Enhancement of Graft-versus-leukemia Effects by Mesenchymal Stem Cells in Mixed Chimerisim after a Murine Non-myeloablative Hematopoietic Stem Cell Transplantation

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Background: Mesenchymal stem cells (MSCs) may be useful for reducing graft-versus-host disease (GVHD) after allogeneic hematopoietic stem cell transplantation (allo-HSCT). The GVHD and a graft-versus-leukemia (GVL) effect are inversely related. We therefore wanted to determine whether MSCs can preserve the GVL effect following experimental allo-HSCT.

Methods: After non-myeloablative allogeneic hematopoietic stem cell transplantation (NM-HSCT) using C57BL/6 (H-2^b)→B6D2F1 (H-$2^{b/d}$), some mice received donor lymphocyte infusion (DLI) for induction of GVL effects by virtue of complete chimerism (CC), while the other groups did not receive DLI with persistence of mixed chimerism (MC). All mice were inoculated subcutaneously with P815 tumor cells and were intravenously treated with either donor MSCs or diluents.

Results: Between the DLI-treated groups with CC, tumor-related deaths and tumor growths were comparable irrespective to the infusion of MSCs. On the contrary, among mice without DLI which showed MC, the administration of MSCs significantly delayed tumor-related deaths compared to those without the administration of MSCs (50-day percent survival, 54.5% vs. 18.1%, P=0.0225). In the MC animals, tumor growth seemed to be more delayed in the mice injected with MSCs than in the controls (P=0.09). Donor MSCs injection was associated with increased donor effector/memory CD62L- T cells in MC but not in CC.

Conclusion: In spite of the observed immunosuppressive effects of donor MSCs, our results indicate that the GVL effects were not influenced by the injection of MSCs but that under a given condition such as MC, the injection of donor MSCs could result in enhanced GVL effects. ***(Korean J Hematol 2008;43:219-231.)***

Key Words: Mesenchymal stem cell, Graft-versus-host disease, Graft-versus-leukemia effect, Allogeneic hematopoietic stem cell transplantation

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INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is a potentially curative treatment for hematological malignancies. However, the relapse of the underlying disease and graft-versus-host disease (GVHD) are major obstacles. The therapeutic potential of allo-HSCT relies on the graft-versus-leukemia (GVL) effect, which eradicates the residual malignant cells via immunological mechanisms.[1] Unfortunately, the GVHD and GVL effects are tightly linked, as demonstrated by the inverse correlation between the leukemia relapse rate and the severity of GVHD.[2]

In addition to hematopoietic stem cells, the bone marrow also contains mesenchymal stem cells (MSCs). These cells, described by Friedenstein et al,[3] have the capacity of prolonged self-renewal and, in an inductive microenvironment, differentiate into specialized mesenchymal tissues, such as hematopoietic stroma, bone, cartilage, adipose tissue, and others.[4-8] MSCs have immunomodulatory properties and have been reported to inhibit T cell proliferation *in vitro*.[9-12]

An immunosuppressive effect of MSCs *in vivo* has been shown in a baboon model, in which an infusion of *ex vivo*-expanded matched donor or third-party MSCs delayed the time of rejection of a histo-incompatible skin graft.[13] The immunosuppressive effect of MSCs in human beings, as a corollary to the immunosuppressive effect of MSCs *in vitro* and in preclinical animal models, suggests that MSCs may be useful for preventing and treating GVHD after allo-HSCT.[14] One concern with use of MSCs, however, is the potential to increase the incidence of relapse as a result of severe immunosuppression. The efficacy of MSCs in GVHD has unfortunately revealed the expected drawback of increased leukemia relapse.[15] Hence, preserving the beneficial control of GVHD without affecting GVL effect remains a challenge for future studies with regard to the use of MSCs.

Using a murine model of non-myeloablative allo-HSCT (NM-HSCT) and donor lymphocyte infusion (DLI), this study investigated whether or not the primary MSCs affect the GVL effect. Establishment of a state of mixed chimerism (MC) followed by delayed DLI has been shown to be associated with a reduced risk of GVHD. In this model, the GVL effect can be induced faster than the anticipated onset of overt clinical manifestation of acute GVHD. In contrast to expectations based on *in vitro* and isolated clinical observations, our data demonstrate that, in this murine NM-HSCT followed by DLI model, the influence of MSCs on GVL effects differed according to the status of chimerism. These findings may have clinical implications for the use of MSCs-based immunosuppressive therapeutic strategies after allo-HSCT.

MATERIALS AND METHODS

1. Mice

Female C57BL/6 (B6, H-2^b) and B6D2F1 (F1, H-$2^{b/d}$) mice were purchased from Japan SLC Inc. (Shizuoka, Japan). The age of the mice ranged from 8 to 12 weeks. The mice were housed in sterilized microisolator cages and received filtered water and normal chow or autoclaved hyperchlorinated drinking water for the first 3 weeks after allo-HSCT.

2. Isolation and proliferation of MSCs from bone marrow (BM)

The BM cells, which were collected by flushing the femurs and tibias with the medium, were cultivated in 75 cm^2 tissue culture flasks at a concentration of 1×10^6 cells/mL using complete Dulbecco modified Eagle medium (WelGENE Inc., Daegu, South Korea) supplemented with 10% heat-inactivated fetal bovine serum (FBS, WelGENE Inc.), 2mM glutamine, 100U/mL penicillin and 100 μg/mL streptomycin (Gibco BRL, Gaithersburg, MA, USA). No cytokines were added at any stage of the experiment. The cultures

were cultured at 37°C in a 5% CO_2 atmosphere. After 72 hours, the non-adherent cells were removed. When the cells had reached 70～80% confluence, the adherent cells were trypsinized (0.05% trypsin at 37°C for 5 minutes), harvested and expanded in larger flasks. A homogenous cell population was obtained after culturing for 3～5 weeks. The MSCs were maintained in culture for no more than 15 *in vitro* passages.

3. Experimental NM-HSCT with DLI and tumor cell inoculation

F1 recipients were given a single dose of 400 cGy nonmyeloablative total body irradiation (TBI) (cesium Cs 137 [^{137}Cs] source). BM cells obtained by flushing the long bones of B6 donor mice with RPMI (WelGENE Inc.) supplemented with 10% FBS (WelGENE Inc.) were injected into the lateral tail vein of F1 recipients on the day of TBI. For DLI, spleen cells from B6 donors were resuspended in RPMI 1640 supplemented with 10% BCS washed twice, resuspended in the same medium, and injected into tail vein of recipient mice 14 days after NM-HSCT. Subcutaneous (*s.c.*) injection of 1×10^6 cells of P815 (H-2^d) mastocytoma line into F1 mice induced locally fast growing tumors. These have been used extensively as a cancer model to analyze and manipulate the relationship between tumor cells and the GVL effect.[16] Survival was monitored daily. The degree of clinical GVHD was assessed every 3 or 4 days based on weight loss, posture, presence of diarrhea, and skin lesions such as alopecia and dermatitis. Tumor growth was inspected every 3 or 4 days by measuring the largest perpendicular diameters with a caliper, which was thus recorded as the tumor area (mm^3). In some experiments, tumor acceptance was finally determined on day 35.

4. Histology and fluorescent detection of MSCs

The tumors, liver, spleen and lungs were removed 3 weeks after the *s.c.* inoculation and were then fixed in 10% buffered formalin, embedded in paraffin, and cut into 5 μm slices. The sections were stained with H&E. For a fluorescent histologic analysis, MSCs were fluorescence-labeled using preincubation with PKH-26 (Sigma-Aldrich, St. Louis, USA) according to the manufacturer's instructions as described.[17] Briefly, 10^5 MSCs were washed two times in HBSS (WelGENE Inc.) supplemented with 2% FBS (WelGENE Inc.), and the pellet was suspended in 600 μL diluent buffer. One μL of PKH-26 solution (10^{-3} mol/L) prediluted into 100 μL diluent buffer was added, and the mixture was left at room temperature for 3 minutes with gentle agitation for several times. The labeling reaction was stopped by the addition of 1mL heat-inactivated FCS (Pan Systems) for 1minute. This mixture was then carefully underlayered with 1mL heat- inactivated FCS. Cells were cytocentrifuged for 5 minutes at 400g, the supernatant was removed, and the pellet was washed three times in serum-containing medium (HBSS/2% FCS) in a fresh tube for every washing step. Twenty-one days after PKH-labeled MSC injection, mice were sacrificed and various organs as well as tumor tissues were harvested. Tissues were immediately embedded in OCT (Cellpath) embedding matrix, placed on dry ice and stored at −80°C until required. OCT embedded tissues were cryosectioned into 5 μm thick sections and allowed to air dry while protected from light. Sections were fixed for 15min in 4% paraformaldehyde, followed by three 5min washed in PBS. Cells were then stained using 4',6-diamidino-2-phenylindole (DAPI; 1 μg/mL) for 4 min in foil and washed thrice for 5min in PBS. Sections were then mounted in DPX mounting medium and examined using a wavelength for PKH26 and DAPI are 551nm/567nm and 358nm/461nm, respectively. Individual single-channel image were capturedusing the appropriate filters and merged to create composite images with analysis software.

5. Flow cytometric analysis

Fluorescein isothiocyanate-conjugate monoclo-

nal antibodies to mouse H-2^{b+}, FITC-conjugated H-2^{d+} were purchased from Pharmingen (San Diego, CA, USA). Blood samples collected in heparin (50 μL) were incubated on ice for 15 minutes with mouse anti-mouse monoclonal antibodies. The samples were washed with phosphate-buffered saline (PBS, WelGENE Inc.) containing 0.05% sodium azide and hemolized with ammonium chloride potassium carbonate to remove red cells. All the samples were analyzed using a FACScan cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA). Normal F1 mice were all stained positive for H-2^{b} and H-2^{d}, in contrast to all normal B6 cells staining positive for H-2^{b}. The percentage of circulating B6 cells in F1 recipients was determined by cal-

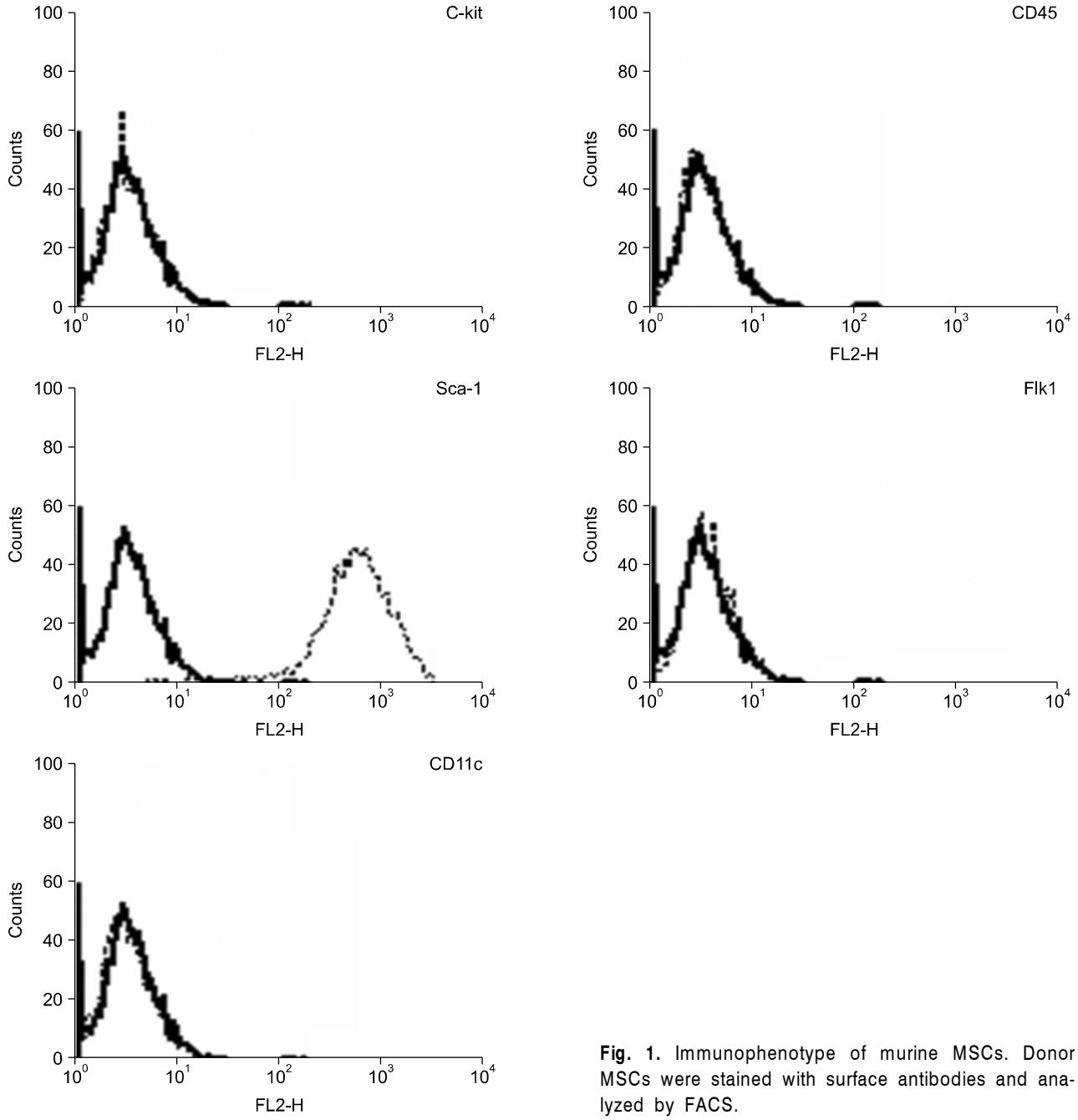

Fig. 1. Immunophenotype of murine MSCs. Donor MSCs were stained with surface antibodies and analyzed by FACS.

culating the number of cells negative for H-2^d. Donor effector/memory T cells were identified as the expression of CD62L-$CD4^+$ or $CD8^+$ from B6 cells.

The identity of the MSCs was confirmed using the immunophenotype criteria, based on the expression of $Sca1^+$ and the absence of hematopoietic (with anti-CD45, -CD11c and -CD117 antibodies) or endothelial cell (with anti-flk1 antibodies) markers. The proportion of $CD45^+$ cells in the MSCs preparations used in the various experiments never exceeded 2%.

6. Mixed lymphocyte reaction (MLR)

The splenocytes were isolated from a mouse spleen by disaggregation into RPMI 1640 medium (WelGENE Inc.) The cell count and viability were assessed by trypan blue dye exclusion. The stimulator splenocytes from F1 mice were treated with 50 μg/mL mitomycin C (Sigma Aldrich Co., MO, USA) for 45 minutes at 37°C, followed by five extensive washes with FBS-containing RPMI 1640 medium. The responder splenocytes from the B6 or F1 mice and the stimulator splenocytes from the F1 mice were resuspended at a concentration of 10^5 cells/100 μL/well. The MSCs (10^5 cells) were added to the mixture to obtain a final volume of 300 μL.

7. Statistical analysis

A Mann-Whitney U-test was used for the statistical analysis of the culture data, clinical GVHD scores and the number of cells, whereas the log rank test was used to analyze the survival data. A P-value <0.05 was considered significant.

RESULTS

1. Isolation, *in vitro* expansion, phenotypic and immunologic characterization of MSCs

MSCs were isolated from BM cells of B6 mice by plastic adherence in long-term culture as reported previously.[18] BM cells were initially cultured at a concentration of 10^6 cells/mL. Each time cells reached confluence, they were detached and replated in culture. The MSCs were negative for CD45, CD11c, c-kit and flk1 but most of them expressed Sca1 (Fig. 1). Using a MLR, the MSCs suppressed the proliferation of responder B6 splenocytes that had been elicited by allogeneic splenocytes from F1 (Fig. 2).

2. NM-HSCT followed by DLI is a model for the GVL effect against P815 tumor cells

Slavin *et al*[19-21] and Sykes and Sachs[22,23] have documented in preclinical animal models that establishing a state of MC was associated with a reduced risk of GVHD. Slavin *et al*[20] and Weiss *et al*[24] reported that as the time interval between HSCT and DLI increased, resistance to GVHD increased. We reasoned that DLI to the tolerant mice may be able to induce a GVL effect before the occurrence of GVHD in this NM-HSCT model. A total 15 sublethally irradiated (TBI 400 cGy) F1 mice for each group (group A~D) were reconstituted with 10^7 B6 BM cells. All mice were found to be chimeric and seemed healthy, with no clinical signs of early GVHD. Considering the clinical application, we started the tumor treatment after establishing the *s.c.* injected P815 tumors.[16] At 14 days following BMT, all mice were inoculated *s.c.* with 10^6 P815 cells, to mimic

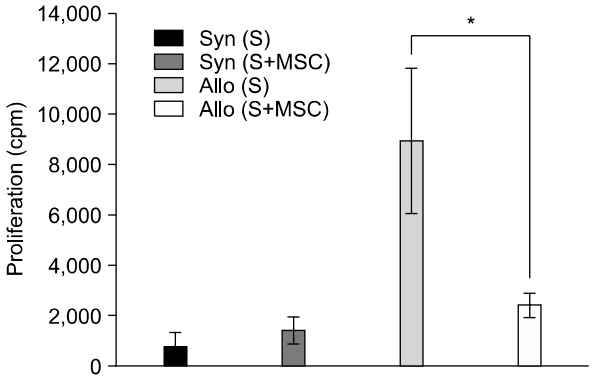

Fig. 2. Responding B6 splenocytes (5×10^5 cells/well) were incubated for 4 days with mitomicin-treated B6 (Syngeneic) or F1 splenocytes (Allogeneic) in the presence of 1×10^5 MSCs or diluent alone (Control). Addition of MSCs inhibited T cell proliferative response compared to the controls (*$P=0.005$).

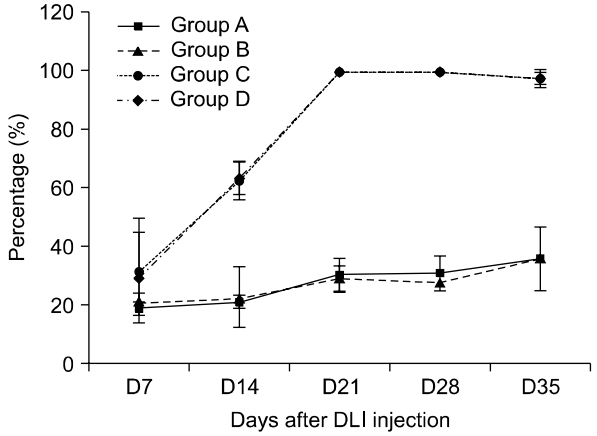

Fig. 3. The percentage of H-2^d negative donor cells in peripheral blood following donor lymphocyte infusion.

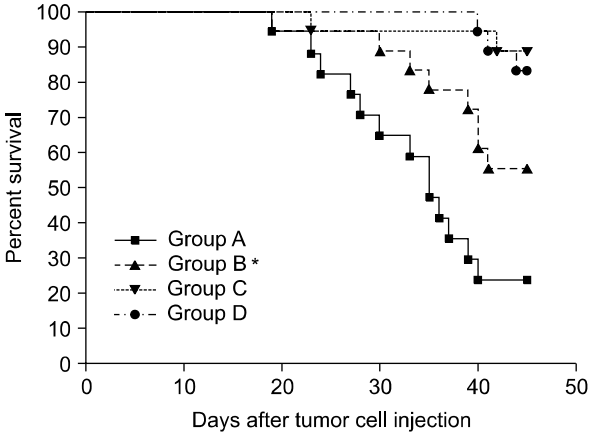

Fig. 4. Effect of MSCs on survival according to the chimeric status. Sublethally irradiated (400cGy) B6D2F1 (H-$2^{b/d}$) mice were reconstituted with 10^7 C57BL/6 (H-2^b) bone marrow cells. All mice were found to be a state of mixed chimeric (MC). At 14 days after NM-HSCT, group C and D mice received DLI at a dose of 20×10^6 spleen cells from donor mice for induction of GVL effects by virtue of complete chimerism (CC), while groups A and B did not receive DLI with persistence of MC. At the same, all mice were inoculated subcutaneously with 1×10^6 P815 cells and then the recipients were intravenously treated on days 15, 18 and 21 with either donor MSCs (group B and D, 5×10^5/day) or diluents (group A and C). Kalan-Meier survival curves demonstrate a difference between the MC and CC groups (group A vs. B, *P=0.0225; group C vs. D, P=0.371).

a state of minimal residual disease following HSCT. Group A and B did not receive additional DLI to remain mixed chimeric, while group C and D received DLI (20×10^7 splenocytes) for induction of GVL effects by achieving a state of complete chimerism (CC). As shown in Fig. 3, the recipients injected with DLI achieved a state of CC within 3 weeks, while those without DLI remained mixed chimeric.

In order to examine the immunosuppressive effect of MSCs on GVL effects, group B (MC) and D (CC) were given a total of three *i.v.* injections of 5×10^5 MSCs on days 15, 18 and 21 (total dose of 1.5×10^6). Each control group, group A (MC) and C (CC), received PBS injection instead of MSCs with no further treatment. As shown in Fig. 4, which pooled the results of three similar experiments, the GVL effects in the state of CC induced with DLI in C57BL/6 → B6D2F1 chimeras (group C and D) could suppress the growth of *s.c.* injected P815 cells up to 6 weeks after DLI. Between the DLI-treated groups with CC, tumor-related deaths were comparable irrespective to the infusion of MSCs (P=0.371). Among the mice with MC in group A, the injection of P815 cells into the recipient skin led to progressive tumor growth and death of about 80% 50 days after the P815 cell injections. On the contrary, among mice with group B, the administration of MSCs significantly delayed tumor-related deaths compared to those without the administration of MSCs (group B vs. A; 50-day percent survival, 54.5% vs. 18.1%, respectively, P= 0.0225).

Table 1. Effect of injected MSCs on tumor growth according to the chimeric status 28 days after tumor inoculation

Group	Chimerism	Treatment		Tumor size on day 42
		DLI	MSC	
(A)	MC	−	−	1.68±0.35
(B)	MC	−	+	1.25±0.22*
(C)	CC	+	−	0.29±0.08
(D)	CC	+	+	0.26±0.09

Attributions: MC, mixed chimerism; CC, complete chimerism. *P=0.09 between group A and B.

Tumor growth was obviously suppressed in the recipients that had been treated with DLI. In the animals with MC, tumor growth observed 28 day after the P815 cell injection seemed to be more

delayed in the mice injected with MSCs than in the controls (group A vs. B, P=0.09), whereas among the mice with CC after DLI, tumor growth was comparable between the two groups (group C vs. D, P=0.8) (Table 1).

3. Donor MSCs injection was associated with increased CD62L- T cells in MC but not in CC

In mice, naïve T cells, which have never encountered antigens specific for their T cell receptors, express CD62L$^+$. This distinguishes them from antigen-experienced effector/memory T cells, which are usually CD62L$^-$. It has been shown that since donor T cells have never encountered host allogeneic antigens, GVHD-inducing T cells should reside in the naïve T cell compartment[25]. Conversely, effector/memory T cells should not induce GVHD since they were not elicited by allo-antigens. When CD62L$^+$ and CD62L$^-$ cells were separately tested in a transplant model, GVHD induction *in vivo* was retained by the naïve phenotype, whereas memory cells did not cause GVHD. Moreover, memory cells immunized against BCL1 (a leukemia/lymphoma cell) retained the ability to proliferate and protect the animal against a challenge of the tumor cells. We examined if donor effect/memory cells in the recipients could be changed after injections of MSCs. As shown in Fig. 5, circulating donor cy-

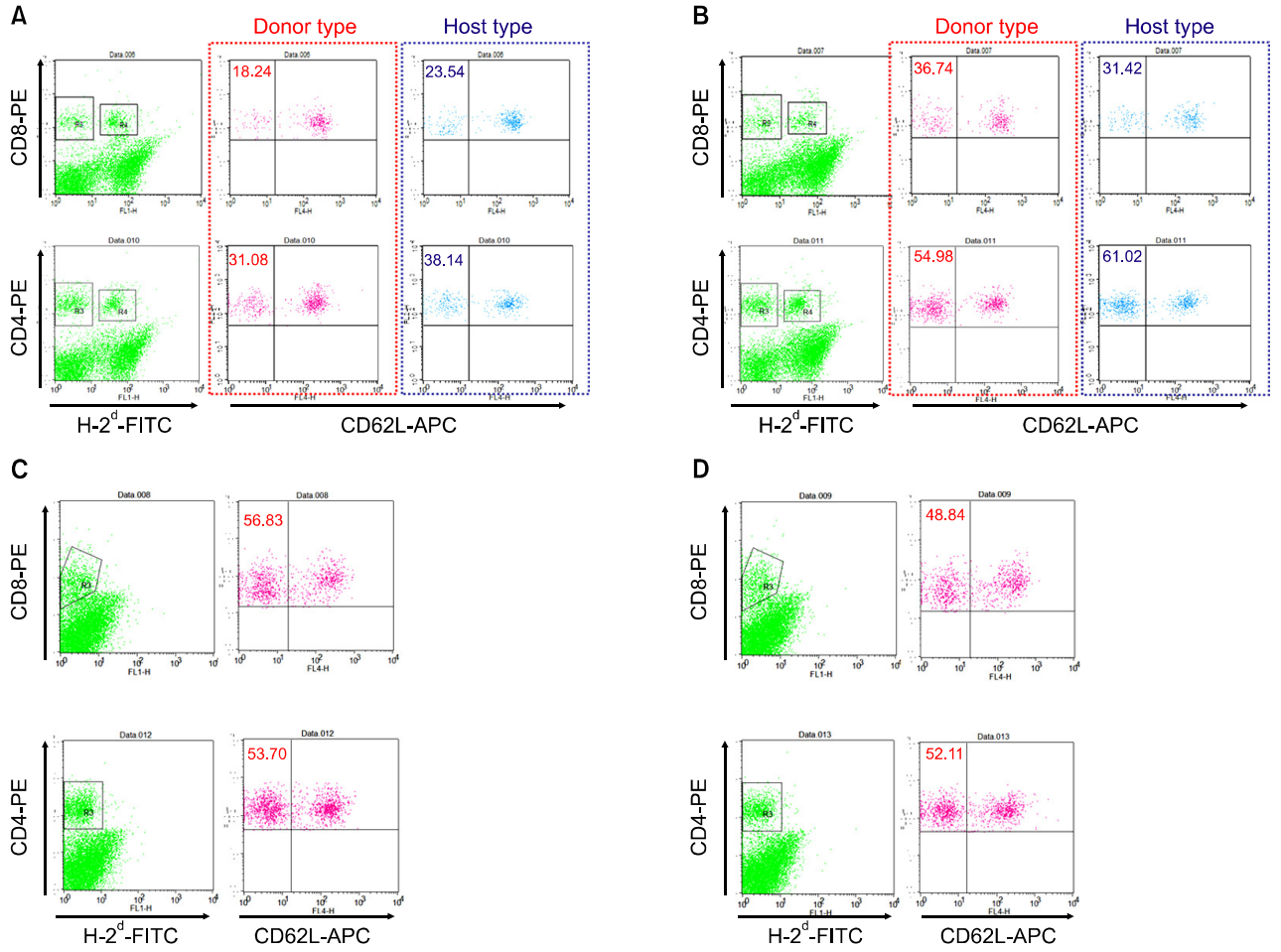

Fig. 5. Flow cytometry analysis of donor and recipient CD62L$^-$ memory/effector CD4$^+$ or CD8$^+$ T cells in peripheral blood obtained 21 days after the injection of MSCs. The percentage of circulating donor cells was determined by calculating the number of cells negative for H-2^d. Each graph represents one of two similar experiments. (A), Group A; (B), Group B; (C), Group C; (D), Group D.

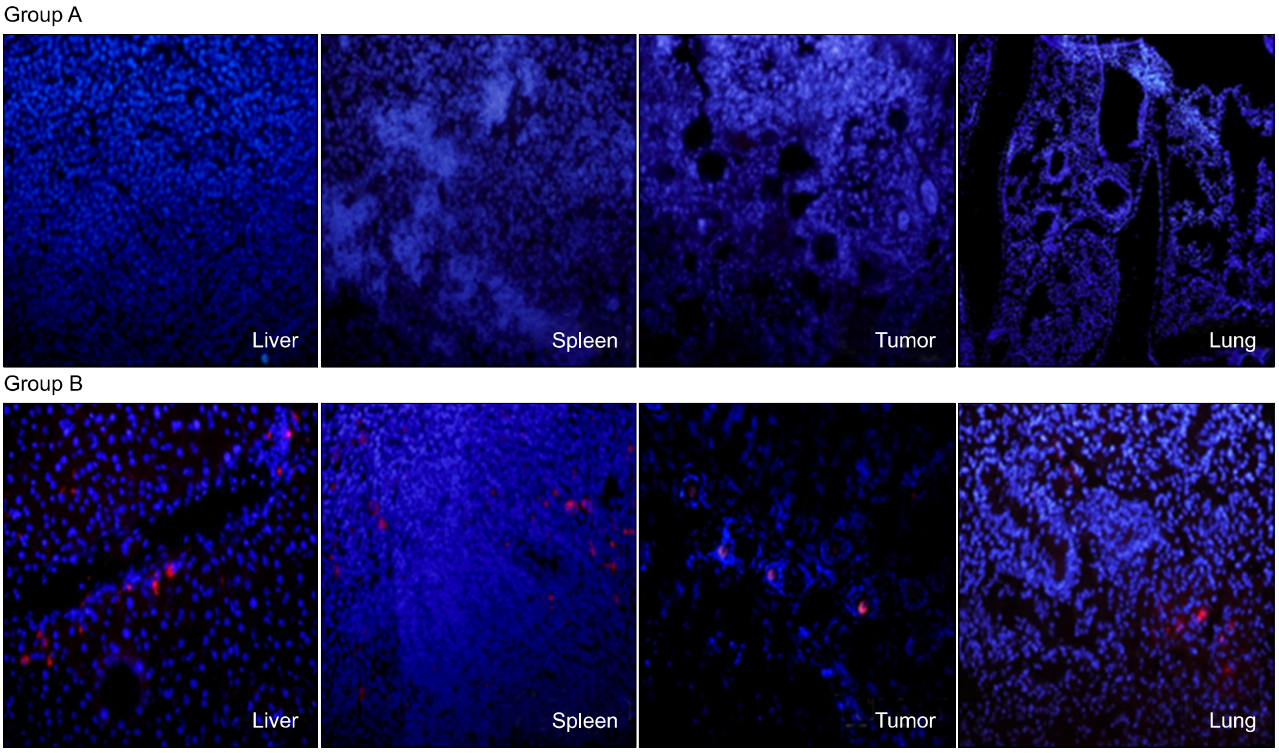

Fig. 6. Tissue distribution of MSCs injected into the recipients with MC (group B). In addition to the tumor tissue, immunohistochemical analysis of lungs, liver, and spleen sections was used to visualize the prelabeled MSCs by their PKH^+ red fluorescence (magnification: ×100). Mice in Group A did not receive the prelabeled MSCs.

totoxic effector/memory $CD4^+CD62L^-$ or $CD8^+CD62L^-$ cells were significantly increased in mice with MC after the injection of MSCs compared to the controls (54.98% vs. 31.08% or 36.74% vs. 18.24%, respectively) but those are comparable in mice with CC after DLI regardless of MSC administration (52.11% vs. 53.70% or 48.84% vs. 56.83%, respectively) on day 21 after the P815 cell injection. The CD25 expression on donor CD8 or CD4 cells were not different (data not shown). These data suggest that the enhanced GVL effect by the injection of MSCs in the recipients with MC was associated with the increased number of donor effector/memory cells with down-regulation of CD62L. Moreover, these results indicate that it may be possible to endow the recipient with T cells retaining memory specific to tumor antigen but incapable of causing GVHD.

4. Migration of donor MSCs in the recipients with MC

The ability of fluorescently labeled MSCs to preferentially home in on the site of a tumor or each organ including liver, lungs, and spleen was determined in the mixed-chimeric recipients with localized (flank tumor) disease. Twenty-one days after the injection of PKH-labeled MSCs, microscopic analysis showed that a substantial number of the fluorescently labeled cells were detected in liver, spleen and lungs as well as the tumor tissue of the mice with MC that had been injected PKH-labeled MSCs (group B, Fig. 6). These data demonstrate that the injected MSCs had an influence on the GVL effects undergoing a regulated homing process *in vivo*.

DISCUSSION

The immunosuppressive properties of MSCs

have been established[26], and recently, the treatment of GVHD with MSCs has been reported,[14,27] which suggests that MSCs can inhibit undesirable immune responses. One concern with use of MSCs after allo-HSCT is the potential to increase the incidence of relapse as a result of severe immunosuppression. Therefore, this study investigated whether or not the injection of MSCs affects the GVL effects. Our results demonstrate that the injection of donor MSCs did not have an influence on the GVL effects induced by a state of CC following DLI. Unexpectedly, the administration of donor MSCs into the recipients with MC strikingly decreased tumor-related deaths as well as tumor growths compared to the control group. The increased number of donor effector/memory T cells observed after the injection of MSCs suggests that MSCs may be immunogenic and the infusion of allogeneic MSCs can trigger a memory T-cell response in a state of MC. Furthermore, MSCs labeled with fluorescence were observed in MC recipients for extended periods after administration.

In clinical studies, delayed transfer of T cells to allogeneic chimeras is also associated with a low risk of GVHD.[28] The precise mechanisms underlying the lack of GVHD are not known but may relate to the resolution of the early inflammatory cascade induced by the conditioning protocol.[29,30] It has been previously shown that, in the well-established mouse models of human GVHD the early administration of the primary MSCs after the transplant failed to show a GVHD protection effect.[31,32] Therefore, this study utilized NM-HSCT followed by DLI to accurately observe the influence of MSCs on GVL effect. It is by now well established that the main benefits of allo-HSCT can be ascribed to the antileukemia effect exerted by donor alloreactive T cells[2,33] and partially, especially following haploidentically mismatched transplants, also by alloreactive natural killer cells,[34] rather than to the effects of myeloablative conditioning *per se*. Therefore, adoptive immunotherapy mediated by infusion of alloreactive lymphocytes after HSCT, also referred to as DLI, has been used as an effective clinical tool following HSCT for treatment[35-37] or for prevention[38] of recurrent leukemia. Slavin *et al* have recently documented that in mice with minimal number of tumor cells, GVL effects can be induced faster than the anticipated onset of overt clinical manifestations of acute GVHD.[39] Hence, GVL effects induced with DLI in recipients with MC could result in successful elimination of tumor cells before death from GVHD in this combination occurs. In our experiments, the model was modified to reveal the efficacy of GVL effects while reducing the occurrence of GVHD by increasing the time interval between HSCT and DLI. A state of MC was established when sublethally irradiated F1 mice were reconstituted with B6 BM cells. All recipients were found to be chimeric and seemed healthy, with no clinical signs of early GVHD. After DLI, GVL effects can be induced faster than the anticipated onset of overt clinical manifestation of acute GVHD in the MC mice with minimal number of P815 tumor cells. We could observe the effect of MSCs on *s.c.* injected tumor growth according to the recipients chimeric status. A recently-published clinical report demonstrated that early co-injection of MSCs with HSCT reduced the occurrence of GVHD but significantly increased the incidence of relapse.[15] However, our experiments showed that the early injection of allogeneic donor MSCs did not hamper the GVL effects induced by DLI.

Surprisingly, injected MSCs suppressed tumor growth and reduced tumor-related deaths in the recipients with MC. Our observations demonstrate that the GVL effects enhanced by the administration of MSCs into the recipients with MC were associated with the emergence of effector/memory cells not expressing CD62L ($CD62L^-$). Naive T cells, which express CD62L, are T cells that have never encountered antigens specific for their T-cell receptors. One of the possible explanations is that MSCs had triggered the onset of a memory T cell response. As described by

many investigators,[40,41] $CD62L^-$ T cells belong to a subset of memory T cells called "effector/memory T cells". This subset of T cells is able to mediate functional memory immune responses. It has been shown that $CD62L^-$ T cells from immunized animals carry highly potent antitumor activity.[42,43] These data strongly suggest that MSCs are not intrinsically immunoprivileged in the chimeric recipients. The putative immunomodulatory effect of allogeneic MSCs that promotes the GVL effect may be mediated by activation of host T cells since the injection of MSCs downregulated CD62L of recipient T cells (Fig. 5A and B).

Nauta *et al*[44] demonstrated that the infusion of allogeneic MSCs can prime allogeneic T cells in naive immunocompetent mice, indicating that MSCs are immunogenic under these conditions. Furthermore, no memory response was observed after the infusion of syngeneic cultured MSCs. In support of these observations are recent findings demonstrating that allogeneic MSCs can be immunogenic in immunocompetent animals.[45,46] The immune response elicited by the infusion of allogeneic donor MSCs into the chimeric recipients may enhance the response of the donor against host antigens, resulting in increased antileukemia effect of alloreactive donor T cells.

Studies have shown that *i.v.* administered MSCs do not engraft in healthy organs, e.g., liver, spleen[47,48] and, in the case of breast cancer, have been shown to migrate to pulmonary metastases of the disease in an animal model.[49] Although the authors reported little or no MSC engraftment in normal areas of the lung, another study reported pooling of MSCs in the healthy lung of an animal after *i.v.* administration, which raised a question as to whether engraftment had been tumor-targeted or as a result of MSCs getting trapped in the capillary network as a result of their relatively large size.[50] In the current study, the ability of fluorescently labeled MSCs to home in on the tumor site was confirmed after systemic administration. The MSCs were shown to be morphologically intact and to have survived up to 21 days after administration. Further studies will be required to evaluate the mechanisms of the cellular interaction between engrafted MSCs and immune cells.

In summary, our results show that MSCs did not hamper the GVL effect induced by DLI following MC in a murine HSCT model and are capable of modulating immune responses *in vivo*. The key finding of the study is the strikingly decreased frequency of tumor growths in the recipients with MC when infused with MSCs compared to the control group. Allogeneic MSCs can not be intrinsically immunoprivileged and that under appropriate conditions, such as MC, allogeneic MSCs induce a memory T-cell response resulting in enhancement of the GVL effect. Increased understanding of the pathways involved in stimulating the GVL effect will potentially provide for translation of this novel therapeutic approach to the clinical setting.

요 약

배경: 중간엽줄기세포(mesenchymal stem cell, MSCs)는 실험실 및 일부 생체 내 연구 결과 강력한 면역억제 효과를 보여주었으며 동종조혈모세포이식(allogeneic hematopoietic stem cell transplantation, allo-HSCT)후의 이식편대숙주질환(graft-vs.-host disease, GVHD)의 예방과 치료 목적으로 이용되고 있다. 그러나 GVHD와 이식편대백혈병(graft-vs.-leukemia, GVL) 효과는 서로 밀접하게 연관되어 GVHD를 억제하면 GVL 효과가 약화되어 이식 후 백혈병 재발이 증가된다. 따라서 본 연구에서는 MSCs가 GVL효과에 미치는 영향을 동물실험을 이용하여 관찰하였다.

방법: 저용량 전처치 allo-HSCT 모델에서 공여자 림프구수혈(donor lymphocyte infusion, DLI) 방법을 이용하여 GVL 효과를 직접적으로 관찰하였다. C57BL/6(H-2^b)→C57BL/6DBA/2(B6DF1, H-$2^{b/d}$)모델에서 수혜자 마우스를 TBI 400cGy로 전처치한 후 공여자 골수세포(10×10^6)를 주입하여 혼합 키메라(mixed chimerism, M.C) 상태를 유도하였으며 모든 마우스들은 MC상태를 유지하였고 GVHD없이 건강함을 확인하였다. 이식 2주 후에 공여자의 비장세포(20×10^6)를 투여

하여 DLI를 시행하고 동시에 백혈병세포주인 P815 (H-2^d)세포를 피하주사하고 주기적으로 종양부피와 종양관련사망을 측정하였다. DLI 후 1, 4, 7일에 공여자의 골수에서 배양된 MSCs (5×10^5/day)를 수혜자 마우스 꼬리정맥으로 주입하였으며 대조군은 동일한 양의 희석물을 투여하였다.

결과: MSCs 투여 후에 종양의 성장을 확인한 결과 DLI를 시행한 완전 키메라(complete chimerism, CC)군에서는 MSCs의 투여에 따라 종양으로 인한 사망과 종양 성장의 차이를 보이지 않았다. 반면 MC군에서는 MSCs를 투여한 마우스에서 종양으로 인한 사망이 지연되었고(50-day percent survival, 54.5% vs. 18.1%, P=0.0225), MSCs를 주입하지 않은 군에 비하여 종양성장이 지연되는 경향을 보여주었다(P=0.09). MC군에서 MSCs를 투여한 이후 공여자 유래 effector/memory $CD62L^-$T cells의 비율이 증가되었으나, CC군에서는 차이가 없었다. MC군의 조직에서 형광염색을 통하여 MSCs 투여 후 21일에도 MSCs가 존재함을 확인하였다.

결론: CC 상태에서 GVL 효과는 MSCs의 투여에 의해서 영향을 받지 않았다. 그러나 MC군에서 MSCs 투여 후에 GVL 효과가 향상되는 것으로 나타났으며 MSCs가 일정한 조건하에서는 면역자극 효과를 발휘할 가능성이 있다. 본 연구 결과는 이식 후 GVHD는 감소시키면서 GVL 효과를 증대시킬 수 있는 치료법 개발에 이용할 수 있을 것으로 기대된다.

REFERENCES

1) Barrett AJ. Mechanisms of the graft-versus-leukemia reaction. Stem Cells 1997;152:48-58.
2) Horowitz MM, Gale RP, Sondel PM, et al. Graft-versus-leukemia reactions after bone marrow transplantation. Blood 1990;75:555-62.
3) Friedenstein AJ, Piatetzky-Shapiro II, Petrakova KV. Osteogenesis in transplants of bone marrow cells. J Embryol Exp Morphol 1966;16:381-90.
4) Friedenstein AJ, Chailakhyan RK, Latsinik NV, Panasyuk AF, Keiliss-Borok IV. Stromal cells responsible for transferring the microenvironment of the hemopoietic tissues. Cloning in vitro and retransplantation in vivo. Transplantation 1974;17:331-40.
5) Caplan AI. Mesenchymal stem cells. J Orthop Res 1991;9:641-50.
6) Prockop DJ. Marrow stromal cells as stem cells for nonhematopoietic tissues. Science 1997;276:71-4.
7) Pittenger MF, Mackay AM, Beck SC, et al. Multilineage potential of adult human mesenchymal stem cells. Science 1999;284:143-7.
8) Bianchi G, Muraglia A, Daga A, Corte G, Cancedda R, Quarto R. Microenvironment and stem properties of bone marrow-derived mesenchymal cells. Wound Repair Regen 2001;9:460-6.
9) Di Nicola M, Carrio-stella C, Magni M, et al. Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. Blood 2002;99:3838-43.
10) Le Blanc K, Tammik L, Sundberg B, Haynesworth SE, Ringdén O. Mesenchymal stem cells inhibit and stimulate mixed lymphocyte cultures and mitogenic responses independently of the major histocompatibility complex. Scand J Immunol 2003;57:11-20.
11) Tse WT, Pendleton JD, Beyer WM, Egalka MC, Guinan EC. Suppression of allogeneic T-cell proliferation by human marrow stromal cells: implications in transplantation. Transplantation 2003;75:389-97.
12) Krampera M, Glennie S, Dyson J, et al. Bone marrow mesenchymal stem cells inhibit the response of naive and memory antigen-specific T cells to their cognate peptide. Blood 2003;101:3722-9.
13) Bartholomew A, Sturgeon C, Siatskas M, et al. Mesenchymal stem cells suppress lymphocyte proliferation in vitro and prolong skin graft survival in vivo. Exp Hematol 2002;30:42-8.
14) Le Blanc K, Rasmusson I, Sundberg B, et al. Treatment of severe acute graft-versus-host disease with third party haploidentical mesenchymal stem cells. Lancet 2004;363:1439-41.
15) Ning H, Yang F, Jiang M, et al. The correlation between cotransplantation of mesenchymal stem cells and higher recurrence rate in hematologic malignancy patients: outcome of a pilot clinical study. Leukemia 2008;22:593-9.
16) Kamate C, Baloul S, Grootenboer S, et al. Inflammation and cancer, the mastocytoma P815 tumor model revisited: triggering of macrophage activation in vivo with pro-tumorigenic consequences. Int J Cancer 2002;100:571-9.
17) Spyridonidis A, Bernhardt W, Behringer D, et al. Proliferation and survival of mammary carcinoma cells are influenced by culture conditions used for ex vivo expansion of CD34(+) blood progenitor cells. Blood 1999;93:746-55.
18) Min CK, Kim BG, Park G, Cho B, Oh IH. IL-10-transduced bone marrow mesenchymal stem cells can attenuate the severity of acute graft-versus-host disease after experimental allogeneic stem cell transplantation. Bone Marrow Transplant 2007;39:

637-45.
19) Slavin S, Strober S, Fuks Z, Kaplan HS. Induction of specific tissue transplantation tolerance using fractionated total lymphoid irradiation in adult mice: long-term survival of allogeneic bone marrow and skin grafts. J Exp Med 1977;146:34-48.
20) Slavin S, Fuks Z, Kaplan HS, Strober S. Transplantation of allogeneic bone marrow without graft-versus-host disease using total lymphoid irradiation. J Exp Med 19781;147:963-72.
21) Slavin S, Reitz B, Bieber CP, Kaplan HS, Strober S. Transplantation tolerance in adult rats using total lymphoid irradiation: permanent survival of skin, heart, and marrow allografts. J Exp Med 1978;147: 700-7.
22) Sykes M, Sachs DH. Mixed allogeneic chimerism as an approach to transplantation tolerance. Immunol Today 1988;9:23-7.
23) Sykes M, Sachs DH. Bone marrow transplantation as a means of inducing tolerance. Semin Immunol 1990;2:401-17.
24) Weiss L, Reich S, Slavin S. Use of recombinant human interleukin-2 in conjunction with bone marrow transplantation as a model for control of minimal residual disease in malignant hematological disorders: I. treatment of murine leukemia in conjunction with allogeneic bone marrow transplantation and IL-2-activated cell-mediated immunotherapy. Cancer Invest 1992;10:19-26.
25) Chen BJ, Cui X, Sempowski GD, Liu C, Chao NJ. Transfer of allogeneic CD62L- memory T cells without graft-versus-host disease. Blood 2004;103:1534-41.
26) Le Blanc K, Ringdén O. Immunobiology of human mesenchymal stem cells and future use in hematopoietic stem cell transplantation. Biol Blood Marrow Transplant 2005;11:321-34.
27) Le Blanc K, Frassoni F, Ball L, et al. Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study. Lancet 2008;371:1579-86.
28) Kolb HJ, Schmid C, Barrett AJ, Schendel DJ. Graft-versus-leukemia reactions in allogeneic chimeras. Blood 2004;103:767-76.
29) Xun CQ, Thompson JS, Jennings CD, Brown SA, Widmer MB. Effect of total body irradiation, busulfan-cyclophosphamide, or cyclophosphamide conditioning on inflammatory cytokine release and development of acute and chronic graft-versus-host disease in H-2-incompatible transplanted SCID mice. Blood 1994;83:2360-7.
30) Hill GR, Crawford JM, Cooke KR, Brinson YS, Pan L, Ferrara JL. Total body irradiation and acute graft-versus-host disease: the role of gastrointestinal damage and inflammatory cytokines. Blood 1997;90:3204-13.
31) Sudres M, Norol F, Trenado A, et al. Bone marrow mesenchymal stem cells suppress lymphocyte proliferation in vitro but fail to prevent graft-versus-host disease in mice. Immunol 2006;176:7761-7.
32) Badillo AT, Peranteau WH, Heaton TE, Quinn C, Flake AW. Murine bone marrow derived stromal progenitor cells fail to prevent or treat acute graft-versus-host disease. Br J Haematol 2008;141: 224-34.
33) Weiden PL, Sullivan KM, Flournoy N, Storb R, Thomas ED. Antileukemic effect of chronic graft-versus-host disease: contribution to improved survival after allogeneic marrow transplantation. N Engl J Med 1981;304:1529-33.
34) Ruggeri L, Capanni M, Urbani E, et al. Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. Science 2002; 295:2097-100.
35) Kolb HJ, Schattenberg A, Goldman JM, et al. Graft-versus-leukemia effect of donor lymphocyte transfusions in marrow grafted patients. Blood 1995; 86:2041-50.
36) Slavin S, Naparstek E, Nagler A, Ackerstein A, Kapelushnik J, Or R. Allogeneic cell therapy for relapsed leukemia after bone marrow transplantation with donor peripheral blood lymphocytes. Exp Hematol 1995;23:1553-62.
37) Slavin S, Naparstek E, Nagler A, et al. Allogeneic cell therapy with donor peripheral blood cells and recombinant human interleukin-2 to treat leukemia relapse after allogeneic bone marrow transplantation. Blood 1996;87:2195-204.
38) Naparstek E, Or R, Nagler A, et al. T-cell-depleted allogeneic bone marrow transplantation for acute leukaemia using Campath-1 antibodies and post-transplant administration of donor's peripheral blood lymphocytes for prevention of relapse. Br J Haematol 1995;89:506-15.
39) Weiss L, Lubin I, Factorowich I, et al. Effective graft-versus-leukemia effects independent of graft-versus-host disease after T cell-depleted allogeneic bone marrow transplantation in a murine model of B cell leukemia/lymphoma. Role of cell therapy and recombinant IL-2. J Immunol 1994;153:2562-7.
40) Mobley JL, Rigby SM, Dailey MO. Regulation of adhesion molecule expression by CD8 T cells in vivo.

II. Expression of L-selectin (CD62L) by memory cytolytic T cells responding to minor histocompatibility antigens. J Immunol 1994;153:5443-52.
41) Ahmadzadeh M, Hussain SF, Farber DL. Heterogeneity of the memory CD4 T cell response: persisting effectors and resting memory T cells. J Immunol 2001;166:926-35.
42) Kagamu H, Touhalisky JE, Plautz GE, Krauss JC, Shu S. Isolation based on L-selectin expression of immune effector T cells derived from tumor-draining lymph nodes. Cancer Res 1996;56:4338-42.
43) Kagamu H, Shu S. Purification of L-selectin(low) cells promotes the generation of highly potent CD4 antitumor effector T lymphocytes. J Immunol 1998; 160:3444-52.
44) Nauta AJ, Westerhuis G, Kruisselbrink AB, Lurvink EG, Willemze R, Fibbe WE. Donor-derived mesenchymal stem cells are immunogenic in an allogeneic host and stimulate donor graft rejection in a nonmyeloablative setting. Blood 2006;108:2114-20.
45) Grinnemo KH, Månsson A, Dellgren G, et al. Xenoreactivity and engraftment of human mesenchymal stem cells transplanted into infarcted rat myocardium. J Thorac Cardiovasc Surg 2004;127: 1293-300.
46) Eliopoulos N, Stagg J, Lejeune L, Pommey S, Galipeau J. Allogeneic marrow stromal cells are immune rejected by MHC class I- and class II-mismatched recipient mice. Blood 2005;106:4057-65.
47) Pereboeva L, Curiel DT. Cellular vehicles for cancer gene therapy: current status and future potential. BioDrugs 2004;18:361-85.
48) Studeny M, Marini FC, Dembinski JL, et al. Mesenchymal stem cells: potential precursors for tumor stroma and targeted-delivery vehicles for anticancer agents. J Natl Cancer Inst 2004;96:1593-603.
49) Stoff-Khalili MA, Rivera AA, Mathis JM, et al. Mesenchymal stem cells as a vehicle for targeted delivery of CRAds to lung metastases of breast carcinoma. Breast Cancer Res Treat 2007;105:157-67.
50) Nakamizo A, Marini F, Amano T, et al. Human bone marrow-derived mesenchymal stem cells in the treatment of gliomas. Cancer Res 2005;65:3307-18.