Journal of Immunology and Regenerative Medicine 1 (2018) 45–56

Contents lists available at ScienceDirect

Journal of Immunology and Regenerative Medicine

journal homepage: www.elsevier.com/locate/regen

Dual IFN-γ/hypoxia priming enhances immunosuppression of mesenchymal stromal cells through regulatory proteins and metabolic mechanisms

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ARTICLE INFO

Article history:
Received 2 October 2017
Received in revised form
28 December 2017
Accepted 6 January 2018
Available online 27 March 2018

Keywords:
Mesenchymal stromal cells
Immunosuppression
Priming
Metabolism
Hypoxia
Interferon gamma

ABSTRACT

The immunosuppressive capacity of human mesenchymal stromal cells (MSCs) renders them promising candidates for treating diverse immune disorders. However, after hundreds of clinical trials, there are still no MSC therapies approved in the United States. MSCs require specific cues to adopt their immunosuppressive phenotype, and yet most clinical trials use cells expanded in basic culture medium and growth conditions. We propose that priming MSCs prior to administration will improve their therapeutic efficacy. Interferon-gamma (IFN-γ) priming are cues common to situations of immune escape that have individually shown promise as MSC priming cues but have not been systematically compared. Using mixed lymphocyte reactions, we show that priming MSCs with either cue alone improves T-cell inhibition. However, combining the two cues results in additive effects and markedly enhances the immunosuppressive phenotype of MSCs. We demonstrate that IFN-γ induces expression of numerous immunosuppressive proteins (IDO, PD-L1, HLA-E, HLA-G), whereas hypoxia switches MSCs to glycolysis, causing rapid glucose consumption and production of T-cell inhibitory lactate levels. Dual IFN-γ/hypoxia primed MSCs display both attributes and have even higher induction of immunosuppressive proteins over IFN-γ priming alone (IDO and HLA-G), which may reflect another benefit of metabolic reconfiguration.

Introduction

Over the last 15 years, there have been over 800 clinical trials registered on clinicaltrials.gov for evaluating mesenchymal stromal cells (MSCs) in a range of conditions such as autoimmune disease, inflammation, transplant rejection, and tissue repair.[1] These trials have been motivated by numerous *in vitro* and *in vivo* studies demonstrating that MSCs can be immunosuppressive, as they suppress inflammatory immune cells while promoting regulatory immune cell phenotypes.[2–4] Conveniently, since the MSCs also hypoimmunogenic, they can also be used allogeneically.[2–4]

While clinical trials have clearly demonstrated a strong safety record,[5] the efficacy of MSCs has been modest and inconsistent.[6,7] For example, one of the first MSC products, Prochymal (currently owned by Mesoblast), showed promise in its Phase II trial for treatment of acute graft-vs-host-disease, but it ultimately failed in Phase III, despite showing suggestions of benefit in subsets of patients.[1,8] While some countries have still approved MSC therapies based on safety data, none are approved in the United States for immunosuppression.

Given that there have been many preclinical studies showing

Abbreviations: MSC, mesenchymal stromal cell; IDO, indoleamine-2,3-dioxygenase; PD-L1, programmed death ligand 1; HLA, human leukocyte antigen.

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https://doi.org/10.1016/j.regen.2018.01.001
2468-4988/

the functional efficacy of MSC immunosuppression using *in vitro* assays and animal models of inflammatory disorders,[9,10] this begs the question – why have the human trials not lived up to the preclinical promise? An excellent review by Jacques Galipeau draws attention to how many MSC products used in clinical trials like Prochymal are cryopreserved and thawed right before patient administration, whereas animal studies generally use fresh, culture-expanded MSCs.[11] Damage upon immediate thawing, especially of MSCs that were over-expanded to begin with, could lead to cells that have diminished therapeutic capacity.[12]

We further posit that lack of MSC priming (a.k.a. licensing) may also be an important factor in explaining their inconsistent therapeutic utility. Over the past 10 years it has been uncovered that MSCs are minimally immunosuppressive at baseline and must be educated to adopt this behavior by specific environmental cues (e.g. inflammation).[10,13–15] Nevertheless, to this day, clinical trials still use naïve MSCs grown in basic culture medium, which do not express immunosuppressive proteins at the time they are injected into patients.[16] This means that the cells rely only on the patient's *in vivo* cues to gradually develop an immunosuppressive phenotype, making the therapy suboptimal and less predictable, since the MSC transplant may not yield the same therapeutic benefit from patient to patient.

Our goal was to design an optimal *in vitro* priming regimen that could eventually be developed for testing in clinical trials. We first examined the microenvironmental cues common to biological scenarios where immune escape and immune tolerance are present, such as solid tumors.[17–19] Across diverse malignancies, hypoxia and inflammation are commonly present, suggesting that the combination of these two environmental cues may be ideal for inducing immunosuppressive cell phenotypes. In support of this notion, there have been several studies of priming MSCs with one of these two priming cues.[10,20,21]

The pro-inflammatory cytokine interferon-γ (IFN-γ) has been the most extensively investigated factor for priming MSCs.[10,13,22] Indeed, the International Society for Cellular Therapy (ISCT) recommends it as a standard priming method for evaluating the immunosuppressive capacity of MSCs *in vitro,* with induction of the tryptophan catabolizing enzyme indoleamine-2,3-dioxygenase (IDO) as a readout.[13] In addition to IFN-γ, hypoxia priming has been shown to influence the immunomodulatory capacity of MSCs, enhance their ability to promote angiogenesis and tissue repair, and improve their survival in animal models of ischemia.[20,23–25]

We hypothesized that combining IFN-γ and hypoxia priming would lead to a stronger immunosuppressive MSC phenotype than if either cue were used alone. We thus compared the effects of individual and dual IFN-γ/hypoxia priming on MSC immnuosuppressive function and explored the underlying mechanisms. Given the trend towards using adipose-derived MSCs in clinical trials, we used cells from this source for our studies.[12] However, many previous studies of MSC priming have used MSCs from alternative tissue sources,[9,11,13,16] and so we will retain the acronym “MSC” to emphasize the likely generalizability of our findings.

Materials and methods

MSC culture and priming

Frozen vials of MSCs from de-identified human lipoaspirates (LaCell, New Orleans, LA) were tested for tri-lineage differentiation as well as positive expression of *in vitro* MSC surface markers. MSCs from 8 different donors were used in experiments to demonstrate the generalizability of the cell responses to priming regimens. Cells were cultured in MSC media (DMEM 11965 (Thermo Fisher, Bridgewater, NJ) with 10% FBS and 1% Penicillin/Streptomycin (Thermo Fisher) and plated into 6-well plates at 5000 cells/cm^2 for priming experiments. MSCs were routinely tested as mycoplasma-free using the MycoAlert kit from Lonza (Allendale, NJ).

Passage 5 MSCs were grown to confluence in and subsequently exposed to: control conditions (normoxia, regular MSC media), individual IFN-γ or hypoxia priming, or dual IFN-γ/hypoxia priming (4 different conditions). IFN-γ (Peprotech, Rocky Hill, NJ) was used at a concentration of 100 ng/mL. A hypoxic culturing environment was achieved using a Galaxy 14S CO_2 incubator (New Brunswick Scientific, Edison, NJ) at 37 °C, 5% CO_2, and 1% O_2. After pilot studies testing gene expression kinetics (data not shown), we chose a priming regimen of 48 h that was used in all experiments. Regardless of priming condition, MSCs always had a viability of >95%. A schematic of the experimental design is shown in Fig. 1.

Mixed lymphocyte reactions (MLRs)

For MLRs, a PBMC cryo-bank was made using fully de-identified samples from 10 different donors, to generate different sets of stimulator-responder pairs. PBMCs were isolated from fresh leukopaks (New York Blood Center, New York, NY) using Histopaque-1077 (Sigma, St. Louis, MO) based on density gradient centrifugation, washed twice with bone marrow medium (BMM; Media 199 (Thermo Fisher) containing 1% HEPES, 1% Penicillin/Streptomycin, and 20 kU DNAse I (Sigma), treated with ACK lysis buffer (Thermo Fisher) for red cell lysis, and cryopreserved.

MSCs were trypsinized after 48-h priming and seeded at either 1 × 106/mL or 2 × 106/mL in 40 μL (i.e. 40,000 or 80,000 cells total) in 96-well U-bottom plates in complete AIM-V (Thermo Fisher) supplemented with 5% heat-inactivated human AB serum (Sigma), 1% Penicillin/Streptomycin, 1% HEPES, and 50 μM 2-mercaptoethanol (cAIM-V). Two batches of allogeneic PBMCs were thawed in 1:1 BMM:cAIM-V and washed twice. Responder PBMCs were stained with BD Violet Proliferation Dye (Becton Dickson (BD) Canaan, CT) per manufacturer's instructions (final concentration: 1 μM). Stimulator PBMCs were inactivated using 30 Gy X-ray irradiation with an X-RAD 320 irradiator (Precision X-ray Inc. North Branford, CT) or 10 mg/mL Mitomycin C (always provided similar results in comparison studies). Stimulator and responder PBMC cell concentrations were adjusted to 2.5 × 106/mL, and 80 μL of each cell suspension (i.e. 200,000 cells) was layered on top of previously plated MSCs. Thus, the stimulator to responder ratio was 1:1 and the MSC to responder PBMC ratio was 1:2.5 or 1:5.

MLR experiments were run for 5 days, with 50 μL of cAIM-V added halfway through. For end-point analysis, two antibody

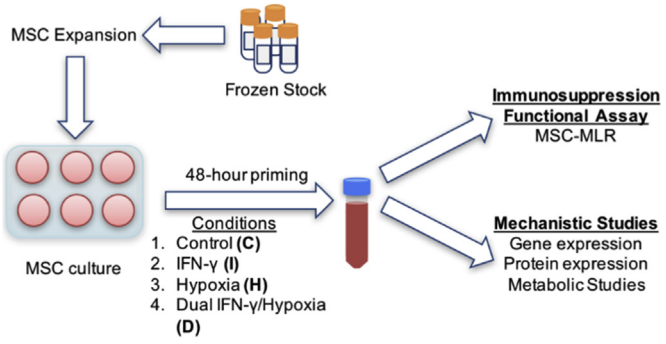

Fig. 1. Experimental Design. Primed MSCs were evaluated for their ability to inhibit T-cells in MSC-mixed lymphocyte reaction co-cultures (MSC-MLR). To then discern the origin of group differences in immunosuppressive capacity, numerous mechanistic studies were pursued.