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Safety Profile of Good Manufacturing Practice Manufactured Interferon γ Primed Mesenchymal Stem/Stromal Cells for Clinical Trials

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ABSTRACT

Mesenchymal stem/stromal cells (MSCs) are widely studied by both academia and industry for a broad array of clinical indications. The collective body of data provides compelling evidence of the clinical safety of MSC therapy. However, generally accepted proof of therapeutic efficacy has not yet been reported. In an effort to generate a more effective therapeutic cell product, investigators are focused on modifying MSC processing protocols to enhance the intrinsic biologic activity. Here, we report a Good Manufacturing Practice-compliant two-step MSC manufacturing protocol to generate MSCs or interferon γ (IFNγ) primed MSCs which allows freshly expanded cells to be infused in patients on a predetermined schedule. This protocol eliminates the need to infuse cryopreserved, just thawed cells which may reduce the immune modulatory activity. Moreover, using (IFNγ) as a prototypic cytokine, we demonstrate the feasibility of priming the cells with any biologic agent. We then characterized MSCs and IFNγ primed MSCs prepared with our protocol, by karyotype, in vitro potential for malignant transformation, biodistribution, effect on engraftment of transplanted hematopoietic cells, and in vivo toxicity in immune deficient mice including a complete post-mortem examination. We found no evidence of toxicity attributable to the MSC or IFNγ primed MSCs. Our data suggest that the clinical risk of infusing MSCs or IFNγ primed MSCs produced by our two-step protocol is not greater than MSCs currently in practice. While actual proof of safety requires Phase I clinical trials, our data support the use of either cell product in new clinical studies. STEM CELLS TRANSLATIONAL MEDICINE 2017;00:000–000

SIGNIFICANCE STATEMENT

Mesenchymal stem/stromal cells (MSCs) are one of the most widely studied cells for the development of cellular therapies. While these cells are unequivocally safe to use in humans, the efficacy of MSCs to treat diseases has not been established. Hence, current efforts are focused on developing the technology to enhance the efficacy of MSCs. Here, we report a novel strategy to prepare MSCs that would mitigate the potential reduction of cell potency observed when infusing freshly thawed, cryopreserved cells. Additionally, we show extensive preclinical data to support the safety of interferon γ primed MSCs, which are designed to enhance the immune modulatory potency of MSCs.

INTRODUCTION

Mesenchymal stem/stromal cells (MSCs) are spindle-shaped, morphologically nondescript, adherent cells with the remarkable capacity to stimulate the body's endogenous reparative mechanisms and modulate the immune response. These properties offer countless opportunities for development of novel therapeutics. Indeed, over the last 2 decades, MSCs have emerged as one of the most commonly studied cell therapies worldwide [1]. This enthusiasm is based largely on the ease of isolation and ex vivo expansion as well as the capacity to infuse cells across HLA barriers rendering implementation of MSC studies far less complex than other cell therapies. Moreover, the encouraging results of innumerable preclinical studies have prompted applications in a vast array of disorders encompassing the spectrum of regenerative medicine and immune modulation. Perhaps

Table 1. Release criteria for primary and secondary expansions

Release test and methods	Primary expansion sample	Secondary expansion sample	Release criteria
Microbial culture—Bactec automated culture system, aerobic, and anaerobic	Fresh MSCs after addition of DMSO, Precryopreservation	Spent culture media 2 days prior to harvest	No growth
Mycoplasma—MycoAlert luminescent assay	Spent culture media day of harvest	Spent culture media day of harvest	Negative (borderline result retested by PCR)
Endotoxin—Endosafe LAL kinetic chomogenic	Test-thawed MSCs	Final MSC product	≤5 EU/ml, primary; ≤5 EU/kg recipient weight, secondary
Trypan Blue Viability	Pre-cryopreservation MSCs	NA	Information only
Trypan Blue Viability	NA	Final MSC product	≥90%
%CD45+—flow cytometry	Fresh MSCs Precryopreservation	Final MSC product	≤20%
%CD105+—flow cytometry	Fresh MSCs Precryopreservation	Final MSC product	≥70%
Gram Stain	NA	Final MSC product	No organisms identified

Abbreviations: MSCs, mesenchymal stem/stromal cells; NA, not applicable.

most importantly, the extraordinary safety profile in clinical trials to date has created a favorable risk-benefit analysis for most disorders lowering the threshold to launch pilot trials. The net result is an abundance of early phase studies suggesting favorable response; however, phase III studies in several diseases have failed to demonstrate a significant therapeutic benefit [2]. These seemingly conflicting results underscore the importance of the method of isolation, expansion, and storage of the MSCs, as well as selection of the targeted disease, eligibility criteria, and endpoints for clinical trials.

Another arena of research that has gained momentum as a consequence of early phase successes is the effort to augment the intrinsic biologic activity of MSCs or engineer the cells to confer novel biologic activity. The high efficiency of gene transfer has led to applications using MSCs to delivery gene therapy. The apparent plasticity of MSCs in vitro opens the door to developing tissue culture conditions to shape the final cell product for a specific application.

The immune suppressive properties of MSCs have garnered considerable attention. Much effort is directed toward enhancing this activity by treating MSCs with modulating cytokines, for example, interferon γ (IFNγ) [3] or interleukin(IL)-17 [4]. These agents explicitly enhance the immune suppressive potential of MSCs in vitro and possibly in vivo. However, such ex vivo manipulation may also fundamentally alter the cells in ways we have not yet recognized possibly posing new, ill-defined risks to study subjects.

Only through human trials will the full range of potential toxicities be revealed. Nonetheless, in an effort to lessen the prospect of an unpredicted toxicity in a human subject, we sought to comprehensively assess the safety of IFNγ-primed MSCs prepared according to current Good Manufacturing Practice (cGMP) protocols with animal and in vitro preclinical analyses. We assessed MSCs and IFNγ-primed MSCs prepared from the same donor in parallel allowing us to attribute any identified toxicities specifically to the IFNγ priming distinct from the general manufacturing protocol.

MATERIALS AND METHODS

Isolation, Primary Ex Vivo Expansion, and Cryopreservation

Bone marrow (~20 ml) was collected by routine aspiration according to a protocol approved by the Institutional Review Board and was promptly mixed with heparin/plasmalyte to a final concentration of 12 U/ml heparin. The marrow, which did not pass through particle filters, was promptly transferred to the cell therapy laboratory where mononuclear cells (MNC) were isolated by density centrifugation over Ficoll at 750g for 20 minutes. After washing, MNC were transferred to CellSTACK culture vessels (Corning, New York, www.corning.com) at a target density of 1.6 E05 cells per cm^2 (range, 0.5–2) in D5 culture media consisting of low-glucose Dulbecco's Modified Eagle's medium (DMEM) supplemented with 5% human platelet lysate (PLTmax, Mill Creek, Rochester, MN, www.millcreekls.com), 2 mM GlutaMax (Gibco/Thermo Fisher Scientific, Waltham, MA, www.thermofisher.com), 2 U/ml preservative-free Heparin (USP), 10 mM N-Acetylcysteine (USP), and 40 μg/ml gentamycin (USP). The cultures were washed at 2–3 days to remove nonadherent cells and replated after 7–10 more days to disperse the adherent cells evenly over the surface. When the adherent cells had expanded to approximately 80% confluence (2–3 weeks in culture), these Passage 0 (P0) cells were either cryopreserved in D4 culture media (D5 minus the gentamycin) with 10% DMSO and 20% PLTmax or split into CellSTACK culture vessels at a target density of 2,000–3,000 cells/cm^2 (range, 1,000–5,000) and continued in culture for 1–2 weeks. When the P1 cells attained about 80% confluence, the cells were collected from culture and cryopreserved. Depending on the size of the marrow harvest, the yield of MSCs and the number required, either P0 or P1 cells may be cryopreserved at 0.9–1.1 E06 cells/ml and stored in liquid nitrogen vapor phase storage to support a single trial. A sample of the cryopreserved population of MSCs underwent release testing according to criteria developed in accordance with recommendations of the Food and Drug Administration (http://www.fda.gov/cber/guidelines.htm, Table 1) to validate our preclinical cell stock.

Secondary Ex Vivo Expansion and Interferon γ Priming

To allow the cells to recover from the detrimental effects of cryopreservation [5], MSCs were thawed in a 37°C water bath, diluted 10-fold with prewarmed 37°C D4 culture media and washed once before being placed into tissue culture in D4 culture media at a target density of 2,000–3,000 cells per cm^2 (range, 1,000–5,000). This culture will always be planned for a 7- or 9-day culture prior to a patient's scheduled infusion. Two days before the expansion was complete, cells were fed with fresh D4 culture media containing 500 U/ml GMP-grade recombinant human IFNγ (R&D

Systems, Minneapolis, MN, www.rndsystems.com). A sample of spent media from each culture vessel was collected 2 days prior to completion and tested for microbial contamination to satisfy the appropriate release criteria. On the day of the planned infusion, the cells were collected by trypsinization (Gibco), washed to remove IFNγ and then resuspended in infusion media (Plasma-Lyte A [USP], 0.22% NaCl [USP], 2.5% Dextrose [USP], 0.5% human serum albumin [USP]. This final cell product or spent media was tested and met all release criteria which will be required in clinical trials before transport to the subject's bedside.

Cell Culture for In Vitro Malignant Transformation

GMP-prepared MSCs were seeded in culture with DMEM-LG (Mediatech, Manassas, VA) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) and 1% Penicillin-Streptomycin (Mediatech) at a density of 3,000 cells/cm^2. The cells were monitored twice per week and the media was changed weekly. When the MSC cultures were estimated to be 80% confluent, the cells were collected by trypsin-EDTA (0.25%) (Mediatech), washed, and reseeded at 3,000 cells per cm^2. The MSCs were serially passaged until senesce. All other studies, in vitro or in vivo, used cells prepared in human platelet lysate.

Trilineage Differentiation Assay

GMP-prepared MSCs and IFNγ primed MSCs were differentiated to osteoblasts, adipocytes, and chondrocytes as determined by staining with Alizarin Red S, Oil Red O, and Alcian Blue, respectively, as previously described [6].

Animals

C57BL/6J and NOD.Cg-$Prkdc^{scid}$ $Il2rg^{tm1Wjl}$/SzJ (NSG) mice were purchased form Jackson Laboratories (Bar Harbor, ME) and transgenic C57BL6 mice with pancellular expression of the green fluorescent protein (GFP) under control of the H2K promoter were obtained from our laboratory colony [7]. Mice were maintained under specific pathogen free condition in our AAALAC-accredited animal research facility. All animal studies were approved by our Institutional Animal Care and Use Committee at The Research Institute at Nationwide Children's Hospital.

Transplantation

Total body irradiation of wild type C57BL/6J mice (500 cGy × 2, 3 hours apart) was performed with an x-ray source (X-RAD 320: Precision x-ray Inc, North Branford, CT). After 48 hours, mice were transplanted with 2×10^6 unfractionated GFP-expressing C57BL/6 marrow cells and 250,000 human [8] or murine [9] MSCs via a tail vein injection. Hematopoietic reconstitution was monitored by serial complete blood counts (Hemavet 950FS, Drew Scientific, Miami Lakes, FL) and flow cytometric analysis for GFP expression (donor cells).

Quantitative PCR

Human MSCs were detected in mouse tissues using the quantitative polymerase chain reaction (qPCR) technique originally described by Francois et al. 2006 [10]. Genomic DNA was extracted from mouse tissue using the QIAamp DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The amount of human DNA in each sample was quantified by amplification of the human beta-globin gene (HBB), while amplification of the endogenous mouse Receptor-Associated Protein at the Synapse gene (RAPSYN) served as an internal control.

One hundred nanograms of DNA extracted from bone, brain, skin, stomach, heart, muscle, bone marrow, lung, liver, kidney, and spleen was amplified using Taqman Fast Advanced Master Mix and a Step-One Plus Real Time PCR system (Applied Biosytems, Foster City, CA, www.thermofisher.com) according to the manufacturer's instructions. The following primers and probes were used: HBB-forward 5′-GTGCACCTGACTCCTGAGGAGA-3′, HBB-reverse 5′-CCTTGATACCAACCTGCCCAGG-3′, HBB-probe 5′-FAM-AAGGTGAACGTGGATGAAGTTGGTGG-TAMRA-3′, RAPSYN-forward 5′-ACCCACCCATCCTGCAAAT-3′, RAPSYN-reverse 5′-ACCTGTCCGTG CTGCAGAA-3′, RAPSYN-probe 5′-FAM-CGGTGCCAGTGATGAGGTTG GTC-TAM RA-3′.

Karyotype Analysis

Cells were harvested from confluent flasks, using Hanks Balanced Salt Solution (Gibco/Life Technologies, Thermo Fisher Scientific) and Trypsin-EDTA 1X (Gibco), and transferred to petri dishes with cover slips. Once the cells had reached 50%–60% confluency, the cover slips were harvested using standard methods (ACT Cytogenetics Laboratory Manual, 2nd edition, Barch, MJ, ed. Raven Press, 1991); briefly, exposure to colcemid for 30 mintes (Gibco), followed by warm hypotonic solution (0.7% sodium citrate) for 20 minutes, then by a 2-minute pre-fixation in 3:1 Carnoy's fixative (3:1 methanol: glacial acetic acid) and then three additional, 10-minute washes with the same fixative. The metaphase spreads were then GTG-banded using standard methods [11]. The cultured cells were analyzed following the College of American Pathologist clinical guidelines for non-neoplastic disorders; namely, 20 metaphase cells were counted and a minimum of five cells were analyzed band for band and karyotyped.

Soft Agar Colony Forming Assay

Anchorage-independent colony formation was assayed in six-well tissue culture plates containing 0.4% agarose medium over a 0.8% agar base layer. MSCs, both with and without IFNγ–priming, as well as the ES-2 carcinoma cell line, were seeded at 20,000 cells per well and incubated at 37°C. Twice weekly, 0.5 ml complete media was added to each well. After 14 days, colonies were stained with 0.01% Crystal Violet for 1 hour.

Gene Expression

Following assessment of the quality of total RNA using Agilent 2100 bioanalyzer and RNA Nano Chip kit (Agilent Technologies, Santa Clara, CA, www.agilent.com), RNA was DNAse treated and 2.5 μg was subjected to rRNA with Ribo-ZeroTM rRNA removal kit for human/mouse/rat (Illumina). To generate directional signal in RNA seq data, libraries were constructed from first strand cDNA using ScriptSeqTM v2 RNA-Seq library preparation kit (Epicentre Biotechnologies, WI, www.epibio.com). Briefly, 50 ng of rRNA-depleted RNA was fragmented and reverse transcribed using random primers containing a 5′ tagging sequence, followed by 3′ end tagging with a terminal-tagging oligo to yield di-tagged, single-stranded cDNA. Following purification by a magnetic-bead based approach, the di-tagged cDNA was amplified by limit-cycle PCR using primer pairs that anneal to tagging sequences and add adaptor sequences required for sequencing cluster generation. Amplified RNA-seq libraries were purified using AMPure XP System (Beckman Coulter, Brea, CA, www.beckman.com). Quality of libraries were determined via Agilent 2200 Tapestation using High Sensitivity D1000 screen tape, and quantified by Qubit flourometer with dsDNA BR assay (Invitrogen by Thermo Fisher Scientific).

Paired-end 150 bp sequence reads were generated using the Illumina HiSeq 4000 platform.

Bioinformatic Analysis

A median of 65 million paired-end 150 bp RNA-Seq reads were generated for each sample (range, 57 to 72 million). Each sample was aligned to the GRCh38.p6 assembly of the Homo Sapiens reference from NCBI (http://www.ncbi.nlm.nih.gov/ assembly/ GCF_000001405.31/) using version 2.5.0c of the RNA-Seq aligner STAR (http://bioinformatics.oxfordjournals.org/content/early/ 2012/10/25/bioinformatics.bts635). Transcript features were identified from the GFF file provided with the GRCh38.p5 assembly from NCBI and raw coverage counts were calculated using HTSeq (http://www-huber.embl.de/users/anders/HTSeq/doc/count.html). The raw RNA-Seq gene expression data was normalized and post-alignment statistical analyses performed using *DESeq2* [12] (http:// genomebiology.com/2014/15/12/550) and custom analysis scripts written in R. Comparisons of gene expression and associated statistical analysis were made between different conditions of interest using the normalized read counts. All fold change values are expressed as test condition/control condition, where values less than one are denoted as the negative of its inverse (note that there will be no fold change values between −1 and 1, and that the fold changes of "1" and "−1" represent the same value). Transcripts were considered significantly differentially expressed using a 10% false discovery rate (DESeq2 adjusted p value $\leq$ .1) and a fold-change cut-off of 2 between the control and test samples.

Flow Cytometry

Flow cytometry analysis was performed on an LSR II (BD Biosciences, San Jose, CA, www.bdbiosciences.com) cytometer using the following antibodies: anti-mouse CD45-APC, Sca1-PerCP/Cy5.5, CD150-PE (eBioscience/Thermo Fisher Scientific), Ter119-APC, lineage cocktail-Pacific Blue, c-Kit-APC; anti-human HLA-DR-PE, HLA-ABC-APC, PD-L1-PE, PD-L2-APC, B7-H2-PE, B7-H3-APC, CD80-PE/ Cy5, CD86-PE/Cy7, CD40-AlexaFluor647 (Biolegend, San Diego, CA, www.biolegend.com). Data were analyzed using FlowJo version 7.6.5 (Tree Star, Inc., Ashland, OR, www.scientificcomputing.com).

Clinical and Anatomic Pathology

Mice were euthanatized by carbon dioxide asphyxiation. Whole blood was collected by percutaneous cardiac puncture following euthanasia. Complete blood counts with 6-part white blood cell differential were performed on a portion of EDTA anti-coagulated whole blood (FORCYTE Autosampler 10, Oxford Science, Inc., Oxford, CT). Following coagulation of the remaining whole blood at room temperature for 30 minutes, the clotted blood was centrifuged at 3,000 rpm for 5–10 minutes at 4°C. Biochemical profiles were performed on serum samples (VetACE, Alfa Wasserman, West Caldwell, NJ). Complete postmortem evaluations were performed, and body and organ (thymus, heart, lungs, liver, spleen, kidneys, adrenals, testes and epididymides, ovaries and uterus, brain) weights were obtained on all mice. All tissues were fixed in 10% neutral buffered formalin with the exception of the skull, sternum, vertebral column and rear legs which were fixed in Decalcifier I (Leica Biosystems, Wetzlar, Germany, www.leicabiosystems.com) for 48 hours. All tissues were processed by routine methods and embedded in paraffin wax. Sections (4 μm) were stained with hematoxylin and eosin (HE), and evaluated with an Olympus BX45 light microscope with attached DP25 digital camera (B & B Microscopes Limited, Pittsburgh, PA, www.bbmicro.com,

and Nikon Instruments, Elgin, IL, www.nikoninstruments.com) by a veterinary pathologist (KMDL) certified by the American College of Veterinary Pathologists (ACVP).

Statistical Analysis

Data were analyzed for statistical significance with a Student *t* test for two group comparisons and one-way ANOVA (with Tukey's posttest analysis when appropriate) for multiple comparisons. Analyses were performed with Prism, version 6.03 (GraphPad Software, Inc., San Diego, Ca, www.graphpad.com). $p < .05$ was taken to be statistically significant.

MSC Manufacturing Process Validation

We developed a 2-step manufacturing process to prepare the clinical cell product (Fig. 1). To validate the manufacturing protocols and assess pharmacology/toxicology of the clinical product, we prepared MSCs from a single donor with and without IFNγ–priming of the cells. MSCs isolated from a donor and expanded ex vivo in parallel tissue culture flasks exhibit indistinguishable gene expression profiles [13] indicating any observed differences between the cell populations in this study must be due to the effect of IFNγ. For MSC manufacturing intended for clinical trials, the bone marrow donor must satisfy the hematopoietic cell donor criteria described in the current standards of Federation for Accreditation of Cellular Therapy (FACT).

Second Expansion and IFNγ Priming

Recovery of viable MSCs from cryopreserved stocks was 97% ± 9% (mean ± standard deviation, $n = 6$). Over the 7-day expansion, the MSCs underwent 3.1 ± 0.9 ($n = 5$) population doublings with a doubling time of 2.5 ± 0.8 days ($n = 5$). The final yield of cells was 23,300 ± 11,000 MSCs per cm^2 ($n = 5$) and the viability was uniformly ≥93%. There was no significant difference in any of these expansion parameters or viability following IFNγ exposure for the last 2 days of culture. At the completion of the 7-day expansion, with/without INFγ exposure, the cells were collected from the culture vessels by trypsinization, washed twice in phosphate buffered saline to remove residual IFNγ and resuspended in infusion media. ELISA measurement of IFNγ (linear range 15–1,000 pg/ml, Table 2) of samples taken at multiple steps during trypsinization and wash showed that the PBS rinse of the culture vessel after removal of the IFNγ-containing media contained 86 pg/ml (approximately 300-fold reduction), while both cell washes as well as the γMSCs in infusion media contained undetectable levels (calculated level of <1 pg/ml which is below the level of assay sensitivity).

MSC Stability

At the conclusion of the expansion, MSCs and γMSCs were each collected by trypsinization, washed, and suspended in infusion media at a concentration of 4×10^6 cells per ml. The cell suspensions (MSCs, $n = 2$; γMSCs, $n = 2$) were each individually drawn up into a syringe and maintained on a vertical rotator at room temperature. Due to the indistinguishable results, we analyzed the 4 groups together. After 4 hours, 93.3% ± 10.2% of cells were recovered and the viability was equivalent from the initial suspension (95.5% ± 5.1% vs. 98.2% ± 2.1%, respectively, $p = .22$, $n = 4$). These data indicate that the cells remain stable for ≥4 hours after collection from the expansion culture and suspension
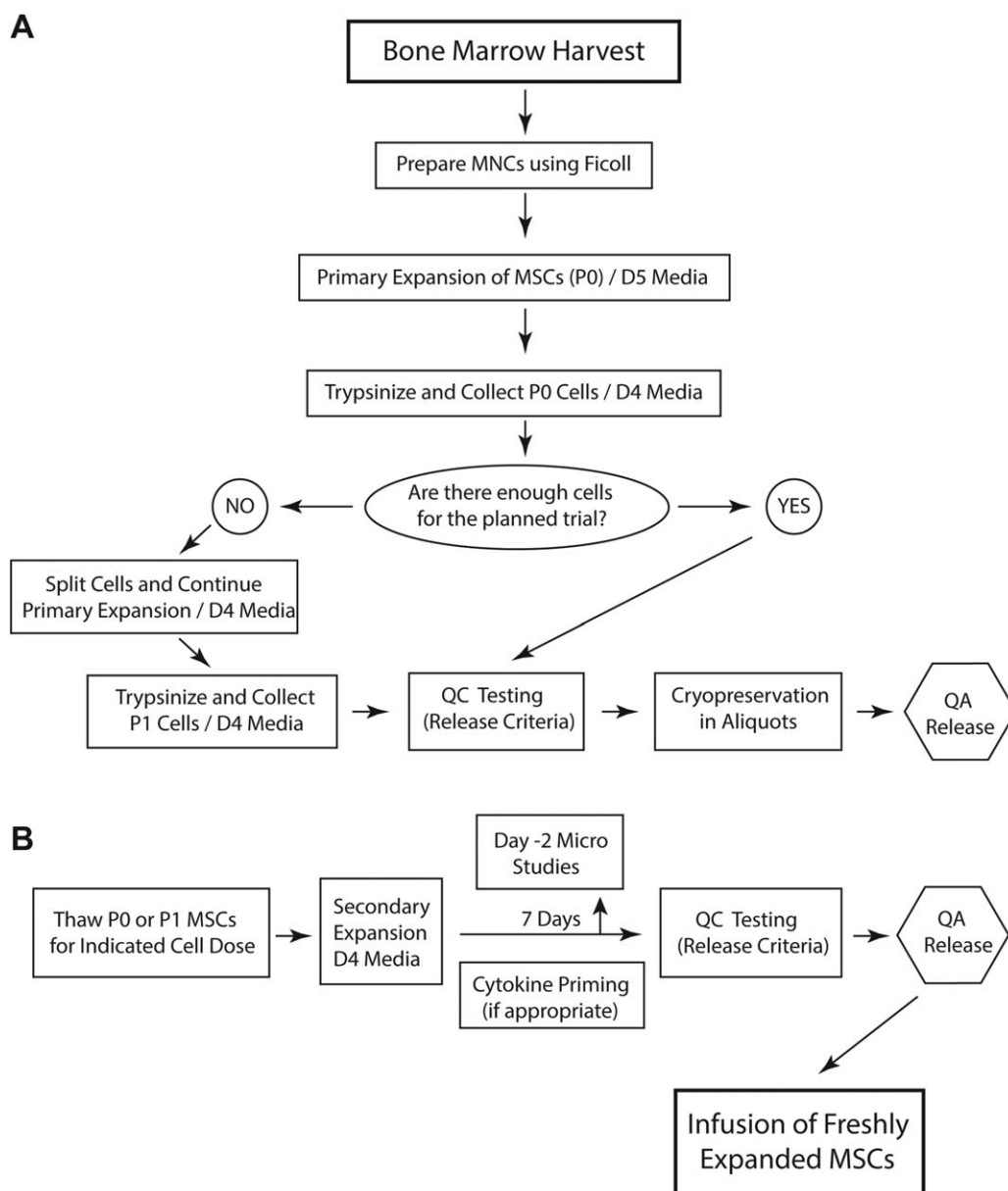
A

B

Figure 1. Schematic representation of the 2-step GMP manufacturing process. **(A):** The isolation and primary expansion of MSCs through cryopreservation **(B)** the thawing and secondary expansion of MSCs. the opportunity for cytokine priming, if appropriate, is shown. See text for details. Abbreviations: MNC, mononuclear cells; MSCs, mesenchymal stem/stromal cells.

in infusion media which is sufficient time to complete release testing and transport to the subjects' bedside.

Next, MSCs ($n = 2$) and γMSCs ($n = 2$) were slowly passed through a syringe and tubing to simulate a clinical slow intravenous "push" infusion. We found 94.6% ± 3.2% of cells were recovered without loss of viability (97.7% ± 1.5% vs. 98.2% ± 1.1%, $p = .56$, $n = 4$) in the effluent supporting the assertion that a subject will receive the intended dose of viable cells.

Product-to-Product Variability

MSCs obtained from three different healthy donors were isolated and IFNγ-primed according to our GMP-protocol (Fig. 1). RNA isolated from the MSCs and the corresponding γMSCs from the same donor was assessed by RNA Seq. Principal component analysis (PCA) using the normalized expression values for the 500 genes

with the highest variance, shows distinct clustering of the three γMSC samples from MSCs, with the first principal component accounting for 86% of the variance (Fig. 2A). This analysis also showed a separation of the three patients' samples, but this only accounted for 8% of the variance. While the preponderance of variation was due to IFNγ priming, the cytokine produces a similar variance in each of the patients; thus, IFNγ priming does not introduce additional interpatient variation than what intrinsically exists in MSCs. Like PCA, Euclidean distance was calculated to assess the overall similarity between samples (Fig. 2B). This analysis revealed tight hierarchical clustering of the three treated or untreated samples and distinct separation was clearly visible between the two groups. To illustrate the relative gene expression profile of the γMSC versus MSCs, the 20 most differentially expressed genes are represented graphically as a heat map (Fig.

Table 2. Residual Interferon γ in cell washings and Infusion medium[a]

Test Media	Expanded MSCs	Expanded MSCs with (48 hours) IFNγ priming
Fresh media ± IFNγ, 500 U/ml (control)	NA[b]	23,489[c]
Final culture medium	7	22,481[c]
PBS plate rinse	ND[d]	86
Wash #1	ND	ND
Wash #2	ND	ND
Infusion medium (0 hours)	ND	ND
Infusion medium (6 hours)	ND	ND

[a]Interferon γ was measured by ELISA and reported as pg/ml.
[b]NA, not applicable. Interferon γ was not added to this fresh medium.
[c]Determined by diluting media 1:50; measured value was in the linear range.
[d]ND, not detected.
Abbreviations: IFNγ, interferon γ; MSCs, mesenchymal stem/stromal cells.

2C). Supporting Information contains a complete list of differentially expressed genes when comparing IFNγ-primed MSCs with conventional MSCs (Supporting Information Fig. 1). Overall, the most highly upregulated pathway is associated with antigen presentation. Of the immune modulating cytokines, IL6, IL15, and IL32 were upregulated while IL16 and IL11 were downregulated; however, only IL6 was expressed at a substantial level. We did not identify upregulation or downregulation of proto-oncogenes or tumor suppressor genes, respectively.

Karyotype

The final MSC product that would be infused in a patient was assessed for the integrity of the chromosomes (Supporting Information Fig. 2). Twenty cells in metaphase from the standard MSC preparation and IFNγ-primed MSCs were examined. All exhibited modal number of 46 chromosomes including two X chromosomes. No consistent structural or numerical abnormalities were detected. All five cells assessed for G-banding from each preparation displayed a normal pattern.

γMSC Phenotype

IFNγ-primed MSCs express HLA Class I and II molecules, as well as PD-L1 and PD-L2, but not CD80 or CD86 as expected (Fig. 3). γMSCs did not express B7-H2, but highly expressed B7-H3, an immune regulating molecule that may provide positive or negative costimulatory signals [14]. Finally, γMSCs expressed low level CD40.

MSCs and γMSCs underwent in vitro differentiation to osteoblasts, adipocytes, and chondroblasts. Both cell preparations differentiated similarly as determined by Alizarin Red S, Oil Red O, and Alcian Blue histochemical staining, respectively.

In Vitro Malignant Transformation

To further asses the propensity of the clinical product to undergo malignant transformation during cell manufacturing, samples of MSCs and γMSCs, obtained at the completion of cGMP-production that would be infused into a human subject, were placed in routine tissue culture (DMEM-LG, 10% FBS) and serially passaged until senescence. Initially, the doubling time was 2–3 days and then slowed to 19–21 days by 3 months. At 2 months

(10 passages), the cells were observed to spread on the plastic tissue culture surface assuming widened morphology, in contrast to the spindle shape of younger, actively replicated cells (Supporting Information Fig. 3). Subsequently, the cells progressively spontaneously released from the plastic surface. Throughout the 6-month interval of in vitro observation, MSCs and γMSCs similarly expanded showing no effect of IFNγ exposure during the cell manufacturing. Moreover, both cells populations expanded evenly without emergence of a rapidly replicating subpopulation.

Next, clinical GMP-manufactured products were assessed by soft agar colony forming assay to detect anchorage-independent clonal proliferation (Fig. 4). The positive control (ES-2 carcinoma) demonstrates robust colony formation with several large colonies and too numerous to count small colonies. Agar plates with MSCs or γMSCs were devoid of any colony formation similar to the negative control.

Biodistribution

To determine the biodistribution of the clinical product in healthy NSG mice, we infused 250,000 MSCs ($n = 6$) or γMSCs ($n = 6$), or 200 μl saline ($n = 6$, controls). At graded intervals, we assessed DNA isolated from freshly harvested tissues for the evidence of MSCs (human DNA) with a quantitative PCR assay using primers specific for a single copy human (HBB) or mouse (RABSYN) gene (Supporting Information Table 2). The assay was considered negative if a threshold (Ct) was not attained by 36 cycles of amplification (limit of linear response). Analysis of standards, comprised of mixing DNA derived from human MSCs with murine DNA demonstrated a quantitative sensitivity of 0.05% human tissue (MSCs) within murine tissue samples and complete specificity (no false positives, $n = 5$) within the experimental limits. We intermittently detected 0.01% human tissue, but the Ct ($>$36) deviated from linearity. Assay of tissues obtained from NSG mice without MSC infusions (negative controls) confirmed the specificity. At 24 hours after infusion, we unambiguously identified lung-derived DNA as human representing MSCs that had, as expected, presumably lodged in the pulmonary capillary beds. Human DNA (MSCs) was undetectable in all other tissues. By day 7, all tissues were devoid of detectable human cells.

Engraftment and Hematopoietic Reconstitution After Marrow Cell/MSC Cotransplantation

Five cohorts of C57BL/6J mice were transplanted 48 hours after total body irradiation (500 cGy × 2 doses, 3 hours apart) with 1×10^6 GFP+ marrow cells and 250,000 ex vivo expanded murine MSCs, murine γMSCs, GMP human MSCs, or GMP human γMSCs. The final cohort did not receive MSCs (negative control). All cohorts recovered leukocytes by day 17 (Fig. 5A) with $>$98% donor-derived hematopoiesis indicating that neither the murine nor human cells impaired engraftment or the kinetics of hematopoietic reconstitution (Fig. 5B). Unexpectedly on day 21, the four cohorts of mice co-transplanted with one of the MSC preparations, had significantly more leukocytes than the mice transplanted only with hematopoietic cells (Fig. 5A). The increased count was specifically due to increased lymphocytes (Fig. 5C) but the CD4:CD8 ratio did not vary among the five cohorts (3–5:1) and CD4+CD25+ cells were consistently <1% of all T cells.

In Vivo Toxicity of MSCs

NSG mice were intravenously (tail vein) infused with 250,000 MSCs ($n = 6$), γMSCs ($n = 6$), or saline ($n = 6$) without any
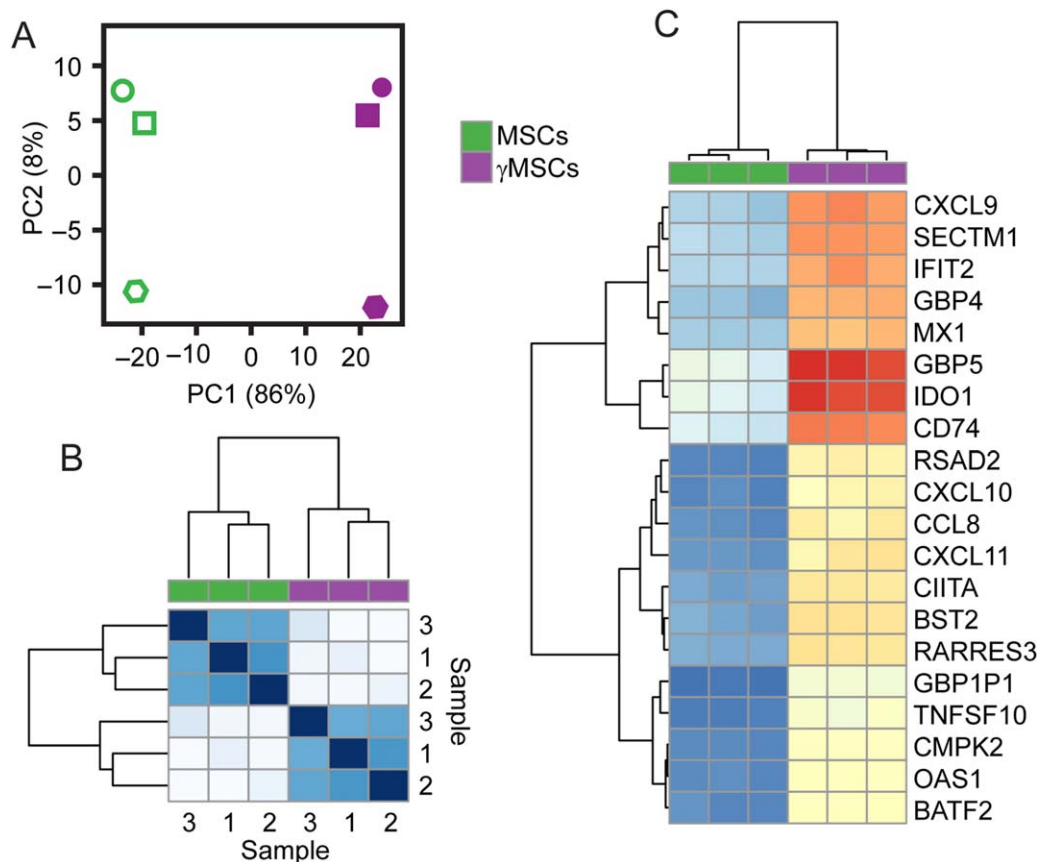
Figure 2. Clustering of global transcriptional responses of MSCs following priming with IFNγ. **(A):** Principal Component Analysis (PCA) of differential gene expression of MSCs (open shapes) and γMSC (filled shapes) from each donor (same color). **(B):** Euclidean distance between samples to assess overall similarity among the samples. The darker blue indicates a shorter distance, more similar; lighter blue indicates greater distance, less similar. **(C):** Heat map analysis of RNA-Seq gene expression data from MSCs and γMSC. The heatmap was generated using the normalized expression values for the 20 most upregulated and downregulated genes following interferon γ priming. Color code indicates relative expression; red highest, blue lowest. Abbreviation: MSCs, mesenchymal stem/stromal cells.

preparative regimen. This dose approximates 1×10^7 MSCs/kg. There were no peri-infusion adverse events. All mice thrived without evidence of toxicity. The heightened human IL6 secreted from the γMSCs, which can react with murine tissues, did not cause any discernable toxicity. At 10 weeks after infusion, 3 mice from each group were euthanized and underwent a comprehensive post mortem examination. The remaining 3 mice in each group were followed for long-term evaluation. One mouse which was infused with MSCs died of undetermined causes at 21 weeks. The 3 control mice, 2 remaining MSC-infused mice, and all 3 γMSC-infused mice are alive and well 16 months after infusion suggesting that the infused cells have not given rise to a malignancy up to this time point.

Post-Mortem Examination MSC- and γMSC-Infused NSG Mice

In an effort to identify occult organ toxicity or tumor formation, three mice from each group (controls, MSC- and γMSC-infused) were assessed by post-mortem examination. At the time of euthanasia (10 weeks after cell infusion), all mice were active, healthy-appearing, and feeding well. The complete blood counts and white blood cell subset proportions were within the expected range for NSG mice and the three groups were not statistically different. Similarly, serum chemistries were within the normal range for all mice. The mean body weight did not differ among the groups.

We did not find any abnormalities by gross examination of the internal organs. Moreover, there were no abnormal tissue masses that could represent a tumor indicating the absence of occult malignancy at 10 weeks post-infusion. The mean weight as a percentage of body weight for each organ was comparable among the groups.

By microscopic examination of paraffin embedded tissue sections, we did not find any pathologic lesions or any histologic lesions attributable to either cell treatment. Furthermore, we did not find evidence of evolving malignancies in situ. Importantly, examination of the lungs revealed an unequivocal absence of histopathology in all mice (cell infused mice and controls) demonstrating the lack of occult pathology which would be potentially attributable to the MSCs (Fig. 6A). Histologic examination of the spleen revealed the expected lymphoid hypoplasia consistent with the NSG phenotype and extramedullary hematopoiesis which is physiologic in mice. In two MSC-infused mice, but none among the controls or γMSC-infused animals, we identified splenic foci of osseous metaplasia (Fig. 6B), which is not uncommon in murine tissues, including NSG mouse spleens [15–18].

DISCUSSION

The principal conclusion from our preclinical data is that the risk of cell therapy using γMSCs is no greater than MSCs, which have
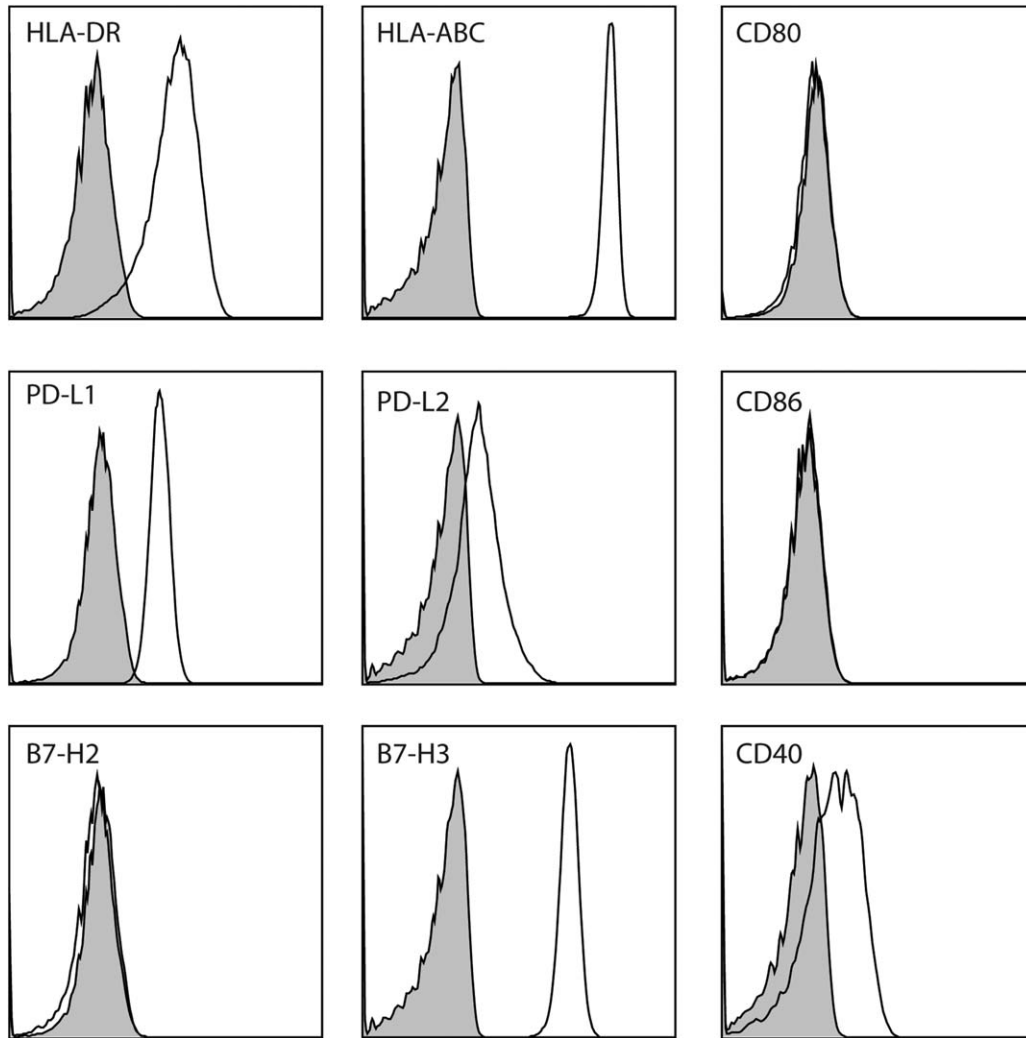
Figure 3. Immunophenotype of interferon γ primed Mesenchymal stem/stromal cell (MSCs). Flow cytometric analysis of select surface marker expression on γMSCs (solid line) and the isotype control (shaded peak).

an outstanding, well-established safety prolife. Moreover, MSCs prepared according to our two-step protocol and infused intravenously do not show new, previously unrecognized risks to cell therapy. Lacking identified toxicity, or even implications of adverse outcomes, our data provide compelling support to proceed with clinical investigation to assess γMSCs in human subjects for appropriate target disorders.

We have described a two-step cGMP-compliant manufacturing protocol to generate IFNγ-primed MSCs for clinical trials. Our protocol overcomes two current major barriers in the production of MSC-based cellular therapeutics: (a) cells must be available on demand and (b) cryopreserved, freshly thawed cells may have reduced potency as the infusion product. In actuality, these two challenges are closely related. The most common approach to providing cells on demand is to cryopreserve manufactured cells which can be thawed and infused at the designated time. However, previously cryopreserved, freshly thawed MSCs may lack the requisite immune suppressive activity to be of therapeutic value [5, 13]. Thus, we isolate, partially expand, and cryopreserve MSCs specified for a given protocol. MSC isolation by methods [19, 20] other than Ficoll and adherence will not detract from the value of the two-step protocol. Moreover, cells remaining cryopreserved

for the duration of typical early phase clinical trial will not affect the secondary expansion potential or potency of the freshly expanded product [21]. Upon scheduling a patient for MSC therapy, we can thaw and further expand for 7 days, which is sufficient to abrogate the detrimental effects of cryopreservation [5]. Then, we can culture in the presence of IFNγ if appropriate and collect freshly expanded, IFNγ primed MSCs for infusion. Thus, we can reliably infuse a patient/subject at a previously scheduled time, that is, on demand, with freshly expanded MSCs.

Ideally, a cryoprotectant would be identified allowing cryopreserved, freshly thawed MSCs to maintain the immune modulatory activity for therapeutic applications. In lieu of such an agent, our manufacturing protocol will subvert these obstacles allowing for unimpeded patient-based MSC research.

The most worrisome complication of any cell therapy is the risk of malignant transformation of the therapeutic cell product after infusion into a patient. We have presented six lines of evidence to address this issue. First, RNA Seq analysis did not show upregulation of a proto-oncogene or downregulation of a tumor suppressor gene. Second, karyotype analysis did not show evidence of any abnormality, random or clonal. Third, prolonged in vitro culture did not reveal outgrowth of a predominate clone.
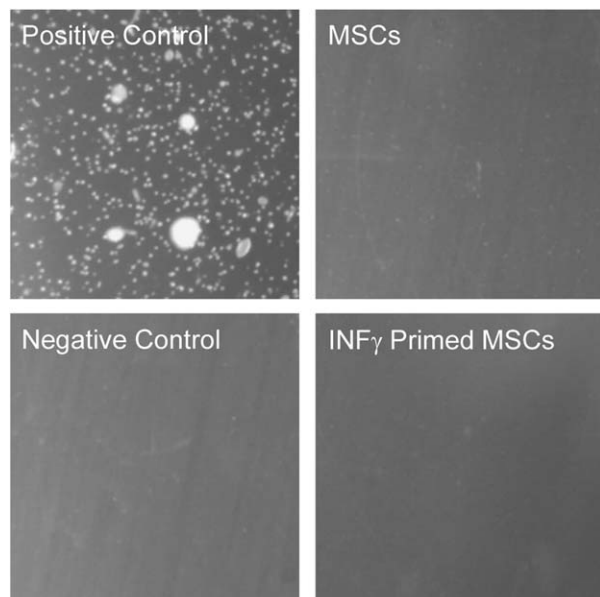
Figure 4. Soft agar colony forming assay to detect malignant transformation. Representative photographs of triplicate agar plates for each condition are shown. The positive control was a human ES-2 ovarian clear cell carcinoma cell line. The negative control was saline. Original magnification was ×46. Abbreviations: IFNγ, interferon γ; MSCs, mesenchymal stem/stromal cells.

Fourth, soft agar colony forming assay definitely showed the absence of anchorage-independent clonal proliferation. Fifth, at 10 weeks after infusion into NSG mice, post-mortem examination did not find evidence of occult tumors. Finally and most importantly, we have not identified clinical evidence of tumor formation in NSG mice 16 months after infusion. Collectively, these data comprise a compelling package to suggest that the risk of malignant transformation of γMSC is not different than that of MSCs, for which this catastrophic complication has never been reported.

Our biodistribution data suggest that both MSCs and γMSC lodge in the pulmonary capillary beds with the first 24 hours after intravenous infusion and then either are cleared by pulmonary macrophages or pass through the capillaries. We did not find evidence of specific or random tissue localization subsequent to 24 hours at the limit of our assay sensitivity; however, our findings differ from the tissue distribution of intravenously infused MSCs often reported [22]. Seeking to emulate a clinical study, we infused 250,000 MSCs/animal which is approximately 10×10^6 MSCs/kg (~25 g/mouse), a large but clinically relevant dose. Moreover, the quantitative sensitivity of our assay was 0.05% and the absolute sensitivity was 0.01%, similar to others [10] and a clinically relevant level of detection. By contrast, studies focused on the biology of MSCs have infused 5×10^6 MSCs/animal which is approximately 200×10^6 MSCs/kg or used an assay with far greater sensitivity [22, 23]. We suggest our findings indicate any MSCs that may reside throughout the body after our clinical-like infusion exist below the clinically relevant detection, except for the risk of malignant transformation which we have shown is no greater than for MSCs in current clinical trials. Thus, the biodistribution of γMSC is not predicted to confer any additional risk compared to MSCs.

The manufacturing protocol we describe includes the preparation of a supply of MSCs cryopreserved at very low passage (typically P0 or P1) earmarked for a restricted number of subjects enrolled in a specific clinical trial. This approach allows for all cells to be used in an early phase clinical trial to be obtained from a single or very few donors and limits the extent of ex vivo expansion. Establishing a master cell bank will likely be required for Phase II and certainly Phase III trials. However, preparing a dedicated supply of cells for a single protocol without preparing a master cell bank, as we describe, is feasible for smaller Phase I trials and should facilitate implementation of formal protocols. The key advance for the MSC field is that such manufacturing should eliminate the need for anecdotal reports of MSC therapy as prospective hypothesis-driven early phase clinical trials are needed to identify the most promising directions for cell therapy research. Of note, samples of cells from the product intended for infusion should be archived for a complete biologic characterization (e.g., transcriptomics, proteomics, and/or metabolomics) in anticipation of a successful Phase I trial creating the need to more rigorously understand the biology of the specific therapeutic cells and reproducibly manufacture the cell product.

A fundamental concern when processing cells using a cytokine treatment is whether residual cytokine will be infused into the human subject. For IFNγ, this concern is heightened because of the potential impact on hematopoiesis [24], such as triggering aplastic anemia [25]. A normal range for serum IFNγ is not available; however, a recent study reported healthy control subjects with a median serum concentration of 11.40 pg/ml (95% confidence interval of 7.80–31.75, $n = 44$, >21 years, M:F, 1:1) [26]. A second study reported serum level of 23 ± 13 pg/ml (mean $\pm$ standard deviation, $n = 11$ ages 13–40) [27]. In our study, we found undetectable levels of IFNγ after the first cell washing (Table II). Even if we assume 10 pg/ml residual IFNγ in the infusion media (an enormous overestimation), a dose of 2×10^6 MSC/kg (infusion medium cell concentration of 4×10^6 MSCs/ml, assume distribution in the blood only) would raise the subject's serum IFNγ level less than 0.2 pg/ml, a clinically inconsequential variation.

Two findings on the post-mortem exam merit consideration. First, the lungs in all mice lacked clinically-silent histopathologic lesions. Since MSCs transiently lodge in the lungs after intravenous infusion [28, 29], investigators are often especially concerned about pulmonary complications. Our data show that infusion of human MSCs and γMSCs into mice do not seem to induce occult changes in the lungs up to 10 weeks after infusion. Thus, the risk of long-term complications would seem to be exceedingly low.

The second interesting finding is the foci of osseous metaplasia in the spleen of two MSC-infused mice but none of the controls or γMSC-infused animals. This murine observation is especially notable given that ectopic ossification is a long-standing concern with MSC therapy. The National Toxicology Program (Department of Health and Human Services) advises preclinical toxicology investigators to remain cognizant of pathology intrinsic to the animal model to avoid incorrectly attributing an observed event to the investigational agent [30]. Osseous metaplasia is not uncommon in murine tissues, especially in lung and kidney of various strains of mice, but has also been reported in the spleen of NSG mice [15–18]. Moreover, our veterinary pathology service has frequently observed splenic osseous in mice of multiple strains (unpublished observation, Krista LaPerle, D.V.M., Ph.D.). Importantly, osseous metaplasia attributable to ex vivo expanded, intravenously-infused MSCs has never been reported in a human
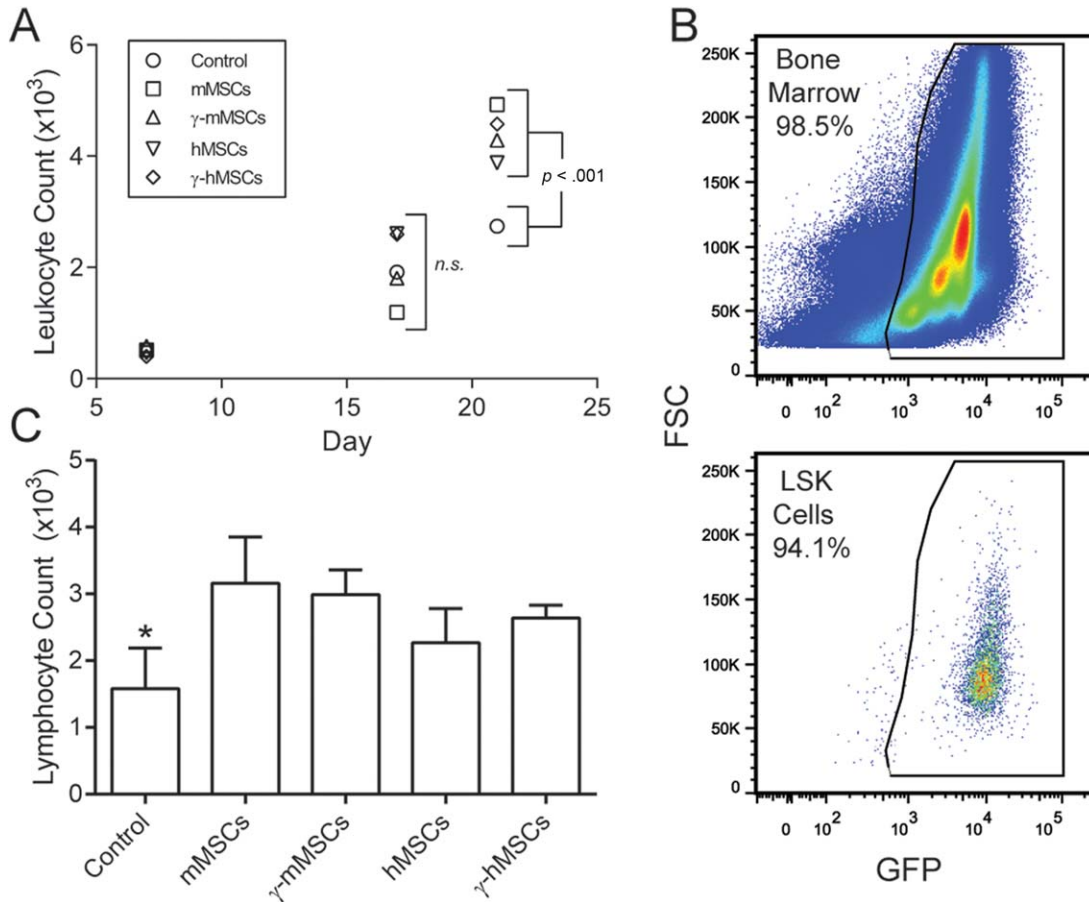
Figure 5. Analysis of hematopoietic engraftment after cotransplantation of hematopoietic cells and MSCs or γMSCs. C57BL/6 mice were lethally irradiated and then transplanted with 2×10^6 unfractionated GFP-expressing C57BL/6 marrow cells and 250,000 GMP-manufactured human MSCs or γMSCs (both clinical products) or 250,000 murine MSCs or γMSCs. **(A):** Kinetics of leukocyte recovery after transplantation for each cohort of mice. The control mice underwent hematopoietic cell transplantation, but did not receive MSC/γMSC cotransplantation. n.s., not significant. **(B):** The absolute lymphocyte count of each cohort assessed 21 days after transplantation. The values represent the mean ± standard deviation for each cohort, $n = 5$, *, $p < .05$ versus each cohort, one-way ANOVA with Tukey's post hoc test. **(C**, top) Representative dot plot of flow cytometric analysis of unfractionated marrow harvested 21 days after transplantation revealing the percentage of donor (GFP-expressing) marrow cells. (bottom) Similar analysis of the lin-sca-1+c-kit+ (LSK) cells which demarcate the primitive stem/progenitors cells from marrow. Abbreviations: GFP, green fluorescent protein; FSC, forward scatter; LSK, lin-sca-1+c-kit+; mMSCs, murine mesehcymal stromal cells; hMSCs human mesenchymal stromal cells; γ-hMSCs, interferon gamma primed human mesenchymal stromal cells.
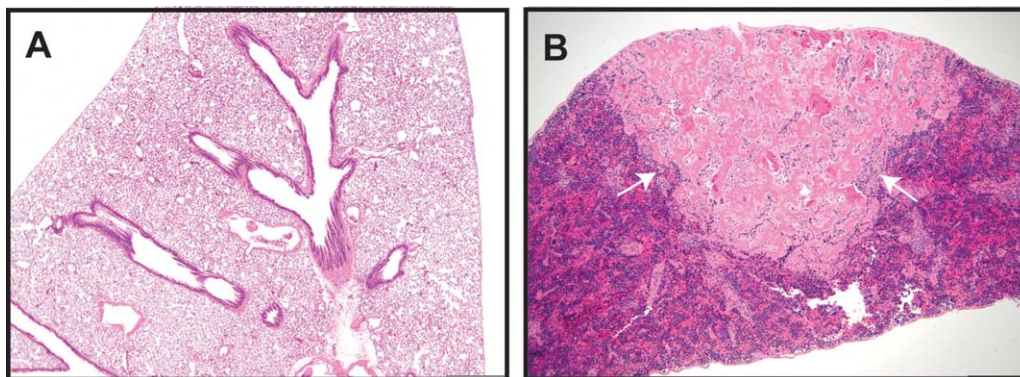

Figure 6. Post-mortem examination. Ten weeks after NSG mice were infused with mesenchymal stem/stromal cells (MSCs)/γMSCs, animals were sacrificed and underwent complete post-mortem examination. **(A):** Representative photomicrograph of a histologic section of lung tissue taken at ×40 original magnification. This section was taken from a mouse which received γMSCs. **(B):** Photomicrograph of a histologic section of spleen showing the osseous metaplasia (white arrows) taken at ×100 original magnification. This section was taken from one of the two MSC-infused mice where this finding was observed.

subject. Hence, we regard this observation as an incidental finding in mice and do not attribute it to the MSCs.

Finally focusing on hematopoietic cell transplantation, our data suggest that infusing MSCs or γMSCs proximate to hematopoietic stem cells did not impair hematopoietic reconstitution, consistent with MSC clinical data. In an effort to thoroughly assess the impact of our clinical cell product (human) cells in a murine transplant model, we assessed both the clinical and similarly prepared murine MSCs/γMSCs. Interestingly, a significantly greater lymphocyte count was observed in all cell infused mice compared to controls at 21 days after transplantation. The observation was most likely due to peripheral expansion of transplanted lymphocytes as donor stem cell-derived, newly differentiated lymphocytes would not be expected to be circulating at the early time point. Moreover, the higher cell count must be due to an INFγ-independent activity as both MSC- and γMSC-infused mice had similarly elevated lymphocytes. Despite the intriguing preclinical murine observation, the clinical significance, if any, remains to be determined.

CONCLUSION

Our study has demonstrated a GMP-manufacturing process to prepare MSCs or γMSCs that can be reliably available at a time designated in advance while avoiding the potential pitfall of using cryopreserved, freshly thawed cells. Moreover, for γMSCs, there seems to be negligible residual INFγ after the cell washes obviating concerns regarding cytokine-related toxicity. While we suggest our preclinical data is compelling, the definitive proof of safety will require Phase I clinical trials for specific disease indications.

AUTHOR CONTRIBUTIONS

A.J.G.: ran analytical experiments contributed to preparation of manuscript; B.D., R.W., and H.B.: prepared MSCs; KMDLaP: supervised the post-mortem exam, analyzed data, contributed to preparation of the manuscript; S.L.H., E.H., and C.A.: performed the cytogenetics; H.R. and S.M.D.: analyzed data; S.O. and M.D.: analyzed data and contributed to preparation of the manuscript; L.O.: developed and supervised MSC preparation, analyzed data, contributed to preparation of the manuscript; E.M.H.: designed and oversaw entire project, developed MSC preparation protocols, analyzed data, prepared manuscript.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

Dr. White is a consultant for GenomeNext LLC. The other authors indicated no potential conflicts of interest.

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