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# Development of a flow cytometry-based potency assay for measuring the *in vitro* immunomodulatory properties of mesenchymal stromal cells

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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 1 ng/mL ultrapure-LPS added to 10-fold diluted, heparin anti-coagulated whole blood incubated for 6 h at 37 °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.



## 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

*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, tumortumour necrosis factor-α.

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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 from years was collected into BD Vacutainer tubes (Sodium heparin ref. 367876, $K_2$EDTA 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 h 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 min 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 h with 0.6 μg/mL Brefeldin A, with or without addition of 1 ng/mL of LPS and in the presence or absence of 2 mM ethylene glycol tetra acetic acid (EGTA, Sigma-Aldrich). After activation, cells were stained, fixed and permeabilized as described above. In other experiments $1 \times 10^6$ 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 min with or without 1 μg/mL biotinylated LPS ($LPS^{biotin}$) in the presence or absence of 2 mM 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 min 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 min. 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 30 mL 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 70–100 μm 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 $5 \times 10^4$ 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 (250*g* for 5 min at room temperature) and counted.

MSC: were cryopreserved at $1 \times 10^6$ 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 $5 \times 10^3$ 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 (250*g* for 5 min at room temperature), counted and seeded again at $3 \times 10^3$ cells/cm$^2$. To use the cells for co-culture with periph-