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Development of a flow cytometry-based potency assay for measuring the *in vitro* immunomodulatory properties of Mesenchymal Stromal Cells

By

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[1] **List of abbreviations**

APC: allophycocyanin; Cy: intracytoplasmic; EGTA: ethylene glycol tetraacetic acid; FBS: fetal bovine serum; FITC: fluorescein isothiocyanate; FSC: forward scatter; GvHD: graft

versus host disease; hBM MSC: human bone marrow mesenchymal stromal cells; HGF: hepatocyte growth factor; HLA: human leukocyte antigen; IDO: indoleamine 2, 3-dioxygenase; IL: interleukin; LPS: lipopolysaccharide; MAMP: microbial–associated molecular patterns; MCP-1: monocyte chemoattractant protein-1; MDP: N-acetylmuramyl-L-alanyl-D-isoglutamine; MSC: mesenchymal stromal cells; NK: natural killer; PBMC: peripheral blood mononuclear cells; PBS: phosphate-buffered saline; PE: phycoerythrin; PerCP Cy5.5: peridinin chlorophyll protein complex with cyanin 5.5; PMA: phorbol 12-Myristate 13-Acetate; PolyIC: Polyinosinic-Polycytidylic acid; SSC: side scatter; TCR: T cell receptor; TGF-β: transforming growth factor-β; TLR: Toll-like receptor; TNF-α: tumor necrosis factor-α.

Abstract

Human bone marrow-derived mesenchymal stromal/stem cells (MSC) have well-documented modulatory effects on multiple immune cell types. Although these effects are linked to their therapeutic benefit in diverse diseases, a reliable, quantitative assay of the immunomodulatory potency of individual human MSC preparations is lacking. The aims of this study were to develop an optimised rapid turnaround, flow cytometry-based whole-blood assay to monitor MSC potency and to validate its application to MSC immunomodulation. A protocol for short-term LPS stimulation of anti-coagulated whole blood samples followed by combined surface CD45/CD14 and intracellular TNF-α staining was initially developed for analysis on a 4 colour desktop cytometer. Optimal monocyte activation was dependent on the presence of extracellular calcium ions thereby precluding the use of EDTA and sodium citrate as anticoagulants. Optimal assay conditions proved to be 1ng/mL ultrapure-LPS added to 10-fold diluted, heparin anti-coagulated whole blood incubated for 6 hours at 37^{o}C. Under these conditions, addition of human bone marrow-derived MSC (hBM-MSC) from multiple donors resulted in a reproducible, dose-dependent inhibition of LPS-stimulated monocyte TNF-α expression. We conclude that this protocol represents a practical, quantitative assay of a clinically relevant functional effect of hBM-MSCs as well as other immunomodulatory agents.

Keywords: Mesenchymal stromal cells, monocytes, TNF-α, immunomodulatory potency, flow cytometry, whole blood assay.

1. Introduction

In the last decade, several studies have emerged demonstrating that autologous and allogeneic culture-expanded mesenchymal stromal cells (MSC) from different sources possess immunomodulatory properties [1–3] . It has been convincingly shown that such immunomodulatory properties of MSC play specific roles in the maintenance of peripheral transplantation tolerance, autoimmunity and tumour evasion [1] . The anti-inflammatory activities of both autologous and allogeneic MSC are being exploited clinically with administration of MSC now being used to treat or prevent a range of immune/inflammatory diseases such as Graft-versus-Host-disease (GvHD), inflammatory bowel disease, diabetes mellitus, multiple sclerosis, organ transplant rejection, myocardial infarction and stroke [4–6] .

Currently, there are at least 572 on-going clinical trials worldwide which aim to exploit the anti-inflammatory and immunomodulatory properties of MSC (www.clinicaltrials.gov). Global commercialization activities in the stem cells market have increased dramatically during the past decade with the establishment of several heavily capitalized companies focusing on MSC manufacturing and cryopreservation [2] . As more individual MSC sources and products are developed and trialled as clinical products and as the regulatory framework for clinical trials of stem cell products continues to evolve, there will be a clear need to confirm and compare the potency of the immunomodulatory/anti-inflammatory effects of each product for optimal treatment of disease [7–9] .

The immunomodulatory activities of MSC *in vitro* have been measured on

different immunological cell types, including T cells, B cells, NK cells and monocytes [10–13] . Monocytes have critical roles in innate and adaptive immunity during infection and sterile inflammation and respond rapidly to activation signals via an array of pattern recognition receptors [14–16] . Monocytes circulate in the peripheral blood and upon stimulation, transmigrate into injured or infected tissues where they contribute immediately to early inflammatory responses and subsequently may differentiate into mature myeloid effector cells including macrophages and dendritic cells [17] . During inflammation, monocytes produce several key pro-inflammatory mediators including tumour necrosis factor alpha (TNF-α), interleukin 12 (IL-12), interleukin 6 (IL-6) and monocyte chemo-attractant protein 1 (MCP-1/CCL2) [4, 14] . TNF-α is involved in the pathogenesis of several diseases such as arthritis, sepsis, acute tissue ischemia, inflammatory bowel disease and GvHD. MSC administration could be used to decrease the severity of inflammation [18–21] .

In this study we describe the development of a flow cytometry-based whole blood assay to screen for potency of human bone marrow-derived MSC (hBM-MSC) to suppress innate immune responses. A key goal was to develop an assay methodology with potential to be rapidly and practically employed in cell manufacturing facilities to allow for the optimal selection of MSC donors or at point of care to facilitate "personalized" matching of a cell product to each patient.

2. Methods

2.1 Optimization of monocyte activation to use by flow cytometry

Peripheral blood from a total 10 healthy adult volunteers ranging in age from 24-64 years was collected into BD Vacutainer tubes (Sodium heparin ref. 367876, K_2EDTA ref. 367873 and Sodium Citrate ref. 363095), according to the protocol approved by the ethics committee of the National University of Ireland in Galway. Once collected, blood was normally used within three hours. However, blood could be stored up to 72 hr prior to testing (see results). In deep round bottom 96 well plates (736-0339 VWR) was added RPMI 1640 media (Gibco), Brefeldin A (eBioscience), ultrapure LPS-EB (InvivoGen) and blood at concentrations indicated below. The plates were sealed and incubated for different lengths of time at 37°C in a humidified incubator containing 5% CO_2 in air. Then, cells were surface stained for 10 minutes at room temperature in the dark with the following monoclonal antibodies (all from eBioscience): CD16 FITC (clone eBioCB16), CD45 PerCP Cy5.5 (clone 2D1), and CD14 APC (clone 61D3). Following washing, fixation and permeabilization using the IntraPrep Kit from Beckman Coulter, cells were stained intra-cytoplasmically with PE labelled monoclonal anti-TNF-α antibody (clone MAb11). In some experiments, cells were labelled with PE-labelled anti-IL-12/IL-23 p40 (clone C8.6), anti-CCL2 (MCP-1; clone2H5) or anti-IL-10 (cloneJES3-9D7). Subsequently, samples were washed, resuspended in FACS buffer (1X PBS, 2% FBS, 0.05% NaN_3) and acquired using the BD Accuri C6 (Becton Dickinson) 4 colour flow cytometer. Data were analysed with BD CSample Analysis software (Becton Dickinson) or FlowJo version 10 (Tree star). As outlined in results, experiments were designed to determine the optimal

conditions of anticoagulants, LPS dose, incubation time and blood dilution to use in the immunosuppressive assay.

For T cell activation, three activation protocols were used, namely i) PMA (5 ng/ml) plus Ionomycin (0.5 μg/ml), ii) PHA (10 μg/ml) or iii) anti-CD3 + anti-CD28 ®Dynabeads.

2.2 Calcium chelation and analysis of LPS surface binding

To determine the effect of calcium chelation, heparinized blood diluted 2 times was cultured for 4 hours with 0.6 μg/mL Brefeldin A, with or without addition of 1ng/mL of LPS and in the presence or absence of 2mM ethylene glycol tetra acetic acid (EGTA, Sigma-Aldrich). After activation, cells were stained, fixed and permeabilized as described above. In other experiments $1x10^6$ MSC or peripheral blood mononuclear cells (PBMC) isolated by Ficoll density gradient centrifugation were resuspended in Dulbecco's phosphate buffered saline without $CaCl_2$ and $MgCl_2$ (DPBS, Gibco) and stimulated at room temperature for 30 minutes with or without 1μg/mL biotinylated LPS (LPS^{biotin}) in the presence or absence of 2mM EGTA. Ultrapure LPS-EB was biotin labelled using Biotin amidocaproate N-Hydroxysuccinimide ester (Sigma-Aldrich), according to the protocol described by Brunialti *et al*. 2002 [22] . After activation, PBMC were washed twice using DPBS, incubated with CD45 PerCP Cy5.5, and CD14 APC for 10 minutes at room temperature and light protected. After washing twice PBMC and MSC, the bound LPS^{biotin} was revealed by adding Streptavidin PE for 10 minutes. Following an additional wash, cells were finally resuspended in DPBS for acquisition on the BD Accuri C6 and LPS

binding to monocytes or MSC, the latter distinguished by their larger FSC/SSC profile, recorded.

2.3 Isolation and expansion of hBM MSC

Bone Marrow (BM) aspirates were obtained from the iliac crest of healthy donors between the ages of 19 and 24. From each donor, a trained physician collected 30mL of BM aspirate into sodium heparin tubes under sterile conditions in a clinical procedure room at Galway University Hospital. Enrolment of healthy adult volunteers and collection of bone marrow samples for the purpose of generating culture-expanded MSC was approved by the Research Ethics Committee of Galway University Hospitals. The bone marrow aspirate was diluted with DPBS and filtered through 70-100μm cell strainer (BD Falcon). Mononuclear cells (MNC) were isolated from BM aspirates by Ficoll density gradient centrifugation. Viable cell numbers in MSC suspensions were calculated using Trypan Blue exclusion (Sigma-Aldrich). The first plating was at a cell density of $5x10^4$ cells/cm^2 in Nunc™ EasYFlasks, cell culture flasks (Thermo Scientific) with complete medium, namely MEM Alpha with Glutamax (Gibco) supplemented with 10%(v/v) Fetal Bovine Serum (FBS – HyClone; Thermo Scientific) and 1% (v/v) Penicillin/Streptomycin (P/S - Life Technologies), for 4 days at 37°C in a humidified incubator containing 5% CO_2 in air. After 4 days, non-adherent cells were gently removed with DPBS and fresh complete medium added. When cells reached 80-90% confluence in passage 0 (P_0) or P_1, MSC were detached using 0.25% Trypsin-EDTA (Life Technologies), and the Trypsin inactivated by adding 10X volume of complete medium.

Detached cells were then centrifuged (250g for 5 minutes at room temperature) and counted.

MSC were cryopreserved at $1x10^6$ cell/mL with freezing medium: FBS (HyClone; Thermo Scientific) containing 10% (v/v) DMSO (Sigma-Aldrich D2650). When required, MSC were thawed, washed extensively and seeded at a density of $5x10^3$ cells/cm^2 into cell culture flasks with complete medium and cultured as before. The medium was renewed every two days until the cells reached 80-90% confluence. For passaging, MSC were detached, centrifuged (250g for 5 minutes at room temperature), counted and seeded again at $3x10^3$ cells/cm^2. To use the cells for co-culture with peripheral blood monocytes, MSC were kept in culture until they reached 90-100% confluence and then detached and counted.

2.4 Immunophenotyping

The immunophenotypic characterization of hBM-MSC was performed on the day they were used for co-culture. Using the human MSC analysis kit (562245) from BD Stemflow™, $2x10^5$ cells were stained according to the manufacturer's protocol with antibodies against CD44, CD73, CD90, CD105, CD11b, CD19, CD34, CD45, HLA-DR and Propidium Iodide (Sigma-Aldrich). The samples were acquired in the BD Accuri C6 flow cytometer and analysed with FlowJo version 10.

2.5 Optimised immunosuppressive assay protocol

This assay is divided in 2 parts. Firstly, MSC were co-cultured with peripheral blood and then monocytes stained intra-cytoplasmically for TNF-α expression, using the same protocol as described above. To prepare the co-culture, peripheral blood collected

as described above was diluted 10X with RPMI 1640 media (Gibco). To each well was added 50μl of this diluted blood. Based on the average monocyte counts from n=10 donors, obtained using data from the Accuri C6 cytometer, this corresponded to 7,500 monocytes, a figure sufficient to obtain adequate events from subsequent flow cytometric analysis. The blood was cultured with 0.6μg/mL Brefeldin A (eBioscience), with or without Ultrapure LPS-EB (InvivoGen) at 1ng/mL. hBM-MSC at passages P_2 - P_7 were added to the culture in different numbers of viable cells ($2.5x10^3$, $5x10^3$, $1x10^4$, $2.5x10^4$, $5x10^4$, $1x10^5$, $2x10^5$, $4x10^5$, $5x10^5$ cells/well) and incubated for 6 hours at 37°C in a humidified incubator containing 5% CO_2 in air. For flow cytometry experiments, the viability of monocytes and MSC was determined by Propidium Iodide exclusion.

As positive controls for the inhibition of monocyte activation by LPS, either 10ng/mL of Dexamethasone (Sigma-Aldrich) or $5x10^5$ immortalized hBM-MSC (TERT cells [23]) were added to cultures containing blood cells. As a negative control, equivalent numbers of the Multiple Myeloma (MM) cell line (ATCC® ＃CRL-2975 ™) or the Jurkat T cell line (ATCC® ＃TIB-152 ™) were added. To test the potency of different MSC on monocytes from different donors, $4x10^5$ MSC preparations from different donors and at different passage number were simultaneously tested on different blood donors.

2.6 Cell contact-dependence of Immunosuppression

To determine if MSC immunosuppression was contact dependent instead of using 96 round bottom plates we used 24 well plates with transwell inserts (Greiner bio-

one # 662 640). For these experiments, $4x10^5$ MSC where placed in the bottom chamber with PBMC, LPS and Brefeldin A in the top chamber. Three separate experiments were carried out using 7 different healthy blood donors and 2 MSC donors $P_2 – P_5$ (n=10). To determine if supernatants from MSC had immunosuppressive activity, 3 different MSC preparations were seeded in 6 well plates ($4x10^5$ MSC/well) for 48 hours in the presence or absence of LPS (1ng/mL) and supernatants collected and added to the whole blood assay.

2.7 Statistical analysis

Results are expressed as average and standard deviation of percentage of TNF-α production by monocytes, using Microsoft Excel. To determine the statistical significance of the differences observed between different conditions, paired parametric t-Tests were performed, using GraphPad Prism software (version 6, San Diego, California, USA). Statistically significance differences were considered * when $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

3. Results and Discussion

3.1 Optimization of monocyte activation

Monocytes within whole blood cultures were distinguished from other PBMC by a combination of CD45 and CD14 staining (Fig. 1A) and by their distinct light scatter profile (Fig. 1B). Gated monocytes were then analysed for intra-cytoplasmic TNF-α with a clear LPS dose-dependent increase in the proportion staining positively at concentrations between 0 and 5ng/ml LPS (Fig. 1C). Monocytes were used as the target population because they are known to respond rapidly to activation by pathogen-associated and damage-associated molecular patterns (PAMPs and DAMPs respectively). The stimulus used in experiments reported here, LPS (or endotoxin), is one of the most potent pathogen-associated stimuli for monocytes and mediates its effect via binding to LPS receptor complex consisting of Toll-like receptor (TLR)-4 and its co-associated proteins CD14 and LPS binding protein (LBP). However, we have tested other activation stimuli including pam3CysSerLys4, phorbol 12-Myristate 13-Acetate, polyinosinic-polycytidylic acid (PolyIC) and N-acetylmuramyl-L-alanyl-D-isoglutamine in this assay and found that all ligands were capable of inducing TNF-α expression by monocytes within 6 hours of activation (data not shown).

One limitation of the assay is that individual monocyte subpopulations cannot be analysed. Currently, 3 monocyte subsets are defined, namely so-called classical ($CD14^{++}CD16^{-}$) non-classical ($CD14^{+}CD16^{++}$) and intermediate ($CD14^{+}CD16^{+/++}$) monocytes [4, 14, 17] . However, upon *in vitro* culture and particularly after LPS

activation, CD16 expression is down regulated (not shown), rendering it impossible to identify non-classical and intermediate subpopulations.

TNF-α was chosen for the readout because the kinetics of production following activation is extremely rapid [24] . In addition, TNF-α is an important cytokine that is involved in the regulation of a wide spectrum of biological processes and regulates the immune response by activating cell proliferation, receptor expression, and migration. In addition, TNF-α has been shown to regulate the production of other cytokines [4] . In the context of the therapeutic use of MSC, important immunosuppressive activities of MSC are mediated via the TNFα/TNFαR [18–20] . This emphasizes the potential of MSC to modulate inflammatory lesions such as GvHD and inflammatory bowel diseases.

In order to optimize the assay, variations of multiple parameters were investigated to determine optimal efficiency and sensitivity. To optimize intra-cytoplasmic accumulation of TNF-α protein, Brefeldin A was added to cultures. Brefeldin-A inhibits protein transport from the endoplasmic reticulum to the Golgi apparatus. Results indicated that concentrations from 0.6 to 3.0μg/mL did not significantly alter the proportion of monocyte expressing TNF-α and their staining intensity was indistinguishable (data not shown). Therefore, 0.6μg/mL was used for all subsequent experiments. It should be noted that monocyte viability, determined by Propidium Iodide exclusion, was not compromised during the assay.

Next, blood was collected in three different anti-coagulants, namely sodium heparin, K_2EDTA and sodium citrate, diluted 5X in RPMI and the % TNF-α^+ monocytes quantified following 6 hours stimulation with 1ng/ml LPS (Supplementary Fig 1). As

shown, there was a strikingly lower expression of TNF-α^+ detected when blood was collected in EDTA. Because EDTA is a divalent cation chelator binding Ca^{2+}, Mg^{2+} and Zn^{2+} we wished to determine whether Ca^{2+} was involved in LPS-mediated monocyte activation and therefore used the Ca^{2+}-specific chelator EGTA as anticoagulant. As shown in Supplementary Fig. 2, where heparinised whole blood diluted 2X in RPMI was activated with 1ng/ml LPS, cytoplasmic TNF-α was readily detectable in heparin (Panel A), while there was no TNF-α staining in cells cultured in the presence of 2mM EGTA (B). To investigate whether Ca^{2+} was involved in LPS binding to monocytes, we incubated PBMC with biotin-labelled LPS (LPS^{biotin}) and revealed bound LPS with Streptavidin-PE. As shown in panels C and D, EGTA did not affect the binding of LPS to monocytes. In addition, LPS did not bind to gated $CD3^+$ T lymphocytes in the same PBMC preparation (E). Taken together, these results indicate that extracellular calcium is not involved in LPS binding but plays a critical role in monocyte activation by TLR-4 ligands. Indeed, a recent publication by Rossol *et al*. shows that, via a G-protein coupled calcium receptor, extracellular calcium has a role as a danger signal activating inflammasomes in monocytes and macrophages [25] .

Dilution of whole blood prior to addition of LPS was found to significantly enhance the sensitivity of the assay as reflected in the proportion of monocytes staining positively for TNF-α for a given concentration of LPS (Supplementary Fig. 3). Thus at a 5X dilution of blood in RPMI, heparin and sodium citrate but not EDTA resulted in optimal stimulation (Panel A). However, when blood was diluted 10X, optimal stimulation was achieved in all three anticoagulants (B). The most likely explanation for these results is

calcium availability. Thus, the serum calcium concentration (0.85–1.05mg/mL) will be further reduced in the presence of either citrate or EDTA anticoagulants [26] . However, EDTA is a more efficient calcium chelator [27] . Dilution of the blood in RPMI 1640, in which the calcium concentration is 100mg/mL, will saturate the calcium chelating activity of the added anticoagulants and therefore at a 5X dilution monocyte activation is achieved in citrate but not in EDTA. At a 10X dilution, the chelating capacity of available EDTA is also compromised and the free calcium concentration becomes sufficient to activate monocytes.

Thus, one of the most critical parameters that we identified during the optimization phase of assay development was the type of anti-coagulant in which blood was collected. As has been previously reported, the selection of anti-coagulant may significantly impact the accuracy of whole blood-based diagnostic tests for hematologic disease since different anti-coagulants have varying effects on blood cells for immunophenotying, morphology or other parameters [28] . Indeed, in a recent publication by Duffy *et al*. [29] where multiple stimuli were used in a human whole blood assay to standardize stimulation systems and define boundaries of a healthy human immune response, the three anticoagulants used herein were also tested. Duffy and colleagues also found that activation by a multiplicity of stimuli, including bacterial, viral, cytokine, TCR and microbial–associated molecular patterns (MAMP) were optimally achieved when blood was collected in heparinized tubes. However, these authors made no further comments on this finding.

Based on the combined results to this point and on the previously unappreciated calcium dependency of LPS-stimulated TNF-α production, heparin was used preferentially for anticoagulation, the LPS concentration was fixed at 1ng/mL and whole blood diluted 10 fold in RPMI. The optimum time for LPS stimulation was determined to be 4-6 hours (Supplementary Fig. 4). Note that after 8-24 hours, some degree of spontaneous TNF-α expression was seen. Blood could be collected in heparin and stored at ambient temperature for 3 days and used successfully in the assay (Supplementary Fig. 5). As an alternative to TNF-α expression, IL-12 (Supplementary Fig. 6), MCP-1 and IL-10 (not shown) were assayed. As confirmed by intra-cytoplasmic staining (data not shown), production of IL-12 by activated monocytes was delayed compared with TNF-α. In addition, there was considerable variability in the proportion of IL-12-expressing cells (Supplementary Fig. 6). Therefore, to shorten the duration of the assay and to maintain reliability, TNF-α production only was monitored. Regarding to MCP-1 and IL-10, it was not possible to have reproducible MCP-1 and detectable IL-10 expression using intra-cytoplasmic expression and flow cytometry (not shown).

3.2 T cell stimulation assay

In parallel with studies investigating the quantitative inhibition of monocyte activation by MSC, studies were also carried out on T cells. To measure the inhibitory activity of MSC on T cells, CFSE-labeled activated T cells are normally used as indicator cells with added MSC inhibiting this proliferation. Results from this assay are rarely quantitated. In addition, this assay is very time-consuming and we wished to develop an assay with a rapid turnaround. Therefore, we investigated

TNF-α and IFN-γ expression and acquisition of early (CD69) and later (CD25) activation markers by freshly-activated T cells. Three activation protocols were used, namely i) PMA (5 ng/ml) plus Ionomycin (0.5 μg/ml), ii) PHA (10 μg/ml) or iii) anti-CD3 + anti-CD28 beads. PHA activation was found to be inferior to the other protocols and was not used further. Preliminary experiments demonstrated that after 24 hours of stimulation by PMA plus Ionomycin, addition of Brefeldin-A prevented up-regulation of CD69 and CD25 after 24 hours by $CD8^+$ T cells (Supplementary Figure 7); similar results were also seen with $CD4^+$ T cells. Therefore, in order to develop an assay investigating cytokine expression by both monocytes and T cells for which Brefeldin-A is necessary, expression of CD69 and CD25 was not pursued further.

Preliminary experiments with the anti-CD3 and anti-CD28 bead activation kit revealed that despite testing at several bead:T cell ratios, T cells could not be activated in whole blood cultures. However, the kit could be used to activate T cells in peripheral blood mononuclear cell preparations following Ficoll density gradient centrifugation. With such cell preparations, it was possible to observe an increase in TNF-α expression by T cells in a time and dose dependent manner. Thus, at a bead:T cell ratio of 10:1, the relatively low TNF-α expression among $CD8^+$ T cells peaked at 6 hours (Supplementary Figure 8, panel A) and in $CD4^+$ cells at 24 hours (Panel B); IFN-γ expression by $CD3^+$ cells was barely detectable (panel C).

Following optimization, PBMC were routinely activated for 24 hours with a 1:1 bead:T cell ratio and co-cultured with graded doses of TERT cells. As shown for monocyte assays (see Fig 2E below), the TERT MSC line consistently inhibited

monocyte activation and was used as positive control. However, as shown in Supplementary Figure 9, TERT cells were unable to inhibit cytokine expression by activated T cells. Similar results were obtained with fresh and cultured MSC.
In summary, despite being able to develop a rapid turnaround assay for the inhibition of monocyte activation by MSC, development of a similar assay for the inhibition of T cell activation was unsuccessful.

3.3 Immunosuppressive assay

Having optimised the various parameters for the assay, experiments were conducted to see whether MSC would inhibit monocyte activation. Human BM-MSC preparations were characterized for expression of CD73, CD90, CD105 and CD44 and for the lack of expression of CD19, CD34, CD45 and HLA-DR on gated viable cells (Supplementary Figure 10). Addition of graded numbers of hBM-MSC resulted in a dose-dependent reduction in monocyte %TNF-α^+ expression (Fig 2A). As can be seen, on addition of MSC up until about 10^5 cells per well, there is an approximately 2.5-fold (157,000 to ~60,000) decrease in the mean fluorescence intensity (MFI) of TNF-α expression with a slight reduction in % TNF-α positive cells. Then on addition of 2.5 to $5x10^5$ MSC, the % TNF-α positive cells decreases, as does the MFI of TNF-α expression by an additional 2-fold (~60,000 to ~30,000). Titration experiments from 5 different blood donors and three MSC preparations plotted as % TNF-a positive cells (Fig 2B) indicated that measurable inhibition of monocyte TNF-α production occurred when between 2.5 and $5x10^4$ MSC were added per sample and increased progressively with higher cell numbers. In

contrast, addition of equivalent numbers of the human Multiple Myeloma (MM) or Jurkat T cell lines as negative controls did not result in inhibition of monocyte TNF-α production (Fig. 2C). As expected, MSC had a mean FSC (a parameter related to relative cell size) value 2.06 times (7.24/3.52) that of MM cells (Supplementary Figure 11). In titration experiments, (Fig 2 B and C), $5x10^5$ MM cells did not inhibit monocyte activation already seen using 10-fold fewer, namely $5x10^4$ MSC making it unlikely that cell size alone was responsible for inhibition. This is a short-term (6 hour) assay in 1ml cultures in round-bottom plates; metabolic arguments are unlikely to be an explanation for the inhibitory effects of MSC on monocyte activation. As a positive control for inhibition, dexamethasone was used. As shown (Fig 2D), a dose-dependent reduction of monocyte %TNF-α expression was observed and was maximal at 10ng/ml dexamethasone – a concentration that was subsequently used in all experiments involving hBM-MSC. As an additional positive control, the hBM-MSC line TERT was used. As shown (Fig 2E), addition of $5x10^5$ TERT cells to the whole blood culture inhibited monocyte activation. Since TERT cells were routinely available, they were used as cellular positive control in all assays.

Monocytes that had been pre-activated by LPS for 1 hour prior to MSC addition were also susceptible to inhibition of TNF-α production (data not shown). Control experiments where monocytes and allogeneic hBM-MSC were co-cultured in the absence of LPS stimulation did not result in any TNF-α expression by monocytes (Fig 2E). Taken together, this indicates that monocyte inhibition is MSC-specific, there is no

activation of monocytes by MSC alone and that LPS stimulation is necessary for monocyte activation.

A series of experiments was subsequently performed in which the monocyte TNF-α inhibition assay was used to compare simultaneously the potency of different MSC preparations on monocytes from different blood donors. Results for MSC from three different BM donors at three different passage numbers assayed on blood samples from two individual volunteers are shown in Figure 3A and 3B. Results indicated comparable suppressive potency of all three MSC cultures with variability across passage number for two of the three cultures. Of note, the suppression patterns observed for the three MSC cultures and their different passage numbers were very similar for both blood donor samples assayed. When suppressive potencies for the P_4 MSC cultures from the three different BM donors were averaged from assay results for four separate blood samples, a greater inhibition of monocyte %TNF-α^+ was seen for one (MSC A) compared to the other two (MSC B and C). Consistent with previous reports [30] , we observed some variations in MSC potency (Fig. 3). Ongoing clinical trials in which the potency assay is being used will attempt to correlate *in vitro* results with clinical outcome.

3.4 Mechanism of action of MSC

Given that MSC inhibited monocyte activation in a dose-dependent manner, we wished to investigate their mechanism of action. One possibility was that MSC in the whole blood culture would remove sufficient LPS so that monocyte activation would not occur. Therefore, we used biotinylated LPS to stain PBMC and MSC as well as cells in the

whole blood assay. The biotinylated LPS used was as biologically active as the unlabelled material (not shown). As shown in Fig. 4A, LPS bound to monocytes in PBMC preparations (solid line) and addition of MSC reduced staining slightly (dotted line). As shown in figure 4B, LPS did not bind to MSC (solid line) even in the presence of PBMC (dotted line). T cells in the same PBMC preparation did not bind LPS (Supplementary Fig 2E). Thus, sequestration of LPS by added MSC was not the cause of reduced monocyte activation.

Release of soluble immune-regulatory factors by MSC such as indoleamine 2, 3-dioxygenase (IDO), hepatic growth factor (HGF), transforming growth factor-β (TGF-β), interleukin (IL)-10, prostaglandin E2 (PGE2) and human leukocyte antigen (HLA) have been implicated in their ability to inhibit of T cell proliferation [31, 32] . However, how MSC inhibit monocyte activation is currently unknown. In these experiments, transwell cultures are frequently used in which cells are cultured in flat bottom wells of plates with porous filter inserts. In carrying out these experiments, we noted that despite MSC being capable of inhibiting monocyte activation in round bottom wells of microtiter plates (Fig. 5, left four histograms), no such inhibition was seen when similar cell densities were cultured in flat-bottom plates (right two histograms). Control experiments where monocytes and allogeneic hBM-MSC were co-cultured in the absence of LPS stimulation did not result in any TNF-α expression by monocytes. Culture well geometry is known to affect cellular interactions, as for example in mixed lymphocyte cultures [33] and these results would indicate a similar phenomenon for the inhibition of monocyte activation by MSC. Based on these results, we conclude that cell

contact is most likely necessary for the mediation of the immunosuppressive activity on monocyte activation. Further investigation of the mechanism of MSC action on monocytes using transwell cultures were deemed inappropriate.

4. Conclusion

Using one of the best-described immune-modulatory activities of MSC, we were able to develop a simple, rapid whole blood quantitative flow cytometry based assay with which to measure their potency. The process of developing the assay conditions also highlighted the importance of considering a range of key technical variables and carefully optimizing each of these. Currently, MSC preparations are being supplied commercially for infusion into patients for a variety of diseases. However, to our knowledge, the only other MSC potency assay so far described measures cytokine release by activated blood cells [8] . This is a more time-consuming and expensive assay than the one described herein. Thus, the whole assay from blood collection to obtaining results, takes only 8-9 hours. The rapidity of this assay therefore has the potential of providing on the same day confirmatory information regarding the potential immunosuppressive potency of a specific batch of MSCs. Such an assay could be used to screen the recipients' blood cells for inhibition by the MSC preparation that will be injected, thereby contributing to personalized medicine. Finally, correlations need to be sought between results obtained *in vitro* with clinical outcome *in vivo*. Such clinical trials are currently being initiated.

Competing interests

The authors declare they have no competing financial interests. This assay is patented under PCT international application number 14/787,367.

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Authors' contributions

AR was responsible for all the experimental work, analysis and data interpretation, as well as manuscript drafting. TR, MG and RC obtained funding and contributed to the study design and coordination. All authors read and approved the final manuscript.

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Figures legends

Figure 1 - Gating strategy used to identify monocytes and their intracytoplasmic TNF-α expression. Panel **A** shows the gating strategy used to identify monocytes by $CD14^+$ and $CD45^+$ expression (black dots), and panel **B** shows the FSC and SSC characteristics of $CD14^+CD45^+$ gated monocytes (black dots) amongst total cells (grey dots). Panel **C** shows histograms of a titration of LPS dose versus intracytoplasmic TNF-α staining. The figure is a representative result of heparinized blood diluted 10X and stimulated with the indicated doses of LPS for 6 hour.

Figure 2 - **Inhibition of monocyte activation by MSC.** Panel **A** shows results obtained measuring TNF-α expression by activated monocytes in the presence of the indicated numbers of MSC. The %TNF-α^+ cells as well as the mean fluorescence intensity (MFI) of positive cells is indicated in the legend. Panel **B** shows the summary of titration experiments where monocytes from 5 different blood donors were stimulated with 1ng/mL LPS in 10X diluted heparinized blood for 6 hours to which were added the indicated numbers of 3 different MSC preparations plotted as %TNF-α^+ cells versus MSC dose. All statistical differences are compared with the control (0 MSC) group. Panel **C** shows the negative control for lack of TNF-α inhibition by the indicated numbers of either MM myeloma or Jurkat T cells added per well for 6 hours to 10X diluted heparinized blood. Panel **D** shows the positive control of inhibition of monocyte TNF-α expression by the indicated doses of dexamethasone added during 6 hours activation with 1 ng/ml LPS in 10X diluted heparinized blood. Compared with control (0ng/mL), all values, shown as mean % ± SD TNF-α expression, were statistically significant. Panel **E**

shows that in addition to BM MSC, TERT cells (immortalized hBM-MSC) can be used as positive control; in both cases, $5x10^5$ cells were added.

Figure 3 - **Differences between hBM MSC donors and passage number on monocytes from different blood donors**. Panel **A** and **B** show TNF-α expression of blood donor 1 and 2, respectively, in the presence of three different MSC preparations at three different passage numbers. Panel **C** shows mean % ± SD TNF-α expression in monocytes from 4 blood donors in the presence of 3 MSC donors, all at P_4. Heparinized blood was diluted 10X and activated for 6 hours with 1ng/mL LPS.

Figure 4 - **Biotinylated LPS binds to monocytes but not to MSC**. Panel **A** shows LPS^{biotin} binding to monocytes in PBMC revealed with strepyavidin-PE (solid line), whereas when co-cultured with MSC, LPS^{biotin} binding is slightly reduced (dotted line). The shaded histogram shows control strepyavidin-PE staining. Panel **B** shows that LPS^{biotin} does not bind to MSC alone (solid line), even in the presence of PBMC (dotted line). For these experiments, monocytes and MSC at a ratio of 1 : 30 were activated with 1μg/mL LPS^{biotin} for 1hour at 4**°** C.

Figure 5 – **Cell contact is necessary for monocyte inhibition by MSC**. Differences between performing the whole blood assay in a 96 well round bottom plate (left four histograms) or a 24 well flat bottom plate (right two histograms). Results are expressed as % ± mean TNF-α expression in monocytes from 7 blood donors it the presence of 2 MSC donors (P_2-P_5) (n=10). Heparinized blood was diluted 10X and activated for 6 hours with 1ng/mL LPS. Similar numbers of cells were cultured in each format. Monocytes

were cultured alone (control), stimulated with LPS (LPS), with MSC alone (MSC) or with LPS+MSC. All statistical differences were compared with the LPS alone group for each plate. Unpaired multiple t-tests, **** P<0.0001.

Supplementary Figures

Figure 1S - Differences between anticoagulants. Results shown as % ± mean TNF-α-expressing gated monocytes (n=4) indicate the effect of different anticoagulants on TNF-α expression. None of the anticoagulants used activated monocytes alone (left three histograms), but on addition of 1 ng/ml LPS (right three histograms), monocytes collected in eithe Heparin or Citrate responded well whereas those in EDTA responded poorly.

Figure 2S - Role of calcium in monocyte activation. Histograms in **A** and **B** show TNF-α expression by monocytes in heparinized blood stimulated with 1ng/mL LPS in absence (**A**) or presence (**B**) of 2mM EGTA. Blood was diluted 2X in RPMI and incubated for 4 hours at 37°C. In the bottom histograms (**C-E**) monocytes (PBMC) were stained for 30 minutes, at room temperature, with 1μg/mL biotin-labeled LPS revealed by streptavidin PE. Histograms show the binding in the absence (**C**) and presence (**D** and **E**) of 2mM EGTA. **E** shows LPS binding to monocytes but not to T cells. This figure is representative of one of four experiments.

Figure 3S - Effect of blood dilution and anticoagulant on monocytes TNF-α expression. Monocyte TNF-α expression (% ± mean positive cells) from blood collected in the

indicated anticoagulants and diluted 5X (**A**) or 10X (**B**) and stimulated for six hours with the indicated LPS concentrations (n=3).

Figure 4S - Comparison between incubation times on monocyte TNF-α expression. Heparinized blood was diluted 10X and activated for six hours with 1ng/mL of LPS for 4, 6, 8 and 24 h (n=4). Results show % ± mean TNF-α expressing cells.

Figure 5S – Effect of blood storage. Heparinized blood was collected and stored at room temperature for 0, 1, 2 and 3 days. Following storage or not, whole blood was diluted 10X and activated for six hours with 1ng/mL LPS. Control samples contained no LPS (n=3). Results show % ± mean TNF-α expressing cells.

Figure 6S - Monocyte IL-12 expression. Heparinised blood was diluted 10X in RPMI and activated for 24 hours with different LPS concentrations (n=4). Results show % ± mean IL-12 expressing cells.

Figure 7s **Brefeldin-A inhibits up-regulation of CD69 and CD25 expression by PMA + Ionomycin-activated CD8$^+$ T cells**. Heparinized blood was diluted 10X in RPMI and T cells were stimulated with 5 ng/mL PMA and 0.5 μg/mL Ionomycin for 24 h, in presence or absence of 0.6 μg/mL Brefeldin. Histograms on the left represent CD69 expression and histograms on the right CD25 expression. The figure is a representative result of one experiment from three.

Figure 8s **Cytokine expression by T cells activated with CD3/CD28 beads for 6, 24 and 48h.** A and B show TNF-α expression by CD8$^+$ (A) and CD8$^+$ (B) T cells

respectively, while C shows IFN-γ expression by $CD3^+$ T cells. $CD3^+$ T cells in PBMC preparations were stimulated with the indicated ratios of beads, in the presence of 0.6μg/mL Brefeldin A (n=3). No statistical significance was found between time points on $CD8^+$ and $CD4^+$. Comparison between "No beads" and 1:1, 1:5 and 1:10 ratios gives statistical significance at the three ratios.

Figure 9s **TERT cells do not immunosuppress T cells activated with CD3/CD28 beads.** PBMC were stimulated with CD3/CD28 beads at 1:1 ratio ($5x10^4$ $CD3^+$:beads), in the presence of $5x10^5$ TERT cells (1:10) (P_{43}-P_{44}) and 0.6 μg/mL Brefeldin A, for 24 h (n=3). Statistical significance compared to "No beads".

Figure 10s **Phenotypic analysis of hBM MSC**. Histograms show the immunophenotyping of bulk human BM MSC labeled with the indicated monoclonal antibodies (grey line) and corresponding isotype control (dark line). The lower middle panel shows staining with all negative markers.

Figure 11s **Cell size analysis of MM, JURKAT and MSC.** Shown are histograms of FSC (a parameter proportional to cell size) of MM (blue), JURKAT (black) and MSC (red, filled) analysed on an BD ACCURI cytometer. The figures in parentheses represent the relative FSC value in arbitrary units.

Vitae

Rhodri Ceredig is a research scientist with over 35 year of experience in mouse lymphocyte development. In 2008, he joined the Regenerative Medicine Institute (REMEDI) as Professor of Immunology and in collaboration with Prof Noel Lowndes has been investigating the role of hypoxia in the DNA damage response of mesenchymal stromal cells. From the early '80's he was involved in using flow cytometry as an experimental tool in immunology and is currently the academic in charge of the flow cytometry core facility at the National University of Ireland, Galway.

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