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Optimization of Human Mesenchymal Stem Cells for Rheumatoid Arthritis: Implications for Improved Therapeutic Outcomes

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Objective. Seropositive rheumatoid arthritis (RA) is a chronic autoimmune disease that is rarely "cured." Human mesenchymal stem cells (hMSCs) are known to reduce inflammation and restore immune homeostasis. However, methods for predicting therapeutic hMSC potency have not been established. The goal of these studies was to use and refine an *ex vivo* functional assay that determines potency of hMSCs and can then be validated in clinical trials as a potency measure of hMSCs used therapeutically to treat RA.

Methods. Allogeneic hMSCs were cytokine-stimulated, and a conditioned medium (CM) was harvested. The CM was tested for the potential to attenuate RA CD4+ T cell proliferation using suppression assays. Indoleamine 2, 3-dioxygenase (IDO) mRNA, and protein were quantified in hMSCs as a measure to compare hMSCs across (prior) studies.

Results. To mimic a proinflammatory environment that resembles that in RA, interleukin-1(IL1β), tumor necrosis factor α (TNFα), and interferon γ (IFNγ) (alone or in combination) were used to precondition hMSCs. Treating hMSCs with a combination of these cytokines generated a CM "secretome" that suppressed T cell proliferation between 70 and 83%. Forty-eight hours of cytokine preconditioning hMSCs was required to maximize this effect. T cell suppression positively correlated with increases in hMSC cellular IDO mRNA and protein.

Conclusion. By standardizing assays to measure hMSC effects, their potency on T cell suppression can be quantified. These studies demonstrate that hMSCs can be compared functionally to identify optimal preparation(s) for therapeutic use in RA and that the potency of hMSC-dependent T cell suppression may differ between hMSC donors. Clinical studies are warranted to validate the hypothesis that ex vivo potency in suppressing T cells will positively correlate with a reduction in RA disease activity and increase in immunological quiescence.

INTRODUCTION

Seropositive RA is a chronic inflammatory disease that reduces the quality of life and, if untreated, leads to joint damage, resulting in disability (1). Characterized by the presence of synovitis, systemic inflammation, and autoantibodies, RA is the most common systemic autoimmune disease, with a worldwide prevalence of approximately 1% (2,3). Although the etiopathogenesis of RA is incompletely understood, dysfunction in immune tolerance has been implicated in susceptibility to RA. Derangement in cytokine production by T cells along with abnormal T-effector function and persistence of abnormal T cell populations in synovial infiltrates have all been observed (4). RA is associated with autoantibodies such as rheumatoid factor (RF) and anticitrullinated protein antibodies, which themselves may react with structural proteins found in joints. Therapy for seropositive RA is generally lifelong and has undesirable side effects (5,6). Many patients respond to conventional and/or biologic disease-modifying antirheumatic drugs

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(DMARDs) (7), but over time up to 35% of these responders to therapy experience loss of clinical effectiveness of DMARDs (conventional and biologic), and experience damage (8,9). Finding alternative therapies that induce long-term immune tolerance with few or no unwanted side effects is desirable.

The use of human mesenchymal stem cells (hMSCs) is an emerging therapeutic strategy that appears to be promising in several autoimmune and nonimmune human diseases. hMSCs have strong anti-inflammatory and immunomodulatory properties (10). The therapeutic effects of hMSCs appear to lie, in part, in their capacity to home to sites of immune dysregulation and their ability to restore immune homeostasis (11).

hMSCs derived from bone marrow, adipocyte tissue, and umbilical cord showed safety and a trend towards efficacy when used therapeutically to treat RA (12). hMSCs treated *ex vivo* with cytokines such as interferon γ (IFNγ) and tumor necrosis factor α (TNFα) mimic conditions that hMSCs encounter in vivo and induce hMSCs to express an immunomodulatory phenotype (13–15). Using hMSCs for therapy in RA is largely empiric. We now demonstrate that the addition of functional assays that may have the potential to predict efficacy *in vivo* could be incorporated for testing as potency measures in clinical trials of RA. Additionally, the studies presented herein report additional establishing standards by which therapeutic hMSCs may be prequalified to be used in RA and related autoimmune disease(s).

PATIENTS AND METHODS

Patients. Female patients with RA (supplementary data; Table S1) and matched healthy control subjects (HCs) were consented and enrolled. The studies presented were approved by the Human Use Committee at the MetroHealth System, Federal Wide Assurance (FWA00003938). The patients with RA met the American College of Rheumatology/European Alliance of Association for Rheumatology. Patients were seropositive for either RF and/or anticitrullinated peptide antibodies, had less than 1 year of doctor-diagnosed RA, and had a mean disease activity score 28 using C-reactive protein score in the moderate to high disease activity range.

hMSC conditioned medium. Bone marrow–derived hMSCs were purchased from the Cell Therapies Integrated Services of Case Comprehensive Cancer Center Core, Case Western Reserve University Medical Center (FWA00003937). hMSCs at passages 3 and 4 were plated at 1×10^6 cells per plate in 10 ml of serum-free mesenchymal stem cell (MSC) medium containing low-glucose dulbecco's modified medium (Corning, NY, USA) supplemented with 5% PLUS Human Platelet Lysate (Compass Biomedical), L-Glutamine (Corning), and 10 ng/ml human fibroblast growth factor (R&D Systems). Once cells reached 70% confluency, the medium was replaced with a serum-free MSC medium containing 20 ng/ml TNFα, or 20 ng/ml IFNγ, or 10 ng/ml IL1β (Thermo Fisher Scientific); medium containing a combination of TNFα, IFNγ, and IL1β (all-3); or in a serum-free hMSC medium without cytokine(s) (unconditioned [UC]) and incubated for 24 or 48 hours. The hMSC conditioned medium (CM) was collected, spun at 450 x g for 10 minutes, filtered through a 0.22-μm filter Millex-GV (Millipore Sigma), aliquoted, and frozen down at −80°C.

CD4+ T cell isolation. Peripheral blood from HCs and patients with early RA (in the first year of disease) were obtained. CD4+ T cell isolation was performed using density gradient medium Lymphoprep in SepMate tubes (STEMCELL Technologies) and RosetteSep Human CD4+ T Cell Enrichment Cocktail (STEMCELL Technologies) according to the manufacturer's directions. Cells were either used immediately in suppression assays or frozen and stored at −80°C for future use.

Suppression assays. CD4+ T cells were seeded in duplicates at 10×10^4 cells/well in a 96-well round bottom plate. Cells were stimulated using biotinylated antibodies directed against human CD2, CD3, and CD28 according to the manufacturer's instructions (Miltenyi Biotec), with the range of CM, and incubated at 37°C in 5% CO_2 for 4 days. CD4+ T cells were loaded with Cell Proliferation Dye eFluor 670 according to the manufacturer's instructions (eBioscience), and proliferation rates were measured by flow cytometer BD LSR II (BD Biosciences) or Attune NxT (Thermo Fisher Scientific). Proliferation was calculated using WinList 7.0 software (Verity Software House). Suppression indices were calculated as previously published (16). Briefly, the proliferation of T cells under the experimental condition was divided by the proliferation of T cells under the control condition and multiplied by 100. This number was then subtracted from 100 to arrive at the percentage suppression of T cells, as follows: 100 − [(proliferation experimental/proliferation control) × 100].

IDO mRNA and protein. hMSCs were plated and treated with or without cytokines for 48 hours. After culture, hMSCs were washed with dulbecco's phosphate-buffered saline and detached using TrypLE (Thermo Fisher Scientific) at 37°C for 5 minutes. RNA and protein were isolated using NucleoSpin RNA/Protein kit (Takara Bio) followed by quantitative real-time polymerase chain reaction using Roche LightCycler 480 System (Roche Diagnostics Corporation). The primers used were 5'-gcccttcaagtgtttcaccaa-3' and 5'-ccagccagacaaatatatgcga-3' for IDO and 5'-ggacttcgagcaagagatgg-3' and 5'-agcactgtgttggcgtacag-3' for β-actin (Integrated DNA Technologies). For Western blot, the antibodies used included mouse antibody (mAb) anti-IDO 1:500 (Cell Signaling Technology) and mAb β-actin (C4) 1:3000 (Santa Cruz Biotechnology). Proteins were quantified and normalized to β-actin using UVP Software VisionWorks LS (Analytik Jena).

Statistical analysis. Statistical analysis was performed using GraphPad Prism 8.1.2 (GraphPad Software). A two-tailed

student's t test was used for comparison between pairs. One-way ANOVA was used for multiple comparisons followed by Dunnett's multiple comparison tests or Tukey's multiple comparison post-test. Significance was denoted as a value of $P \leq 0.05$. Data are presented as mean ± SEM of at least three independent experiments.

RESULTS

Maximizing suppression of CD4+ T cells by hMSC medium. To generate hMSC CM, hMSCs were pretreated for 48 hours with the following conditions: medium containing IFNγ, IL1β, TNFα, a cytokine cocktail containing all three, or UC medium lacking added cytokines. To quantify the effects of hMSCs on CD4+ T cell proliferation, CD4+ T cells were induced to proliferate in the presence of CM or UC as indicated (Figures 1A and 1B). Greater CD4+ T cell suppression was observed using all three CM (76% ± 2% [mean ± SEM]; $P < 0.0001$) compared with IL1β CM (24% ± 7%; $P < 0.05$) or TNFα CM (18% ± 4%; $P < 0.05$). The use of IFNγ CM also resulted in significant CD4+ T cell suppression (43% ± 6%; $P < 0.001$) although not quite at the level seen when using CM obtained from hMSCs treated with the combination of IL-1β, TNFα, and IFNγ together ($P < 0.0001$).

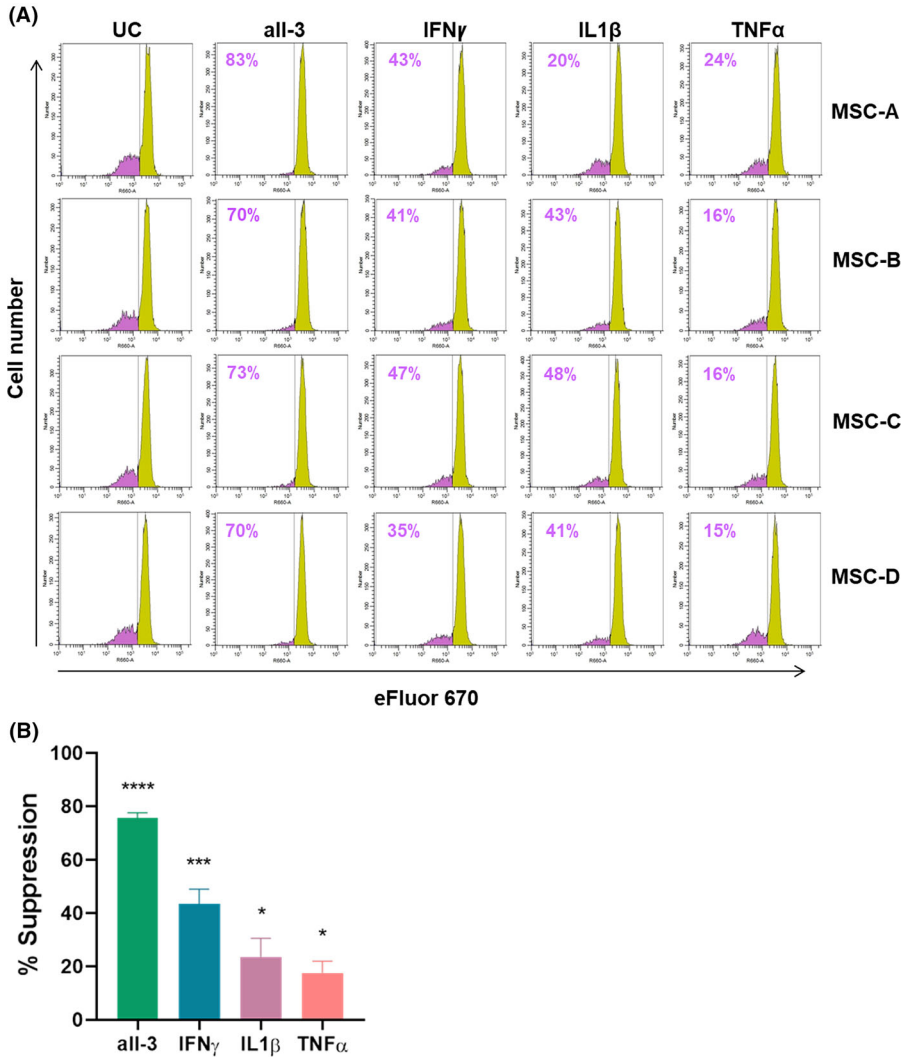

Figure 1. Conditioned medium (CM) from human mesenchymal stem cells (MSCs) treated with cytokines suppresses CD4+ T cell proliferation. **A**, CD4+ T cell proliferation is suppressed by CM from four different human MSC donors preconditioned with interferon γ (IFNγ), Interleukin-1 β (IL1β), tumor necrosis factor α (TNFα), or a combination of the three cytokines (all-3) compared with unconditioned control (UC) human MSCs. eFluor 670-labeled CD4+ T cells were CD2/3/28 activated and cultured in the presence of various human MSC CM. Proliferation was determined under each condition by eFluor 670 dilution assessed by flow cytometry. The yellow peaks represent generation 1 of the CD4+ T cell population, whereas cells in purple have undergone cell division. Percentage suppression is calculated as described in the METHODS (the percentage of suppression cells are indicated in the top left). **B**, Aggregated data from four independent experiments demonstrate the mean level of suppression of healthy control CD4+ T cells incubated with the range of human MSC CM. All conditions are compared with reference UC human MSC. Statistical analysis using one-way ANOVA and Dunnett's Multiple Comparison post-test demonstrated significance as indicated. *$P < 0.05$, ***$P < 0.001$, and ****$P < 0.0001$.

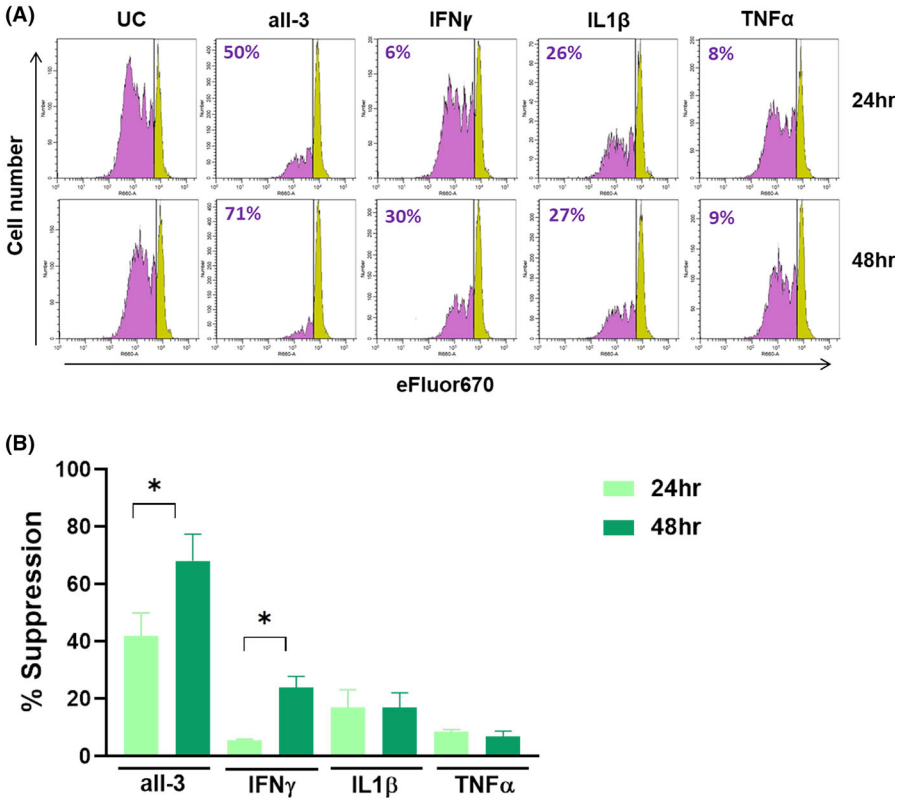

Figure 2. CD4+ T cell proliferation is suppressed to a greater extent by human mesenchymal stem cells (hMSCs) preconditioned with cytokines for 48 hours than at 24 hours. **A**, eFluor 670-labeled healthy CD4+ T cells were CD2/3/28-activated and cultured in the presence of various hMSC conditioned medium (CM) harvested from hMSCs at 24 and 48 hours. Proliferation was determined under each condition by eFluor 670 dilution assessed by flow cytometry. Representative histograms from three independent experiments are shown (the percentages of suppression cells are indicated in the top left). **B**, CD4+ T cell suppression in the presence of hMSC CM under each condition, as described in **A**. Combined data from three hMSC donors. A paired t-test was used to compare 24 hours with 48 hours. Data are given as mean ± SEM. *$P < 0.05$. all-3, a combination of the three cytokines; IFNγ, interferon γ; TNFα, tumor necrosis factor α.

The effect of cytokine preconditioning hMSC for 48 hours is superior to 24 hours in suppressing CD4+ T cell proliferation. To produce hMSCs that maximally suppress CD4+ T cell proliferation, *ex vivo* experiments were designed to test which cytokine(s) maximize suppression of CD4+ T cell proliferation by hMSC secretomes. The time of hMSC exposure to cytokine(s) was tested first as a variable (Figures 2A and 2B). Cytokine preconditioning of hMSCs was performed for 24 and 48 hours. As shown in a representative experiment, CD4+ T cell proliferation was suppressed by CM harvested at both 24 and 48 hours (Figure 2A). CD4+ T cell suppression was 1.4-fold greater with treatment with all three cytokines for 48 hours compared with 24 hours. Furthermore, aggregated data of the three experiments using CM from hMSCs that were treated with all three cytokines showed 1.6-fold greater suppression at 48 hours compared with 24 hours (Figure 2B). Similarly, CM from hMSCs treated with IFNγ alone for 48 hours suppressed CD4+ T cells fivefold more than IFNγ CM from 24 hours (Figure 2A), and aggregated data from three experiments demonstrated 4.2-fold more suppression at 48 hours compared with the 24-hour time point. These data provided the rationale for standardizing hMSC activation conditions at 48 hours for the remainder of the experiments described. In contrast, CM derived from hMSCs treated singly with either IL1β or TNFα alone did not suppress CD4+ T cells at 24 or 48 hours (Figure 2B).

Allogeneic hMSCs from distinct donors differ in their potency for suppressing CD4+ T cells. To determine whether hMSCs from donors varied in their capacity to suppress CD4+ T cells, hMSCs from three donors (MSC-A-C) were compared using suppression assays. RA and control CD4+ T cells were used to detect whether both were susceptible to hMSC secretome–induced CD4+ T cell suppression. As previously observed, hMSC secretomes from cytokine-treated hMSCs were superior in suppressing CD4+ T cell proliferation to UC hMSCs. Further, CM was superior to the UC medium regardless of whether CD4+ T cells were isolated from patients with RA or HCs (Figure 3A). Suppression rates of 59% ± 0.3% (mean ± SEM) (MSC-A) and 62% ± 3% in (MSC-C) were observed for RA CD4+ T cells when incubated with CM (all-3) compared with UC medium (hMSC using media alone). Suppression rates of 59% ± 0.1% (MSC-A) and 73% ± 3% (MSC-C) were observed using HC CD4+ T cells (all three CM compared with UC medium). In

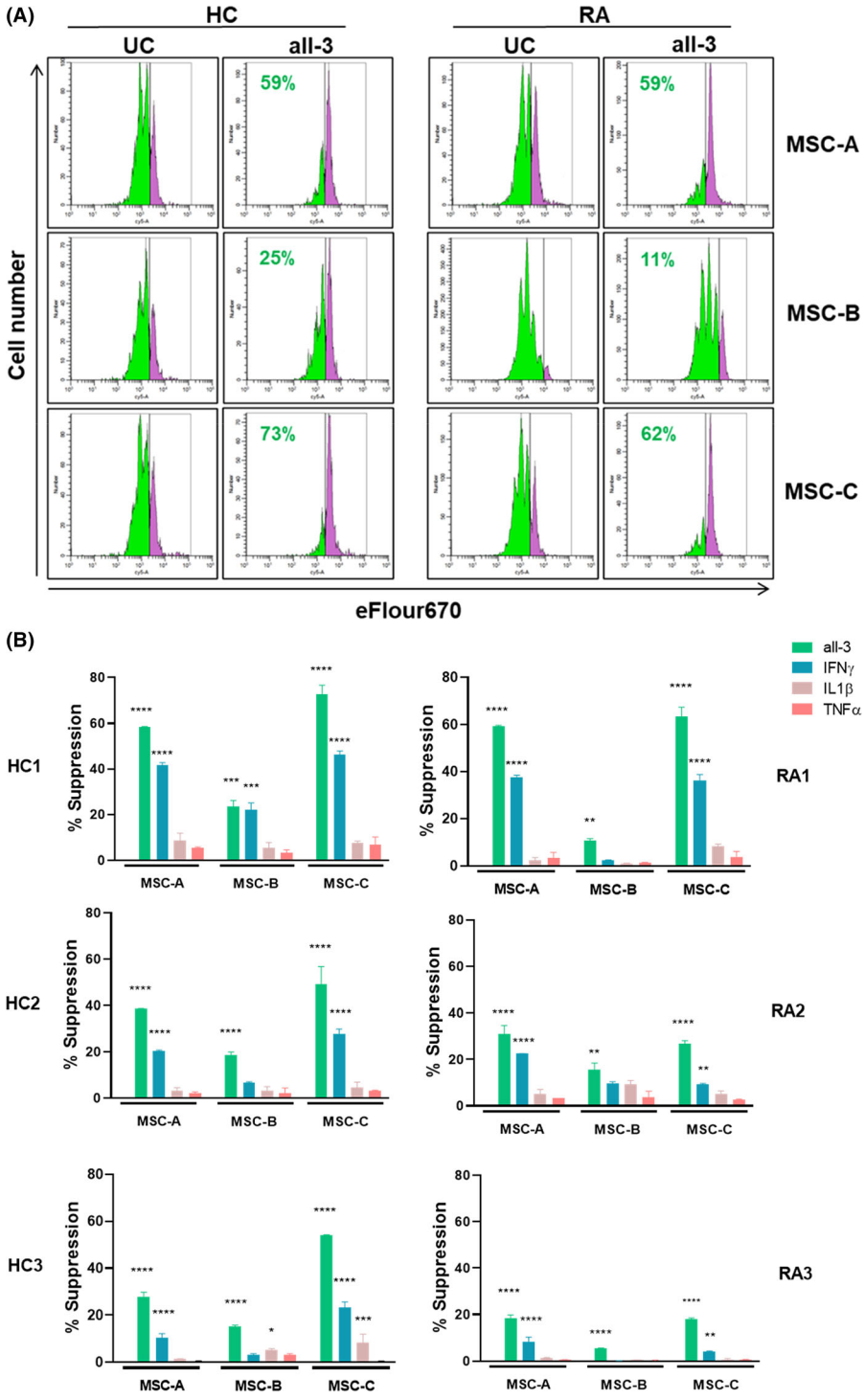

Figure 3. Variability within suppression assays can be seen by varying the human mesenchymal stem cell (hMSC) donors and CD4+ T cells donors. **A**, Secretomes from hMSC donors A and C effectively suppress healthy control (HC) and rheumatoid arthritis (RA) CD4+ T cell proliferation. CM from hMSC donor B incompletely suppresses RA CD4+ T cells. Proliferating cells are shown in green, and nonproliferating G0 cells are shown in purple. **B**, Both RA and HC CD4+ T cells were suppressed by CM, but the magnitude varied depending on which of the three distinct hMSC donor cells were used. T cell suppression (mean ± SEM) was calculated by using a one-way ANOVA followed by Tukey's multiple comparisons test. *$P < 0.05$, **$P < 0.005$, ***$P < 0.001$, and **** $P < 0.0001$.

contrast, MSC-B CM treated as above was only suppressed by 25% ± 0.%6 (HCs) and 11% ± 1% (patients with RA) (Figure 3A). The MSC-A and MSC-C secretomes tended to outperform the MSC-B secretome in magnitude; however, this did not reach statistical significance. The percentage suppression for each of the cytokines alone is shown in the supplementary data (Table S2).

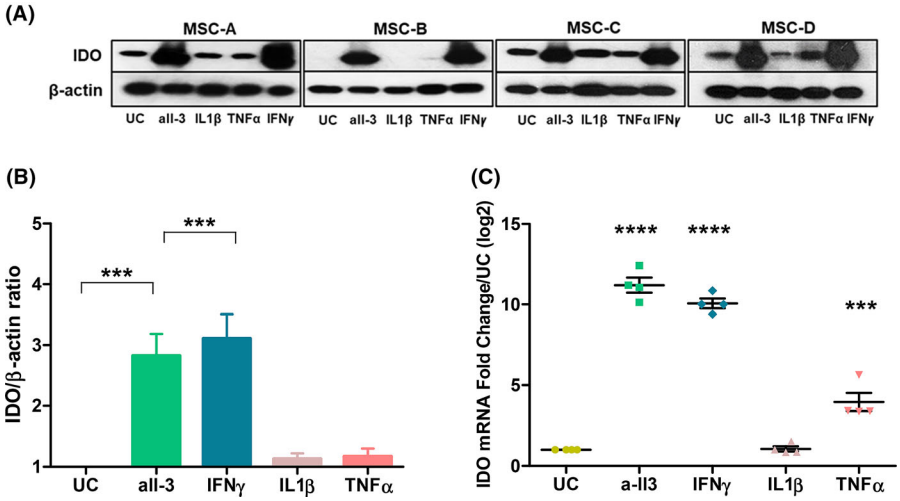

Figure 4. Human mesenchymal stem cell (hMSC) indoleamine 2, 3-dioxygenase (IDO) protein was upregulated by the combination of all three cytokines (all-3) or interferon γ (IFNγ) alone. **A**, hMSCs were preconditioned with the combination of IL1β, tumor necrosis factor α (TNFα), and IFNγ or each cytokine alone for 48 hours. Western blot of total protein extracts probed with antibodies for IDO (top) and β-actin (bottom) in hMSCs. **B**, Densitometry was used to quantify protein levels and obtained levels were normalized to β-actin (the normalized values were related to IDO expression for untreated control [UC], which was set to 1). Combined data from three independent experiments are shown. ***$P < 0.0001$. **C**, IDO mRNA expression was upregulated as measured by quantitative real-time polymerase chain reaction at 48 hours after cytokine stimulation. Fold change was normalized to β-actin (the normalized values were related to IDO expression for UC, which was set to 1). To determine significance, one-way ANOVA followed by Dunnett's Multiple Comparison post-test was used. ****$P \leq 0.0001$.

RA and HC T cells vary in their response to hMSC CM. Significant suppression of CD4+ T cell proliferation occurred in all experiments using CM (all-3) and in IFNγ CM from MSC-A and MSC-C ($P < 0.0001$). For HC CD4+ T cells, only one of three experiments using IFNγ CM (Figure 3B; HC1, MSC-B) showed a significant difference compared with UC ($P < 0.001$). For RA CD4+ T cells, IFNγ CM from MSC-B was not different compared with UC (Figure 3B; RA1-3, MSC-B). Individual suppression rates for individual assays are shown in the supplementary data (Table S2).

Preconditioning hMSCs upregulates IDO mRNA and protein. hMSCs were treated with cytokines for 48 hours as previously described. To evaluate the impact of cytokine treatment on a known immune modulator secreted by hMSC, RNA and total protein were measured. Both IDO mRNA and protein are constitutively expressed by hMSCs (Figures 4A-4C). All four donors' (A-D) hMSCs displayed an increase in IDO protein expression in response to treatment with IFNγ or a combination of the three cytokines in comparison with the unconditioned hMSCs (Figure 4A). hMSC IDO mRNA increased by 13.5-fold using CM (all-3) compared with UC medium, accompanied by IDO protein level increases of 2.80-fold. Similarly, IFNγ alone induced a 13.2-fold increase in hMSC IDO mRNA ($P < 0.0001$) and a 3.1-fold increase in IDO protein expression ($P < 0.0001$) (Figures 4B and 4C). In contrast, the treatment of hMSCs with TNFα alone increased IDO mRNA by fourfold compared with control without a significant increase in IDO protein. Treatment of the hMSCs with IL1β alone did not substantially increase hMSC IDO gene expression or protein over its constitutive levels (Figures 4B and 4C). Of the four donors whose hMSCs were tested, a positive correlation between IDO mRNA levels and suppression of CD4+ T cell proliferation was observed (Spearman $r = 0.8$; $P = 0.33$).

DISCUSSION

In this report, we designed ex vivo assays with the potential to serve as surrogate measures for how therapeutic hMSCs will act *in vivo*. By using inflammatory cytokines that are found in RA, we sought to recapitulate the environment that hMSCs are exposed to during RA. We hypothesized that by mimicking conditions that hMSCs encounter *in vivo*, an assay could be developed to predict the potency, and ultimately the efficacy, of hMSCs delivered therapeutically. In these studies, we used T cell suppression as a functional assay to estimate and eventually predict the potency of hMSCs *in vivo*. Although RA is a multisystem and complex disease, many of the pathophysiological mechanisms are thought to be mediated by alterations in CD4+ effector function. hMSCs have been demonstrated to have efficacy in mouse models of RA (17,18) and are being tested therapeutically to treat human RA (NCT03186417). Currently, the use of hMSCs to treat human RA is largely empirical with regard to dose, hMSC source, frequency of administration, and stage of RA. Factors such as tissue origin of hMSC (eg, bone marrow, fat, umbilical cord, etc.), donor health and genetic characteristics, tissue processing, culture conditions, and pretreatment differ between reports and thus may affect clinical outcomes. hMSCs have

strong anti-inflammatory and immunomodulatory properties; the extent to which hMSCs sense and respond to immune derangement and restore immune homeostasis is a matter of controversy (19). Studies show that treatment with hMSCs reduces the inflammatory response of peripheral blood mononuclear cells from patients with RA *ex vivo* (20). The ultimate goal of the studies described is to validate a potency assay for cellular products (hMSCs delivered by local injection or systemic infusion, or hMSC CM or exosomes delivered similarly) to be used therapeutically in RA and related autoimmune disorders. Several key questions about using hMSCs for cellular therapy have been posed in the literature, and an ex vivo model that can predict a clinical outcome is essential for developing pathways that can facilitate United States Food and Drug Administration approval of cellular products.

Preconditioning hMSCs with proinflammatory cytokines and/or growth factors is a known strategy to improve their immunosuppressive function ex vivo and increases their secretion of anti-inflammatory and immunomodulatory factors (21–24). The combination of the proinflammatory cytokines IL1β, TNFα, and IFNγ is known to be present in the blood and joints of patients with RA (7,25–28). Preconditioning hMSCs generated secretomes that in turn suppress CD4+ T cell proliferation to a greater extent than secretomes from hMSCs cultured with single cytokines do. The three-cytokine combination appears to be synergistic; neither TNFα nor IL1β alone was as effective as the combination of the three cytokines. Although IFNγ upregulates major histocompatibility complex (HMC) Class II in hMSCs (29,30), CM from hMSCs pretreated with IFNγ was more suppressive in comparison with CM from hMSCs pretreated with IL1β or TNFα, especially when RA CD4+ T cells were tested. IFNγ and the combination of all three cytokines may, in part, explain the short-lived therapeutic hMSCs in vivo after infusion (31). The secretomes of hMSCs conditioned ex vivo appear to affect CD4+ T cells in distinct but reproducible ways. Data demonstrating that cytokines act in synergy on hMSCs raise the possibility that hMSCs pretreated *ex vivo* with one or more cytokines can be used *ex vivo* to maximize therapeutic hMSC effectiveness for use *in vivo*.

The hMSC potency assay was optimized to determine the T cell suppressive response in milieus mimicking the RA environment. In these studies, we tested a variety of hMSC donor cells using a variety of cytokines alone and in combination. In theory, when preparing clinical-grade products for therapeutic use, minimally manipulating the product and minimizing the time in culture for such products is desirable to reduce the potential for infection and other changes in their molecular phenotype. In our hands, the minimal amount for hMSC preconditioning was chosen on the basis of published reports, and 24- and 48-hour timepoints were targeted to see which better distinguished allogeneic hMSC donors from each other (32,33). Using patient and HC CD4+ T cell suppression signatures, cellular products could be distinguished from each other.

The observed variability in suppression assays appears to depend on the individual hMSC donors versus the source of the CD4+ T cells. This suggests that not all hMSC products are identical even when controlled for age, harvest and culture conditions, and preparation of the cellular product. Further, the data demonstrate that, even when the source of the CM is held constant, differences in the levels in ex vivo hMSC-dependent T cell suppression persist and vary between individuals. Taken together, the data suggest that hMSC therapy can be optimized for a specific disease-associated pathophysiology, and therapeutic "matching" off-the-shelf hMSCs for recipients with RA should be the goal.

The relative effectiveness of CM from hMSC treated with IFNγ or in combination (all three) makes it likely that the induction of IDO mediates some of the CD4+ T cell suppression that was observed. These data are compatible with published data that IDO is a major mediator of the immunosuppressive activity of hMSCs, lending face validity to our approach (34–36). IFNγ-induced expression of IDO is important for hMSC-dependent suppression of T cell proliferation and activation (37). Differences in IDO may be, in part, responsible for the differences in suppression of CD4+ T cells by hMSC-B compared with hMSC-A and hMSC-C. However, a rise in IFNγ-induced MHC Class II cell surface expression is observed at 48 hours. Initially, the absence of MHC Class II may render nonimmunogenic hMSCs, but once MHC Class II increases, it is plausible that this may contribute to allogeneic hMSC rapid removal during treatment of human disease (38) along with complement fixation. This may improve their safety by eliminating the threat of their abnormal implantation of allogeneic hMSCs into the host immune system. There are other molecules secreted by hMSCs, such as prostaglandin E2 and cyclooxygenase 2, that may also contribute to hMSC immunomodulatory activity and tune the magnitude of CD4+ T cell suppression (34,35). The hMSC CM can be further interrogated to identify additional bioactive substances (eg, secretome). However, the functional assay presented reflects the summative outcome of the biological variables of hMSC products and the T cells they suppress. Clearly, not all hMSC secretomes suppress T cells equally, and not all T cells from HC or patients with RA are equally susceptible to immunomodulation by hMSCs. The data presented show that the effects of hMSCs differ in magnitude (potency) but not in directionality, making it likely that the observations can be generalized to more subjects and diseases. It appears possible to optimize the potential of cellular therapy between diseases, and the data suggest that the choice of allogeneic hMSCs can be personalized. The suppression assays have excellent potential for use as a potency assay to predict hMSC therapeutic efficacy.

A limitation of our studies is that the timing of when the hMSC therapy should be delivered to induce immune tolerance in RA is not addressed. Evidence that RA is not a single disease, but rather at least two or more distinct entities differing in factors including biological susceptibility and pathogenetic drivers, led

us to study more homogenous seropositive individuals with early, active disease (39). We hypothesize that, early in disease, a "window of opportunity" exists in seropositive RA, during which hMSC-based treatment is more likely to induce immune quiescence. During later stages of RA, any number of therapies may incompletely control the disease. Whether at a more refractory stage of RA, failure to suppress CD4+ T cell proliferation *ex vivo* distinguishes patients who are more likely to be hMSC nonresponders remains an open question. Defining the RA phenotype being studied in clinical trials is likely to be of prime importance.

A major challenge in evaluating the utility of cell-based therapy in immune disease is measuring the potency of cellular products. We now present evidence to suggest that a simplified functional assay using hMSC secretomes can be used to test and potentially predict the effectiveness of cellular therapies in RA. The results suggest that, if we can generate "off-the-shelf" hMSC products and use the functional potency assay reported here to tailor hMSCs to recipients with RA, then improved therapeutic efficacy can be achieved. Nevertheless, this potency assay is based on functional measures and still needs to be validated in a clinical study. If an association can be demonstrated, this simple assay could become a new standard of how cell-based therapies are qualified therapeutically in arthritis and arthritis-related disease.

The issues above are important regarding the utility of cell-based therapy in autoimmune diseases overall and in RA in particular. Additional issues that need to be addressed include whether cell therapy can and should be used as primary or as adjunctive therapy in autoimmune disease, whether hMSCs should be preconditioned *ex vivo* prior to use in humans, whether there is an optimal time for when hMSCs should be used therapeutically, and whether they should be used alone or in combination with other therapies. hMSCs can mediate immunoregulatory effects on both innate and adaptive immunity (12) through the secretion of soluble factors (indirect effects) and/or via cell-cell contact (direct effects) in mice, but this is not as widely studied in humans. Our studies cannot address the overarching issues that ultimately require additional research, including the possibility that hMSCs may be effective in some diseases and/or clinical scenarios but not in others. We do, however, hope to help the field move forward in understanding how we can compare cellular products with each other *ex vivo* that have clinically meaningful correlates *in vivo*. We have already undertaken addressing the harder question in our area of interest, namely, whether hMSCs can be administered therapeutically to induce biological and clinical improvement in RA. Others are performing similar studies in systemic lupus erythematosus, multiple sclerosis, and other inflammatory disorders. It is critically important that these studies will be performed rigorously in the context of an approved clinical trial to yield credible data, whether it is negative or positive, and, most importantly, to assure patient safety.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Drs. Breitman and Singer had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Breitman,. Bonfield, Caplan, Lazarus, Singer.

Acquisition of data. Breitman, Bonfield, Caplan, Haghiac, LaSalvia, Reese-Koc, Singer.

Analysis and interpretation of data. Breitman, Bonfield, Caplan, Lazarus, Singer.

REFERENCES

1. Calabresi E, Petrelli F, Bonifacio AF, Puxeddu I, Alunno A. One year in review 2018: pathogenesis of rheumatoid arthritis. Clin Exp Rheumatol 2018;36:175–84.
2. Cojocaru M, Cojocaru IM, Silosi I, Vrabie CD, Tanasescu R. Extra-articular manifestations in rheumatoid arthritis. Maedica (Buchar) 2010; 5:286–91.
3. Gabriel SE, Michaud K. Epidemiological studies in incidence, prevalence, mortality, and comorbidity of the rheumatic diseases. Arthritis Res Ther 2009;11:229.
4. Cope AP. T cells in rheumatoid arthritis. Arthritis Res Ther 2008;10 Suppl 1:S1.
5. Lin YJ, Anzaghe M, Schulke S. Update on the pathomechanism, diagnosis, and treatment options for rheumatoid arthritis. Cells 2020;9:880.
6. Guo Q, Wang Y, Xu D, Nossent J, Pavlos NJ, Xu J. Rheumatoid arthritis: pathological mechanisms and modern pharmacologic therapies. Bone Res 2018;6:15.
7. Smolen JS, Aletaha D, McInnes IB. Rheumatoid arthritis. Lancet 2016;388:2023–38.
8. Zhou ZY, Griffith J, Du EX, Chin D, Betts KA, Ganguli A. Economic burden of switching to a non-tumor necrosis factor inhibitor versus a tumor necrosis factor inhibitor biologic therapy among patients with rheumatoid arthritis. Adv Ther 2016;33:807–23.
9. Finckh A, Simard JF, Gabay C, Guerne PA, SCQM Physicians. Evidence for differential acquired drug resistance to anti-tumour necrosis factor agents in rheumatoid arthritis. Ann Rheum Dis 2006;65:746–52.
10. Weiss AR, Dahlke MH. Immunomodulation by mesenchymal stem cells (MSCs): mechanisms of action of living, apoptotic, and dead MSCs. Front Immunol 2019;10:1191.
11. Prockop DJ, Oh JY. Mesenchymal stem/stromal cells (MSCs): role as guardians of inflammation. Mol Ther 2012;20:14–20.
12. Wang L, Wang L, Cong X, Liu G, Zhou J, Bai B, et al. Human umbilical cord mesenchymal stem cell therapy for patients with active rheumatoid arthritis: safety and efficacy. Stem Cells Dev 2013;22: 3192–202.
13. De Witte SF, Franquesa M, Baan CC, Hoogduijn MJ. Toward development of imesenchymal stem cells for immunomodulatory therapy. Front Immunol 2015;6:648.
14. Sivanathan KN, Rojas-Canales DM, Hope CM, Krishnan R, Carroll RP, Gronthos S, et al. Interleukin-17A-induced human mesenchymal stem cells are superior modulators of immunological function. Stem Cells 2015;33:2850–63.
15. Prasanna SJ, Gopalakrishnan D, Shankar SR, Vasandan AB. Pro-inflammatory cytokines, IFNgamma and TNFalpha, influence immune properties of human bone marrow and Wharton jelly mesenchymal stem cells differentially. PLoS One 2010;5:e9016.
16. McMurchy AN, Levings MK. Suppression assays with human T regulatory cells: a technical guide. Eur J Immunol 2012;42:27–34.

17. Swart JF, de Roock S, Hofhuis FM, Rozemuller H, van den Broek T, Moerer P, et al. Mesenchymal stem cell therapy in proteoglycan induced arthritis. Ann Rheum Dis 2015;74:769–77.
18. Gonzalez MA, Gonzalez-Rey E, Rico L, Buscher D, Delgado M. Treatment of experimental arthritis by inducing immune tolerance with human adipose-derived mesenchymal stem cells. Arthritis Rheum 2009;60:1006–19.
19. Bernardo ME, Fibbe WE. Mesenchymal stromal cells: sensors and switchers of inflammation. Cell Stem Cell 2013;13:392–402.
20. Zeng J, Wang F, Mao M. Coculture of fibroblastlike synoviocytes with umbilical cordmesenchymal stem cells inhibits expression of proinfl ammatory proteins, induces apoptosis and promotes chondrogenesis. Mol Med Rep 2016;14:3887–93.
21. Zhou Y, Tsai TL, Li WJ. Strategies to retain properties of bone marrow-derived mesenchymal stem cells ex vivo. Ann N Y Acad Sci 2017;1409:3–17.
22. Baldari S, di Rocco G, Piccoli M, Pozzobon M, Muraca M, Toietta G. Challenges and Strategies for Improving the Regenerative Effects of Mesenchymal Stromal Cell-Based Therapies. Int J Mol Sci 2017;18: 2087.
23. Petrenko Y, Sykova E, Kubinova S. The therapeutic potential of three-dimensional multipotent mesenchymal stromal cell spheroids. Stem Cell Res Ther 2017;8:94.
24. Najar M, Krayem M, Merimi M, Burny A, Meuleman N, Bron D, et al. Insights into inflammatory priming of mesenchymal stromal cells: functional biological impacts. Inflamm Res 2018;67:467–77.
25. Brennan FM, McInnes IB. Evidence that cytokines play a role in rheumatoid arthritis. J Clin Invest 2008;118:3537–45.
26. McInnes IB, Schett G. The pathogenesis of rheumatoid arthritis. N Engl J Med 2011;365:2205–19.
27. Md Yusof MY, Emery P. Targeting interleukin-6 in rheumatoid arthritis. Drugs 2013;73:341–56.
28. Firestein GS. Evolving concepts of rheumatoid arthritis. Nature 2003; 423:356–61.
29. Le Blanc K, Tammik L, Sundberg B, Haynesworth SE, Ringden O. Mesenchymal stem cells inhibit and stimulate mixed lymphocyte cultures and mitogenic responses independently of the major histocompatibility complex. Scand J Immunol 2003;57:11–20.
30. Grau-Vorster M, Laitinen A, Nystedt J, Vives J. HLA-DR expression in clinical-grade bone marrow-derived multipotent mesenchymal stromal cells: a two-site study. Stem Cell Res Ther 2019;10:164.
31. Eggenhofer E, Benseler V, Kroemer A, Popp FC, Geissler EK, Schlitt HJ, et al. Mesenchymal stem cells are short-lived and do not migrate beyond the lungs after intravenous infusion. Front Immunol 2012;3:297.
32. Redondo-Castro E, Cunningham C, Miller J, Martuscelli L, Aoulad-Ali S, Rothwell NJ, et al. Interleukin-1 primes human mesenchymal stem cells towards an anti-inflammatory and pro-trophic phenotype in vitro. Stem Cell Res Ther 2017;8:79.
33. Fan H, Zhao G, Liu L, Liu F, Gong W, Liu X, et al. Pre-treatment with IL-1beta enhances the efficacy of MSC transplantation in DSS-induced colitis. Cell Mol Immunol 2012;9:473–81.
34. Krampera M, Cosmi L, Angeli R, Pasini A, Liotta F, Andreini A, et al. Role for interferon-gamma in the immunomodulatory activity of human bone marrow mesenchymal stem cells. Stem Cells 2006;24: 386–98.
35. Ryan JM, Barry F, Murphy JM, Mahon BP. Interferon-gamma does not break, but promotes the immunosuppressive capacity of adult human mesenchymal stem cells. Clin Exp Immunol 2007;149: 353–63.
36. Su J, Chen X, Huang Y, Li W, Li J, Cao K, et al. Phylogenetic distinction of iNOS and IDO function in mesenchymal stem cell-mediated immunosuppression in mammalian species. Cell Death Differ 2014; 21:388–96.
37. Zimmermann JA, Hettiaratchi MH, McDevitt TC. Enhanced immunosuppression of T cells by sustained presentation of bioactive interferon-gamma within three-dimensional mesenchymal stem cell constructs. Stem Cells Transl Med 2017;6:223–37.
38. Saat TC, van den Engel S, Bijman-Lachger W, Korevaar SS, Hoogduijn MJ, JN IJ, et al. Fate and effect of intravenously infused mesenchymal stem cells in a mouse model of hepatic ischemia reperfusion injury and resection. Stem Cells Int 2016;2016: 5761487.
39. Stanich JA, Carter JD, Whittum-Hudson J, Hudson AP: Rheumatoid arthritis: disease or syndrome? [Review]. Open Access Rheumatol 2009;1:179–92.