Freshly Thawed and Continuously Cultured Human Bone Marrow-Derived Mesenchymal Stromal Cells Comparably Ameliorate Allergic Airways Inflammation in Immunocompetent Mice

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Key Words. Mesenchymal stromal cells • Cryopreservation • Asthma • Mouse

ABSTRACT

Recent data suggest that freshly thawed previously frozen mesenchymal stromal cells (MSCs) may not have the same effectiveness or breadth of anti-inflammatory activities as do continuously cultured MSCs. This has significant implications for clinical use, in which many infusion schemes use frozen cells thawed at the bedside for administration. The available data, however, predominantly evaluate in vitro MSC properties, and so far there has been limited in vivo analysis. To further assess this issue, we compared freshly thawed (thawed) versus continuously cultured (fresh) human bone marrow-derived MSC (hMSC) administration in a mouse model of mixed Th2/Th17 allergic airway inflammation induced by Aspergillus hyphal extract (AHE) exposures in immunocompetent C57Bl/6 mice. Control cell populations included fresh versus thawed murine bone marrow-derived MSCs (mMSCs) and human lung fibroblasts (HLFs). Systemic administration of both thawed and fresh hMSCs and mMSCs, but not HLFs, at the onset of antigen challenge in previously sensitized mice significantly ameliorated the AHE-provoked increases in airway hyper-reactivity, lung inflammation, and antigen-specific CD4 T-cell Th2 and Th17 phenotype. Notably, there was no difference in effects of fresh versus thawed hMSCs or mMSCs on any outcome measured except for some variability in the effects on the bronchoalveolar lavage fluid composition. These results demonstrated potent xenogeneic effects of human MSCs in an immunocompetent mouse model of allergic airways inflammation and that thawed MSCs are as effective as fresh MSCs. The question of fresh versus thawed MSC effectiveness needs to be investigated carefully and may differ in different in vivo disease-specific models. Stem Cells Translational Medicine 2015;4:1–10

SIGNIFICANCE

This study addressed whether freshly thawed mesenchymal stromal cells (MSCs) are as effective in in vivo settings as those that have been continuously cultured. It also provided further data demonstrating that xenogeneic use of MSCs in immunocompetent mice is as effective as murine MSCs. This information provides further support and direction for potential clinical use of MSCs in patients with severe asthma.

INTRODUCTION

Mesenchymal stromal cells (MSCs) isolated from bone marrow, adipose tissue, umbilical cord blood, and other sources secrete a range of anti-inflammatory mediators and have other anti-inflammatory actions in response to different inflammatory stimuli [1–3]. As such, when isolated from the different sources and readministered either systemically or directly in an organ-specific manner, they can have significant anti-inflammatory and disease-ameliorating actions in a wide variety of preclinical inflammatory and autoimmune disease models [4–6]. This has led to an increasing number of clinical investigations of both autologous and allogeneic MSC administration and some suggestions of successful desired anti-inflammatory actions in clinical use, most notably in refractory pediatric graft-versus-host disease [4–7]. However, despite abundant literature describing the actions of MSCs in both in vitro assays and preclinical disease models, fundamental questions remain about the mechanisms of MSC actions in clinical applications. Issues include a relative lack of information correlating in vitro MSC potency on any given inflammatory pathway with the desired disease-specific actions for a given clinical indication. In parallel, optimal
approaches for isolating, expanding, preparing, and administering MSCs for disease-specific clinical indications have not yet been determined [8–10].

A simple yet fundamental approach for MSC use has recently come under renewed scrutiny. For practical reasons, systemic administration of MSCs, particularly non-human leukocyte antigen-matched allogeneic MSCs, has involved freezing of cell preparations after isolation and expansion. The frozen MSCs, suspended at the desired concentration in a cryopreservation medium suitable for infusion, are then thawed just prior to administration. However, a significant number of the frozen MSCs may undergo apoptosis during the freeze-thaw process, although much subsequent study has attempted to minimize this occurrence with improved freeze-thaw approaches and cryopreservatives [11–14]. A recent report demonstrated that freshly thawed MSCs were not as effective in in vitro potency assays as continuously cultured MSCs of the same passage number [15]. Data from this study further demonstrated that it took up to 24 hours for the thawed MSCs to regain potency in the in vitro assays. A second recent study found that freeze-thawed MSCs demonstrated reduced initial responsiveness to proinflammatory stimuli, impaired production of anti-inflammatory mediators, and strong activation of the complement cascade compared with continuously cultured cells [16]. This report included a retrospective analysis of systemic MSC administration in patients with complications of hematopoietic stem cell transplantation at the Karolinska Institute between 2002 and 2007 and demonstrated that the use of continuously cultured MSCs at low passage had a response rate twice that observed in a comparable group of patients treated with freshly thawed cells at higher passage (100% vs. 50%) [16]. These results suggest potential disadvantages to the use of freshly thawed MSCs and may significantly affect clinical investigations and uses of MSCs for which it is not uncommon to use freshly thawed cells. However, there have been no other detailed investigations of potential effects of thawing on the immediate anti-inflammatory actions of MSCs either in vitro or in vivo or of whether this might differ depending on the nature of the disease-specific inflammatory environment.

To address this issue, we investigated the question of freshly thawed (thawed) versus continuously cultured (fresh) MSC potency in an in vivo mouse model of allergic airway inflammation. We and others have demonstrated that systemic administration of either syngeneic or allogeneic MSCs during either sensitization or challenge ameliorates airways hyper-reactivity and lung inflammation provoked by a variety of different antigens [17–27]. We have most recently demonstrated the efficacy of MSC administration in a mucosal immunization model involving intratracheal administration of *Aspergillus* hyphal extract (AHE) [28]. This provokes a mixed Th2/Th17 model of eosinophilic and neutrophilic allergic airway inflammation and is used as a mouse model of severe refractory neutrophilic asthma [29, 30]. Furthermore, because an increasing number of studies have demonstrated efficacy and thus potential usefulness as preclinical models of human MSC (hMSC) administration in immunocompetent mouse models of lung and other diseases [31–35], both fresh and thawed human and syngeneic mouse MSCs were assessed in AHE sensitized and challenged immunocompetent C57Bl/6 mice.

**MATERIALS AND METHODS**

**Mice**

C57Bl/6 mice (male, 8–12 weeks, n = 72; Jackson Laboratories, Bar Harbor, ME, http://www.jax.org) were housed in microisolator cages and used in accordance with the University of Vermont (UVM) institutional animal care and use committee under applicable Association for Assessment and Accreditation of Laboratory Animal Care guidelines.

**Cells and Cell Culture**

Murine bone marrow-derived mesenchymal stromal cells (mMSCs) from C57Bl/6 mice were obtained from the Texas...
A&M stem cell core facility [36]. Human mesenchymal stem cells derived from bone marrow of normal human volunteers were obtained from the National Heart, Lung, and Blood Institute’s Production Assistance for Cellular Therapies program (D.H.M.). These cells have been extensively characterized previously for cell surface marker expression and differentiation capacity [36–38].

mMSCs were expanded in culture using Iscove’s Modified Dulbecco’s Medium (Hyclone; GE Healthcare Bio-Sciences, Pittsburgh, PA, http://www.gelifesciences.com), 10% fetal bovine serum (FBS; Hyclone), 10% horse serum (Hyclone), 1% penicillin/streptomycin (Invitrogen, Life Technologies; Thermo Fisher Scientific, Waltham, MA, http://www.thermofisher.com), and 2 mM l-glutamine (Invitrogen) and used at passages 4–6. hMSCs were cultured in Minimal Essential Medium with Earle’s Balanced Salts (Hyclone; GE Healthcare Bio-Sciences, Pittsburgh, PA, http://www.gelifesciences.com), 20% FBS, 1% penicillin/streptomycin, and 2 mM l-glutamine and used at passage 6 or lower. Human and mouse MSCs were passaged every 3 days for these studies. We routinely used mouse and human bone marrow-derived MSCs in passages 2–6 and in previous studies [23, 28]; these cells have been considered low passage, and anything beyond is considered high passage and is not used for in vivo studies. We have never observed any significant difference in behavior of the MSCs in the in vivo studies within this range of passages. Moreover, we were careful to not let individual culture plates go beyond 70% passage to minimize any potential paracrine signaling, so the cells are still actively growing at the time of harvest. Normal adult HLFs were expanded in culture with Dulbecco’s Modified Eagles Medium: Nutrient Mixture F-12 (Sigma-Aldrich, St. Louis, MO, https://www.sigmaaldrich.com), 10% FBS, 1% penicillin/streptomycin, and 2 mM l-glutamine and used at passage 6 or lower.

For use in experiments, the cells were harvested for injection using 2.5% Trypsin/EDTA (Invitrogen). Cell density and viability was determined after washing using trypan blue staining and counted using a hemocytometer. Cell pellets were then resuspended in sterile...
phosphate-buffered saline (PBS) to a final concentration of $1 \times 10^6$ cells per 200 ml immediately prior to injection. Cells suspended in sterile PBS were kept on ice until administration within 15 minutes of thawing and washing. Cells (hMSCs, mMSCs, HLFs) in parallel plates from the same batches and passagenumber as those harvested from continuous culture were similarly harvested and then cryopreserved at $-80^\circ$C for 48 hours at $1.5 \times 10^6$ cells in 1 ml of freezing solution (50% medium, 40% FBS, and 10% dimethyl sulfoxide), followed by 7 days of storage in liquid nitrogen. Cells were thawed immediately prior to injection and washed three times with PBS. Cell viability, density, and final concentration ($1 \times 10^6$ viable cells per 200 ml PBS) were determined after washing by trypan blue exclusion and counting using a hemocytometer, as described for cultured MSC preparations [23, 28]. Cells were suspended in sterile PBS and kept on ice until administration within 15 minutes of thawing and washing.

**Induction of Allergic Airway Inflammation**

The study design is shown in schematic form in Figure 1. AHE aliquots at a concentration of 1.466 mg/ml in 1× PBS, generously provided by the Whittaker laboratory at UVM and previously used by us, were thawed and vortexed immediately prior to use, diluted to a final concentration of 5 μg AHE in 40 μl sterile 1× PBS [28–30]. Mice were anesthetized by isoflurane inhalation and received an oropharyngeal administration of PBS (naïve) or AHE solution on days 0 and 7 to initiate the immune response (sensitization), then challenged for 3 days on days 14–16 with oropharyngeal inoculations using the same AHE preparation (Fig. 1A) [28].

**Cell Administration**

On day 14, immediately after the AHE inoculation, mice were systemically administered by a tail vein injection of fresh or recently thawed hMSCs or mMSCs ($1 \times 10^6$ viable cells in 200 ml PBS) or of 1× PBS control (Fig. 1B). Mice were euthanized on day 19, and lung function, lung inflammation, and antigen-specific T-cell activity were measured, as described below. Because viability was ~70% in the thawed cells, 30% more cells were used in mice receiving thawed cells to have ~1 million viable cells injected. In real numbers, this equates to ~1.3 million thawed versus 1 million fresh cells.

**Respiratory Mechanics**

Pulmonary function was analyzed using the forced oscillation technique (flexiVent; SCIREQ Scientific Respiratory Equipment, Montreal, Canada, http://www.scireq.com) as previously described [23, 28, 39, 40]. The peak responses for airway resistance, overall tissue resistance, and elastance within the lung were determined in response to sequential inhalation of nebulized saline, followed by 3.125 mg/ml, 12.5 mg/ml, and 25 mg/ml of methacholine (MCh) diluted in saline.

**Assessment of Airway Inflammation**

Following evaluation of lung mechanics, mice were euthanized by lethal intraperitoneal injection of sodium pentobarbital. Bronchoalveolar lavage fluid (BALF) was collected by administering 1 ml of sterile 1× PBS to the airways through a tracheal cannula.
and rinsing the lungs three times prior to recovery. BALF was centrifuged at 2,000 \( \text{g} \), for 5 minutes at 4°C, and the supernatant was collected in separate tubes and stored at 280°C. A Bio-Plex cytokine assay system (Bio-Rad, Hercules, CA, http://www.bio-rad.com) was used to examine undiluted BALF samples for soluble inflammatory cytokines using a mouse 23-plex panel. Concentrations were determined using the Bio-Plex Manager software. The cell pellet was resuspended, and an aliquot was used to determine total cell count with an ADVIA hematology analyzer (Siemens, Munich, Germany, http://usa.healthcare.siemens.com). Cytospins were made using 3 \( \times \) 10^4 cells centrifuged onto precleaned, pretreated glass slides (Corning, Corning, NY, http://www.corning.com) at 300g for 8 minutes, dried overnight, and stained using Hema 3 Manual Staining System (Fisher Scientific, Pittsburgh, PA, http://www.thermofisher.com). Different cell populations were determined by blinded manual count of 200 cells performed by three separate persons. Following BALF collection, the trachea and heart/lung block were removed and placed in a nitrogen. The left lobe was then gravity fixed (20 cm H\(_2\)O) for 1 hour with 4% paraformaldehyde and 5-\( \mu \text{m} \) paraffin sections subsequently stained with hematoxylin and eosin. In a blinded fashion, 3 separate individuals evaluated airways inflammation, 10 airways per animal, based on the presence and intensity of peribronchial cell infiltrates compared with positive and negative controls using an established semiquantitative scoring system, using a 0–3 range as previously described. No individual knew which group was analyzed [23, 28].

**Mediastinal Lymph Node Mixed Lymphocyte Assessments**

Mediastinal lymph nodes (MLNs) were isolated by dissection from each mouse and placed in T-cell medium (RPMI, 5% FBS, 1X penicillin/streptomycin, 2 mM L-glutamine, 2,500 mg/ml glucose, 1 mg/ml folate in 2 g/l sodium bicarbonate, 1 mM sodium pyruvate, and 50 \( \mu \text{M} \) β-mercaptoethanol). To ensure enough cells for assay, MLN cells from mice of the same experimental group were pooled and pressed through a 40-\( \mu \text{m} \) mesh filter into a single-cell suspension. Cells were then washed twice in 1X PBS and resuspended for counting. One million cells per time point (24, 48, and 72 hours) were plated in duplicate for each group in a 24-well dish.

**Figure 4.** Systemic administration of fresh or thawed hMSCs or mMSCs significantly reduces increases in BALF inflammatory cells provoked by AHE sensitization and challenge. (A): Total BALF cell numbers in N and A mice treated with fresh and thawed HLF, hMSC, and mMSC. (B): Differential BALF cell populations normalized to total cell numbers: 17 N, 15 A-P, 6 A-HLF-F, 6 A-HLF-T, 10 A-hMSC-F, 6 A-hMSC-T, 6 A-mMSC-F, 6 A-mMSC-T. Data are presented as mean ± SD. \( p < .05 \). Significance compared with N and A-P. \#F versus T. Abbreviations: A, AHE-exposed; A-P, AHE-exposed mice treated with PBS; AHE, Aspergillus hyphae extract; BALF, bronchoalveolar lavage fluid; F, fresh; HLF, human lung fibroblasts; hMSC, human mesenchymal stromal cells; mMSC, murine mesenchymal stromal cells; N, naive; P, vehicle (PBS); PBS, phosphate-buffered saline; T, thawed.
in 500 µl of T-cell medium. In half of the wells, cells were stimulated with 1 µg of AHE in the medium for 24 or 48 hours; the other wells were left unstimulated for the same time points. Total contents of each well were collected at the indicated time points and were centrifuged for 5 minutes at 5,000 rpm to pellet cells and debris. Supernatants were moved to a new tube and frozen at −20°C. Content of representative Th1, Th2, and Th17 soluble mediators (IL-4, IL-5, IL-17a, and IFN-γ) were assessed by commercially available enzyme-linked immunosorbent assay (ELISA) kits (BioLegend, San Diego, CA, http://www.biolegend.com). Sensitivity of ELISA kits was IL-4, 1 pg/ml; IL-5, 4 pg/ml; IL-17, 8 pg/ml; and IFN-γ, 4 pg/ml.

Statistical Analyses
All data were graphed and analyzed using the GraphPad Prism v6.0 statistical software package (GraphPad Software, La Jolla, CA, http://www.graphpad.com). The normality of the data (Kolmogorov-Smirnov test with Lilliefors correction) and the homogeneity of variances (Levene median test) were tested. Parametric data are expressed as mean ± SD. Differences between the groups were evaluated by one-way analysis of variance (ANOVA) followed by Tukey’s test. Nonparametric data were analyzed using ANOVA on ranks followed by Dunn’s post hoc test. Statistical significance was established at p ≤ .05.

RESULTS
Systemic Administration of Fresh or Thawed hMSCs and mMSCs Comparably Ameliorate AHE-induced Airway Hyper-Responsiveness
AHE sensitization and challenge resulted in a significant increase compared with naive mice in each measure of methacholine-stimulated airway hyper-reactivity (AHR): large airway resistance, lung elastance, and tissue resistance (Fig. 2). Administration of either mMSCs or hMSCs significantly decreased each measure of AHR, whereas administration of the HLF control cell population had no effect. Notably, hMSCs were as effective as mMSCs, and no differences were observed in effects of fresh versus thawed for mMSCs, hMSCs, or HLFs.

Systemic Administration of Fresh or Thawed hMSCs and mMSCs Comparably Ameliorate AHE-induced Lung Inflammation
AHE sensitization and challenge resulted in a significant increase in histologic and BALF inflammation compared with naive mice (Fig. 3–5). Administration of either mMSCs or hMSCs comparably and significantly decreased both histologic inflammation (Fig. 3) and total and differential BALF cell counts (Fig. 4). No difference was observed in effects of fresh versus thawed for mMSCs, hMSCs, or HLFs.
pronounced reduction of lymphocytes using fresh mMSCs compared with thawed cells (Fig. 4B). HLF administration had no effects on the AHE-provoked histologic or BALF inflammation.

Administration of hMSCs, mMSCs, and HLFs had more mixed effects on levels of BALF cytokines (Fig. 5). HLFs generally had no effect on levels of any cytokine except for increases in IL-5, IL-6, and IL-12 produced by thawed but not fresh cells over those produced by AHE exposure alone. Both fresh and thawed hMSCs and mMSCs had similar effects in decreasing AHE-provoked increase in BALF IL-4 and RANTES. Fresh mMSCs were less effective than either thawed mMSCs or either fresh or thawed hMSCs in reducing IL-5, whereas both fresh and thawed hMSCs were both comparably more potent in reducing IL-12p40. In contrast, mMSCs, both fresh and thawed, were more potent in increasing IFNγ levels. Thawed mMSCs were more potent in reducing IL-5 than fresh mMSCs, and thawed hMSCs were more potent in reducing both IL-17 and keratinocyte chemoattractant compared with fresh hMSCs. No effect of any cell types was observed on levels of IL-3 or IL-13. The number of experimental samples in which measurable levels of cytokines were detected is included in supplemental online Table 1.

### Systemic Administration of Fresh or Thawed hMSCs and mMSCs Comparably Ameliorate Antigen-Specific CD4 T-cell Release of Th2 and Th17 Mediators

AHE sensitization and challenge resulted in a significant increase in release of IL-4, IL-5, and IL-17 by mixed MLN cultures following ex vivo antigen stimulation (Fig. 6). This was most notable at 48 hours, particularly for the increase in IL-17. Administration of either mMSCs or hMSCs, but not HLFs, comparably decreased levels of all three cytokines, and no difference was observed in effects of fresh versus thawed cells. In contrast, both fresh and thawed mMSCs were more potent that hMSCs in increasing IFNγ release.

### DISCUSSION

The first notable finding of these studies is that hMSCs were as effective, if not more so, than syngeneic mMSCs in ameliorating experimentally induced mixed Th2/Th17 AHR and lung inflammation in an immunocompetent mouse model. The second notable finding is that freshly thawed MSCs were as effective overall as freshly harvested continuously cultured MSCs in decreasing the AHE-induced AHR, lung inflammation, and antigen-specific CD4 Th2/Th17 phenotype.
A growing number of preclinical studies demonstrate that xenogeneic administration of human MSCs is feasible and can be effective in mitigating disease-specific endpoints in different preclinical disease models [41, 42]. Whether these reflect specific anti-inflammatory actions of the administered hMSCs or the newly appreciated instant blood-mediated inflammatory reaction or other reactions to the hMSCs is not yet clear [16, 43, 44]. Whatever the underlying mechanisms, use of xenogeneic hMSC administration in immunocompetent mice provides a novel model with which to investigate these pathways. These approaches may also provide more direct approaches for determining in vivo potency measures of hMSCs for any disease indication. Recent data suggest that in vitro data and a retrospective analysis of clinical MSC investigations [5, 6]. A number of factors could potentially explain these differing observations that will need further investigation. In the current study, cell viability was ∼70% after thawing and harvest compared with 100% viability of the freshly harvested, continuously cultured cells. These are comparable to experiences in other studies, as are the freezing medium and the freeze-thaw technique used [11–14]. As such, there is no obvious discrepancy in the technical approaches. We used equal numbers of viable cells because it seemed an apt comparison. As such, 30% more cells were used in mice receiving thawed cells so as to have ∼1 million viable cells injected. In real numbers, this equates to ∼1.3 million thawed versus 1 million fresh cells. Acknowledging the differences, the total cell numbers are still well within the range of 1 million to 2 million cells per mouse administered in previous studies [23, 28], and so we do not think the total cell numbers accounted for any significant effects in the outcome measures. Although the increased number of cells and perhaps the presence of apoptotic and likely necrotic cells may have stimulated inflammatory host reactions, they did not appear to influence effects on the AHE-stimulated...
AHR, lung inflammation, and antigen-specific Th phenotype. The length of freezing prior to thaw in the current studies was relatively short (7 days) and may not have had the same effect as longer freezing. Longer freeze time prior to thawing and use has been demonstrated to have detrimental effects on initial cell viability after thawing and on proliferative and differentiation capacities [11–14]. However, length of freeze time on anti-inflammatory actions is not yet well understood.

It is noteworthy that similar effects of fresh versus thawed cells were seen not just with syngeneic mMSC but also with xenogeneic hMSC administration. This suggests that whatever actions that MSCs are exerting to ameliorate inflammation in this specific disease model are not affected by species difference or by any potential detrimental effects of the freeze-thaw on relevant anti-inflammatory actions of the MSCs. As such, these results complement increasing data demonstrating that effects such as those of xenogeneic versus syngeneic or allogeneic cells and the use of fresh versus thawed MSCs will be disease specific. This reflects the increasing recognition of the complexity of potential MSC actions and will need to be carefully investigated in appropriate disease-specific contexts.

CONCLUSION

Even accounting for some number of the actual numbers of cells administered, freshly thawed bone marrow-derived MSCs were as potent as, if not more potent than, continuously cultured MSCs in ameliorating airways hyper-responsiveness, lung inflammation, and activity of antigen-specific CD4 T lymphocytes in an in vivo model of mixed Th2/Th17 allergic airway inflammation. These data suggest that administration of freshly thawed MSCs may be effective in appropriate clinical scenarios. However, this needs to be investigated in each disease system for which the MSCs might conceivably be used. In parallel, human bone marrow-derived MSCs were as, if not more, potent than mouse MSCs in this model. This observation adds to a growing number of reports demonstrating efficacy of xenogeneic human MSC administration in immunocompetent mouse models of disease. However, the mechanisms by which the human MSCs act have not yet been elucidated and may be different from those by which mouse MSCs act.

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Freshly Thawed and Continuously Cultured Human Bone Marrow-Derived Mesenchymal Stromal Cells Comparably Ameliorate Allergic Airways Inflammation in Immunocompetent Mice

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Stem Cells Trans Med  published online April 29, 2015

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