# Mesenchymal Stem Cells Prolong Composite Tissue Allotransplant Survival in a Swine Model

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**Background.** This study investigated whether mesenchymal stem cells (MSCs) combined with bone marrow transplantation (BMT), irradiation, or short-term immunosuppressant therapy could prolong composite tissue allotransplant survival in a swine hind-limb model.

**Methods.** Heterotopic hind-limb transplantation was performed in outbred miniature swine. Group I (n=5) was the untreated control. Group II (n=3) received MSCs alone (given on days −1, +3, +7, +14, +21). Group III (n=6) received cyclosporine A (CsA days 0 to +28). Group IV (n=4) received preconditioning irradiation (day −1), BMT (day +1), and CsA (days 0 to +28). Group V (n=5) received irradiation (day −1), BMT (day +1), CsA (days 0 to +28), and MSCs (days +1, +7,+14). The expression and localization of $CD4^+/CD25^+$ T cells and MSCs were assessed using flow cytometry and immunohistochemistry.

**Results.** The allografts survival with MSCs alone revealed a significant prolongation, when compared with the controls ($P=0.02$). Allografts with CsA treatment exhibited delayed rejection. Irradiation and BMT-CsA treatment revealed no significant allograft survival benefit when compared with the CsA treatment group, but graft-versus-host disease (GVHD) was evident. However, combination of MSCs-BMT-CsA treatment demonstrated significant prolongation of allograft survival (>200 days, $P<0.001$) and no signs of GVHD with the lowest degree of rejection in the allo-skin and interstitial muscle layers. The $CD4^+/CD25^+$ regulatory-like T-cell expression in the circulating blood and allo-skin significantly increased in the MSC-BMT-CsA group. Examination of bromodeoxyuridine-labeled MSCs revealed donor MSC engraftment into the recipient and donor skin and the recipient liver parenchymal tissue.

**Conclusion.** These results suggested that the regulatory activity of MSCs on T cells and GVHD might contribute to significant prolongation of composite tissue allotransplant survival in the MSC-BMT-CsA treatment.

**Keywords:** Composite tissue allotransplant, Mesenchymal stem cells.

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Composite tissue allotransplantation (CTA; consisting of tissues such as skin, muscle, and bone) may serve as an ideal solution for the replacement or repair of certain tissues after traumatic loss, tumor resection, or repair of congenital abnormalities (*1*, *2*). Recently, important advances have been made in the field of transplantation: the first successful human hand transplantation was performed in 1998 (*3*, *4*), and the first partial face allotransplantation in humans was undertaken in November 2005 (*5*). Since this time, CTA has gained popularity as a viable alternative for tissue reconstruction. However, CTA transplants are not routinely performed for tissue repair and reconstruction because life-long administration of immunosuppressive agents with potentially harmful adverse effects is required to prevent the immune system from rejecting this antigenic tissue constructs (*6*). Rejection episodes of skin containing CTA often occur because it is more antigenic than solid-organ transplantation (*7*). Furthermore, these immunosuppressive agents have also been shown to be associated with an increased rate of neoplasia and opportunistic infections. Even in the presence of excellent patient compliance, conventional immunosuppressive protocols may not be sufficient to prevent delayed rejection (*8*).

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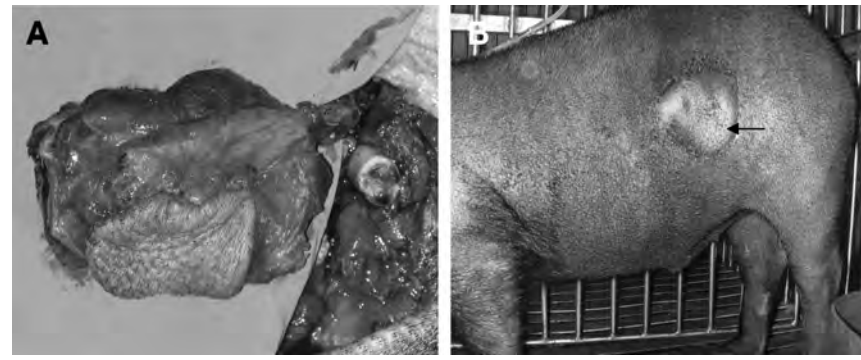


**FIGURE 1.** (A) A heterotopic hind-limb composite tissue allotransplantation model consisting of the distal femur, knee joint, tibio-fibula, and surrounding muscle with a vascularized skin paddle was harvested. The limb graft was placed in a subcutaneous abdominal wall pocket, and the vessels were anastomosed. (B) The allograft revealed strong circulation posttransplantation.

Consequently, researchers have been actively seeking alternative methods for establishing life-long tolerance while minimizing toxicity.

It has been previously demonstrated that bone marrow transplantation (BMT) combined with immunosuppressant administration prolongs organ transplant survival (*9*). Despite the promising potential of mixed allogeneic chimerism in inducing CTA tolerance, graft-versus-host disease (GVHD) secondary to introduction of donor BMT and toxicity from ablative host conditioning are considered to be the main hurdles in widespread acceptance of this technique (*10*). Bone marrow stroma contains multipotent nonhematopoietic progenitor cells that are capable of differentiating into various mesenchymal cell types. These cells have been characterized as marrow stromal cells, mesenchymal progenitor cells, or mesenchymal stem cells (MSCs; *11*, *12*). Previous publications revealed that MSCs do not express immunogenic costimulatory molecules such as B7-1, B7-2, or the CD40; therefore, they are most likely unable to stimulate alloreactive T cells (*13–15*). MSCs have been successfully used in the treatment of acute GVHD resulting from BMT (*16*). Previous studies have also found that MSCs inhibit T-cell proliferation in mixed lymphocyte cultures and prolonging skin allograft survival in a rodent model (*17*, *18*).

This study investigated whether MSCs combined with irradiation-BMT and short-term administration of cyclosporine A (CsA) could permit prolonged allograft survival and induce immune tolerance in a miniature swine hind-limb CTA model.

## MATERIALS AND METHODS

### Animals

Twenty-four outbred domestic miniature swine (Lan-Yu strain; age 3 months; weight 12–20 kg) were included in the study. The Lan-Yu strain is an indigenous breed from Lau-Yu Islet, southeast of Taiwan, with genotypes GPI-BB and PGD-AA. The inherited differences in donors and recipients from the original parental generation were identified (*19*, *20*) and reported by the National Tai-Tung Veterinary Research Institute, Taiwan. The study was conducted in accordance with the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health, Bethesda, MD. Experiments were conducted using the Institutional Animal Care and Use Committee protocol approved by the Chang Gung Memorial Hospital in Kaohsiung, Taiwan.

### Animal Model: Heterotopic Hind-Limb Swine Model

Heterotopic hind-limb transplantation was performed as previously described (Fig. 1; *21*, *22*). In brief, animals were premedicated with an intramuscular injection of ketamine (10 mg/kg) and xylazine (1.5 mg/kg) and then placed in the supine position and intubated. Anesthesia was maintained with pentobarbital (50 mg/kg) and oxygen inhalation throughout the procedure. A composite tissue skeletal graft consisting of tissues from the tibia, fibula, knee joint, distal femur, and surrounding muscle was harvested from the donor swine. A skin paddle measuring approximately $8\times8$ cm$^2$ was preserved on the medial aspect of the knee supplied by the superficial femoral vessels. The tibia and fibula were divided at approximately 5 cm below the knee, and the femur was divided 5 cm above the knee. The thigh muscles were divided at the midfemur level. On division of the vascular pedicle, heparinized saline was flushed through the femoral artery. Once the limb harvest was complete, the donor animal was killed with an overdose of pentobarbital. The recipient animal was prepared in a similar fashion as described for the donor swine. A subcutaneous pocket was created in the anterolateral abdominal wall, and the limb graft was placed in the pocket. The vessels were anastomosed in an end-to-end fashion onto the host femoral vessels with a 9-0 nylon interrupted suture under microscopic magnification. A defect was created in the skin of the host's flank, and the skin paddle was sutured into place on the donor limb.

### Experimental Design

In this study, miniature swine underwent heterotopic hind-limb transplantation. Group I (n=5) was the control cohort and hence did not undergo immunosuppressive therapy. Group II (n=3) received $1\times10^7$ MSCs/dose (given on days −1, +3, +7, +14, +21). Group III (n=6) received CsA for 4 weeks (days 0 to +28; 10 mg/kg for 2 weeks, followed by 5 mg/kg for 2 weeks). Group IV (n=4) received preconditioning irradiation (day −1; 150 cGy for total body irradiation and 700 cGy for intrathymus irradiation), $1\times10^8$ cells BMT (day +1), and CsA (same protocol as group III; days 0 to +28). Group V (n=5) received irradiation, BMT (day +1), CsA (same protocol as group III; days 0 to +28), and $1\times10^7$ MSCs/dose (given on days +7, +14, +21). In groups III to V, the CsA dosage after the first 2 weeks was decreased to investigate the effects of MSCs on allograft survival under low-dose immunosuppression for future clinical application. For imi-

tation of clinical instances, the levels of CsA (all trough level between 100 and 300 ng/mL) have been monitored by regularly drawing the recipient blood per week till 4 weeks postoperatively. The viability of the swine and signs of allograft rejection were continuously monitored postoperatively. The experimental endpoint was defined as desquamation and necrosis of the entire area of donor skin.

### Culturing of MSCs

Bone marrow cells from the donor were harvested and isolated 2 weeks before CTA by using previously described methods (*12*, *23*). Briefly, the bone marrow cells were suspended in Dulbecco's minimal essential medium with low glucose, 10% fetal bovine serum, antibiotic/antimycotic, and glutamax, and they were plated in a 6-well dish. Cultures were incubated at 37°C in a 5% $CO_2$ atmosphere. After 4 hr of subtraction and removal of adherent cells, the nonadherent cells were transferred to a 25-T subculture flask. When they were 70% to 80% confluent, adherent cells were trypsinized (0.05% trypsin at 37°C for 5 min), harvested, and expanded in 75-T flasks. A homogenous cell population was obtained after 3 weeks of tissue culture. The cultures were maintained at 37°C in a humidified atmosphere containing 95% ambient air and 5% $CO_2$ and subcultured before confluency. The MSCs were expanded in culture and characterized by flow cytometry after surface staining for CD90 (Fitzgerald, Concord, MA), CD44 (BD Pharmingen, San Diego, CA), major histocompatibility complex (MHC) class I (Serotec, Oxford, UK), CD106 (vascular adhesion molecule-1; VCAM-1, Lifespan, Seattle, WA), CD45 (Serotec), MHC class II (Serotec), and the B7 costimulatory molecules CD80/B7-1 (Lifespan) expressions. The reactivity of MSCs to other antibodies representing different lineage phenotypes could not be examined because of the limited availability of commercial antibodies raised against swine. MSCs were routinely frozen in a medium containing 10% dimethyl sulfoxide and 90% fetal bovine serum. Before experimental use, MSCs were tested for their ability to differentiate into different mesenchymal lineages, including adipocytes, osteoblasts, and chondrocytes. To induce adipogenic differentiation, MSCs were cultured with $10^{-8}$ M dexamethasone and 5 $\mu$g/mL insulin, and droplet staining was performed using oil-red O. Osteogenic differentiation was induced by treating MSCs with $10^{-8}$ M dexamethasone, 10 mM $\beta$-glycerol-phosphate, and 50 $\mu$g/mL ascorbic acid, and differentiated cells were identified by von Kossa staining. For chondrocyte differentiation, a pellet culture system was used. Chondrogenic differentiation medium was composed of high-glucose Dulbecco's minimal essential medium (Gibco Laboratories, Grand Island, NY) supplemented with 40 $\mu$g/mL proline, 50 mg/mL ITS-plus (final concentration 6.25 mg/mL insulin, 6.25 mg/mL transferrin, 6.25 mg/mL selenous acid, 5.33 mg/mL linoleic acid, and 1.25 mg/mL bovine serum albumin), 100 $\mu$g/mL sodium pyruvate, glutamax, 50 $\mu$g/mL ascorbate-2-phosphate, 10 ng/mL transforming growth factor-$\beta$3, and $10^{-7}$ M dexamethasone. Chondrogenic differentiation was visualized by Alcian blue staining.

### Histologic Evaluation of Graft Rejection

The transplanted limb was observed daily for signs of rejection as defined by the well-characterized sequence of epidermilysis, dyskeratosis, and necrosis. Biopsies of donor skin and muscle were obtained at predetermined time points (postoperative days 14, 28, 42, 100, and 200) or at the time of clinically evident rejection. The reason why we biopsy only the skin and muscle is, from our previous experience, that the skin demonstrates the highest antigenicity and the joint cartilage is less antigenic than the skin (*6*, *21*). The other reason is that these tissues can easily be monitored to determine whether or not early rejection occurs to the allograft. To obtain a biopsy sample, tissues were harvested, fixed in 10% neutral buffered formalin, sectioned, and stained with hematoxylin-eosin (H&E). The animals were killed at the clinically defined endpoint. Histologic evaluation of the graft biopsy for CTA rejection was referred to previously reported consensus scheme (*24–26*). According to the severity of pathologic changes, the following rejection grades using a Banff classification were applied: grade 0, no or rare inflammatory infiltrates; grade I, mild, mild perivascular infiltration; grade II, moderate, moderate-to-severe perivascular inflammation, no epidermal dyskeratosis, or apoptosis; grade III, severe, dense inflammation and epidermal involvement with epithelial apoptosis, dyskeratosis, and keratinolysis; and grade IV, necrotizing acute rejection (*26*).

### Flow Cytometry Flow Cytometric Assessment of $CD4^+/CD25^+$ T Cells

Flow cytometric analysis was performed on the peripheral blood samples of recipients collected on specified days posttransplant. The whole blood was incubated for 20 to 30 min in the dark (room temperature) with 5 $\mu$L of mouse anti-porcine CD25-fluorescein isothiocyanate (Pharmingen) and combinations of mouse anti-pig $CD4^-$ phycoerythrin. After incubation, red blood cells were lysed (FACS Lysing Solution; Pharmingen), centrifuged at 1500 rpm for 5 min for two times. Cells were analyzed by flow cytometry (FACS Scan, Becton Dickinson, San Jose, CA).

### Immunohistochemical Staining

The tissue sections were subjected to immunohistochemical (IHC) staining $CD25^+$ T cells to investigate the T-cell regulation after transplant specified days (*27*). For IHC staining, the horseradish peroxidase-diaminobenzidine (HRP-DAB) system staining kit was used (BioGeneX, San Ramon, CA; *27*). After endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 10 min at room temperature, the tissue sections were stained, respectively, with mouse anti-porcine CD25 (Serotec). The antibody was allowed to bind at a 1:50 dilution in phosphate-buffered saline at 4°C in the dark overnight. The reaction sections were incubated with biotinylated anti-mouse antibody as a secondary antibody for 30 min. Visualization of the specific binding on the sites of primary antibodies was developed by an enzymatic conversion of the chromogenic substrate 3, 3′ diaminobenzidine into a brown precipitate by horseradish peroxidase. After counterstaining with hematoxylin, donor tissue sections were mounted, cleared, and cover slipped.

### BrdU Labeling of MSCs to Trace Donor MSC Engraftment

MSCs were labeled with two rounds of 7.5 $\mu$g/mL bromodeoxyuridine (BrdU) in culture medium (−3 and −1 days before injection) and then injected into the recipient

animals to assess proliferation and mitosis of the MSC population. We did not use green fluorescent protein transduction with lentivirus to MSCs as an indicator because we have tried to establish this trafficking but inconsistent expression by our technique. Biopsy samples of recipient tissue (obtained from the bone marrow, liver, muscle, and skin) and donor tissue (obtained from skin and muscle) were harvested at 3 and 10 days after injection. IHC staining was performed on biopsy samples using a HRP-DAB staining kit (BioGeneX).

### Histomorphometric Analysis

The tissue sections obtained from different treatment specimens were analyzed. For immunostaining quantification, sections were analyzed by using a Zeiss Axioskop 2 plus microscope (Carl Zeiss, Gottingen, Germany). Areas (3 $mm^2$) containing positive immunostained cells were analyzed. Four random selected areas were then taken under 400× magnifications. All images of each specimen were captured using a Cool CCD camera (SNAP-Pro cf Digital kit; Media Cybernetics, Silverbernetics Spring, MD). Images were analyzed by using Image-Pro Plus image analysis software (Media Cybernetics, Silverbernetics Spring, MD).

### Statistical Analyses

Student's *t* test or analysis of variance was used to assess the statistically significant differences between the experimental groups. Graft survival was compared between the different groups of transplanted animals using a Kaplan-Meier analysis and the log-rank test. A $P$ value of $<0.05$ was considered to be statistically significant.

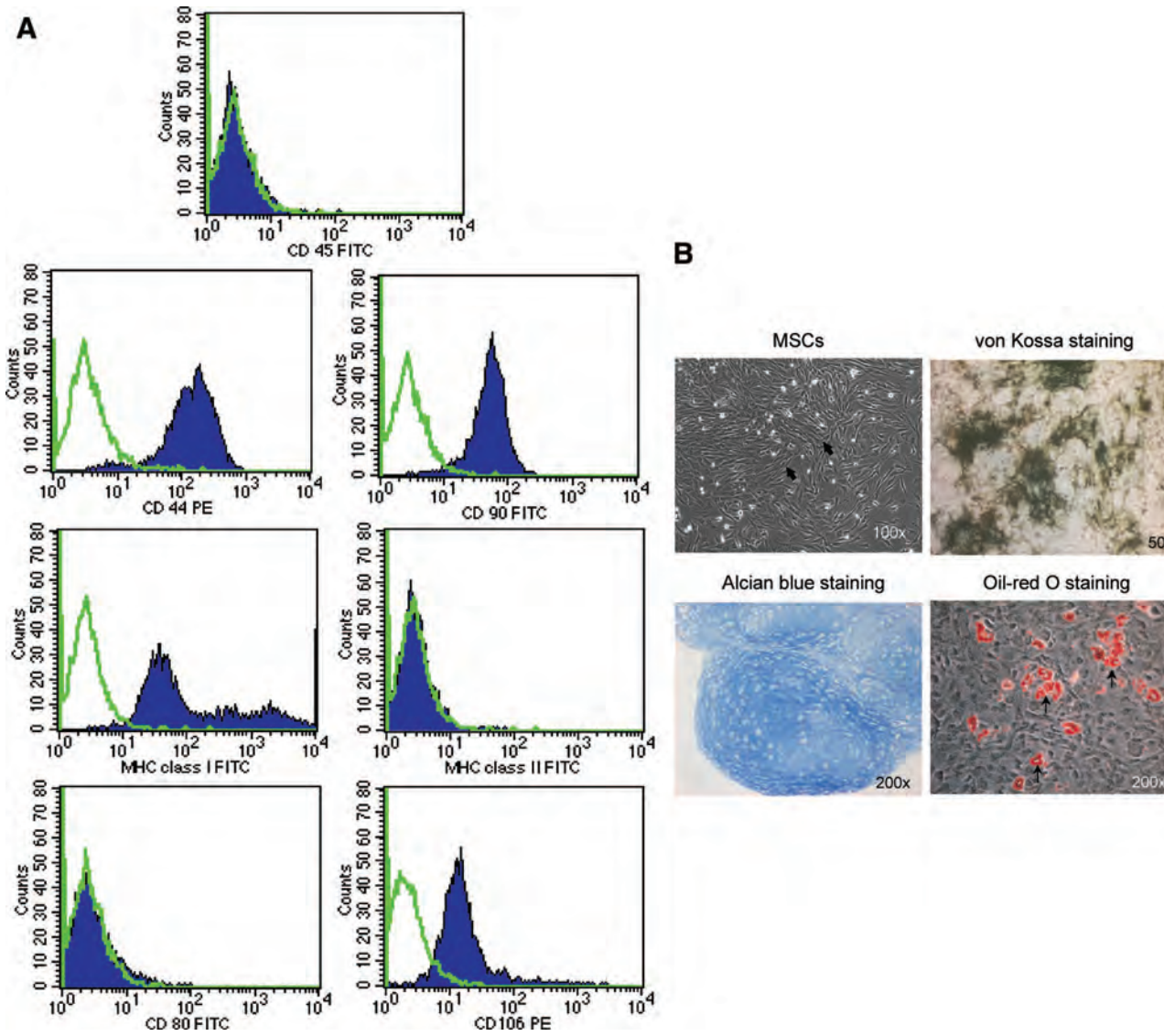


**FIGURE 2.** Donor bone marrow cells were harvested and isolated for mesenchymal stem cell culture. (A) MSCs were expanded in the culture and demonstrated positive surface staining for CD44, CD90, major histocompatibility complex class I, and CD106; but not for CD45, major histocompatibility complex class II, and CD80/B7-1 expression, as revealed by flow cytometry. (B) Mesenchymal stem cells were tested for their ability to differentiate into adipocytes, osteoblasts, and chondrocytes. Osteoblasts were identified by von Kossa staining, oil-red O was used for lipid droplet staining, and chondrogenic differentiation was visualized by Alcian blue staining.

## RESULTS

### Characterization and Differentiation of MSC In Vitro

Donor bone marrow cells were harvested and isolated for MSC culture. MSCs were expanded in the culture and demonstrated positive surface staining for CD44, CD90, MHC class I, and CD106; but not for CD45, MHC class II, and CD80/B7–1 expression, as revealed by flow cytometry (Fig. 2A). MSCs were tested for their ability to differentiate into adipocytes, osteoblasts, and chondrocytes. Osteoblasts were identified by von Kossa staining, oil-red O was used for lipid droplet staining, and chondrogenic differentiation was visualized by Alcian blue staining (Fig. 2B).

### MSCs in Combination With BMT and Short-Term Immunosuppressant Therapy Prolong Allograft Survival

Our results demonstrate progressive rejection of the swine hind-limb allograft by postoperative days 9 to 14 in group I (control). Allograft transplantation in combination with short-term multiple MSC injections in the absence of immunosuppressant (group II) revealed significant prolongation of allograft survival by postoperative days 15 to 25 when compared with controls ($P=0.02$). These results suggest that MSCs may act in a positive manner to prolong CTA survival. Allograft transplantation along with CsA treatment in group III resulted in delay rejection between postoperative days 28 and 45 ($P<0.01$). Irradiation in conjunction with BMT and CsA treatment in group IV provided no statistically significant prolongation in allograft survival when compared with CsA treatment group. In this group, GVHD-related symptoms were observed, including progressive weight loss and diarrhea, bone marrow failure with severe T-cell destruction and pancytopenia, and, ultimately, death. However, irradiation combined with MSCs, BMT, and short-term CsA treatment as in group V resulted in a significant prolongation of allograft survival (>200 days, $P<0.001$) in 60% recipients when compared with allograft survival in the other experimental groups and without signs of GVHD (Fig. 3).

### MSCs in Combination With BMT and Short-Term Immunosuppressant Therapy Suppress Allograft Rejection

Histopathologic evaluation of allograft biopsy samples revealed severe signs of graft rejection (grades III–IV), including inflammatory cell infiltrates in the allo-skin and muscle samples of group I at 2 weeks posttransplantation. The allograft biopsies of group II, which received MSCs alone, revealed moderate to severe rejection (grades II–III) in the allo-skin and interstitial muscle layers at 2 weeks posttranaplantation. Histologic examination of allograft biopsies from group III revealed mild rejection (grade I) in the interstitial muscle layers, but mild to moderate rejection (grades I–II) with lymphocyte infiltration was observed in the allo-skin at 2 weeks posttransplantation. Donor skin and muscle biopsies from the BMT-CsA treated group (group IV) revealed milder rejection signs than those

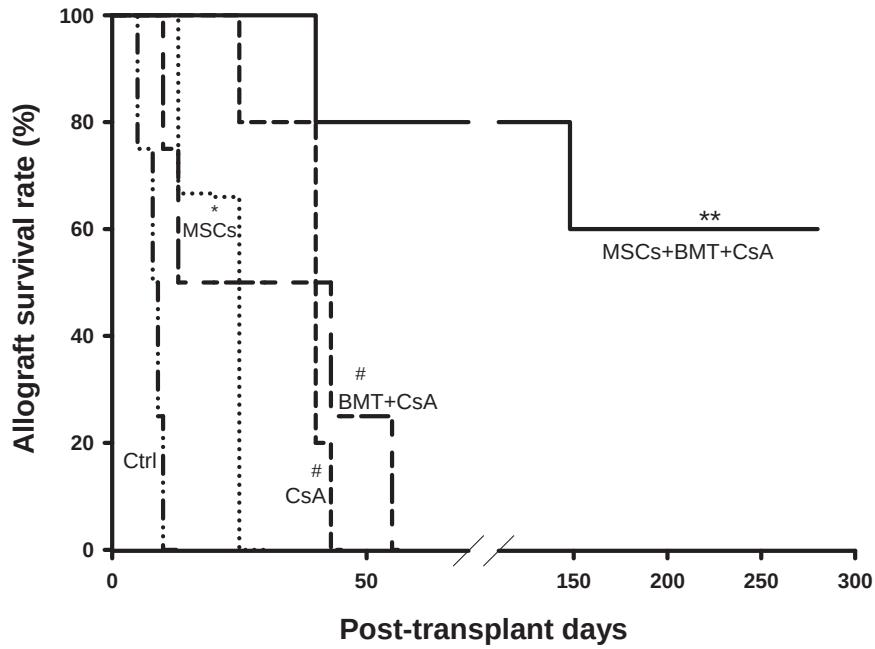


**FIGURE 3.** Mesenchymal stem cells (MSCs) in combination with short-term immunosuppressive therapy prolonged allograft survival. The allograft was rejected by days 9 to 14 in the control group. Short-term multiple MSC injections in the absence of immunosuppression (group II) revealed an increase in allograft survival to days 15 to 25 (*$P$ value=0.02 vs. control). The allograft from the group receiving short-term cyclosporine A (CsA) treatment (group III) resulted in delayed rejection by postoperative days 28 to 45 ($^{\#}P$ value <0.01 vs. control). Irradiation in combination with bone marrow transplantation-CsA therapy (group IV) yielded no significant prolongation of allograft survival (days 13–57) when compared with group III. The combination of MSCs with irradiation and bone marrow transplantation-CsA significantly prolonged allograft survival when compared with survival in the other groups (**$P<0.001$). The indicated factors were statistically significant ($P<0.05$) when compared with controls.

seen in the group treated with CsA alone. However, at 2 weeks posttransplantation, histologic examination of the MSC-BMT-CsA treated group (group V) revealed the lowest degree of rejection in the allo-skin (grade 0–I) and no changes (grade 0) in the interstitial muscle layer when compared with other groups (Fig. 4). Allo-skin and muscle tissue biopsies of group V animals at 35 weeks posttransplantation revealed no signs of rejection (Fig. 4).

### $CD4^+/CD25^+$ T-Cell Expression in Circulating Blood and Allo-Tissues

Flow cytometric analysis of the recipients' peripheral blood revealed that the expression of $CD4^+/CD25^+$ regulatory-like T cells was significantly increased in group V with MSCs-BMT-CsA treatment, when compared with that in control group (Fig. 5). Immunohistochemical staining of the biopsy tissue of the donor allo-skin from MSCs-BMT-CsA treatment group revealed a significant number of $CD25^+$ T cells in the subcutaneous and dermis layers when compared with those revealed in the controls and other groups (Fig. 6). Significant expression of $CD25^+$ cells was not found in the biopsy donor muscle. This demonstrated that MSCs along with short-term immunosuppressant therapy could increase the expression of regulatory T cells.

### Homing of BrdU-Labeled MSCs to Recipient Tissues

BrdU-labeled donor MSCs were intravenously injected into the recipient swine. We investigated MSC homing and engraftment at 3 and 10 days postinjection. Our data revealed a significant population of BrdU-labeled donor MSCs in the subcutaneous layers of both the donor and recipient skin and the perivascular parenchyma of the recipient liver, as detected by HRP-DAB staining. However, no significant expression of donor-derived MSCs was seen in the donor or recipient muscle tissue or in the recipient bone marrow tissue (Fig. 7).

## DISCUSSION

Unlike many other solid-organ transplants that prolong the patient's lifespan, CTA is an elective procedure performed with the goal of improving the patient's quality of life. Numerous antirejection and immunosuppressive therapies have been pursued to induce immune tolerance toward the graft or decrease CTA antigenicity (*27–29*). However, complications secondary to chronic immunosuppression remain an obstacle. Therefore, the development of novel nontoxic tolerance strategies which circumvent the long-term use of immunosuppressants is critical.

Recent studies reported that donor MSCs do not express costimulatory molecules and are potent in inhibiting

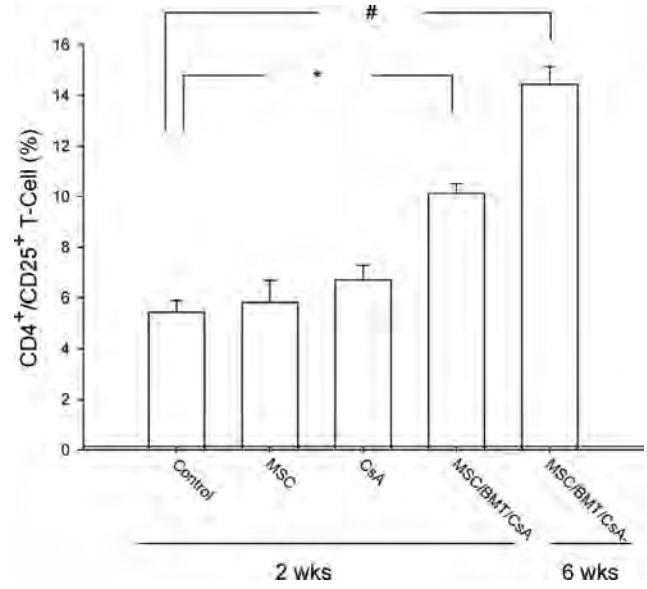


**FIGURE 5.** Mesenchymal stem cells (MSCs) with short-term immunosuppressants could increase regulatory T-cells expression. The $CD4^+/CD25^+$ T-cells expression in circulating blood was detected by flow cytometry. The data analysis revealed the $CD4^+/CD25^+$ regulatory-like T-cells expressions significantly increased in group with MSC-bone marrow transplantation-cyclosporine A treatment when compared with the controls. The indicated signals were statistically significant ($^*P<0.05$, $^\#P<0.001$).

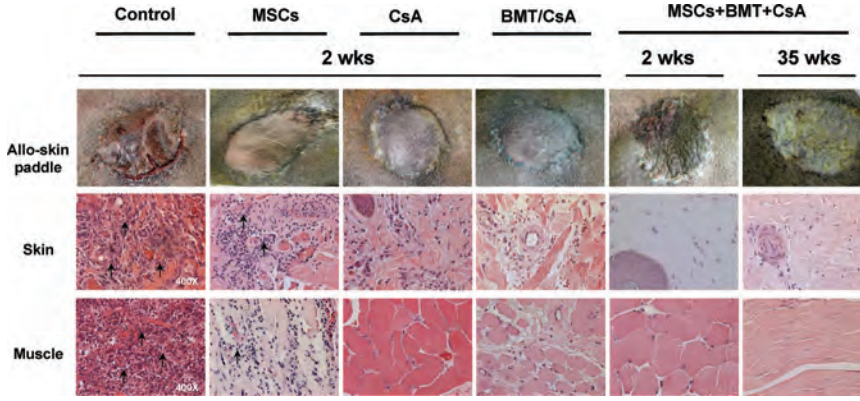


**FIGURE 4.** Mesenchymal stem cells (MSCs) in combination with short-term immunosuppressive therapy decreased allograft rejection. At 2 weeks posttransplantation, histologic examination of allograft biopsies from the MSC-bone marrow transplantation-cyclosporine A group demonstrated decreased inflammatory cell infiltrates in the allo-skin and interstitial muscle layers when compared with the groups not treated with MSCs. The allo-skin and muscle biopsies from the MSC-bone marrow transplantation-cyclosporine A group revealed no apparent rejection signs even at 35 weeks posttransplantation. Magnification is ×400 in biopsy tissue.

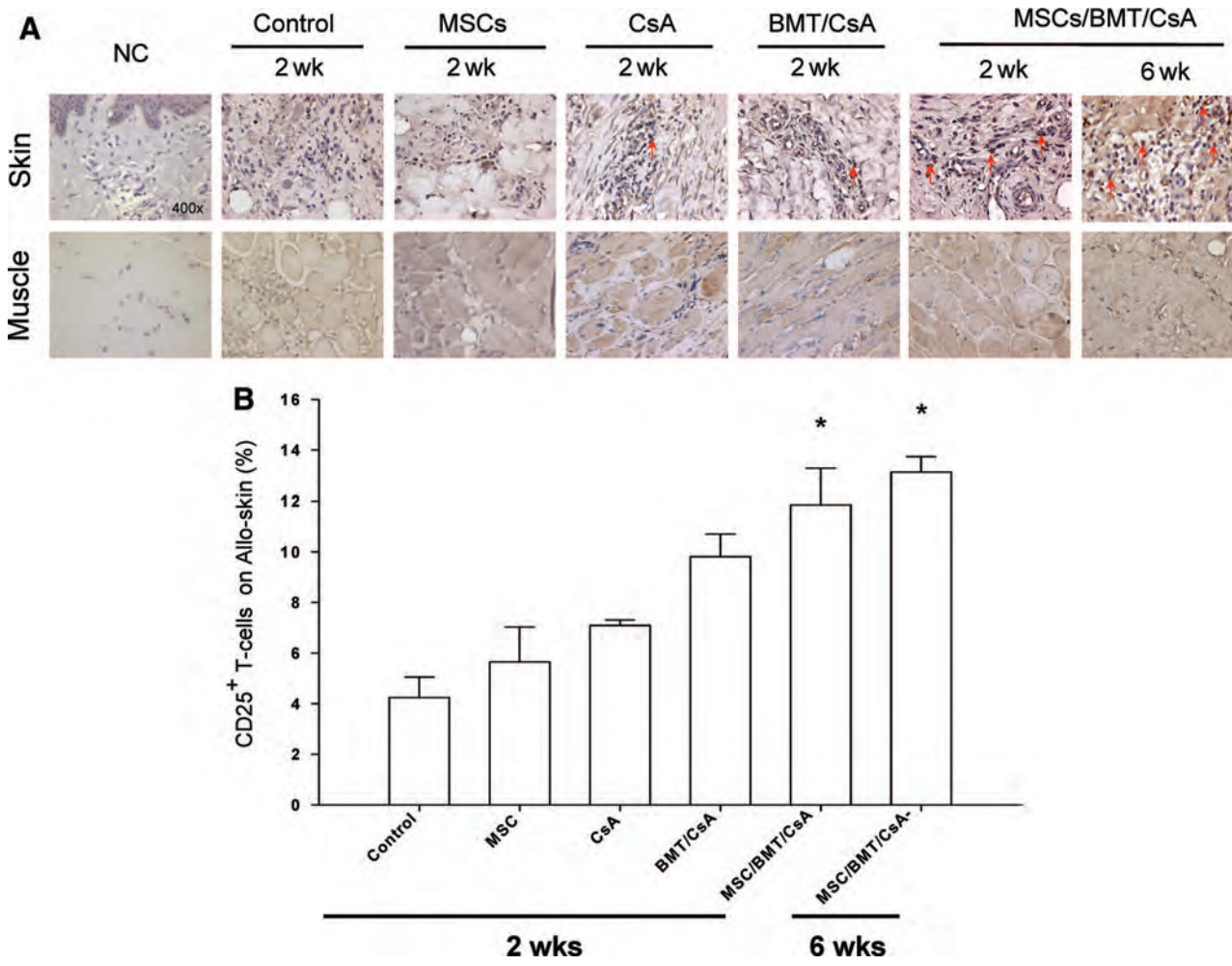


**FIGURE 6.** Mesenchymal stem cells (MSCs) with short-term immunosuppressants increase the $CD25^+$ T-cells expression in allo-transplant tissues. Immunohistochemical staining of biopsy tissue in donor allo-skin of the group with MSC-bone marrow transplantation-cyclosporine A treatment revealed significant $CD25^+$ T-cells expressions in the subcutaneous and dermis layers when compared with that rejection in the other groups. Significant expression of $CD25^+$ cells was not found in the biopsy donor muscle. The indicated signals were statistically significant when compared with controls (*$P<0.05$). Magnification $\times 400$

T-cell proliferation in mixed lymphocyte cultures, preventing GVHD resulting from BMT and prolonging skin allograft survival in rodent models (*13–18*). The results of this study indicated animals treated with multiple MSC injections in the absence of immunosuppressants revealed significant prolongation of allograft survival, when compared with the controls. These data demonstrate that MSCs alone have a positive effect on allograft survival. The limb allografts in recipients of short-term CsA monotherapy were delayed rejection posttransplantation. Interestingly, irradiation in conjunction with BMT-CsA treatment did not significantly prolong in allograft survival when compared with CsA treatment alone. Moreover, in irradiation combined with BMT-CsA treated group, GVHD-related symptoms were observed. In contrast, recipients treated with MSCs combined with BMT-CsA accepted swine hind-limb allograft throughout the follow-up period of more than 200 days posttransplantation and without signs of GVHD. This demonstrated multiple MSCs infusions in conjunction with irradiation, BMT, and short-term immunosuppressions represent a novel strategy to substantially prolong allograft survival.

Skin is considered to be the most antigenic in CTA (*6*). Histopathologic analysis of allograft biopsy tissue from the control group 2 weeks posttransplant demonstrated substantial skin necrosis with severe acute rejection signs in the dermal-epidermal junction. Donor skin biopsies from the BMT-CsA group revealed more mild rejection signs than the group that received CsA alone. Interestingly, the biopsy of allograft muscle demonstrates much less cellular rejection when compared with the allo-skin in all groups at 2 weeks posttransplant, except for the control and MSC-treatment group. This indicated that the antigenicity of skin is a major obstacle to the induction of CTA tolerance. The split tolerance observed in a nonmyeloablative chimerism strategy (*30*) supports this hypothesis. Nevertheless, the recipients receiving combination of MSCs and BMT-CsA therapy demonstrated no signs of rejection in the donor skin and muscle biopsies at 35 weeks posttransplantation. These results indi-

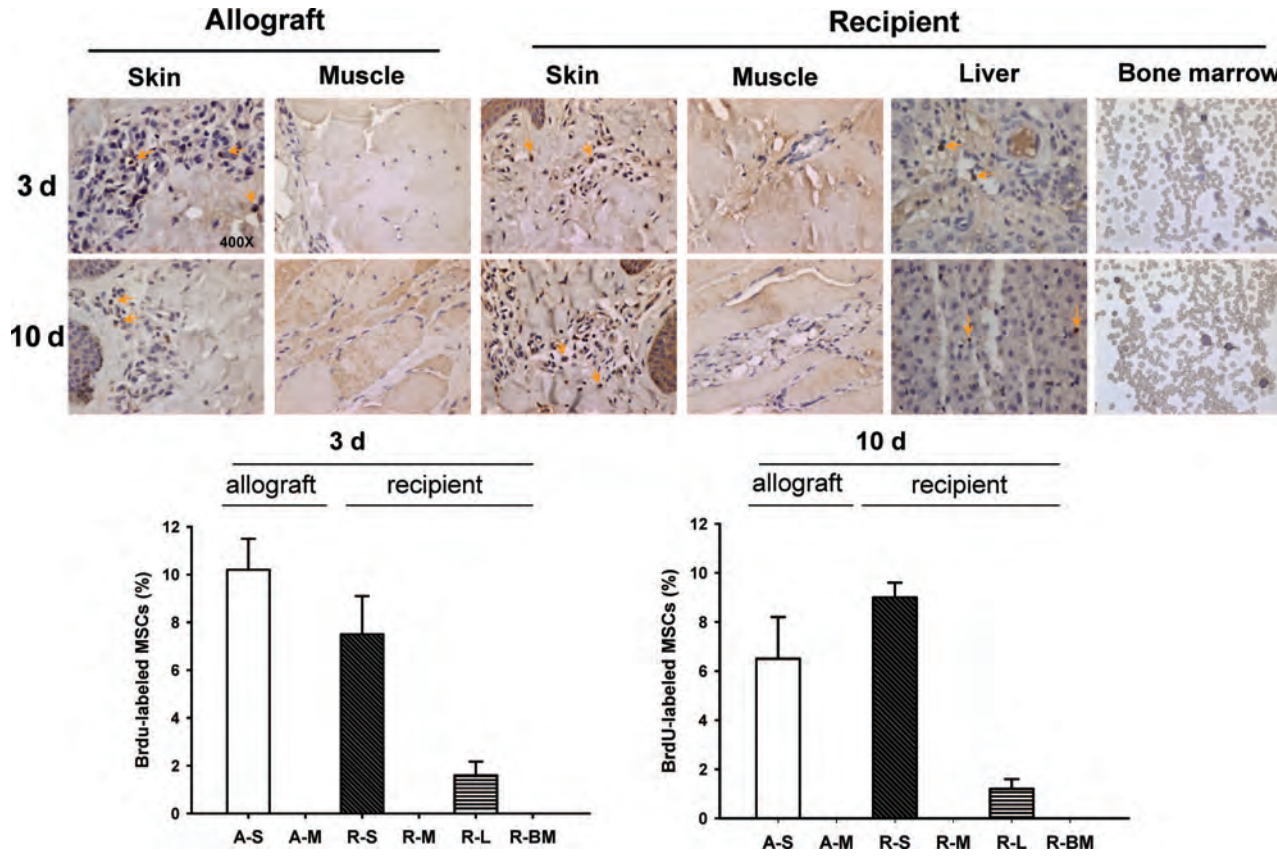


**FIGURE 7.** Evaluation of the homing and engraftment of bromodeoxyuridine-labeled donor mesenchymal stem cells (MSCs) at 3 and 10 days postinjection in recipient and donor tissues. These data revealed significant expression of bromodeoxyuridine-labeled donor MSCs in the subcutaneous layers of donor and recipient skin and in the perivascular parenchyma of the recipient liver as detected using horseradish peroxidase-diaminobenzidine staining. However, no significant expression of donor-derived MSCs was found in the donor or recipient muscle or in the recipient bone marrow tissue. Magnification ×400.

cated that MSCs with irradiation and BMT-CsA treatment prevent allo-skin rejection, and induce CTA tolerance.

To explore the mechanisms of how MSC modulates allograft survival, the homing of MSC in CTA recipients was tracked by using BrdU-labeled donor MSCs. Immunohistochemical staining data revealed that donor MSCs were preferentially present in the subcutaneous layers of both the donor and recipient allo-skin and the recipient perivascular liver parenchyma. Interestingly, no BrdU-labeled cells were observed in the recipient muscle or bone marrow tissue. This result indicated that the hematogenous spread of MSCs could enable engraftment and proliferation of these cells in the recipient tissue.

Previous studies have reported that BMT combined with immunosuppressive therapy could prolong organ-transplant survival (*9*, *31*). However, GVHD still remains the most frequent complication associated with transplantation of allogeneic hematopoietic grafts (*10*). Some studies have demonstrated that administration of irradiation-BMT and short-term immunosuppression may induce donor-specific tolerance without GVHD in a rodent CTA model (*32*, *33*). Nevertheless, our large animal study did not yield such results. In this swine CTA study, the combination of BMT-CsA after preconditioning total body irradiation exerted no positive effect of allograft survival when compared with CsA alone. Rather, GVHD symptoms were apparent in the recipient swine. A possible explanation of the contradictory results seen in the rodent and swine CTA models may be related to the differences between MHC and non-MHC in inbred rodents and pigs. As noted earlier, the miniature swine used in our study may have various MHC and non-MHC haplotypes, and it is possible that the phenomenon observed in the rodent model may not be directly applicable to clinical settings. Another reason might be the severe T-cell destruction and pancytopenia secondary to bone marrow failure in the recipient swine. These possibilities may help explain why the BMT-CsA group did not exhibit prolonged allograft survival.

Recently, the immunoregulatory properties of an MSC infusion have been proposed both in vitro and in vivo (*14*, *17*). Additional studies have suggested that MSCs may inhibit T-cell activation, thus prolonging skin allograft survival and eventually inducing immune tolerance in a rodent model (*17*, *18*). In this study, the fluorescence-activated cell sorter analysis of the $CD4^+/CD25^+$ regulatory-like T-cells expression in recipient peripheral blood revealed significant increase in MSCs with preconditional irradiation and BMT-CsA therapy, when compared with controls. Immunohistochemical staining of allograft tissue showed significant increase of $CD25^+$ T-cells

in subcutaneous and dermis layers in MSCs with preconditional irradiation and BMT-CsA therapy. These indicated that the regulation activity of MSCs on T cells might contribute to CTA survival.

However, there are still some limitations of this study. First, our results revealed BrdU-labeled MSCs could indicate as a semiquantification of trafficking the homing of MSCs. Further studies using green fluorescent protein transduction with lentivirus should be more reliable and more visualizable assays than BrdU-labeled MSCs to traffic the engraftment of MSCs. Second, in this study, the biomechanisms of MSC inducing immune tolerance of CTA are still unclear and further investigations are essential to explore the biosignals in immunomodulatory effects between MSCs and T-cells. Additionally, we could not provide a group in which the swine received bone marrow cells alone instead of MSCs although treatment with MSCs alone could significantly prolong allograft survival. Therefore, this study does not include the ultimate proof of the superiority of MSCs over bone marrow cells to permit CTA engraftment in immune tolerance. Further investigations are currently underway to examine whether repeated infusions of MSCs might be effective in inducing tolerance in CTA.

In summary, this study indicated that multiple infusions of donor MSCs combined with BMT, preconditioning irradiation, and transient immunosuppression could effectively prevent GVHD and prolong the survival of a miniature swine hind-limb allotransplant model. This prolongation might occur because MSCs increase the engraftment of donor progenitor cells and modulate host immune function. MSC infusion provides a potential novel strategy for clinically improving allograft survival and inducing immune tolerance.

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