Cell Transplantation, Vol. 15, pp. 711–721, 2006
Printed in the USA.

0963-6897/06 $90.00 + .00
E-ISSN 1555-3892
www.cognizantcommunication.com

Immunologic Consequences of Multiple, High-Dose Administration of Allogeneic Mesenchymal Stem Cells to Baboons

Kirstin J. Beggs,* Alex Lyubimov,† Jade N. Borneman,* Amelia Bartholomew,† Annemarie Moseley,* Robert Dodds,* Michael P. Archambault,* Alan K. Smith,* and Kevin R. McIntosh*

*Osiris Therapeutics, Inc., Baltimore, MD, USA
†University of Illinois, Chicago, IL, USA

Mesenchymal stem cells (MSCs) express low immunogenicity and demonstrate immunomodulatory properties in vitro that may safely allow their transplantation into unrelated immunocompetent recipients without the use of pharmacologic immunosuppression. To test this hypothesis, three groups of baboons (three animals per group) were injected as follows: group 1 animals were injected with vehicle; group 2 animals were injected IV with DiI-labeled MSCs (5×10^6 MSCs/kg body weight) followed 6 weeks later by IM injections of DiO-labeled MSCs (5×10^6 MSCs/kg) from the same donor; and group 3 animals were treated similarly as group 2 except that MSCs were derived from two different donors. Muscle biopsies, performed 4 weeks after the second injection of MSCs, showed persistence of DiO-labeled MSCs in 50% of the recipients. Blood was drawn at intervals for evaluation of basic immune parameters (Con A mitogen responsiveness, PBMC phenotyping, immunoglobulin levels), and to determine T-cell and alloantibody responses to donor alloantigens. Host T-cell responses to donor alloantigens were decreased in the majority of recipients without suppressing the overall T-cell response to Con A, or affecting basic parameters of the immune system. All recipient baboons produced alloantibodies that reacted with donor PBMCs. Two of six animals produced alloantibodies that reacted with MSCs. We conclude that multiple administrations of high doses of allogeneic MSCs affected alloreactive immune responses without compromising the overall immune system of recipient baboons. The induction of host T-cell hyporesponsiveness to donor alloantigens may facilitate MSC survival.

Key words: Mesenchymal stem cells; Baboons; Immunogenicity; Allogeneic; T-cell hyporesponsiveness; Alloantibody

INTRODUCTION

Bone marrow-derived mesenchymal stem cells (MSCs) have the capacity to differentiate into a variety of tissues including bone, cartilage, stroma, fat, muscle, and tendon (13,29,30). These cells exist at very low frequency in bone marrow, and can be isolated and greatly expanded (billions of MSCs can be produced from a small bone marrow aspirate) without losing the ability to differentiate into multiple lineages. Thus, these cells have clinical potential for repair or replacement of damaged tissues (7). The clinical application of MSCs for tissue regeneration could be most readily achieved with an allogeneic product; that is, cells derived from one donor that could be used in multiple recipients. A potential limitation to this "universal donor" concept is rejection of donor cells by the recipient's immune system, particularly after multiple administrations.

MSCs exhibit certain properties that suggest they may be transplanted across genetic barriers. These cells do not express cell surface markers that are highly stimulatory to the immune system, such as MHC class II and costimulatory molecules comprising CD40, CD80, and CD86 (18,32). Furthermore, MSCs do not stimulate alloreactive T-cell proliferation in one-way mixed lymphocyte reaction (MLR) cultures (9,11,18,20,27,32). Furthermore, human MSCs actively suppressed primary MLR responses when added at the initiation of culture or during an ongoing response (9,11,18,20,27,32). Suppression was not genetically restricted because MSCs from third-party donors were as suppressive as MSCs matched to responder or stimulator cells used in the MLR. The suppressive characteristic of MSCs is not unique to human MSCs. Suppression of alloreactivity by MSCs derived from mice (12,14) and baboons (2) has been reported previously and we have documented allo-

Received October 6, 2005; final acceptance May 29, 2006.
Address correspondence to Kevin R. McIntosh, Ph.D., Cognate BioServices, Inc., 1448 S. Rolling Road, Baltimore, MD 21227, USA. Tel: 410-455-5550; Fax: 410-455-5551; E-mail: kmcintosh@cognatebioservices.com

suppression by MSCs from rats, dogs, and goats (unpublished observations). The mechanism of suppression by MSCs involves a soluble molecule(s) as MSCs suppressed mixed lymphocyte cultures across a transwell membrane (11,18,32). Candidate molecules implicated in suppression include transforming growth factor-β and hepatocyte growth factor (11), prostaglandin E_2 (1), and indoleamine 2,3-dioxygenase (28).

Several reports suggest that MSCs may mediate suppression in vivo. Bartholomew et al. demonstrated that baboons injected with MSCs exhibited delayed rejection responses to donor-matched and third-party skin grafts (2). Intriguing data from phase I clinical trials and a case report suggest that MSCs may suppress graft-versus-host disease (GVHD) in humans. Patients undergoing HLA-identical, sibling-matched hematopoietic stem cell transplants plus MSC infusion (up to 5×10^6 MSCs/kg body weight) exhibited a lower incidence of both acute and chronic graft versus host disease than expected (19). Similarly, a patient with severe acute GVHD recovered after he was treated with haploidentical MSCs (21). Recently, MSCs have been reported to prevent the onset of experimental autoimmune encephalomyelitis in mice with the subsequent induction of T cell anergy (33).

Can the low immunogenicity of MSCs, coupled with their immunosuppressive properties, permit their survival in genetically disparate immunocompetent recipient animals? Few studies have addressed this point. Allogeneic baboon MSCs were detected in various tissues in a nonconditioned recipient animal 9 months following IV infusion (10). Xenotransplantation of human MSCs into immunocompetent fetal lambs has been successful, as the cells engrafted and exhibited site-specific differentiation into chondrocytes, adipocytes, myocytes, cardiomyocytes, and stroma (23). Similarly, in nonconditioned rodent models, mouse MSCs have been shown to engraft in rat bone marrow and heart muscle for at least 13 weeks (31).

The purpose of the present study was to investigate the survival of transplanted allogeneic MSCs in nonconditioned baboons, and to evaluate the cellular and humoral immune responses evoked in these animals from primary and secondary challenge of cells. A secondary goal of the study was to determine the safety of multiple injections of clinically relevant doses of allogeneic MSCs by assessing broad immune parameters such as mitogen responsiveness, PBMC phenotyping, and immunoglobulin levels. MSCs were administered by IV and IM routes; the latter in an attempt to maximize induction of an immune response. We chose the baboon model for these experiments, both for clinical relevance and data showing that baboon MSCs exhibited similar immunosuppressive properties to human MSCs (2).

MATERIALS AND METHODS

Animals

Nine female baboons (*Papio anubis*), at least 11 years old, were obtained from Charles River Breeding Laboratories (Fort Washington, NY or Houston, TX), Health Sciences Center (University of Oklahoma, Oklahoma City, OK), and Columbia University (New York, NY) and used as recipients. All animals in this study were quarantined for at least 2 months in the University of Illinois, Chicago (UIC) baboon breeding colony. Animal weights at the initiation of study ranged from 13.5 to 25.3 kg. Six juvenile male baboons were bone marrow donors for the MSCs. All of the donors were spuma negative and were housed at the Southwest Foundation for Biomedical Research (San Antonio, TX). All animal work was performed under institutionally approved animal protocols.

Study Design

MSC donors were purposefully mismatched to recipient animals by HLA typing (minimum of three mismatches at HLA-A, HLA-B, and HLA-DR). Molecular HLA typing was performed by the Tissue Typing Laboratory, Department of Transplantation Medicine at UIC. Human class I and class II leukocyte antigen typing was performed via sequence specific primer amplification PCR (SSP UniTray, PelFreez Clinical Systems, Brown Deer, WI). Typing results for the donor–recipient combinations of animals used in this study are shown in Table 1.

The nine animals were divided into three groups of three animals per group (see Fig. 1).

Group 1. Control baboons received an initial IV injection of 95% Plasma-Lyte® (Baxter, Deerfield, IL) plus 5% human albumin (American Red Cross, Rockville, MD) in a volume of 20 ml. Six weeks later the animals received a second IM injection of vehicle. The IM injections were administered to eight sites at 2 ml/site as follows: one injection bilaterally into the triceps, two injections bilaterally into the quadriceps, and one injection bilaterally into the biceps femoris (hamstring) muscles. Each injection site was marked with a tattoo to localize subsequent biopsy.

Group 2. Baboons received an initial IV injection of allogeneic MSCs in vehicle at a dose of 5×10^6 MSCs/kg body weight (approximately 100×10^6 MSCs/animal). Six weeks later, the animals received a second IM injection of MSCs (5×10^6 MSCs/kg body weight) from the same donor as the cells used for the IV injection. We have designated this group "AA," because both injections of MSCs were derived from the same donor.

Table 1. HLA Designations of Baboons Used as Donors and Recipients

Group	Animal No.	Class 1A	Class 1B	Class IIDR	Class IIDQ
2 (AA)	6214 (recip)	66, unk	15,44	6 or 8, unk	5, unk
	14682 (dnr A)	23 or 24, 32	37, unk	14 or 18, 14	5, unk
	6309 (recip)	unk, unk	44,unk	14 or 18, 6 or 8	5, 6
	14862 (dnr A)	24, unk	37, 44	14,18	5, unk
	6908 (recip)	66,unk	35, 44	14 or 18, unk	5, 6
	14891 (dnr A)	unk, unk	37, 35	14 or 18, 14	6, unk
3 (AB)	5500 (recip)	66, unk	35, unk	6 or 8, unk	5, unk
	14518 (dnr A)	24, unk	44, unk	14, unk	6, unk
	14485 (dnr B)	unk, unk	55, unk	14, unk	5,unk
	6115 (recip)	11, 66	15, unk	14 or 18, 6 or 8	5, 9
	14682 (dnr A)	23 or 24, 32	37, unk	14 or 18, 14	5, unk
	14485 (dnr B)	unk, unk	55, unk	14, unk	5, unk
	6907 (recip)	66, unk	unk, unk	14 or 18, 6 or 8	5, 6
	14403 (dnr A)	24, unk	44, unk	14, unk	5, 8
	14485 (dnr B)	unk, unk	55, unk	14, unk	5, unk

Baboon recipients in group 2 (AA) received two injections from the same donor "A." Recipients in group 3 (AB) received a first injection from donor "A" and a second injection from an unrelated donor "B." Nonreactivity to available primer pairs is depicted as unknown (unk); multiple reactivities to different primer pairs are shown with an "or" spacer.

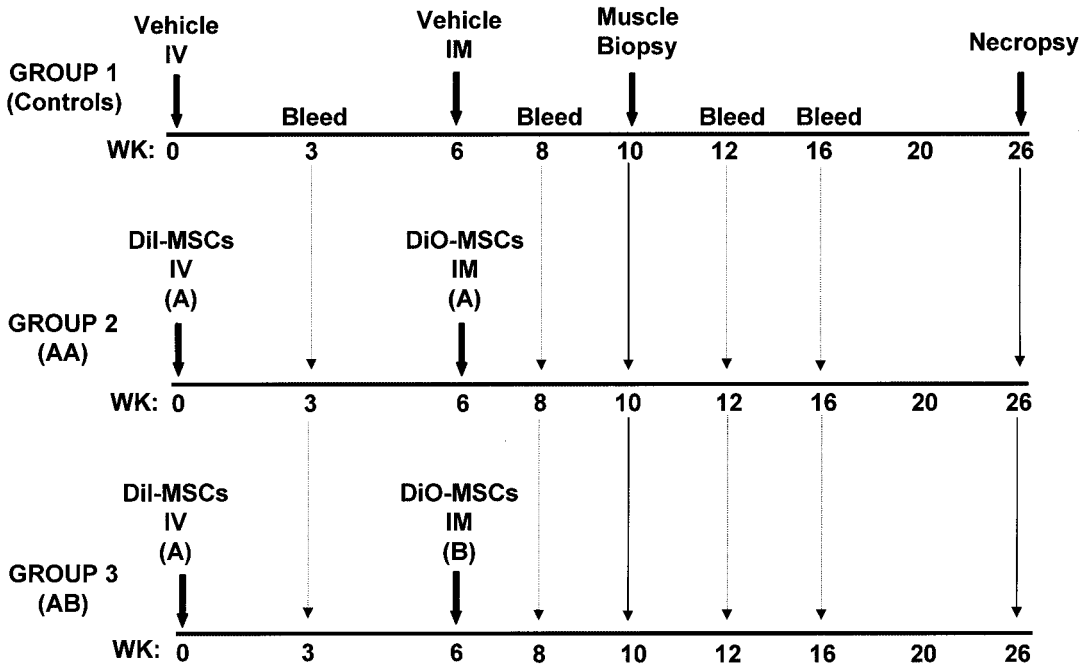

Figure 1. Study design. See text for details.

Intramuscular injections were distributed over eight sites as described for group 1.

Group 3. Baboons were treated as described for group 2 with the exception that the second IM injection consisted of MSCs from a different donor than was used for the IV injection. We have designated this group "AB," because MSCs were derived from different donors. Donor "B" was HLA disparate at a minimum of three loci with both the recipient and donor "A."

Recipient baboons were bled prior to the initial vehicle or MSC injections, and at intervals thereafter to assess T-cell reactivity to donor antigens, alloantibody, and peripheral blood phenotype. Muscle biopsies were performed at 10 weeks and 6 months (at necropsy) at the site of injection.

Production of Dye-Labeled Baboon MSCs

Aspirates (2–4 ml) were drawn from the iliac crest in heparin and processed as described for human cells (30). Cells were cultured in Dulbecco's low-glucose medium containing 10% selected fetal bovine serum (HyClone, Logan, UT) plus 1% antibiotic/antimycotic (all culture reagents from Invitrogen, Carlsbad, CA, except as indicated). At the end of primary culture (P0), MSCs were cryopreserved in 90% FBS plus 10% DMSO. To produce cells for experiments, P0 cells were thawed and subsequently expanded through three passages in MSC medium using Nunc 10-stack cell factories (VWR Scientific Products, West Chester, PA). Greater than 95% of the cells stained positively with SH3 antibody, similar to human MSCs (15), and less than 5% of the cells stained positive for myeloid and lymphocyte-specific antibodies. Baboon MSCs were labeled with the fluorescent dyes DiI or DiO (Molecular Probes, Eugene, OR), according to the manufacturer's instructions prior to cryopreservation in a cocktail of 85% Plasma-Lyte®A, 10% DMSO (Sigma, St. Louis, MO), and 5% human serum albumin. Cells were cryopreserved in 50 ml cryocyte bags (Baxter) using a programmable Cryomed freezer (Forma Scientific, Marietta, OH). MSCs were shipped to UIC in a dry shipper (Custom Biogenic Systems, Shelby Township, MI) that maintains liquid nitrogen vapor temperatures for at least 1 week. MSCs, recovered after thawing the bags in a water bath at 37°C, were greater than 85% viable by trypan blue exclusion, with a recovery efficiency greater than 85%.

Biopsy Processing

At 10 weeks, 4 weeks after the IM injections of MSCs, surgical muscle biopsies were performed at four of the eight injection sites. The remaining four sites were biopsied at necropsy. The biopsy specimen was divided for confocal microscopy (Nikon PCM 200) and general histology. For confocal microscopy, the tissue was placed into cold PBS and examined as soon as possible (within 2 days). For histology, the tissue was fixed in 10% formalin overnight at room temperature and subsequently placed in cold PBS. The tissue was sectioned and stained with hematoxylin and eosin by traditional methods.

Mixed Lymphocyte Reaction

Baboon peripheral blood mononuclear cells (PBMCs) were prepared from blood samples by centrifugation over Ficoll Hypaque (Amersham-Pharmacia Biotech, Piscataway, NJ) according to the manufacturer's instructions. One-way MLRs were performed in flat-bottom 96-well microtiter plates by culturing recipient PBMCs (2×10^5/well) with irradiated donor PBMCs (1×10^5/well) or autologous PBMCs (1×10^5/well) as controls. PBMCs were irradiated with 4000 rads of X-irradiation (Faxitron X Ray, Buffalo Grove, IL) to prevent T cells in the stimulator PBMC population from reacting against responder cells. Recipient responder cells (2×10^5/well) were also cultured with the T-cell mitogen, concanavalin A (Con A, 10 μg/ml, Sigma) to evaluate the proliferative activity of total T cells. Cultures were performed in Iscove's modified Dulbecco's medium (IMDM, Invitrogen) containing 5% human AB serum (Pel-Freez Biologicals, Rogers, AK), 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 5.5×10^{-5} M 2-mercaptoethanol, and 1% antibiotic/antimycotic (all culture reagents from Invitrogen, except as indicated). MLR assays were set up in duplicate for harvesting at two time points, days 3 and 7, so as not to miss peak MLR activity due to potential recipient priming. Mitogen (Con A) stimulation was measured at the typical peak of the response, on day 3. T-cell proliferation to alloantigens or mitogen was determined by addition of [^{3}H]thymidine ([^{3}H]TdR, Amersham-Pharmacia Biotech) to wells at 1 μCi/well for the final 18 h of culture. The cells were harvested using a Mach III 96-well cell harvester (Tomtec, Hamden, CT) and radioactivity incorporated into cellular DNA was determined by scintillation counting using a Microbeta Trilux liquid scintillation counter (Wallac Inc., Gaithersburg, MD). MLR data were calculated as Δcpm $\pm$ SD of three to four wells, subtracting autologous counts (autologous irradiated PBMCs as stimulators) from allogeneic counts (donor irradiated PBMCs as stimulators). Mitogen data were calculated as Δcpm $\pm$ SD of three to four wells, subtracting spontaneous proliferation of responder cells cultured in medium alone.

To determine whether significant differences existed between pretreatment and posttreatment responses, analyses of variance were conducted and used to perform *t*-tests to determine whether MLR and Con A responses

at each time point were significantly different ($p < 0.05$) from their pretreatment levels. Due to large differences in these responses between individual animals, data were log-transformed prior to statistical analysis. Data representing group responses were normalized as "percent of control" where the control was the pretreatment MLR response.

Alloantibody Assay

Sera were collected from recipient baboons prior to MSC injection and at intervals after injection as depicted in the tables. These sera were assessed for binding to either donor PBMCs or donor MSCs by a flow cytometry method (17). Briefly, donor cells were incubated with various dilutions of recipient serum (1:10, 1:100, 1:1000) for 1 h at 4°C, washed, and reincubated with a secondary biotinylated mouse anti-baboon immunoglobulin antibody for an additional 60 min at 4°C. Isotype controls were run at each dilution using biotinylated normal mouse immunoglobulin. After 1-h incubation with secondary antibody, FITC-streptavidin, was added for 30 min at 4°C. Cells were washed and fluorescence was determined on a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA). The percentage of positive cells was determined by analysis using CellQuest software. Positive titers were defined as exceeding a threshold of mean baseline titers plus 3 SD. Baseline titers were determined for each target cell population using naive serum obtained from nine animals. Sera that did not exhibit significant binding to target cells at a 1:10 dilution were considered negative.

Additional Immunological Parameters

Blood samples were obtained from recipient baboons prior to MSC treatment, and at 6, 12, and 26 weeks posttreatment and evaluated for immunoglobulin levels, antinuclear antibodies, and percentage of various mononuclear cell populations. IgG and IgM immunoglobulins were quantified by immunoprecipitate turbidity on a Hitachi 717 analyzer using the SPQ Kit System (Diasorin, Stillwater, MN). Antinuclear antibodies were assessed by ELISA (EL-ANA Screen ELISA kit, TheraTest Labs, Lombard, IL). Analysis of cell surface markers on PBMCs was performed using a panel of fluorochrome-labeled monoclonal antibodies diluted and used according to the manufacturer's instructions (BD Biosciences, San Diego, CA). The antibody panel consisted of individual or combinations of antibodies directed to CD3, CD19, CD14, CD56, CD3/CD25, CD3/CD4, and CD3/CD8. For all experiments, nonspecific fluorescence was determined by substitution with appropriate isotype-matched control monoclonal antibodies. Data were analyzed by collecting 10,000 events on a Becton Dickinson Vantage instrument using Cell-Quest software. Analysis of variance followed by Dunnett's procedure was used to evaluate the differences in means for each parameter analyzed between control and experimental groups.

RESULTS

Persistence of Donor MSCs in Muscle

Four weeks after the second administration of allogeneic MSCs, muscle biopsies were performed at four of eight sites of IM injections. The biopsies were evaluated for the presence of DiO-labeled cells using confocal microscopy. Positive biopsies containing dye-labeled cells were found for baboons in group 2 (#6309, #6908) and group 3 (#5500), or 50% of the treated animals. In each animal, only one of four biopsy specimens showed evidence of donor cells. The histological results from recipient #5500 were typical of the positive biopsies and are shown in Figure 2. Serial sections stained with H&E and an unstained section viewed under fluorescent light in the green channel demonstrated DiO-positive donor MSCs between muscle bundles that were homogenous in appearance and uniformly labeled with dye. There was no evidence of a host cell response to the allogeneic MSCs in the form of lymphocyte, macrophage, or neutrophil infiltration. Viewing the section in the red channel showed no fluorescence, indicating both the absence of DiI-labeled MSCs from the initial IV injection, and the lack of autofluorescence. Muscle samples taken from the injection sites at necropsy (20 weeks after IM injection) were negative for DiI- and DiO-labeled cells.

T-Cell Reactivity to Donor Alloantigens

To determine whether allogeneic MSCs induced a systemic T-cell response to donor alloantigens, one-way MLR assays were performed on blood samples obtained prior to cell infusion and at various intervals after infusion. Each MLR assay was harvested at two time points, days 3 and 7, to assess forward shifts in temporal kinetics due to T-cell priming in vivo. However, we observed no shift in kinetics: day 3 responses were always less than day 7 responses, usually by 75% or greater. Therefore, only day 7 results are reported.

Group 1 vehicle control animals did not show significant increases or decreases in MLR or Con A activity relative to pretreatment values throughout the initial 12-week period (Fig. 3A). At 16 weeks, there was a significant drop in both MLR and Con A responsiveness, suggesting that the blood samples had been compromised, possibly during shipping. Therefore, the 16-week samples were omitted from analysis.

Group 2 (AA) and group 3 (AB) baboons showed decreasing MLR responses to donor alloantigens after the second injection of MSCs that attained statistical significance at 12 weeks (Fig. 3B and C, respectively). The suppressed MLR response was not reflected in the mito-

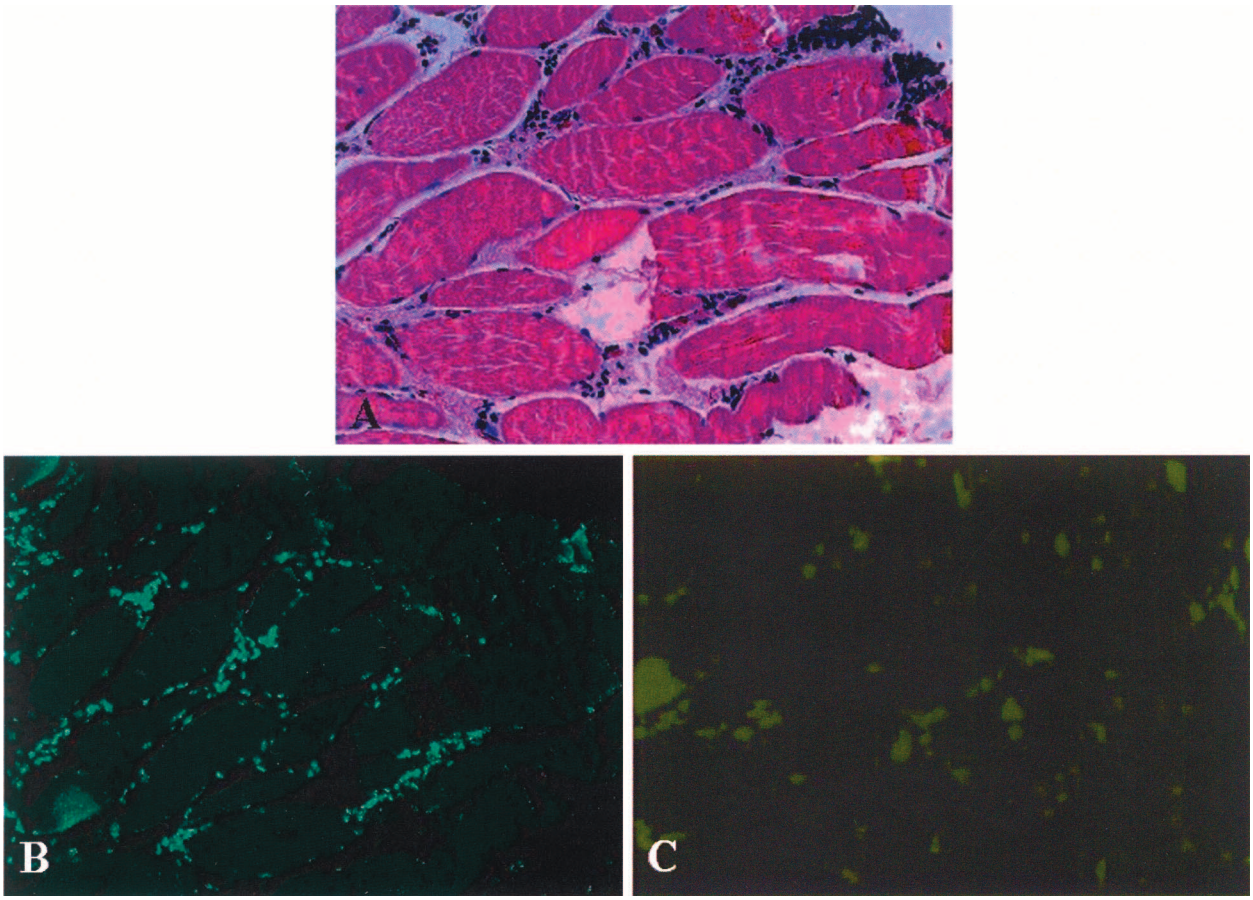

Figure 2. Presence of DiO-labeled MSCs in muscle. Histological evaluation of a muscle biopsy from a group 3 animal (#5500) shows cells between muscle bundles by H&E staining (A, original magnification 20×). A serial section viewed under the green channel demonstrates that the cells are DiO-labeled MSCs (B, original magnification 20×). The same section viewed under the red channel shows no DiI-labeled cells or autofluorescence (C, original magnification 20×). Similar results were found for two additional group 2 baboons (#6309 and #6908).

genic response to ConA, suggesting an allospecific effect.

T-cell hyporesponsiveness to donor alloantigens did not occur in all recipients. The individual responses for the time period bracketing the biopsy at 10 weeks (i.e., 8 and 12 weeks) are shown in Figure 4. If T-cell hyporesponsiveness is defined as a response that is less than 30% of the pretreatment response at either time point, four of six treated animals were hyporesponsive to donor alloantigens. None of the control animals (group 1) were hyporesponsive. Note that responses were similar or increased between weeks 8 and 12 in control and nonhyporesponsive animals, whereas the response decreased dramatically in the hyporesponsive animals.

Antibody Production to Donor Alloantigens

Sera from all nine baboons were evaluated for the presence of antibodies binding to either donor PBMCs or MSCs. None of the animals in the study exhibited a preexisting alloantibody titer (≥1:10) to donor PBMCs or MSCs (data not shown). With the exception of one serum sample that exhibited some binding to PBMCs at 8 weeks, group 1 vehicle control sera were negative for binding to either cell population, as expected (Table 2).

Group 2 (AA) baboons developed variable alloantibody titers to PBMC targets (Table 2). Titers ranged from 1:10 to greater than 1:1000, reached peak levels prior to or the second MSC injection, and were present at single or multiple time points. None of the group 2 animals exhibited a demonstrable alloantibody titer (≥1:10) to MSC target cells.

Group 3 (AB) animals produced alloantibody titers more consistently than group 2 baboons (Table 2). All three animals produced antibodies that bound to either donor "A" or donor "B" PBMCs. Most of the animals produced antibodies that bound to both donors. Recipient #6115 showed a definite boosting response with donor "B" cells, likely to cross-reacting antigens found on

both "A" and "B" cells. Also supporting the notion of cross-reactivity, administration with "A" induced a higher titer to "B" than "A" from 3 to 6 weeks in two out of three animals, prior to injection with "B" cells. When MSCs were used as targets, low alloantibody titers were exhibited by two out of three animals, and in one of these animals the titer was present at only one time point.

Other Immunological Parameters

There were no significant treatment-related changes in hematology, IgM, and IgG immunoglobulin levels, or antinuclear antibodies between any of the groups, at any time point (data not shown). FACS analysis of PBMCs showed one minimal, but statistically significant, change in the form of higher percentage of $CD3^+CD4^+$ cells ($48.8 \pm 4.7\%$) and lower percentage of $CD3^+CD8^+$ ($34.0 \pm 4.8\%$) in group 3 baboons at 12 weeks compared to group 1 control animals ($35.9 \pm 6.6\%$ and $45.3 \pm 4.5\%$, respectively). The ratio of these subsets was maintained and the total number of lymphocytes was unchanged, compared to the vehicle control group. Conditions associated with immunoincompetence are usually associated with a decrease in the $CD3^+/CD4^+$ subsets, and a decrease in the $CD3^+CD4^+/CD3^+CD8^+$ ratio (16), unlike the results reported here. The percentage of $CD3^+$, $CD3^+/CD25^+$, $CD14^+$, $CD19^+$, and $CD56^+$ cells was similar between all groups at all time points.

DISCUSSION

This study was undertaken to determine the immunologic consequences and safety of administering multiple doses of allogeneic MSCs to normal, immunocompetent animals at clinically relevant doses. Routes of MSC administration, particularly the IM route, were chosen to maximize activation of the immune system. We found that, after injection of MSCs, alloreactive T-cell responses to donor alloantigens decreased in most recipients whereas alloantibody responses were induced. Multiple injections of MSCs did not affect the overall immune system, however, as reflected by broader immunologic parameters including T-cell responsiveness to mitogen, peripheral blood mononuclear cell phenotype, immunoglobulin levels, and generation of aberrant antibodies

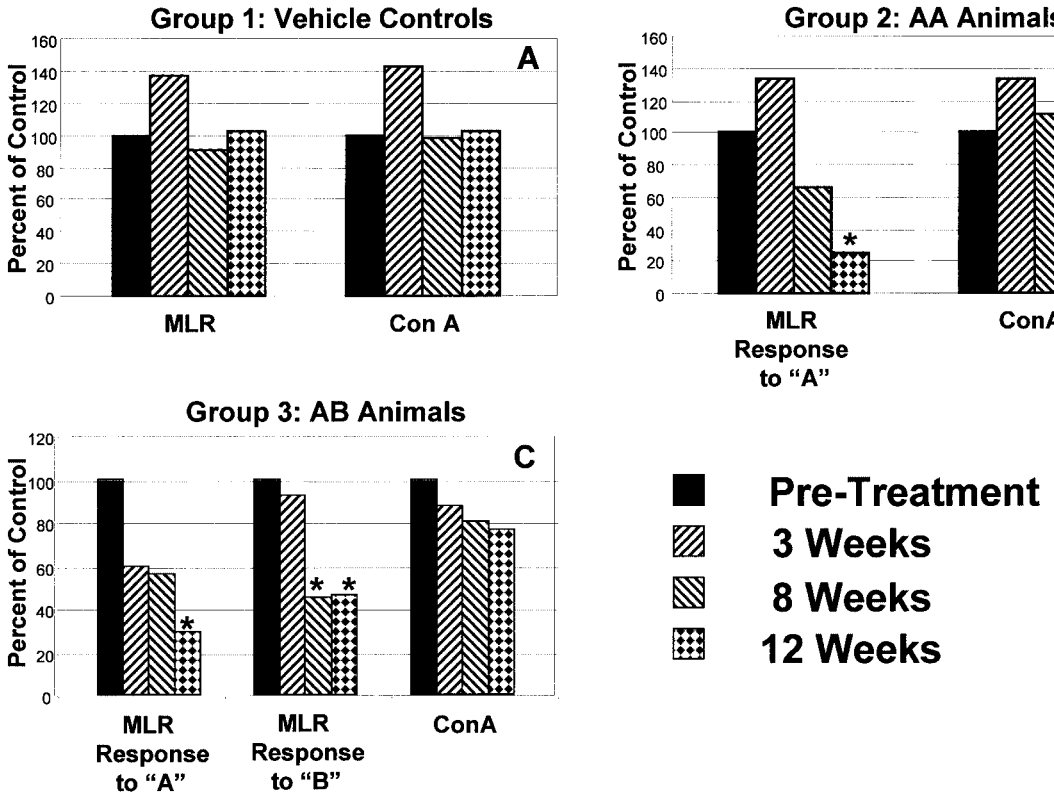

Figure 3. Multiple injections of MSCs induce T-cell hyporesponsiveness to donor alloantigens. Recipient baboon PBMCs were obtained prior to treatment with MSCs, and at 3, 8, and 12 weeks after treatment. PBMCs were cultured with irradiated donor PBMCs for one-way MLR assays or they were cultured with Con A to determine overall T-cell reactivity. Data are expressed as percent of pretreatment values, averaged from three animals per group, for group 1 (A), group 2 (B), and group 3 (C). Bars labeled with an asterisk represent significantly lower mean responses compared to pretreatment values ($p < 0.05$).

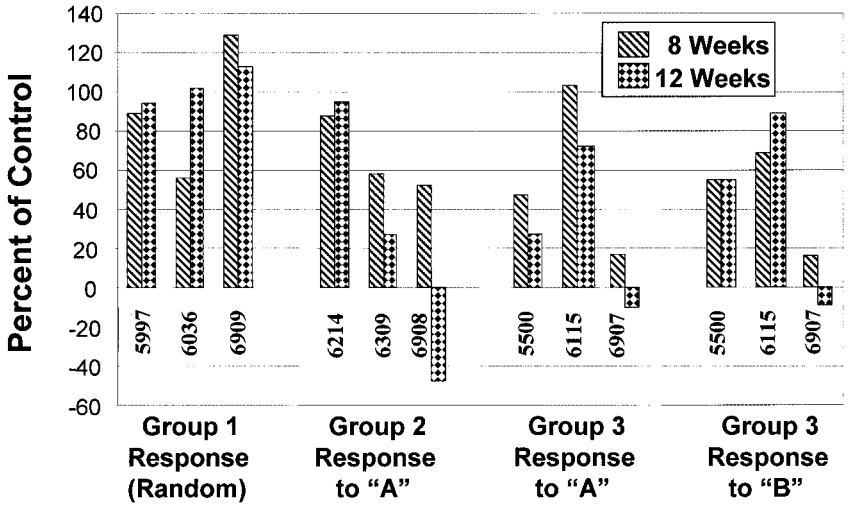

Figure 4. Individual MLR responses to donor alloantigens. Recipient baboon responses are shown for 8 and 12 weeks after MSC treatment (see Fig. 3 legend). Data are expressed as percent of pretreatment values, averaged from triplicate wells. Negative values are due to the response being higher to autologous PBMCs than to allogeneic PBMCs. Individual pretreatment responses (allogeneic CPM response minus autologous CPM response), which represent 100% of the control response, are as follows (results expressed as mean ΔCMP ± SD): group 1—#5997: 21,051 ± 7592, #6036: 69,696 ± 17,128, #6909: 75,393 ± 15,398; group 2—#6214: 15,300 ± 5570, #6309: 33,056 ± 6088, #6908: 41,022 ± 3369; group 3 versus A—#5500: 18,929 ± 650, #6115: 21,537 ± 6612, #6907: 125,441 ± 22,071; group 3 versus B—#5500: 14,092 ± 2705, #6115: 12,489 ± 2072, #6907: 124,291 (no SD, 1 well).

(antinuclear antibodies). The more specific effects of MSCs on cellular and humoral alloreactivity did not cause any apparent health problems throughout the 6-month study period, indicating that the treatment regimen used in this study was safe. MSCs were found in half of the treated recipients at 1 month postinjection, which is considerably longer than the 1–2-week period expected for rejection of differentiated mesenchymal lineage cells (4,25). All muscle samples taken at necropsy at 6 months were negative for donor MSCs.

Using the MLR assay as an indicator of T-cell alloreactivity, we did not observe enhanced recipient T-cell responses to donor alloantigens in any of the MSC-treated animals that would suggest priming; in fact, most recipients demonstrated a progressive loss of alloreactivity with time after treatment while maintaining an overall normal T-cell response to Con A. The suppressed response to donor cells suggests that the recipients were potentially tolerized by treatment with MSCs, but this remains to be further elucidated as the specificity of the hyporesponsive state was not determined by analyzing responses to third-party cells. The results showing that T-cell hyporesponsiveness developed after administration of MSCs was not expected, as previous in vitro studies have shown that MSCs did not tolerize naive T cells (11,18), and even primed them for secondary responses (18). However, several recent studies have indicated that MSCs can induce T-cell hyporesponsiveness in vivo. In one study, tolerance developed as a consequence of MSC engraftment and the development of mixed chimerism in lethally irradiated mice reconstituted with syngeneic bone marrow (8). In a second study, T-cell anergy developed in mice with experimental autoimmune encephalomyelitis that were treated with MSCs (33). Unresponsiveness to restimulation in vitro was reversed by the addition of IL-2 to the cultures. The discrepancy between in vitro and in vivo results may be attributed to the complexity of whole animal and lymphoid organ systems as well as long-term consequences of donor cell engraftment. Our results support the existing data that MSCs can induce T-cell hyporesponsiveness in vivo.

Alloantibodies were produced by baboons injected with MSCs. Although the role of alloantibodies has been well established in solid organ allograft rejection [reviewed in (26)], the role of alloantibodies in stem cell rejection is only starting to be investigated (3). Antibodies can destroy a graft by direct lysis involving the complement system or indirectly via antibody-dependent, cell-mediated cytotoxicity (ADCC) mechanisms. Preexisting antibody can mediate hyperacute and accelerated/acute rejection, which was not the situation in our animals because they did not have alloantibodies reactive to donor strain antigens in their pretreatment sera but

developed them after immunization. This response may be species related as rats injected intravenously with allogeneic MSCs did not develop alloantibodies when evaluated 4 weeks after injection (22).

In our study, most of the baboons produced alloantibodies in response to injection with allogeneic MSCs. The single exception was baboon #6907 (group 3), which did not produce any antibody to the immunizing alloantigen from donor "A." In most animals, there was not a boosting effect on antibody titer after the second injection of MSCs (i.e., antibody titers remained consistent). Interestingly, all three animals in group 3 had similar or higher titers to donor "B" PBMCs than to donor "A" PBMCs, even prior to being injected with donor "B" cells. This is probably due to a higher level of expression of MHC molecules on donor "B" PBMCs, which were used for the second injection in all three animals (donor #14485, see Table 1). Antibody titers were always higher against PBMC targets than MSC targets, likely due to higher expression of MHC molecules

Table 2. Alloantibody Response to Donor Cells

	Baboon #5997		Baboon #5036		Baboon #6909	
Group 1 (Random)	PBMCs	MSCs	PBMCs	MSCs	PBMCs	MSCs
3 weeks	<1:10	<1:10	<1:10	<1:10	<1:10	<1:10
6 weeks	<1:10	<1:10	<1:10	<1:10	<1:10	<1:10
8 weeks	<1:10	<1:10	**1:10**	<1:10	<1:10	<1:10
12 weeks	<1:10	<1:10	<1:10	<1:10	<1:10	<1:10
16 weeks	<1:10	<1:10	<1:10	<1:10	<1:10	<1:10
	Baboon #6214		Baboon #6309		Baboon #6908	
Group 2 (Donor 11A")	PBMCs	MSCs	PBMCs	MSCs	PBMCs	MSCs
3 weeks	**≥1:1000**	<1:10	**1:10**	<1:10	<1:10	<1:10
6 weeks	**1:10**	<1:10	**1:10**	<1:10	<1:10	<1:10
8 weeks	**1:100**	<1:10	<1:10	<1:10	**≥1:1000**	<1:10
12 weeks	<1:10	<1:10	<1:10	<1:10	<1:10	<1:10
16 weeks	**1:10**	<1:10	<1:10	<1:10	<1:10	<1:10
	Baboon #5500		Baboon #6115		Baboon #6907	
Group 3 (Donor "A")	PBMCs	MSCs	PBMCs	MSCs	PBMCs	MSCs
3 weeks	**1:10**	<1:10	**1:100**	<1:10	<1:10	<1:10
6 weeks	**1:10**	<1:10	<1:10	<1:10	<1:10	<1:10
8 weeks	**1:10**	<1:10	**≥1:1000**	**1:10**	<1:10	<1:10
12 weeks	<1:10	<1:10	**≥1:1000**	**1:10**	<1:10	<1:10
16 weeks	<1:10	<1:10	**≥1:1000**	<1:10	<1:10	<1:10
	Baboon #5500		Baboon #6115		Baboon #6907	
Group 3 (Donor "B")	PBMCs	MSCs	PBMCs	MSCs	PBMCs	MSCs
3 weeks	**1:100**	<1:10	**1:100**	<1:10	**1:10**	<1:10
6 weeks	**1:100**	<1:10	<1:10	<1:10	**1:100**	<1:10
8 weeks	**1:100**	**1:10**	**≥1:1000**	**1:10**	**1:10**	<1:10
12 weeks	<1:10	<1:10	**≥1:1000**	<1:10	**1:10**	<1:10
16 weeks	**1:100**	<1:10	**≥1:1000**	<1:10	**1:10**	<1:10

Alloantibody binding titers were determined from blood samples obtained from recipient baboons at the times indicated post-MSC treatment. PBMCs and MSCs were used as target cells in the assay. Group 1 sera were evaluated using target cells from random animals, group 2 sera were tested against cells from donor "A," and group 3 sera were evaluated against both donors "A" and "B" because these animals were injected with MSCs derived from both donors.

Table 3. Individual Responses of Baboons to Allogeneic MSC Transplantation

Group	Baboon No.	MSC in Biopsy	T-Cell Hyporesp. State		Alloantibody Titers			
					PBMC		MSC	
			8 Weeks	12 Weeks	8 Weeks	12 Weeks	8 Weeks	12 Weeks
2 (AA)	6214	Neg	Neg	Neg	1:100	Neg	Neg	Neg
	6309	Pos	Inter	Inter	Neg	Neg	Neg	Neg
	6908	Pos	Inter	High	1:1000	Neg	Neg	Neg
3 (AB)	5500	Pos	Inter	Inter	1:100	Neg	1:10	Neg
	6115	Neg	Neg	Neg	1:1000	1:1000	1:10	Neg
	6907	Neg	High	High	1:10	1:10	Neg	Neg

MSC biopsy positivity was determined on week 10 of study by confocal microscopy to DiO-labeled MSCs. T-cell hyporesponsive state on weeks 8 and 12 was arbitrarily rated according to the following scale: Negative (Neg, >75% of control), Intermediate (Inter, 25–75% of control), and High (<25% of control). Alloantibody titers are shown to PBMC and MSC target cells (donor "A" for group 2 and donor "B" for group 3).

on the PBMC population. Alternatively, it is possible that contaminating PBMCs present in the MSC population were responsible for inducing PBMC-specific antibodies.

Does tolerance or alloantibody response account for the success or failure of survival of the injected MSCs? A summary of data for the most relevant time points bracketing the muscle biopsy for individual baboons is shown in Table 3. In all three animals in which cell survival was demonstrated, T-cell hyporesponsiveness to donor alloantigens was induced, whereas in two out of three animals in which MSCs were not detected in the biopsy specimens, hyporesponsiveness was not induced. In the single animal in which MSCs were not detected but hyporesponsiveness was induced (group 3 baboon #6907), biopsy sampling error may have resulted in missing the intramuscular depot of cells as only one in four biopsies taken from positive baboons showed evidence of donor MSCs. Overall, our results suggest a relationship between hyporesponsiveness and cell survival.

The relationship between MSC survival and alloantibody production is more tenuous. Two of three animals with MSC-positive biopsies had sera at 8 weeks that contained alloantibodies that bound to PBMCs; one of these sera contained antibodies that bound to donor MSCs (Table 2). We speculate that the antibodies may not have inflicted damage to the MSCs due to low titer (1:10) or to antibody-mediated enhancement of graft survival (6).

In summary, IV infusion of high, clinically relevant doses of mismatched, allogeneic MSCs followed by IM injection of MSCs from the same mismatched donor or a third mismatched donor did not result in any changes in the overall health or immune status of the recipient animals. Transplanted allogeneic MSCs were detected for at least 1 month in muscle biopsies of half of these normal recipient animals. It seems likely that the immunomodulatory properties of MSCs, such as the previously reported ability of MSCs to suppress alloreactive T cells in vitro and in vivo, and the newly described ability of these cells to induce T-cell hyporesponsiveness to donor alloantigens in vivo, contributed to the maintenance of the allogeneic MSCs. Our failure to demonstrate MSCs in the muscle biopsies at extended time points could be due to cell migration, loss of fluorescent label, chronic rejection response by cells or antibodies, or lack of engraftment into a normal, noninjured environment. The baboon model used in our study was not one of functional tissue repair; it is possible that in an injury model, MSCs may be recruited to the site of injury and persist for longer periods of time in this environment. Clinically, these results are encouraging for a safe "universal cell" approach in which MSCs expanded from a single donor could be used in a variety of patients for tissue repair, particularly in acute settings when autologous isolation and expansion is not feasible.

ACKNOWLEDGMENTS: The authors would like to thank Dr. Marla McIntosh for performing statistical analyses on the MLR data. We are indebted to Mark Pittenger for review of the manuscript.

REFERENCES

1. Aggarwal, S.; Pittenger, M. F. Human mesenchymal stem cells modulate allogeneic immune cell responses. Blood 105:1815–1822; 2005.
2. Bartholomew, A.; Sturgeon, C.; Siatskas, M.; Ferrer, K.; McIntosh, K. R.; Patil, S.; Hardy, W.; Devine, S.; Ucker, D.; Deans, R.; Moseley, A.; Hoffman, R. Mesenchymal stem cells suppress lymphocyte proliferation in vitro and prolong skin graft survival in vivo. Exp. Hematol. 30:42–48; 2002.

3. Bradley, J. A.; Bolton, E. M.; Pedersen, R. A. Stem cell medicine encounters the immune system. Nat. Rev. Immunol. 2:859–871; 2002.
4. Camirand, G.; Caron, N.; Asselin, I.; Tremblay, J. P. Combined immunosuppression of mycophenolate mofetil and FK506 for myoblast transplantation in mdx mice. Transplantation 72:38–44; 2001.
5. Caplan, F Mesenchymal stem cells. J. Orthop. Res. 9: 641–650; 1991.
6. Carpenter, C. B.; d'Apice, A. J. F.; Abbas, A. K. The role of antibodies in the rejection and enhancement of organ allografts. Adv. Immunol. 22:1–65; 1976.
7. Deans, R. J.; Moseley, A. B. Mesenchymal stem cells: Biology and potential clinical uses. Exp. Hematol. 28: 875–884; 2000.
8. Deng, W.; Han, Q.; Liao, L.; Li, C.; Ge, W.; Zhao, Z.; You, S.; Deng, H.; Zhao, C. H. Allogeneic bone marrow-derived flk-1+Sca-1- mesenchymal stem cells leads to stable mixed chimerism and donor-specific tolerance. Exp. Hematol. 32:861–867; 2004.
9. Devine, S. M.; Peter, S.; Martin, B. J.; Barry, F.; McIntosh, K. R. Mesenchymal stem cells: Stealth and suppression. Cancer J. 7:S76–82; 2001.
10. Devine, S. M.; Cobbs, C.; Jennings, M.; Bartholomew, A.; Hoffman, R. Mesenchymal stem cells distribute to a wide range of tissues following systemic infusion into nonhuman primates. Blood 101:2999–3001; 2003.
11. Di Nicola, M.; Carlo-Stella, C.; Magni, M.; Milanesi, M.; Longoni, P. D.; Matteucci, P.; Grisanti, S.; Gianni, A. M. Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. Blood 99:3838–3843; 2002.
12. Djouad, F.; Plence, P.; Bony, C.; Tropel, P.; Apparailly, F.; Sany, J.; Noël, D.; Jorgensen, C. Immunosuppressive effect of mesenchymal stem cells favors tumor growth in allogeneic animals. Blood 102:3837–3844; 2003.
13. Friedenstein, A. J. Precursor cells of mechanocytes. Int. Rev. Cytol. 47:327–359; 1976.
14. Glennie, S.; Soeiro, I.; Dyson, P. J.; Lam, E. W.; Dazzi, F. Bone marrow mesenchymal stem cells induce division arrest anergy of activated T cells. Blood 105:2821–2827; 2005.
15. Haynesworth, S. E.; Baber, M. A.; Caplan, A. I. Cell surface antigens on human bone marrow-derived mesenchymal stem cells are detected by monoclonal antibodies. Bone 13:69–80; 1992.
16. Huston, D. P. The biology of the immune system. JAMA 278:1804–1814; 1997.
17. Kawai, T.; Poncelet, A.; Sachs, D. H.; Mauiyyedi, S.; Boskovic, S.; Wee, S. L.; Ko, D. S.; Bartholomew, A.; Kimikawa, M.; Hong, H. Z.; Abrahamian, G.; Colvin, R. B.; Cosim,i A. B. Long-term outcome and alloantibody production in a non-myeloablative regimen for induction of renal allograft tolerance. Transplantation 68:1767–1775; 1999.
18. Klyushnenkova, E.; Mosca, J. D.; Zernetkina, V.; Majumdar, M. K.; Beggs, K. J.; Simonetti, D. W.; Deans, R. J.; McIntosh, K. R. T cell responses to allogeneic human mesenchymal stem cells: Immunogenicity, tolerance, and suppression. J. Biomed. Sci. 12:47–57; 2005.
19. Lazarus, H. M.; Koc, O. N. Culture-expanded human marrow-derived MSCs in clinical hematopoietic stem cell transplantation. Graft 3:329–333; 2000.
20. Le Blanc, K.; Tammik, L.; Sundberg, B.; Haynesworth, S. E.; Ringden, O. Mesenchymal stem cells inhibit and stimulate mixed lymphocyte cultures and mitogenic responses independently of the major histocompatibility complex. Scand. J. Immunol. 57:11–20; 2003.
21. Le Blanc, K.; Rasmusson, I.; Sundberg, B.; Götherström, C.; Hassan, M.; Uzunel, M.; Ringdén, O. Treatment of severe acute graft- versus-host disease with third party haploidentical mesenchymal stem cells. Lancet 363:1439–1441; 2004.
22. Li, Y.; McIntosh, K.; Chen, J.; Zhang, C.; Gao, Q.; Borneman, J.; Raginski, K.; Mitchell, J.; Shen, L.; Zhang, J.; Lu, D.; Chopp, M. Allogeneic bone marrow stromal cells promote glial-axonal remodeling without immunologic sensitization after stroke in rats. Exp. Neurol. 198:313–325; 2006.
23. Liechty, K. W.; MacKenzie, T. C.; Shaaban, A. F.; Radu, A.; Moseley, A. M.; Deans, R.; Marshak, D. R.; Flake, A. W. Human mesenchymal stem cells engraft and demonstrate site-specific differentiation after in utero transplantation in sheep. Nat. Med. 6:1282–1286; 2000.
24. Majumdar, M. K.; Keane-Moore, M.; Buyaner, D.; Hardy, W. B.; Moorman, M. A.; McIntosh, K. R.; Mosca, J. D. Characterization and functionality of cell surface molecules on human mesenchymal stem cells. J. Biomed. Sci. 10:228–241; 2003.
25. Malejczyk, J.; Osiecka, A.; Hyc, A.; Moskalewski, S. Effect of immunosuppression on rejection of cartilage formed by transplanted allogeneic rib chondrocytes in mice. Clin. Orthop. 269:266–273; 1991.
26. Mason, D. W.; Morris, P. J. Effector mechanisms in allograft rejection. Annu. Rev. Immunol. 4:119–145; 1986.
27. McIntosh, K.; Bartholomew, A. Stromal cell modulation of the immune system. A potential role for mesenchymal stem cells. Graft 3:324–328; 2000.
28. Meisel, R.; Zibert, A.; Laryea, M.; Göbel, U.; Däubener, W.; Dilloo, D. Human bone marrow stromal cells inhibit allogeneic T-cell responses by indoleamine 2,3-dioxygenase-mediated tryptophan degradation. Blood 103:4619–4621; 2004.
29. Owen, M. E. Marrow stromal cells. J. Cell Sci. 10:63–76; 1988.
30. Pittenger, M. F.; Mackay, A. M.; Beck, S. C.; Jaiswal, R. K.; Douglas, R.; Mosca, J. D.; Moorman, M. A.; Simonetti, D. W.; Craig, S.; Marshak, D. R. Multilineage potential of adult human mesenchymal stem cells. Science 284:143–147; 1999.
31. Saito, T.; Kuang, J-Q.; Bittira, B.; Al-Khaldi, A.; Chiu, R. C-J. Xenotransplant cardiac chimera: immune tolerance of adult stem cells. Ann. Thorac. Surg. 74:19–24; 2002.
32. Tse, W. T.; Pendleton, J. D.; Bever, W. M.; Egalka, M. C.; Guinan, E. C. Suppression of allogeneic T-cell proliferation by human marrow stromal cells: Implications in transplantation. Transplantation 75:389–397; 2003.
33. Zappia, E.; Casazza, S.; Pedemonte, E.; Benvenuto, F.; Bonanni, I.; Gerdoni, E.; Giunti, D.; Ceravolo, A.; Cazzanti, F.; Frassoni, F.; Mancardi, G.; Uccelli, A. Mesenchymal stem cells ameliorate experimental autoimmune encephalomyelitis inducing T cell anergy. Blood 106: 1755–1761; 2005.