Stromal cell therapy

Lysis and phenotypic modulation of mesenchymal stromal cells upon blood contact triggers anti-inflammatory skewing of the peripheral innate immune repertoire

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ABSTRACT

Background aims: Mesenchymal stromal cells (MSCs) are used to treat immune-related disorders, including graft-versus-host disease. Upon intravenous infusion, MSCs trigger the instant blood-mediated inflammatory response, resulting in activation of both complement and coagulation cascades, and are rapidly cleared from circulation. Despite no/minimal engraftment, long-term immunoregulatory properties are evident. The aim of this study was to establish the effects of blood exposure on MSC viability and immunomodulatory functions.

Methods: Human, bone marrow derived MSCs were exposed to human plasma +/- heat inactivation or whole blood. MSC number, viability and cellular damage was assessed using the JC-1 mitochondrial depolarization assay and annexin V staining. C3c binding and expression of the inhibitory receptors CD46, CD55 and CD59 and complement receptors C3aR and C5aR were evaluated by flow cytometry. MSCs pre-exposed to plasma were cultured with peripheral blood mononuclear cells (PBMCs) and monocyte subsets characterized by flow cytometry. The PBMC and MSC secretome was assessed using enzyme-linked immunosorbent assays against tumor necrosis factor alpha, interleukin (IL)-6 and IL-10. Monocyte recruitment towards the MSC secretome was evaluated using Boyden chambers and screened for chemotactic factors including monocyte chemoattractant protein (MCP)-1. MSC effects on the peripheral immune repertoire was also evaluated in whole blood by flow cytometry.

Results: Plasma induced rapid lysis of 57% of MSCs, which reduced to 1% lysis with heat inactivation plasma. Of those cells that were not lysed, C3c could be seen bound to the surface of the cells, with a significant swelling of the MSCs and induction of cell death. The MSC secretome reduced monocyte recruitment, in part due to a reduction in MCP-1, and downregulated PBMC tumor necrosis factor alpha secretion while increasing IL-6 levels in the co-culture supernatant. A significant decrease in CD14+ monocytes was evident after MSC addition to whole blood alongside a significant increase in IL-6 levels, with those remaining monocytes demonstrating an increase in classical and decrease in non-classical subsets. This was accompanied by a significant increase in both mononuclear and polymorphonuclear myeloid-derived suppressor cells.

Conclusions: This study demonstrates that a significant number of MSCs are rapidly lysed upon contact with blood, with those surviving demonstrating a shift in their phenotype, including a reduction in the secretion of monocyte recruitment factors and an enhanced ability to skew the phenotype of monocytes. Shifts in the innate immune repertoire, towards an immunosuppressive profile, were also evident within whole blood after MSC addition. These findings suggest that exposure to blood components can promote peripheral immunomodulation via multiple mechanisms that persist within the system long after the infused MSCs have been cleared.

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Introduction

The use of mesenchymal stromal cells (MSCs) as a cellular therapeutic has demonstrably grown and evolved over the last 20 years. MSC therapies have shown great promise, and their use for a variety of disorders, especially within the realm of immune dysregulation, has grown exponentially. Despite both pre-clinical studies and clinical trials reporting safe administration of MSCs through a number of routes, including intravenous (IV), our understanding as to the mode of action and fate of the cells upon infusion remains limited [1].

A large disparity in efficacy of therapy has been reported within the wider literature, a phenomenon that has been attributed, in part, to different sources of MSCs, production methods, the use of freeze–thawed versus fresh products, in vitro age or passage of the cells, cellular dosage and mode of administration [2]. Such studies using MSCs from different tissues have demonstrated that one name does not fit all, and that there is a need to understand each cell product in terms of its therapeutic potential and effect upon administration [2].

The literature has further highlighted the stark difference in blood compatibility in terms of safety between different MSC sources, with bone marrow (BM) MSCs triggering minimal clotting upon IV administration compared with, for example, decidual stem cells [3,4].

Previous studies have highlighted the potential for MSCs to trigger the instant blood-mediated inflammatory reaction upon infusion [3,5]. This theory is supported by evaluation of patient plasma, indicating a significant increase in complement C3a levels and thrombin–anti-thrombin complex formation, and a drop in platelet count, indicative of activation of both the complement and coagulation cascades [3]. Clinical studies have demonstrated fast clearance of infused MSCs from the periphery, with cells traveling to the lungs and secondary lymphoid organs exhibiting no/little engraftment [1,6]. Despite this, MSCs exert long-term therapeutic effects in multiple diseases, including type 1 diabetes and graft-versus-host disease [7–9]. Hence, engraftment is not a requirement for effect; it is rather the interaction with the immune system and endogenous tissue resident cells, including stroma, that skew inflammation away from tissue-destructive pathways towards an anti-inflammatory and long-term tolerogenic profile [10]. This phenomenon is one of the reasons that IV infusion remains the most widely used form of administration of MSC therapy, with a recent meta-review demonstrating the safety of such clinical protocols, with no elevated risk of tumor development or ectopic tissue formation compared with controls [1].

Loss of cells from the periphery, while promoting an anti-inflammatory/tolerogenic response, has been linked to MSC interaction with the innate immune repertoire [11–13]. Different mechanisms have been proposed including phagocytosis of MSCs by monocytes, as well as peripheral cytotoxic cells [11,14]. Increased recognition of the cells may occur through binding of complement fragments, such as C3b to their surface [3,15–17]. Interaction of the innate immune cells, and their subsequent skewing to an anti-inflammatory state, is proposed as one of the mechanisms resulting in a sustained therapeutic effect after MSC clearance, and the later induction of tolerance, associated with increased levels of peripheral inducible regulatory T cells [16,17].

In addition to their direct immunomodulatory effects, MSCs have been suggested to exert orchestrating effects on the peripheral immune repertoire through their release of paracrine factors, either freely, within extracellular vesicles (EVs) or by fusion of membrane particles (MPs) with specific subsets of pro-inflammatory monocytes [18,19]. We hypothesize that blood contact may result in direct lysis of MSCs, resulting in destruction of the cell membrane, and direct spillage of stored immunomodulatory factors. Although rapidly lost upon blood contact, multiple pathways for MSC-mediated immunomodulation of the peripheral immune repertoire can then culminate in a systemic anti-inflammatory effect, long surviving the clearance of infused MSCs.

Methods

Ethical approval

Human BM-derived MSCs and peripheral blood drawn for plasma and whole-blood experiments detailed herein were obtained from healthy donors who provided written consent in line with the Helsinki convention. The study was approved by the local ethical review board at Karolinska University Hospital, Huddinge, Sweden.

Isolation and culture maintenance of MSCs

Human BM-derived MSCs were isolated, expanded and characterized as previously described [3,20]. BM mononuclear cells were separated over a Percoll gradient (Redigrad; GE Health Care, Uppsala, Sweden), washed and reconstituted in Dulbecco’s Modified Eagle’s Medium low-glucose medium (Invitrogen, Waltham, MA, USA) supplemented with 9% pooled human platelet rich plasma and 100 IU/mL penicillin, 0.1 mg/mL streptomycin and 0.25 μg/mL Fungizone (Invitrogen; herein termed culture media) and plated at a density of 1.6 × 10^6 cells/cm^2. Adherent MSCs were expanded to 80% confluence, before the cells were detached with 0.05% trypsin–ethylenediaminetetraacetic acid (Invitrogen) and replated at a density of 4 × 10^5 cells/cm^2. MSCs were cultured for up to three passages for use in these experiments. Expanded cells were cryopreserved in culture media supplemented with 10% (v/v) dimethyl sulfoxide (Sigma-Aldrich Sweden AB, Stockholm, Sweden). All MSCs used in the experiments that follow were freeze–thawed directly before use.

Exposure of MSCs to peripheral blood plasma

Peripheral blood was drawn from a healthy donor (n = 1 donor with blood drawn on three separate occasions) using non-heparinized blood tubes without vacuum to limit cell activation. Lepirudin (Refudran; 50 μg/mL; Celgene Europe, Windsor, UK) was immediately added to the blood to prevent coagulation. For isolation of plasma, the blood was kept on ice until centrifugation at 2000g for 10 min at 4°C. Heat inactivation (HI) of the plasma (60°C for 30 min), followed by a second centrifugation step at 2000g for 10 min at 4°C, was used as a negative control.

Freeze–thawed MSCs (n = 8 donors) were washed and centrifuged at 500g for 7 min before resuspending in Dulbecco’s Modified Eagle’s Medium low-glucose media, counted and viability assessed using the Viability and Cell Count Assay for the NC-3000 (Chemometec A/S, Allerod, Denmark). MSCs (2 × 10^5 cells in a total volume of 100 μL) were added to an equal volume of media, plasma or HI plasma. Cells were incubated for 1 h at 37°C with 5% CO2 for all experiments unless otherwise specified. Cultures were centrifuged at 400g for 5 min at room temperature (RT) and the supernatant removed. MSCs were subsequently assessed for viability and cell number as described previously. Plasma exposed MSCs were also processed for the following assays:

Assessment of MSC mitochondrial membrane potential: JC-1 was used to assess cell damage and early-stage cell death. Treated MSCs (n = 4 donors) were incubated with 2.5 μg/mL JC-1 (ChemoMetec) for 10 min at 37°C. Stained cells were centrifuged for 5 min at 400g. The MSCs were washed twice in phosphate-buffered saline (PBS) before being resuspended in 1 μg/mL 4,6-diamidino-2-phenylindole diluted in PBS (ChemoMetec) and analyzing using the NC-3000. Gated cells were further evaluated for average diameter.

Evaluation of apoptosis using annexin V: Levels of MSC apoptosis were evaluated on the NC-3000 using annexin V and propidium iodide staining. To summarize, MSCs (n = 4 donors) were washed in PBS, centrifuged at 400g and resuspended in annexin V binding buffer (BioLegend, Koblenz, Germany) before staining with annexin V
Flow cytometry for C3c and its respective inhibitors and receptors: Binding of C3c, and cell surface expression of the complement inhibitors CD46, CD55 and CD59 and receptors C3aR and C5aR, were evaluated on MSCs exposed to plasma +/- HI. C3c binding was assessed using a time course of exposure to plasma from 0 (media control), 10, 30 and 60 min (n = 3 donors). C3c binding results at 60 min were further validated in a total of 8 MSC donors. CD46, CD55, CD59, C3aR and C5aR levels (n = 3 donors for complement inhibitors and n = 8 donors for complement receptors) were assessed at 60 min. For all antibodies, MSCs +/- plasma exposure, were washed in PBS, centrifuged at 400g for 5 min, resuspended in 1 mL of PBS and stained for 25 min with 1 mL of LIVE/DEAD Fixable Aqua-Dead Cell Stain Kit as per the manufacturer's instructions (Thermo Fisher Scientific, Stockholm, Sweden). Cells were washed in PBS, resuspended in buffer (PBS/0.1% [w/v] bovine serum albumin) and stained with the aforementioned cell surface markers or their respective fluorochrome immunoglobulin controls for 15 min at RT protected from light (Table 1). Stained cells were washed in PBS and resuspended in 1× CellFIX (BD Biosciences, Stockholm, Sweden). MSCs were run on a BD LSRII Fortessa (BD Biosciences) or CytoFLEX S (Beckman Coulter AB, Bromma, Sweden) with 5000 events recorded.

Effects of plasma-exposed MSCs on monocyte recruitment and phenotype

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats (n = 2) as previously described [5,21]. To summarize, buffy coats were diluted 1:3 with PBS, layered over Lymphoprep (STEMCELL Technologies, Cologne, Germany) and centrifuged at 500g for 30 min with no brake.

Recruitment of monocytes in response to plasma exposed MSC conditioned media

MSCs incubated, as described previously, with media, plasma or HI plasma were centrifuged at 500g for 5 min (n = 4 donors). Cells were counted and plated in 2.5 mL of culture media, in a six-well plate and incubated for 18 h at 37°C/5% CO₂. Conditioned media was harvested, centrifuged at 500g for 5 min to remove any cellular debris and stored at −80°C until further analysis.

CD14⁺ monocytes were isolated from the derived PBMC fraction using the Pan Monocyte Isolation Kit (n = 1 buffy coat; Miltenyi Biotec Norden AB, Lund, Sweden). Purity of the derived monocyte population was evaluated by flow cytometry (94% CD14⁺). To assess the directed migration of monocytes towards the conditioned media derived from MSCs exposed to culture media (control) or plasma +/- HI, 7.5 × 10⁵ CD14⁺ monocytes were loaded onto 3-μm pore-sized polyethylene terephthalate membrane plate inserts (BD Biosciences). Three hundred microliters of MSC-conditioned media was added to the bottom of the Boyden chamber. Monocytes were co-cultured with the conditioned media for 3 h at 37°C/5% CO₂. Media was subsequently removed from the bottom of the chamber and a cell count performed using the Viability and Cell Count Assay for the NC-3000 (ChemoMetec) as described previously.

Skewing of monocyte phenotype by MSCs exposed to plasma: MSCs (n = 4 donors) +/- pre-exposure to plasma +/- HI were washed, counted and viability assessed using the NC-3000 (ChemoMetec) before setting up co-cultures with PBMCs (n = 2 buffy coats) at a 10:1 (PBMC:MSC) ratio for 18 h in RPMI 1640 media supplemented with 10% (v/v) fetal calf serum and 100 IU/mL penicillin, 0.1 mg/mL streptomycin (Invitrogen). Co-cultures were performed in ultralow attachment round bottomed 96-well plates (Thermo Fisher Scientific) and incubated at 37°C/5% CO₂. Conditioned media was separated from the co-cultured cells by centrifugation at 400g for 5 min, before being stored at −80°C before analysis. The harvested cells were washed in PBS, centrifuged at 400g for 5 min and stained with LIVE/DEAD Fixable Aqua-Dead Cell Stain Kit as described previously. PBMCs were washed in PBS before staining with an antibody cocktail of CD206, CD163, CD14, CD16 and human leukocyte antigen class II (Table 1) for 15 min at RT. Cells were run on a CytoFLEX S, with 40 000 events recorded within the live cell gate. Control PBMC-only wells were run in technical repeats of four per buffy coat.

Immunomodulatory effects of MSCs in whole blood

Peripheral blood drawn from healthy volunteers (n = 2) was incubated with freeze-thawed MSCs (n = 4) at a concentration of 5 × 10⁴ cells/200 μL of blood. These calculations were based on the average clinical dose of 2 × 10⁶ cells/kg body weight (assuming an average weight of 70 kg and a total blood volume of 5 liters). MSCs were
incubated with the blood for 6 h in static conditions in LoBind 0.5 mL microfuge tubes (Eppendorf Nordic AS, Sverige Filial, Solna, Sweden) with no lid, but loose covering with Parafilm M (Sigma-Aldrich Sweden AB) to prevent evaporation at 37°C/5% CO₂. Samples were centrifuged at 400g for 5 min and the supernatant transferred to a new tube. Plasma was harvested from the supernatant fraction by further centrifugation at 2000g for 10 min at 4°C, before snap-freezing and storage at −80°C before analysis.

**Immune profiling of whole blood exposed to MSCs:** Cell pellets derived from the aforementioned experiments were resuspended in 1 × red blood cell lysis buffer (Thermo Fisher Scientific) and incubated at RT for 10 min. The reaction was stopped by the addition of 2 volumes of PBS. Cells were centrifuged at 400g for 10 min, resuspended in 1 mL of PBS and stained using the LIVE/DEAD Fixable Aqua-Dead Cell Stain Kit as described previously. Cells were washed and stained with a panel of antibodies for the evaluation of T cells, B cells, natural killer cells, natural killer T cells, monocytes, myeloid-derived suppressor cells (MDSCs) and granulocytes (Table 1) for 15 min at RT. Cells were washed in PBS and resuspended in 1 × CellFIX before analysis. Cells were run on a CytoFLEX S with 8000−100 000 total events recorded. Whole blood only controls were run in four technical repeats per blood donor used.

**Enzyme-linked immunosorbent assay (ELISA)**

Levels of monocyte chemoattractant protein (MCP)-1, interleukin (IL)-6, IL-10 and tumor necrosis factor (TNF)α were evaluated within plasma samples by ELISA according to the manufacturer’s protocol (R&D Systems, Abingdon, UK; n = 4). MCP-1 values were normalized to pg/mL per 1 × 10⁶ MSCs based on the numbers of cells seeded for conditioned media generation (see above).

**Statistics**

Data were assessed for normality using the Shapiro–Wilk test and equal variances using the F-test. Parametric data were statistically evaluated using Student’s t-test, with non-parametric data assessed using the Mann–Whitney U test (where data were unpaired), or Wilcoxon matched-pairs signed test (where data was paired). Statistical significance was assumed at P < 0.05.

**Results**

**Exposure to blood components results in necrotic cell death and rapid lysis of MSCs**

MSCs were exposed to plasma +/- HI for 1 h. Assessment of cell number demonstrated a significant drop in detectable MSCs (P = 0.0003; mean of 60.63% ± 18.29% standard deviation of the mean [SD]) upon exposure to active plasma (Figure 1A; data shown as percentage change in MSC compared with media controls set at 0% as represented by the dotted line). No significant reduction in cell number was seen upon incubation with HI plasma (Figure 1A; mean 0.1337% ± 16.87% SD). Of those detectable cells, a significant reduction in viability was evident in the active plasma group (Figure 1B; P = 0.0078; mean 86.16% ± 7.388% SD) compared with HI plasma (Figure 1B; mean 92.25% ± 5.38% SD). Data in Figure 1B are shown as percentage viability compared with media controls set at 100%.

To assess whether the remaining detectable cells were negatively impacted by exposure to plasma, MSCs from each treatment group were investigated using the JC-1 mitochondrial depolarization assay as an indicator of early-stage cell damage (Figure 1C), and annexin V as a measurement of apoptosis (Figure 1E). JC-1 staining revealed a significant induction in mitochondrial membrane depolarization, indicating damage to the MSCs in both the plasma and HI plasma groups compared with media controls (Figure 1C; P = 0.001 media versus plasma; P = 0.0035 media versus HI plasma). No significant difference was seen between plasma and HI plasma groups, indicative of a damage mechanism independent of active complement (Figure 1C). In line with this observation, exposure to plasma significantly increased the average MSC diameter, indicating cellular swelling and induction of oncosis (Figure 1D; P < 0.05; **P < 0.01; ***P < 0.001; +++P < 0.0001).

Annexin V staining demonstrated a trend towards increased apoptosis with exposure of the MSCs to active plasma, but this was not statistically significant. These data suggest that the majority of cell death occurring within this 1 h incubation time frame was not predominantly the result of apoptosis (Figure 1E). A significant reduction in the number of viable MSCs was seen with active plasma treatment compared with media controls and HI plasma (Figure 1E; P = 0.0295 media versus plasma; P = 0.0378 plasma versus HI plasma). No significant difference in the number of live cells was evident between media and HI plasma groups (Figure 1E). These findings were coupled with a significantly greater number of dead (LIVE/DEAD+) MSCs upon treatment with active plasma compared with both media controls and HI plasma (Figure 1E; P = 0.0280 media versus plasma;
increase in C3c+ MSCs between the active plasma groups at 10 min; with significantly greater levels of C3c in MSCs in the active plasma group compared with both media controls and HI plasma (Figure 2A(ii); P = 0.0155 media 30 min versus plasma 30 min; P = 0.0156 plasma 30 min versus HI plasma 30 min). There was a significant increase in C3c+ MSCs between the active plasma groups at 10 and 30 min (Figure 2A(i); P = 0.0038). This was maintained at 60 min, with significantly greater levels of C3c+ MSCs in the active plasma group compared with both media controls and HI plasma (Figure 2A(i); P = 0.0262 media 60 min versus plasma 60 min; P = 0.0267 plasma 60 min versus HI plasma 60 min).

These data were further validated with a greater number of MSC donors (n = 8 donors) within the 60-min incubation group, confirming significant binding of C3c to the cell surface of MSCs in the active plasma group compared with media controls and HI plasma (Figure 2A(ii); P = 0.0154 media versus plasma; P = 0.0177 plasma versus HI plasma). With the greater number of donors, a small, but significant increase in C3c binding was seen between the media and HI plasma groups (Figure 2A(ii); P = 0.0416).

Evaluation of the complement inhibitors CD46, CD55 and CD59 on the cell surface of MSCs +/- exposure to plasma or HI plasma for 60 min confirmed that MSCs express all three inhibitors on their cell surface, but the level of expression, as evaluated by measurement of median fluorescence intensity did not alter with C3c binding upon exposure to plasma (Figure 2B). Expression of the complement receptors C3aR and C5aR were modulated by exposure to plasma (Figure 2C). Variability in expression and response to plasma exposure was seen between donors, but significantly higher numbers of C3aR+ MSCs were seen after active plasma exposure compared with HI plasma (Figure 2C(i); P = 0.0091). In contrast, the frequency of C5aR+ MSCs significantly decreased after both active and HI plasma exposure (Figure 2C(ii); P = 0.0078 media versus plasma; P = 0.0318 media versus HI plasma).

MSCs exposed to plasma modulate the recruitment and phenotype of CD14+ monocytes

The immunomodulatory action of IV infused MSCs has been reported to be primarily induced through their early interaction with bloodborne CD14+ monocytes, resulting in MSC phagocytosis and skewing of monocytes to an anti-inflammatory phenotype. Here we demonstrate that MSCs, exposed to plasma or HI plasma, significantly reduce their secretion of the monocyte recruitment factor, MCP-1 (Figure 3A(i); P = 0.008 media versus plasma; P = 0.0319 media versus HI plasma). No significant difference in MCP-1 secretion was evident between MSCs exposed to active or HI plasma (Figure 3A(ii)).

To confirm whether this change in the secretory profile of the MSCs was functionally relevant in the chemotaxis of monocytes, purified CD14+ monocytes were incubated in Boyden chambers, with conditioned media from MSCs +/- pre-exposure to plasma or HI plasma. In line with the MCP-1 secretion analyses, a significant reduction in monocyte chemotaxis was evident in response to conditioned media from MSCs pre-exposed to active or HI plasma compared with control MSC conditioned media (Figure 3A(ii); P = 0.0357 media versus plasma; P = 0.0211 media versus HI plasma). No significant difference in the numbers of recruited monocytes were seen between active and HI plasma groups (Figure 3A(iii)).

MSCs previously exposed to plasma, whether HI or active, significantly reduced the numbers of CD14+ monocytes upon co-culture with PBMCs (Figure 3B(i); P = 0.018 PBMCs only versus plasma; P = 0.035 PBMCs only versus HI plasma). MSCs previously exposed to culture media had no impact on the frequency of CD14+ monocytes (Figure 3B(i)). Subset analysis of the monocytes revealed that those incubated with MSCs pre-exposed to plasma matured in phenotype, with a significant decrease in the number of CD14+CD16- classical monocytes (Figure 3B(ii); P < 0.0001 PBMCs only versus plasma; P = 0.0002 PBMCs only versus HI plasma). The effect was greater with MSCs exposed to active plasma compared with HI plasma (Figure 3B(ii); P = 0.0003 plasma versus HI plasma). This shift in classical monocyte phenotype was not observed with MSCs pre-incubated in media (Figure 3B(ii)). A transition to an intermediate CD14+CD16+ phenotype was observed with MSCs pre-treated with media, plasma or HI plasma (Figure 3B(iii); P = 0.0053 PBMCs only versus media; P < 0.0001 PBMCs only versus plasma and P = 0.0001 media and HI plasma versus plasma). A reduction in CD14+CD16- non-classical monocytes was evident with MSCs pre-treated with media or plasma, but not HI plasma (Figure 3B(iv); P < 0.0001 PBMCs only versus media and P = 0.003 PBMCs versus plasma). Here, the reduction was greater in the media control group compared with the active plasma pre-treated group (Figure 3B(iv); P = 0.0018).

Skewing of monocytes by MSCs away from the immature dendritic cell lineage, and toward that of anti-inflammatory primed monocytes has been reported to occur principally through IL-6 signalling [13]. Within the conditioned media of MSC:PBMC co-cultures, significantly elevated levels of IL-6 were detected in the presence of MSCs compared with PBMCs alone (Figure 3C(i); P < 0.0001 PBMCs only versus media, plasma or HI plasma). Cultures supplemented with MSCs pre-exposed to HI plasma contained significantly lower levels of IL-6 compared with the active plasma group (Figure 3C(i); P = 0.0065).

To evaluate whether the skewing of the monocytes evidenced in Figure 3B led to a significant shift in the function of the cells, levels of TNFα and IL-10 were evaluated within the culture media. TNFα secretion was significantly downregulated in co-cultures of PBMCs with MSCs compared with PBMC only controls (Figure 3C(ii); P < 0.0001 PBMCs only versus media, plasma or HI plasma). MSCs pre-exposed to active plasma were significantly more effective in downregulating TNFα levels compared with those pre-incubated with media or HI plasma (Figure 3C(ii); P = 0.0012 media versus plasma; P = 0.0007 plasma versus HI plasma). No significant difference in the downregulation of TNFα was observed between MSCs pre-exposed to media or HI plasma (Figure 3C(iii)). Interestingly, the decrease in TNFα upon exposure to MSCs was not accompanied by a switch to IL-10 production. MSCs pre-exposed to plasma, either active or HI, did not significantly modulate IL-10 production within the PBMCs (Figure 3C(iii)). MSCs pre-exposed to media in the control group did, however, significantly induce IL-10 production in PBMC co-cultures (Figure 3C(iii); P = 0.007 PBMCs only versus media). In line with these findings, levels of IL-10 in MSC:PBMC co-cultures were significantly greater where media control MSCs were used compared with both plasma and HI plasma MSCs (Figure 3C(iii); P = 0.0078 media versus plasma; P = 0.0011 media versus HI plasma). Levels of IL-10 were greater in the HI plasma group compared with active plasma (Figure 3C(iii); P = 0.0156 plasma versus HI plasma). Taken together with the data observed in the media control group, regulation of IL-10 production in monocytes by MSCs seemingly requires metabolically active, healthy MSCs.
To establish whether this functional shift in phenotype to that of an anti-inflammatory profile, correlated with levels of cell surface anti-inflammatory M2 markers, monocyte subsets were further evaluated by flow cytometry for the cell surface expression of CD206 and CD163. Classical monocytes exposed to MSCs demonstrated a greater cell surface expression of the scavenger receptor CD163, either alone or in combination with CD206 expression (supplementary Figure 1A(iii) P = 0.0049 media versus PBMCs only; P = 0.0030 plasma versus PBMCs only; P = 0.0004 HI plasma versus PBMCs only and (iv) P = 0.0090 media versus PBMCs only; P = 0.0084 plasma versus PBMCs only; P < 0.0001 HI plasma versus PBMCs only). This was evident irrespective of the MSC pre-treatment, although plasma exposure, either active or HI significantly increased this effect compared with media controls (supplementary Figure 1A(i)) P = 0.0078 media versus HI plasma, (iii) P = 0.0235 media versus plasma; P = 0.0013 media versus HI plasma and (iv) P = 0.0015 media versus HI plasma). In the predominant intermediate monocyte phenotype seen with MSCs exposed to active plasma, increased levels of CD206−CD163− monocytes were observed compared with media

**Figure 2.** Complement fragments bind to the cell surface of MSCs, regulating their expression of C3aR and C5aR. MSCs were evaluated for their responsiveness to complement fragments within plasma. (A (i)) Complement fragment C3c bound to the cell surface of MSCs in a time-dependent manner. HI plasma and culture media served as controls. Data are expressed as mean percentage C3c positive MSCs ± standard error of the mean (SEM) using a scatter graph with bars (n = 3 donors). (A (ii)) Data were further validated at the 1-h incubation time point. Data are expressed as percentage C3c positive MSCs using a box and whiskers plot showing min to max values (n = 8 donors); (B) Expression of the complement inhibitors (i) CD46, (ii) CD55 and (iii) CD59 were evaluated upon exposure to plasma for 1 h. Data are presented as median fluorescence intensity (MFI) ± SEM (n = 3 donors) using scatter graphs with bars. (C) Expression of the complement receptors (i) C3aR and (ii) C5aR were assessed on the surface of MSCs after exposure to plasma for 1 h. Data are expressed as percentage positive MSCs using box and whiskers plots showing min to max values (n = 8 donors). *P < 0.05; **P < 0.01.
and HI plasma pre-treated MSCs (supplementary Figure 1B(i)) $P = 0.0019$ media versus plasma; $P < 0.0001$ plasma versus HI plasma). This was in parallel to reduced numbers of CD206+ intermediate monocytes upon co-culture with active plasma treated MSCs compared with media control exposed MSCs (supplementary Figure 1B(ii) $P = 0.0181$ and (iv) $P = 0.0098$). In contrast, HI plasma decreased the frequency of CD206-CD163- intermediate monocytes and increased CD206+CD163+ intermediate monocytes compared with
PBMC only controls (supplementary Figure 1B(i) P = 0.0005 and (iv) P < 0.0001). In the small percentage of non-classical monocytes, active plasma generated a significant increase in CD206-CD163- expression compared with media controls (supplementary Figure 1C(i) P=0.0004), while decreasing the frequency of the CD206+CD163- subset (supplementary Figure 1C (ii) P = 0.0017 media versus plasma).

The introduction of MSCs to whole blood increases plasma levels of IL-6, modulating the phenotype of CD14+ monocytes and myeloid-derived suppressor cell subsets

To validate observations from the aforementioned experiments within a more clinically applicable model, MSCs were incubated for 6 h in whole blood. The introduction of MSCs to the blood rapidly elevated plasma levels of IL-6 (Figure 4A; P = 0.002). In line with this finding, levels of CD14+ monocytes within the blood were significantly decreased in response to the MSCs, as previously seen with MSCs pre-exposed to plasma (Figure 4B; P = 0.0402). Of the remaining CD14+ monocytes, significant skewing toward a classical monocyte phenotype was seen upon exposure to MSCs (Figure 4C(i); P = 0.0286), accompanied by a significant decrease in the number of non-classical CD14+CD16+ monocytes (Figure 4C(ii); P = 0.0286). It should be noted that the proportion of non-classical monocytes within analyzed whole blood exposed to MSCs remained greater than the classical subset (0.61% ± 0.76% classical monocytes versus 2.88% ± 0.5% non-classical monocytes of total events recorded). Further evaluation of monocyte subsets using CD206 and CD163 highlighted a significant increase in the CD206-CD163- fraction of classical, intermediate and non-classical monocytes (supplementary Figure 2A(i) P = 0.0242, B(i) P=0.0002 and C(i) P < 0.0001). In contrast, a consistent decrease in CD206+ monocytes was evident with exposure to MSCs (supplementary Figure 2A(ii) P = 0.0208 CD206+CD163- classical and B(ii) P = 0.0286 intermediate CD206+CD163- and C(ii) P = 0.018 non-classical CD206+CD163- and (iv) P = 0.0228 CD206+CD163+ non-classical monocytes). Increased expression of CD163 in CD206- intermediate and non-classical monocytes was however observed upon the addition of MSCs (supplementary Figure 2B(iii) and C(iii) P = 0.0286). No expression of classical CD206+CD163+ or CD206+CD163+ monocytes was observed by flow cytometry.

MSCs significantly induced both mononcytic myeloid-derived suppressor cells (Mo-MDSCs) (Figure 4D(i)); P = 0.0286) and polymorphonuclear (PMN)-MDSCs (Figure 4D(ii); P = 0.0077) within the model.

Discussion

The principle aim of this study was to delineate the effects of blood components on MSC viability and phenotype and establish how this interaction may downstream modulate the innate immune response. The cells used in this investigation were treated as they would be for clinical use, i.e., freeze–thawed and added directly to plasma or whole blood without recovery time, so we could establish how these cells respond to IV delivery in a clinically relevant model. Although the focus of this particular study is on recapitulating preparation of an “off-the-shelf” freeze–thawed MSC product that does not undergo culture recovery before administration, it is acknowledged that the impact of whole blood or plasma exposure on these cells may be altered by a change in processing/production procedure. The impact on freeze–thawing on increasing the vulnerability of MSCs to its environment is well documented and may contribute to the extent of cell lysis and death we report in this study [22–24].

We demonstrate that, on average, 57% of MSCs exposed to active plasma are rapidly lysed and are undetectable. This loss of cells was not seen in H1 plasma, demonstrating that this was an active process, not linked to immune cells, but rather bioactive molecules, such as complement fragments within the plasma. Those cells that survived the initial insult of active plasma exposure were not unscathed however, demonstrating a time dependent increase in the binding of C3c fragments to the cell surface of the MSCs accompanied by increased membrane damage (Figure 5).

Significant increases in the cell area, or swelling, of the MSCs seen upon exposure to active plasma or H1 plasma indicate that complement binding is not the sole factor mediating damage to the MSCs and early-stage oncosis. These data are supported by the observation of increased membrane damage, as assessed by mitochondrial depolarization. Evaluation of phosphatidylinserine on the cell membrane using annexin V demonstrated a trend toward increased numbers of apoptotic cells, indicative that although the primary mode of cell death is onotic, there is also apoptotic cell death induction. Galleu et al. [11] reported in a murine model of acute graft-versus-host disease that MSCs undergo caspase activation and apoptosis after infusion, and that this is essential for their immunosuppressive function. This observation would strongly suggest that multiple mediators induce damaging effects upon contact with blood components, from which these cells would inevitably become vulnerable to elimination by the immune system.

Interestingly, increasing evidence suggests that damage and death of MSCs upon infusion may be central to their therapeutic effect. Apoptotic bodies derived from MSCs have demonstrated beneficial effects in promoting cutaneous wound healing and in type II diabetes [25,26]. In addition to apoptotic bodies, lysis of the MSCs and damage to the cell membrane would inevitably result in the spillage of cellular contents and release of MPs and EVs. The therapeutic effects of MSC MPs and EVs have themselves been reported to trigger immunomodulatory effects, with the former selectively binding to monocytes and inducing apoptosis within the pro-inflammatory, classical monocyte subset [18,19]. A recent report has also demonstrated that although MSCs rarely migrate to the site of injury, the released EVs after infusion can reach the injury site and enhance functional recovery of immunocompetence using a rat model of severe spinal cord injury [27].

MSCs are poorly equipped for whole blood exposure, and it has been previously reported that they are injured upon complement exposure [15,16]. Within this study, we demonstrate that although MSCs, as previously described, express complement inhibitors CD46, CD55 and CD59 on their cell surface, this expression level remains unaltered by exposure to active plasma and its complement components. Interestingly, differential regulation of the complement receptors C3aR and C5aR was seen, with an upregulation of the former and a downregulation of the latter upon plasma exposure. C5a complement fragments have been described to specifically induce apoptosis in MSCs, and downregulation or blockade of the C5a:C5aR pathway inhibits cell death [17]. This may explain, in part, why cell death seen in our system appeared to be primarily onocitic. Furthermore, MSC administration also has been demonstrated to result in decreased C5a levels in serum, as well as C5aR levels in kidney tissue after ischemia reperfusion, confirmed in vitro to be mediated by the modulation of pro-inflammatory macrophages that decrease C5aR expression and TNFα secretion [28]. In contrast, the C3a:C3aR pathway has been implicated in anti-inflammatory effects, by way of downregulating pro-inflammatory responses in macrophages and facilitating phagocytosis. Furthermore, it has been previously demonstrated that C3 binding to the MSC cell surface enhances the suppression of lymphocyte proliferation, indicating that while perhaps sealing the fate of the infused MSC, this plays a significant role in their therapeutic effect [10].

Multiple papers have highlighted the importance of MSC products having high viability upon infusion [11,29], that although the ultimate fate of the MSC may be to die rapidly upon administration, both therapeutic efficacy and safety of the MSC drug product are reliant upon the cells being alive and healthy at the point of delivery. This may, in part, be due to the need for the cells to release stored immuno-modulatory factors. The short lifespan of the MSCs upon infusion challenges their ability to modulate their secretome toward an anti-
inflammatory profile with the upregulation of indoleamine-2,3 dioxygenase, for example. Factors, such as IL-6, are constitutively produced by the MSCs though and have been previously indicated to play a significant role in the skewing of the monocyte compartment [13,14,18].

Using a combination of plasma conditioning of MSCs and whole-blood experiments, we were able to delineate the direct effects on the monocyte compartment and how blood exposure modulated the phenotype of the MSCs. In both systems, we observed a significant release of IL-6 by MSCs that was not detrimentally affected by blood component contact. Intriguingly, exposure to plasma resulted in a decrease in the secretion of the monocyte recruitment chemokine, MCP-1, which led to a suppression in the directed recruitment of monocytes in culture. These data demonstrate that those cells surviving blood contact can significantly modulate their secretome within 24 hours.

Co-culture of MSCs with PBMCs demonstrated that MSCs modulate monocyte maturation, with those previously exposed to active plasma reducing the numbers of classical monocytes, while increasing those with an intermediate phenotype. Irrespective of the culture system, previous exposure of MSCs to whole blood or plasma significantly reduced the frequency of CD14+ monocytes, specifically

Figure 4. MSCs exposed to whole blood suppress CD14+ monocytes while increasing levels of myeloid-derived suppressor cells. (A) MSCs exposed to whole blood trigger a rapid upregulation in plasma concentrations of IL-6. Data are expressed as pg/mL. (B) This was accompanied by a decrease in the frequency of CD14+ monocytes. The phenotype of the remaining monocytes was skewed by the presence of MSCs, with (C(i)) an increase in the frequency of classical monocytes and decrease in those with an (ii) intermediate phenotype, although this did not reach statistical significance. (iii) A significant decrease in the frequency of non-classical monocytes was also evident. With the overall decrease in frequency of CD14+ monocytes, small but significant increases in the frequency of (D(i)) Mo-MDSCs and (ii) PMN-MDSCs were observed. Data are expressed as percentage of cells. All figures are presented as box and whiskers plots showing min to max values. *P < 0.05; **P < 0.01.
where remaining cells are then cleared from the system.

MSCs surviving their peripheral interactions are trafficking milieu able to skew the adaptive T-cell repertoire to a regulatory phenotype, resulting in the activation of heme-oxygenase 1 and the production of anti-inflammatory heme metabolites. Heme-oxygenase 1 itself has been implicated in triggering the first wave of immunomodulatory effects by MSCs, ultimately resulting in a suppressive effect on T-cell proliferation and induction of IL-10+ and transforming growth factor β+ T cells [37].

CD206 is also a scavenger receptor, primarily functioning by binding to glycoproteins including myeloperoxidase, tissue plasminogen activator and lysosomal hydrolases released during inflammatory events [38,39]. Interestingly, although previous exposure to active plasma modulated the ratio of monocyte subsets with respect to this receptor, no upregulation in any monocyte subset within the whole blood system was evident. It is interesting to hypothesize why such a difference would be seen but indicates that the cascade of multiple anti-inflammatory signaling pathways initiated in whole blood by different cell populations may differentially shift the profile of monocytes.

Conclusions

Freeze–thawed MSCs exposed to whole blood, during the process of IV administration, are largely lost through cell lysis. Those remaining cells demonstrate cellular damage, which presents an altered phenotypic profile, characterized by the binding of C3c to their cell surface, modification to their secretome, and a vulnerability to downstream cell death (Figure 5). The sacrificial effects on the MSCs, however, form the innate immune trigger, resulting in an anti-inflammatory skewing of the monocyte profile, with elevated levels of classical monocytes and an increase in the frequency of both Mo-MDSCs and PMN-MDSCs. This shift in the peripheral immune repertoire provides the foundation for modulation of both the T- and B-cell compartment, and induction of tolerogenic responses that can be maintained long after the MSCs have been cleared.

Declaration of Competing Interest

LCD and TPF are employed by NextCell Pharma AB, Sweden, Sweden. LCD owns and acts as consultant for CellTherEx Consulting AB, Stockholm, Sweden. All other authors have no commercial, proprietary or financial interest in the products or companies described in this article.

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Author Contributions

Conception and design of the study: LCD and KLB. Acquisition of data: LCD, SQ, CEJ, ATA and TPF. Analysis and interpretation of data: LCD, CEJ, ATA and TPF. Drafting or revising the manuscript: LCD and SQ. All authors have approved the final article.

Supplementary materials

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