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Human Mesenchymal Stem Cell–Educated Macrophages Are a Distinct High IL-6–Producing Subset that Confer Protection in Graft-versus-Host-Disease and Radiation Injury Models

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A B S T R A C T

Mesenchymal stem cells (MSCs) have immunosuppressive and tissue repair properties, but clinical trials using MSCs to prevent or treat graft-versus-host disease (GVHD) have shown mixed results. Macrophages (MØs) are important regulators of immunity and can promote tissue regeneration and remodeling. We have previously shown that MSCs can educate MØs toward a unique anti-inflammatory immunophenotype (MSC-educated MØs [MEMs]); however, their implications for in vivo models of inflammation have not been studied yet. We now show that in comparison with MØs, MEMs have increased expression of the inhibitory molecules PD-L1, PD-L2, in addition to markers of alternatively activated MØs: CD206 and CD163. RNA-Seq analysis of MEMs, as compared with MØs, show a distinct gene expression profile that positively correlates with multiple pathways important in tissue repair. MEMs also show increased expression of IL-6, transforming growth factor-β, arginase-1, CD73, and decreased expression of IL-12 and tumor necrosis factor-α. We show that IL-6 secretion is controlled in part by the cyclo-oxygenase-2, arginase, and JAK1/STAT1 pathway. When tested in vivo, we show that human MEMs significantly enhance survival from lethal GVHD and improve survival of mice from radiation injury. We show these effects could be mediated in part through suppression of human T cell proliferation and may have attenuated host tissue injury in part by enhancing murine fibroblast proliferation. MEMs are a unique MØ subset with therapeutic potential for the management of GVHD and/or protection from radiation-induced injury.

INTRODUCTION

Cellular therapies such as mesenchymal stem cells (MSCs) have potent immunosuppressive properties and have demonstrated efficacy in radiation protection [1,2] and graft-versus-host-disease (GVHD) in experimental and human studies [3-5]. It has been shown that MSCs isolated from the bone marrow (BM) are effective in treating acute GVHD [6,7], particularly in children with gut GVHD [8]. However, in a phase III trial to treat steroid-resistant GVHD, MSC infusions were found to be safe but did not meet the primary endpoint and were not shown to be superior to pharmacologic immunosuppression [9]. Thus, clinical-grade MSCs are not yet approved in the United States for treatment of acute GVHD. Although MSCs are conditionally approved in Canada and some other countries to treat acute GVHD, their ultimate role in the management of GVHD is still not clear [10-12].

Data have shown that MSCs regulate immunity in part through regulation of monocytes [13] and macrophages (MØs) [14-16]. MØs are recognized for their ability to polarize into subsets of classically activated (M1) MØs, which mediate tissue damage and are "proinflammatory," or alternatively activated (M2) MØs, which contribute to wound healing and tissue repair and are "anti-inflammatory" [17,18]. More recently, MØs are emerging as important players in mediating immunomodulation and tissue homeostasis [19,20]. Previously, we described in vitro a unique population of MØs, human MSC-educated MØs (MEMs), characterized by high levels of expression of the anti-inflammatory/tissue regenerative cytokines IL-10 and IL-6 and low levels of expression of the

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proinflammatory cytokines IL-12 and tumor necrosis factor (TNF)-α [21]. In contrast, M2 MØs typically express high levels of IL-10 but low levels of IL-6 in addition to low levels of IL-12 and TNF-α [18,22]. Since our initial report, other investigators have published on the ability of both murine and human MSCs to recruit monocytes and MØs to sites of inflammation and switch their phenotype to M2 MØs [14,23-25]. However, ex vivo generated MEMs have not been examined for their efficacy in inflammatory models in vivo.

We hypothesized that MEMs could be a potential cellular therapeutic for GVHD. In this report we show that human MEMs have genetic and cell surface molecule expression profiles in vitro consistent with anti-inflammatory cell subsets that facilitate wound healing and tissue repair and are characterized by high IL-6 expression that is controlled in part by redundant but nonoverlapping signaling pathways. We also show that MEMs are also superior to MSCs in managing xenogeneic GVHD in vivo in part by reducing T cell proliferation. MEMs also increase survival from lethal total body irradiation in vivo in part by augmenting fibroblast expansion.

METHODS

Mice

Female NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (NSG) mice were purchased from The Jackson Laboratory (Bar Harbor, ME), and males and females between 8 and 16 weeks of age were used. All animals were bred and housed in a pathogen-free facility throughout the study. The Animal Care and Use Committee at the University of Wisconsin approved all experimental protocols.

Human MØ and MEM Cultures

Healthy donor peripheral whole blood buffy coats were purchased from Interstate Blood Bank, Inc. (Memphis, TN). Peripheral blood mononuclear cells (PBMCs) were isolated from the buffy coats by density-gradient separation, and monocyte isolation followed by MØ generation was performed as previously described [21]. MSCs were obtained from BM filters from University of Wisconsin Hospital and Clinics through an institutional review board–approved protocol. Filters were rinsed in PBS and MSCs were isolated from erythrocytes and platelets in the filters by Ficoll density gradient separation, then cultured in alpha MEM media (CellGro; Corning, Manassas, VA) supplemented with 10% heat-inactivated FBS (Hyclone, Logan, UT), 100X L-Ala-L-Glutamine (GlutaGro; Corning), and 100X NEAA (Corning), and used at passage 4 or 5 [21,26]. MSCs were verified by morphology, adherence onto 75-cm^2 plastic flasks (Greiner Bio-one, Monroe, NC), and flow cytometry.

On day +7, after releasing MSCs using trypsin (TrypLe; Invitrogen, Carlsbad, CA), cells were washed twice with PBS and then added to MØ cultures to develop MEMs, at a ratio of 10:1 of MØs/MSCs, as previously described [21]. On day +10, CD14$^+$ cells were collected using Stem Pro Accutase cell detachment (Gibco Life Technologies, Waltham, MA) and resorted based on CD14 to eliminate the MSCs in the MEM cultures.

Measurement and Inhibition of IL-6 Production

On day +7 of human MØ generation from CD14$^+$ monocytes, MØs were plated at 2×10^5 cells/well in a 6-well plate in media alone or stimulated with 20 ng/mL recombinant human IL-4 (animal free) (BioLegend, San Diego, CA) or 50 ng/mL human IL-13 (animal free) (PeproTech Inc., Rocky Hill, NJ) or co-cultured with BM-MSCs (2×10^4 cells) in direct cell contact to generate MEMs (ratio of MØ/MSC is 10:1). Some groups of MEMs (MØ plus MSCs in direct contact) were treated with NS398, a cyclo-oxygenase (COX)-2 inhibitor, at 5 μM/well (Cayman Chemical, Ann Arbor, MI); NOR-NOHA, an arginase inhibitor, at 300 μM/well (Cayman Chemical); or ruxolitinib, a JAK1/JAK2 inhibitor, at 1 μM/mL (INCB-018424; Active Biochem, Maplewood, NJ).

For transwell experiments, .4-μm pore size transwells (24-mm insert; Corning, Kennebunk, ME) were placed in 6-well plates containing MØs (2×10^5 cells/well) in the lower chamber and allowed to differentiate for 7 days. Then, MSCs (2×10^4 cells/transwell) were seeded in the upper chamber above the MØs and incubated at 37°C for 3 more days as previously described [21]. Supernatant from lower chamber of the transwell system were collected and frozen at −20°C for IL-6 assessment by ELISA.

In other experiments, after generation of MØs or MEMs for 10 days, CD14$^+$ cells were resorted from MEMs to purify the MØ population and eliminate MSCs in the MEM cultures. CD14$^+$ MØs or CD14$^+$ MEMs were replated in 96-well plates in triplicate for each donor at 1×10^5 cells/well in media or stimulated with 1 μg/mL lipopolysaccharide (LPS) from *Escherichia coli* ultrapure 0111:B4 (Sigma Aldrich, St. Louis, MO). Human IL-6 levels in supernatants from human MØ, MEMs, MØ + IL-4, or MØ + IL-13 were quantified using the Legend Max ELISA kit with precoated plates with human IL-6 according to manufacturer's instructions (cat. no. 430507; BioLegend). ELISA plates were read at 450 nm on a VERSAmax Tunable Plate Reader (Molecular Devices, Sunnyvale, CA), and data were collected using SOFTmax PRO software (Molecular Devices).

Reverse Transcriptase-PCR

Total RNA was extracted using the RNeasy mini kit (Qiagen, Germantown, MD) with on column DNAse I treatment (Qiagen) following manufacturer's instructions. Total RNA from each sample was quantified using a Nano Drop (ThermoFisherScientific, Waltham, MA), and the resultant RNA was reverse transcribed to cDNA using the high capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA). cDNA was then used for reverse transcriptase-PCR using AB Step-ONE Plus (Applied Biosystems) in the presence of SYBR Green Supermix (Applied Biosystems, Warrington, UK). Human primer assays for TNF, IFN-γ, IL-6, IL-12α, IL-1α, IL-1β, IL-10, transforming growth factor (TGF)-β, arginase-1, NOS2, NOS2, CD206, CD163, CD39, and CD73 were purchased from Qiagen. Each reaction mixture of primer, cDNA, Syber green, and water was set up 3 to 5 replicates per treatment group for each of those primers.

mRNA levels were calculated using the comparative threshold cycle method (C_t). C_t values for the housekeeping gene (*GAPDH*) and for the genes of interest were determined, and the difference between the C_t values of each gene of interest and the mean GAPDH C_t was calculated (ΔC_t). Differences in ΔC_t ($\Delta\Delta C_t$) of genes of interest in MEMs were normalized to MØ control group as shown in the following equation: $\Delta\Delta C_t = \Delta C_t(\text{MEMs}) - \Delta C_t(\text{mean of MØs})$. Reverse transcriptase-PCR data are presented as fold change expression = $2^{-\Delta\Delta Ct}$ of each gene in comparison with the MØ group. A dissociation melt curve at the end of reverse transcriptase-PCR was also run to verify the homogeneity of the PCR products and absence of primer-dimers.

Rnaseq Library Preparation

MØs or MEMs were generated for 10 days as described above. RNA was extracted from frozen cell pellets of MØ-derived from blood (MØ-PBMC) or MEMs (also blood derived) or MØ derived from BM (MØ-BM) using Qiagen's RNeasy kit. RNA was treated with DNase I (New England Biolabs, Ipswich, MA) and then analyzed for quality using the RNA 6000 Pico Kit and the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). One to 5 μg of total RNA was treated with RiboZero rRNA Removal Reagent (Epicentre, Madison, WI), and then qualitative analysis was performed on the Bioanalyzer to ensure 18S and 28S rRNA peaks were no longer present. rRNA-depleted total RNA (.5 to 50 ng) was used to generate RNA sequencing libraries using the ScriptSeq v2 RNA-Seq Library Preparation Kit (Epicentre). Libraries were prepared to a mean size of 300 to 500 base pairs and analyzed using the high sensitivity DNA Kit (Agilent Technologies) with the Bioanalyzer. Libraries were then diluted 1:10,000 and analyzed using the Library Quant Kit (Kapa Biosystems, Wilmington, MA) on the LightCycler96 (Roche Diagnostics, Indianapolis, IN). The samples were then normalized for accurate loading on the Hiseq2000 platform (Illumina, San Diego, CA).

In Vivo Xenogeneic GVHD Model

In the established GVHD model, on day +0 male and female NSG mice received 30×10^6 human PBMCs previously frozen and thawed and injected in .2 mL i.v. in the absence of any conditioning. Engraftment of human CD45$^+$ cells were assessed by flow cytometry and staining for human anti-CD45 in the spleen, peripheral blood, and BM [27] before starting treatment with PBS, MEMs, or MSCs. On day +18, when mice showed clinical evidence of GVHD, mice were randomized to receive PBS, 5×10^5 human BM-derived MSCs (passage 4 or 5), or 5×10^5 MEMs in .2 mL PBS i.v to treat GVHD and monitored for survival.

In Vivo Lethal Radiation Model

On day 0 NSG male and female mice received 3 Gy lethal total body irradiation followed by (3 hours later) PBS, 5×10^5 human MØs, 5×10^5 human BM-derived MSCs (passage 4 or 5), or 5×10^5 MEMs treatment i.v. Mice were monitored 3 times a week for survival, weight change, and clinical sickness scores, similar to GVHD scoring, based on weight loss, posture, activity, fur grooming, and skin texture, as previously described [28].

Murine Fibroblast Proliferation Assay

After 10 days of MØ generation, human CD14$^+$ sorted MØ and CD14$^+$ sorted MEMs (to eliminate MSCs) were seeded at 2.5×10^5 cells with 1×10^4 NIH-3T3-GFP murine fibroblasts (Cell Biolabs Inc, San Diego, CA) in MØ media in T-25 flasks for 7 days at 37°C. NIH-3T3-GFP cells were also labeled with Violet Proliferation Dye 450 (VPD450, cat. no. 562158; BD Biosciences, San Jose, CA) when adding them with MØ or MEMs to check fibroblast proliferation by flow cytometry on day 7 of co-cultures. Proliferating NIH-3T3 were gated on GFP$^+$ VPD450$^-$ cells.