Potency Analysis of Mesenchymal Stromal Cells Using a Combinatorial Assay Matrix Approach

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Graphical Abstract

Highlights
- T cell suppression by MSCs correlates with cytokine and morphogen expression
- MSC and responder PBMC interactions are bidirectional
- MSC potency affects the secretome and correlates with T cell suppression
- The matrix response of MSCs to PBMCs is replicated by IFNγ stimulation

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In Brief
Assays that inform on mesenchymal stromal cell (MSC) immune potency need to be defined in advanced clinical trials. Chinnadurai et al. tested an in vitro assay matrix approach combining molecular genetic and secretome analysis, elements of which could be deployed to define MSC immune modulatory potency.
Potency Analysis of Mesenchymal Stromal Cells Using a Combinatorial Assay Matrix Approach

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SUMMARY

Assays that can characterize MSC immune potency need to be identified for use in advanced clinical trials. MSCs possess a number of putative regenerative and immunomodulatory properties, and an assay matrix approach may best capture involved effector pathways. We have tested two assay systems to measure the potency of MSCs derived from human subjects: MSC secretome analysis and a quantitative RNA-based array for genes specific to immunomodulatory and homing properties of MSCs. Secretome analysis identified a unique cytokine signature that is upregulated by MSCs or downregulated in responder PBMCs and correlated with T cell suppression. Use of interferon-γ as a surrogate for the action of activated PBMCs on MSCs served as an alternative for the use of human PBMCs as responder cells in a potency assay. Our approach and results define and simplify the multifunctional or matrix responses of MSCs and may serve as a platform for robust potency analysis.

INTRODUCTION

The United States Food and Drug Administration and European Medicines Agency classify cell culture-expanded mesenchymal stromal cells (MSCs) as more than minimally manipulated cellular and gene therapy (CGT) products (Mendicino et al., 2014). For early-phase human clinical trials, release criteria for cellular biopharmaceuticals include identity, viability, and sterility as set parameters (Dominici et al., 2006). However, for advanced-phase clinical trials, regulatory authorities mandate the development of potency assays as part of the release criteria (de Wolf et al., 2017). The challenges of developing potency assays for MSC-like products include the variability of tissue sources, largely undefined mechanisms of action in humans, and lack of reference standards (Bianco et al., 2013; Deskins et al., 2013; Galipeau and Krampera, 2015; Hematti, 2016; Krampera et al., 2013; Menard and Tart, 2013; Radizzani et al., 2016). In addition, analyzing a single effector pathway as a surrogate measure of potency might be misleading, considering that MSCs deploy a plurality of immune-modulatory and regenerative properties that are induced upon interaction with host microenvironmental and immune status cues (Bernardo and Fibbe, 2013; Keating, 2012; Shi et al., 2012). The International Society for Cell Therapy (ISCT) released a consensus statement that a matrix assay approach could be developed that best captures the summation of effector pathways significant to MSC immunomodulation, regeneration, and homing properties (Galipeau et al., 2016). In an effort to develop such an approach and to define matrix responses of MSCs to physiological inflammatory cues, we have developed two complementary analytical systems: a secretome-based assay that defines MSC and peripheral blood mononuclear cell (PBMC) responses to each other upon reciprocal interactions and a transcriptome-based assay that defines RNA expression of selected gene products expressed by MSCs upon interaction with either PBMCs or interferon-γ (IFNγ). In both assays, the guiding principles are to compare resting MSCs with their activated counterparts and quantitate the dynamic response of MSC immune effector pathways and correlate that response with the suppression of PBMC proliferation.

RESULTS

MSC and PBMC Interactions Lead to the Induction and Inhibition of Unique Secretome Signatures

We analyzed the comparative suppressive effect of MSCs derived from human subjects with Crohn’s disease (n = 5) or graft versus host disease (GvHD) (n = 5) on the proliferation of staphylococcal enterotoxin B (SEB)-activated PBMCs from three independent, unrelated, normal volunteer donors. Our results demonstrate that both MSC populations inhibited CD3+Ki67+ T cell proliferation efficiently in a dose-dependent manner in all the three PBMC donors tested (Figures 1A and 1B). We performed a focused analysis of 29 biologically relevant cytokines and morphogens released in the supernatant derived from cocultures of activated PBMCs and MSCs. Under all conditions, PBMC numbers were kept constant as MSC numbers were escalated, resulting in a differential MSC-to-PBMC ratio that...
Figure 1. Secretome Matrix Signature Responses of GvHD and Crohn’s Disease MSC Interaction with PBMCs

MSCs derived from Crohn’s disease (n = 5) and GvHD (n = 5) patients were cultured with SEB-stimulated independent PBMC donors (n = 3; green, blue, and red) at the indicated ratios, where PBMCs were kept constant with titration of MSC numbers. 4 days after culture, CD3+ T cell proliferation was measured by Ki67 intracellular staining and acquired through flow cytometry. (A and B) Representative fluorescence-activated cell sorting (FACS) plot (A) and cumulative dose-dependent effect of GvHD and Crohn’s disease MSCs on T cell proliferation (B). The supernatant of SEB-activated PBMCs (n = 3; green, blue, and red) co-cultured with and without MSCs (Crohn’s disease and GvHD, n = 5 each) and reference MSC cultures without PBMCs (black) were collected at the indicated ratios. Quantitative levels of 29 cytokines were assayed (picograms per milliliter) through luminex xMAP (multi-analyte profiling) technology.

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allowed for a dose/effect analysis of MSCs on PBMCs. We also analyzed the secretome of supernatant derived from cultures with an identical MSC density but in the absence of PBMCs, which served as a reference control. Dose dependency analysis identified at least eight PBMC-derived cytokines (of 29 tested) that displayed significantly correlated downregulation ($R^2 \geq 0.5$) upon interaction with MSCs. The ranking of these was as follows: interleukin-13 (IL-13), tumor necrosis factor alpha (TNF-$\alpha$), CCL3, IL-5, IL-2R, IFN$\gamma$, CCL4, and IL-12 (Figure 1C). None of the downregulated cytokines were produced in MSC cultures without PBMCs, and, hence, the downregulation was due to the direct effect of MSCs on SEB-activated PBMC responders and reflects downregulated responses of PBMCs upon interaction with MSCs. These results suggested that a focused analysis of PBMC-sourced cytokines that are both highly correlated (e.g., $R^2 \geq 0.5$) and substantial in magnitude ($>3$-fold in suppression) may be the most robust predictors of MSC suppression of PBMC functionality, especially when testing clinically relevant matched pairs (e.g., in vitro assessment of the therapeutic MSCs tested against PBMCs from a recipient patient). We identified 8 cytokines (vascular endothelial growth factor [VEGF], IFN$\alpha$, GCSF, CXCL9, CCL2, IL-7, fibroblast growth factor (FGF)-basic, and CXCL10) that were secreted at low/no levels in activated PBMC cultures alone but, upon interaction with MSCs, were upregulated in a dose-dependent manner (Figure 1D). Based on the secretome analysis of resting MSCs without PBMCs, we have grouped upregulated cytokines into two overlapping categories: basal and inducible. In the basal situation, cytokines (VEGF, IFN$\alpha$, CCL2, IL-7, and FGF-basic) are secreted by MSCs constitutively independent of PBMCs, and their increased detection in MSC and PBMC ratios correlated with MSC input cell number in a dose/effect manner. In contrast, induced upregulated cytokine response (GCSF, CXCL9, CXCL10, CCL2) reflects de novo MSC and PBMC functionality upon their interaction (Figure 1D). We also examined the expression levels of 13 additional cytokines that did not show significant ($R^2 \leq 0.5$) dose-dependent up- or downregulation in MSC and PBMC cultures, suggestive of cytokines that are unmodulated by MSC and PBMC interaction (Figure 1E). To describe the magnitude of responses of MSC and PBMC interaction, we have calculated the fold induction and downregulation of cytokine levels in comparison with PBMC controls without MSCs (Table S1). Although suppressed cytokines were ranked based on these correlitive $R^2$ values (with the MSC/PBMC ratio as a surrogate of MSC dose/effect on PBMCs), the magnitude of that suppression varied widely for different cytokines; for example, the level of TNF-$\alpha$ suppression identified TNF-$\alpha$ as a sizable responder (Table S1). Similarly, with the upregulated cytokine levels that are highly correlated with PBMC suppression, the magnitude of that upregulation varies, with GCSF standing out as being nearly 25-fold more abundant than most other factors (Table S1).

MSCs' Suppressive Effect on T Cell Proliferation Correlated with Basal and Inducible MSC Secretome Signature

We analyzed T cell proliferation and cytokine levels with and without MSCs at MSC:PBMC ratios of 1:8 to 1:2 by linear regression analysis to identify correlations between MSC dose and PBMC response. Using this approach, we demonstrated that suppression of PBMC proliferation was significantly correlated ($R^2 \geq 0.5$) with a suppression of TNF-$\alpha$, IFN$\gamma$, IL-13, IL-5, IL-2R, CCL3, and CCL4 cytokine levels (Figures 2A and 2C). Reciprocally, increased levels of VEGF, IFN$\alpha$, CXCL10, GCSF, CXCL9, IL-7, and CCL2 were correlated with PBMC suppression (Figures 2B and 2D). We have stratified the $R^2$ values in descending hierarchical order and have highlighted the more robust correlations ($R^2 \geq 0.5$), considering that $R^2 = 1.0$ and $R^2 = 0$ are the best and no correlation, respectively. CCL2 display $R^2$ values of 0.4788, and, because of decimal rounding, we have included them within the ($R^2 \geq 0.5$) range. In addition, 15 cytokines did not show significant correlation with T cell suppression (Figure 2E). We have also tested a supplemental correlation model with a best fit sigmoidal relationship curve and identified a similar pattern, although there are some minor variations in the rankings (Figure S1; Table S2). We have also tested MSCs derived from the bone marrow of healthy individuals, and our results demonstrate that healthy MSCs also exhibited up- and downregulated secretome signatures and were correlated with T cell suppression consistent with that observed with MSCs derived from Crohn’s disease and GvHD patients (Figure S2).

Secretome Responses of MSC and PBMC Interaction Are Bidirectional

To identify the source of the upregulated cytokines VEGF, GCSF, CXCL9, IL-7, CCL2, and CXCL10, we investigated their mRNA transcripts in MSCs and PBMCs that were either cultured alone or together in a two-chamber transwell system. We identified two patterns of cytokine expression in MSCs and PBMCs: constitutive expression by MSCs (VEGF, CCL2, and IL-7) (Figure 3A) and PBMCs (VEGF and CXCL10) (Figure 3B) and upregulated in MSCs (GCSF, CXCL9, and CXCL10) (Figure 3A) and PBMCs (GCSF, CXCL9, IL-7, and CCL2) (Figure 3B) upon their mutual interaction. These results demonstrate that MSC and PBMC reciprocal interaction leads to the induction of unique cytokine and chemokine expression by both cell types, which cumulatively reflect consensual immune modulation.

PBMC Donors Largely Determine Variations in Matrix Responses of MSC and PBMC Interactions

Two-way ANOVA multiple comparisons analysis demonstrated that the secretome changes arising from MSC and PBMC interplay are remarkably consistent when comparing MSCs derived from subjects with GvHD or Crohn’s disease, with an exception made for the expression of CXCL9, CCL2, and CXCL10 (Table 1).

(C and D) Dose-dependent (C) downregulation and (D) upregulation of cytokines in MSC and PBMC cultures. Cytokine levels in MSC cultures at different densities (black line) are shown as a reference. Down- and upregulated cytokines are organized based on descending hierarchical dose dependency $R^2$ values.

(E) Cytokine secretion levels that are not modulated dose-dependently by MSC additions. Cytokines are organized based on descending hierarchical dose dependency $R^2$ values.
However, the variance in secretomes derived from distinct PBMC responders grouped and compared with each other (PBMC 1 versus 2, 2 versus 3, and 1 versus 3) is more substantial (Table 1). In fact, CXCL9, CCL2, and CXCL10 are upregulated after PBMC interactions, and, thus, the variability in PBMC responders accounts for such differences (Table 1). Regardless, the most robust secretome correlates across MSC sources and suppression of PBMC proliferation were suppression of IFNγ production by activated PBMCs and MSC-derived expression of VEGF and IFNα. Altogether, variations in the responses of MSC and PBMC interaction largely stemmed from differences among individual PBMC responder cells. This observation highlights the fact that most variance observed is not from MSC biology but, rather, PBMC responders, suggesting that lympho-myeloid responders may dictate the disparity in outcomes following use of MSCs.

Fitness of MSCs Dictates the Correlation of Secretome Signature with T Cell Suppression

Thawed MSCs undergo a heat shock response that, as a whole, mitigates their potency as a cell pharmaceutical agent. To test whether thawing affects the responsiveness of MSC preparations, we examined the secretome signature of MSCs previously cryopreserved and then thawed and activated by co-culture with PBMC responders. Consistent with previous studies (Chinna-durai et al., 2016; Moll et al., 2014), thawed MSCs were defective in inhibiting T cell proliferation (Figure 4A), and here we further show that thawed MSC secretome response failed to exhibit any correlation with T cell proliferation (Figures 4B–4F; Figure S3; Table S3), and the R² correlative values of T cell proliferation with secretome signature are significantly lower than the values derived from fit MSCs (Figures 4G and 4H).

Matrix Molecular Genetic Responses of MSCs to PBMCs Are Highly Conserved

We further complemented the secretome analysis of MSC/PBMC co-cultures with a comprehensive MSC RNA transcriptional analysis of more than 40 genes that have been invoked in MSC immune functionality, as described previously (Chinna-durai et al., 2015; Galipeau et al., 2016). Crohn’s disease and GvHD MSCs were co-cultured with SEB-activated PBMCs in a two-chamber transwell assay system, and RNA expression derived from MSCs exposed to activated PBMC medium (but no contact) through a transwell system was compared with

Figure 2. Correlation of MSC-Mediated T Cell Suppression with Matrix Secretome Signature

Individual cytokine levels in MSC and PBMC co-culture were subjected to linear regression analysis with the corresponding T cell proliferation values (% CD3⁺Ki67⁺) to obtain R² values. (A and B) Descending hierarchical organization R² values of (A) downregulated and (B) upregulated secretome signature. (C and D) Correlation plots of (C) downregulated and (D) upregulated cytokines with T cell proliferation with appropriate R² and p values. (E) Unmodulated cytokines of MSC and PBMC co-cultures were tested for their linear correlation with the respective % CD3⁺Ki67⁺ T cell proliferation. Unmodulated cytokine plots were also hierarchically organized based on descending R² values and were split based on the pattern of downregulated (left) or upregulated (right) cytokine signature. Data were transformed to logarithmic scale wherever necessary to best fit the regression line. Regression analysis was performed in Prism software.
that of control MSCs cultured in parallel. We observed that, in response to the PBMC secretome, MSCs altered the expression levels of several genes that are deemed important for MSC function (Figure 5A). Volcano plot analysis demonstrates that transcriptional activation of indoleamine 2,3 dioxygenase (IDO), CXCL9, CXCL10, CXCL11, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), CIITA, IL-8, ICAM-1, HLADR, CCL5, and CCL7 was upregulated substantially and significantly upon interaction with the secretome of all three PBMC donors tested (Figure 5B). No significant differences were observed between the transcriptional responses of MSCs derived from subjects with Crohn’s disease and GvHD (Figure 5C). Similarly, no differences were observed in MSC responses to three distinct PBMC donors (Figure 5C).

### IFNγ Stimulation Recapitulates PBMC-Induced Matrix Responses in MSCs

Considering that PBMC-derived IFNγ is a key cytokine leading to MSC licensing in vitro, we examined the output of a simplified assay stimulating MSCs with recombinant IFNγ as a substitute for SEB-activated PBMCs (Galipeau and Krampera, 2015; Klinker et al., 2017). We observed that both Crohn’s disease and GvHD MSCs responded to IFNγ and upregulated the expression of IDO, CXCL9, CXCL10, CXCL11, CIITA, TRAIL, ICAM-1, HLADR, CCL5, and CCL7 in a manner closely paralleling that of the response to PBMCs (Figures 5D and 5E). No significant differences in the matrix responses were observed between Crohn’s disease and GvHD MSCs (Figure 5F), suggesting that MSCs’ response to inflammatory cues are conserved irrespective of the two ailments (GvHD and Crohn’s disease) tested here. Altogether, these results demonstrate that MSCs’ intrinsic responses to interactions with the PBMC secretome or IFNγ are highly correlated.

### IDO Is the Dominant Determinant of MSC-Mediated Inhibition of T Cell Proliferation

To define the mechanistic contribution of cytokines correlating with MSC-mediated inhibition of T cell proliferation, we blocked the function of VEGF, GCSF, CXCL9, CXCL10, IFNα, CCL2, and IL-7. Neutralizing antibodies to the cytokines VEGF, GCSF, CCL2, and IL-7 and the receptors of CXCL9 (CXCR3), CXCL10 (CXCR3), and IFNα (IFNαR1) were used to block the effect of these cytokines individually in MSC and PBMC co-cultures. We also used 1-methyl tryptophan (1-MT), which is a pharmacological inhibitor of IFNγ/C0 inducible IDO in MSCs. Our results demonstrated that blocking of these effector cytokines did not reverse/attenuate either Crohn’s disease or GvHD MSC-suppressive activity (Figures 6A and 6B). However, 1-MT completely reversed Crohn’s disease and GvHD MSC suppressive activity (Figures 6A and 6B). Next, to define the role of IDO in MSC-mediated contact and non-contact inhibition of T cell proliferation, we utilized 1-MT in co-culture of MSCs and PBMCs and a two-chamber transwell culture system of MSCs and PBMCs in which cells were separated by a cytokine- or metabolite-permeable, 0.4 μm membrane. Our results demonstrated that 1-MT reversed both contact- and non-contact-mediated inhibition of T cell proliferation by Crohn’s disease and GvHD MSCs (Figures 6C and 6D). To further authenticate the uniqueness of IDO’s central role in MSC-mediated inhibition of T cell proliferation, we performed a

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1:2, 1:4, and 1:8, MSC:PBMC ratio; ns, non-significant; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

*Consistent in responses irrespective of the source of MSC and PBMC donors.
small interfering RNA (siRNA) knockdown approach to silence IDO in MSCs (Figure 6E). Our results demonstrated that silencing IDO in either Crohn’s disease or GvHD MSCs substantially attenuated their suppressive properties regarding T cell proliferation (Figures 6F and 6G). Altogether, these results demonstrate that IDO is the key mechanism by which MSCs inhibit T cell proliferation in vitro.

**DISCUSSION**

In response to an ISCT position statement (Galipeau et al., 2016), we rigorously analyzed the MSC secretome and transcriptome matrix response of MSCs to PBMCs and IFNγ and identified biological outputs that correlate with suppression of PBMC proliferation in vitro. The induction of GCSF, CXCL9, IL-7, and CCL2 expression by MSCs interacting with SEB-activated PBMCs clearly indicates that the mechanism of action of MSCs involves the modification of active regulatory pathways in immune responders that play a major role in host tissue immune modulation. GCSF, CXCL9, and IL-7 play a major role in hematopoiesis and T cell recruitment, and their role in MSC therapeutic activity has yet to be defined. VEGF is secreted by MSCs innately and is not modulated upon PBMC interaction but still correlated with PBMC suppression. However, the correlation between VEGF secretion and suppression of T cell proliferation is lost in frozen-thawed MSCs. Thus, VEGF expression serves as a surrogate of the innate fitness of MSCs and PBMC suppression. In addition, downregulation of the inflammatory cytokines IL-13, TNFα, CCL3, IL-5, IL-2R, IFNγ, CCL4, and IL-12 and their direct correlation with the percentage of CD3+Ki67+ T cells reflects the multifunctional response of PBMCs to the suppressive effect of MSCs and serves as a measure of lymphomyeloid host response to MSCs. These effector pathways, as documented by protein-based assays of the secretome, complement the transcriptome data that further identified the expression upregulation of transcripts encoding for non-secreted or cell-bound proteins involved in modulating the immune response, including IDO, ICAM1, PDL1, HLADR, and CIITA. As an aggregate, transcriptome and secretome analysis likely captures the bulk of the dynamic response of MSCs to a defined stimulatory cue. In addition, our mechanistic data strengthen the concept that MSCs’ in vitro suppressive effect on T cell proliferation is largely mediated by IDO because blocking of IDO completely abolished MSCs’ in vitro suppressive activity on T cell proliferation in both contact-dependent and contact-independent cultures. Sole blocking of VEGF, GCSF, CXCL9, CXCL10, IFNα, CCL2, and IL-7 did not reverse MSCs’ ability to block T cell proliferation. However, it remains to be determined whether the observed matrix response may play an important role in vivo upon other lymphoid subsets other than T cells as well as on myeloid cells such as monocytes and granulocytes. Altogether, our results demonstrate that, although the matrix responses of MSC and PBMC interaction correlate with MSCs’ in vitro suppressive activity, unlike IDO, they do not solely dictate MSCs’ suppressive activity. Our data further support the ISCT recommendation of immunological characterization of MSCs by measuring IDO induction and function as a potent readout of MSC functionality on T cell biology.

Several studies address the potential utility of a cellular universal ruler for MSC potency assays (Bloom et al., 2015; Deans, 2015; Salem et al., 2015; Tanave et al., 2015; Viswanathan et al., 2014). However, MSCs’ mechanism of action in vivo may well involve the effect of a plurality of effector pathways with synergistic and overlapping functionalities whose integrative effects are not completely understood when examined for use in distinct clinical uses. For example, dominant MSC functionalities in immune modulation may overlap with those relevant in tissue injury response, with some relevant unique components to each. Considering this, it has been a challenge to define and identify a cellular universal reference standard that would contemporaneously display all of the potential functions of MSCs identified to date. Indeed, many MSC functionalities are revealed as part of a dynamic response to environmental cues reflective of the pathophysiology to which they are responding. Our approach obviates the significance of universal reference by introducing an internal reference control: using unstimulated MSCs as the baseline for a measured dynamic response to a relevant environmental cue such as SEB-activated PBMCs or, in a more reductionist manner, recombinant interferon-γ. With this approach, it is feasible, as we show here, to interrogate quantitatively the dynamic response of clinical-grade MSCs to a stimulus reasonably reflective of the targeted disease process at hand. A potential weakness of this approach arises from its reductionist formulation. We have focused on suppression of T cell proliferation as the mean output of our matrix analysis. It is now well established that MSCs can directly interact with a multiplicity of cell effectors of innate, adaptive, and regulatory immunity whose intrinsic functionalities are not well represented in the PBMC suppressor assay. This weakness notwithstanding, the basic principle of interrogating the dynamic range of MSC responses to defined cues (immune, tissue damage) while examining functionalities with a hypothesis-driven likelihood of affecting responder cell (immune or somatic) physiology remains valid.

**Figure 4.** Frozen-Thawed Human MSC and PBMC Co-culture Displays Defective Matrix Responses

MSCs thawed from cryopreservation (Cryo) were co-cultured with SEB-stimulated PBMCs. Four days after culture, T cell proliferation was measured by Ki67 intracellular staining, and cytokines were measured by luminex xMAP (multi-analyte profiling) technology.

- **A** Cumulative percentage of T cell proliferation (CD3+Ki67+) in the presence of frozen-thawed Crohn’s disease and GvHD MSCs is shown with mean and SD.
- **B** and **C** R^2 values, which represent the correlation between T cell proliferation and cytokine levels in thawed MSC and PBMC co-cultures, are split based on (B) downregulated and (C) upregulated cytokine signatures that are identified in live MSCs cultures.
- **D**–**F** Correlation plots of % CD3+Ki67+ (x axis) and cytokine levels (y axis) in (D) downregulated, (E) upregulated, and (F) unmodulated cytokine signatures are shown.
- **G** and **H** R^2 values obtained for each (G) downregulated and (H) upregulated cytokine were compared with the values derived from fresh MSCs. Paired analysis was performed to obtain p values.

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Figure 5. Matrix Molecular Genetic Responses of MSCs to Independent PBMC Donors and IFN\(\gamma\)

MSCs derived from Crohn’s disease and GvHD patients were stimulated with SEB-activated PBMC donors in a two-chamber transwell assay plate, and MSCs without PBMCs were kept as reference cultures. Total RNA was generated from the lysates of MSCs and converted into cDNA. The transcriptional profile of over 40 genes was investigated in Fluidigm nanoscale qPCR 48 \times 48 array plates.

(A) Heatmap representing the relative CT values of individual targets in resting and PBMC-treated MSCs. CT values are normalized based on endogenous control GAPDH. Blue, low expression; red, high expression; G, GvHD MSCs; C, Crohn’s disease MSCs; #1, #2, and #3, independent PBMC donors. Arrow marks indicate substantial and significant change in expression.

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It has been demonstrated that freezing and thawing affects MSC functionality (Chinnadurai et al., 2016; François et al., 2012; Hoogduijn et al., 2016; Kadekar et al., 2016; Moll et al., 2014, 2016; Nold et al., 2015; Pogozhykh et al., 2017; Pollock et al., 2015; Quimby et al., 2013). Here we further show that thawed MSCs display a defective secretome signature with T cell proliferation. However, some studies indicate that thawed MSC functionality is similar to that of fresh cells (Bárcia et al., 2017; Burand et al., 2017; Cruz et al., 2015; Gramlich et al., 2016; Luetzkendorf et al., 2015). A recent study demonstrated that transient temperature fluctuations under frozen cell conditions severely impairs the functionality of the cells immediately post-thawing, despite these cells exhibiting high viability (Chabot et al., 2017). We and others also demonstrated that replicative exhausted MSCs display poor functionality despite exhibiting high viability (Loisel et al., 2017; Sepúlveda et al., 2014). In clinical trials where MSCs are administered as a pharmaceutical agent, 70% viability is used as the major release criterion, and in laboratory studies, the normalization of fresh and thawed cells was performed with trypan blue viability, as is routine in clinical trials. We had demonstrated that thawed MSCs display some level of a suppressive effect at a high MSC and PBMC ratio, although these showed inferiority compared with fresh cells because of their poor survival upon contact with inflammatory immune responders (Chinnadurai et al., 2016). This lysis of thawed cells by activated PBMCs varies with the level of immune mismatch between MSC and PBMC donors. Hence, suppressive variations of thawed MSCs could arise depending on the PBMC donor tested. We have demonstrated here that the source of the variance in potency assays is largely due to the variations in PBMC donors rather than the MSCs themselves. Altogether, these data provide insights into fitness and suggest that the potency of MSCs is not solely dependent on their viability.

In the present study, we have used two complementary approaches, secretome (29 cytokines) and selective genomics (~40 genes), to capture the summation of effector pathways of MSCs that are regulated by activated PBMCs and IFN-γ. Because of the differences in cell-bound and secreted expression, all of the factors were not tested in both of these approaches, although some (such as CXCL9, CXCL10, CCL2, hepatocyte growth factor (HGF), VEGF, IL-8, and CCL5) were tested in both methods. Both secretome and gene expression data confirm the upregulated expression of CXCL9 and CXCL10 by MSCs upon interaction with PBMCs or IFN-γ stimulation, and expression correlated with T cell suppression. In addition, both of these approaches demonstrated that VEGF, HGF, and CCL2 are constitutively secreted by MSCs and are not modulated by IFN-γ or PBMCs, whereas VEGF and CCL2 predicts T cell suppression. Similarly, although CCL5 mRNA was inducible by IFN-γ or PBMCs on MSCs, its secretion did not show a robust correlation with T cell proliferation. In addition, gene expression data demonstrate that PBMCs, but not IFN-γ, upregulate IL-8 by MSCs, and, in support, secretome analysis demonstrates that resting MSCs do not express IL-8. Because activated PBMCs without MSCs secrete IL-8, their upregulation was not detected in the secretome of MSC and PBMC coculture. Altogether, our study defines that both selective secretome and gene expression analyses are comparable with each other in determining MSC potency.

We also employed a focused analysis with IFN-γ that simplifies the matrix response approach relative to the use of random donor PBMC responder cells. The mechanism of action of MSCs in suppressing T cell functionality goes beyond that ofIDO and includes other synergistic responses, including PD-L1/L2 and likely others (Chinnadurai et al., 2014). IFN-γ stimulation of MSCs faithfully recapitulates the molecular genetic changes that are observed in MSCs co-cultured with activated PBMCs, except for a few, such as TNF-stimulated gene 6 protein (TSG-6), IL-8 (which could be activated through TNF-α), IL-1, or other PBMC-derived cytokines independent of IFN-γ (Fan et al., 2012; Ko et al., 2016; Prockop, 2013; Yun et al., 2017). In conclusion, interrogating the secretome and transcriptome dynamic response of MSCs to defined morphogen or leukine cues may well serve as the best means of interrogating the fitness and potency of MSC products for advanced clinical trials.

**EXPERIMENTAL PROCEDURES**

**Bone Marrow MSC Isolation**

MSCs were obtained from consenting subjects with GvHD or Crohn’s disease (n = 5 each) according to Emory University Institutional Review Board-approved guidelines. Patients enrolled in this study had active GvHD or inflammatory bowel disease at the time of bone marrow harvest, as described previously (Copland et al., 2015; Dhere et al., 2016). Bone marrow aspirates were subjected to a Ficoll density gradient to obtain mononuclear cells, as described previously (Chinnadurai et al., 2014). Bone marrow mononuclear cells were plated on alpha-minimal essential medium (αMEM) containing 10% human platelet lysate (Copland et al., 2013) and 100 U/mL penicillin/ streptomycin/Amphotericin B (200,000 cells/cm²). Non-adherent cells were removed from culture after 3 days, and MSCs were allowed to expand for an additional 7 days (passage 0). Subsequently, MSCs were passaged weekly and replated at a seeding density of 1,000 cells/cm². All assays were performed using MSCs between passages 3 and 5.

**Freezing and Thawing**

MSCs at 70%–80% confluence were trypsinized and washed with complete medium. Cell pellets were slowly resuspended with freezing medium (5% human serum albumin [has] in αMEM [HyClone, USA] with 10% DMSO [Celgro, 

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(B) Volcano plots representing the differential matrix expression of genes on MSCs to independent PBMC donors.

(C) Differences in MSC responses to PBMCs based on the source of MSCs (GvHD versus Crohn’s disease) and stimulator PBMC donors #1, #2, and #3. The x-axis CT difference and y-axis negative log p value represent substantiality and significance, respectively. Crohn’s disease and GvHD MSCs were stimulated with ± 20 ng/mL IFN-γ for 48 hr. A nanoscale qPCR array was performed as described above.

(D) Heatmap representing the relative CT values of individual targets in resting and IFN-γ-treated GvHD and Crohn’s disease MSCs. CT values are normalized based on endogenous control GAPDH. Blue, low expression; red, high expression. Arrow marks indicate substantial and significant change in expression.

(E and F) Volcano plots representing (E) differential matrix expression of genes on MSCs to IFN-γ and (F) the CT differences in MSC responses to IFN-γ based on the source of MSCs (GvHD versus Crohn’s disease).

Results were analyzed in JMP software.
MSC and PBMC Co-culture
Crohn’s disease (n = 5) or GvHD (n = 5) MSCs were seeded into 96-well plates at a density of 50,000, 25,000, 12,500, or 0 cells per well, and 100,000 human PBMCs (n = 5) were added to each well. 1000 ng/mL SEB (Toxin Technology, Sarasota, FL) was used to activate T cells. MSCs at a similar density without PBMCs were used as a reference control. To test the effect of freezing/thawing on MSC fitness, thawed MSCs from cryopreservation were used in similar assays. Thawed and fresh MSC viability count was determined by mixing equal volumes of 0.4% trypan blue and cell mixture and analyzed either using a hemacytometer or by automated cell counting (Invirotex Countess, USA). Both fresh and thawed cell concentrations were normalized based on the viability count. Subsequently, they were seeded into 96-well plates as above, and 1,000 ng/mL SEB and PBMCs were added immediately. For blocking experiments, anti-VEGF (Thermo Fisher, USA), anti-GCSF, anti-CXCR3, anti-IFNα(1R1), anti-CCL2, and anti-IL-7 or isotype control antibodies (R&D Systems, USA) were added to MSC and SEB-activated PBMC co-cultures at concentrations of 25 ng/mL. Four days after culture, a Ki67 flow cytometric proliferation assay was performed according to the manufacturer’s instructions with CD3-APC-Cy7 and Ki67-PE antibodies (BD Biosciences, San Jose, CA).

Assays to Block IDO

10,000 or 20,000 MSCs were seeded into 96-well plates 1 day before transfection. MSCs were then transfected with non-targeting control or IDO siRNA (Dharmacon, USA). Briefly, 2 µL of 100 µM siRNA solution (A) or 3 µL Dharmafect 1 reagent (B) was added to 250 µL siMEM containing 10 mM HEPES. A and B were mixed and incubated at room temperature for 30 min. 50 µL of the siRNA transfection cocktail was added to each well. The cells were then incubated for 5 hr, and the transfection medium was replaced with MSC culture medium. siRNA knockdown MSCs were treated with IFNγ (20ng/mL) for 72 hr for RNA extraction. Total RNA was extracted using the RNeasy Plus Mini Kit (Qiagen, USA) and converted into cDNA using the Quantitect reverse transcription kit (Qiagen, USA). cDNA samples were pre-amplified with a 14-cycle qRT-PCR reaction for each sample with the pooled primers as described by the TaqMan Pre-Amp Mastermix (Fluidigm BioMark) manufacturer protocols. 2, 304 parallel qRT-PCR reactions were performed for each primer pair on each sample on a 48 × 48 array. Amplification was detected using an Eva Green detection assay on a Biomark I machine based on standard Fluidigm protocols.

Multiple Assays

Supernatants from co-culture of MSCs and/or PBMCs were collected after 4 days and stored at ~80°C. Stored supernatants were thawed and centrifuged at 500 x g for 3 min to eliminate cell debris and analyzed by magnetic bead-based multiplex luminex assays for cytokines, chemokines, and growth factors, including FGF-basic, IFNγ, IL-12(p40/p70), IL-13, CCL5(regulated on activation, normal T cell expressed and secreted or CCL5 [RANTES]), CCL3(MIP-1α), CXCL9(monokine induced by gamma interferon or CXCL9 [MIG]), CCL4(MIP-1β), VEGF, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-2R, CCL2(MCP-1), CCL11(Eotaxin), IL-8, IL-10, IL-15, IL-17, IL-1RA, granulocyte-macrophage colony-stimulating factor [GM-CSF], granulocyte-colony stimulating factor (G-CSF), epidermal growth factor (EGF), HGF, TNF-α, IL-7, CXCL10(IP-10), and IFN-α (Human Cytokine 30-plex Panel, Life Technologies), according to the manufacturer’s instructions using luminex xMAP (multi-analyte profiling) technology. Results were plotted as picograms per milliliter.

Quantitative Nanoscale PCR Array

Quantitative RT-PCR was performed using Fluidigm 48 x 48 nanofluidic arrays. MSCs from GvHD and Crohn’s disease patients were stimulated with IFNγ (20 ng/mL) for 48 hr. For non-contact MSC and PBMC culture, MSCs and SEB-activated PBMCs were cultured in the bottom and top, respectively, of transwell plates using Corning Costar 0.4 µm transwell cell culture plates. RNA was prepared from independent cell lysates (RNeasy Plus Mini Kit, Qiagen, USA) and converted into cDNA using the Quantitect reverse transcription kit (Qiagen, USA). cDNA samples were pre amplified with a 14-cycle PCR reaction for each sample with the pooled primers as described by the TaqMan Pre-Amp Mastermix (Fluidigm BioMark) manufacturer protocols. 2, 304 parallel qRT-PCR reactions were performed for each primer pair on each sample on a 48 x 48 array. Amplification was detected using an Eva Green detection assay on a Biomark I machine based on standard Fluidigm protocols. PCR data were normalized and analyzed with SAS/JMP Genomics software.

Statistical Methods

Data were analyzed with GraphPad Prism 5.0 software for statistics. Bonferroni multiple comparisons were performed to define whether cytokine responses were either from MSC or PBMC donors. Dose dependency and correlation between secretome and T cell proliferation were determined using linear regression analyses to obtain R² and p values using GraphPad Prism 5.0 software. For non-linear sigmoid regression analysis, the program R was used.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures and three tables and can be found with this article online at https://doi.org/10.1016/j.celrep.2018.02.013.

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AUTHOR CONTRIBUTIONS

R.C. designed the research plan, performed most experiments, analyzed results, and wrote the manuscript. D.R. and L.J.A. helped with the cytokine multiplex experiments. M.G. helped with MSC preparations. S.K. and M.Q. provided bone marrow aspirates from patients with Crohn’s disease and GvHD, respectively. D.A. and G.G. helped with the qPCR array. Y.L. helped with statistical analysis. J.G. designed the research plan, analyzed results, and wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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