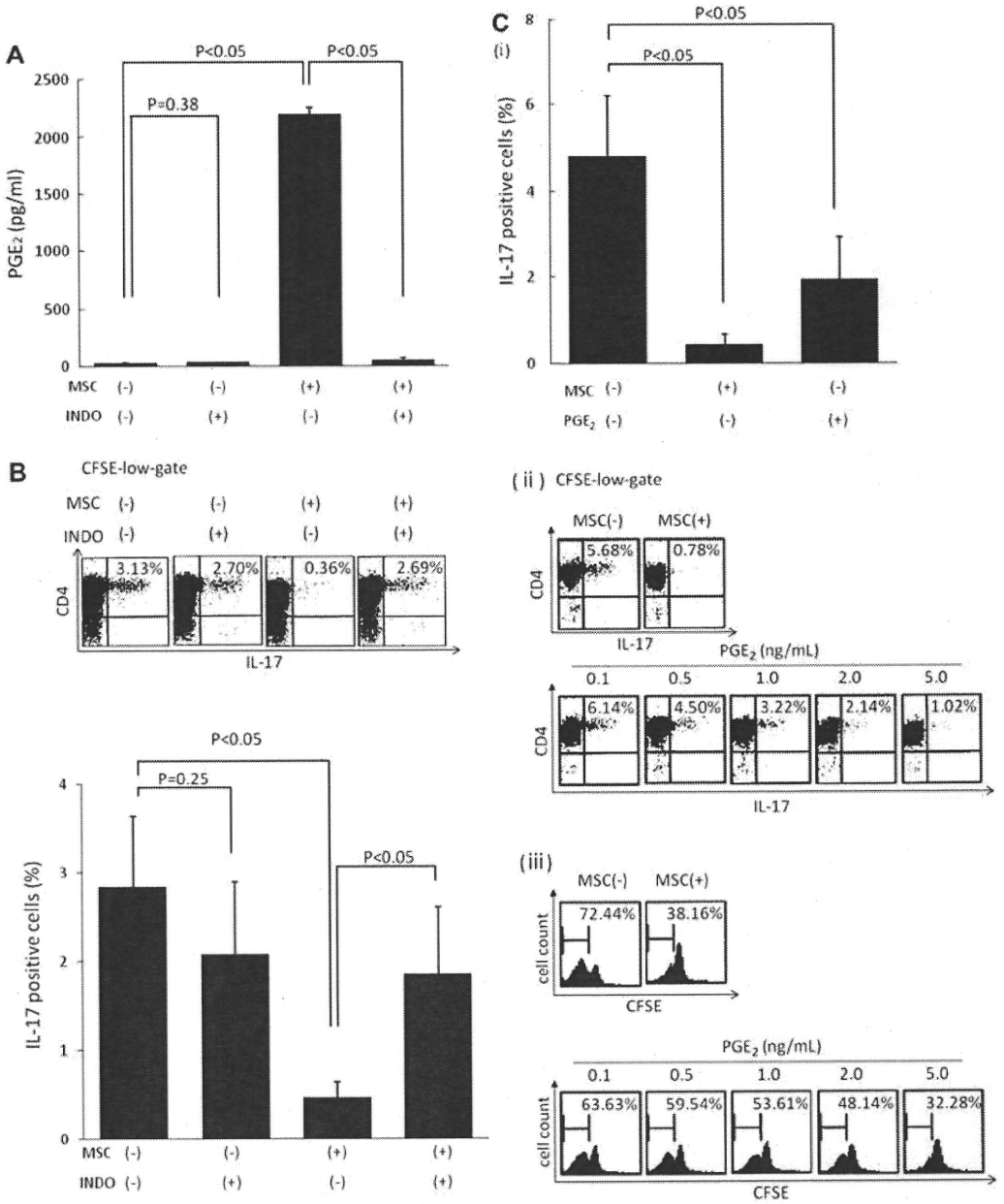

Figure 5. PGE2 partly mediates MSC-induced Th17 differentiation. (A) PGE2, produced in media following culture of CD4 T cells in the absence or presence of MSC with or without the prostaglandin inhibitor indomethacin (INDO, 5 μM), was assayed by ELISA. (B) Th17 differentiation of the cells in (A) was analyzed by flow cytometry and a representative result is shown (upper panel). The average percentage of IL-17-positive cells of three independent experiments is indicated in the lower panel. (C) (i) The effect of direct addition of PGE2 (2 ng/mL) into co-cultures of MSC and CD4 T cells was assessed by the percentage of IL-17-positive CD4 T cells using flow cytometric analysis. The average of three independent experiments is shown. (ii) Effects of titration of PGE2 on Th17 differentiation were investigated as in (i). The representative result from three experiments is shown. (iii) Effects of titration of PGE2 on proliferation were analyzed using CFSE and flow cytometry. The representative result from three experiments is shown.

effect of PGE2 on secondary expansion after primary differentiation rather than directly on primary differentiation, which was the focus of our study. In our study, the addition of PGE2 to the primary differentiation assay inhibited Th17 cell differentiation and proliferation in a dose-dependent manner (Figure 5C). We believe that the different timing of PGE2 addition was the cause of the different results.

The observed inhibition of MSC suppression of Th17 differentiation by PGE2 and IDO only partly explains the mechanism of MSC suppression. Therefore a future search for other mediators of MSC suppression remains to be carried out. Nevertheless, our results provide new insight into the mechanism of MSC immunomodulation that might help us understand the underlying signaling pathways by

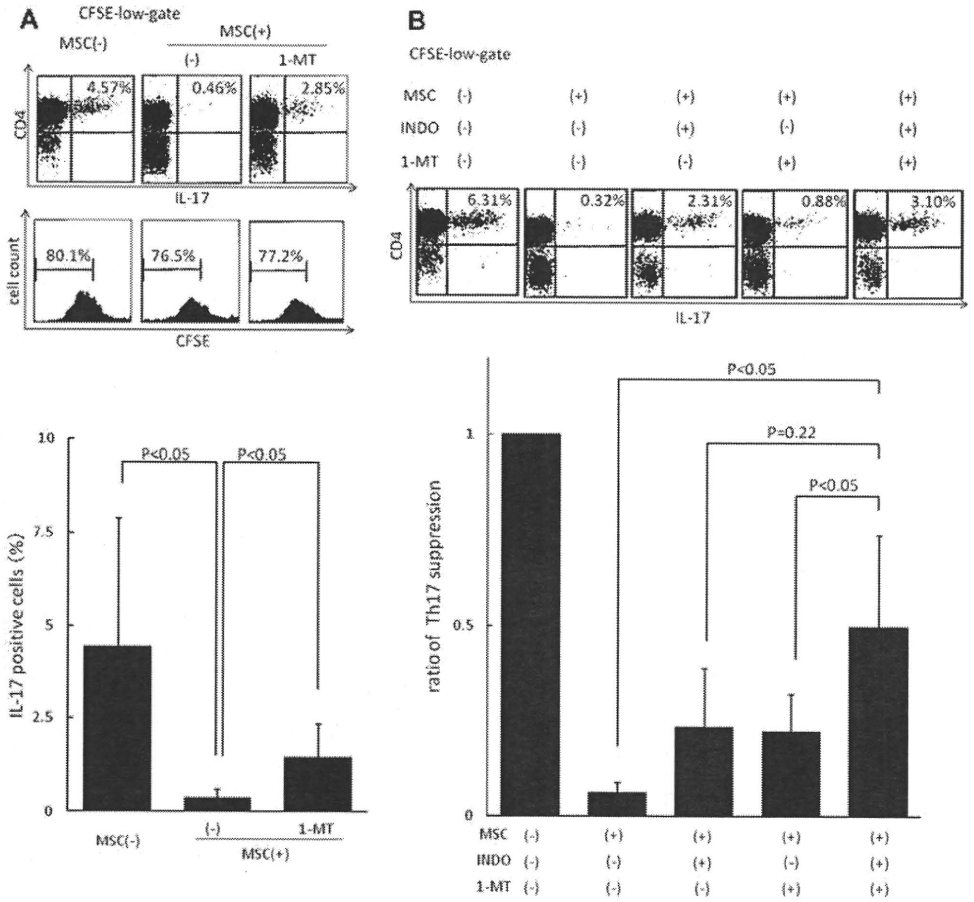

Figure 6. The effect of IDO on MSC suppression of Th17 differentiation. (A) CD4 T cells were cultured for 4 days in the absence of MSC, or in the presence of MSC with or without the IDO inhibitor 1-MT (1 mM), under Th17 conditions. The effect of 1-MT in co-cultures of MSC and CD4 T cells was assessed by the percentage of IL-17-positive CD4 T cells (upper panel) and cell division (middle panel) using flow cytometric analysis. The lower panel indicates the average percentage of IL-17-positive cells. (B) The effect of simultaneous addition of two inhibitors, INDO (5 μM) and 1-MT (1 mM), on CD4 T cells cultured for 4 days in the presence or absence of MSC under Th17 conditions. CD4 T cells were stained with anti-IL-17 and analyzed using flow cytometry (upper panels). The lower panel depicts the ratio of IL-17-positive cells relative to a positive control from three independent experiments.

which MSC modulate GvHD. The combination of our data and previously published studies suggest that immunomodulation by MSC may be mediated by different mechanisms under different conditions.

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Review Article

Mesenchymal Stromal Cells for Graft-Versus-Host Disease : Basic Aspects and Clinical Outcomes

Kazuya Sato, Katsutoshi Ozaki, Masaki Mori, Kazuo Muroi, and Keiya Ozawa

Mesenchymal stromal cells (MSCs) have unique characteristics such immune suppression by inhibiting T cell proliferation, tissue-repair ability and acceleration of hemopoietic stem cell engraftment. The cells are rare in bone marrow, but easily cultured under standard culture conditions. Soluble factors and cells are implicated in the MSC-mediated T cell suppression and numerous clinical trials using MSCs to prevent and treat graft-versus-host disease (GVHD) have been reported. MSCs are suggested to suppress acute GVHD without impairing graft-versus-leukemia effects and increasing systemic infections. In this review, we focus on basic aspects of MSC-mediated T cell suppression and clinical trials using MSCs for GVHD and related conditions. 〔*J Clin Exp Hematopathol 50(2) : 79-89, 2010*〕

Keywords: mesenchymal stromal cells, bone marrow, immunosuppression, graft-versus-host disease

INTRODUCTION

Mesenchymal stromal cells (MSCs) are non-hemopoietic cells with the capacity to self-renew and differentiate into various cell lineages of mesenchymal origin.[1] These cells can be obtained from bone marrow, adipose tissues, fetal liver, and umbilical cord blood.[2-4] MSCs have great expansive potential under optimal conditions *in vitro*. After a 2-3 day incubation of human bone marrow aspirate, colonies of plastic-adherent spindle-shaped cells can be found (Fig. 1). Functionally, adult MSCs are characterized by rapid proliferation (a doubling time of 33 hr).[5] Although it has been estimated that MSCs constitute only 0.01%-0.001% of bone marrow cells, as many as 50-375 million MSCs can be generated by the passages from a 10-mL human bone marrow aspirate, and still retain their capacity for differentiation.[1] MSCs are expected to be a source of regenerative medicine for repairing defects in a variety of diseases. In children with osteogenesis imperfecta, allogeneic bone marrow-derived MSCs engrafted and stimulated growth.[6] Also, MSCs play a key role in the maintenance of the bone marrow microenvironment and regulate the maturation of hemopoietic stem cells by providing various growth factors. Promotion of engraftment and hematological recovery after the co-infusion of autologous hematological stem cells and MSCs were reported.[7-9]

More recently, the immune regulatory potential of MSCs has been focused on. MSCs have been found to suppress inflammation by inhibiting T cell proliferation, representing a novel treatment for graft-versus-host disease (GVHD). Le Blanc *et al.* described a patient with severe refractory stage IV GVHD of the gut and liver who was infused with MSCs in 2004.[10] His GVHD improved dramatically and rapidly following 2 infusions, and no significant side effects occurred. In a multicenter phase II study by the European Group for Blood and Marrow Transplantation, the response rate to treatment of GVHD with MSCs was over 70%, and treatment efficiency was not related to a donor human leukocyte antigen (HLA)-match.[11] However, the molecular mechanisms by which MSCs suppress immune responses *in vivo* and *in vitro* are poorly understood. We here review the molecular mechanisms of immunomodulation by MSCs and results of clinical trials using the cells.

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BASIC ASPECTS

Immune regulation by MSCs

First, it should be emphasized that there are distinct differences in immune suppressive activity between human and non-human derived MSCs.[12] Regardless of species though, MSCs exert strong immune suppressive activity against a broad range of immune cells. However, the rate of cell growth, cell surface antigens, and soluble factors implicated in MSC-mediated immune suppression vary (data not

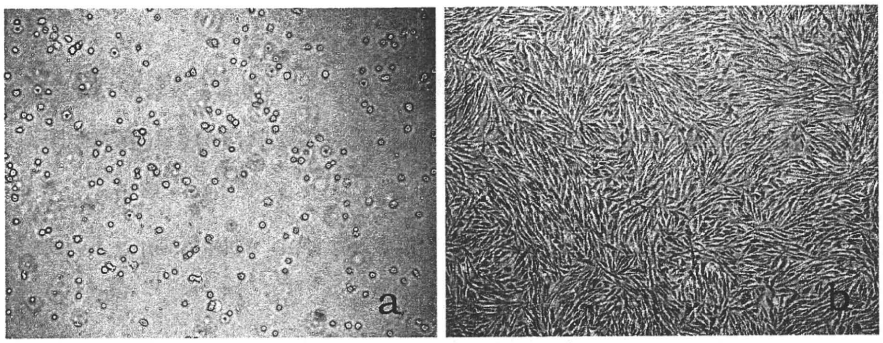

Fig. 1. Bone marrow-derived mesenchymal stromal cells on phase contrast microscope. After incubation of human bone marrow aspirate for 2 days, adherent cells appear (***1a***) and they rapidly grow at 14 days (***1b***). (***1a***) & (***1b***) ×40.

shown).[12,13] Despite the great interest in MSCs, a clear definition of MSCs has not been established, and plastic-adherent cells from bone marrow cultures are highly heterogeneous. Human MSCs can be relatively easily isolated and rapidly expanded. In contrast, murine MSCs are difficult to propagate and usually contaminated by hemopoietic precursors (data not shown).[14] Furthermore, methods of isolation and expansion differ among investigators. Therefore, results regarding the immune suppressive mechanisms of MSCs should be interpreted carefully. MSCs have been shown to inhibit not only T cells[15-20] but also B cells,[18,20,21] natural killer cells,[22] and monocyte-derived dendritic cells.[23] As the T cell inhibition by MSCs has been investigated, we focus here on the molecular mechanism of this inhibition.

Conventional T cells

The idea of investigating the immune suppressive effects of MSCs on T cell responses comes from the role of the thymic epithelium in T cell development.[24] Hemopoietic stem cells reside in bone marrow niches surrounded by MSCs which regulate the self-renewal and differentiation. However, little has been investigated about T cell regulation by MSCs. In the presence of MSCs, T cell responses stimulated by alloantigens (e.g., mixed lymphocytes),[15,17,19] peptide antigens,[16,18] mitogens,[15,19,20] and a CD3/CD28 antibody[20] have been tested, suggesting that the immune suppressing effects of MSCs are not antigen-specific. The inhibitory effects of MSCs on T cell proliferation are dose-dependent.

Phorbol 12-myristate 13-acetate and ionomycin are known to act downstream of the T cell receptor complex by activating protein kinase C and inducing Ca^{2+} influx, respectively. T cell proliferation stimulated by these mitogens was also suppressed by MSCs, suggesting that the T cell receptor complex is not a target for the suppression and that MSCs influence signals downstream of protein kinase C and Ca^{2+} influx.[20] As MSCs equally inhibit the proliferation of both CD4 and CD8-positive T cells as well as unfractionated T cells, the inhibitory effects of MSCs do not target any specific T cell subpopulations.[15,18,20] The transwell system, by which one can physically separate T cells and MSCs with a finely textured permeable membrane, has been used to confirm the necessity of cell-contact. However, T cell-MSC-contact dependency is still controversial.[15-20] Di Nicola *et al.* initially reported that T cell proliferation was also significantly inhibited using the transwell system, thus suggesting that a soluble factor is involved. However, the rate of T cell inhibition increased when contact between MSCs and T cells was allowed.[15] These results have been also confirmed in our laboratory.[20] Taken together, cell-contact could be required for maximum T cell suppression by MSCs, but soluble factors secreted by MSCs have recently been considered to play a key role in MSC-mediated immune suppression.

So far, transforming growth factor-β1, hepatocyte growth factor, indoleamine 2, 3-dioxygenase, which induces tryptophan's catabolism, prostaglandin E2 (PGE2), and nitric oxide (NO) have been reported to mediate the T cell inhibition by MSCs (Table 1).[15,17,19,20] Djouad *et al.* found that conditioning medium obtained from MSCs cocultured with "activated (stimulated)" splenocytes suppressed T cell proliferation, whereas the supernatant from neither cultures of MSCs alone nor MSCs cocultured with "resting (non-stimulated)" splenocytes inhibited T cell proliferation, suggesting that the "activation" of MSCs by T cells was required for T cell suppression.[25] Some reports have shown that MSCs suppressed the expression of the early activation markers CD25 (IL-2 receptor) and CD69,[15,18,20] whereas others have demonstrated that MSCs had little or no effect on the activation markers.[26,27] Division arrest anergy of activated T cells induced by MSCs was also reported. Glennie *et al.* have shown that the expression of cyclin D2 was prevented, whereas the expression of the negative cell cycle regulatory protein $p27^{kip1}$ was strongly downregulated in stimulated T cells co-cultured with MSCs.[18] Analysis of the cell cycle showed that T cells,

Table 1. Mesenchymal stromal cell (MSC)-mediated immune suppression

1st author	Origin of MSCs	Source of MSCs	Necessity of cell-contact	Immunosuppressive factor(s) or mechanism	Reference
Di Nicola	human	bone marrow	partially required	TGF-β, HGF	15
Krampera	mouse	bone marrow	required	unmentioned	16
Meisel	human	bone marrow	not examined	IDO	17
Glennie	mouse	bone marrow	not examined	division arrest anergy	18
Aggarwal	human	bone marrow	not examined	PGE2	19
Sato	mouse	bone marrow	partially required	nitric oxide, PGE2	20

TGF-β, transforming growth factor-β ; HGF, hepatocyte growth facto ; IDO, indoleamine 2, 3-dioxygenase, PGE2, prostaglandin E2

stimulated in the presence of MSCs, were arrested at the G1 phase.[18] These investigators argued that the inhibition of T cell proliferation was profound and irreversible.[18] However, Krampera *et al.* and we have shown that although the presence of MSCs inhibited the first antigenic stimulation, when MSCs were removed the response to the second stimulation was restored.[16,20] Recently, we have reported that the STAT5 phosphorylation in T cells was suppressed in the presence of MSCs and that NO is involved in the suppression of STAT5 phosphorylation and T cell proliferation.[20] However, MSCs from inducible NO synthase knockout mice could still suppress T cell proliferation. Furthermore, indomethacin (inhibitor of PGE2 production) also restored T cell proliferation, but the effects of a specific inhibitor of NO synthase and indomethacin were not additive. These findings suggest that the molecular mechanisms of T cell inhibition by MSCs involve various factors in response to inflammatory cytokines, and that the cell-signaling pathway is also complicated.

Th1/Th2 and Th17

The importance of the T helper (Th)1/Th2 balance has been well established in GVHD. In some experimental models, Th1 cells augment and Th2 cells ameliorate acute GVHD.[28,29] A previous report by our colleagues confirmed that mouse MSCs suppressed both the proliferation and differentiation of Th1 cells, whereas the suppression of Th2 cells was mild.[30] Aggarwal *et al.* also showed that human MSCs caused Th1 cells to secret less interferon-γ and caused Th2 cells to increase secretion of interleukin (IL)-4.[19] These results suggested that MSCs interact with T cells and induce a Th1 to Th2 shift. Recently, we identified a novel T cell subset, namely, CD4 T cells which produce the proinflammatory cytokine IL-17. Regulatory T (Treg) cells positive for CD4 and CD25 are another newly recognized subset, in which the CD4 T cells have high levels of Foxp3 expression and inhibit T cell proliferation. Treg cells prevented GVHD by inhibiting the proliferation and function of conventional T cells in a murine model,[31] whereas the role of Th17 cells in the pathogenesis of GVHD is still unknown.[32,33] Very recently, we showed that MSCs block the differentiation of Th17 cells through PGE2 production.[34]

CLINICAL OUTCOMES

MSCs for steroid-resistant acute GVHD

A summary of published reports on the treatment of steroid-resistant acute GVHD (aGVHD) with MSCs is shown in Table 2. The first case of severe aGVHD successfully treated with MSCs was reported by LeBlanc *et al.*[10] The patient, a 9-year-old boy with acute lymphoblastic leukemia (ALL) in his third remission, received a peripheral blood stem cell transplant from an HLA-identical unrelated female donor. After the transplantation, the patient developed grade IV aGVHD of the liver and gut, which did not respond to conventional doses of steroid, bolus steroid, infliximab, daclizmab, and mycophenolate mofetil or other treatments. MSCs were prepared from his haploidentical mother's bone marrow and infused twice into the patient. The patient's aGVHD completely disappeared. Importantly, in the authors' institution, this individual was the only surviving patient among 25 patients with grade IV aGVHD after hemopoietic stem cell transplantation (HSCT). Ringden *et al.* that reported eight adults received MSCs for steroid-resistant aGVHD.[35] The MSCs were prepared from a median of 50 ml of bone marrow from HLA-identical siblings, haploidentical donors, and HLA-mismatched donors. They were infused at a median of 77 days after HSCT. Five patients showed a complete response (CR). The survival of patients with gut aGVHD who received MSCs was significantly better than that of the untreated patients. Prasad *et al.* reported the treatment of 12 pediatric patients with steroid-resistant aGVHD with MSCs.[36] MSCs derived from bone marrow of HLA-mismatched unrelated donors (third-party MSCs, Prochymal) were provided by Osris Therapeutics, Inc. MSC therapy was started at a me-

Table 2. Treatment of steroid-resistant acute graft-versus-host disease with mesenchymal stromal cells

1 st author	Year of publication	Ref	No. of pts	Age (Years)	No. of pts with aGVHD II/III/IV	No. of MSC donors S/Haplo/U	No. of pts for MSC passages Once/Twice/More	No. of pts for MSC infusions Once/Twice/More	1 st MSC infusion Days after HSCT	No. of infused MSC doses (× 10^6/kg)	No. of pts with response CR/PR/Others (%)	Outcome (No.) Alive/Death
Le Blanc	2004	10	1	9	0/0/1	0/1/0	0/1/0	0/1/0	73	1.5	1 (100)/0/0	1/0
Ringden	2006	35	8	56	0/6/2	4/0/4	3/4/4	5/3/3	77	1.3	5 (63)/0/3 (38)	5/3
Ringden[#]	2006	35	16	40	2/10/4	ND	ND	ND	ND	ND	ND	ND
Prasad	2007	36	12	6	0/7/5	0/0/12	ND	0/0/12 (8 times)	119	2 (10 pts), 8 (2 pts)	6 (50)/6 (50)/0	6/6
Fang	2007	37	6	40	0/2/4	0/2/4	ND	5/1/0	71	1	5 (83)/0/1 (17)	4/2
Müller	2008	38	2	9	1/1/0	0/1/1	ND	2/0/0	89	1.7	0/0/2 (100)	1/1
Le Blanc	2008	11	55	22	5/25/25	5/18/69	14/42/36[¶]	27/22/6	ND	1.4	30 (55)/9 (16)/16 (29)	21/34
von Bonin	2009	39	13	58	0/2/11	0/0/13	ND[§]	0/13/0	41[/]	0.9	1 (8)/1 (8)/11 (85)	4/9
ND	2009	40	260	ND	ND	0/0/260	ND	ND	ND	2	CR (40)	ND
Muroi	2009	41	2	37.5	0/0/2	2/0/0	0/0/2	0/2/0	42	1.0	0/0/2 (100)	0/2
Kebriaei[†]	2009	42	31	52	21/7/3	0/0/31	ND	0/31/0	ND	2 (16 pts), 8 (15 pts)	24 (77)/5 (16)/2 (6)	22/9
ND[†]	2009	43	192	ND	ND	0/0/192	ND	ND	ND	2	ND	ND

Ref, references ; Pts, patients ; No., number ; aGVHD, acute graft-versus-host disease ; MSC, mesenchymal stromal cells ; S, sibling donors ; Haplo, haploidentical donors ; U, unrelated donors ; HSCT, hemopoietic stem cell transplantation ; CR, complete response ; PR, partial response ; Others ; response including minimal response, no response, progression, and no evaluable cases ; ND, not done or not shown ; [#], controls ; [¶], no. of MSC infusions for MSC passages ; [/], including after donor lymphocyte infusion and cessation of immunosuppressants ; [†], MSC with or without steroid as a first line therapy for aGVHD

dian of 81 days after HSCT. All patients responded to the therapy with 6 patients having a CR and the rest, a partial response (PR). The application of MSCs derived from adipose tissue to 6 patients with steroid-resistant aGVHD was reported.[37] The median age of the patients was 40 years. The MSCs were obtained from either haploidentical or unrelated donors. The cells were isolated from abdominal adipose tissue of the donors by lipectomy, and cultured with an expansion medium. Five patients showed a CR, four of which were alive and disease-free following infusions of the adipose-derived MSCs. Müller *et al.* reported the response of bone marrow-derived MSCs to various conditions after HSCT.[38] MSCs were isolated with 20 mL of bone marrow and cultured in an expansion medium. One of two pediatric patients with steroid-resistant aGVHD did not develop chronic GVHD (cGVHD). The European Group for Blood and Marrow Transplantation reported a phase II study of bone marrow-derived MSCs for steroid-resistant aGVHD.[11] The pediatric and adult patients numbered 25 and 30, respectively. The median age of all the patients was 22 years. MSC donors included HLA-identical siblings, haploidentical donors, and HLA-mismatched unrelated donors. Mononuclear cells were isolated from a median of 60 mL of bone marrow collected from MSC donors and cultured to obtain MSCs in an expansion medium. The MSCs were passaged once for 14 infusions, two or three times for 47 infusions, and three or four times for 29 infusions. The number of infusions was one for 27 patients, two for 22 patients, and more than two for 6 patients. A median number of 1.4×10^6 MSCs/kg was infused. A CR was obtained for 68% of the pediatric patients and 43% of the adult patients. The overall response rate of the patients was 70%. The 2-year survival rate of complete responders was significantly better (53%) than that of partial responders plus non-responders (16%). There was no difference in response rates between patients who received MSCs from third-party donors and those who received MSCs from other sources. von Bonin *et al.* reported the treatment of steroid-resistant aGVHD with MSCs.[39] Thirteen patients with a median age of 58 years were treated with MSCs for steroid-resistant aGVHD. MSCs from unrelated donors' bone marrow were expanded in a medium containing 10% human platelet lysate instead of fetal calf serum (FCS). The median time of the first MSC infusion after HSCT was 41 days. A CR, PR, and mixed response were obtained in one patient, one patient, and five patients, respectively. Osiris conducted a phase III study of Procymal for patients with steroid-resistant aGVHD (protocol 280). This trial was a double-blind, placebo controlled study and patients were randomly allocated treatment with Procymal and a placebo at a proportion of two to one. The total number of patients enrolled was 260. MSCs were administered twice a week for 4 wk at 2×10^6 cells/kg per infusion. Recently, Osiris published preliminary results of the phase III study.[40] Although there was no difference between Procymal and placebo at the primary endpoint, the rate of CR was better in the Prochymal group than in the control group (40% and 28%, respectively). Procymal significantly improved response rates to liver and gut aGVHD (29% and 88%, respectively). Notably, the Procymal group had more severe GVHD (28%) than the control group (16%).

We conducted a pilot study of the use of MSCs for steroid-resistant aGVHD after HSCT, which was approved by an institutional review board.[41] The MSC donors were only relatives. Eight patients with steroid-resistant aGVHD were enrolled. About 10 mL of bone marrow was aspirated from each donor. Mononuclear cells were isolated using Ficoll-hypaque density gradient centrifugation and suspended in a human MSC expansion medium containing 10% FCS. Cells were cultured at a density of 1×10^6/mL at 37°C in a 5% CO_2 incubator and non-adherent cells were removed. When adherent cells became confluent, they were detached with trypsin and ethylenediaminetetraacetic acid and passaged. The supernatant of harvested MSCs was checked for bacteria, fungi, endotoxin, hepatitis B antigen, hepatitis C antibody, Epstein-Barr virus DNA, cytomegalovirus DNA, and human herpesvirus 6 DNA. A chromosomal analysis of the MSCs was performed. Of eight patients with steroid-resistant aGVHD, the GVHD in five patients was resolved slowly by steroid or by the addition of a bolus of methylprednisolone and/or mycophenolate mofetil. One patient was excluded due to viral pneumonia. Two patients were administered MSCs, one of whom showed a minimal response. This patient was a 42-year-old male who had acute myeloblastic leukemia (AML) which progressed from myelodysplastic syndrome and did not enter into complete remission (Fig. 2). He received a peripheral blood stem cell transplant from his HLA-identical sister after myeloablative conditioning. The GVHD prophylaxis was short-term methotrexate and cyclosporine treatment. On day 14 after transplantation, a donor-cell engraftment was observed using fluorescent *in situ* hybridization for X and Y chromosomes. On day 18, 1 mg/kg/day of prednisolone was started for grade II aGVHD of skin, liver and gut. On day 22, the aGVHD had progressed despite of prednisolone treatemnt. Therefore, mycophenolate mofetil was added and the dose of prednisolone was increased. The aGVHD worsened and bloody diarrhea with abdominal cramps occurred. The pathological findings of the colon mucosa were compatible with aGVHD. A bolus of methylprednisolone was given and cyclosporine was changed to tacrolimus. Following these treatments, the skin and liver aGVHD were resolved. However, the gut aGVHD persisted with bloody diarrhea and severe abdominal cramps. Therefore, MSCs were prepared from bone marrow of the same peripheral blood stem cell transplant donor. On day 58, 0.06×10^6/kg of thawed MSCs were infused, however, the abdominal cramps and bloody stools persisted. On day 74, the patient complained of severe abdominal pain. Computed tomography showed free-air in