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T cell responses to allogeneic human mesenchymal stem cells: immunogenicity, tolerance, and suppression

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Summary

Human mesenchymal stem cells (MSCs) were evaluated for their ability to activate allogeneic T cells in cell mixing experiments. Phenotypic characterization of MSCs by flow cytometry showed expression of MHC Class I alloantigens, but minimal expression of Class II alloantigens and costimulatory molecules, including CD80 (B7-1), CD86 (B7-2), and CD40. T cells purified from peripheral blood mononuclear cells (PBMCs) did not proliferate to allogeneic MSCs. Lack of response was not due to a deficiency of costimulation, since retroviral transduction of MSCs with either B7-1 or B7-2 costimulatory molecules did not result in lymphoproliferation. Although these results suggested that MSCs were immunologically inert or potentially tolerogenic, T cells cultured with MSCs produced IFN-γ and displayed secondary kinetics to restimulation with PBMCs, indicating alloantigen priming rather than tolerance induction by the MSCs. To determine whether MSCs suppressed alloreactive T cells, MSCs were added to primary mixed lymphocyte reaction (MLR) cultures. MSCs suppressed cell proliferation when added at the initiation of culture or when added to an ongoing MLR culture. Suppression was dose-dependent, genetically unrestricted, and occurred whether or not MSCs were pretreated with IFN-γ. MSCs in transwell chambers suppressed primary MLR cultures, indicating that suppression was mediated by soluble molecules. Analysis of cytokines in suppressed MLR cultures demonstrated up-regulation of IFN-γ and IL-10, and down-regulation of TNF-α production relative to control cultures. We conclude that MSCs can initiate activation of alloreactive T cells, but do not elicit T cell proliferative responses due to active suppressive mechanisms.

Introduction

Human mesenchymal stem cells (MSCs) have the capacity to differentiate into a variety of tissues including bone, cartilage, stroma, fat, muscle, and tendon [1–3]. Although these cells exist at very low frequency in bone marrow, they can be isolated by simple procedures and greatly expanded without losing the ability to differentiate into multiple lineages. Thus, these cells have clinical potential for repair or replacement of damaged tissues [4]. The clinical application of MSCs for tissue regeneration could be most readily achieved with an allogeneic product; that is, a large number of cells derived from a single 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.

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Although the requirement for host conditioning for allogeneic hematopoietic stem cell transplantation has been understood for many years, the parameters for successful transplantation of non-hematopoietic stem cells are currently unknown. Generally, cells that express MHC molecules can stimulate T cells directly if they possess appropriate costimulatory molecules such as B7-1 or B7-2 [5], or they can activate T cells through the indirect pathway, by cross presentation of their MHC antigens by professional antigen presenting cells (APCs). Either way, the cells will be rejected by the immune system unless suppressive or tolerogenic mechanisms are employed.

The purpose of the current study was to investigate immunological properties of human MSCs to assess their suitability for allogeneic transplantation. We assessed MSCs for immunologically relevant cell surface molecules, determined whether they could function as stimulatory cells in one-way mixed lymphocyte reaction (MLR) assays, evaluated their ability to suppress the MLR response, and determined whether MSCs tolerized T cells in primary cultures. Our results demonstrate that MSCs do not induce T cell proliferation. The latter finding can be attributed to suppressive mechanisms, rather than lack of immunogenicity or induction of tolerance.

Materials and methods

Isolation, expansion, differentiation, and transduction of human MSCs

Bone marrow samples were collected from healthy human adult donors under an Institutional Review Board-approved protocol. Human MSCs were isolated and culture-expanded according to previously reported methods [3, 6]. The cells were routinely subcultured every 7–10 days. By the second passage, MSCs appeared as a homogenous population, by morphological and FACS criteria. The expanded population was uniformly positive for SH2, SH3, CD29, CD44, CD71, CD90, CD106, CD120a, and CD124 and uniformly negative for hematopoietic markers CD34, CD14, and CD45 [3, 4, 6]. MSCs were used from passage 3 to 5 for experimentation. To differentiate MSCS along the osteogenic pathway, cells were cultured in medium supplemented with 100 nM dexamethasone, 0.05 mM L-ascorbic acid-2 phosphate, and 10 mM β-glycerophosphate [7]. Human B7-1 and B7-2 cDNAs were amplified from human bone marrow and cloned into a retroviral vector containing the neomycin phosphotransferase gene. Centrifugal procedures were used to optimize transduction of MSCs with amphotropic retrovirus [8]. Transduced MSCs were selected for 2 weeks with G418 (0.5 mg/ml), pooled, expanded, and passaged at confluency.

Preparation of peripheral blood mononuclear cells and T cells

Leukopheresis samples were collected from healthy human donors by voluntary consent under an Institutional Review Board-approved protocol or purchased from the American Red Cross (Rockville, MD). Peripheral blood mononuclear cells (PBMCs) were prepared from the samples by density gradient centrifugation over Ficoll-Hypaque according to the manufacturer's instructions (Amersham-Pharmacia Biotech, Piscataway, NJ). PBMCs were further fractionated into T cell enriched populations by negative depletion of B cells and monocytes accomplished by magnetic sorting. Briefly, PBMCs (100×10^6 cells/ml) were labeled with antibodies (1.0 $\mu g/10^6$ cells) directed against MHC Class II, CD14, and CD19 followed by incubation with biotinylated rat anti-mouse IgG (all antibodies from Pharmingen, San Diego, CA). The antibody-labeled cells were removed by tagging them with magnetic streptavidin beads and running the cells through a magnetic field (MACS system, Miltenyi Biotec, Auburn, CA). The enriched T cell population contained at least 85% CD3 positive cells as determined by flow cytometry.

Mixed lymphocyte reaction (MLR) cultures

The one-way MLR was used to assess T cell reactivity against allogeneic cell populations. For cultures in flat-bottom microtiter plates (96 well culture clusters with low evaporation lids, Corning, Inc., Corning, NY), purified human T cells (10^5/well) or unfractionated PBMCs (2×10^5/well) were added to inactivated allogeneic PBMCs (2×10^5/well) or MSCs (2×10^4/well). The difference between stimulator cell numbers reflects the large size of MSCs – these cells are approximately 30 μm in diameter and

2×10^4 MSCs are confluent in a microtiter well. Inactivated PBMCs were prepared by treating the cells 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. For larger cultures in 6 well plates (T cell activation studies, cytokine analysis, tolerance experiments), purified T cells (2×10^6/well) were added to irradiated PBMCs (5×10^6/well) or MSCs (2×10^5/well). In some experiments, monocytes were enriched as stimulator cells by adhering PBMCs (same cell concentrations as above) to the wells (2×10^5/well or 5×10^6/well for microtiter or 6 well cluster plates, respectively) for a 24 h period and washing out the non-adherent cells. To fully express MHC molecules, MSCs were treated with IFN-γ (100 U/ml, Boehringer Ingelheim, San Diego, CA) in MSC medium for the 3 day period prior to addition of T cells. Cells treated with IFN-γ were always washed thoroughly (minimum of 5 washes) prior to co-culture with lymphocytes. MLR cultures were performed in complete culture medium: Iscove's modified Dulbecco's medium (IMDM, Life Technologies) containing 5% human AB serum (Sigma, St. Louis, MO), 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 5.5×10^{-5} M 2-mercaptoethanol, and 1% antibiotic/antimycotic (all culture reagents from Life Technologies, except as indicated). T cell proliferation to alloantigens 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). T cell proliferation is expressed as mean counts per minute of 3–4 wells ± standard deviation.

Transwell cultures

MSCs were plated into the lower chamber of 24 well transwell plates (Corning #3415, 3.0 μm pore size) at a confluent density of 10^5 MSCs/well. The MSCs were treated with IFN-γ for 3 days. After washing the cells thoroughly to remove IFN-γ, MLRs were set up in the upper chambers between purified T cells (2×10^5/well) and irradiated PBMCs (2×10^5/well). After 7 days of culture, cells from the upper chamber were transferred to microtiter wells in triplicate (100 μl/well), pulsed with ^{3}H-TdR, and harvested 18 h later. T cell proliferation is expressed as mean counts per minute of 3–4 wells ± standard deviation.

Fluorescence-activated cell sorting analysis

Analysis of cell surface molecules on MSCs was performed using a panel of fluorochrome-labeled monoclonal antibodies diluted according to the manufacturer's instructions (Pharmingen). Non-specific fluorescence was determined by substitution with appropriate isotype-matched irrelevant monoclonal antibodies. Data were analyzed by collecting 10,000 events on a Becton Dickinson Vantage instrument using Cell-Quest software.

Measurement of cytokines by ELISA

Supernatants from cultures were collected at intervals after antigen-stimulation and assayed in duplicate for the presence of cytokines using Predicta™ cytokine kits (Genzyme Diagnostics, Cambridge, MA). Manufacturer's protocol was followed for each kit. Optical density at 450 nm was read on a Bio-Rad, Model 3550 Microplate Reader (Richmond, CA).

Results

Immune phenotype of human MSCs

Human MSCs were evaluated for the expression of immunologically relevant cell surface molecules by flow cytometry. MSCs were positive for MHC Class I, weakly positive for MHC Class II, and negative for costimulatory molecules CD80 (B7-1), CD86 (B7-2), and CD40 (Figure 1). It was of interest to evaluate expression of these molecules in the presence of the pro-inflammatory cytokine, IFN-γ, since MSCs may be used clinically in sites of inflammation. When treated with IFN-γ for 3 days, MSCs up-regulated the expression of both MHC Class I and Class II molecules. Although CD40 expression was weakly positive after treatment with IFN-γ, CD80 and CD86 costimulatory molecules were not affected.

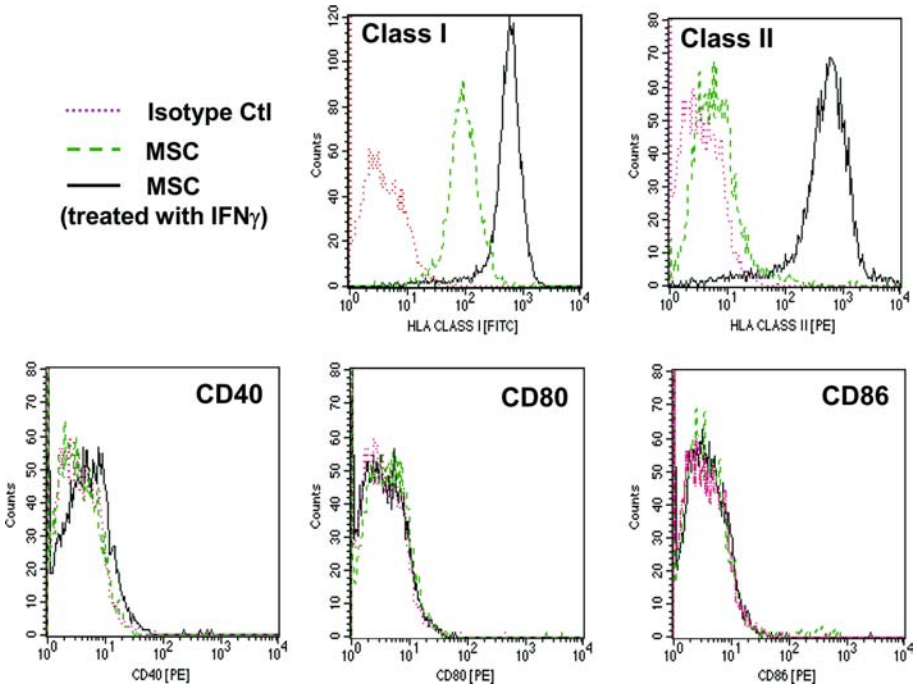

Figure 1. Human MSCs do not express costimulatory molecules. MSCs were cultured with or without human IFN-γ for 3 days, trypsinized, and stained with specific fluorochrome-labeled antibodies or isotype control antibodies for flow cytometry. Similar results were obtained from 3 additional donors.

T cell responses to allogeneic MSCs

To determine whether MSCs could elicit a proliferative response from allogeneic T cells, unfractionated PBMCs or purified T cells were co-cultured with MSCs in microtiter wells and pulsed with ^{3}H-TdR at intervals to determine lymphoproliferation (Figure 2). Unfractionated PBMCs responded vigorously to irradiated allogeneic PBMCs on day 6 (Panel a). The proliferative response to MSCs from the same donor was 50–60% lower and peaked on day 8. When purified T cells were used as responder cells in the mixed cell cultures, very high responses were generated to allogeneic PBMCs whereas negligible responses were observed to MSCs (Panel b). Pretreatment of MSCs with IFN-γ to up-regulate MHC molecules did not enhance immunogenicity. Nearly identical results were obtained using a different PBMC donor. We also analyzed expression of activation markers on CD3-positive T cells in co-cultures of T cells and allogeneic MSCs by flow cytometry. Few T cells expressed CD25 or CD134 (2–3%) in response to allogeneic MSCs, whereas the response to allogeneic PBMCs was considerably higher (15–19%, data not shown).

Since poor stimulatory function could be due to a deficiency of costimulatory molecules, MSCs were transduced with either human B7-1 or B7-2. As shown in Figure 3, Panel a, purified T cells responded vigorously to allogeneic PBMCs but they did not respond well to non-transduced MSCs, control-transduced MSCs (with the mouse antigen Lyb486), or to MSCs transduced with B7-1 or B7-2. The highest response was to B7-2 transduced MSCs, but even this response was less than 20% of the response to PBMCs at the peak of the response (6 days). The failure of transduced cells to stimulate T cells was not due to technical considerations, as both B7-1 and B7-2 were highly expressed on MSCs as assessed by flow cytometry (data not shown). To determine whether the response was dose-dependent, MSCs were titrated from 2×10^{4}/well (confluence) down to 625/well without greatly affecting the response (data not shown). Thus, the expression of B7 costimulatory molecules on MSCs did not endow them with the capacity to function efficiently as stimulator cells in the MLR.

To determine whether differentiation of MSCs resulted in the loss of 'immune privilege', MSCs were differentiated along the osteogenic (OS)

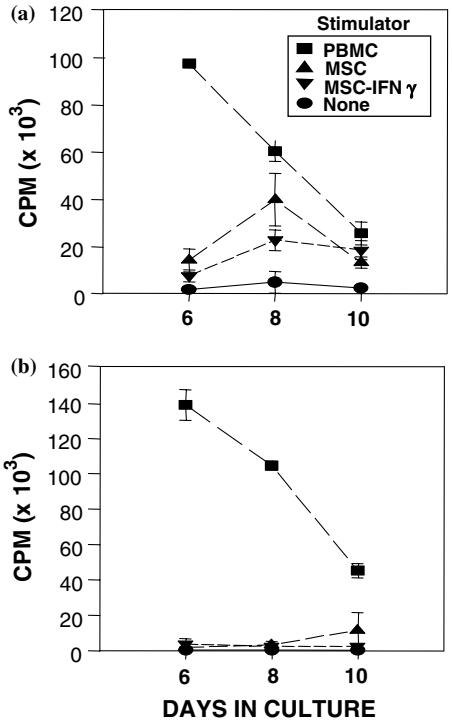

Figure 2. T cell proliferative responses to allogeneic MSCs. To determine proliferative responses to MSCs, unfractionated PBMCs (Panel a) or purified T cells (Panel b) were cultured in microtiter wells with allogeneic irradiated PBMCs, untreated MSCs, or MSCs that were cultured with IFN-γ for 3 days prior to co-culture with responder cells (see legend on Panel a). Cells were pulsed with ^{3}H-TdR for the final 18 h of the culture period to assess T cell proliferation.

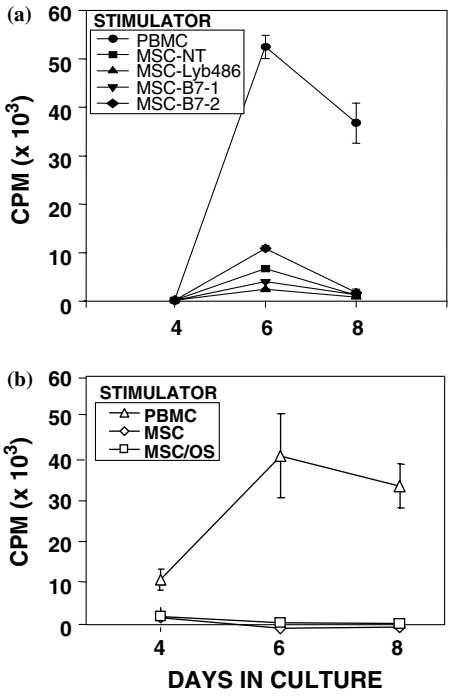

Figure 3. T cell proliferative responses to manipulated allogeneic MSCs. (a) Purified T cells were cultured with the adherent fraction of irradiated, allogeneic PBMCs or MSCs that were non-transduced (NT), transduced with a control murine gene (Lyb486), or transduced with human B7-1 or B7-2. MSCs were treated with IFN-γ for 3 days prior to culture with T cells. Cultures were pulsed with ^{3}H-TdR for 18 h prior to harvesting cells on the days shown. Results were typical of 3 additional experiments. (b) MSCs were differentiated along the osteogenic lineage *in vitro* for 16 days. OS-differentiated MSCs (MSC/OS) and non-differentiated MSCs were harvested by trypsinization and plated at 2×10^5 cells/well in 6-well plates. MSCs were treated with IFN-γ for 3 days before adding allogeneic T cells (1.5×10^6/well). Positive control cultures were set up using irradiated PBMCs as stimulator cells (5×10^6/well). Non-adherent T cells were collected from the cultures on days 3, 5, and 7, and distributed into triplicate microtiter wells. The cells were pulsed with ^{3}H-TdR for 18 h to determine proliferation. Similar results were obtained in 2 additional experiments.

lineage prior to co-culture with purified allogeneic T cells. Osteogenic differentiation was demonstrated by elevated alkaline phosphatase activity (60.5 nmol pNP/min/10^6 cells vs. 0.8 nmol pNP/min/10^6 cells for uninduced controls) and calcium deposition (40.3 μg/well vs. 2.9 μg/well for uninduced controls). Treatment of the OS-differentiated MSCs with IFN-γ resulted in up-regulation of MHC Class II molecules, similar to what was observed with non-OS treated MSCs (data not shown). Osteogenic differentiation of MSCs did not result in a T cell proliferative response whether or not the cells were treated with IFN-γ (Figure 3, Panel b).

Tolerance

MSCs have the potential to behave as tolerance-inducing APCs due to their expression of MHC molecules (particularly after treatment with IFN-γ) and deficiency of B7-1 and B7-2 costimulatory molecules. To determine whether MSCs induce tolerance, T cells were cultured with irradiated allogeneic PBMCs or MSCs for 7 days. As expected, T cells responded vigorously to allogeneic PBMCs whereas they did not respond to allogeneic MSCs (Figure 4, Panel a). The T cells were recovered, rested for 3 days in medium, and re-cultured with irradiated PBMCs from the original donor. Our results indicate that tolerance was not induced by MSCs since the T cell

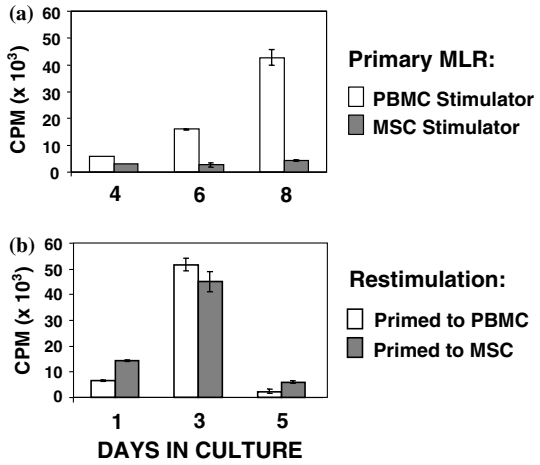

Figure 4. T cells cultured with allogeneic MSCs exhibit secondary kinetics upon restimulation with PBMCs. (a) Purified T cells were seeded in 6 well cluster plates with irradiated, allogeneic PBMCs or IFN-γ-treated MSCs for 7 days. On days 3, 5, and 7, the cultures were suspended and 200 μl aliquots of cells were transferred to microtiter plates and pulsed with ^{3}H-TdR for 18 h to assess T cell proliferation. (b) Non-adherent cells were recovered from the bulk cultures and rested in medium for 3 days. After rest, T cells were collected and cultured with irradiated PBMCs from the original stimulator cell donor in microtiter plates. Cultures were pulsed with ^{3}H-TdR for 18 h on days 0, 2, and 4 to determine the restimulation response.

restimulation response was nearly identical to that of T cells precultured with PBMCs (Figure 4, Panel b). The accelerated kinetics of the restimulation response (peak activation by 3 days instead of 7 days) indicated that T cells had been primed by exposure to either PBMCs or MSCs. These results have been repeated in 7 additional experiments using 4 different MSC donors.

Suppression

In tolerance experiments, T cells were unresponsive in mixed cell cultures containing MSCs but responsive in secondary cultures in the absence of MSCs. This suggested that MSCs may have suppressive activity. To test this possibility, MSCs were titrated into primary MLR cultures at the initiation of culture. MSCs were matched to responder cells (Figure 5, Panel a, top), stimulator cells (Figure 5, Panel a, bottom), or were used as third party cells to the MLR (Figure 5, Panel b). MSCs suppressed the primary MLR in dose-dependent fashion, regardless of MHC matching. Pre-treatment of the MSCs with IFN-γ for 3 days prior to co-culture with lymphocytes did not affect suppression at low and intermediate doses of MSCs (Panel a); IFN-γ treatment enhanced suppression at the highest dose of cells. Mean percent suppression by untreated MSCs at confluence (22,500 MSCs/well) from 2 donors in 6 different MLR combinations, regardless of matching, was 77 $\pm$ 7.0% for donor 1 and 79 $\pm$ 2.2% for donor 2. Mean percent suppression by IFN-γ-treated MSCs was 94 $\pm$ 2.0% for donor 1 and 88 $\pm$ 5.3% for donor 2.

MSCs from multiple donors were tested for their ability to suppress an ongoing primary MLR when added on the 4th day of a 7-day culture. MSCs from all donors suppressed the ongoing MLR (Figure 5, Panel c). One of the MSC donors (155) was unrelated to either donor of the responder or stimulator cells participating in the MLR indicating lack of MHC restriction in suppression of ongoing responses. Results obtained using MSCs from 5 different donors indicate that treatment with IFN-γ did not enhance suppression of multiple ongoing MLRs (mean suppression by untreated MSCs = 85.0 $\pm$ 12.7%, n = 7; mean suppression by IFN-γ-treated MSCs = 62.8 $\pm$ 33.9%, n = 16).

Experiments were performed in transwell chambers to determine whether MSCs could suppress an MLR response through a membrane that prevents cell–cell contact. As shown in Figure 5, Panel d, MSCs from two different donors suppressed T cell proliferation through the membrane, indicating production of a soluble suppressive molecule(s). Pre-treatment of the MSCs with IFN-γ for 3 days prior to co-culture with lymphocytes did not affect suppression. This experiment, repeated with 4 additional MSC donors, showed a mean suppression of 72 $\pm$ 24.1%. Mean suppression of ongoing cultures through a transwell membrane was 81 $\pm$ 3.2% (3 different MSC donors). In the latter experiments, T cells were cultured with allogeneic stimulator PBMCs for 4 days prior to adding the cells to the transwells.

Cytokine response

Secretion of cytokines IFN-γ, TNF-α, IL-2, IL-4, and IL-10 was evaluated during a primary MLR using allogeneic PBMCs or MSCs as stimulator cells, or during suppression of a primary MLR by MSCs. T cell responses to MSC stimulators resulted in elevated levels of IFN-γ and greatly

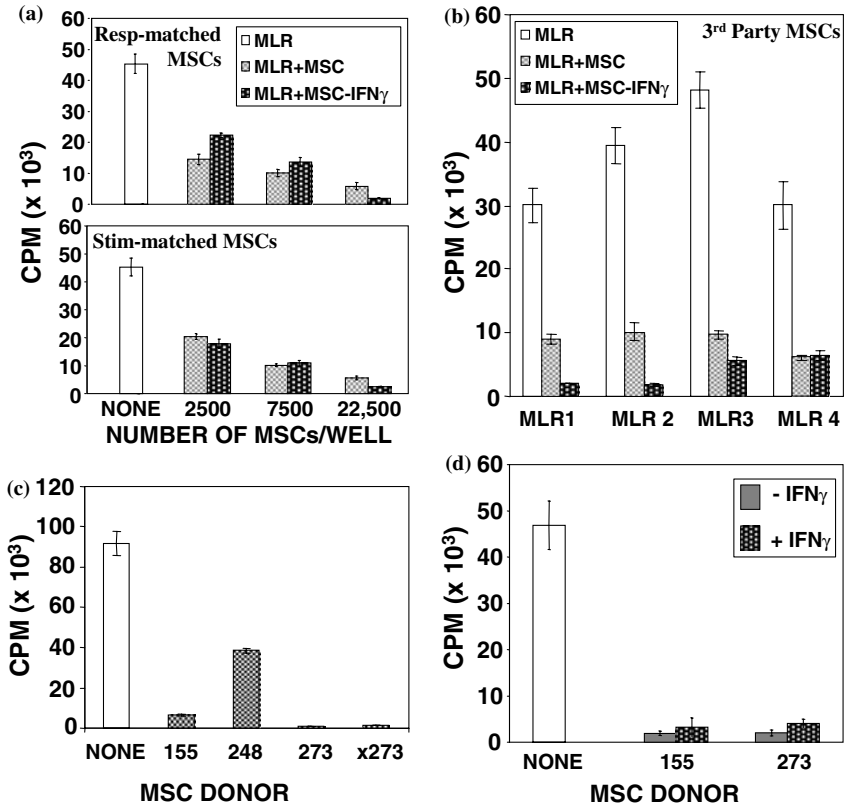

Figure 5. MSCs suppress primary MLR cultures. (a) MSCs were plated in microtiter wells with or without IFN-γ for 3 days at the numbers indicated. The wells were washed extensively to remove IFN-γ prior to addition of PBMCs for two-way MLRs (10^5 PBMCs from each donor/well). For experiments shown in the upper panel, responder PBMCs were matched to the MSC donor. In the lower panel, stimulator PBMCs were matched to the MSC donor. Lymphoproliferation was determined by pulsing the cultures with ^{3}H-TdR on day 6 and harvesting the cells 18 h later. (b) Experiments were performed as described in Panel a with the exception that MSCs were derived from a donor that was not matched to either the responder or stimulator cell donors. Third party MSCs were plated at a single density of 2×10^4 MSCs/well. (c) Purified T cells from donor 273 were cultured with irradiated PBMCs from donor 248 in microtiter wells. On the 4th day of culture, freshly trypsinized MSCs from the indicated donors were added to the ongoing cultures at 2×10^4 MSCs/well. The cultures were pulsed with ^{3}H-TdR on day 7 and harvested 18 h later for determination of T cell proliferation. (d) MSCs from donor 273 were plated into the lower chamber of transwell plates and treated with IFN-γ for 3 days. MLRs were set up in the upper chambers between purified T cells from donor 248 and irradiated PBMCs from donor 273. After 7 days of culture, cells from the upper chamber were transferred to microtiter wells in triplicate (100 μl/well), pulsed with ^{3}H-TdR, and harvested 18 h later.

reduced levels of TNF-α compared to PBMC stimulators (Figure 6). Low levels of IL-10 were produced to both populations. In MLR cultures suppressed by MSCs, high levels of IFN-γ and IL-10 were observed relative to control (non-suppressed) MLR cultures, whereas very low amounts of TNF-α were produced. IL-2 and IL-4 were undetectable under any culture conditions.

Discussion

Our data demonstrate that MSCs do not induce a proliferative response from alloreactive T cells. This was not due to a deficiency of alloantigen or costimulatory molecules, but to an active suppressive mechanism that interferes with T cell proliferation. Experiments designed to enhance the allogenicity of MSCs by replacing or up-regulating potentially deficient molecules failed. MSCs were pre-treated with IFN-γ to represent a 'worst case scenario' in which MSCs are implanted into sites of inflammation. This treatment, which up-regulated alloantigen expression, did not enhance the stimulatory capacity of the MSCs. Transduction with B7-1 or B7-2 did not appreciably enhance the ability of these cells to stimulate alloreactive T cell proliferation, indicating that the lack of response

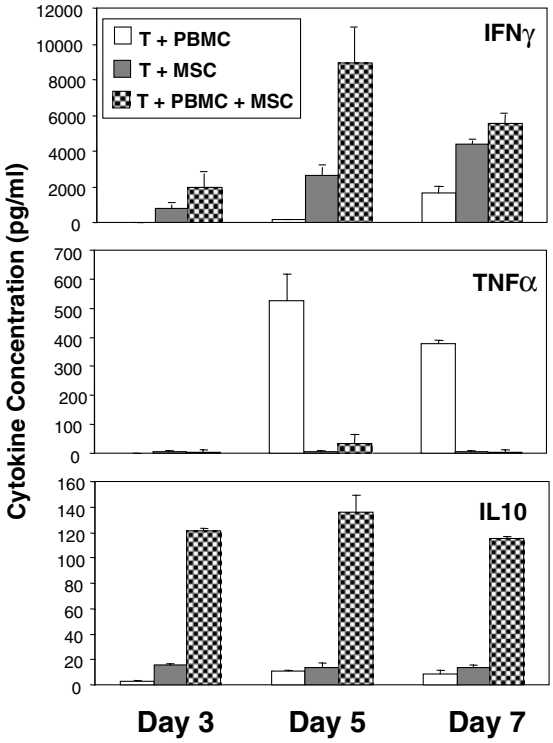

Figure 6. Cytokine production in co-cultures. T cells were cultured with irradiated allogeneic PBMCs and/or MSCs in 6 well cluster plates. Supernatants were collected at intervals (not more than 10% of total volume) and assessed for the indicated cytokines by ELISA. Cytokine production by T cells cultured alone, irradiated PBMCs cultured alone, or MSCs cultured alone was less than 5 pg/ml. Similar results were observed in at least 3 additional experiments.

was not due to a deficiency of costimulatory molecules. Supplementing the cultures with cytokines that have been shown to be important in APC function (IL-1α, IL-1β, and TNF-α) [9] was also ineffective in generating T cell proliferative responses to allogeneic MSCs (data not shown). We also attempted to block IFN-γ with neutralizing monoclonal antibodies since the levels of IFN-γ were high in co-cultures of T cells and allogeneic MSCs, and this cytokine has anti-proliferative effects [10]. Neutralizing antibodies did not enhance T cell proliferation to MSCs (data not shown). Finally, MSCs induced to differentiate down the osteogenic lineage did not induce T cell proliferation. This result was unexpected since differentiated osteoblasts have been reported to be immunogenic [11]. It is possible a sufficient number of MSCs remain as undifferentiated cells in these cultures to suppress T cell proliferation.

MSCs did not tolerize resting T cells. These results were unexpected since the phenotype of IFN-γ-treated MSCs suggested that these cells should be tolerogenic as they express MHC Class I and Class II alloantigens in the apparent absence of B7 costimulatory signals [5]. We found that MSCs primed alloreactive T cells to respond with secondary kinetics to restimulation with PBMCs. Thus, MSCs can function as APCs for T cell activation which suggests that they express co-stimulatory molecules. Interestingly, addition of CTLA-4/Fc to primary cultures of MSCs and allogeneic T cells prevented T cell priming in two experiments (data not shown). Whether MSCs express low, but functionally significant, amounts of B7 (below the level of detection by flow cytometry) or that CTLA-4/Fc prevented T cell costimulation between activated T cells [12] has not been determined. MSCs also express adhesion molecules such as ICAM-1/LFA-1 [4, 6] that have costimulatory properties [13] which could participate in T cell priming and interfere with the induction of tolerance.

Our experiments showing that T cells did not proliferate in the presence of MSCs, but responded to PBMCs with secondary kinetics after being removed from MSCs, strongly suggested that MSCs were immunosuppressive. Indeed, MSCs suppressed alloreactive proliferation whether the cells were added at the initiation of MLR cultures or during ongoing responses. Suppression of alloreactivity by MSCs has been reported for human [14–17] as well as baboon [18] and mouse MSCs [19]. Moreover, suppression of MLR cultures by non-stem cells of mesenchymal lineage has been reported previously for human gingival fibroblasts [20] and human corneal fibroblasts [21], although there are numerous reports of fibroblasts performing an accessory function in T cell responses as well [22–24]. MSCs did not need to be treated with exogenous IFN-γ in order to be suppressive unlike other mesenchymal cells that have been reported to express suppressive activity [20, 21]. The mechanism of suppression by MSCs involved a soluble molecule(s) as demonstrated by the ability of MSCs to suppress mixed lymphocyte cultures across a transwell membrane. Analysis of cytokines in the supernatants of suppressed mixed lymphocyte cultures showed moderate to high levels of most cytokines tested, relative to non-suppressed MLR cultures. Data were

presented for IFN-γ and IL-10, but similar increases were also found for IL1-β, TGF-β, IL-11, and IL-12 (preliminary experiments). The exception to this trend was TNF-α which was found in greatly reduced levels in suppressed cultures. We have failed to block suppression in the suppressed cultures by adding neutralizing antibodies specific for cytokines known to have antiproliferative activity, including IFN-γ [10], IL-10 [25], and TGF-β [26]. Attempts to block inhibitory molecules commonly produced by monocytes/macrophages such as prostaglandins (blocked with indomethacin), nitric oxide (blocked with L-leucine methyl ester), and indoleamine 2,3-dioxygenase [27] (blocked with 1-methyl-tryptophan) also failed to diminish suppression by MSCs. Finally, blockade of apoptosis pathways by TRAIL-Fc and Fas-Fc fusion proteins did not have an effect on suppression. Thus, we could not identify the soluble suppressor factor produced by MSCs. It has been reported that TGF-β and HGF secreted by MSCs function synergistically to suppress T cell proliferation [14].

Although the lack of a vigorous T cell response to allogeneic MSCs is a positive sign for survival of transplanted MSCs, it does not guarantee site-specific survival of these cells. For example, chondrocytes and myoblasts do not activate alloreactive T cells *in vitro* [28, 29] but these cell types are rapidly rejected after transplantation to immunocompetent recipients [30, 31]. Furthermore, our own studies showed that MSCs primed alloreactive T cells and they had a partial stimulatory effect on unfractionated PBMCs *in vitro*. In studies recently completed, allogeneic baboon MSCs (minimum 3/6 mismatch at HLA A, B, and DR) survived at least 16 weeks in immunocompetent recipients after transplant in hydroxyapatite matrices as shown by dye-tracking and PCR studies, as well as by the formation of bone in the implants [32]. In a separate study, sequential administration of large numbers of allogeneic baboon MSCs by intravenous and intramuscular injections did not induce systemic T cell alloreactivity, and the cells were shown to persist for at least 4 weeks at the site of the second intramuscular injection (manuscript in preparation). Long-term cell engraftment has been reported in a xenogeneic transplant model in which human MSCs were administered *in utero* to fetal lambs before or after development of the immune system [33, 34]. Human MSCs persisted for 13 months and exhibited site-specific differentiation into chondrocytes, adipocytes, myocytes, cardiomyocytes, and stroma [33]. Similarly, embryonic stem-like cells derived from rats have been shown to engraft in allogeneic recipients without host conditioning, resulting in mixed chimerism [35]. These results suggest that MSCs, as well as other types of stem cells, can be transplanted successfully across MHC barriers. Not all stem cells exhibit immune privilege, however, as purified hematopoietic stem cells express MHC class II antigens and are fully capable of presenting alloantigen to T cells and stimulating their proliferation in MLR assays [36].

Long-term persistence of allogeneic MSCs in immunocompetent recipients suggests that the immunoregulatory properties of MSCs may be operational *in vivo*. This is supported by preliminary results from ongoing clinical trials [37, 38] and a skin graft study in baboons [18]. In clinical trials, MSCs were evaluated for their ability to enhance engraftment of unselected HLA-matched peripheral blood or bone marrow stem cell transplants. The incidence of Grade 3-4 acute GVHD and chronic GVHD in treated patients was considerably lower than expected [37], suggesting that MSCs suppressed donor T cell alloreactivity against the recipient. Similarly, baboon MSCs, administered intravenously, delayed rejection of donor and third party skin grafts from 7 to 11 days, indicating that suppression was operational *in vivo* and non-specific. The immunoregulatory properties of MSCs may prove useful in treating diseases that are mediated by alloreactive T cells such as graft vs. host disease and transplant rejection [39]. Additional benefit may be derived if MSCs alter cytokine production in patients, resulting in decreased levels of TNF-α and less tissue damage. The ability of MSCs to regulate the immune system, coupled with their capacity for tissue regeneration, opens exciting possibilities for treating both the underlying cause of immune-mediated diseases and correcting damage resulting from these diseases.

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