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Monocytic myeloid-derived suppressor cells generated from rhesus macaque bone marrow enrich for regulatory T cells

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Abstract

Putative monocytic myeloid-derived suppressor cells (mMDSC; lineage[−]HLA-DR^{−/lo}) were generated in 7-day cultures from normal rhesus macaque bone marrow (BM) cells in GM-CSF and IL-6. Three subsets were identified based on their differential expression of CD14, CD33, CD34 and CD11b. Following flow sorting, assessment of the capacity of these subsets to suppress anti-CD3/CD28-stimulated CD4 and CD8 T cell proliferation revealed that the most potent population was CD14^{hi}CD33^{−/lo}CD34^{lo}CD11b^{hi}. These BM-derived mMDSC markedly increased the incidence of CD4⁺CD25⁺CD127[−]Foxp3⁺ regulatory T cells in responder T cell populations. They offer potential value in testing the therapeutic efficacy of immunoregulatory mMDSC for the promotion of tolerance in nonhuman primate transplant models.

Keywords

myeloid-derived suppressor cells; bone marrow; nonhuman primate; T cells; immune regulation

1. Introduction

Myeloid-derived suppressor cells (MDSC) comprise a heterogeneous population of regulatory innate immune cells [1, 2] defined initially by their absence of MHC class II and lymphoid-associated antigens. They arise from bone marrow (BM) precursors during acute and chronic inflammation, including transplantation and can potently inhibit T cell activation and proliferation [3, 4]. MDSC expansion is driven by hematopoietic growth

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Conflict of interest

The authors declare no financial or commercial conflict of interest.

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factors (granulocyte colony-stimulating factor [G-CSF], macrophage [M]-CSF and GM-CSF) and other molecules [4] that act together with pro-inflammatory cytokines to prevent their terminal differentiation into macrophages, dendritic cells (DC) or granulocytes [5]. Phenotypic criteria for identification and characterization of subpopulations of human MDSC (polymorphonuclear [PMN]-MDSC, monocytic [m]MDSC and early-stage MDSC) have recently been defined [6].

MDSC expansion appears to be required for the induction of organ transplant tolerance in rodents [7, 8], while accumulation of MDSC that mediate forkhead box p3 (Foxp3)⁺ regulatory T cell (Treg) expansion has been described in the blood of stable renal transplant patients [9]. In rodents, MDSC have also shown promise as cellular therapeutic agents in inhibiting experimental graft-versus-host disease (GVHD) lethality after hematopoietic stem cell transplantation [10] and in prolonging pancreatic islet or heart allograft survival by enhancing Treg [11, 12]. Significantly, mMDSC, that are the subject of this study, have been shown to be more suppressive than PMN-MDSC in mice [13, 14] and efficient suppressors of human CD4⁺ T cell proliferation [9].

While MDSC are well-characterized in mice and humans, only limited information is available regarding MDSC in nonhuman primates (NHP). These species, in particular rhesus macaques, serve as important pre-clinical models for testing of new anti-viral vaccines and of innovative tolerogenic therapies, including regulatory immune cell therapy in transplantation [15]. Recently, we reported [16] that mMDSC isolated from cytokine (G- and GM-CSF) - mobilized rhesus macaque PBMC could suppress autologous T cell proliferation and enhance Treg. Given the very limited number of mMDSC that can be flow-sorted from normal or mobilized peripheral blood for systemic adoptive cell transfer, we examined whether mMDSC could be generated from rhesus BM cells. Our results show that mMDSC can be generated readily ex vivo from femoral BM of healthy (non-mobilized) rhesus, providing an additional source of these immunoregulatory myeloid cells for potential transfer to hematopoietic stem cell or organ transplant recipients. These BM-derived mMDSC (BM-mMDSC) exhibited potent T cell suppressive activity and enhanced numbers of Treg relative to T effector cells in the responder cell population.

2. Materials and methods

2.1. Rhesus macaques

Captive-bred, simian immunodeficiency virus-negative, herpes B virus-negative, male juvenile Indian rhesus macaques (*Macacca mulatta*) weighing 5–7 kg were used. They were obtained from the National Institute of Allergy and Infectious Disease (NIAID)-sponsored rhesus macaque colony (Yemassee, SC) and maintained in the NHP Research Facility of the Department of Laboratory Animal Resources at the University of Pittsburgh School of Medicine. All procedures were approved by the University of Pittsburgh Institutional Animal Care and Use Committee and conducted according to the guidelines set forth in the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2. Generation of mMDSC from rhesus BM

Femoral BM cells (20×10^6) were cultured in T75cm² flasks for 7 days (d) in DMEM media (ThermoFisher Scientific, Waltham, MA) supplemented with 7% v/v human AB serum (Gemini Bio-Products, Sacramento, CA), recombinant human (rh) GM-CSF (80 ng/ml) and rhIL-6 (15 ng/ml) (RD Systems, Minneapolis, MN). Fresh cytokines were added on d 3 and 5 of culture. mMDSC flow-sorted from the peripheral blood of G- and GM-CSF-treated rhesus macaques as described [16] were used in functional comparison experiments.

2.3. Antibodies and fluorescence-activated cell sorting (FACS)

The cultured cells were stained for 15 min with the following fluorochrome-conjugated cross-reactive anti-human monoclonal antibodies (mAbs): CD3, CD20, HLA-DR, CD14 and CD33. After washing, putative mMDSC with the surface phenotype CD3⁺, CD20⁺ (lin⁺), HLADR^{-/lo} were flow-sorted based on CD14 and CD33 expression. Thereafter, they were further stained with the cross-reactive anti-human mAbs: CD34 and CD11b. Further details of the mAbs used and their sources are shown in Table 1.

2.4. Analysis of mMDSC suppressive function

Autologous normal rhesus peripheral blood T cells were isolated as described [17], using a NHP Pan T cell Isolation Kit (Miltenyi Biotec, Auburn, CA) according to the manufacturer's protocol. The T cells were then stained with 1 μ M Violet Proliferation Dye (VPD) 450 (BD Biosciences, San Jose, CA) per the manufacturer's protocol. The ability of putative mMDSC to suppress proliferation of these labelled T cells was assessed by co-culture of 50,000 (1:2) or 25,000 (1:4) flow-sorted mMDSC (added at the start of culture) per 100,000 T cells for 4 d in the presence of anti(α)-CD3/CD28 beads (Miltenyi Biotec) (1 bead: 15 T cells). VPD450-labelled T cells incubated in media alone or with α CD3/CD28 beads were included as controls. Proliferation was estimated by VPD450 dilution analysis.

2.5. Regulatory T cell (Treg) analysis

Putative mMDSC were co-cultured with VPD450-labelled autologous T cells as described in 2.4 (above). After 4 d, the cells were harvested and surfaced stained with cross-reactive anti-human mAbs: CD3, CD4, CD25 and CD127, as detailed in Table 1. After washing, the cells were stained intracellularly for forkhead box p3 (Foxp3), as described [18].

2.6. Statistics

Data are expressed either as means $\pm$ 1 standard error or shown as results obtained from individual animals (n=2–3 monkeys).

3. Results

3.1. Flow sorting and characterization of putative rhesus BM-derived mMDSC (BM-mMDSC)

The phenotype of putative mMDSC that could be generated from normal rhesus BM were uniformly lin⁺, HLA-DR^{-/lo} and expressed CD33 and CD14, as shown in Fig. 1, confirming their mMDSC phenotype [6]. Three putative mMDSC subsets: Group 1 (Gp1), Group 2

(Gp2) and Group 3 (Gp3) were generated from fresh BM (Fig. 1A). All three groups were lin^- and $\text{HLA-DR}^{-/\text{lo}}$, but differed in their expression of markers CD33 and CD14. The three putative mMDSC populations were flow sorted based on CD14 and CD33 expression and afterwards stained for markers CD34 and CD11b. Gp1 cells were CD14^{hi} , $\text{CD33}^{-/\text{lo}}$, CD34^{lo} and CD11b^{hi} , Gp2 cells were CD14^+ , CD33^+ , CD34^+ and CD11b^+ and Gp3 cells were $\text{CD14}^{-/\text{lo}}$, CD33^{hi} , CD34^+ and $\text{CD11b}^{-/\text{lo}}$ (Fig. 1B, C).

Flow-sorted, BM-derived, putative mMDSC cell subsets from 2 unrelated, MHC-incompatible monkeys were examined for their ability to suppress $\alpha\text{CD3-CD28}$ -stimulated autologous T cell proliferation and to ascertain whether they increased the incidence of proliferating $\text{CD4}^+\text{CD25}^+\text{CD127-Foxp3}^+$ Treg. Gp1 cells demonstrated the most potent ability on a per cell basis to suppress both CD4^+ and CD8^+ T cell proliferation (Fig. 2A and Supplementary Fig. 1) and to increase the incidence of proliferating Treg (Fig. 2B and Supplementary Fig. 2). Whereas Gp2 cells showed a reduced ability to suppress T cell proliferation and increase the incidence of proliferating Treg compared with Gp1 cells, Gp3 cells showed almost no ability to perform either function (Fig. 2A–D). Based on their surface phenotype and suppressive function, flow-sorted Gp1 cells were deemed the most robust BM-mMDSC population.

We then compared directly the suppressive ability of flow-sorted Gp1 BM-mMDSC to that of rhesus peripheral blood mMDSC mobilized in vivo by administration of G- and GM-CSF and flow-sorted as described [16]. Similar suppressive function on a per cell basis was observed Supplementary Fig. 3).

3.2. Yields of flow-sorted (Gp1) BM-mMDSC

Twenty million femoral BM cells isolated from each of 3 normal rhesus monkeys were cultured for 7 d, as described in the Materials and Methods. The mean percentage of putative Gp1 mMDSC as shown in Fig. 1 that could be flow-sorted from the harvested cells was $5.1 \pm 0.5\%$ (Table 2). Since the mean total number of BM cells that could be isolated from the 2 femurs of rhesus macaques was 757 ± 38.10^6 , the total theoretical number of Gp1 BM-mMDSC that could be generated and sort-purified was $38 \pm 2.4.10^6$. If it is assumed that the mMDSC were required for adoptive transfer into a 5 kg young adult recipient monkey, then the maximum number of Gp1 BM-mMDSC that could be infused per kg body weight would be $7.7 \pm 0.4/\text{kg}$ (Table 2).

4. Discussion

There is currently considerable interest in regulatory immune cell therapy for the promotion of allograft survival and transplant tolerance [19]. Based on studies in small animal models, mMDSC are potentially valuable regulatory innate myeloid cells for adoptive cell therapy testing in large animal models, particularly NHP [4, 16] and ultimately in humans. Our data show, for the first time to our knowledge, that mMDSC can be generated from the BM of healthy (non-mobilized) rhesus macaques. Like mMDSC mobilized with G- and GM-CSF into rhesus peripheral blood [16], these BM-mMDSC (particularly the CD14^{hi} , $\text{CD33}^{-/\text{lo}}$, CD34^{lo} CD11b^{hi} Gp1 population) proved to be potent suppressors of autologous CD4^+ T cell and to a less marked extent, CD8^+ T cell proliferation. They were also very effective in

promoting increased numbers of CD4⁺Treg relative to T effector cells. The BM could therefore serve as an alternative or additional source of these regulatory immune cells for therapeutic evaluation. Numbers of BM cells that can be harvested from macaque long bones and ileum by perfusion have been documented [20], whereas vertebral bodies of organ donors can also serve as a source of BM cells for use in combination with organ transplantation [21]. Thus, the potential numbers of mMDSC that can be generated from collective BM sources may markedly exceed those that can be isolated from blood or generated from circulating precursors.

Our studies provide new information on the characteristics of ex vivo-generated rhesus mMDSC, that complements that we have described previously for mMDSC isolated from PBMC of rhesus monkeys treated with G- and GM-CSF. As with mouse mMDSC [10], arginase-1 appears to play an important role in their T cell inhibitory function [16]. Other effector molecules, including iNOS, heme oxygenase-1 and IDO have been implicated in suppression of alloimmune or anti-tumor immune responses by MDSCs [7, 22, 23]. Increases in circulating mMDSC have been reported recently in simian immunodeficiency virus (SIV)- or influenza A-vaccinated monkeys [24, 25]. Paradoxically however, reductions in these cells have been observed in the BM of chronically SIV-infected macaques [26]. Comparison of the phenotype and function of mMDSC isolated from virally-infected monkeys and those generated in this study from normal BM cells would be of interest.

MDSC have been generated previously ex vivo from mouse BM cells in response to G- and GM-CSF and shown to both depress allogeneic T cell responses in an L-arginine-dependent manner and inhibit GVHD [10]. Cells resembling mMDSC and with ability to reduce T cell proliferation have also been generated ex vivo from PBMC of healthy rhesus macaques in response to GM-CSF, IL-6, IL-1, prostaglandin (PG) E2 and IL-11 [25], a more elaborate proinflammatory cytokine cocktail than that used to generate mMDSC from BM cells in the present study. Furthermore, addition of PGE2 to GM-CSF/IL-4-supplemented human blood monocyte cultures has been shown to induce an MDSC phenotype and cytotoxic T lymphocyte-suppressive function [27]. The present observations offer an approach to ex vivo generation of potentially suppressive NHP mMDSC that may prove valuable in assessing their therapeutic efficacy for promotion of transplant tolerance in the important pre-clinical rhesus macaque model.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

BM	bone marrow
Foxp3	forkhead box p3

(m) MDSC (monocytic) myeloid-derived suppressor cells
Treg regulatory T cells

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Highlights

- Monocytic myeloid-derived suppressor cells (mMDSC) can be generated ex vivo from precursors in nonhuman primate (NHP) bone marrow using GM-CSF and IL-6
- mMDSC with the phenotype lineage⁻HLA-DR⁻CD14^{hi}CD33^{-/lo}CD34^{lo}CD11b^{hi} potently suppress both CD4 and CD8 T cell proliferation
- These mMDSC enrich for CD4⁺ regulatory T cells
- They can be generated in adequate numbers for potential therapeutic application

Fig. 1. Putative mMDSC can be generated ex vivo from rhesus macaque BM cells using GM-CSF and IL-6.

(A) Femoral BM cells (n=3 normal monkeys) were cultured for 7 days in the presence GM-CSF and IL-6, as described in the Materials and Methods. After harvesting, the cultured cells were stained for CD3, CD20 (lin), HLA-DR, CD33 and CD14 and flow-sorted. Three putative mMDSC populations were identified as groups (Gp) 1, 2 and 3. The black open histogram denotes HLA-DR expression and the dashed histogram denotes isotype expression on the lin⁻ population. (B) The sorted cells were further stained for CD34 and CD11b and putative mMDSC further defined as lin⁻, HLA-DR^{-/lo}, CD14^{hi}, CD33^{-/lo},

CD34^{lo} and CD11b^{hi}, which correlated with Gp1 cells. The black solid histogram represents CD34 and CD11b marker expression on Gp1 cells. The dark grey and light grey histograms represent Gp2 and Gp3 CD34 and CD11b marker expression, respectively. (C) Aggregate data (means $\pm$ SEM) for CD11b and CD34 expression obtained from n=3 normal monkeys.

Fig. 2. The putative BM-derived mMDSC cell population Gp1 potently suppresses autologous T cell proliferation and increases the incidence of proliferating Treg. VPD450-labeled autologous T cells (T) were stimulated with α CD3/CD28 stimulatory beads (15 beads: 1T cell), as described in the Materials and Methods. Flow-sorted, BM-derived, putative mMDSC populations (Gp1, Gp2 and Gp3; as defined in Fig. 1) were added to the bead-stimulated T cells (1T: 2 mMDSC) at the start of 4-day co-cultures. (A) Representative images of T cell suppression. (B) Representative images of the incidences of Treg in the proliferating T cell populations. (C) Summary data (n=2 monkeys) of putative BM-mMDSC-induced suppression of T cell proliferation. (D) Summary data (n=2 monkeys)

of the incidence of proliferating Treg in the various mMDSC-treated cultures. Data are shown as means $\pm$ SE.

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Table 1

Cross-reactive anti-human mAbs used for phenotypic characterization and flow sorting of rhesus mMDSC and T cells

mAb	Clone	Vendor
HLA-DR	G46-6	BD Bioscience, San Jose, CA
CD14	M5E2	BD Bioscience, San Jose, CA
CD11b	ICRF44	BD Bioscience, San Jose, CA
CD20	2H7	BioLegend, San Diego, CA
CD34	563	BD Bioscience, San Jose, CA
CD33	AC104.3E3	Miltenyi Biotec, Auburn, CA
CD3	SP34-2	BD Bioscience, San Jose, CA
CD4	L200	BD Bioscience, San Jose, CA
CD25	BC96	ThermoFisher Scientific, Waltham, MA
CD127	HIL-7R-M21	BD Bioscience, San Jose, CA
Foxp3	PCH101	Miltenyi Biotec, Auburn, CA

Table 2:

Yields of flow-sorted mMDSC from cultured BM cells

Monkey ID	BM cells isolated (x10 ⁶)	Gp1 (CD33 ⁺ CD14 ⁺) BM-mMDSC (%) [*]	Theo. # of mMDSC (x10 ⁶)	Theo. # of mMDSC (x10 ⁶)/kg body weight [†]
RM120–15	740	5.1	38	7.5
RM123–15	700	6.1	43	8.5
RM122–15	830	4.2	35	7.0
Mean (3)	757	5.1	38	7.7
SE	38	0.5	2.3	0.4

^{*} For characterization of Gp1 BM-mMDSC, see text and Fig. 1 legend

[†] Assuming a recipient body weight of 5 kg