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Enhancement of the immunoregulatory potency of mesenchymal stromal cells by treatment with immunosuppressive drugs

JOHN GIRDLESTONE[1,2,*], JEFFREY PIDO-LOPEZ[1,*], SAKET SRIVASTAVA[1,2], JIANGUO CHAI[3], NEIL LEAVER[4], ANTONIO GALLEU[5], GIOVANNA LOMBARDI[3] & CRISTINA V. NAVARRETE[1,2]

[1]*Histocompatibility and Immunogenetics Research Group, NHS Blood and Transplant, Colindale, London, United Kingdom,* [2]*Division of Infection & Immunity, University College, London, United Kingdom,* [3]*MRC Centre for Transplantation, King's College, London, United Kingdom,* [4]*UK National Monitoring Service for Sirolimus, Royal Brompton & Harefield NHS Foundation Trust, Harefield, United Kingdom, and* [5]*Regenerative and Haematological Medicine, Rayne Institute, King's College, London, United Kingdom*

Abstract

Background aims. Multipotent mesenchymal stromal cells (MSCs) are distinguished by their ability to differentiate into a number of stromal derivatives of interest for regenerative medicine, but they also have immunoregulatory properties that are being tested in a number of clinical settings. *Methods.* We show that brief incubations with rapamycin, everolimus, FK506 or cyclosporine A increase the immunosuppressive potency of MSCs and other cell types. *Results.* The treated MSCs are up to 5-fold more potent at inhibiting the induced proliferation of T lymphocytes *in vitro*. We show that this effect probably is due to adsorption of the drug by the MSCs during pre-treatment, with subsequent diffusion into co-cultures at concentrations sufficient to inhibit T-cell proliferation. MSCs contain measurable amounts of rapamycin after a 15-min exposure, and the potentiating effect is blocked by a neutralizing antibody to the drug. With the use of a pre-clinical model of acute graft-versus-host disease, we demonstrate that a low dose of rapamycin-treated but not untreated umbilical cord–derived MSCs significantly inhibit the onset of disease. *Conclusions.* The use of treated MSCs may achieve clinical end points not reached with untreated MSCs and allow for infusion of fewer cells to reduce costs and minimize potential side effects.

Key Words: *immunoregulation, immunosuppression, mesenchymal stromal cells, rapamycin, sirolimus*

Introduction

Mesenchymal stromal cells (MSCs) are defined by their ability to differentiate into osteoblasts, chondrocytes and adipocytes [1], but much of the current clinical interest is aimed at exploiting their immunoregulatory properties [2,3]. A prevailing hypothesis is that MSCs exert their beneficial effects on tissue regeneration, not through replacement of damaged cells, but by providing anti-inflammatory signals and growth factors that promote the regeneration process [4]. MSCs have been shown to actively suppress the function or differentiation of all immune cell types tested (monocytes, dendritic cells, B and T lymphocytes and natural killer cells), and multiple mechanisms appear to be involved, including cell-cell contact and secretion of agents such as prostaglandins, transforming growth factor, indoleamine oxidase, TSG6, or heme oxygenase [4–7].

Hundreds of clinical trials have been registered that involve infusion of autologous, second-party or third-party MSCs derived from bone marrow, adipose tissue or umbilical cord (Clinicaltrials.gov). Many of the applications are directed at inhibition of undesirable immune responses such as acute graft-versus-host disease (aGVHD) after hematopoietic stem cell transplantation, rejection of solid-organ transplants or autoimmune diseases such as multiple sclerosis and Crohn's disease [8,9]. Current protocols use relatively small numbers of MSCs per treatment, on the order of 1×10^6/kg body weight [10,11]. It is surprising that positive responses have been reported from such low doses, particularly because tracking experiments indicate that MSCs

*These authors contributed equally to this work.

Correspondence: **John Girdlestone**, H&I R&D, NHSBT Colindale Centre, Charcot Road, London, NW9 5BG, UK. E-mail: john.girdlestone@nhsbt.nhs.uk

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act in a transient manner, and few are detected even several days after infusion [12]. The ability of intravenously injected MSCs to localize at sites of tissue damage is one mechanism by which such limited cell numbers could promote repair [13], and the release of exosomes with immunoregulatory potential could allow for disseminated effects [14]. Although the properties of MSCs make them attractive for treating inflammatory conditions, they can also be co-opted by tumors, in which their trophic and immunosuppressive functions could promote disease [15].

There is interest in exploiting the homing ability of MSCs to use them as drug delivery systems and, to this end, they have been genetically modified to produce cytokines and enzymes for anti-cancer pro-drug conversion [16–18]. Although MSCs are not thought to persist long after infusion, any genetic manipulation introduces the potential for oncogenic or other undesirable changes and complicates their clinical application. In the current report, we describe a method by which the immunoregulatory potency of MSCs, as well as other cell types, can be increased without genetic modification simply by brief exposure to immunosuppressive drugs (ISDs). The ability to combine the homing and suppressive activities of MSCs with ISDs has the potential to increase the therapeutic potential of this experimental cell therapy and to reduce production costs if fewer MSCs are required per treatment.

Methods

MSCs and fibroblasts

Umbilical cord MSCs (UC-MSCs) were generated as described [19] from fresh cord segments collected from full-term births by NHS Cord Blood Bank (NHS-CBB) staff (Colindale, United Kingdom) after informed ethical consent was obtained. Cells were cultured at 37°C with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM):F12 (Lonza) with penicillin/streptomycin (Sigma) and 10% fetal calf serum (FCS) (Life Technologies) and were passaged with the use of 0.125 % trypsin (Sigma). Bone marrow (BM)-MSCs were generated through the use of standard methods from frozen aliquots of mononuclear cells (MNCs) purchased from DV Biologics. Briefly, the MNCs were thawed and plated in a tissue culture flask with the medium as above. After colonies of MSC-like cells were observed, they were passaged, and expanded and their phenotype was assessed by means of flow cytometry for the presence and absence of surface markers (cluster of differentiation [CD]73, CD90, CD45 and CD34; Biolegend) as described [19].

The HS27 human foreskin fibroblast cell line (ECACC) and primary human dermal fibroblasts (TCS Cellworks) were grown under the conditions used for MSCs. Human umbilical vein endothelial cells (HUVECs) were purchased from ECACC and Life Technologies and expanded in endothelial cell growth medium (TCS Cellworks).

Mononuclear cells

Adult peripheral blood (AB) MNCs from consenting platelet donors were prepared from apheresis cones [20] provided by NHSBT. The contents of the cones were diluted with calcium and magnesium-free phosphate-buffered saline (PBS) and were centrifuged over Lymphoprep (Axis-Shield); cells at the interphase were then subjected to a 200*g*, 12-min spin to deplete platelets. Aliquots were frozen in 10% dimethyl sulfoxide (DMSO), 20% FCS and 70% DMEM:F12 and stored in a −150° freezer (Panasonic). Purified CD4+ responder T-cell populations were prepared by means of incubation of MNCs with biotinylated antibodies against CD8, CD14, CD15, CD16, CD19, CD56 and HLA-DR (Biolegend); depletion with streptavidin-coated magnetic beads (Sigma) was then performed. CD4+ populations were depleted of T-regulatory cells (Tregs) with the use of magnetic beads to remove CD25+ cells (Miltenyi Biotec), with the efficiency tested by means of staining for CD25 and CD127, and for FoxP3 after perm/fix treatment (eBioscience) and incubation with PE-labeled anti-FoxP3 (eBioscience clone PCH101) (Supplementary Figure 1). Antigen-presenting cells (APC) were generated through magnetic bead depletion of MNCs with biotinylated anti-CD2 and anti-CD3 and used in a 1:1 ratio with CD4+ T cells as described [21]. For cell proliferation assays, MNCs and lymphocytes were labeled with carboxyfluorescein succinimidyl ester (CFSE) (Sigma) as described [21].

Drug treatment and suppression assays

Rapamycin was purchased as a 2.5 mg/mL DMSO solution; cyclosporine A (CsA), everolimus, azathioprine, mycophenolate mofetil and FK-506 monohydrate (all from Sigma) were dissolved in DMSO, with aliquots stored at −20° until use. For drug pretreatment of MSCs and fibroblasts, cells were cultured in T25 flasks with 5 mL of standard growth medium until near confluence. Drug stock solutions were diluted in DMSO such that they were added to the cultures at ≤10 μL. These volumes of DMSO were shown to have no effect on MSC function in control experiments (data not shown). At times indicated in the text, the medium was removed from

the cells, which were then rinsed with 5 mL of PBS. Trypsin was added in a 1-mL final volume (0.125%) at room temperature until cells detached; cells were then neutralized with 250 μL of FCS and diluted with 5 mL of PBS before centrifugation. After complete aspiration of the supernatant and a second PBS wash, the cell pellet was resuspended in growth medium at an initial concentration of 2.5×10^5 cells/mL, with two further 5-fold dilutions made in medium to distribute 1000, 5000 and 25,000 cells/well in 100-μL aliquots to U-bottomed 96-well plates (BD Falcon). Where noted, some treatments were carried out on trypsinized cells in suspension in 5 mL of standard growth medium with 50 ng/mL of the indicated drug. Control co-culture wells were prepared with 100 μL of growth medium alone. After 2 to 4 h was allowed for cell adhesion, CFSE-labeled responder lymphocytes (MNCs or CD4) were resuspended at 5×10^5 cells/mL MSC growth medium and 100-μL aliquots distributed to the MSC and control plates. Where indicated in the text, T-cell activation was induced with phytohemagglutinin-L (Sigma) at 0.5 μg/mL final concentration, T-cell activation beads (Miltenyi) at 1 bead/2 MNCs or 1:1 co-cultures of CD4 T cells and allogeneic APC (5×10^4 cells each).

Cell proliferation was monitored with the use of CFSE dye dilution after 3 to 4 days of culture with phytohemagglutinin (PHA) or activation beads or 6 days for allostimulation, with 3 to 4 wells for each condition pooled and the cells stained with antibodies to CD3 (PerCP), CD25 (Pe-Cy7) and CD4 (APC-Cy7) (Biolegend) for analysis on a FacsCanto II (Becton Dickinson). Infinicyt cytometry software was used to determine the cell numbers in each division cohort. Proliferation indices (PI) were calculated in Excel (Microsoft) by use of the formula: (P0 + P1 + P2 + …)/(P0 + 0.5 P1 + 0.25 P2 + …), where P0 is the number of cells undivided, P1 is one division and so forth. To normalize samples for comparison, the PI of samples stimulated in the absence of MSC (PI^{stim}) were defined as 1 and unstimulated controls (PI^{unstim}) were 0: $PI^{norm} = [(PI^{n} - PI^{unstim})/(PI^{stim} - PI^{unstim})]$. MNCs from at least three different donors were used for each experiment, and Student's *t*-tests were performed in Excel for determining significance.

Washout experiments

UC-MSCs were treated with 100 or 500 ng/mL of rapamycin in six-well plates with 2.5 mL of medium per well. After 2 h, the medium was removed from all wells, which were then rinsed several times with PBS. One set of wells was trypsinized and the MSCs were plated as above for suppression assays. The remaining wells were prepared with 2.5 mL of fresh growth medium without drug and returned to the incubator for 24 h of further culture; cells were then trypsinized and plated for suppression assays with the use of aliquots of frozen MNCs from the same donors as used on the previous day.

Drug neutralization

UC-MSCs at near confluence in T25 flasks in standard growth medium were treated for 75 min with 50 ng/mL of rapamycin; cells were then distributed to 96-well plates as described above for suppression assays. Fifteen minutes before addition of CFSE-labeled reporter MNCs, dilutions of a sheep anti-rapamycin immunoglobulin (Ig) fraction or a pre-immune control (Aalto Bio Reagents) were added to the MSC-containing wells or to control wells previously prepared to provide a final concentration of 0.5 or 2.5 ng/mL rapamycin (200 μL, final volume). The "×1" condition corresponded to 2 μg/mL of immune immunoglobulin (0.4 μg/well), which, in prior experiments, was sufficient to neutralize the anti-proliferative effect of 0.5 ng of rapamycin in the standard 200-μL suppression assay volume (Supplementary Figure 2). T-cell stimulation was performed with the use of PHA, and proliferation was monitored as above.

Rapamycin measurements

Triplicate cultures of UC-MSCs in T25 flasks were decanted, and 5 mL of fresh standard growth medium or medium containing 50 ng/mL of rapamycin was added. After 15 min at 37°, the medium was removed and stored in two aliquots at −80°. The cultures were rinsed with 5 mL of PBS; the cells were then detached in 1 mL of trypsin (0.125%). The suspension (1.2×10^6 cells) was distributed equally between two conical tubes, and each was made up to 15 mL with 100 μL of FCS and PBS. The tubes were centrifuged to pellet the cells, and all supernatant was aspirated off. The cells were resuspended in 500 μL DMEM:F12 without FCS and stored at −80°. One of each pair of aliquots was used to determine that the levels of rapamycin were within the dynamic range for the rapamycin assay. Briefly, 150-μL samples were mixed with 450 μL of methanol:zinc sulphate extraction reagent containing an internal standard, desmethoxyrapamycin (Pfizer), and quantified by means of mass spectrometry.

Humanized mouse model of aGVHD

Male and female BALB/c RAG2−/−common gamma chain (γc)−/− mice (Charles River) between 8 and 15 weeks old were used for an aGVHD xenogeneic model

[modified from those described by Ali *et al.* [22] and Moncrieffe *et al.* [23]]. Mice were maintained under pathogen-free conditions at the Biological Science Unit Animal Facility King's College London. All animal experiments were specifically approved by the Institutional Committees on Animal Welfare of the United Kingdom Home Office (the Home Office Animals Scientific Procedures Act, 1986).

Xenogeneic aGVHD was induced by the intravenous injection of adult human MNCs prepared as above: 1.5×10^7 MNCs in 200 μL of PBS or 200 μL of PBS alone (ie, no aGVHD control) were injected through the tail vein 24 h after total body irradiation at 400 cGy. At day 9 after irradiation, mice given human MNCs were intravenously injected with 0.5×10^6 or 2×10^6 UC-MSCs; 0.5×10^6 UC-MSCs pre-treated for 16 h with 100 ng/mL of rapamycin; or 1×50 ng or 3×50 μg rapamycin (ie, 50 μg injected on 3 consecutive days), all in 200 μL of PBS. A control group of aGVHD mice was injected with 200 μL of PBS alone. Animal weights were measured and recorded every 2 to 3 days after irradiation. Animals that developed clinical symptoms of severe aGVHD (>15% weight loss, hunched posture, fur loss, reduced mobility, tachypnea) were euthanized, and an end point of survival was recorded for all mice. Surviving mice were euthanized at the termination of the experiments. Experiments were undertaken twice, and the results of the two experiments were subsequently pooled together. Survival analysis was performed with the use of Prism (GraphPad), with a log-rank (Mantel-Cox) test used for comparisons.

Tissue samples (spleen, lung, liver, gut and kidney) were extracted for analysis at the time of euthanasia. Approximately 0.5cm^2 of sample from each tissue was excised and passed through 50-μm cell strainers (BD Falcon) to obtain single-cell suspensions. Red blood cells (RBCs)were removed from the cell suspensions with RBC lysis solution (eBioscience). Approximately 0.5×10^6 cells were stained with fluorescently labeled mouse antibodies to human CD3, CD4, CD8 and CD45 (Biolegend) and analyzed by means of flow cytometry for their human MNC content. All mice given human MNCs and killed or surviving at day 69 after irradiation had ≥8% of cells from at least one of their tissues analyzed that were positive for human CD45.

Results

Pre-treatment of MSCs from different sources with rapamycin increases their immunosuppressive potency

In a screen of modifiers of signaling pathways potentially involved in MSC-monocyte interactions [21], it was observed that pre-treatment of MSCs with rapamycin for 24 to 48 h led to a dose-dependent increase in their ability to inhibit T-cell proliferation. The increased suppression was seen with treated MSCs derived from bone marrow (Figure 1A) or umbilical cord (Figure 1B), as measured by proliferation of T lymphocytes in MNC preparations stimulated with PHA. With a pre-treatment of 50 ng/mL there was a consistent and significant inhibition even at the lowest ratio of MSCs to MNCs (1:50). Incubation of MSCs with higher doses of rapamycin (100, 500 ng/mL) did not lead to substantial increases in the effect (data not

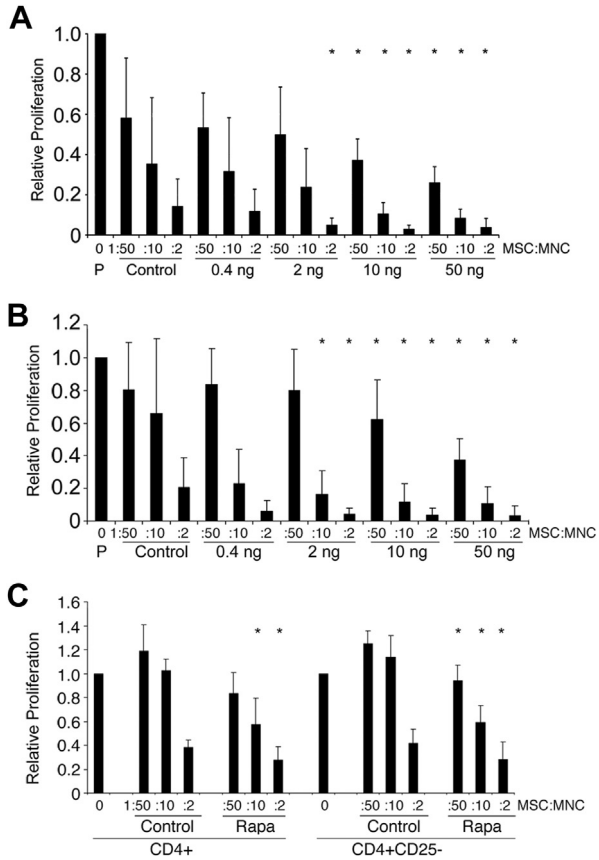

Figure 1. Rapamycin pre-treatment increases the immunosuppressive potency of MSCs. (A) BM-MSC cultures were incubated for 24 h with 0 (control), 0.4, 2, 10 or 50 ng/mL rapamycin before re-plating for co-culture at 1 k, 5 k and 25 k with 50 k PHA-stimulated MNCs. The ratios of MSCs:MNCs are indicated. "P" represents the proliferation index of CD3+ cells in the absence of MSCs and is defined as 1. Error bars in this and subsequent figures represent standard deviation of the mean; asterisks indicate $P < 0.05$ as compared with the same number of untreated MSCs ($n = 6$). The raw and normalized data for one experiment are provided in Supplementary Figure 3. (B) Representative UC-MSC line was used in a suppression assay as above with gating performed on CD3+CD4+ T cells ($n = 3$). (C) CD3+CD4+ lymphocytes and parallel preparations depleted of CD25+ cells (see Supplementary Figure 1) were stimulated with anti-CD3, −CD28 beads in the presence of control MSCs or cells pre-treated with rapamycin ($n = 6$).

shown), and 50 ng/mL was used for subsequent experiments unless noted. Purified CD4+ T cells stimulated with anti-CD3, anti-CD28 activation beads were also inhibited to a greater degree by rapamycin—pre-treated MSCs, and the effect did not require the presence of CD25+ Tregs (Figure 1C). The relatively weaker inhibition of proliferation of the purified CD4+ cells is consistent with our earlier finding that monocytes are required for maximal suppression by MSCs [21]. Enhanced suppression was also seen with CD4+ T cells stimulated by allogeneic APCs in the presence of rapamycin-treated MSCs (data not shown).

Enhancement by rapamycin is rapid, transient and additive to immunosuppression by MSCs

Initial experiments used 24- to 48-h pre-treatments on the hypothesis that sustained inhibition of the mTOR pathway in the MSCs might lead to a strengthening or induction of immunosuppressive mechanisms. However, time-course studies showed that equivalent effects were achieved with incubations as short as 5 min (Figure 2A). With such brief exposures, the MSCs can be incubated with drug while in suspension after trypsinization (data not shown). MSCs could also be frozen after drug treatment with retention of their enhanced suppressive activity upon thawing (Supplementary Figure 4), further simplifying their preparation for potential clinical use.

To examine the persistence of the rapamycin effects, we performed wash-out experiments (Figure 2B). When MSCs were treated, washed and cultured for 24 h in fresh medium without drug, the rapamycin effect was diminished but still significant. An increase to 500 ng/mL of rapamycin pre-treatment did not prolong the drug's effect (data not shown). After several passages, the drug-treated cells showed no increased suppressiveness as compared with controls, but re-treatment restored the effect (data not shown). Wash-out experiments were also used to test the possible effects of rapamycin exposure on the proliferation and differentiation of MSCs. After 24-h treatment with levels of rapamycin shown to increase suppressiveness (50—100 ng/mL), subsequent rates of expansion or adipogenic differentiation [19] were not inhibited significantly (data not shown).

Previous reports on the interactions between ISDs and MSCs have suggested that the suppressive effects of rapamycin on T-cell proliferation are inhibited by the presence of MSCs [24]. We did not find significant inhibition of rapamycin effects, but there was a trend to a slightly less-than-additive effect when T cells were activated in the presence of both

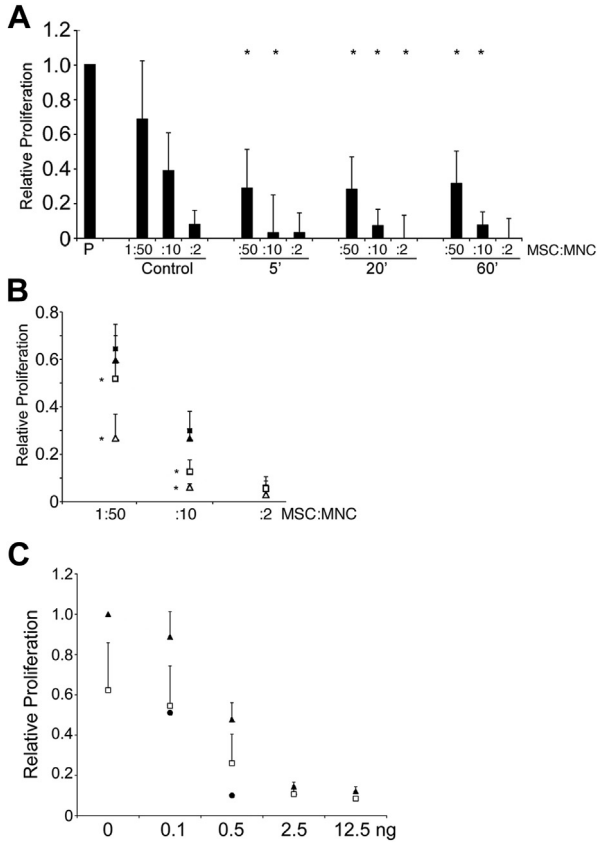

Figure 2. Time course of rapamycin effect. (A) Parallel cultures of UC-MSCs were incubated with rapamycin at 50 ng/mL for 5, 20 or 60 min and used in suppression assays as above ($n = 8$). (B) Parallel cultures were treated with/without rapamycin at 100 ng/mL for 2 h; one control/treated pair was used immediately to set up a suppression assay (day 0, triangles), whereas a second pair was washed and then cultured for a further 24 h in fresh medium without drugs (day +1, squares). Solid figures: control MSCs, open figures: rapamycin-treated MSCs. MNC aliquots from the same donors were used to monitor suppression for the two sets of treated MSCs ($n = 6$). *$P < 0.05$ as compared with the same number of untreated MSCs. (C) Titration of rapamycin (0 to 12.5 ng/mL) was carried out in the absence (triangles) or presence (open squares) of 1 k untreated MSCs (1:50 ratio to MNCs) to assess their possible interactions in the inhibition of PHA-stimulated CD4+ T-cell proliferation. Induced proliferation in the absence of MSCs or drugs was assigned to be 1; the calculated additive effect of MSCs and drugs is shown with circles ($n = 4$).

the drug and MSCs that had not been pre-treated (Figure 2C). These results suggest that MSCs and rapamycin act to inhibit T-cell proliferation through largely independent mechanisms, and there is no evidence for synergy or rapamycin-induced stimulation of MSC-suppressive mechanisms.

Rapamycin is taken up by MSCs and the enhancement effect is blocked by a neutralizing anti-serum

Rather than modifying the physiology of the MSCs by inhibition of mTOR, another possibility is that increased suppression of T-cell proliferation is due to

the action of rapamycin itself in the assay cultures. We calculated that the trypsinization and washing steps involved in re-plating the MSCs after drug treatment would reduce any carry-over in the medium to levels below that seen to inhibit T cells (Figure 2C). However, the lipophilic nature of rapamycin is known to result in significant partition into red and white blood cells *in vivo* [25]. Mass spectrometry measurements showed that after a 15-min incubation with 5 mL of medium containing 250 ng of rapamycin, an average of 79 ± 28 ng (n = 3) was present in a pellet of 1.2 × 10^6 cells. Therefore, transfer of 5000 treated cells into a 200-μL suppression assay would deliver approximately 0.3 ng of rapamycin, resulting in a concentration (1.5 ng/mL) sufficient for significant inhibition of T-cell proliferation if it was available to diffuse from the MSCs to the lymphocytes.

To test the possibility that the treated MSCs were adsorbing the drug and introducing it into the suppression assay cultures, neutralization experiments were performed. In control experiments with MNC cultures treated with rapamycin in the absence of MSCs, a pre-determined "×1" amount of antibody was able to block the inhibition caused by 2.5 ng/mL of drug in a 200-μL culture volume and did not affect suppression by untreated MSCs (Supplementary Figure 2). As seen in Figure 3, inclusion of an anti-serum directed against rapamycin but not a pre-immune control inhibited all of the drug-dependent increase in suppressive activity. Together with detection of rapamycin in the MSCs, this indicates strongly that the drug-induced increase in suppression is mediated by the drug itself and that the rapamycin

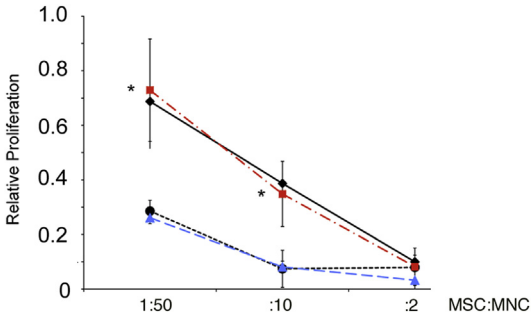

Figure 3. The increased suppression by pre-treated MSCs is blocked by an anti-serum against rapamycin. T-cell proliferation induced by PHA (=1 without MSC) was measured in co-cultures with 1 k, 5 k and 25 k untreated UC-MSCs (solid black line, diamonds) or those pre-treated for 75 min with 50 ng/mL rapamycin (dotted black line, circles). Pre-immune serum (dashed blue lines, triangles) or a ×1 amount of anti-rapamycin (dash-dot red line, squares) was added to the MSC cultures 15 min before addition of 50 k MNCs. When anti-rapamycin was added to reactions containing rapamycin-treated MSCs, the resulting T-cell proliferation was equivalent to that seen with control MSCs and significantly different from the treated MSCs at 1:50 and 1:10 (*$P < 0.05$, $n = 4$).

effect is additive to the immunosuppression caused by the native MSCs. Observations that MSCs inhibit rapamycin's effects on T-cell proliferation could therefore be due to buffering of the drug by the MSCs in co-cultures.

Other cell types are made more suppressive by rapamycin

If rapamycin is taken up by MSCs because of their lipophilic nature, then pre-treatment of other cell types might also make them more immunosuppressive. Indeed, primary and permanent fibroblastic lines became more suppressive when pre-treated with rapamycin (Figure 4A,B). Rapamycin also enhanced the suppressiveness of mouse embryo fibroblasts, showing that the effect is not specific for human cells (data not shown). HUVECs did not suppress T-cell proliferation substantially except at the highest ratios (1 HUVEC:2 MNCs), but treatment with rapamycin made them significantly suppressive even at 1:50 (Figure 4C). As seen with MSCs, the drug-dependent increase in suppression was totally blocked by anti-rapamycin (Figure 4C), indicating that the enhanced effect was due primarily to adsorption and release of the drug rather than to induction of immune-inhibitory mechanisms in the HUVECs.

Pre-treatment of dendritic cells for 24 h with rapamycin has been reported to increase their tolerogenic function without inhibiting their migratory ability [26]. In addition to the reported reduction in co-stimulatory molecules caused by the drug, we hypothesized that absorption and release of rapamycin by APCs could also contribute to their reduced activation of T cells. As shown in Figure 4D, incubation of APCs with rapamycin significantly reduces their allo-stimulatory function, and this decrease is fully reversed by neutralization with anti-rapamycin. Therefore, under our experimental conditions, the effect of the drug itself on T-cell proliferation appears to be the dominant mechanism of suppression.

Other ISDs can increase the potency of MSCs

Several other ISDs were tested to determine if they could also increase the immunosuppressive potency of MSCs. As shown in Figure 5A, the rapalogue everolimus increases the potency of MSCs with a similar dose profile as seen with rapamycin. FK506 (tacrolimus) binds to the same cellular target protein (FKBP12) as does rapamycin but acts through a different downstream mechanism [27]. Orange *et al.* [28] have reported that FK506 is adsorbed rapidly and then released by dendritic cells in a manner analogous to our observations for rapamycin.

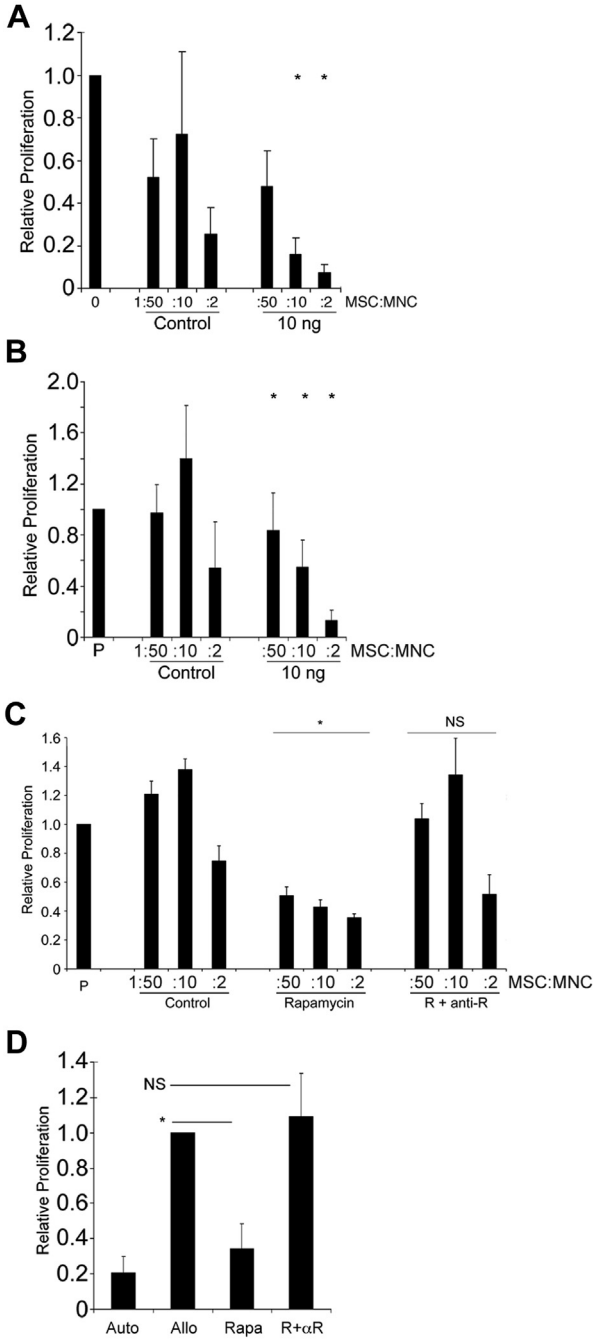

Figure 4. Rapamycin pre-treatment of other cell types. (A) Human dermal fibroblasts ($n = 5$) and (B) the HS27 fibroblastic cell line ($n = 8$) were pre-treated with rapamycin for 2 h at 10 ng/mL and were then plated at 1, 5 and 25 k for suppression assays of PHA-induced CD4+ T-cell proliferation as for MSCs. (C) Untreated HUVECs (control) or those pre-treated with rapamycin for 15 min at 50 ng/mL were plated for suppression assays with (R+anti-R) or without anti-rapamycin. In comparison with control HUVECs, the treated cells were more suppressive than the equivalent number of control cells ($P < 0.05$) at all ratios, but there was no significant difference (NS) when anti-rapamycin was added to the co-cultures ($n = 3$). (D) APC preparations from three adult donors were incubated with or without rapamycin at 50 ng/mL for 1 h and were then extensively washed before co-culture in a mixed lymphocyte reaction (MLR). Each CD4+ responder was set up against autologous APCs (Auto) and each of the other two allogeneic APCs (Allo). Anti-rapamycin was included in one set of rapamycin pre-treated APC reactions, as for previous neutralization experiments (R+ anti-R). The relative proliferation on allo-stimulation was defined as 1 to normalize responses. NS, non-significant. *$P < 0.05$ ($n = 6$, 3 responders ×2 allo-stimulators).

Indeed, FK506 also increased suppressiveness of both MSCs (Figure 5A) and HUVECs in the 10- to 50-ng/mL range (Figure 5B). The action of FK506 was also rapid, allowing for treatment of cells in suspension (Figure 5B). CsA did not show any effect when used to pre-treat MSCs at 50 ng/mL (data not shown), but therapeutic doses are substantially higher than those for rapalogues and tacrolimus. When MSCs were pre-treated at 5 μg/mL CsA, they were significantly more immunosuppressive than were controls (Figure 5C).

Rapamycin-treated MSCs show increased potency in a pre-clinical aGVHD model

Acute GVHD is a serious cause of morbidity and mortality in patients receiving hematopoietic stem cell transplants and has been one of the main applications for MSCs in clinical trials [9]. As a stringent test of whether pre-treatment of MSCs improves their potency *in vivo*, we tested a low dose (5×10^5) of control UC-MSC−treated or rapamycin-treated cells and a higher dose (2×10^6) of untreated cells for their ability to inhibit the onset of aGVHD in a xenogeneic model. The low dose of untreated UC-MSCs showed no significant benefit, but, when pre-treated with rapamycin, they were superior to the higher dose as measured by survival or weight loss (Figure 6A,B). Whereas the higher dose of MSCs showed a trend toward promoting greater survival ($P = 0.24$), only the cohort receiving rapamycin-treated cells showed a significant survival advantage over the non-treated xenogeneic group ($P = 0.03$). From our measurements of rapamycin taken up by MSCs (see above) we estimated that 30 to 40 ng of drug would be contained in 5×10^5 treated cells, >1000 times less than therapeutic doses used by others [29−31]. Indeed, even when animals were treated with three doses of 50 μg of rapamycin, there was no significant inhibition of GVHD in our model, indicating that there is a strong synergistic action with MSCs pre-treated with drug. Analysis of tissues showed that all animals induced for aGVHD contained human CD45+ cells in all tissues tested, confirming that engraftment had occurred in all mice and negating the possibility that some mice survived because the infused human MNCs did not engraft. The proportion of human MNCs in spleen was

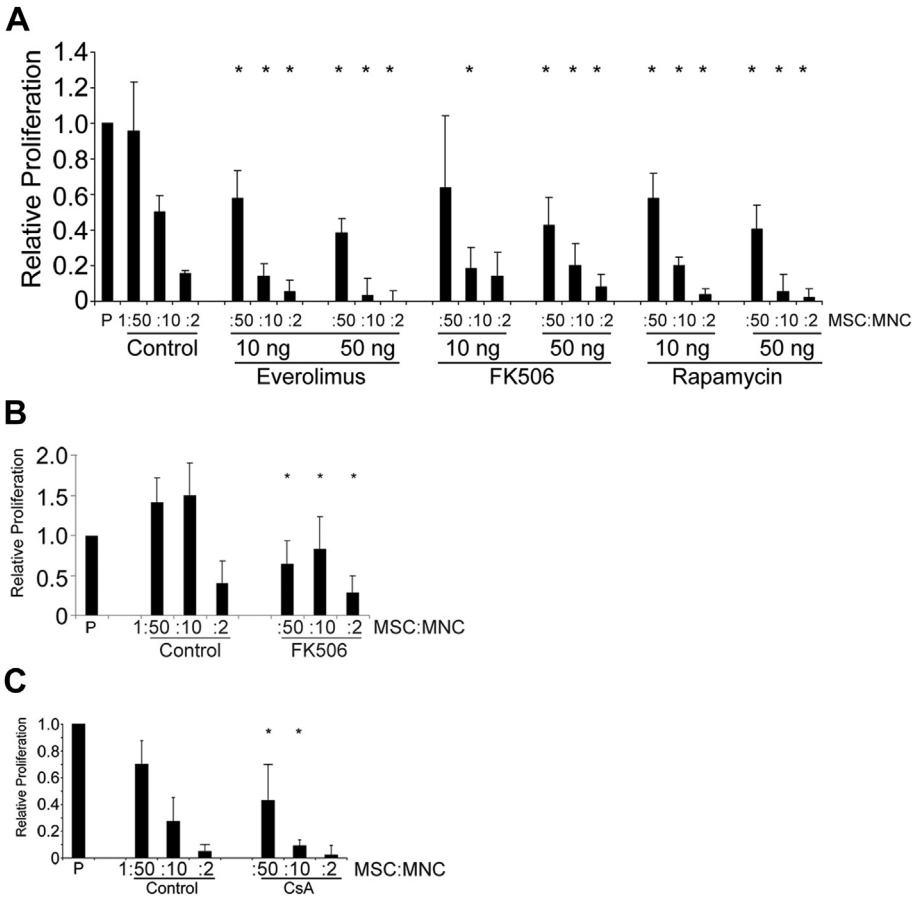

Figure 5. Pre-treatment of MSCs with other immunosuppressive drugs. (A) UC-MSC were pre-treated for 2 h with the indicated doses (10, 50 ng/mL) of everolimus, FK506 and rapamycin and were then plated with 50 k MNCs at the indicated ratios for suppression assays ($n = 3$). (B) HUVECs were treated for 15 min in suspension with 50 ng/mL FK506 and were then washed twice before plating for suppression assays as above ($n = 5$). (C) UC-MSCs were pre-treated for 1 h with CsA at 5 μg/mL and were then plated for assays as above ($n = 9$). *$P < 0.05$ compared with the equivalent number of control cells.

significantly lower in the animals receiving rapamycin-treated cells, but this difference was not evident in other tissues such as liver (Figure 6C), gut, lung and kidney (not shown).

Discussion

MSCs have intriguing immunosuppressive properties that make them promising candidates as an off-the-shelf cell product for regenerative medicine and for treatment of autoimmune diseases and immune complications of stem cell and solid-organ transplantation [8,9]. For these latter indications, relatively low numbers of MSCs have been infused under current protocols, but insufficient dose-escalation studies have been performed to know the optimal dosage and schedule of treatments. Some of the most promising clinical responses have been reported in children, in whom higher doses per kilogram are more easily achieved, but a regimen of eight injections at 2×10^6/kg [11] would provide logistical and financial challenges for adult patients. Although MSCs can be expanded readily and no serious adverse events have been associated with their administration, there are concerns regarding problems arising from extensive passaging [32].

In studying the mechanisms by which MSCs mediate their immunosuppressive effects with a view to enhance their activity [21], we observed that pre-treatment with rapamycin significantly increased their ability to inhibit T-cell proliferation. Rapamycin is the canonical inhibitor of the mTOR pathway that integrates sensing of nutrient and growth factor signals to regulate cell metabolism and proliferation [33]. Lymphocytes are particularly sensitive to the anti-proliferative actions of rapamycin, although other cell types can be inhibited at higher doses. Although mTOR inhibition is likely to alter MSC metabolism to some degree, subsequent experiments indicated that the primary basis of the enhanced suppression is the action of the drug itself in *trans*, with the MSCs acting as a drug delivery system.

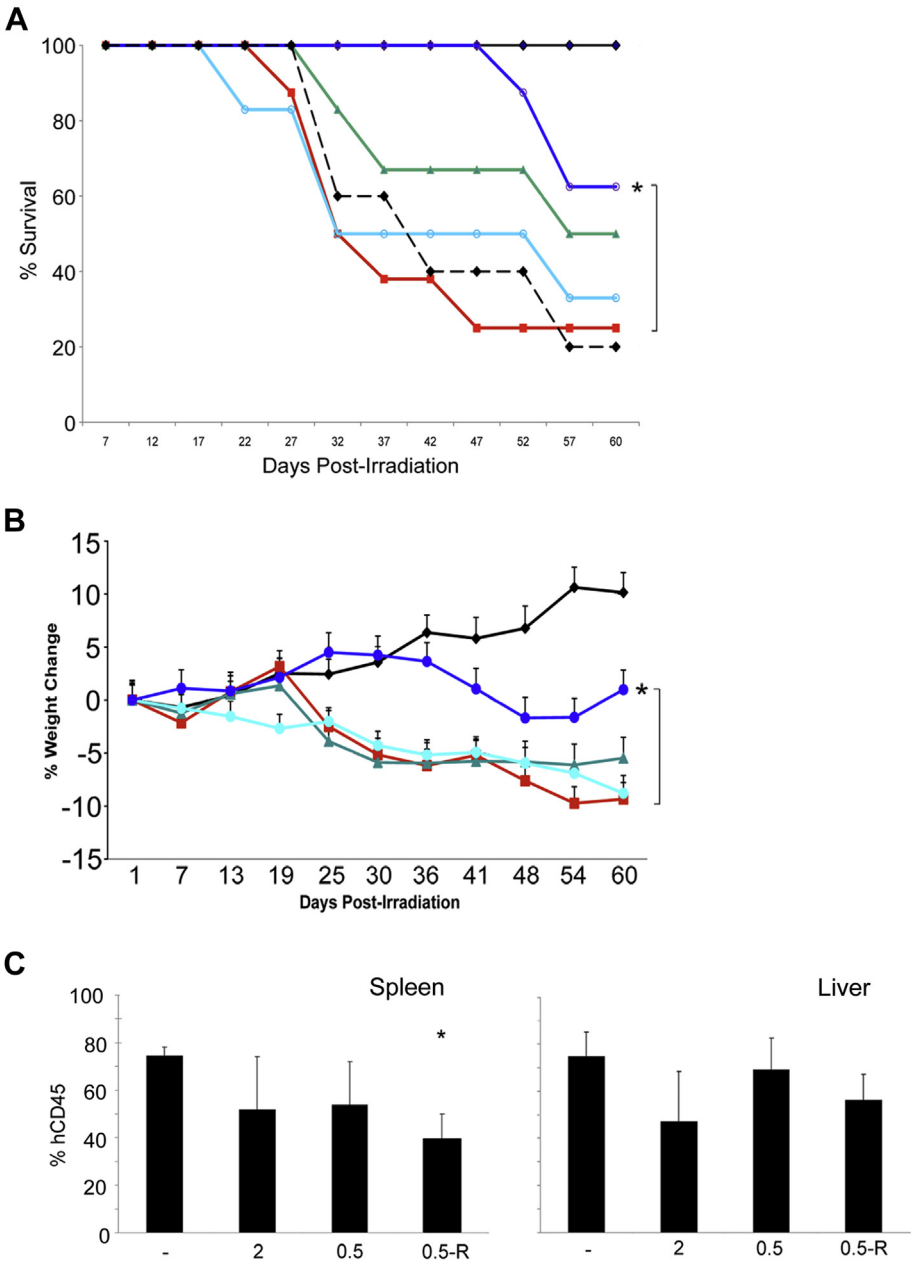

Figure 6. Pre-treatment of UC-MSCs increases their ability to inhibit onset of aGVHD. (A) Control mice (no MNCs: solid black line, diamonds, $n = 5$) or groups that were injected with human MNCs with or without UC-MSCs (MNC alone: red squares, $n = 8$; MNC + 0.5×10^6 MSCs, light blue circles, $n = 6$; MNCs + 0.5×10^6 MSC pre-treated with rapamycin, dark blue circles, $n = 8$; MNCs + 2×10^6 MSCs, green triangles, $n = 6$; 3×50 μg rapamycin, black diamonds dotted line, $n = 5$) were followed for 60 days and monitored for survival and (B) body weight. Only the group receiving low-dose treated MSCs showed a significant increase in survival over the xenogeneic control (Mantel-Cox $P = 0.03$) and also showed significantly less weight loss (*$P < 0.05$). (C) The percentage content of human CD45+ cells was determined in multiple tissues at euthanasia or at the end of the experiment, and results for spleen (left) and liver (right) are presented for groups receiving human MNCs without UC-MSCs (−; $n = 5$), 2×10^6 untreated MSC (2; $n = 4$) and 0.5×10^6 MSCs that were untreated (0.5; $n = 4$) or pre-treated with rapamycin (0.5-R; $n = 7$). Error bars represent standard deviation; asterisk indicates statistical significance compared with "−" animals ($P < 0.05$).

FK506 showed a similar potentiating effect despite acting through a distinct pathway to rapamycin, although it does bind to FKBP12, the same cellular target as the rapalogues [34]. However, because CsA binds to cyclophilin rather than to FKBP12 [27] yet also increased the suppressive activities of MSCs, the accumulated results are consistent with the enhancement effect being due to the drugs rather than to regulation of a specific signaling pathway within the MSC. Not all ISDs were found to enhance the suppressive activity of MSCs; there was no significant change when azathioprine or mycophenolate

mofetil was used for pre-treatment at 5 μg/mL (data not shown). It is not known whether the biophysical characteristics or metabolism of these compounds prevent their effectiveness in pre-treatment of MSCs.

There are precedents for the loading of cells with hydrophobic drugs, with Pessina *et al.* [35] reporting that sufficient amounts of the anti-cancer drug paclitaxel were adsorbed by MSCs in a 24-h incubation to reduce tumor growth *in vivo* and Orange *et al.* [28] finding that FK-506–loaded dendritic cells could inhibit autoimmune arthritis. A rapid partition of ISDs into cells would appear to explain our observations of increased suppression by brief pre-treatments of MSCs, fibroblasts, APCs and HUVECs and may explain at least part of the increased suppression reported for rapamycin-treated endothelia and Tregs [30,36].

It is well documented that rapamycin partitions into blood cells [25], and measurement of rapamycin in our treated MSCs showed sufficient levels of drug even after extensive washing to inhibit T-cell proliferation in co-culture. The ability of a neutralizing anti-serum to block the enhanced suppression induced by pre-treatment of MSCs is strong evidence that diffusion of the drug into the co-culture is responsible for their increased potency. It is difficult to exclude the possibility that MSCs require the continuous presence of rapamycin acquired in their pre-treatment to maintain an enhanced suppressive activity. However, the ability to neutralize the effect shows that rapamycin is available for binding by antibody in the medium and therefore could also be free to act on lymphocytes in the co-cultures. Although it is conceivable that rapamycin and other ISDs may be inducing immunosuppressive factors in MSCs and all the other cell types tested, the lack of synergism when rapamycin and MSCs are added at the same time argues against this mechanism as a significant contribution to the enhanced suppression [24]. Therefore, the simplest explanation supported by the results is that the enhanced suppression is due to uptake and release of the drugs by the cells.

Wash-out experiments indicated a half-life of approximately 1 day for the rapamycin effect on MSCs *in vitro*, which raised questions as to whether it would persist long enough to have an impact in therapeutic situations. *In vivo* tracking indicates the MSCs themselves have a half-life on the order of a few days [12]; therefore, it is possible that even a transient boost to their immunosuppressive activity might be sufficient to produce significant clinical benefits. This was effectively demonstrated by the ability of a single low dose (0.5×10^6) of rapamycin-treated UC-MSCs to significantly inhibit the onset of xenogeneic GVHD. The same number of untreated cells had no apparent effect, and, even a dose of 2×10^6, similar to that used in other studies [37,38], showed only a trend toward greater survival. Whereas rapamycin alone has been used for prevention of solid-organ transplant rejection and aGVHD in mice transplant models [30,31], doses of 3×50 μg were insufficient in our experiments, as was a single dose of 50 ng that we calculate to be the approximate amount of drug introduced by the pre-treated MSC (data not shown). Therefore, whereas the suppressive effects of MSCs and rapamycin were seen to be additive *in vitro*, synergism is indicated by the *in vivo* model. Synergism has also been reported with systemic administration of much higher doses of rapamycin (2 mg/kg per day) together with MSCs in a heart transplant model [29]. We postulate that the enhancement in our *in vivo* model is due to the MSCs acting as delivery vectors, targeting the drug as well as their own repertoire of immunosuppressants to sites of inflammation where they have been reported to localize [39]. Our GVHD protocol involved only a single injection of UC-MSCs, and it remains to be determined if multiple doses of cells pre-treated with rapamycin, or other ISDs, would further inhibit the mortality and weight loss seen in the second month after initiation of aGVHD.

MSCs and rapamycin have both been reported to increase the proportion of Tregs through direct or indirect mechanisms [40,41]. Although we found that Tregs were not required for the rapamycin effect on MSCs *in vitro* and do not appear to be necessary for suppression by MSCs of solid-organ graft rejection [42], further experiments are required to determine their potential contribution to the rapamycin-treated MSC effect *in vivo*. There was a trend toward lower proportions of human MNCs in animals receiving MSCs in our GVHD model, but the decrease was not substantial, and it remains to be determined whether the treated MSCs inhibit disease through promotion of Tregs and/or anergy of effector lymphocytes.

The method that we have reported here for combining a promising cellular therapy with standardly used ISDs has the potential to increase significantly the clinical utility of MSCs. The results of our pre-clinical GVHD model indicate that it may be possible to reduce the number of cells that are required for infusion into a patient, thereby reducing the costs of production and risks from over-expansion. The use of pre-treated cells may also allow for achievement of clinical end points that are not reached with standard dosing regimens. Although enhanced MSCs are unlikely to eliminate the need for systemic administration of drugs that have undesirable side effects, they may contribute significantly to their reduced use through targeted delivery. More broadly, the demonstration that all

cell types tested appear to sequester hydrophobic ISDs opens up interesting possibilities for pre-treatment of cells and tissues before infusion or transplantation to reduce inflammation and immune-mediated rejection.

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Supplementary data

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